



Assessment of food microbiological indicators applied on poultry carcasses by culture combined MALDI-TOF MS identification and 16S rRNA amplicon sequencing

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ABSTRACT

Examination of the bacterial contamination on food products is still largely performed by standardized culture methods, though culture-independent methods are suggested as a more reliable approach. Knowledge of the diversity of bacteria isolated from food as well as the impact of the plate incubation conditions applied are still understudied. The impact of incubation at 7 °C and 30 °C on total aerobic bacterial count and diversity, and the performance of ISO methods generally applied in microbiological quality examination were assessed by culture combined MALDI-TOF MS identification and 16S rRNA amplicon sequencing. Examining breast skin of 16 chicken carcasses, no significant impact of the incubation temperature on the total aerobic bacteria level and diversity was detected, limiting the usefulness of additional psychrophilic examination. Bacteria phenotypically similar to *Pseudomonas*, were identified on selective CFC plates, and on MRS agar plates for lactic acid bacteria, *Escherichia coli* and *Staphylococcus* were commonly present. Application of 16S rRNA amplicon sequencing revealed a higher bacterial diversity, but the impact of the DNA extraction kit applied, and the detection of non-viable bacteria should be taken into account to interpret the final outcome.

1. Introduction

Poultry meat, including cuts and carcasses, has become one of the most consumed meat products worldwide (OECD, 2017). Its consumption is still increasing regardless of region or income level, and has already reached a mean of 13.9 kg per capita per year, surpassing pork as the preferred animal protein. However, consumption levels vary depending on the regions, ranging from 12.3 kg per capita in China, 24.2 kg in the European Union, to 48.8 kg per capita in United States and 56.9 kg in Israel (OECD, 2017).

Compared with beef and pork, fresh poultry meat has a higher initial bacterial contamination level, and is more associated with the presence of foodborne pathogens (Meredith et al., 2014). Therefore, it needs to be microbiologically monitored, and several criteria are in place to control pathogenic bacteria. For instance, *Salmonella* should be absent in 25 g poultry meat according to EU regulation (EC, 2073/2005), and no *Salmonella* should be present in poultry meat according to Chinese legislation (GB 16869-2005). Also, *Campylobacter* spp.

should be less than 1000 CFU/g on broiler carcasses according to EU 2017/1495, which is amending regulation EC 2073/2005. Besides specific pathogen detection, total aerobic bacteria (TAB) counting as a general microbiological indicator is routinely performed on poultry products. According to GB 16869-2005, TAB should not exceed 1×10^6 CFU/g in fresh poultry meat in China, whereas relevant criteria are lacking in the EU. As for the method used, TAB should be determined at 30 °C incubated for 72 h (ISO 4833) or at 36 °C \pm 1 incubated for 48 h \pm 2 h (GB 4789.2-2016). However, after slaughter, processing and storage temperature is not allowed to exceed 4 °C (EC 853/2004) or 7 °C (GB 31621) till consumption. Currently, industry would prefer more to determine the bacterial load at a low, more relevant temperature. Low processing and storage temperatures benefit psychotropic bacteria, including major spoilage related microorganisms (Mann et al., 2016; Nychas et al., 2008), but are not targeted by current methods nor legislations. At present, there is a lack of statistically based comparative studies determining the cultivable bacterial populations at different temperatures on food, and poultry products in particular

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(Chaiba et al., 2007). Moreover, general, non-selective counting agar plates are routinely used for TAB and total anaerobic bacteria (TANAB), and results obtained are often considered decision values for the level of hygiene during processing. However, information on the bacterial identity and diversity present is completely lacking.

Microbial diversity has been already studied a lot in all different kind of matrices, and both culture-dependent and culture-independent methods are applied (Höll et al., 2016; Rouger et al., 2018). However, this is rarely performed in a comparative set-up in food matrices. In food microbiology, culture-dependent methods are still the most direct way to study the presence of microorganisms, but are nowadays considered as labor-intensive and biased, due to the isolation conditions and media applied, and the potential stress situation of the targeted microorganisms (Cocolin et al., 2013). Furthermore, routine microbiological analysis of food is based on rapid determination of selected general parameters, among which those already mentioned above, but without further identification nor confirmation of the isolates. With the development of matrix-assisted laser desorption ionization time of flight mass spectrum (MALDI-TOF MS), rapid identification of a large amount of colonies present on cultivation plates has become attainable. This fast, reliable and powerful tool has been limited applied to study the microbiological quality in food so far (Höll et al., 2016), and no study has aimed to retrospect the microbial identify and diversity on those routine isolation plates using MALDI-TOF MS. Culture-independent methods have become the major trend for microbiota research (Cauchie et al., 2017; Ercolini, 2013; Yang et al., 2016). These techniques rely on the combination of genome and bioinformatics analysis, and provide more detailed microbial information without the need of cultivation. Already some studies applied 16S rRNA amplicon sequencing to examine the microbial diversity on isolation plates originating from environmental samples, but not yet in food microbiology (Ellis et al., 2003).

In addition to TAB and TANAB, other specific groups of bacteria are also regularly determined as hygiene or spoilage indicators such as, *E. coli*, lactic acid bacteria (LAB) and *Pseudomonas*. For this, in the context of food safety control and international trade, normalized methods, such as ISO are applied, though data about the bacterial specificity and selectivity are also largely lacking (Säde et al., 2013).

The aim of the present study was to assess the impact of both temperature incubation and analytical method on bacterial levels, identification and diversity detected in a food matrix. Therefore, as a proof of principle, poultry carcasses were examined using commonly applied ISO-methods combined with MALDI-TOF MS identification, as well as 16S rRNA amplicon sequencing.

2. Materials and methods

2.1. Sampling

From October 2016 to September 2017, 16 fresh air packed chicken carcasses were purchased 5–7 days before expiration on different occasions and in different local supermarkets in Ghent, Belgium. The left side of the breast skin of each carcass (12 ± 2 g) was taken aseptically, and homogenized with 1:10 (W/W) Buffer Peptone Water (BPW, 3564684, Bio-RAD, France) in a sterile stomacher bag for 2 min at 230 rpm using a peristaltic homogenizer (Stomacher® 400 Circulator machine, Seward, UK).

2.2. 16S rRNA amplicon sequencing

To assess the microbial diversity, from 8 randomly chosen chicken carcasses (C1 to C8), 15 ml of the corresponding stomached homogenate (SH) samples were centrifuged at $8000 \times g$ for 15 min, and prepared for DNA extraction. Two widely used DNA extraction kits (Table 1) were used following the manufacture instructions: 1) DNeasy PowerFood Microbial Kit (Qiagen, Germany); 2) FastDNA® SPIN Kit for

Table 1
Differences in the DNA extraction kits protocols and outcomes.

	DNeasy PowerFood Microbial Kit	FastDNA® SPIN Kit for soil
Sample amount	1.8 ml	0.5 ml
Lysis method	Beads, Vortex Adapter, maximum speed 10 min	Beads, Mixer Mill MM400, 30HZ/s 90s
DNA elution metrics	EB	DES
DNA concentration (ng/ul)	SH 19.50 ± 22.86 PW 305.08 ± 166.25 PW + 1 410.15 ± 125.36	4.10 ± 3.11 94.85 ± 103.79 125.77 ± 74.41
Reads number	SH 64085 ± 9673 PW 51920 ± 6576 PW + 1 58546 ± 4346 G(+)* 740102 G(-)* 1737963	49851 ± 6009 52510 ± 5014 51210 ± 5800 331132 1968958

*: $p < 0.01$ indicating the reads number of Gram positive and Gram negative is significantly dependent on the DNA extraction kits.

SH: stomached homogenate samples.

PW: plate washing samples with the same dilution as counting plates.

PW + 1: plate washing samples with 1 fewer dilution fold than counting plates.

Soil (MP Biomedicals, US). DNA quantity and quality were checked by Quantus™ Fluorometer (Promega, USA) and NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher, USA) respectively. DNA samples were shipped to Novogene Institute (Hongkong, China) for 16S rRNA amplicon sequencing. The V3-V4 region of 16S rRNA gene was amplified using primer 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGTATCTAAT). DNA libraries of paired ends with single index were constructed using Truseq-dna-pcr-free-library-prep kit. Sequencing was performed on Illumina 2500 platform (Q30 > 80%).

Qiime2 (version 2017.10) software pipeline (<https://qiime2.org>) was used for data analysis. Reads were demultiplexed with q2-demux (<https://github.com/qiime2/q2-demux>). Then q2-dada2 plugin was implemented to quality control process, and all phiX reads and chimeric sequences were filtered. Based on demux summary, sequences at 154 bases of both forward and reverse reads were truncated. After denoising the data using dada2 denoise-paired method, representative sequences of each sample were retained and then assigned to taxa using Naive Bayes classifiers pre-trained on Greengenes 13.8 99% OTUs full-length sequences (<https://docs.qiime2.org/2017.12/data-resources/>).

2.3. Bacterial isolation

Ten-fold serial dilutions of each stomached homogenate (SH), in total 16, were prepared in BPW, followed by quantitative bacterial isolation in duplo: i) total aerobic bacteria isolation was performed according to ISO 4833 part 2 on Plate Count Agar (PCA, 3564475, Bio-RAD), once incubated at 30 °C for 72 h, and once at 7 °C for 96 h; ii) total anaerobic bacteria isolation was performed on PCA as described above, but incubated in a anaerobic cabinet at 30 °C for 48 h; iii) Lactic Acid Bacteria (LAB) isolation was done according to ISO 15214 on De Man, Rogosa and Sharpe medium (MRS, 3564244, Bio-RAD), incubated at 30 °C for 72 h aerobically; presumptive *Pseudomonas* spp. were isolated according to ISO 13720 on Cephalothin-Sodium Fusidate-Cetrimide Agar with Modified CFC Selective Supplement (CFC, CM0559B with SR0103E, OXOID, Basingstoke, UK), incubated at 30 °C for 48 h aerobically, and *E. coli* was selectively isolated on Tryptone Bile X-glucuronide (TBX, 3564035, Bio-RAD), incubated at 37 °C for 24 h aerobically according to ISO 16649-2. Colonies were counted from the plates with a bacterial growth between 30 and 300 CFU/plate on PCA, and between 15 and 150 CFU on all others.

From all PCA and other selective isolation plates selected for counting, a part of each individual colony was picked and pure-cultured on Trypticase Soy Agar (TSA, CM0131, OXOID) or MRS, and incubated

as before.

2.4. Plate growth profiling using 16S rRNA amplicon sequencing

To identify the bacterial population with a prior cultivation by 16S rRNA amplicon sequencing, the remaining bacterial growths after picking on the PCA plates, as well as on the corresponding PCA plates inoculated with one dilution higher, were collected by a plate washing technique. For this, from the PCA plates of the 8 chosen chicken carcasses (C1 to C8) (see 2.2), the remaining bacterial mass left on the three PCA plates (aerobic 7 °C, 30 °C and anaerobic, each condition including 2 plates) was harvested after prior counting and picking (plate wash, PW), as well as the total biomass from the same PCA plates but one dilution higher (PW + 1), if available. In brief, 5 ml of BPW was brought onto each agar surface, the biomass recovered from 2 plates resuspended using a cell scraper, and pooled into a single centrifuge tube per sample for subsequent 16S rRNA amplicon sequencing (following the description as 2.2).

2.5. MALDI-TOF MS identification of colonies

One colony of each pure-culture on each TSA and MRS plate was picked using a sterilized toothpick and smeared gently onto a MALDI-TOF MS target plate (Bruker Daltonics, Bremen, Germany). After air-drying, the sample was covered with 1 µl matrix solution containing 10 mg/ml α -cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoroacetic acid (50:47.5:2.5, v v^{-1}). Each series of measurements was preceded by a calibration step with a bacterial test standard (BTS 155 255343; Bruker Daltonics) to validate the run. Mass spectra were generated by a Micro-flex LT MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (11/4337 nm) operating in linear positive ion detection mode under the Bruker flex Control software (Bruker Daltonics). Identifications were obtained by comparing the mass spectra to the Bruker MSP database (version DB5989) using the Bruker Compass software (Bruker Daltonics) at default settings. Identifications at species or genus level were considered if scores were respectively above 2.0 and 1.7 according to the report generated by Bruker Compass.

2.6. 16S rRNA gene sequencing identification of colonies

Isolates for which no spectra was obtained or not identified by the Bruker MSP database were analyzed again using the more extensive extraction method based on Microorganism profiling 'Ethanol/Formic Acid extraction procedure' from Bruker Daltonics (03.04.2006). Briefly, a loop of bacterial growth was suspended in 300 µl milli-Q water and 900 µl ethanol followed by centrifugation at 14,000 $\times g$ for 3 min twice, the supernatant was discarded and the ethanol-free pellet was suspended in 20–50 µl 70% formic acid and equal volume of acetonitrile followed centrifugation at 14,000 $\times g$ for 3 min. The supernatant was collected and 1 µl was spotted twice on a MALDI-TOF MS target plate, covered by matrix solution. The measurement was performed as described above. When still no identification was obtained, dereplication was performed by importing the mass spectra into the BioNumerics 7.2.6 software (Applied Maths, Belgium) to select isolates for further identification by 16S rRNA gene sequencing. A curve-based analysis was performed using the UPGMA clustering algorithm, and the similarity between the spectra was expressed using Pearson's product moment correlation coefficient. Representative isolates per cluster were visually selected for subsequent 16S rRNA gene sequencing. Hence, DNA was extracted by alkaline lyses where one colony was suspended into 20 µl of lysis buffer (2.5 ml 10% SDS, 5 ml 1N NaOH and 92.5 ml Milli-Q water) and heated for 15 min at 95 °C. After a short spin, 180 µl Milli-Q water was added. Subsequently, the suspension was collected after centrifugation for 5 min at 10,000 $\times g$ at 4 °C. For 16S rRNA gene partial sequencing, the oligonucleotide primers pA (5'-AGA GTT TGA

TCC TGG CTC AG-3') and the pH (5'-AAG GAG GTG ATC CAG CCG CA-3') were used to amplify a 1.5 kb fragment (Edwards et al., 1989). The PCR mixture (final volume, 25 µl) contained 2.5 µl template DNA, 0.25 µl of each primer at concentration of 10 µM, 2.5 µl of each deoxynucleoside triphosphate at a concentration of 2 µM each, 0.5 µl AmpliTaq DNA polymerase (1 U/µl) and 16.5 µl of Milli Q water. PCR conditions consisted of 30 cycles. Amplicons were collected and submitted for Sanger sequencing (Eurofins), and V1-V3 region was sequenced. Subsequently, to obtain the identification, the sequences were blast towards the gene bank database (<https://www.ncbi.nlm.nih.gov>). For the taxa identification, the cut-off value for species is 98.65% and for genus is 94.5% (Kim et al., 2014; Yarza et al., 2014).

2.7. Statistic analyses

Coverage, diversity indices and richness of community information obtained from 16S rRNA amplicon sequencing and MALDI-TOF MS were calculated by PAST software package (<https://folk.uio.no/ohammer/past/>), as well as the rarefaction curves (Hammer et al., 2001). Abundance significance was calculated using Welch's *t*-test with no correction by STAMP (<http://kiwi.cs.dal.ca/Software/STAMP>). Rstudio 1.0.153 (<https://www.rstudio.com>) was applied for data visualization. Statistical differences were determined by *t*-test and Chi square test (5% significance level).

3. Results

3.1. Bacterial isolation and identification

Results of the quantitative microbial analysis of the breast skin from 16 poultry carcasses are presented in Fig. 1. In general, the contamination levels of TAB incubated at 30 °C and 7 °C, TANAB, LAB, presumptive *Pseudomonas* spp. and *E. coli* were 4.78 ± 0.53 (mean \pm SD, log 10 CFU/g), 4.78 ± 0.83 , 4.36 ± 0.66 , 4.02 ± 0.42 , 4.73 ± 0.77 and 2.91 ± 0.52 , respectively. Mean TAB levels determined at 30 °C and 7 °C showed no significant difference ($p > 0.05$), though a larger distribution range was observed for the incubation temperature at 7 °C (Fig. 1). For each sample, total aerobic bacteria levels after incubation at 7 °C and 30 °C, ranged from 1.09 to 0.03 CFU/g.

For the further identification by MALDI-TOF MS, a total of 6188 isolates were collected from all isolation plates selected for counting, of which 1726 and 1508 from the PCA plates incubated at 30 °C and 7 °C aerobically, as well as 1097, 795, 1020 and 42 isolates from the anaerobic incubated PCA, MRS, CFC and TBX plates, respectively

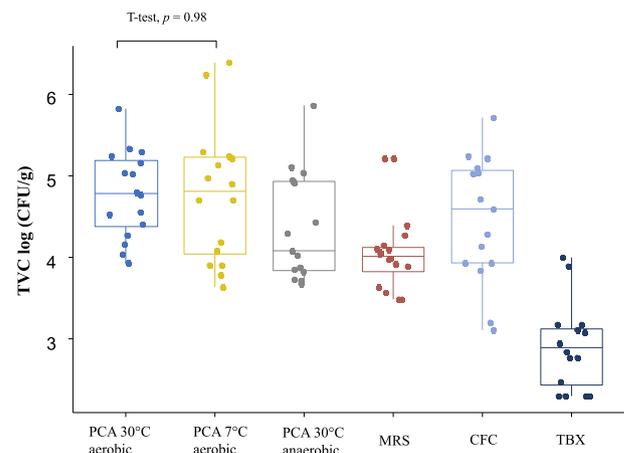


Fig. 1. Total viable counting on analysis plates. Total aerobic bacteria (TAB) counting incubated at 30 °C and 7 °C on PCA plates has no significantly difference. Each spot represents a sample.

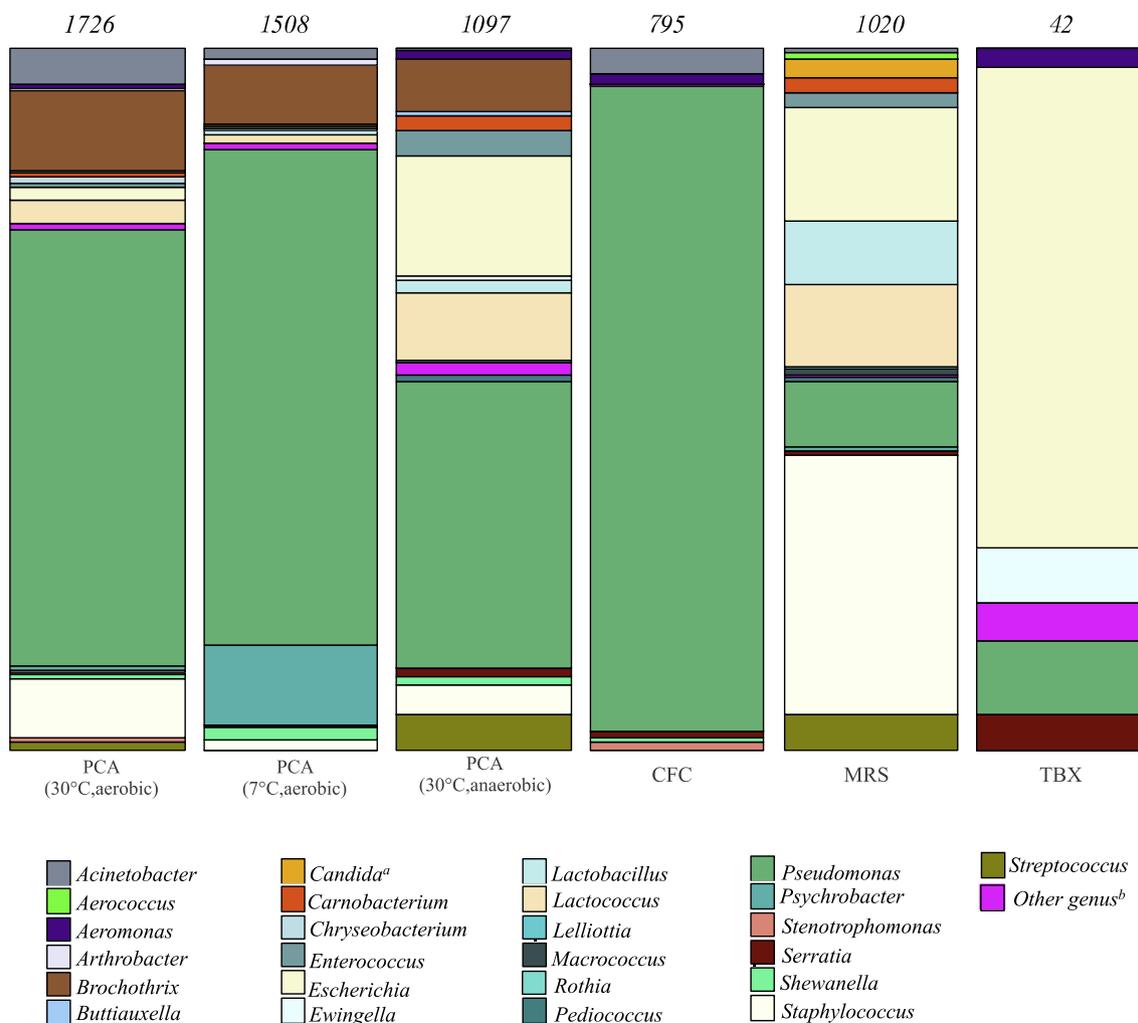


Fig. 2. Composition of bacterial genera isolated from 16 chicken carcasses using different incubation medium and conditions identified using MALDI-TOF MS (relative abundance > 0.5%).

^a: Fungal genus

^b: Other genus (relative abundance < 0.5%) including *Janthinobacterium*, *Flavobacterium*, *Enterobacter*, *Kluyvera*, *Leucobacter*, *Pantoea*, *Comamonas*, *Citrobacter*, *Wautersiella*, *Rahnella*, *Raoultella*, *Cronobacter*, *Yersinia*, *Vagococcus*, *Providencia*, *Delftia*, *Rhizobium*, *Morganella*, *Klebsiella*, *Hafnia*, *Cryptococcus*, *Gluconobacter*, *Corynebacterium*, *Glutamicibacter*, *Pseudoalteromonas*, *Pseudochrobactrum*, *Rouxiella*.

(Fig. 2). After preparation with the rapid smear or extraction method, MALDI-TOF MS spectra were obtained for 5636 isolates (= 91.1%), of which 4827 were identified at genus ($n = 1580$) and/or species ($n = 3247$) level using the Bruker MSP database. All other 809 isolates were identified after subsequent 16S rRNA gene sequencing. Those isolates were identified as belonging to *Pseudomonas* ($n = 347$), *Psychrobacter* ($n = 177$), *Staphylococcus* ($n = 156$) and 20 other genera ($n = 129$).

3.2. Microbial community profiling of the breast skin

Using 16S rRNA amplicon sequencing, in total 911,485 reads were generated from the 16 stomached homogenate samples (C1 to C8 x 2 extraction kits, Fig. 3.), of which 2726 exact amplicon sequence variants (OTUs) were picked out. These OTUs were assigned into 324 taxa at species level. Only taxa accounting for more than 0.5% of the total reads calculated were retained in the profiling of the microbial dominance diversity.

The bacterial diversity of the chicken breast skin, profiled with 16 stomached homogenate (SH) samples, according to each DNA extraction kit is presented in Fig. 3. In total 21 taxa were retained, of which 15 were identified at genus level, 2 at family level, 1 at order

level, 1 at phylum level, 1 at class level, and 1 at kingdom level. In the stomached homogenate samples using the Qiagen food DNA kit, the top three abundant genera were *Psychrobacter*, *Brochothrix* and *Pseudomonas* (Fig. 3). Using the MP soil kit, *Pseudomonas*, a group of unassigned Enterobacteriaceae and *Brochothrix* were detected as the dominant microorganisms in the stomached homogenate samples (Fig. 3). When comparing differences in abundance of bacterial genera in the stomached homogenate samples between both extraction methods, the Gram-positive genera *Enterococcus*, *Lactobacillus*, *Brochothrix* and *Lactococcus* were significantly more frequently detected when the Qiagen food kit was used, whereas the Gram-negative genera *Pseudomonas*, *Shewanella* and *Rahnella* were more present when the MP soil kit was applied ($p < 0.05$).

3.3. Bacterial diversity on general counting plates

Bacterial diversity on general counting plates (PCA) was determined with both MALDI-TOF MS and 16S rRNA amplicon sequencing (Fig. 4.). Using MALDI-TOF MS, on the TAB counting plates (PCA) incubated at 30 °C aerobically, with 1646 isolates identified, members of the genus *Pseudomonas* were most abundant followed by members of the genera *Brochothrix* and *Staphylococcus*. Additionally, *Pseudomonas* species were

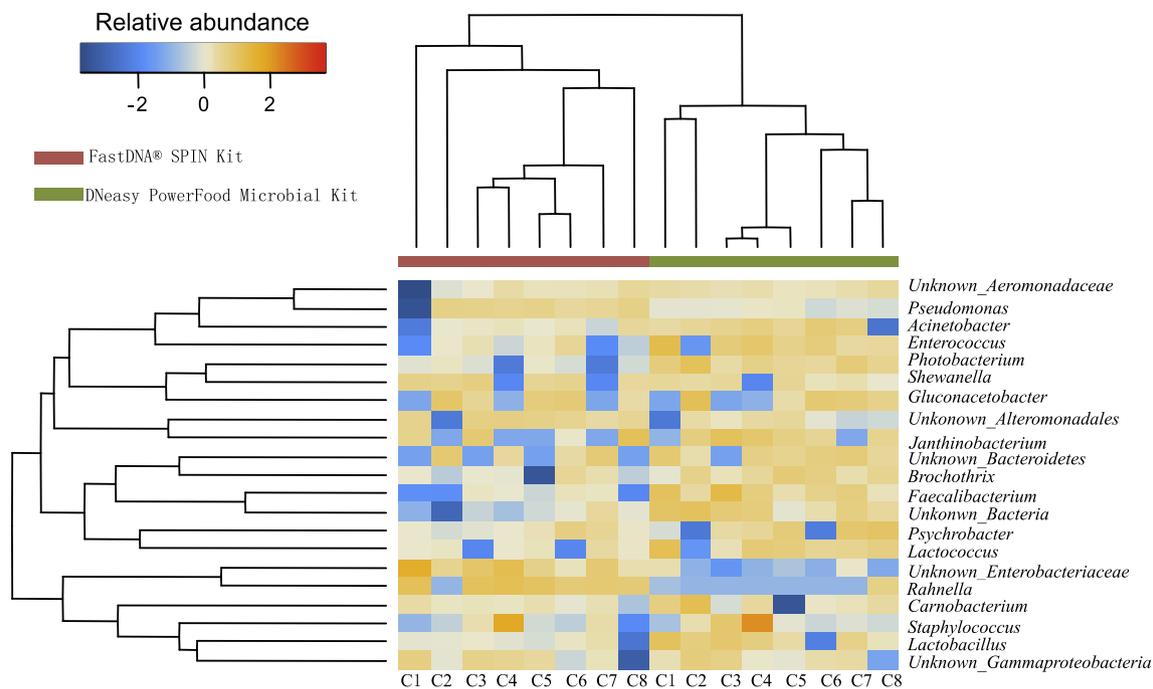


Fig. 3. Relative abundance of genera from 8 stomacheder homogenate (SH) samples with DNA extracted using both DNeasy PowerFood Microbial kit (Qiagen, green group) and FastDNA® SPIN Kit for Soil (MP, red group). All sequences with abundance above 0.5% of the total sequences were included in the analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

also the most occurring on the PCA plates incubated at 7 °C aerobically (n = 1429) as well as anaerobically (n = 939), though followed by *Psychrobacter* and *Brochothrix* species at 7 °C aerobically, and by *Escherichia* and *Lactococcus* at 30 °C anaerobically. Under aerobic incubation, the genera *Escherichia*, *Aerococcus* and *Rothia* were only isolated at 30 °C whereas the genera *Lactobacillus*, *Candida* and *Ewingella* only at 7 °C. Of the 18 genera present on the TANAB plates, the genera *Pediococcus*, *Enterobacter*, *Kluyvera*, *Raoultella*, *Cronobacter*, *Morganella*, *Hafnia* and *Gluconobacter* were only isolated under anaerobic incubation, although they are all facultative anaerobic. As for plate washing samples using 16S rRNA amplicon sequencing including both dilutions, 3,936,828 reads were generated from the plate washing samples (PW and PW + 1, Fig. 4.), of which 1965 exact amplicon sequence variants (OTUs) were picked out. These OTUs were assigned into 243 taxa at species level. Only taxa accounting for more than 0.5% of the total reads calculated were retained in the profiling of the microbial dominance diversity. In total 20 taxa were retained, of which 16 taxa were identified at family level, 1 at order level, 1 at class level, 1 at phylum level and 1 at kingdom level (Fig. 4). Regardless of the DNA extraction kits, Pseudomonadaceae, Enterobacteriaceae, Moraxellaceae, Aeromonadaceae and Listeriaceae were the top 5 families present on PCA plates incubated at 30 °C aerobically whereas Pseudomonadaceae, Listeriaceae, the group of unassigned Alteromonadales, Enterobacteriaceae and Moraxellaceae were commonly present on the aerobic PCA plates incubated at 7 °C. On the PCA plates incubated at 30 °C anaerobically, the members of the families Pseudomonadaceae, Enterobacteriaceae, Listeriaceae, Carnobacteriaceae and Aeromonadaceae were the most occurring families (Fig. 4). In Fig. 4, the comparisons of bacterial diversity identified between MALDI-TOF MS analysis and 16S rRNA amplicon sequencing with different dilution folds are also shown.

3.4. Performance of selective medium

In general (shown in Fig. 2.), on the CFC plates (n = 931), besides *Pseudomonas* species, there were also a few other genera present potentially leading to misidentification, including the oxidase positive genera *Aeromonas* and *Shewanella*, and the oxidase negative genera

Acinetobacter, *Serratia* and *Brochothrix*. On the MRS plates (n = 653) included for the isolation of LAB, the genera *Lactococcus* (n = 77) and *Lactobacillus* (n = 58) were most frequently isolated, but bacteria belonging to the genera *Escherichia* (n = 106) and *Staphylococcus* (n = 241) were present as well. On the selective TBX plates (n = 38) for *E. coli* isolation, all typical blue colonies were confirmed as *E. coli*, while other, non-typical colonies present were identified as bacteria belonging to the genera *Pseudomonas*, *Janthinobacterium*, *Serratia*, *Pantoea*, *Comamonas* and *Aeromonas*.

3.5. Diversity and richness comparison

In the alpha-diversity analysis, the depth of all the sequencing reads was cutoff to the same level, of which a total of 37,696 reads was retained for all samples covered. The rarefaction curves are presented in Supplementary Fig. S1 and the alpha-diversity index in Table 2. For the samples identified with 16S rRNA amplicon sequencing, independently of the extraction kit applied, the richness from the plate washing samples were both lower than the stomacheder homogenate (SH), among which richness of the stomacheder homogenate extracted using Qiagen food kit near 3 times higher than other samples. On the PCA plates from which individual colony was identified, when the Qiagen kit was applied, the highest richness was observed on the plates incubated at 30 °C aerobically, followed by those incubated at 30 °C anaerobically and 7 °C aerobically. However, using the MP soil kit examining the same sample, richness of all plates was lower compared to when Qiagen food kit was applied, and anaerobic incubation yielded the lowest richness. For the stomacheder homogenate samples, Shannon index was higher when the Qiagen food kit was used. The diversity within samples examined by MALDI-TOF MS and PW+1 amplicon sequencing was always highest on anaerobic plates in contrast to PW samples, where it showed to be kit-dependent (Table 2). The evenness index of these samples was similar, but bacterial communities on PCA plates using the Qiagen kit were mildly more variable than those analyzed with the MP kit. Compared with MALDI-TOF MS based identification, 16S rRNA amplicon sequencing identified a more diverse and even bacterial composition (Table 2) as well as family diversity

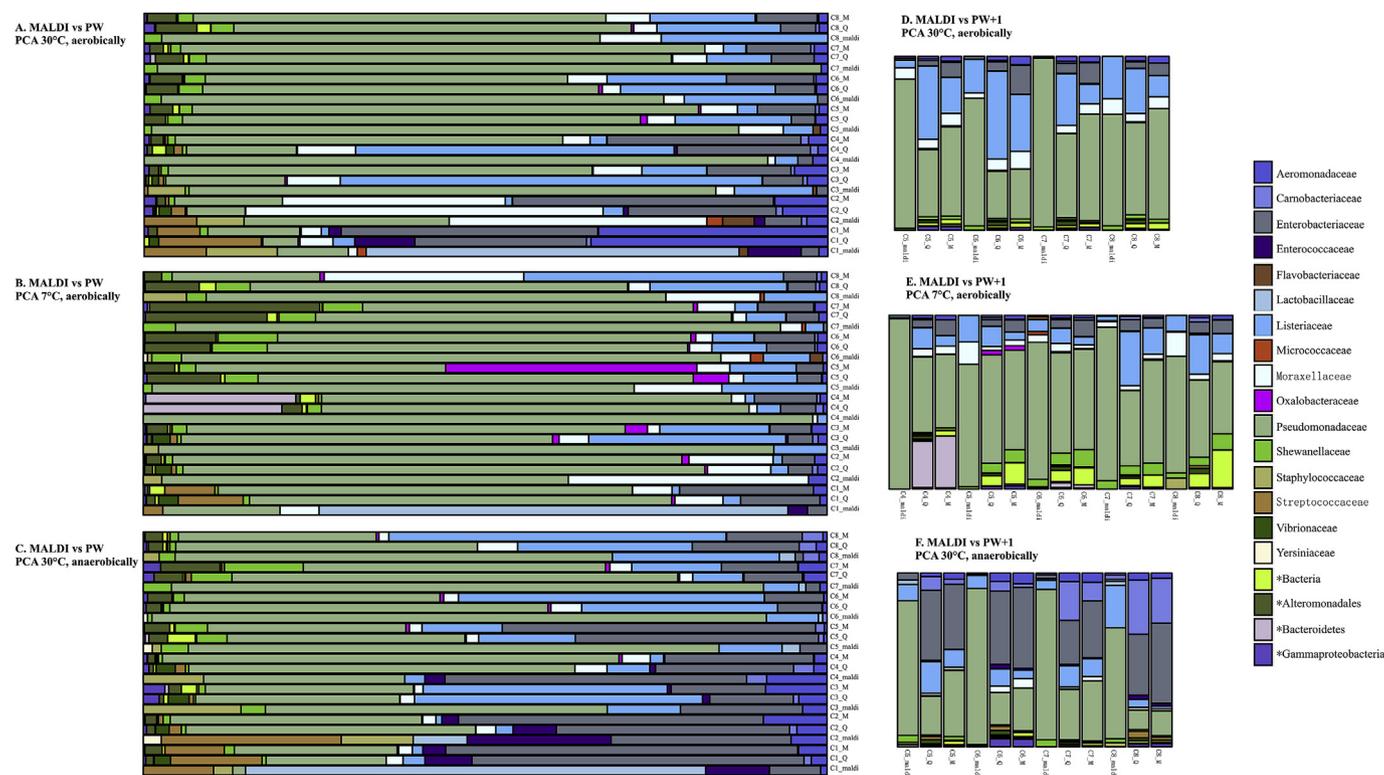


Fig. 4. Relative abundance of bacterial diversity on PCA plates incubated at three conditions (30 °C aerobically: A and D; 7 °C aerobically: B and E; 30 °C anaerobically: C and F) using MALDI-TOF MS based analysis (MALDI) and plate washing amplicon sequencing for different sample dilution (PW and PW + 1).

C: Chicken carcass.

maldi: colonies identified using MALDI-TOF MS.

Q: DNA extracted using DNeasy PowerFood Microbial kit (Qiagen).

M: DNA extracted using FastDNA[®] SPIN Kit for Soil (MP).

PW: plate washing samples with the same dilution as counting plates.

PW + 1: plate washing samples with 1 fewer dilution fold than counting plates.

Families abundance exceed 0.5% of total reads or isolates were taken into account.

*: unassigned taxonomy level.

A, B, C: Comparison of samples with same dilution between being identified with MALDI-TOF MS and plate washing amplicon sequencing (MALDI vs PW).

D, E, F: Comparison of samples identified with MALDI-TOF MS to samples with less dilution identified using plate washing amplicon sequencing (MALDI vs PW + 1).

Table 2

Alpha Diversity metrics of three analysis types (mean ± SD).

Alpha Diversity metrics		Chao1_Richness	Shannon_Diversity	Equitability_Evenness
MALDI-TOF MS ^a	30 °C	45.75	1.51	0.14
	7 °C	44.33	1.15	0.12
	Anaerobic	37.20	2.07	0.26
SH ^b		Q:468.75 ± 115.59	Q:7.52 ± 0.67	Q:0.85 ± 0.05
		M:181.38 ± 31.26	M:5.83 ± 0.38	M:0.78 ± 0.04
		Q:172.00 ± 57.90	Q:5.78 ± 0.40	Q:0.79 ± 0.05
PW ^b	30 °C	M:142.25 ± 17.43	M:5.77 ± 0.28	M:0.81 ± 0.03
		Q:147.00 ± 34.12	Q:5.74 ± 0.11	Q:0.80 ± 0.04
	7 °C	M:137.38 ± 14.36	M:5.71 ± 0.18	M:0.81 ± 0.04
		Q:160.25 ± 35.84	Q:5.90 ± 0.33	Q:0.81 ± 0.02
	Anaerobic	M:126.38 ± 20.66	M:5.56 ± 0.38	M:0.80 ± 0.04
		Q:186.50 ± 10.66	Q:5.73 ± 0.21	Q:0.76 ± 0.03
PW + 1 ^b	30 °C	M:167.50 ± 8.43	M:6.04 ± 0.12	M:0.82 ± 0.01
		Q:195.40 ± 26.71	Q:6.12 ± 0.19	Q:0.81 ± 0.01
	7 °C	M:135.20 ± 13.52	M:6.01 ± 0.15	M:0.85 ± 0.02
		Q:204.25 ± 30.97	Q:6.34 ± 0.20	Q:0.83 ± 0.02
	Anaerobic	M:150.50 ± 23.98	M:6.12 ± 0.25	M:0.85 ± 0.02

Q: DNA extracted using DNeasy PowerFood Microbial Kit.

M: DNA extracted using FastDNA[®] SPIN Kit for soil.

SH: stomached homogenate samples.

PW: plate washing samples with the same dilution as counting plates.

PW + 1: plate washing samples with 1 fewer dilution fold than counting plates.

^a Calculation on genus level using PAST software.

^b Calculation on dada2-feature using inner program of QIIME2.

(Fig. 4.). As for the conditions of incubation temperatures and anaerobic atmosphere, bacterial diversity on anaerobic plates incubated at 30 °C was less rich but more diverse and equal (even), compared with the flora isolated under other incubation conditions, independently of the identification method. Bacterial diversity related to primary sample dilution is also shown in Fig. 4. The diversity of PW was lower than PW + 1 ($p < 0.05$). Regardless of the DNA extraction kit and incubation conditions, 16S rRNA amplicon sequencing always detected a higher bacterial diversity than MALDI-TOF MS based analysis within the same fold diluted samples. Even so, dominated families detected were the same between both identification methods except for sample 1, in which Lactobacillaceae was as most dominant identified using MALDI-TOF MS based analysis. The 16S rRNA amplicon sequencing for plate washing samples enables the comparison of bacterial diversity among differently diluted samples. A more diverse composition was found with sequencing compared to MALDI-TOF MS based analysis for the isolates from more diluted samples. The impact of the primary dilution is represented by 9 genera responsible for the significantly different abundance ($p < 0.05$). Except for the genus *Pseudomonas*, which was significantly dominant in the more diluted samples identified using MALDI-TOF MS, other genera were detected as the more abundant taxa in the less diluted samples, including *Photobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Faecalibacterium*, *Rahnella* and *Staphylococcus*. Redundancy analysis result for beta-diversity is shown in Supplementary Fig. S2. In this, bacteria isolation, identification techniques and DNA extraction kits for amplicon sequencing were taken into account for various bacterial diversity of the same sample.

4. Discussion

Determination of the number of total aerobic bacteria as first indicator of bacterial contamination and estimation of shelf life is routinely performed for poultry products. In the present study, the TAB levels determined at 30 °C are in line with those reported in other studies (Balamatsia et al., 2007; Chaiba et al., 2007; del Río et al., 2007). As shown in other studies, broiler skin has a higher bacterial contamination than breast meat or legs, suggesting that the findings for carcasses can be extrapolated to other poultry products than carcasses alone. Levels of total anaerobic bacteria on poultry carcasses are rarely reported, and levels in the present study were lower than the one reported for raw meat by Pothakos et al. (2012). Concerning the *E. coli*, presumptive *Pseudomonas* spp. and lactic acid bacteria, levels were slightly higher than in other reports (Balamatsia et al., 2007; Chaiba et al., 2007; del Río et al., 2007). As in all referred studies standard isolation methods have been applied, reasons for the various contamination levels probably include food product and processing related issues such as age and breed of the birds, slaughter and processing techniques, storage time and temperature conditions before analysis.

In the present study, besides the level of bacterial contamination, also the bacterial diversity was assessed. For TAB, regardless of the incubation temperature applied, *Pseudomonas* always turned out to be the dominant genus, and was also confirmed by culture-independent approach (Fig. 4.). *Pseudomonas* as abundant genus on poultry products has been reported before (Höll et al., 2016; Rouger et al., 2018), but the results of the present study question the added value of additional examination for presumptive *Pseudomonas* species on selective plates. *Pseudomonas* is regarded as an indicator organism for spoilage, though the taxonomy of the genus is complicated, and not well defined. As also experienced in the present study, clear identification at species level remains difficult, even when MALDI-TOF MS and 16S rRNA gene sequencing are applied (Mulet et al., 2010). The species *Pseudomonas extremorientalis*, *Pseudomonas fluorescens* and *Pseudomonas fragi* were mostly reported as reliable identification (score > 2.3) by MALDI-TOF MS, and are in line with previous identifications by Rouger et al. (2017). *Brochothrix*, the second most common genus in the present study, was reported as the most dominating genus in the study of

Rouger et al. (2018) in which chicken drumsticks were examined, and identification was performed using V1-V3 amplicon sequencing. Interestingly, in the present study, the group of the stomacheder homogenate samples analyzed using the Qiagen extraction kit also identified *Brochothrix* as the dominant genus, whereas this genus was found less dominant on isolation plates than *Pseudomonas*. According to these findings, culture methods, DNA extraction as well as molecular analysis, all seem to influence the final outcome. Distinguishing 'mesophilic' from 'psychrophilic' bacterial flora showed to be impossible with current procedures, as *Pseudomonas* and *Brochothrix* exceeded more than 80% of the total bacterial isolated growth, regardless of the incubation temperature. In a limited number of samples, *Psychrobacter* was only present on PCA plates incubated at 7 °C. A few genera detected as highly abundant on PCA plates incubated at 7 °C using 16S rRNA amplicon sequencing, were not identified by MALDI-TOF MS, but only after additional 16S rRNA gene sequencing. For the case of *Psychrobacter*, there are only two strains in the Bruker commercial library, (*Psychrobacter lutiphocae* and *Psychrobacter* sp.), and this library seems unable to identify the broiler isolates, even not at genus level. A potential explanation for this is the reported the Lipid A Acyl structure consisting of outer membrane of some *Psychrobacter* species, which can change depending on the incubation temperature, possibly resulting in an altered spectrum (Sweet et al., 2015). According to the information in the Bruker database, incubation at 37 °C was applied, in contrast to 30 °C in the present study. The genus *Photobacterium* was also not identified by MALDI-TOF MS, although 16S rRNA amplicon sequencing showed that 0.7% of the total sequences detected with the analyses strategies we applied, could be attributed to this genus. Considering the correlation between initial contamination level and bacterial diversity, the contamination level of C1 to C4 is at least 1 log lower than C5 to C8. However the dominant bacteria and general diversity were similar among the samples, even when lower dilutions were taken into account with plate washing amplicon sequencing (Fig. 4.).

Concerning food safety, only the potential foodborne pathogen *Yersinia enterocolitica* was isolated on the general counting plates, and also detected by 16S rRNA amplicon sequencing analysis, but additional virulence analysis was not performed.

For the culture-dependent assessment of bacterial diversity on food matrices, massive identification of isolates by MALDI-TOF MS showed to be a much more rapid and valuable alternative than the commonly applied phenotypic based test, such as biochemical galleries or serological agglutination (Höll et al., 2016). In the present study, around 78% of the isolates could be identified using smearing as first and rapid inoculation method, or subsequently by the more time-consuming extraction. Identification even increased up to 90% when the remaining isolates when examined by 16S rRNA gene sequencing. The reason for the near 10% isolates not identified is mainly due to their pinpoint growth and consequently loss during subculture. With MALDI-TOF MS, the score for species level identification defaulted as above 2.0 showed not to be always correct as sometimes the same colonies (analysis in duplo) were identified as different species, even if the scores were above 2.0. In hence, the present study adopted the score of > 2.3 when species level was applied, as previously reported in other studies (Jeong et al., 2016).

The present study also examined the specificity of frequently used selective medium during microbiological food examination. On the *Pseudomonas* CFC plates, a few other genera were present besides *Pseudomonas*. Among these bacteria, some of them can be differentiated using classical oxidase test, as for example the genus *Serratia* (Tryfinopoulou et al., 2001). Even though the confirmation with oxidase test was performed in some studies, the presence of other oxidase positive isolates on the CFC plates, such as *Aeromonas* and *Shewanella*, could lead to the overestimation of the presence of *Pseudomonas*. On the lactic acid bacteria MRS plates, the proportion of *E. coli* and *Staphylococcus* together exceeded more than 50% resulting that the targeted LAB group were not the most abundant isolates. The presence of *E. coli* and

Staphylococcus can be explained as they can grow at pH 6.5 (Glass et al., 1992; Notermans and Heuvelman, 1983). Moreover, according to Fang et al. (1996), isolation of members of the lactic acid bacteria group can even be suppressed in the presence of *E. coli* and *Staphylococcus*. The yeast *Candida* was also present on the MRS plates and on the PCA plates incubated at 7 °C. It has been reported that when MRS plates are incubated in aerobic conditions, growth of yeasts is possible (Fitzsimmons and Berry, 1994). On the TBX plates, all typical colonies were confirmed as *E. coli*, meaning that this selective medium is reliable to perform quantitative analysis of *E. coli*. Limited presence of other bacteria (Fig. 2.) attested the powerful selectivity of TBX as reported in a previous study (Taormina et al., 1998).

Several comparisons have been performed in the present study, including the comparison of TAB level determined at 30 °C and 7 °C, the comparison of bacterial diversity identified using individual colony identification using MALDI-TOF MS and plate washing amplicon sequencing and the comparison of variations in methodology. For the initial incubation temperature to determine the contaminants on food products, there is an ongoing discussion in food industry that 30 °C incubation does not represent the relevant bacterial flora. However statistically based comparison between mesophilic and psychrophilic incubation conditions have not been performed. In the present study, TAB levels determined at 30 °C and 7 °C showed no significant difference ($p > 0.05$), implying that in contrast to this current opinion in industry, incubation temperature has no significant impact on the TAB results, at least not for broiler carcasses. Furthermore, time of analysis, and consequently time of reporting, is at least 24 h longer at 7 °C, and colonies often stay pinpointed hampering fast or automated reading. So from the point of pure quantitative bacterial analysis, there is no added value. Compared with individual colony identification by MALDI-TOF MS to examine the bacterial diversity on the plates, combination of washing the bacterial growth from the plates combined with 16S rRNA amplicon sequencing provided similar dominance profiling, but with more detailed taxonomic information. In this way, this approach could also be promising in food microbiology, as already previously reported in environmental research (Ellis et al., 2003). However, for food control, bacteria detected by culture independent methods do not necessarily prove the viability of the microorganism, and especially the presence of pathogens detected should be interpreted with caution. The present study also assessed some variations in methodology, including different DNA extraction kits and sample serial dilution. The differences in relative abundance of the stomached homogenate samples, examined directly using 16S rRNA amplicon sequencing with different DNA extraction kits (Fig. 3.), showed that DNA extraction methods have a significant impact on the final outcome of bacterial diversity and abundance, as also reported in a metagenomic methodology study for gut flora (Costea et al., 2017) and food microbiota (Rouger et al., 2017). In the present study, the Qiagen food kit provided a more diverse result, of which more abundant Gram-positive bacteria were detected compared to the MP soil kit (Fig. 3 and Table 2). An explanation might be the difference of the physical disruption between the two kits. The study of Kennedy et al. (2014) claimed that enzymatic treatment without enough physical disruption leads to reduced recovery of Gram-positive organisms and artificially elevated levels of Gram-negative bacteria. However, the bacterial diversity identified from the plate washing samples using 16S rRNA amplicon sequencing was similar, independently of the DNA extraction kits used (Fig. 4 and Table 2). This might be due to the high concentration of biomass washed liquid (PW + 1) reducing the bias during PCR processing. As reported by Chandler et al. (1997), low template concentrations were reported to generate more random fluctuations in primers efficiency. In hence, thorough cell wall disruption during the DNA extraction is necessary, especially for the low concentration samples. Evaluating the impact of dilution, one should expect the proportion of each taxa to be similar between the serial dilutions, but this was not what was observed. Some low concentration bacteria were missed along the serial dilution.

In conclusion, the present study demonstrated that determination of TAB level at 30 °C up for 72 h (ISO 4833) is best fitted to determine both the bacterial contamination level as the diversity, with *Pseudomonas* and *Brochothrix* as the dominant genera on poultry carcasses. To reveal additional psychrophilic bacteria, not only longer incubation time is needed, but the benefit is limited. For the examination of specific bacterial groups on food products, currently applied selective media might overestimate the contamination level due to the presence of non-targeted bacteria, especially for LAB. To avoid this overestimation, further confirmation should be performed. Even so, misidentification is inevitable, as for example, the oxidase positive genus *Aeromonas* isolated on CFC plates. The incorporation of MALDI-TOF MS showed to be a valid and fast method. When culture-independent methods are preferred, the impact of the DNA extraction kit applied should also be taken into account, as well as the fact that it not necessarily reflects the still viable bacterial community present.

Declarations of interest

None.

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Appendix A. Supplementary data

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