



## Microbiota encompassing putative spoilage bacteria in retail packaged broiler meat and commercial broiler abattoir

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### ABSTRACT

It is well established, that certain bacteria within the *Brochothrix*, *Carnobacterium*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* genera have an important role in the spoilage of chill stored poultry meat packaged in modified atmosphere. However, little is known about the role of microorganisms that are difficult to culture and the microbiota during poultry spoilage. We combined traditional cultivation and culture-independent 16S rRNA amplicon sequencing to investigate the microbiota encompassing putative bacteria of whole broiler meat, packaged in modified atmosphere, during and exceeding shelf-life. Samples were taken from 6 flocks during independent slaughter days. Additional samples were analysed from the production line. There was a significant difference in the microbial community structure of 80%O<sub>2</sub>/20%CO<sub>2</sub> retail packaged broiler meat during different times of shelf-life, mainly due to an increase of species within the *Brochothrix*, *Carnobacterium*, *Vagococcus*, and *Janthinobacterium* genera. These genera were already detected four to eight days after slaughter. However, no significant difference between flocks with respect to the microbiota encompassing putative spoilage bacteria was observed when examined in retail packaged broilers, slaughtered at the same abattoir on different days. Our study also showed that lactic acid bacteria within the *Vagococcus* genus can constitute a dominating part of the later shelf-life microbiota in fresh whole broiler meat packaged in 80%O<sub>2</sub>/20%CO<sub>2</sub> modified atmosphere. A single operational taxonomic unit (OTU) assigned as *Janthinobacterium lividum*, an occasional spoiler of meat products, was identified as a major part of the microbiota in late shelf life broiler meat and swab samples in the cooling facility at the slaughter house production line. The combination of traditional cultivation and culture-independent methods provided a great insight into the microbiota of broiler meat during shelf-life and identified a potential point of contamination in the production line for cold tolerant *Janthinobacterium*.

### 1. Introduction

Microbial spoilage is a key factor for the limitations in the shelf-life of fresh poultry meat. The shelf-life of retail poultry meat is short, normally up to around 10 days (Jimenez et al., 1997). However, some producers have reported an acceptable shelf-life of more than two weeks for their retail packaged broiler meat. Important parameters when ensuring long shelf-life are production hygiene, storage temperature and packaging method (Doulgeraki et al., 2012). High CO<sub>2</sub> modified atmosphere packaging (MAP) probably inhibits growth of strictly aerobic microorganisms and prolongs the shelf-life of fresh meats. However, some lactic acid bacteria and *Brochothrix*

*thermosphacta* can grow in high CO<sub>2</sub> MAP, and they can spoil meat products by formation of sour smells and sometimes slime (Doulgeraki et al., 2012; Nieminen et al., 2015; Rahkila et al., 2012). Studies on meat spoilage have focused on cultivable microorganisms and have identified Gram negatives such as *Pseudomonas* spp. and *Acinetobacter* spp., together with *Flavobacterium* spp. and some Enterobacteriaceae in aerobically packaged meat (Daud et al., 1979; Doulgeraki et al., 2012; Jimenez et al., 1997; Liang et al., 2012; Sade et al., 2013). In MAP, the predominant microorganisms involved in spoilage of poultry meat have been identified as the Gram positive *Brochothrix thermosphacta* and lactic acid bacteria within the *Carnobacterium*, *Lactococcus*, *Lactobacillus* and *Leuconostoc* genera (Barakat et al., 2000; Jimenez et al., 1997;

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Koort et al., 2005; Sade et al., 2013). Many microorganisms are not easily cultivable and it remains unknown what their role is in poultry meat spoilage, even though they might influence spoilage directly or indirectly. Recent studies of seafood and meat by culture-independent methods have provided a greater insight into their microbiota (Chaillou et al., 2015; Nieminen et al., 2012; Nieminen et al., 2015; Powell and Tamplin, 2012; Rouger et al., 2018). In the present study we aim to determine the microbiota of poultry meat during and exceeding shelf-life, by culture-independent 16S rRNA amplicon sequencing.

We considered two hypotheses to explain the composition of microbiota on fresh whole broiler meat. Hypothesis one being that the microbiota is diverse between retail meat from different broiler flocks, slaughtered and packaged at the same abattoir on different days of slaughter. The idea being that the live broilers carry different initial microbiota, from the farms into the slaughter line, in which case we would investigate, which potential poultry meat spoilers different flocks carry into the production line and what influence it has on the spoilage microbiota during and exceeding shelf-life. The alternative hypothesis is that the microbiota composition is not different between retail meat from different broiler flocks, when slaughtered and packaged at the same abattoir, and under these circumstances we should be able to identify a core microbiota encompassing putative spoilage bacteria and identify critical points in the slaughterhouse production line for important poultry meat spoilers.

To test both hypotheses we collected samples of whole retail packaged broiler meat from different flocks in a single poultry abattoir on different days in which we compared the composition of the microbiota during and exceeding shelf-life by 16S rRNA amplicon sequencing. As the microbiota encompassing putative spoilage bacteria was quite similar between flocks, slaughtered and packaged under similar circumstances over a four month period, we defined a core microbiota encompassing putative spoilage bacteria and tried to identify potential contamination points in the slaughterhouse production line.

## 2. Material and methods

### 2.1.1. Retail sampling

Samples of whole broiler meat from five broiler flocks, six per flock, retail packaged in 80%O<sub>2</sub>/20%CO<sub>2</sub> were collected at five different days of slaughter at a commercial Danish poultry slaughterhouse between February and May 2015. All flocks originated from different primary production farms. The packages were kept cool (5–8 °C) and transported to the laboratory as quickly as possible (6–9 h). At arrival, samples were stored at 6 °C until further sampling. The storage temperature was chosen to represent storage conditions at retail distribution. Broilers were sampled either 0, 4, 8, 10, 12 and 16 days after slaughter, respectively. Six whole retail packaged broilers were sampled from a single flock of a Norwegian slaughterhouse, packaged in 50%N<sub>2</sub>/50%CO<sub>2</sub>. Norwegian broiler carcasses were transported to the laboratory at 5–8 °C as quickly as possible (16 h) to be analysed in Denmark and broilers were sampled either 1, 4, 8, 10, 12 and 16 days after slaughter, respectively.

### 2.1.2. Production line sampling

Four broiler carcasses were collected during the slaughter of the second flock slaughtered on the third sampling day. Broiler carcasses were taken immediately after scalding at 55 °C, directly after feather plucking, immediately after evisceration, and in the middle of the cooling chain. Carcasses were kept cool (5–8 °C) and returned as quickly as possible to the laboratory (12–15 h) for further processing (see Sections 2.3, 2.4).

Swab samples were taken from the production line during the slaughter of the sampled retail broiler flock on the third sampling day at the Danish abattoir, at scalding, feather plucking, evisceration of

intestinal organs, and in the cooling chain. Two additional swab samples were taken in the cooling condenser at sampling day 1 and 4. The 55 °C scalding tub was sampled by dipping sterile tampons (Easy, Dansk Supermarked, Denmark) into the tub for 1 min and thereafter placed in a 50 mL sterile plastic tube. The rubber plucking fingers were sampled by thoroughly rubbing with a sterile cloth soaked with 0.1% peptone solution (A04121A, Food Diagnostics, Denmark) and returned to the sterile resealable packaging. The eviscerator knife was sampled by stopping the machine for 5 min and thoroughly swabbing knives with a cotton swab soaked in maximum recovery diluent (MRD) (MW7969, MWE, England). In the cooling chain, an area of approximately 10 × 10 cm on the grid of the central cooling condenser, was thoroughly swabbed using a cotton swab soaked in MRD (MW7969, MWE, England). Samples were kept cool (5–8 °C) and returned as quickly as possible to the laboratory (12–15 h) for further processing (see Sections 2.3, 2.4).

### 2.2. Sensory evaluation

Organoleptic changes of whole raw broilers were evaluated by a trained person 10 min after opening of the retail packaged broilers. The slime formation, odour, colour, and overall acceptability was evaluated and scored. The slime formation was scored as 1 = No slime, 2 = A little slime, but still acceptable, or 3 = Clear slime layer, unacceptable. Odour was scored as 1 = Fresh, 2 = Some off-odour, but still acceptable, 3 = Clear off-odour, unacceptable, or 4 = Clearly deviating off-odour, unacceptable, and it was noted if the odour could be described as sour, sulphurous, or putrid. Colour, such as browning or whitening of the meat, was scored as 1 = No discolouration, 2 = Starting discolouration, acceptable, 3 = Clear discolouration, unacceptable, or 4 = Clearly deviating colour, unacceptable. The overall acceptability for consumption was then evaluated and scored acceptable/not acceptable.

### 2.3. Cultivation based methods

Carcasses and whole broiler meat samples were rinsed in 500 mL sterile saline water (0.9%) and shaken thoroughly for 1 min. Swab sample replicates were pooled into one sample for each sampling point and flock. Sterile saline water (0.9%) was added to the scalding tampon samples in a 1:1 w/v scale. Cloth samples from the plucking fingers were weighed and sterile saline water added in 1:9 w/v. Each of the cotton swabs was in 10 mL maximum recovery dilutant. The rinse water from each retail broiler, carcass and swab samples, were diluted to a countable range in saline (0.9%) peptone buffered (0.1%) water, and total viable psychrotrophic, and for retail broiler also selective counts, were plated in duplicates for each sample. One ml of appropriate dilutions in plate count agar pour plates (PCA) (CM0325B, Oxoid, Thermo Fisher Scientific Inc., USA) were incubated at 6.5 °C for 10 days. The deep seed in the pour plates will include anaerobic conditions. Selective media were seeded with 100 µL of the appropriate dilutions. MacConkey agar (MC) (105,465, Merck Millipore, Germany) was incubated for 10 days at 6.5 °C. This medium is indicative of Gram negative enteric bacilli. Enrichment for lactic acid bacteria was performed on de Man, Rogosa and Sharpe agar (MRS) (CM0361, Oxoid, Thermo Fisher Scientific Inc., USA) incubated at 20 °C for 3 days in normal atmosphere. Streptomycin thallos acidione agar (STAA) (CM0881B, Oxoid, Thermo Fisher Scientific Inc., USA) with supplement (SR0151E, Oxoid, Thermo Fisher Scientific Inc., USA) with incubation at 20 °C for 3 days was used for indicative enrichment of *Brochothrix thermosphacta*.

Viable counts of whole retail broilers samples were converted to CFU/cm<sup>2</sup>, by using the relationship between carcass weight and surface area (Thomas, 1978).

## 2.4. Microbial community structure

### 2.4.1. DNA extraction

DNA was extracted from 45 samples (Supplementary Table 1) which included rinse water samples from 35 retail chicken, four carcasses and six swab samples. DNA was extracted with the PowerLyzer PowerSoil DNA isolation kit (Mobio, USA) following the manufacturers protocol, except using the pellet from spun down rinse water, beat beading for 10 min using a vortex adaptor (Mobio, USA), and final elution in nuclease free water (Thermo Fisher Scientific Inc., USA). Extracted DNA was stored at  $-20^{\circ}\text{C}$  throughout storage for a maximum of two months prior to PCR amplification.

### 2.4.2. 16S rRNA amplicon sequencing

Microbial community structure was investigated by 16S rRNA amplicon sequencing of total extracted DNA. Samples were sequenced on an Illumina MiSeq (Caporaso et al., 2012) at DNAsense (Aalborg, Denmark), using primers targeting the V1-3 variable region (27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 534R 5'-ATTACCGCGGCTGC TGG-3') on the 16S rRNA gene (Ward et al., 2012). The negative control was without DNA template and showed  $< 0.5\text{ ng}/\mu\text{L}$  measured by Qubit 2.0.

### 2.4.3. Microbiota data analysis

De-multiplexed sequences were received as paired-end reads ( $2 \times 301\text{ bp}$ ) in Fastq format. Sequence data were analysed in Ubuntu terminal using the UPARSE Utax workflow (Edgar, 2013), merging raw reads to a length of minimum 425 bp and maximum 525 bp (maxee 3.0), dereplicating reads to filter away sequences only seen once in the whole dataset, mapping reads of minimum 97% identity (maxaccepts 8, maxrejects 64) into operational taxonomic units (OTU) and in the process also removing chimeras. OTUs were classified in the MiDAS database, a curated version of the SILVA database (McIlroy et al., 2015), using an Utax cut off value of 0.5. Rarefaction, exploratory analysis and visualisation of data, was performed in Rstudio (RStudioTeam, 2015) using the Ampvis package (Albertsen et al., 2015). To investigate relationships between flocks, time after slaughter, and the given microbiota on OTU level, multivariate statistics using the “output = complete” function in Ampvis was used to get the  $p$ -value of correlation between the given variables and microbial community structure. To resolve the classified OTUs obtained by 16S rRNA amplicon sequencing down to species level, sequences of dominating OTUs during shelf-life, were analysed in the EzTaxon-e 16S rRNA gene database (Kim et al., 2012).

## 3. Results

### 3.1. Acceptable shelf-life

Organoleptic acceptable shelf-life varied between 8 and 12 days for the Danish broiler flocks (Fig. 1 and Supplementary Fig. 1). The acceptable organoleptic shelf life for Norwegian broilers was above 12 days. The main reason for rejection was sour smell formation for all six flocks, while additional unacceptable colour change and slime formation spoiled the product after 12–16 days. When the broilers were evaluated as spoiled, the total viable counts had reached around  $10^7$ – $10^8\text{ CFU}/\text{cm}^2$ , a 5–6  $\log_{10}$  increase from day 0 (Fig. 2). Colony counts on MRS, STAA, and MC agars were around  $10^6$ – $10^7\text{ CFU}/\text{cm}^2$  when products were evaluated as spoiled, indicating a high number of lactic acid bacteria, presumptive *Brochothrix*, and presumptive Gram negative enteric bacteria on the spoiled broiler meat (Supplementary Fig. 2).

The Norwegian broilers generally had lower microbial counts than the Danish broilers, only reaching a total viable count  $> 10^7\text{ CFU}/\text{cm}^2$  16 days after slaughter, and also had a longer organoleptic acceptable shelf-life (Fig. 2, Supplementary Fig. 2).

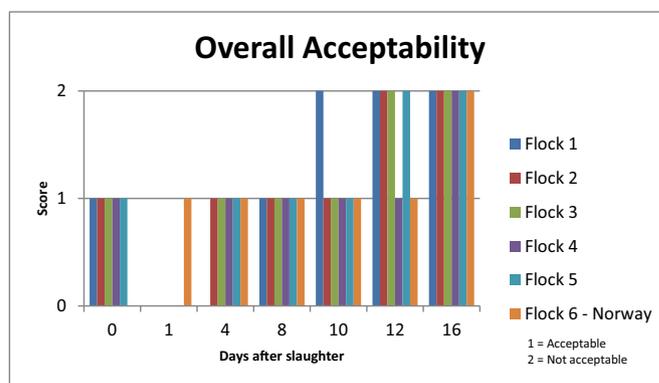


Fig. 1. Overall acceptability (organoleptic shelf life) of retail broiler meat samples. Score 1 = Acceptable, score 2 = not acceptable. Danish broiler flocks were not sampled on day 1, while the Norwegian broiler flock was not sampled on day 0.

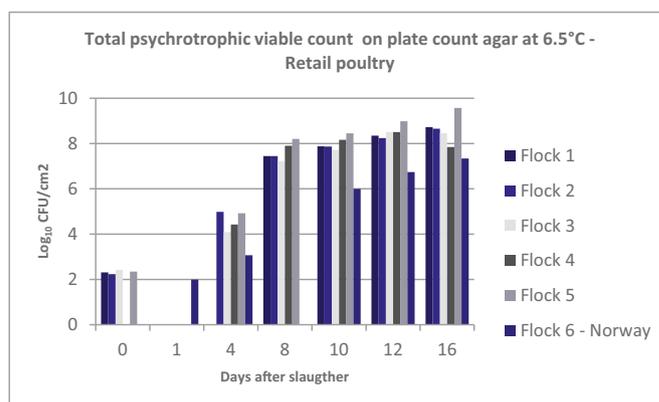


Fig. 2. Total psychrotrophic viable count ( $\log_{10}\text{ CFU}/\text{cm}^2$ ) on retail broiler meat 0–16 days after slaughter. The sample from flock 1 day 4 was not collected. Samples on PCA from flock 4 day 0 (4–0) and flock 6 day 8 (6–8), were contaminated and have been removed from the CFU data.

### 3.2. Microbiota identified by 16S rRNA amplicon sequencing

16S rRNA amplicon sequencing of total DNA extracted yielded a total of 2,378,672 individual raw sequences, with a median of 50,769 raw reads per sample (variation 2,413–255,754) (Supplementary Table 1). The 664,754 read pairs were merged and 89.1% of these were mapped into 734 different OTUs (mean sequences mapped/sample = 12,876, variation 1,753–35,751) after removal of singletons and chimeras (Supplementary Table 1). The sequences have been deposited with SRA at NCBI (<https://www.ncbi.nlm.nih.gov/>) under bioproject accession number SRP186620 and BioSample accession SAMN10986241. Sufficient sampling depth was verified by rarefaction curves (supplementary Fig. 3) which flattened out around 1890 sequences, with around 25 to 250 OTUs represented per sample. The sequence analysis indicated a high number of different OTUs in the fresh retail broiler meat (day 0–4) while the older meat has lower diversity of OTUs (Fig. 3).

#### 3.2.1. Microbiota of retail broilers during shelf-life

The phylogenetic structure of the broiler meat microbiota was investigated by assigning OTUs into bacterial genera. The microbiota on OTU level was significantly different between the days after slaughter ( $p \leq 0.001$ ), while there was no significant difference between flocks for the five broiler flocks on the same day of shelf life after slaughter ( $p = 0.847$ ). Principal component analysis of the microbiota data revealed that for retail broiler meat samples, the different days of

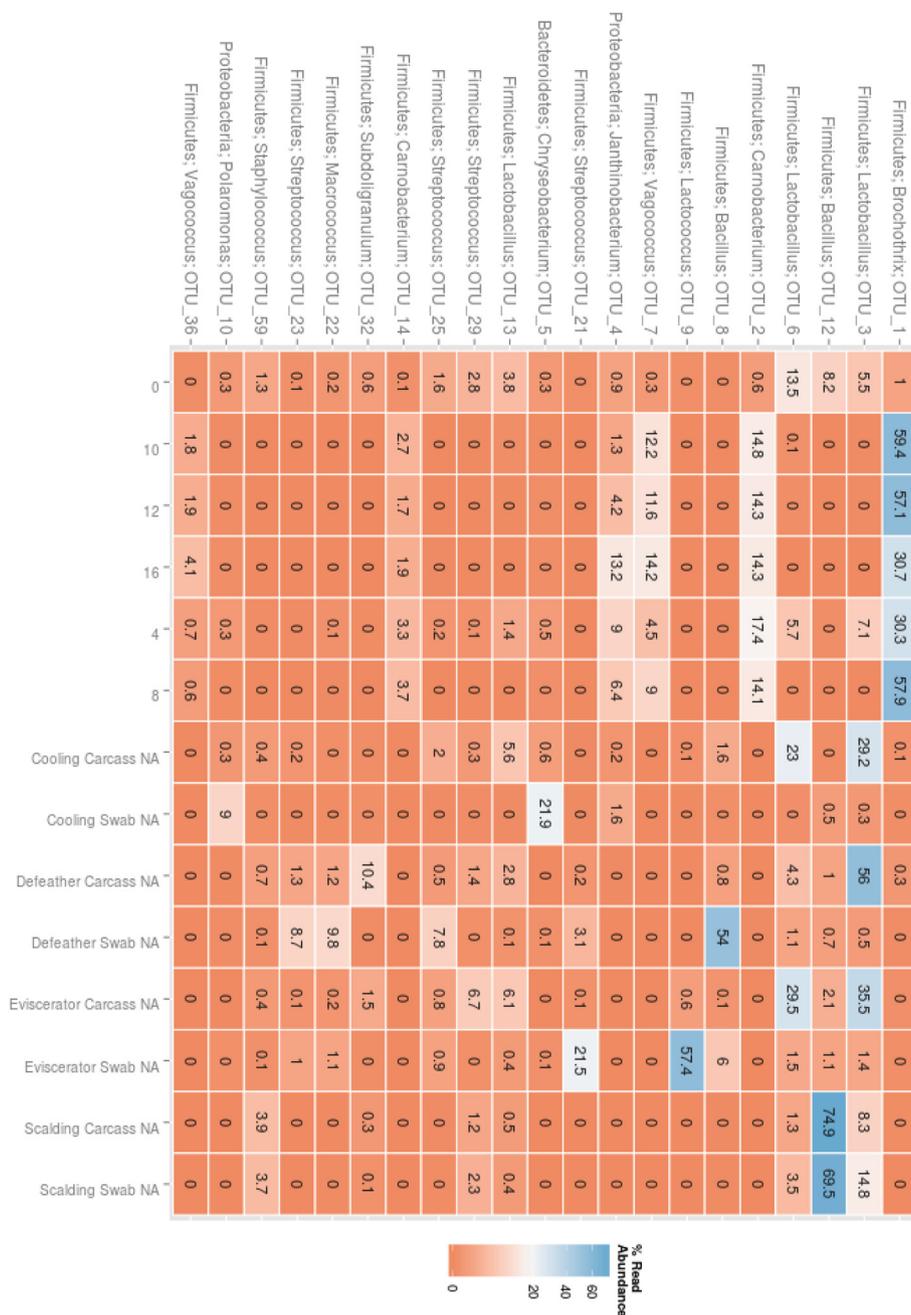


Fig. 3. Heatmap displaying the microbiota relative abundance of top 21 genera, isolated from Danish retail broiler meat and abattoir production line carcass and swab samples. Labeled by day after slaughter (0 = day 0, 4 = day 4, 8 = day 8, 10 = day 10, 12 = day 12, 16 = day 16) (retail meat) or sampling type and place (production line).

slaughter group together in three groups centred around day 0, day 4 and day 8–16 samples (Supplementary Fig. 4).

On the Norwegian broiler flock, lactococci was a dominating part of the microbiota encompassing putative spoilage bacteria, mainly represented by OTU\_11 on day 10–16, which was 98.4% identical to *Lactococcus piscium* CCG 32732<sup>T</sup>, and to a lesser degree an OTU, which was 99.4% identical to *Lactococcus raffinolactis* DSM 20443<sup>T</sup>. On Danish broilers only one lactococcus (OTU\_11) had a relative abundance of 0.7–1.9% four to 16 days after slaughter. While their relative abundance might be different during shelf-life, the same OTU sequences for the different putative spoilage bacteria genera *Lactococcus*, *Carnobacterium*, *Brochothrix*, and *Janthinobacterium*, were identified in both Danish and Norwegian retail broiler samples (data not shown).

On phylum level, the microbiota in all broiler meat samples was

clearly dominated by Firmicutes, and to a lesser degree Proteobacteria and Bacteroidetes (Fig. 4). The initial microbiota was very diverse with > 150 different OTUs represented. A few genera, *Lactobacillus* and *Bacillus* of the Firmicutes, and the Bacteroidetes members *Chryseobacterium* and *Flavobacterium* were relatively more abundant to all flocks on the very fresh broilers (day 0–4), however, their relative abundance rapidly decreased to < 0.5% of the broiler microbiota in older meat samples (Fig. 4). A few genera started to dominate the broiler microbiota already four days after slaughter. They were core OTUs on all flocks but had low relative abundance (≤1%) at day zero. The dominant genera from day eight and exceeding acceptable shelf-life included members of the Firmicutes *Brochothrix*, *Carnobacterium* and *Vagococcus*, and less pronounced the proteobacterial genus *Janthinobacterium*. This genus increased to a high abundance in the spoiled

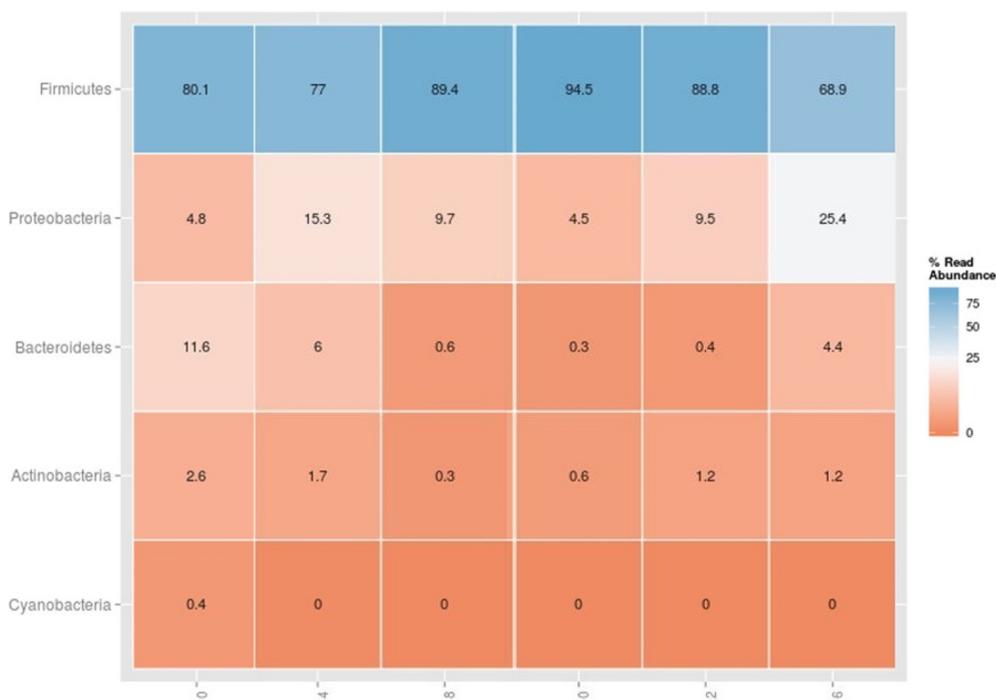


Fig. 4. Heatmap of the relative abundance of top 5 phyla identified in Danish whole broiler meat microbiota during and exceeding shelf-life (X-axis = days after slaughter).

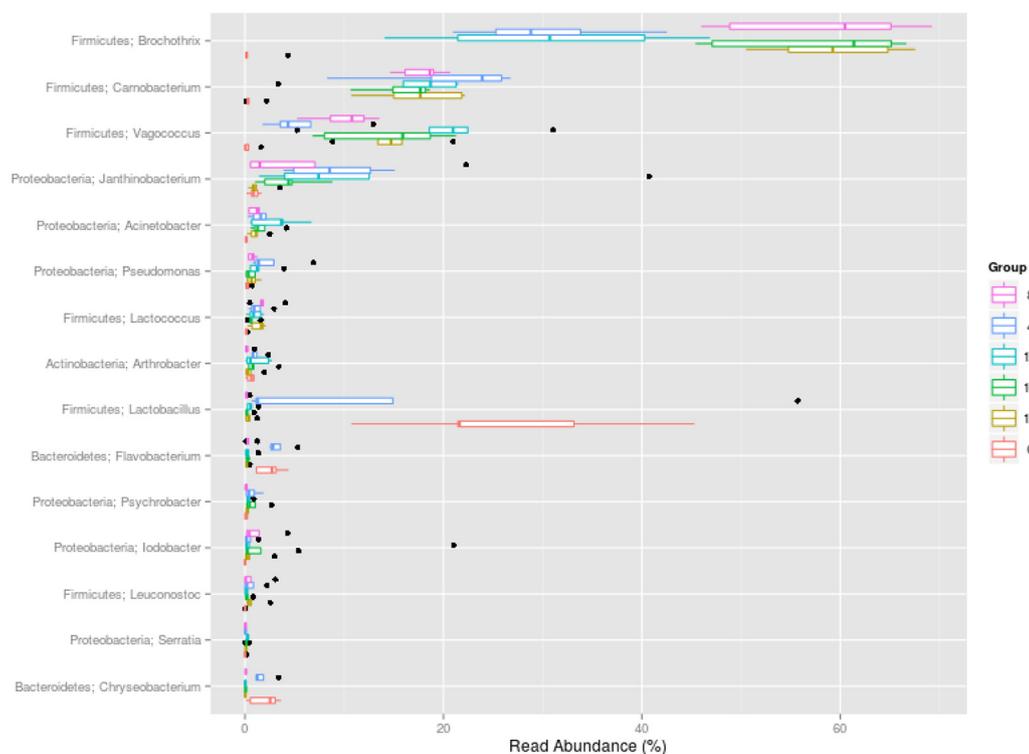


Fig. 5. Boxplot of the relative abundance top 15 genera identified in Danish whole broiler meat microbiota during and exceeding shelf-life, flocks pooled and grouped by days after slaughter. The boxes include the inter-quartile range (25–75% of data), with the median indicated and the whiskers being the lowest inter-quartile – 1.5 × inter-quartile range and highest inter-quartile + 1.5 × inter-quartile range, respectively. The outliers are shown as dots.

broiler meat samples (Fig. 5). The genera *Pseudomonas* and *Acinetobacter* (assigned as 16S rRNA indistinguishable *Pseudomonas fragi* group and *A. harbinensis*), within the Proteobacteria phylum, had a low relative abundance on the broiler meat during the whole sampling period (Fig. 5). One OTU was assigned to the *Leuconostoc* genus and had a low relative abundance which was stable during shelf-life.

The Norwegian flock had a significantly ( $p \leq 0.001$ ) different microbiota compared to the Danish flocks, it is however unknown if the

difference in packaging atmosphere, abattoir production hygiene, and/or primary production differences is the cause. The main difference being, an initial microbiota dominated by the proteobacteria *Polaromonas* and *Janthinobacterium*, and the bacteroidetes *Flavobacterium*, and a later shelf-life microbiota dominated by *Carnobacterium*, *Lactococcus*, and *Janthinobacterium* on the Norwegian broiler meat (Supplementary Fig. 5).

### 3.2.2. Species level

For the Danish samples, only one OTU (OTU\_1) classified as *Brochothrix* in the MiDAS database, had 100% identity to *Brochothrix thermosphacta* type strain DSM 20171<sup>T</sup> in the EzTaxon-e database. Two OTUs were classified in the *Carnobacterium* genus (OTU\_2 and OTU\_14), with the relative abundance of OTU\_2 being around 4–5 times higher than OTU\_14. OTU\_2 had 99.8% identity to *Carnobacterium maltaromaticum* DSM 20342<sup>T</sup>, while OTU\_14 had a 100% identity to *Carnobacterium divergens* DSM 20623<sup>T</sup>. Eight OTUs were classified to the *Vagococcus* genus in the MiDAS database and of these, two OTUs (OTU\_7 and OTU\_36), were much more prevalent in the mid/late shelf-life compared to early shelf-life, especially OTU\_7 (Fig. 3). The remaining six *Vagococcus* OTUs had very low relative abundances during shelf-life ( $\leq 0.8\%$ ). OTU\_7 had 98.4% identity to *Vagococcus fluvialis* CCUG 32704<sup>T</sup> and OTU\_36 99.8% identity to *Vagococcus salmoninarum* CCUG 33394<sup>T</sup>. Only one OTU (OTU\_4) was classified to the Gram negative *Janthinobacterium* genus, and the sequence had 100% identity to *Janthinobacterium lividum* DSM 1522<sup>T</sup>.

The lactobacilli found to be very abundant on the fresh broiler meats (day 0–4), consisted mainly of three OTUs, namely OTU\_3, OTU\_6, and OTU\_13, while they were quickly outgrown and had a relative abundance  $< 0.1\%$  in later and exceeding shelf-life (Fig. 5). OTU\_6 had 100% identity to *Lactobacillus crispatus* DSM 20584<sup>T</sup>, OTU\_3 had 99.6% identity to *Lactobacillus salivarius* ATCC 11741<sup>T</sup>, and OTU\_13 had 99.6% identity to *Lactobacillus johnsonii* ATCC 33200<sup>T</sup>. The relative abundance of two other *Lactobacilli* OTUs increased from  $< 0.1\%$  in fresh broiler meat to around 0.2–0.4% at day 16. One was 100% identical to *Lactobacillus fuchuensis* JCM 11249<sup>T</sup> and the other 100% identical to *Lactobacillus oligofermentans* AMKR18<sup>T</sup>, respectively.

A potential environmental pathogen was identified, but with a low relative abundance. Within the Enterobacteriaceae family, one OTU classified in the genus *Serratia*, was found 99.6% identical to *Serratia liquefaciens* ATCC 27592<sup>T</sup>, within the indistinguishable 16S rRNA liquefaciens group, consisting of *S. liquefaciens*, *Serratia grimesii*, and *Serratia proteamaculans*. Some strains of *Serratia liquefaciens* can be pathogenic, causing urinary tract, bloodstream infections, meningitis, and pneumonia in human, but outbreaks are normally associated with nosocomial infections due to poor hygiene (Mossad, 2000). The *Serratia* OTU had a low relative abundance, but was found highest 16 days after slaughter (0.1–0.4%) (Fig. 5).

### 3.3. Possible contaminations from the production line

The CFUs from the swab samples of the production line were of qualitative nature and originated from different sampling sites in the production chain and different sampling days. We recovered and identified some of the previously described microbiota encompassing putative spoilage bacteria in the production line. There were significant differences in the microbiota of swab samples between the four different sampling places in the production line ( $p \leq 0.001$ ) and principal component analysis clearly separated the different sampling places (Fig. 6). The scalding tub was dominated by *Bacillus* (OTU\_12) and *Lactobacillus* (OTU\_3, OTU\_6, and OTU\_13) OTUs, and to a minor degree also *Staphylococcus* (OTU\_29) and *Streptococcus* (OTU\_59) (Fig. 3). The *Bacillus* (assigned as *B. thermoamylovorans*) and *Lactobacillus* (assigned as *L. salivarius*, *L. crispatus*, and *L. johnsonii*) OTUs identified in the scalding tub were found in all four swab sampling places, but they were only dominating in the scalding tub and only identified on the broiler meat samples on day 0 and 4 after slaughter. The swab sample from the feather plucking fingers was dominated by *Bacillus* (OTU\_8 assigned *B. cereus*) and *Streptococcus* OTUs (OTU\_21, OTU\_23, and OTU\_25) which was different from the scalding tub and an OTU assigned to the *Macrococcus* genus (OTU\_22) (Fig. 3). In the eviscerator swab sample, three OTUs dominated, namely *Lactococcus* (OTU\_9 assigned *L. garvieae*), and to some degree two *Streptococcus* and *Bacillus* OTUs also identified on the plucking fingers (Fig. 3). The cooling condenser swab samples were

dominated by psychrotrophic bacteria. The *Janthinobacterium* OTU (OTU\_4), assigned to *J. lividum*, was found in high abundance on spoiled broiler meat, in the swabs taken from the cooling condenser and on carcasses taken in the cooling chain (Fig. 3). Other major parts of the cooling condenser microbiota identified by 16S rRNA amplicon sequencing, included the genera *Chryseobacterium*, *Polaromonas*, and *Rhodospirillum rubrum* (Figs. 3 and 6), however, these genera were not part of the microbiota on the spoiled broiler meat, and they were quickly outgrown on the fresh retail broiler meat.

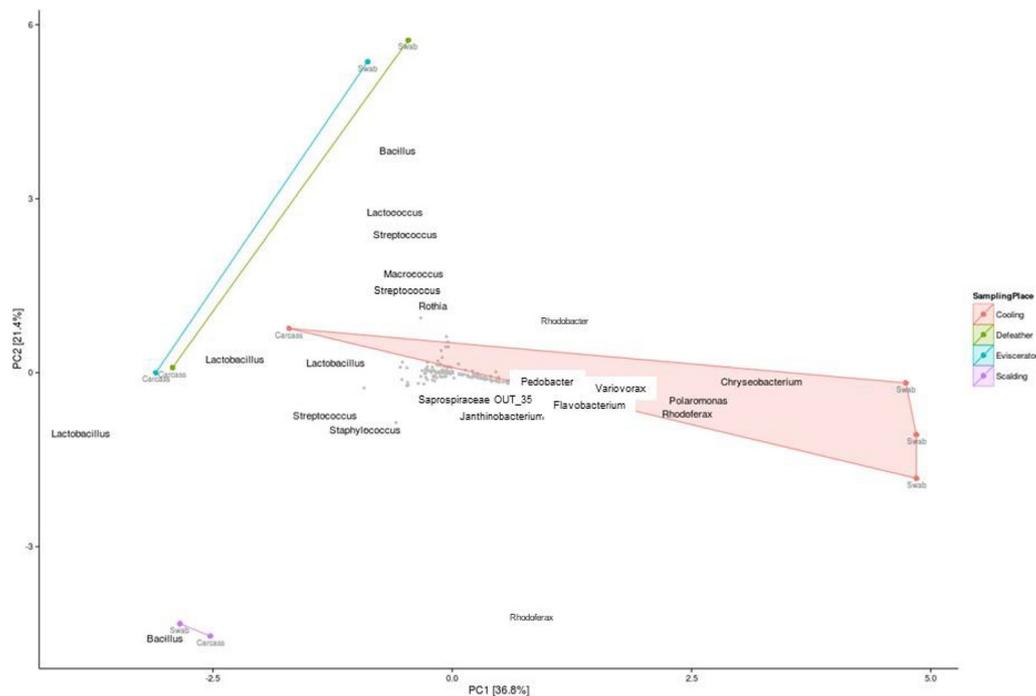
A single OTU (OTU\_12) within the *Bacillus* genus was dominating ( $> 69\%$ ) in the microbiota of carcasses taken from the scalding tub and the scalding tub itself, but already after plucking the relative abundance of *Bacillus* decreased to  $< 2\%$ . The three *Lactobacillus* OTUs (assigned as *L. crispatus*, *L. salivarius*, and *L. johnsonii*) readily identified on the very fresh (day 0) retail broiler meat, were found as a dominating part of the microbiota throughout the production line, especially on the broiler carcasses, but also as part of the production line equipment microbiota, however these were quickly outcompeted on the broiler meat during shelf life (Fig. 3).

The dominating OTUs from the later shelf-life retail broilers, *Carnobacterium*, *Brochothrix* and *Vagococcus* were not readily detected by 16S rRNA amplicon sequencing in the production line on either carcasses or equipment (Fig. 3). The *Brochothrix* OTU was detected in all swab and carcass samples, except the scalding tub swab, with a prevalence of  $< 0.1\%$ . The two *Vagococcus* OTUs and the *Carnobacterium* OTU assigned as *C. divergens*, found in high relative abundance on the late shelf life broiler meat, were not detected in any of the swab or carcass samples. The OTU assigned as *C. maltaromaticum*, found readily on the late shelf life broiler meat, was only identified in the production in a swab sample from the feather plucking fingers, with a prevalence of  $< 0.1\%$ . OTUs within the genera *Pseudomonas*, *Acinetobacter*, and *Serratia* were not detected by 16S rRNA amplicon sequencing in the production line and on slaughter line carcasses.

## 4. Discussion

For the first time, we show that members of the lactic acid bacterial genus *Vagococcus* form a major part of microbiota in fresh whole broiler meat packaged in modified atmosphere. Most likely the species *V. fluvialis* and *V. salmoninarum* were the dominant members of the genus. There has been very limited evidence of the role of members within the *Vagococcus* genus in meat spoilage, but the genus has been identified in MAP packaged fish (Chaillou et al., 2015; Powell and Tamplin, 2012), spoiled cooked shrimp (Jaffres et al., 2010), and in one case also from brined MAP packaged broiler filets (Nieminen et al., 2012). Some lactic acid bacteria can be difficult to cultivate (Leisner et al., 2007), and it might be the reason for the limited knowledge of the spoilage potential of vagococci, but as we found it as a dominating part of the broiler meat late shelf-life microbiota in high O<sub>2</sub>/high CO<sub>2</sub> MAP. Further investigations of the role of members of *Vagococcus* in poultry meat spoilage, direct or indirect, are desirable.

The spoilage potential of *B. thermosphacta* in meat is well known as very potent, and we found it to quickly increase during shelf-life and form a major part of the microbiota in spoiled broiler meat. The spoilage activity of members of the *Carnobacterium* genus can vary, but the species *C. maltaromaticum* and *C. divergens* identified here in high relative abundance on the spoiled broiler meat, have often been associated to meat spoilage (Leisner et al., 2007). The route of contamination for these two *Carnobacterium* species have previously been shown to stem from the abattoir production environment and not the incoming broiler flocks (Vihavainen et al., 2007), however, we were not able to easily detect *Carnobacterium* on the carcasses or equipment in the production line. We were also not able to easily detect *Brochothrix* and *Vagococcus* by 16S rRNA amplicon sequencing of samples taken in the production line on either carcasses or equipment. The relative abundance of *Carnobacterium*, *Vagococcus*, and *Brochothrix* on the very



**Fig. 6.** PCoA of microbiota from abattoir production line, carcass and swab samples, principal component 1 and 2 (PC1 & PC2). Data points with the same colour and connected by lines refer to the same day of sampling. Bacterial names refer to loadings of the top 25 OTUs.

fresh retail broiler meat was low contrary to recent studies that showed a dominance of members of *Brochothrix*, *Pseudomonas* and *Carnobacterium* on French chicken cuts stored at various MAP conditions (Rouger et al., 2018).

Known spoilers within the Gram negative bacteria, such as certain *Pseudomonas* and *Acinetobacter* species, are typically associated with conventionally aerobically packaged meat (Liang et al., 2012), so it is no surprise that we found their relative abundance very low in the MAP packaged retail broiler meat during shelf-life. However, we find the Gram negative Betaproteobacteria *Janthinobacterium*, likely *J. lividum*, as a dominating part of the broiler microbiota under spoilage conditions in both 80%O<sub>2</sub>/20%CO<sub>2</sub> and 50%N<sub>2</sub>/50%CO<sub>2</sub> MAP. *J. lividum* has previously been described as a milk spoiler (Eneroth et al., 2000) and occasionally meat spoiler (Penney et al., 1993), associated with production of the pigment violacein and slime formation. Our results indicated that the cooling condenser in the broiler abattoir production line was potential major route of contamination with *J. lividum*.

The microbiota encompassing putative spoilage bacteria composition was not significantly different between meat from different broiler flocks, when slaughtered and packaged at the same abattoir, independent of day of slaughter. The microbiota encompassing putative spoilage bacteria composition on broiler meat from a different abattoir (Norway), packaged in a different modified atmosphere is, however different. Broiler meat from Norway packaged in 50%N<sub>2</sub>/50%CO<sub>2</sub> MAP, had longer shelf-life, lower CFUs during shelf-life, and a lower relative abundance of *Brochothrix* and *Vagococcus* compared to the Danish samples. It has previously been shown that certain *Lactobacillus* and *Lactococcus* species, such as *L. salivarius*, have a potential as protective cultures against spoilage bacteria, as for instance the potent spoiler *B. thermosphacta* (Rahkila et al., 2012), which together with the lower CFU count could explain the longer shelf life we found in the Norwegian broiler meat. Our findings support that abattoir production line hygiene and composition of packaging gas are important parameters for spoilage microbiota and shelf-life of broiler meat, while the individual broiler flocks display no significant difference in microbiota during shelf-life of the retail packaged broilers if slaughter and packaged under the same conditions. Highlighting the important role of the

production environment, as a route of contamination, for spoilage microorganisms, such as *Brochothrix* and *Janthinobacterium*.

Furthermore, our results indicate that the relative abundance of a potentially pathogenic *Serratia* species increase during shelf-life in high oxygen MAP, even though in a low relative abundance, and it is apparently able to multiply in retail packaged broiler meat kept at refrigeration temperature, which could potentially be a food safety issue. However, we were not able to identify *Serratia* in the abattoir production environment.

Using 16S rRNA amplicon sequencing allowed us easily to identify the microbiota on retail packaged broiler meat during shelf-life, without being reliant on traditional cultivation steps. The 16S rRNA amplicon sequencing method is not absolutely quantitative, making it impossible to translate our relative abundance into actual abundance of the individual genera. However, we can still compare the relative abundance of different OTUs and genera in different samples and how it changes between storage time and different broiler flocks. For interpretation of actual abundance levels we will be reliant on using a supporting quantitative method since an increase of around 6 log<sub>10</sub> CFU on retail broilers from day zero to sixteen was observed which resulted in sensory rejection at around 8–12 days after slaughter that was combined with CFUs higher than 10<sup>7</sup> CFU/cm<sup>2</sup>. The high colony counts of *Brochothrix*, lactic acid bacteria, and Gram negative selective agars fits the findings of very high relative abundance of both *Brochothrix*, Gram negative *Janthinobacterium*, and lactic acid bacteria, such as *Carnobacterium* and *Vagococcus*, identified on spoiled broilers by 16S rRNA amplicon sequencing. Our results show that using 16S rRNA amplicon sequencing can help us identify bacterial genera not previously associated as putative spoilage bacteria of meat which in the future can expand our horizon on the microbial composition of food products of animal origin.

## 5. Conclusion

We confirmed the hypothesis that there is no significant difference in the microbiota between retail broiler meat, when flocks are slaughtered and MAP packaged under the same conditions. The 16S rRNA

amplicon sequencing allowed us easily to identify the broiler microbiota during shelf-life, including well known spoilers *Brochothrix*, *Carnobacterium*, and *Janthinobacterium* species, and for the first time identify *Vagococcus* species as a dominating part of the late shelf-life microbiota on retail whole broiler meat in 80%O<sub>2</sub>/20%CO<sub>2</sub> MAP. Dominating initial whole broiler meat microbiota, including *Lactobacillus*, *Bacillus*, *Chryseobacterium* and *Flavobacterium* species, is rapidly outgrown by *Brochothrix*, *Carnobacterium*, *Janthinobacterium* and *Vagococcus* species during storage in 80%O<sub>2</sub>/20%CO<sub>2</sub> MAP at 6 °C. The microbiota encompassing putative spoilage bacteria was dominant already four to eight days after slaughter, and acceptable shelf-life of whole broiler meat in 80%O<sub>2</sub>/20%CO<sub>2</sub> MAP was around 8–12 days, where total viable count was above 10<sup>7</sup> CFU/cm<sup>2</sup> and sensory rejection was mainly associated with sour smell formation. A potential major route of contamination for *J. lividum* is the cooling condenser in the broiler abattoir production line.

## Declarations of interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2019.04.003>.

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