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Bovine lymph nodes as a source of *Escherichia coli* contamination of the meat

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ABSTRACT

Ground beef contamination with *Escherichia coli* is usually a result of carcass faecal contamination during the slaughter process. Carcasses are contaminated when they come into contact with soiled hides or intestinal leakage content during dressing and the evisceration processes. A more recent and compelling hypothesis is that, when lymph nodes are present in manufacturing beef trimmings, they can be a potential source of *Enterobacteriaceae* contamination of ground beef. The aim of this study was to investigate the occurrence of *E. coli* in lymph nodes from beef carcasses used for ground meat production, in six slaughter plants situated in central Italy A total of 597 subiliac (precrural) lymph nodes were obtained from 597 cattle carcasses and screened for *E. coli* by culture. Furthermore, *E. coli* isolates (one per positive carcass) were tested for *stx1*, *stx2 eaeA* and *hlyA* genes that are commonly used to identify and characterise shiga toxin-producing *E. coli* (STEC). In addition, the *E. coli* isolates were profiled for antimicrobial susceptibility. A proportion of 34.2% (204/597) carcasses were positive for *E. coli*. PCR revealed that 29% (59/204) of *E. coli* possessed *stx1* or *stx2* which corresponded to 9.9% of the cattle sampled. Moreover, a combination of *stx1* or *stx2* and *eaeA* was found in in 4 isolates (2% among *E. coli* positive samples and 1% among cattle sampled) and a combination of *stx1* or *stx2* and *eaeA* and *hly* in 1 isolate (0.5% and 0.2%). More than 95% of isolates were susceptible to gentamicin, ceftriaxone, cyprofloxacin and cefotaxime while high rates of resistance were recorded for cephalotin, ampicillin, tetracycline, tripe sulfa and streptomycin. The multivariate analysis identified "age" as the factor most closely related to *E. coli* positivity (either generic *E. coli* or STEC) in bovine lymph nodes. In conclusion, subiliac lymph nodes represent a source of *E. coli* for ground beef. These results are of major importance for risk assessment and improving good manufacturing practices during animal slaughter and ground meat production.

1. Introduction

Pathogenic STEC are significant causes of foodborne disease throughout the world (Scallan et al., 2011). In fact, the presence of *E. coli* in food or on animal carcasses surfaces after slaughter is an indication of unhygienic slaughter processes and the possible presence of foodborne disease-causing bacteria. "Shiga toxin-producing *Escherichia coli*" (STEC) are considered important foodborne pathogenic *E. coli* characterised by mild watery or severe bloody diarrhoea and complication such as haemolytic uremic syndrome. Cattle are a major reservoir and source of STEC and beef or beef products are commonly identified as a major source of STEC disease in humans. In the United States, consumption of ground beef was associated with 30% of cases of foodborne illness caused by STEC O157 (Heiman et al., 2015). Between April and June 2019, an outbreak of *E. coli* infection was reported from 10 states in the United States with a total of 209 people infected. Twenty-nine people were hospitalised and two cases of haemolytic uremic syndrome were reported. Epidemiologic and laboratory evidence indicated that ground beef was the likely source of this outbreak (CDC, 2019).

Generic and pathogenic *E. coli* can contaminate the animal carcasses during the slaughter process as a result of faecal contamination during

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evisceration and dressing processes especially or as a result of cross contamination due to contact with other carcasses, contaminated slaughter equipment or abattoir personnel along the food chain (Barkocy-Gallagher et al., 2003; Costa et al., 2020; Elder et al., 2000; Greig et al., 2012).

Considerable efforts have been made to prevent or minimise *E. coli* contamination during slaughter or eliminate the bacteria which may be present on the carcasses after slaughter by implementing HACCP systems and good manufacturing practices (GMPs) (Brichta-Harhay et al., 2008; Cenci-Goga et al., 2014; Phillips et al., 2012).

In addition to intestinal content and hides, lymph nodes have been previously identified as a potential source of enteric bacteria including *Salmonella* (Arthur et al., 2008; Webb et al., 2017). A number of studies have also shown that, under certain conditions (such as disruption of the ecologic gastro-intestinal equilibrium to allow intestinal bacterial overgrowth, increased permeability of the intestinal mucosal barrier, and deficiencies in host immune defences), enteric bacteria in the gastrointestinal tract enter the epithelial mucosa, invade the lamina propria and are able to spread to peripheral lymph nodes and other organs, a condition which has been termed "bacterial translocation" (Berg and Garlington, 1979). Moreover, lymph nodes function as a filter mechanism that sequesters pathogens which are eventually destroyed by lymphocytes. It has also been shown that certain bacteria are able to evade the host immune responses and survive within immune cells, including macrophages (Arthur et al., 2010; Azevedo et al., 2016; Bonardi et al., 2007; Poirier et al., 2008; Richter-Dahlfors et al., 1997). Bacterial translocation in lymphocytes, evasion and survival in immune cell such as macrophages, has become a major food safety and public health concern, as lymph nodes can be incorporated into ground beef during processing, and present a major hazard as a potential source of pathogenic foodborne enteric bacteria. Current studies on the contribution of carcass lymph nodes as a potential source of ground beef contamination with *E. coli* are scant (Arthur et al., 2010; Bonardi et al., 2007; Grispoldi et al., 2017).

The aims of this study were to 1) investigate the presence of *E. coli* in lymph nodes from beef carcasses that have been passed for human consumption after meat inspection and determine 2) whether some of the *E. coli* present in lymph nodes are STEC, 3) antimicrobial resistance profiles of *E. coli* recovered from beef carcass lymph nodes and 4) the risk factors associated with the presence of *E. coli* in cattle lymph nodes. The ultimate goal is to ascertain to what extent lymph nodes are a potential source of *E. coli* and/or STEC and pinpoint potential risk factors (sex, age, breed, diet and the slaughter procedures [farm-slaughterhouse distance, ordinary or casualty slaughter]) associated with the prevalence of *E. coli* in subiliac (precrural) lymph nodes.

2. Materials and methods

2.1. Sampling, microbiological analyses and PCR

Samples were collected in central Italy in a period between May 2016 and September 2017. A convenience sample of six commercial processing plants consisting of three plants that primarily harvest feedlot cattle and three that primarily harvest cull cows was enrolled. The sample size was calculated using the formula $n = Z2 * p * (1 - p) / C2$, where Z is the Z-value (e.g. 1.96 for a 95% confidence level), p is the expected prevalence, expressed as a decimal, and C is the absolute precision, expressed as a decimal (Mariano et al., 2009). With approximately 8500 bovines slaughtered per year and an expected prevalence for *E. coli* positive samples of 50% (0.5), a precision of 4% and a confidence level of 0.95, a sample size of approx. 600 samples was then required. Therefore 597 animals were then randomly selected using animal-identification numbers database at the onset of the study (specifically all numbers were printed, cut out and drawn from a "hat" blindly).

Distribution of samples according to risk factors included: "gender" (357 males and 240 females), "age" (438 animals under 2 years of age, 159 older than 2 years), "farm slaughterhouse distance" (157 animals coming from a distance exceeding 350 Km, 164 from distances between 350 and 50 km, 276 from distances $<$ 50 km), "ordinary or casualty slaughter" (105 samples came from casualty slaughter, defined in Italy as "*macellazione d'emergenza*". i.e. the slaughter of compromised animals, with veterinary certification for transportation to slaughterhouses), "organs condemned by veterinary inspection" (118 carcasses had at least one organ condemned), "diet" (157 animals came from farms with a diet where $>$ 70% of feed ratio was done via polyphite grasslands) and "breed".

Lymph nodes were analysed, *more solito*, by our standard laboratory method (Sechi et al., 2012). which is a modification of the procedure originally described by Cobbold (2009). Briefly, while wearing new sterile gloves for each carcass, the subiliac (precrural) lymph nodes and the surrounding fat were dissected with a sterile scalpel from each carcass side. Lymph node samples were placed in individual plastic bags per animal. Data identifying each animal was recorded on each sample bag. Samples were transported to the laboratory in a refrigerated container.

Under sterile conditions lymph nodes were freed from the surrounding fat tissue and from the capsule and flamed for 3 to 5 s for surface sterilization. Lymph nodes were cut into small pieces with a sterile knife and 10 g of tissue were placed in a sterile stomacher bag (PBI International. Milan, Italy) containing 90 ml of Peptone Water (PW, Conda pronadisa, Madrid, Spain) and homogenised for 2 min using a stomacher (PBI International). The homogenised sample was initially pre-enriched by incubation at 35–37 °C for 18–24 h and then inoculated on Violet Red Bile Lactose agar (VRBL, Oxoid, Basingstoke, Hampshire, UK) using the spread plate technique and incubated at 35–37 °C for 18–24 h. Purplish-red colonies with a diameter of at least 0.5 mm were selected and replicated for confirmation on Mac Conkey agar (Conda pronadisa) and Tryptone Bile X-glucuronide agar (TBX, Oxoid) using the replica plating technique (Lederberg and Lederberg, 1952) to verify their presumptive *E. coli* status. All positive colonies on Mac Conkey and TBX were identifies as presumptive *E. coli* and inoculated into Brilliant Green Bile broth (BGB, Oxoid) containing a Durham bell each of and incubated at 37 °C and 44 °C for 24 h to detect gas production. Isolates were also inoculated into PW tubes (Conda pronadisa) and incubated at 37 °C for 24 h. Kovac's reagent (Conda pronadisa) was added to PW for the indole test. One isolates per carcass with phenotypic characteristics corresponding to *E. coli* was stored at −80 °C until further processing.

DNA was extracted according to a protocol previously described by Cenci Goga et al. (2004). STEC were identified by PCR using amplification conditions and primers previously described by Paton and Paton (1998) and Gannon et al. (1997).

2.2. Antimicrobial susceptibility

E. coli isolates were tested for antimicrobial susceptibility against a panel of 19 antimicrobials by the disk diffusion method (Kirby Bauer Test) as described by the Clinical and Laboratory Standards Institute (CLSI, 2015) The following antimicrobials were tested: sulphonamides 300 mg, sulphamethoxazole/trimethoprim 25 mg, ciprofloxacin 5 mg, nalixidic acid 30 mg, enrofloxacin 5 mg, chloramphenicol 30 mg, amoxycillin/clavulanic acid 30 (20 + 10) mg, ampicillin 10 mg, cefotaxime 30 mg, ceftriaxone 30 mg, cephalothin 30 mg, ticarcillin 75 mg, tetracycline 30 mg, amikacyn 30 mg, gentamicin 10 mg, kanamycin 30 mg, neomycin30 mg, streptomycin 10 mg, and colistin 10 mg. This antimicrobial panel was selected to test the major groups of antimicrobials. Briefly, frozen isolates were thawed and cultured in BHI broth (Bio-Rad) at 35 to 37 °C for 24 h. A portion of the culture broth was inoculated into 6 ml of 0.9% sterile physiological saline solution until a turbidity of 2 McFarland was reached. Using a sterile swab, the solution was spread on Muller-Hinton agar plates (Oxoid).

Table 1
Distribution of *E. coli* isolates.

	Tot	Gender		Age (months)		Diet		Distance (km)						Slaughter		Organs[a]	
		m	f	≤24	> 24	F	C	> 350	< 250	< 200	< 150	< 100	< 50	o	c	Yes	No
All samples	597	357	240	208	389	157	440	157	5	5	70	84	276	492	105	118	360
E. coli	204	112	92	56	148	36	168	36	1	1	25	38	103	155	49	44	116
Stx1	41	13	28	11	30	3	38	3	1	0	5	10	22	18	23	8	11
Stx2	23	7	16	7	16	3	20	3	0	0	0	2	18	21	2	10	12
eaeA	11	5	6	5	6	4	7	4	0	0	0	3	4	9	2	3	6
hly	34	26	8	13	21	10	24	10	1	0	4	8	11	31	3	12	21
Stx1 OR Stx2	59	18	41	14	45	5	54	5	1	0	5	12	36	34	25	14	22
Cephalotin	183	98	85	51	132	34	149	34	1	1	23	33	91	139	44	43	101
Ampicillin	85	42	43	19	66	13	72	13	1	0	8	18	45	65	20	16	53
Tetracycline	38	20	18	16	22	9	29	9	0	0	6	8	15	29	9	8	23
Triple sulphonamides	30	16	14	14	16	8	22	8	0	0	4	5	13	24	6	7	17
Streptomycin	23	7	16	8	15	4	19	4	0	0	4	4	11	18	5	4	16
Sulphamethoxazole/trimethoprim	17	10	7	8	9	5	12	5	0	0	3	3	6	14	3	3	11

Gender: male (m) or female (f); diet: mainly forage (f) or concentrate (c); slaughter: ordinary (o) or casualty (c); organs condemned by veterinary inspection: yes or no.

[a] For 119 animals, out 597, no data on organs condemnations were recorded by the official veterinarian.

Antimicrobial disks (Oxoid) were placed on Muller-Hinton agar plates which were incubated at 37 °C for 18 to 24 h. At the end of incubation, the diameters of the growth inhibitory zones were measured, and these were interpreted using specific CLSI tables whereby the bacterium is classified as sensitive, intermediate or resistant (CLSI, 2011).

2.3. Data analysis

To identify the risk factors associated with *E. coli* prevalence, first a univariate analysis of the variables of interest was conducted with binary logistic regression, followed by multiple logistic regression performed with StatView 5 for Mac OS (SAS Inst. Inc., Cary, NC, USA).

3. Results and discussion

Lymph nodes from a total of 597 cattle were analysed (Table 1): 357 (59.8%) were males and 240 (40.2%) females, the age ranged between 1 and 18 years (34.8% [208 animals] were < 24 months old and 65.2% [389 animals] were older than 24 months), 157 had been fed a forage rich diet (26.3%) and 440 high grain rations (73.7%). The distance between the farm and the slaughterhouse ranged between 5 and 400 km, 492 animals (82.4%) were slaughtered according to the ordinary procedure and 105 (17.6%) to casualty slaughter. One hundred and eighteen carcasses had at least one organ condemned after veterinary post mortem inspection (19.8%), while in 360 animals (60.3%) no abnormalities were detected. Most of the subjects were cross-breed (342 animals, 57.3%) or belonged to Chianina (144 animals, 24.1%) or Holstein (42 animals, 7.0%) breeds; the remaining animals belonged to Limousine, pezzata rossa and pezzata nera breeds.

A total of 204 lymph nodes from a total of 597 cattle were positive for *E. coli*, with an overall prevalence of 34.2%. The prevalence of *E. coli* in lymph nodes from males was 31.4% (112 positives out of 357 samples) and in females 38% (92 positives out of 240 samples). The prevalence of positive lymph nodes was 26.9% (56 out of 208) in animals younger than 24 months and 38.0% in animals older than 24 months (148 out of 389). The prevalence of *E. coli* in lymph nodes of animals fed a forage rich diet was 23% (36 out of 157) and in those fed high grain rations was 38.2% (168 out of 440). The distribution of positive samples according to the distance farm-slaughterhouse is shown in Table 1. The prevalence of *E. coli* in lymph nodes from animals slaughtered according to the ordinary procedure was 31.5% (155 positives out of 492 samples), whereas the prevalence of *E. coli* in the lymph nodes from animals slaughtered according to casualty slaughter was 47% (49 positives out of 105 samples). According to organs condemned the prevalence of *E. coli* in lymph nodes from carcasses that had at least one organ condemned was 37% (44 out of 118 positive samples) and 32.2% (116 out of 360) from carcasses where no abnormalities had been detected at the veterinary post mortem inspection.

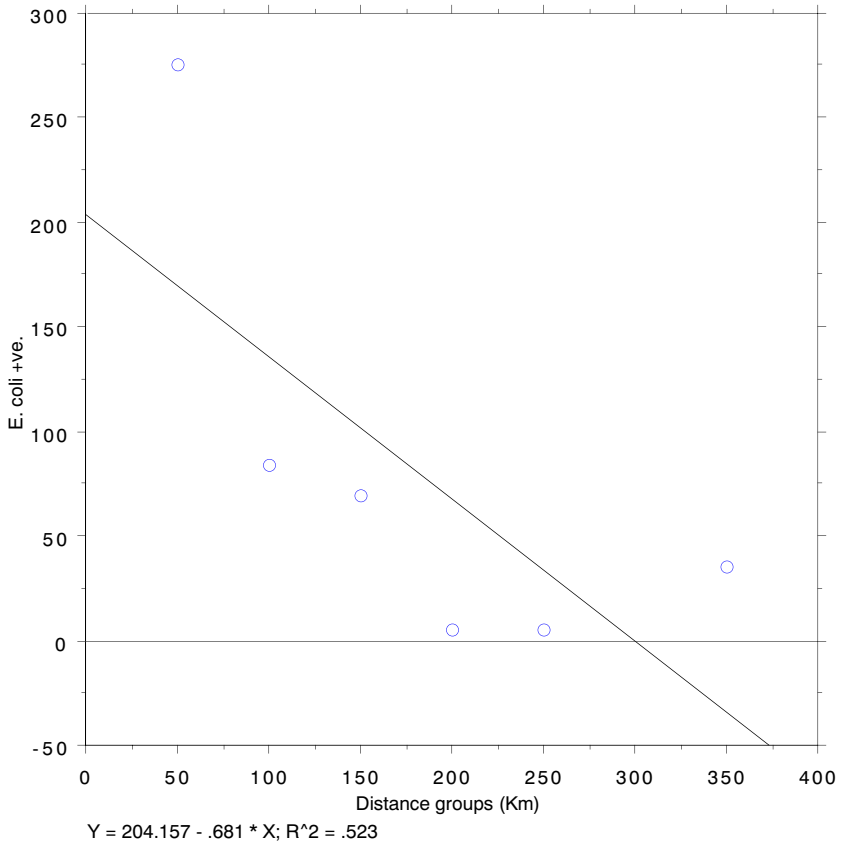
The multivariate analysis (Table 5) identified “age” as the factor most closely related to *E. coli* positivity: OR 1.122 (CI = 1.039–1.211, p = 0.0034) indicates a higher probability of *E. coli* positivity in lymph nodes sampled from older animals. Moreover, the similarity for odd ratio from multiple logistic regression with the simple logistic regression indicates that there is little confounding effect of other factors on the relationship between the age and *E. coli* positivity. On the other hand, the simple logistic regression (Table 4) showed statistically significant differences ($p < 0.05$) also for “distance” where apparently the longer the distance the lower the probability of *E. coli* positivity, “slaughter” with a higher positivity in carcasses from casualty slaughter, and “diet” for a lower positivity from animals fed a forage rich diet (OR 2.076, CI = 1.366–3.156).

The correlation between age or farm-slaughterhouse distance and the prevalence of *E. coli* was also demonstrated with a simple linear regression (Fig. 1). The analysis showed a positive correlation between the animal's age and the prevalence of *E. coli* ($r^2 = 0.744$) with a higher prevalence of positive in older animals and a negative correlation between the farm-slaughterhouse distance ($r^2 = 0.523$) with a lower prevalence in animals transferred over longer distances.

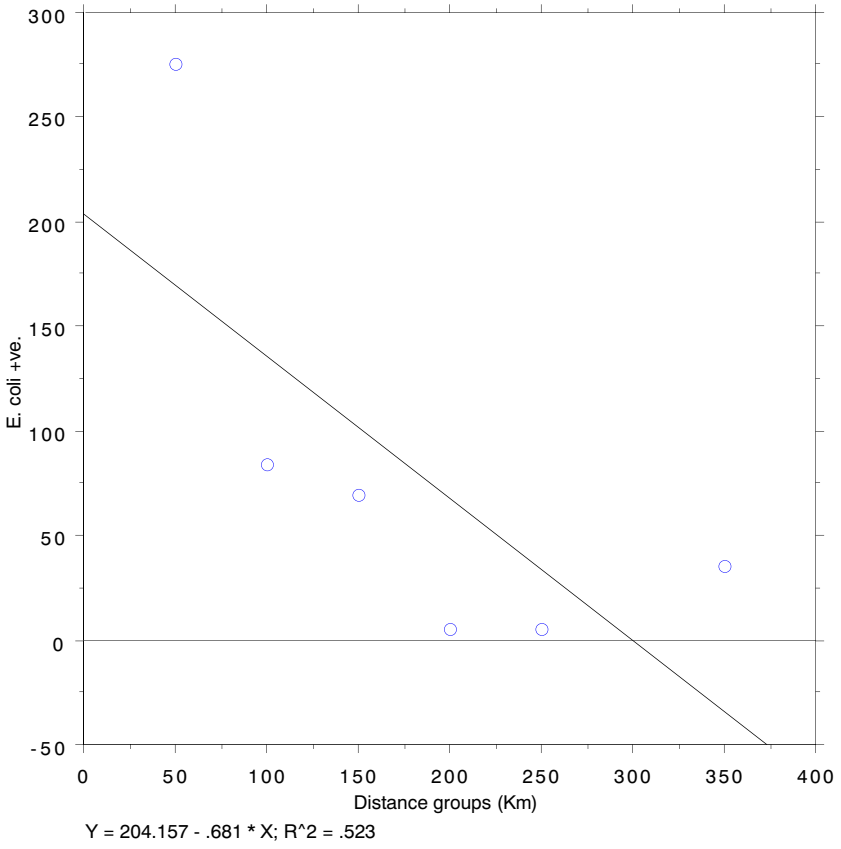
The combined analysis of data from the simple and multiple logistic regression analysis along with the data distribution, help shed some light on the prevalence of *E. coli* in the subiliac lymph nodes of the 597 cattle analysed. From Table 6 it is evident that “age” is a confounding variable for “distance”, “slaughter” and “diet”: the mean age for animals on a forage rich diet was 1.41 years compared to 3.11 for those fed high grain rations, a high proportion of younger animals came from longer distances and the mean age for animals slaughtered by ordinary procedure was 1.86 compared to 6.43 for casualty slaughters.

The fact that the prevalence of *E. coli* is lower in lymph nodes of animals that travelled longer distances could also be explained by the fact that the majority of these animals came from regions in the north of Italy, where cattle receive a forage prevalent diet. There are controversial data in literature regarding the effect of different diets on the *E. coli* population in cattle (Callaway et al., 2009). In particular, some authors suggest that switching from high grain diets to hay can reduce the *E. coli* contamination in the abattoir (Diez-Gonzalez et al., 1998) and other postulates that forage content in the diet does not have significant impact on faecal *E. coli* O157:H7 levels (Biswas et al., 2016). Another hypothesis, given that animals that have travelled for longer journey have had longer feed withdrawal is related to fasting. However, it must be stressed that the dynamics of shedding of any bacterial

a



b



(caption on next page)

Fig. 1. a. Simple linear regression between the animal's age and the prevalence of *E. coli*.
b. Simple linear regression between farm-slaughterhouse distance and the prevalence of *E. coli*.

groups by cattle are complex and can be affected by numerous, inherently variable and inter-related factors such as extent of attachment/detachment to intestinal mucosa or distribution and growth in solid/liquid phases of gut contents, as well as their passage rates through various sections of the gut. Moreover, although increased shedding may be correlated to feed withdrawal or transport, shedding cannot be factored into lymph node positivity over a short span of time. Contrary to the fasting hypothesis, the results of the famous study by Reid et al. (2002) indicate that feed withdrawal for 24 or 48 hour periods can indeed increase the number of total *E. coli* numbers shed by cattle. From the meat safety perspective, therefore, the results of that study do not support the concept of using pre-slaughter fasting of cattle as a measure to reduce excretion of, and subsequent carcass contamination with, enteric *E. coli*. This latest evidence was indeed the final nail in the coffin for the fasting/feed withdrawal theory in cattle.

Higher prevalence of *E. coli* in lymph nodes of animals slaughtered with casualty procedure could be related to concomitant diseases but is more likely due to the fact that this procedure is usually applied in older animals. Here an important comment is essential. Regulation 218/2014 stipulates: "Regulation (EC) No 853/2004 lays down conditions under which meat from animals having undergone emergency slaughter outside a slaughterhouse, is fit for human consumption. As emergency slaughter meat which has successfully passed meat inspection does not constitute a risk to public health, the requirement for a special health mark and the restriction to the national market for the emergency slaughter meat should be deleted from that Regulation and the requirement for a special health mark for the emergency slaughter meat also from Regulation (EC) No 854/2004." However Italian health ministry has identified two different situations: 1) "*macellazione d'urgenza al di fuori del macello*", i.e. emergency slaughter outside a slaughterhouse and 2) "macellazione d'emergenza al macello", i.e. casualty slaughter for compromised animals, which may have veterinary certification for transportation to slaughterhouses (Poeta et al., 2013). So, there is a sort of false friend in the translation because emergency has been translated into "urgenza", and casualty into "emergenza". All animals tested in this study belonged to the "macellazione d'emergenza al macello" (i.e. casualty slaughter). This is a peculiar situation because all casualty slaughter should be classified either as regular slaughter or as on farm emergency slaughter, this middle ground is deceiving because it may include true emergency slaughter that should have been done on farm and other uncertain situations where the veterinarian decision may be questionable. For this reason, the new Regulation 624/2019 stipulates that: "In the event of emergency slaughter, ante-mortem inspection cannot be carried out in the slaughterhouse. In order to avoid causing the animal unnecessary suffering by transporting it to a slaughterhouse, and to limit economic losses for operators and reduce food waste, criteria and conditions should be laid down permitting ante-mortem inspection to be performed outside the slaughterhouse in the event of an emergency slaughter. Animals subject to emergency slaughter may still be fit for human consumption subject to a favourable meat inspection. These inspections should provide maximal guarantees of the fitness for consumption when allowing emergency slaughter outside the slaughterhouse" and that the ante mortem inspection along with the authorization to perform the emergency slaughter outside a slaughterhouse is done only by an official veterinarian.

PCR results (Tables 1 and 2) showed 41 positives for the *stx1* gene (20% among the isolates and 6.9% among all tested animals), 23 positives for the *stx2* gene (11% and 3.9%), 11 positives for the *eaeA* gene (5.4% and 1.8%), 34 positives for the *hly* gene (17% and of 5.7%), and 5 positives for both the *stx1* and *stx2* gene (2% and 0.8%). Moreover, one of the genes stx1 or stx2 were found in 59 out of 204 isolates (29% and 9.9%), a combination of stx1 or stx2 and eaeA in 4 isolates (2% and 0.7%) and a combination of stx1 or stx2 and eaeA and hly in 1 isolate (0.5% and 0.2%). The antimicrobial susceptibility test data are shown in Tables 1 and 3. High percentages (> 90%) of susceptible strains were found for sulphamethoxazole/trimethoprim, chloramphenicol, nalidixic acid, gentamicin, amikacin, cefotaxime, ceftriaxone, ciprofloxacin and enrofloxacin. A high prevalence of resistance strains was observed for cephalotin, ampicillin, tetracycline, triple sulpha and streptomycin.

Table 2
Number of *E. coli* isolates positive for more than one gene.

AND	Stx2	eaeA	Hly (n = 34)
stx1 (n = 41)	5	2	6
stx2 (n = 23)		3	3
eaeA (n = 11)			2
stx1 AND Stx2 (n = 5)		1	0
stx1 OR Stx2 (n = 59)		4	9
stx1 OR Stx2 AND eae (n = 4)			1

stx1 OR stx2 OR eaeA OR hly: 90.

Table 3
Antimicrobial susceptibility data.

Antimicrobial	Resistant (%)	Intermediate (%)	Sensitive (%)
Cephalothin	89.7	7.4	3
Ampicillin	42	18	40
Tetracycline	19	2	79.4
Compound sulphonamides	15	2	83.3
Streptomycin	11	21	68.1
Sulphamethoxazole/trimethoprim	8.3	0.5	91.2
Neomycin	5.9	40	54.4
Amoxycillin/clavulanic acid	5.4	12	82.8
Chloramphenicol	4.9	0.5	94.6
Kanamycin	4	8.3	87.7
Nalixidic acid	4	1	94.6
Gentamicin	3	0	96.6
Ticarcillin	10	1	88.7
Amikacyn	2	0.5	97.5
Cefotaxime	0.5	2	97.1
Ceftriaxone	0.5	0	99.5
Cyprofloxacin	2	0	98.0
Enrofloxacin	0.5	2	97.5
Colistine	0	15	85.3

The multivariate analysis of the isolates, identified "age", "gender" and "slaughter" as the factors most closely related to PCR positivity for stx1 or stx2, where isolates from females and ordinary slaughter carried these genes more frequently. Other factors, such as "diet", and "distance" were linked to PCR positivity only at the simple logistic regression indicating the same confounding effect shown for the logistic regression of *E. coli* positivity.

Similar results were obtained for antimicrobial susceptibility, where "age" was the factor most closely associated with antimicrobial resistant strains for ampicillin, tetracycline, triple sulpha, streptomycin.

Our cross-sectional study is the first original attempt to analyse risk factors associated with *E. coli* prevalence in lymph nodes. In fact a great deal of information is available on these sources of faecal contamination along with the best detection methods (Cenci-Goga et al., 2007) and the physical and chemical decontamination treatments used to minimise them (Barco et al., 2015), there is little information available in literature regarding lymph-nodes as a source of *E. coli* contamination of beef carcasses (Grispoldi et al., 2017; Sofos et al., 1999). It is well known that *Enterobacteriaceae*, especially *E. coli* can contaminate beef carcasses along the slaughter line and that microbial contamination of carcasses may occur from transfer of faecal material from the hide,

Table 4
Factors associated with *E. coli* positivity: results of logistic regression for each variable.

	OR (95% C.I.)	p
Gender		
Male	1[a]	
Female	1.360 (0.965–1.916)	0.0791
Age (years)	1.141 (1.075–1.213)	< 0.0001**
Forage diet		
Yes	1[a]	
No	2.076 (1.366–3.156)	0.0006**
Distance (km)	0.814 (0.721–0.919)	0.0009**
Slaughter		
Regular	1[a]	
Casualty	1.902 (1.240–2.919)	0.0032**
Organs condemned		
No	1[a]	
Yes	1.251 (0.811–1.930)	0.3120
Breed		
Holstein	1[a]	
Chianina	1.751 (0.860–3.564)	0.1225
Half breed	0.653 (0.332–1.282)	0.2154
Others	1.157 (0.522–2.563)	0.7190

OR: odd ratio. p: p-value.
[a] Reference level.
** $p < 0.05$.

Table 5
Factors associated with *E. coli* positivity: results of multiple logistic regression.

	R (95% C.I.)	p
Age (years)	1.122 (1.039–1.211)	0.0034**
Forage diet		
No	1[a]	
Yes	1.919 (0.435–8.463)	0.3894
Distance (km)	1.036 (0.670–1.600)	0.8746
Slaughter		
Regular	1[a]	
Casualty	0.965 (0.550–1.693)	0.9013

R: odd ratio. p: p-value.
[a] Reference level.
** $p < 0.05$.

hooves or ruptured gut at various stages of processing (Blagojevic et al., 2012; Greig et al., 2012; Madden et al., 2004). Lymph nodes, indeed, can be contaminated and can transfer the bacteria that they harbour to ground meat, when they are not removed from the carcasses: for instance, Lowe et al. (2011, 2012); Mann et al. (2014) and Mann et al. (2015) have demonstrated an unexpected bacterial diversity in lymphatic gastro-intestinal tract-associated organs, such as pharyngeal lymphatic tissues and enteric lymph nodes; providing evidence that lymphatic organs are a serious contamination source. The occurrence of translocated, viable bacteria in lymph nodes is therefore of practical relevance for carcass contamination (Berends et al., 1998; Borch et al., 1996; Vieira-Pinto et al., 2005). Subiliac (precrural) lymph nodes, in particular, are often left on the carcass with the fat tissue around them due to their anatomical position, and are incorporated in the production of ground beef. In a study on the tracking of source of *Salmonella* in ground beef Koohmaraie et al. (2012) conclude that since deep tissue lymph nodes are not removed during carcass processing into ground beef, Salmonella could easily be found in ground beef.

The prevalence of *E. coli* contamination of lymph nodes that we found in our study is much higher than the level reported by Bailey et al. (2017). In the latter study, the authors analysed 197 sets of lymph nodes (superficial cervical, iliofemoralis, subiliac, popliteal, ischiatic, axillary, pre-sternal, coxalis and pre-pectoral) from Australian beef cattle. Coliforms were detected in 1.6% of lymph of nodes overall and the anatomical sites had no > 5% of samples positive for *E. coli*. On the other hand, similar levels of *E. coli* contamination of lymph nodes were reported by Cobbold (2009): parotid, submaxillary and retropharyngeal nodes were collected from 534 cattle and *E. coli* was detected in 57% of samples. Based on the detection of shiga toxin genes by PCR, 7.3% of samples had evidence of the presence of STEC. Similar results were reported by Sofos et al. (1999) in a study in the United States on potential sources of beef carcass contamination. In a study on the prevalence of *E. coli* isolates of significant food safety hazard in ground meat, Bosilevac and Koohmaraie (2011) found that 0.24% of 4133 ground beef samples were positive for STEC carrying also the *eae*, *subA*, and *nle* genes.

The presence of drug resistant bacteria on the carcasses raise food safety concerns because there is a potential transfer of resistant foodborne pathogens to humans through the food chain especially in the cases of Salmonella and Campylobacter spp. (Moyane et al., 2013). In our study, the majority of isolates were resistant to cephalothin and sensitive to nalidixic acid. A similar pattern was observed by other authors (Ntuli et al., 2016; Sayah et al., 2005). Cephalothin belongs to the cephalosporin antimicrobial class and is not commonly used in food producing animals as a medicine (Sayah et al., 2005); however, high resistance to cephalothin may be attributed to cross-resistance, which develops through using other cephalosporins (Giguère et al., 2013; Sayah et al., 2005).

4. Conclusions

Our study showed that subiliac lymph nodes can represent a source of contamination by generic *E. coli* and by STEC of ground beef, if they are left on the carcass. The high prevalence of contaminated lymph nodes (an overall prevalence of 34.2%) and the presence of STEC (overall prevalence of 9.9%), demonstrated that they can be an important and neglected alternative source of *E. coli*, besides the transfer of faecal material from the hide, hooves or ruptured gut at various stages of processing. Multiple logistic regression analysis demonstrated a correlation between the age of the animals and the presence of *E. coli* in lymph nodes. Our study has also identified other factors, such as diet (high grain rations vs forage rich diet), distance from slaughterhouse and kind of slaughter (casualty vs ordinary), related to *E. coli* positivity and the presence of genes that code for shiga toxins, however “age” is always a confounding variable for “distance”, “slaughter” and “diet”.

Table 6
Distribution of age of animals.

	Tot	Diet		Distance (km)						Slaughter	
		f	c	> 350	< 250	< 200	< 150	< 100	< 50	o	c
Mean	2,66	1,41	3,11	1,41	1,57	2,02	2,93	3,24	3,17	1,86	6,43
Std. dev.	2,83	0,26	3,18	0,26	0,52	0,13	3,26	2,60	3,37	1,50	4,24
Std. error	0,12	0,02	0,15	0,02	0,23	0,06	0,39	0,28	0,20	0,07	0,41
Count	597	157	440	157	5	5	70	84	276	492	105

Diet: mainly forage (f) or concentrate (c); slaughter: ordinary (o) or casualty (c).

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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