Food Control 146 (2023) 109526

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Assessment of poultry process hygiene and bacterial dynamics along two broiler slaughter lines in Norway

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ARTICLE INFO

Keywords:
Broiler
Poultry
Campylobacter
Bio-mapping
Risk-categorization
16S sequencing
Metagenomics

ABSTRACT

Good process hygiene in broiler slaughter is paramount to achieve safe products with long shelf-lives. Here we investigated changes in bacterial load and diversity on chicken carcasses at selected stages on slaughtering lines in two abattoirs in Norway. Carcasses included in the study, came from flocks that had been classified as either positive or negative for *Campylobacter*. In total, 120 neck-skins were collected at four sampling points: before scalding, after plucking, after evisceration, and after chilling. The bacterial load was analyzed at each sampling point using quantitative and qualitative cultivation while the bacterial composition was determined using amplicon sequencing of the 16S rRNA gene. Bacterial loads on carcasses decreased along the slaughter line by 2.1, 1.1, 1.1, and 1.0 log cfu per g for Total Plate Counts (TPC), *Enterobacteriaceae, Escherichia coli*, and *Campylobacter*, respectively. The largest reduction was observed after washing and chilling. For TPC, a large reduction was also observed after scalding and plucking. Scalding water samples had low amounts of *E. coli* and were negative for *Campylobacter*. Only a weak statistical association was found between indicator counts and *Campylobacter*. The 16S rRNA amplicon sequencing results showed a more diverse bacterial community at the start of the slaughter line, dominated by *Staphylococcus*, *Escherichia-Shigella,* and *Streptococcus,* which altered to a less-diverse community, dominated by *Asinibacterium* spp., *Afipia* spp., *Pseudomonas, Polaromonas*, and *Psychrobacter* after chilling. Both abattoirs were assessed as low risk by a new categorization method. This study contributes to identify factors that increases and decreases levels of *Campylobacter* and other bacteria during slaughter and should enable the implementation of control measures and thus improve meat safety.

1. Introduction

A major challenge for large-scale slaughtering of broilers is to minimise the contamination of meat by maintaining good process hygiene(Mataragas et al., 2012). A slaughter line consists of process stages that perform different operations of slaughtering and carcass preparation, often in carousel systems: stunning, bleeding, scalding, plucking, evisceration, washing and chilling, and are rather similar throughout Europe (Boysen et al., 2016). The slaughter speed is usually 4000–13000 carcasses per hour. Compared with slaughtering of cattle, pigs, and sheep, conventional broiler slaughtering is highly mechanized and involves minimal manual work (Mataragas et al., 2012). Uniform shapes and even sizes of the carcasses are therefore important for the machines to perform as intended and to minimise contamination, mainly of faecal origin, on the meat (Soro et al., 2020).

The initial bacterial loads in the intestines and on the outside of the live chickens that arrive for slaughter, are mainly determined by the bacterial environment on-farm (Ghareeb et al., 2013). Avoiding pathogens on-farm is a huge challenge. Abattoirs can compensate for and minimise bacterial loads on broiler carcasses to some extent through good hygiene practices (GHP), hazard analysis and critical control points (HACCP), and different interventions for reducing contamination of carcass surfaces (Nastasijević, Proscia, et al., 2020). To monitor and control the bacterial loads on carcasses, abattoirs perform

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https://doi.org/10.1016/j.foodcont.2022.109526
Received 6 September 2022; Received in revised form 8 November 2022; Accepted 20 November 2022
Available online 24 November 2022

microbiological testing according to process hygiene criteria (Anon, 2005, 2017).

Broiler meats as a source of human disease are mainly associated with *Campylobacter* and *Salmonella* infections (EFSA, 2012), the two most common causes of bacterial gastroenteritis in Europe (EFSA/ECDC, 2021). Both pathogens can be shed by healthy birds and are therefore difficult to detect on-farm. As neither of these are detected by visual meat inspection, establishing an integrated food safety assurance system, achievable through improved food chain information (FCI) and risk-based interventions, is necessary to improve surveillance and control of meat safety in broiler meat production (Lupo et al., 2013). Abattoirs attempt to improve chicken meat safety, with regards to *Campylobacter* and *Salmonella*, by preventing or reducing transfer of the pathogens from feathers and intestines to carcasses, i.e., abattoir process hygiene (Buncic et al., 2017). Many factors affect hygiene performances in abattoirs, including, for instance, equipment settings and design, maintenance, slaughter speed, staff hygiene training, management motivation, and environmental aspects (Djekic & Tomasevic, 2016; EFSA, 2012; Habib et al., 2012).

Risk categorization of abattoirs according to their capacity to control hazards based on their process hygiene has been suggested as a contribution to risk-based meat safety assurance systems (MSAS) by the European Food Safety Authority (EFSA) (Blagojevic et al., 2021; Cegar et al., 2022; EFSA, 2012), but this is not yet implemented in the EU. Risk categorization may be useful for the abattoirs in sales and marketing, inspire to improve process hygiene, better staff training, and a tool for the competent authorities in assessment of audit frequency. According to EU legislation, hygiene in poultry abattoirs is assessed through absence of *Campylobacter* and *Salmonella* on chilled carcasses (Anon, 2005, 2017). *Escherichia coli* and *Enterobacteriaceae* are widely used indicators of faecal contamination during slaughter of all animal species. In the United States, *E. coli* is used as the indicator of faecal contamination on broiler carcasses, along with monitoring the presence of *Salmonella* (FSIS, 2021). Using indicator bacteria, such as *E. coli* and *Enterobacteriaceae,* as process hygiene criteria in European poultry abattoirs, has been discussed and suggested by EFSA (EFSA, 2012) but so far, *Campylobacter* and *Salmonella* are in use.

In Norway, an "Action Plan against *Campylobacter* (APaC)" has monitored broiler flocks for *Campylobacter* since 2001 (https://www.vetinst.no/overvaking/campylobacter-fjorfe). Norwegian flocks are infrequent carriers of *Campylobacter* (4–8% yearly average), and flocks slaughtered during the summer season, May–October, aged up to 50 days, must be sampled on-farm for caecal *Campylobacter* carriage. Positive flocks are usually slaughtered at the end of the working day, and carcasses are frozen (−18 °C) for more than 3 weeks, before sending the meat to the market. Farms with infected flocks, receive guidance on GHP and biosafety.

To improve meat safety and to extend the shelf-lives of chicken meat by reducing carcass surface contamination, the effectiveness of different measures during slaughter and chilling must be assessed (Belluco et al., 2016; Rosenquist et al., 2006). Evisceration is regarded as the process with the highest risk for contamination of carcass surfaces (Althaus et al., 2017). Investigating which bacteria are present and dominant at different processing stages, such as scalding and chilling, could provide insights regarding which organisms are able to survive different time/-temperature combinations, water pressures, wind speeds, and other interventions. Several studies have shown reductions in bacterial loads during the scalding process (Berrang et al., 2000; 2011; Northcutt et al., 2003) and hot water (Hugas & Tsigarida, 2008; Loretz et al., 2010; Svobodová et al., 2012; Zhang et al., 2013). However, the use of chemical and physical interventions has been controversial and impeded by legislation in Europe, due to concerns that decontamination could mask unhygienic slaughter practices (Hugas & Tsigarida, 2008). Bacterial community profile on broiler carcasses and especially on chilled carcasses, will help the meat industry implementing effective measures to produce safe meat with extended shelf lives (Hansen et al., 2023). Some studies have investigated bacterial population compositions in poultry intestines and on carcass surfaces (Chen et al., 2020; Handley et al., 2018).

The aim of this study was to investigate the effects of selected slaughter operations on *Campylobacter* and other microbiological contamination of broiler carcasses, including bacterial dynamics and diversity, at two commercial broiler abattoirs in Norway. In addition, the study aimed to evaluate the difference between abattoirs and define possible risk characterization for abattoirs according to selected bacterial counts.

2. Materials and methods

2.1. Slaughter lines and carcasses

Sampling was performed at two commercial broiler abattoirs in Norway in August 2020 (single-day pilot study in abattoir A) and in August 2021 (day 1 and 2 in abattoir B) (Table 1). Abattoir A had a slaughter speed of 9000 carcasses per hour and was over 30 years old, while abattoir B was newly built (in 2021) and operated at a speed of 12,500 carcasses per hour.

In both abattoirs, broilers were first stunned with gas, then hung up by their legs, exsanguinated, and scalded in hot water (temperature of 55–56 °C) for approximately 120 s in a double-tank scalder. There was no overflow equipment in the scalders. Following scalding, the feathers were removed by a plucking machine, then evisceration took place using equipment that included vent cutter, opener, and eviscerator (Meyn, Oostzaan, Netherlands). After crop and lung removal, the carcasses were washed under a potable cold-water spray (14–16 °C) for 3 s, before being sent through an air chilling tunnel at 4 °C and air speed of 2–4 m/s. At abattoir A, the carcasses were in the air chilling tunnel for 2 h, whereas in abattoir B the duration was 3 h and 15 min. No water was used on the carcasses during chilling.

At both abattoirs, the carcasses originated from flocks participating in APaC, where faecal samples were collected 4–6 days before slaughter and analyzed using real-time PCR (Lund et al., 2004). As part of the FCI, results of the *Campylobacter* analysis are sent to the abattoir before the flock is slaughtered. All included birds were Hubbard breed and slaughtered at an age of around 46 days. Mean broiler weight was 1.6 kg, and the coefficient of variation (CV%) of carcass weight was 13–15%.

2.2. Sampling

Four sampling points along the slaughter line were selected: 1) before scalding, 2) after plucking (defeathering), 3) after evisceration, and 4) after chilling (Table 1). The sampling was performed on three separate days. At abattoir A (pilot study), 40 samples from two *Campylobacter*-positive flocks were collected, randomly selected from the slaughter line by two persons that sampled from two sampling points each. At abattoir B, 40 samples from *Campylobacter*-positive flocks were collected on day 1 and 40 samples from *Campylobacter*-negative flocks were collected on day 2, in the same way as in abattoir A. Three flocks were slaughtered each day, and sampling was collected from two of them in the middle of the day. Flock sizes were 10,000–25,000 birds. There was no washing and disinfection of the slaughter line between the flocks, only at the end of the day. The day sampling from *Campylobacter*-negative flocks was performed, all flocks were *Campylobacter*-negative.

Neck-skins were excised aseptically using a sterile disposable scalpel or sterile scissors and placed separately into sterile bags (RollBag®, Interscience, France), according to ISO 17604:2015 (ISO, 2015). Each neck-skin was a minimum of 10 g, and 10 neck skins were collected at each sampling point per day, giving a total of 30 neck-skin samples per sampling point and 120 neck-skin samples. All samples were stored at 3–6 °C overnight and analyzed the following day.

In addition, 200 mL of water from the scalding tanks were collected three times per sampling day, one sample at the start and two at the end

Table 1
An overview of neck-skin samples and water samples collected at each abattoir.

Day of sampling	Abattoir	Flock *Campylobacter* status	Scalding water tank [a]	Before scalding	After plucking	After evisceration	After chilling	Analyses
Pilot	A	Positive	3	10	10	10	10	TPC[b], *E. coli*, *Enterobacteriaceae*
1	B	Negative	3	10	10	10	10	TPC[b], *E. coli*, *Enterobacteriaceae*, 16S rRNA gene seq
2	B	Positive	3	10	10	10	10	TPC[b], *E. coli*, *Enterobacteriaceae*, 16 S rRNA gene seq
Total				30	30	30	30	

[a] The water samples were only analyzed for *E. coli* and *Campylobacter*.
[b] TPC – Total Aerobic Plate Count.

of the slaughtering of the trial flocks.

2.3. Microbiological analyses

All samples were analyzed for common process-hygiene indicators; Total Aerobic Plate Count (TPC), *Enterobacteriaceae*, and *E. coli.* A sterile knife was used to excise 10 g of the neck skin, and this was added to 90 mL sterile peptone saline and homogenized for 30 s in a peristaltic blender (Laboratory blender, Stomacher 400, Seward). From this suspension, 10 mL was used for culture-based bacteriological analyses, and approximately 80 mL was used for DNA extraction and subsequent 16S rRNA gene sequencing (see Section 2.4).

Following serial dilutions, one mL of the appropriate dilution was plated onto a 3M Petrifilm™ Select *E. coli* Count Plate (6434, 3M Microbiology, St Paul, MN, USA), an *Enterobacteriaceae* Count Plate (3M 6420), and an Aerobic Count Plate (3M 6400), as described by the manufacturer. The Petrifilms were incubated aerobically at 30 ± 1 °C for 72 ± 3 h, 37 ± 1 °C for 24 ± 2 h, and 42 ± 1 °C for 24 ± 2 h for TPC, *Enterobacteriaceae*, and *E. coli,* respectively, and read according to the manufacturer's instructions.

Quantitative detection of *Campylobacter* from 10 g of neck skins was performed as described in NMKL no 119, 2007. Serial dilutions were plated on mCCDA (Modified Charcoal-Cefoperazone-deoxycholate agar, OXOID Thermo Fisher Scientific) and incubated microaerobically at 41.5 °C for 44 h. After incubation, typical and suspicious colonies were counted, and a selection of colonies was subjected to confirmation using MALDI-TOF (Bruker Daltronics). To be able to quantify numbers <100 cfu per g in the samples collected in abattoir B, one mL was distributed between three mCCDA plates (333 μL on each plate), giving a detection limit of 10 cfu per g.

In order to quantify *E. coli* in scalding water, one mL water was plated directly on 3M Petrifilm™ Select *E. coli* Count Plate and incubated as described above. Quantitative detection of *Campylobacter* in scalding water was conducted in abattoir A samples only, while qualitative detection was done in samples from abattoir B. To enumerate and detect *Campylobacter* in scalding water, five mL of water was filtered and placed on a mCCDA plate and incubated at 41.5 °C for 44 ± 4 h in a microaerophilic atmosphere. For qualitative detection of *Campylobacter* in scalding water, 25 mL water was suspended in 225 mL Bolton broth and incubated at 41.5 °C for 44 ± 4 h in a microaerophilic atmosphere (NMKL no. 119, 2007). After incubation, 10 μL of the enrichment broth was plated onto mCCDA and incubated in a microaerophilic atmosphere as described above. Confirmation of presumptive *Campylobacter* spp. was carried out using MALDI-TOF (Bruker Daltronics).

2.4. 16S rRNA gene sequencing

Of the suspension described in Section 2.3, 80 mL were centrifuged at 10,000×*g* for 20 min at 4 °C in a Multifuge X3R equipped with a Fiberlite F14-6x250LE rotor (Thermo Fisher Scientific). DNA was extracted from the pellet using a Purelink Microbiome DNA Purification Kit (Invitrogen). Each pellet was resuspended in 1.4 mL of S1 buffer and incubated at 37 °C under agitation at 1800 rpm for 10 min in a heating block (Thermomixer Comfort, Eppendorf). The lysate was divided into two equal parts, with 100 μL Lysis Enhancer added to 700 μL, while the other 700 μL retained as a backup. The mixture was incubated at 65 °C with shaking at 1800 rpm for 10 min before the lysate was transferred to a Lysing Matrix E tube (MP Biomedicals). Bead beating was performed in a Precellys Evolution Homogeniser (Bertin Instruments) in 5 cycles of 1 min shaking and 5 min rest. From this step onwards DNA purification performed as described by the manufacturer. The 100 μL of eluate was added 10 μL of 3 M sodium acetate pH 5.2 and 330 μL of 100% ethanol, and the mixture was incubated for 1 h at 20 °C. The eluate was centrifugated for 30 min at 25,000×*g* and 4 °C, and the resulting pellet was washed with 1 mL 70% ethanol and centrifuged at 25,000×*g* and 4 °C for 5 min. The pellet was dissolved in 50 μL IDTE pH 8.0 (Integrated DNA Technologies, IDT).

To determine the bacterial composition at the genus level, the V1–V2 region of the 16S rRNA gene was amplified by PCR and sequenced. PCR was performed using universal primers 16S–27F-YM (5′- AGAGT TTGATYMTGGCTCAG3′) (Frank et al., 2008) and 16S-357R (5′-CTGCTGCCTYCCGTA-3′) (Wilmotte et al., 1993). The forward primer was tailed with a partial Illumina adapter (ACACTCTTTCCCTACACGA CGCTCTTCCGATCT) and the reverse primer was tailed with a partial Illumina adapter (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT). In both oligonucleotides were a spacer inserted between the gene specific sequence and the adapter as described by (de Muinck et al., 2017). The eight different spacers were mixed prior to PCR. An indexing PCR were performed with AATGATACGGCGACCACCGAGATCTACAC <iiiiii>ACACTCTTTCCCTACACGAC and CAAGCAGAAGACGGCATACG AGAT<iiiiii>GTGACTGGAGTTCAGACGTG, where the <iiiiii> is TruSeq Small RNA 6 bp indexes (Illumina). The first PCR was performed with Q5 Hot Start High-Fidelity DNA Polymerase mastermix (New England Biolabs, NEB) and 300 nM primers on a Veriti Thermal Cycler (Thermo Fisher Scientific) with the following PCR program; 30 s at 98 °C before 30 cycles of 98 °C for 15 s and 55 °C for 5 min followed by one cycle of 65 °C for 10 min. The PCR products were purified with AMPure XP SPRI beads (Beckman Coulter Life Sciences) using a bead to sample ratio of 0.9 x.

The indexing PCR was performed with the same mastermix and 100 nM primers with the following PCR program; 30 s at 98 °C before 30 cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 30 s followed by one cycle of 72 °C for 10 min. The PCR products were purified with AMPure XP SPRI beads using a bead to sample ratio of 0.9 x. The purified PCR amplicons were quantified with Qubit dsDNA HS assay (Thermo Fisher Scientific) and with a D1000 TapeStation kit on a 4200 TapeStation System (Agilent). The libraries were pooled in equimolar concentrations to a final pool concentration of 6 nM, based on the TapeStation data. The library pool was denatured and diluted as described by Illumina before loading the 8.5 pM library pool, including 5% phiX on a V3 flowcell and run with 2 x 300 bp chemistry on a MiSeq instrument (Illumina). A cluster density of 990 K/mm^2 was observed.

2.5. Bioinformatic analysis

The demultiplexed sequence dataset was processed using the Dada2

pipeline (Bioconductor version 1.20.0 with R (version 4.1.0). In brief, any reads containing ambiguous bases were removed. Primer sequences were trimmed using Cut-adapt (version 3.4) (Martin, 2011). The paired-end reads were trimmed to a specific length (forward: 240 bp; reverse: 200 bp) with additional settings: truncQ = 2, maxEE = c(2,2), before the error rates of the sequences were determined. Dereplicated reads were used to correct the errors with Dada2 (version 4.0.5) (Callahan et al., 2016), before merging the dereplicated reads of each pair. The resulting contigs were screened and any contigs shorter than 280 bp or longer than 398 bp were removed from the dataset. Chimeric sequences were removed using the command: removeBimeraDenovo with the consensus method applied. The taxonomy was then added to each contig using the SSU rRNA Silva non-redundant (99%) database V138 training set (Quast et al., 2013). The sequence data files, and the metadata were combined into a Phyloseq object (version 1.32.0) (McMurdie & Holmes, 2013).

The Phyloseq object was imported into R-studio (version 1.3.959), and all Amplicon Sequence Variant sequences that were classified as either "Chloroplast" or "Mitochondria" or that were not assigned to the kingdom Bacteria, were removed (Supplementary materials Table S1). All samples were downsampled to 16,324 reads found in the sample with the lowest sequencing effort (sample: 679-34) and the resulting table was used to visualize the diversity among samples and calculate alpha and beta-diversity measures using Phyloseq and tidyverse (version 1.3.1) (Wickham et al., 2019). The results were visualized in R-studio using ggplot2 (version 3.3.5) (Wickham, 2016) with a colour scheme generated by Polychrome (version 1.5.1) (Coombes et al., 2019). The abundance difference between slaughter days for the *Escherichia—-Shigella* genus was tested with the Kruskal-Walli's test as implemented in the R stats package (version 4.0.0), using the scaled abundances for testing.

2.6. Risk categorization of abattoirs

Risk categorization of the two abattoirs was performed according to Cegar et al. (2022): scores of compliances with 1) regulatory criteria (Anon., 2005; 2017) and 2) criteria for three indicator bacteria; TPC (with limits m = 5 log CFU per g, M = 6 log CFU per g), *Enterobacteriaceae* (m = 4, M = 5 log CFU per g) and *E. coli* (m = 3, M = 4 log CFU per g). The scores were 1 for satisfactory results ≤ m, 2 for acceptable results between m and M, and score 3 for unsatisfactory > M. A geometric mean of compliance of the scores from 1) and 2) was calculated and a risk category per abattoir was set (low ≤1.5, medium between 1.5 and 2, high risk >2).

2.7. Statistical analyses of bacterial counts

Count data were transformed to $\log_{10}$ cfu per g. Statistical analyses were conducted in Stata/MP 16.0 (StataCorp, College Station, TX). *Campylobacter* results below the limit of detection were set to 1 cfu per g for neck-skins and 1 cfu per mL for water samples. Descriptive statistics were performed and significant differences between groups were tested by ANOVA. Concordance correlation coefficients were used for testing the closeness of observations for paired results for indicator bacteria and *Campylobacter* counts. The level of significance was set at P ≤ 0.05.

3. Results

3.1. Bacterial loads along the slaughter line

In both abattoirs, the highest TPC means on neck skins were detected at the first sampling point (Fig. 1). TPC means decreased significantly (P < 0.05) along the slaughter lines in both abattoirs (Table 2). For *Enterobacteriaceae* and *E. coli*, the highest means were from sampling

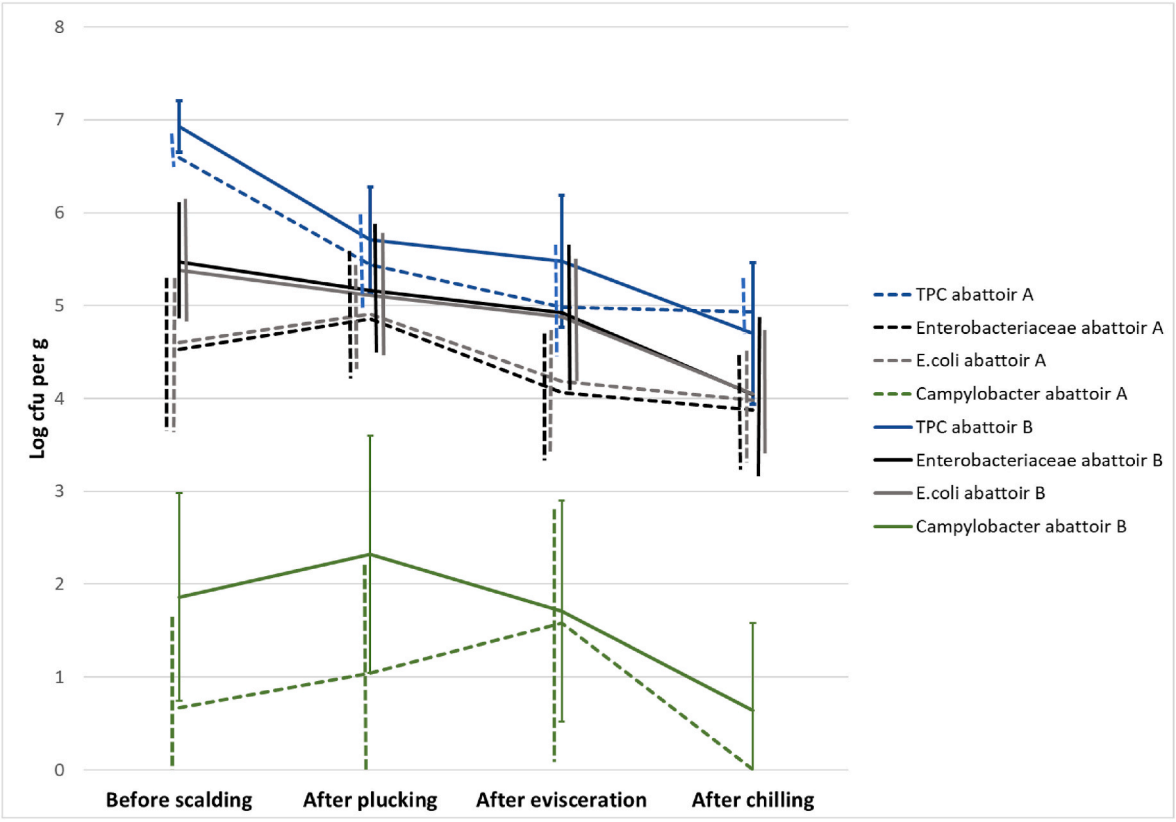

Fig. 1. TPC, *Enterobacteriaceae*, *E. coli* and *Campylobacter* mean log cfu per g from abattoir A (with dotted lines) and abattoir B (solid lines) at four sampling points along the slaughter line. Error bars illustrate ± standard deviation (SD).

point 2 (after plucking) in abattoir A and from sampling point 1 in abattoir B. *Campylobacter* means were low before scalding and increased after scalding and ribbing in both abattoirs, and also after evisceration in abattoir A. The amounts of TPC, *Enterobacteriaceae, E. coli,* and *Campylobacter* were the lowest after chilling ($P < 0.05$, Fig. 1 and Table 2). The stepwise change of indicator bacteria means between each of the four sampling points, showed that TPC had a large decrease during the scalding/ribbing-step and *Enterobacteriaceae* and *E. coli* had a large reduction during washing and chilling (Fig. 2). All samples were above the detection limit for all studied indicator bacteria.

Higher means of TPC, *Enterobacteriaceae*, and *E. coli* was found for *Campylobacter*-negative flocks than for *Campylobacter*-positive flocks at all four sampling points (sampled at different days) (Table 2).

Concordance correlation coefficients (ccc) between *Campylobacter* and *E. coli* were 0.033, −0.023, 0.006, −0.002 at sampling points 1–4, respectively. In the same order, ccc between *Campylobacter* and *Enterobacteriaceae* were 0.031, −0.020, 0.012, and −0.005, and ccc between *Campylobacter* and TPC were 0.008, −0.022, −0.020, and 0.004, at sampling points 1–4, respectively.

3.2. Abattoirs

Comparison of the results between the two abattoirs showed that the mean TPC before scalding was significantly higher in abattoir B (6.9 log cfu per g) compared to abattoir A (6.6 log cfu per g, $P < 0.05$) (Table 2). However, at the remaining sampling points, the results were more similar and after chilling the results did not differ (4.9 and 4.7 log cfu per g, respectively) (Table 2). The same pattern, with higher counts at the start of the slaughter line in abattoir B and then similar levels after chilling, was seen for *Enterobacteriaceae, E. coli,* and *Campylobacter*. Reductions in means from sampling point 1 to 4 along the slaughter lines were larger in abattoir B than in A by 0.6–0.8 cfu per g for TPC, *Enterobacteriaceae* and *E. coli* (Fig. 2).

Table 2
Mean log cfu per g ± standard deviation (SD) for TPC, Enterobacteriaceae, *E. coli* and *Campylobacter* from four sampling points from abattoir A and B, n = 10 per sampling location at abattoir A and n = 20 at abattoir B. Different superscripts in a row indicate significant difference between groups at $P < 0.05$ level by ANOVA.

	Before scalding	After plucking	After evisceration	After chilling
Abattoir A				
TPC	6.59 (0.15) a	5.44 (0.51) b	4.98 (0.56) bc	4.93 (0.31) c
Enterobacteriaceae	4.53 (0.79) ab	4.86 (0.77) a	4.06 (0.76) ab	3.88 (0.59) b
E. coli	4.60 (0.81) ab	4.91 (0.65) a	4.18 (0.71) ab	3.98 (0.57) b
Campylobacter	0.67 (1.09) ab	1.04 (1.36) ab	1.58 (1.49) a	0 b
Abattoir B				
TPC (total) [a]	6.93 (0.27) a	5.71 (0.57) b	5.48 (0.71) b	4.70 (0.76) c
-*Campylobacter*-negative day1	6.99 (0.32)	6.15 (0.33)	6.06 (0.47)	5.11 (0.79)
-*Campylobacter*-positive day2	6.89 (0.23)	5.28 (0.41)	4.92 (0.32)	4.29 (0.47)
Enterobacteriaceae	5.47 (0.69) a	5.16 (0.68) a	4.92 (0.85) a	4.04 (0.88) b
-*Campylobacter*-negative day1	5.89 (0.62)	5.58 (0.47)	5.56 (0.58)	4.68 (0.78)
-*Campylobacter*-positive day2	5.06 (0.48)	4.75 (0.62)	4.30 (0.57)	3.40 (0.34)
E. coli	5.38 (0.73) a	5.11 (0.70) a	4.88 (0.98) a	4.05 (0.86) b
-*Campylobacter*-negative day1	5.82 (0.75)	5.66 (0.52)	5.53 (0.59)	4.68 (0.71)
-*Campylobacter*-positive day2	4.96 (0.42)	4.66 (0.57)	4.24 (0.64)	3.43 (0.43)
Campylobacter[b]	1.86 (1.12) ab	2.32 (1.28) a	1.71 (1.19) ab	0.64 (0.94) b

[a] Mean in abattoir B for both *Campylobacter*-positive and –negative flocks.
[b] Analyzed for *Campylobacter* for only *Campylobacter*-positive flocks.

3.3. Scalding water

Scalding water samples (n = 9) were analyzed for *E. coli* and *Campylobacter*. *Campylobacter* was not detected. One water sample had high levels of *E. coli* (>400 cfu per mL) while remaining samples were below 15 cfu per mL.

3.4. 16S rRNA microbiome composition (abattoir B only)

The demultiplexed sequence data was archived at SRA with project accession: PRJNA873297.

The final Illumina 16s rRNA V1–V2 dataset contained 2,767,195 reads with an average of 34,590 reads (±9168) per sample. A total of 2636 Amplicon Sequence Variants (ASV) were found. The samples were dominated by the phyla Firmicutes, Proteobacteria, Actinobacteria and Bacteriodota, with different abundances of these genera according to sampling point and days of slaughter (Supplementary Materials Fig. S1). At the genus level, the communities changed between sampling points (Fig. 3). The relative abundance of taxa with at least 10% abundance in the 16s rRNA community profiles are illustrated in Fig. 3.

Before scalding, the bacterial communities were dominated by *Staphylococcus* spp. (26.22%), *Escherichia-Shigella* spp. (16.11%) and *Streptococcus* spp. (8.86%) (Table 3). *Escherichia-Shigella* spp. had the highest abundances in samples taken after plucking (39.05%), but this dropped to 7.22% after chilling. In contrast, *Asinibacterium* spp. and *Afipia* spp. increased along the slaughter line, from almost 0%–26.05% and 9.43%, respectively, after chilling (Fig. 3A; Table 3). There were also differences in *Escherichia-Shigella* abundances between the two slaughter days, where only *Campylobacter*-negative flocks were sampled on day 1 and only *Campylobacter*-positive flocks were sampled on day 2 (Kruskal-Wallis' test $p < 0.001$). *Escherichia-Shigella* abundances were higher in the *Campylobacter*-negative broilers (day 1) at all sampling points, except after plucking (sampling point 2) where there was no significant difference in the abundance between the days ($p = 0.82$). *Campylobacter* observed in 16S rRNA reads was at relative low levels (<10% and therefore not included in Fig. 3) at the first three sampling sites, and not detected at all after chilling. All *Campylobacter* colonies were identified as *C. jejuni* by MALDI-TOF.

The bacterial diversity declined along the slaughter line for samples collected on day 2 (Shannon diversity (SD) index sampling point 1; 4.5, SD index 2.8 sampling point 4, Supplementary Materials Fig. S2), while a moderate decrease was seen for samples collected on day 1 (Fig. S2). The bacterial diversity differed between days only at the first sampling point before scalding (Fig. S2), otherwise the samples were of similar high/low diversity at the other sampling points.

A pair-wise comparison of the community composition using PCoA with Bray-Curtis' dissimilarity distances showed that the neck-skin communities differed between the four sampling points, as well as between the two days, but become more similar at sampling point 3 and 4 the results (Fig. 3B). The communities from both days showed a similar change in community composition for each of the sampling points, with the intermediate points showing the most variable community compositions.

3.5. Risk categorization of abattoirs

Risk categorization of the two abattoirs showed that all samples from chilled carcasses in both abattoirs were below the limit of 3.0 log cfu per g for *Campylobacter*. The samples were not analyzed for *Salmonella* in this study, but the abattoirs performed routine microbiological testing, and *Salmonella* was not detected. Thus, the two abattoirs were

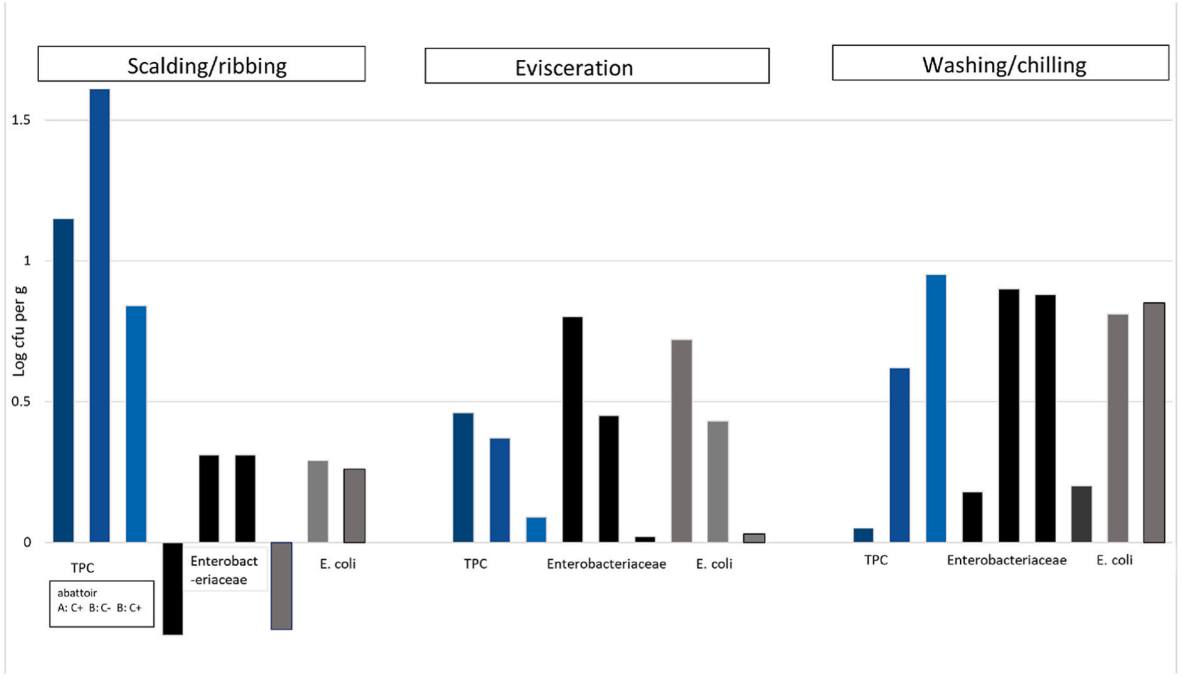

Fig. 2. Stepwise reduction between sampling point 1 and 2, 2 and 3, 3 and 4 in means of TPC (blue bars), *Enterobacteriaceae* (black bars) and *E. coli* (grey bars). For each parameter, the order of the bars is abattoir A (only *Campylobacter*-positive birds) to the left, abattoir B (*Campylobacter*-negative birds day1) in the middle, and abattoir B (*Campylobacter*-positive birds day2) to the right.

categorized as satisfactory (score 1). For all three indicator bacteria, abattoir A were categorized as score 1, and abattoir B was score 1 for TPC and *Enterobacteriaceae* and score 2 for *E. coli*. The geometric mean was 1.0 for abattoir A and 1.33 for abattoir B. Accordingly, both abattoirs were low-risk abattoirs (geometric mean of score $\leq$1.5).

4. Discussion

Our study showed that the initial bacterial loads on broiler neck-skin samples taken before scalding to after chilling along the two slaughter lines, were significantly reduced by 2.1, 1.1, 1.1, and 1.0 log cfu per g for TPC, *Enterobacteriaceae, E. coli,* and *Campylobacter*, respectively. The lowest levels found at the four selected sampling points, were after chilling for all four analytes: TPC, *Enterobacteriaceae*, *E. coli* and *Campylobacter*. Whereas TPC was mainly reduced during scalding and plucking (between sampling sites 1 and 2), the main reductions in *Enterobacteriaceae*, *E. coli,* and *Campylobacter* occurred during cold water-washing and chilling (between sampling sites 3 and 4), especially at abattoir B. These declining contamination trends along the slaughter lines were confirmed by the 16S rRNA gene analyses for samples from abattoir B, by the high abundance of *Escherichia-Shigella* spp. before scalding, especially at day 1 for *Campylobacter*-negative birds, and dropped to a low abundance after chilling.

A study by Althaus et al. (2017) commented that the literature often shows common trends, with reductions in contamination from scalding, increases from plucking, no change or increases from evisceration, and reductions from washing and chilling (Berghaus et al., 2013; Duffy et al., 2014; Guerin et al., 2010; Huang et al., 2017; Projahn et al., 2018; Seliwiorstow et al., 2015). Direct study comparisons are often difficult because the effect of a particular process stage strongly depends on various conditions, such as temperature and time used for scalding, types of washing steps, and variations in chilling methods. Results may be reported in different units, mainly log per ml, log per cm^2, or log per g, according to sampling method used and this hampers comparisons. In our study, count data were transformed to log cfu per cm^2, according to the conversion factor provided by Nagel Gravning et al. (2021); the mean TPC of our study was 6.3 log per cm^2 before scalding and 4.2 log per cm^2 after chilling. Another observation was that many studies report the levels of *E. coli* and *Enterobacteriaceae* of broiler carcass surfaces as being very similar (Althaus et al., 2017; Buess et al., 2019), whereas in beef and pig carcasses, levels of *E. coli* are usually 0.4–0.6 log cfu per cm^2 lower than *Enterobacteriaceae* analyzed from the same samples (Barco et al., 2017).

Process hygiene differs from operation to operation, throughout the slaughter line. The scalding process in warm water loosens the feathers from the skin prior to the defeathering process in the plucking machine. In Europe, "soft scalding" at 51–54 °C for 120–210 s is typically used, and "hard scalding" at temperatures higher than 60 °C for 45–90 s is often used in USA (Projahn et al., 2018). Scalding is reported to reduce the bacterial loads significantly, by 1.0–1.5 log per g, especially when the water temperature is higher than 55 °C (ICMS, 1998). There is a contamination risk during defeathering due to the high pressure of the plucking fingers that may result in faecal leakage (Allen et al., 2003). Another risk is that *Campylobacter* hidden in feather follicles are spread onto the skin during plucking, and studies have found an increase of *Campylobacter* by 0.4 log CFU per g after plucking (Althaus et al., 2017). Also, Zhang et al. (2020) claimed that follicle cavities were filled with fluid, feather fragments, and dirt that cause bacterial cross-contamination in the feather follicle cavity. Our results were in accordance with findings in literature, where there was a high reduction in TPC and a moderate increase of *Campylobacter* from before scalding until after plucking. Evisceration is a challenging process, especially when carcass sizes are uneven, as the process is automated and cannot be adjusted for each carcass. Thus, damaged intestines are regarded as a risk for contamination of the carcass surfaces (Keener et al., 2004). Nevertheless, Zweifel et al. (2015) found that eviscerating operations were performed without extensive additional contamination, contrary to other studies (Abu-Ruwaida et al., 1994; Berrang & Dickens, 2000). Cold spray washing inside and outside the carcasses for 5–6 s can reduce contamination slightly (Oyarzabal et al., 2004), but may also spread contamination from a restricted spot to a larger area (Loretz et al., 2010). There are many methods for chilling carcasses, such as air chilling, with or without additional water spray and different air speed, and chilling in water immersion with or without added chemicals and

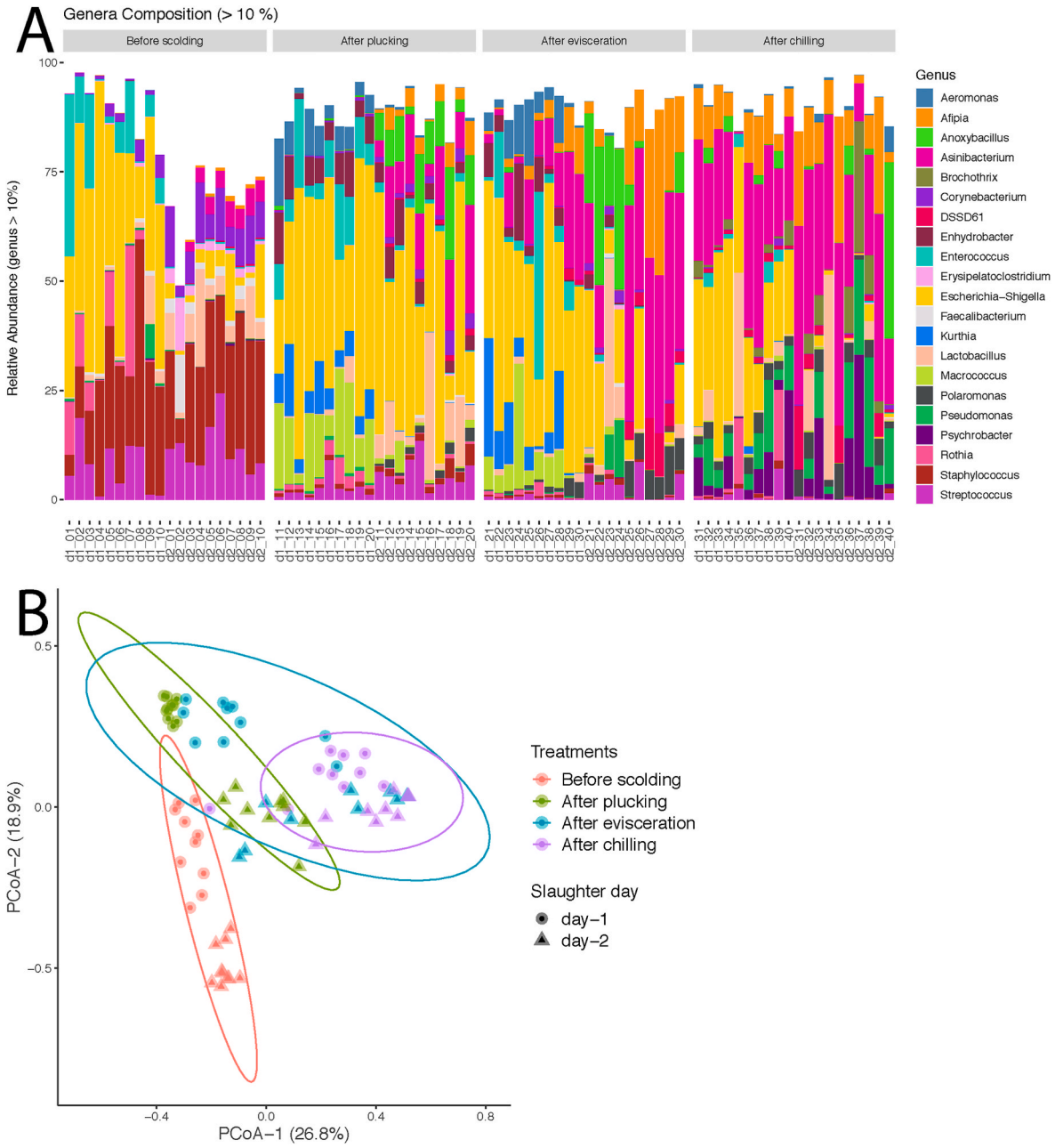

Fig. 3. Bacterial 16s rRNA diversity of poultry neck-skin communities at four steps along the slaughter line in abattoir B. . A) Relative abundance 16s rRNA community profiles including taxa constituting at least 10% abundance in a single sample. Read abundances were normalized to the smallest read number before calculating relative abundances. The samples are indicated on the x-axis, with d1 or d2, to differentiate between the slaughter days (d2 samples were from *Campylobacter*-positive flocks). B) Principal coordinate analysis of bacterial neck-skin community variation at the genus level. The distances between samples are based on Bray-Curtis' dissimilarity. The variation explained by the PCoA-1 and PCoA-2 axis is indicated in percentages on the axis labels. Samples are coloured according to the sampling point along the slaughter line. Dots and triangles represent d1 and d2, respectively.

ice (Chen et al., 2020). Some studies have observed larger reductions in contamination with water immersion chilling than with air chilling (Corry et al., 2007), but also increases (Duffy et al., 2014; Pacholewicz et al., 2015). In our study, the carcass temperature was reduced to 4 °C during air chilling, and water was not added to the carcasses. Thus, the carcass surfaces were dried to some extent. EU legislation does not specify duration requirements for chilling, only that the maximum final temperature should be 4 °C. At abattoir A, the chilling lasted for 2 h, whereas at abattoir B, the duration was 3 h and 15 min. Factors such as air flow rate, humidity, cleaning etc, may be reasons for larger reduction in bacterial counts, but this was not investigated. In total, the reductions in indicator bacteria counts (Fig. 2) from the first to last sampling points along the slaughter lines were larger in abattoir B than in A.

There were only four sampling points along the slaughter lines, and thus, not enough to point out which operation in the carousel system was the most effective in reducing contamination on neck skins, for instance whether scalding and ribbing both reduced the bacteria level or one reduced and one increased the bacteria level. Ideally, the sampling should have been performed between every carousel, to perform bio-mapping; a systematic analysis to measure the microbial recovery pre- and postintervention comprising the whole system (Handley et al., 2018). Larger group sizes and more balanced data between abattoirs would also have improved the study. The study design had limitations due to one-day sampling on separate days, and for risk characterization

Table 3
Median relative abundances for the 21 most-abundant taxa for the sampling points along the slaughter line. The relative abundances (%) are based on scaled counts. The row "Other genera" includes all the remaining identified taxa, including genera such as *Campylobacter*, *Clostridium*, etc.

Genus	Before scalding	After plucking	After evisceration	After chilling
Aeromonas	0.00	0.94	0.57	0.23
Afipia	0.04	0.21	3.52	9.43
Anoxybacillus	0.00	1.39	0.19	0.16
Asinibacterium	0.07	1.39	14.00	26.05
Brochothrix	0.00	0.00	0.00	1.98
Corynebacterium	4.74	0.91	0.27	0.11
DSSD61	0.00	0.06	0.52	0.88
Enhydrobacter	0.00	4.76	1.28	0.05
Enterococcus	0.40	2.41	0.64	0.11
Erysipelatoclostridium	0.76	0.00	0.00	0.00
Escherichia-Shigella	16.11	39.05	25.34	7.22
Faecalibacterium	1.02	0.02	0.00	0.00
Kurthia	0.00	1.71	0.08	0.00
Lactobacillus	2.65	1.28	0.73	0.58
Macrococcus	0.03	3.46	0.62	0.18
Polaromonas	0.01	0.08	1.25	3.76
Pseudomonas	0.15	0.10	0.09	4.89
Psychrobacter	0.04	0.00	0.00	3.68
Rothia	0.15	0.63	0.26	0.06
Staphylococcus	26.22	1.21	0.48	0.11
Streptococcus	8.86	3.18	0.51	0.21
Other genera	22.31	10.44	9.61	10.11

of abattoirs, microbiological results on basis of multiple sampling days in different periods of the year would have improved the evaluation. Also, *Campylobacter*-positive and -negative flocks were sampled different days, and comparison between those two groups will therefore be affected by day-to-day variations in process hygiene (Nagel Gravning et al., 2021).

EFSA listed 13 biological hazards for public health concerning poultry meat in a scientific opinion in 2012, and among these were *Campylobacter* spp., *Salmonella* spp., VTEC, ESBL-Amp C *E. coli*, *B. cereus*, *C. perfringens* (EFSA, 2012). EFSA claimed that a farm-to-fork approach is needed to control those 13 pathogens that pose a threat to public health. This approach includes primary interventions at poultry farms, implementation of effective control measures at abattoirs, and consumer awareness (Nastasijević, Vesković, & Milijašević, 2020; Pessoa et al., 2021). For abattoirs, identifying factors that can reduce the bacterial loads are essential. Also, bio-mapping of bacterial communities on carcasses in their own slaughter line give more detailed information about which operations and exposures are reducing different types of bacteria. We observed that *Campylobacter*-negative birds had higher counts of TPC, *Enterobacteriaceae*, and *E. coli* than *Campylobacter*-positive birds, maybe caused by a change in the microbiota in the intestines of the chickens when infected by *Campylobacter* as described by Awad et al. (2016).

Campylobacter and *Salmonella* on chilled carcasses are set as the criteria for assessing poultry process hygiene in EU legislation (Anon, 2005, 2017). A suggestion, based on our results, is that *E. coli* or *Enterobacteriaceae* are suited as process hygiene criteria, as these bacteria are indicators of faecal contamination and are always present in the process. Faecal contamination is essential for broiler process hygiene and the levels can provide useful information to the abattoir managers about need for improvements of slaughter hygiene. Our results indicate that *E. coli* and *Enterobacteriaceae* both reflect the effectiveness of different measures and level of carcass contamination. However, the correlations between *Campylobacter* and all studied indicator bacteria were low, close to 0 (range from −0.02 to 0.03) for chilled carcasses, and thus, less suitable to reflect the level of *Campylobacter*. Boysen et al. (2016) found that *E. coli* was not suitable for indicator for *Campylobacter*, the opposite to the results from Duffy et al. (2014) and Roccato et al. (2018).

Our results showed that bacterial communities of poultry at two different days can be highly divergent when they arrive at the abattoir (Fig. 3). Alpha diversity indices, such as the Shannon diversity index, which describe the species richness and evenness in a sample, declined along the slaughter line on day 2 for the *Campylobacter*-positive birds. *Escherichia-Shigella* was the dominating genera at the three first sampling stages but was significantly reduced after chilling. Gram-negative bacteria, like *E. coli* and *Campylobacter*, are more heat and cold sensitive than Gram-positive bacteria (Jay, 2005). Wang et al. (2019) found that *Escherichia-Shigella* and *Streptococcus* were present on broiler carcasses in high abundances after defeathering but decreased after washing and chilling. In our study, *Asinibacterium* spp. and *Afipia* spp. dominated on chilled neck skins, among other slow growing bacterial genera such as *Pseudomonas, Polaromonas, and Psychrobacter*. Other studies have also found spoilage bacteria, possibly originated from feather, feet, water supply in abattoir, chill tank, and equipment, and to less extent from intestines, as dominating on chilled carcasses (Chen et al., 2020; Heir et al., 2022; Holck et al., 2014; Rouger et al., 2017; Wang et al., 2016). The bacterial communities on chilled carcasses and storage conditions affect shelf lives of the meat products and meat safety.

Risk-categorization of abattoirs has not yet been standardized. One suggested method is to categorize abattoirs based on compliance with the limits set in microbiological criteria for process hygiene (Anon, 2005, 2017) for *Salmonella* and *Campylobacter* and compliance with limits for indicator bacteria set by Cegar et al. (2022). In our study, the two abattoirs were categorized as low-risk abattoirs. This result reflected the authors' impression of good slaughter hygiene and high technical status in the two abattoirs. In addition to the slaughter hygiene, bacterial status on-farm is also important for meat safety. Flocks that were infected by *Campylobacter* on-farm, had *Campylobacter* on chilled carcasses, however at low levels. Cegar et al. (2022) commented that the level of indicators on chilled carcasses was mainly related to abattoir process hygiene, while the presence of pathogens was also affected by their farm status. Both pathogenic and indicator bacteria should be included in the categorization criteria. A suggestion for improving the categorization criteria is to base the microbiological result on several sampling days in different periods of the year. Another suggestion for improvement of this method, is to include the percentage of log-reduction along the slaughter lines in the criteria. Limits for satisfactory, acceptable, and unacceptable categories must be set on basis of assessment of results in different abattoirs. In our study, the reductions from before scalding to after chilling were 25% and 32% for TPC, 14% and 26% for *Enterobacteriaceae,* and 13% and 25% for *E. coli* in abattoir A and B, respectively. Other processing factors that affect the effectiveness of different measures, such as time/temperature/rate for scalding and chilling etc. may also be included in the categorization criteria. Further investigation for identifying criteria in categorization criteria for risk-based meat safety assurance systems is needed.

5. Conclusion

Reductions in counts of TPC, *Enterobacteriaceae*, *E. coli,* and *Campylobacter* were found in neck-skins on broiler carcasses during slaughtering at four selected stages in two Norwegian abattoirs. The last step, which included washing and chilling, resulted in the largest reduction. Identifying those factors that increases and decreases levels of *Campylobacter* and other bacteria during processing should enable the implementation of control measures and thus improve meat safety.

CRediT authorship contribution statement

Sigrun J. Hauge: Conceptualization, Methodology, Software, Writing – review & editing. **Gro S. Johannessen:** Formal analysis, Writing – review & editing. **Thomas H.A. Haverkamp:** Bioinformatic analysis, Writing – review & editing. **Solfrid Bjørkøy:** Molecular Microbiology, Writing – review & editing. **Ann Katrin Llarena:** Writing

– review & editing. **Bjørn Spilsberg:** Conceptualization, Sampling, Writing – review & editing. **Magnus Leithaug:** Molecular Microbiology. **Marianne Økland:** Formal analysis, Writing – review & editing. **Janne Holthe:** Writing – review & editing. **Ole-Johan Røtterud:** Conceptualization. **Ole Alvseike:** Conceptualization, Writing – review & editing. **Gunvor Elise Nagel-Alne:** Conceptualization, Methodology, Software, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgement

The study was mainly financed by the Research Council of Norway, grant no 296327.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109526.

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