International Journal of Food Microbiology 336 (2021) 108902

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Evaluation of microbial contamination of different pork carcass areas through culture-dependent and independent methods in small-scale slaughterhouses

Maria Francesca Peruzy[a,b,c], Kurt Houf[a,c], Marie Joossens[c], Zhongjia Yu[a,c], Yolande Therese Rose Proroga[d], Nicoletta Murru[b,*]

[a] Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[b] Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Via Delpino 1, 80137 Napoli, Italy
[c] Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, Karel Lodewijk Ledeganckstraat 35, Ghent, Belgium
[d] Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute, 2, Portici, NA, Italy

ARTICLE INFO

Keywords:
Pork carcass
Salmonella spp.
Microbiological community
MALDI TOF MS
16S amplicon sequencing

ABSTRACT

Routine evaluation of the slaughter process is performed by the enumeration of the aerobic colony count, *Enterobacteriaceae* and *Salmonella* spp. on the carcass through destructive or non-destructive methods. With non-destructive methods, bacteria are counted from a minimum area of 100 cm^2 in different sampling sites on the pork carcasses, and the results of these investigated areas are pooled to one value for the complete carcass evaluation (a total of 400 cm^2). However, the composition of the bacterial community present on the different sampling areas remains unknown. The aim of the study was to characterize the microbial population present on four areas (ham, back, jowl and belly) of eight pork carcasses belonging to two different slaughterhouses through culture-dependent (Matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometry MALDI-TOF MS, combined with 16S rRNA gene sequencing) and complementary culture-independent (16S rRNA amplicon sequencing) methods. The presence of *Salmonella* spp. and *Y. enterocolitica* was additionally assessed. Using MALDI-TOF MS, *Staphylococcus*, *Pseudomonas*, and *Escherichia coli* were found to dominate the bacterial cultures isolated from the 8 carcasses. Based on the 16S rRNA amplicon sequencing analyses however, no specific genus clearly dominated the bacterial community composition. By using this culture-independent method, the most abundant genera in microbial populations of the ham, back, jowl and belly were found to be similar, but important differences between the two slaughterhouses were observed. Thus, present data suggests that the indigenous bacterial population of individual animals is overruled by the microbial population of the slaughterhouse in which the carcass is handled. Also, our data suggests that sampling of only one carcass area by official authorities may be appropriate for the evaluation of the hygienic status of the carcasses and therefore of the slaughter process.

1. Introduction

Pork carcasses and pork cuts may support the growth and serve as a source of different microorganisms which may have important consequences for the quality and safety of the product (Koutsoumanis and Sofos, 2004). The microbial load on pork carcasses strongly depends on the spread of microorganisms during the slaughtering process (stunning, bleeding, scalding, dehairing, singeing, evisceration, splitting and cooling) (Mann et al., 2016). In particular, microbial surface contamination may occur from the animals' hide and gastrointestinal tract, or from equipment, contact surfaces and slaughterhouse workers (Mrdovic et al., 2017). To reduce or inhibit the bacterial growth, the EU regulation 853/2004 establish that carcasses must be cooled down to a temperature of no more than 7 °C after the dressing stage, as 7 °C is recognized as the limit temperature below which most pathogens do not grow (Koutsoumanis and Sofos, 2004). However, the growth of some pathogens, as well as a range of food spoilage organisms, is not completely inhibited. Thus, the microbial population on the carcasses after the dressing stage is still composed of a mixture of mesophilic and psychrotrophic bacteria that may affect meat quality and cause spoilage (such as *Aeromonas*, *Brochothrix*, *Serratia*, and *Pseudomonas* spp.) and/or may be responsible of human illness (Mann et al., 2016). Among

* Corresponding author.
E-mail address: murru@unina.it (N. Murru).

https://doi.org/10.1016/j.ijfoodmicro.2020.108902
Received 6 May 2020; Received in revised form 25 September 2020; Accepted 28 September 2020
Available online 08 October 2020

pathogens, a good deal have an enteric origin (such as *Salmonella* and *Yersinia*), and the presence of them on the carcass surface is mainly the result of an improper evisceration (Choi et al., 2013; Mrdovic et al., 2017; Sánchez-Rodríguez et al., 2018). To indirectly evaluate the hygiene of the slaughter process, the EU regulation 1441/07 require the obligatory control through the excision method or the swabbing method of Total Aerobic Bacterial count (TAB) at 30 °C, of the Enterobacteriaceae and of *Salmonella* spp. on different sampling sites on the pork carcasses, sites should be chosen to target the areas with the highest level of contamination (ISO 176604/2015). In particular, the excision method (destructive) is performed incising and removing a specific sampling area of skin or tissue from the carcasses while swabbing is a non-destructive method that includes the use of absorbent material (e.g. sponges, swabs, tampons, cloths).However, while *Enterobacteriaceae* and *Salmonella* spp. indicate fecal contamination and, thus, improper evisceration, the determination of TAB at 30 °C provides only an indication about the level of culturable bacteria present on the carcasses, without giving any additional information at taxonomic level and on the bacterial diversity between the different sampling sites. Thus, as no or only some colonies are picked for further identification, knowledge on the microbial diversity of the counted microorganisms is still lacking.

Culture-independent techniques are commonly used to study complex microbial communities and therefore, to obtain information at taxonomic level on bacteria present in different ecosystems (Ercolini, 2013). Among them 16S amplicon sequencing is the most commonly employed because it is quick, simple and cost-effective (Knight et al., 2018) even if it still subject to some biases and a large number of unknown taxa are produced (Knight et al., 2018). However, culturing in combination with sequencing has been shown to enable the identification of organisms belonging to unknown taxa generated with the culture-independent methods (Lagier et al., 2016).

For the culture-dependent techniques, the 16S gene is also used for the identification of bacterial isolates. Upon isolation and DNA extraction, sequencing of the whole 16S gene of isolates is considered the "gold standard" method for identification, however, it is still expensive and, moreover, time-consuming (Singhal et al., 2015). Identification of bacterial isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid and accurate method for routine identification of clinical isolates (Cherkaoui et al., 2010), however, the database for identification is still limited to these clinical relevant microorganism.

Combined identification of bacterial strains using MALDI TOF MS and 16S gene sequencing with overall community profiling using a culture-independent method is complementary and yields important insights into the complex relationship between microorganisms in a food (Peruzy et al., 2019a; Yu et al., 2019). The aim of the present study was to evaluate the microbial diversity occurring on four sampling sites of pork carcasses slaughtered in two different slaughterhouses through the culture-dependent and independent approaches.

2. Materials and methods

2.1. Sampling

A total of 8 pork carcasses (C1 to C8) were examined. They originated from different Italian farms and were slaughtered in two different abattoirs named SA (C1-C4) and SB (C5-C8) in the Campania region of southern Italy. The slaughterhouses were regularly inspected by the competent authority and the daily production capacity of SA and SB was around 150 and 48 carcasses, respectively. The layout of the slaughter processes was similar, only differing in the singeing step. In SA, singeing was performed mechanically while in SB it was manual. Each slaughterhouse was visited twice, a week apart, to collect samples from four couples of carcasses (C1-C2, C3-C4, C5-C6 and C7-C8). From each carcass, four areas (ham (H), back (B) (loin-near the split surface), jowl (J) and belly (Y) near the split surface) of 100 cm^2 each, were swabbed using three cellulose sponges (WPB01245WA, Sigma-Aldrich) (non-destructive method) (Fig. 1) prehydrated with 10 ml sterilized peptone water (PW; CM0009, OXOID, Basingstoke, UK). One sponge per area was used for both bacterial isolation and for community DNA extraction with subsequent amplicon analyses, and the other two sponges for *Salmonella* spp. and *Yersinia enterocolitica* detection, respectively. All samples were taken from a carcass half after evisceration and trimming, but before cooling. Samples were transported at 4 °C to the laboratory and processed within one hour after sampling.

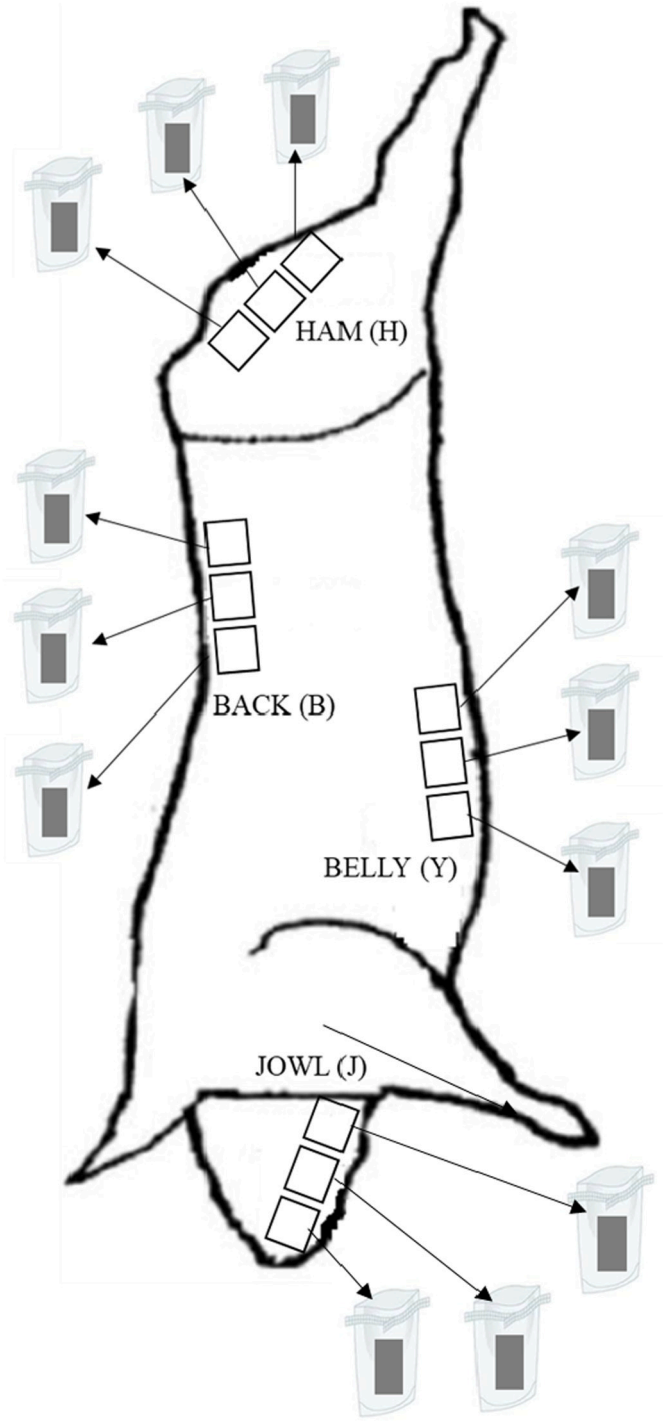

Fig. 1. The four sampling areas on each pork carcass swabbed using three cellulose sponges per area: ham (H), back (B), jowl (J) and belly (Y).

Table 1
Media and conditions used for the quantitative bacterial isolation of: mesophilic bacteria on PCA (TAB 30 °C), psychotropic bacteria on PCA (TAB 7 °C), anaerobic bacteria (TANAB), *Enterobacteriaceae* (EB) on VRBG, *E. coli* on TBX, presumptive *Pseudomonas* spp. on CFC and Lactic acid bacteria on MRS.

Parameter	Medium	Atmospheric condition	T°	Time (hours (h)/ days (d))	Reference
TAB 30 °C	Plate Count Agar (PCA; CM0325, Oxoid)	Aerobic atmosphere	30 °C	48–72-h	ISO 4833-2:2013
TAB 7 °C	Plate Count Agar (PCA; CM0325, Oxoid)	Aerobic atmosphere	7 °C	10-d	Ercolini et al., 2009
TANAB	Plate Count Agar (PCA; CM0325, Oxoid)	Anaerobic atmosphere	30 °C	48–72-h	Peruzy et al., 2019b
EB	Violet Red Bile Glucose Agar (VRBG, CM1082, Oxoid)	Aerobic atmosphere	37 °C	24-h	ISO 21528-2:2017
E. coli	Tryptone Bile X-Glucuronide (TBX, CM0945, Oxoid)	Aerobic atmosphere	44 °C	24–48-h	ISO 16649-2:2001
Pseudomonas spp.	Cephalothin-Sodium Fusidate-Cetrimide Agar with Modified CFC Selective Supplement (CFC, CM0559B with SR0103E, Oxoid)	Aerobic atmosphere	25 °C	48-h	ISO 13720:2010
LAB	De man, Rogosa and Sharpe agar (MRS, CM0361, Oxoid)	Aerobic atmosphere	30 °C	72-h	ISO 15214:1998

2.2. Bacterial isolation

Per carcass, one sponge for each area was soaked in 90 ml PW (1:10 W/W) in a stomacher bag and homogenized for three minutes at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France). An aliquot of homogenates was used for community DNA extractie (see further). In parallel, ten-fold serial dilutions of each homogenate were prepared in PW, followed by bacterial count in duplicate for the different analyses summarized in Table 1.

The colonies were counted, picked from the agar plates, subcultured on Tryptic Soy Agar (TSA, CM0131, Oxoid) or MRS and incubated at the appropriate conditions as described above.

2.3. MALDI-TOF MS identification and data analysis (culture-based)

Isolates were first analyzed using the "direct colony identification method" (Alatoom et al., 2011). In brief, bacterial growth was smeared in duplicate onto a 96-spotsteel plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Subsequently, the sample was covered with 1 μl matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoracetic acid (50:47.5:2.5, [vol/vol/vol]). Bruker's Bacterial Test Standard (BTS155 255343; Bruker Daltonics) was used as mass calibration and reference standard before each series of MALDI measurements.

Samples were processed in the Microflex™ LT MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (l1/ 4337 nm) operating in linear positive ion detection mode using MALDI Biotyper Automation Control 2.0 (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. Identification score criteria were classified according to Jeong et al. (2016) with a log (score) values of ≥2.3 indicating highly probable species identification, between 2.0 and 2.3 secure genus and probable species, a score between 1.7 and 2.0 probable genus and < 1.7, non-reliable identification. Isolates for which a score of less than 2.0 was obtained with the direct colony method, and the isolates on MRS agar plates (Alatoom et al., 2011) were analyzed using the "Ethanol/Formic Acid extraction" procedure (03.04.2006) from Bruker Daltonics, further referred to as "the protein extraction method". Individual colonies were suspended in 800 μl of TSB and onto MRS broth and incubated at 28 °C for 24 h. Subsequently, the samples were centrifuged (1533*g* at 4 °C) for 10 min. The supernatant was discarded and the pellet was washed twice in 500 μl of Milli-Q water and centrifuged (1533*g* at 4 °C) for 10 min. After the second centrifugation, the supernatant was removed and the pellet suspended in 100 μl of Milli-Q water. Next, 50 μl formic acid and 50 μl of acetonitrile were added to the pellet and mixed thoroughly by pipetting, followed by centrifugation (1533*g* at 4 °C) for 10 min. One microliter of the supernatant was spotted onto a 96-spot plate and allowed to dry at room temperature. Afterward, 1 μl of matrix solution was added. The analysis was repeated when the spots resulted in 'no peaks found'.

Spectra of the isolates for which again a MALDI TOF MS score below 1.7 was obtained, were imported into the BioNumerics 7.2.6 software (Applied Maths, Sint-Martens-Latem, Belgium) to perform a dereplication in order to select representatives for further analysis. For this, the Pearson correlation coefficient was applied and curve-based analysis was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm. Based on dendrogram distance level settings and best matches ranking, representative isolates were selected for subsequent 16S rRNA gene sequencing. DNA was extracted using alkaline lyses where one colony was suspended into 20 μl of lysis buffer (2.5 ml 10%SDS, 5 ml 1 N NaOH and 92.5 ml Milli-Q water and incubated for 15 min at 95 °C. After a short spin, 180 μl Milli-Q water) were added. Subsequently, the suspension was centrifuged for 5 min at 10,000 ×*g* at 4 °C. To amplify the 16S rRNA gene, 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. 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 to (Eurofins) for Sanger sequencing. Taxonomic identity was recorded by blasting the sequences to NCBI (https://www.ncbi.nlm.nih.gov).

2.4. Community identification by 16S rRNA amplicon sequencing (culture-independent method)

To characterize the microbial community on the carcass's samples, DNA was extracted from 1.8 ml of each homogenate obtained as described above using the PowerFood Microbial DNA Isolation kit (Qiagen, Germany) following manufacturer's recommendations. The DNA quantity was measured using the dual-channel Quantus™ Fluorometer (Promega USA) and for DNA purity, the ratio of absorbance at 260 nm and 280 nm was evaluated with NanoDrop™ 2000/ 2000c Spectrophotometers (Thermo Fisher Scientific Inc., Waltham, MA, USA).

All DNA extracts fulfilled the quality requirements (amount ≥ 100 ng, concentration ≥ 5 ng/μl and volume ≥ 20 μl (OD260/280 = 1.8–2.0)) therefore, they were all submitted to Novogene (HK) Company Limitation for 16S rRNA amplicon sequencing (https://en.novogene.com). The V3-V4 region of the 16S rRNA gene was amplified with primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) for library construction. Truseq-dna-pcr-free-library-prep kit was used to construct the amplicon libraries of paired-end reads using single index. Amplicons were mixed in equimolar amounts and sequenced on the Illumina2500 platform with Sequencing strategy PE250.

Qiime2 (version 2018.6) 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 for the quality control process, and all phiX reads and chimeric sequences were filtered. Based on demux summary, sequences of 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/).

2.5. Detection of pathogenic bacteria

The detection of *Salmonella* spp. was performed from one sponge from each carcass area using the reference analytical microbiological methods ISO 6579 -1:2017 and the isolates were sent to the *Salmonella* Typing Centre of the Campania Region (Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, NA, Italy) for serotyping following the Kaufmann-White scheme (Le Minor and Popoff, 1987).

The detection of *Yersinia enterocolitica* was performed through Quantitative (q) PCR. In particular, one sponge for each area was homogenized once in 90 ml (1:10 W/W) Peptone Sorbitol Bile Broth (PSB, 17192, Sigma-Aldrich). One volume of the homogenate was used for DNA extraction, the remaining were stored at 4 °C. DNA was extracted using the Chelex-100-resin method (1422822, Biorad, Hercules, CA, USA), where 2 ml of each homogenate was transferred into a 2 ml centrifuge tube, and centrifuged for 10 min at 10,000 ×*g* at 4 °C. The supernatant was discarded, the pellet re-suspended in 300 μl of 6% Chelex 100 by vortexing, and incubated for 20 min at 56 °C and again for 8 min at 100 °C. The suspension was immediately chilled on ice for 1 min, and centrifuged for 5 min at 10,000 ×*g* at 4 °C. In order to evaluate the presence of *Y. enterocolitica* 4/O:3 and Biotype 1A, SYBR green PCR was conducted (Peruzy et al., 2017). The gene *ystA* was used as a target for the pathogenic biotype and three μl of DNA extracted were added to 22 μl of PCR mix. The mastermix contained 12.5 μl of Qiagen QuantiTect SYBR Green PCR Kit (1×), 100 nM of both primers ystA-F (5′-ATCGACACCAATAACCGCTGAG-3′) and ystA-R (5′-CCAATC ACTACTGACTTCGGCT-3′). To evaluate the presence of the biotype 1A, the presence of the target gene *ystB* gene was likewise examined, using 150 nM of both primers ystB-F (5′-GTACATTAGGCCAAGAGACG-3′) and ystB-R (5′-GCAACATACCTCACAACACC-3′) (Peruzy et al., 2017). The amplification protocol consisted of 15 min at 95 °C, followed by 35 cycles of amplification (95 °C for 10 s, 60 °C for 30 s). The melting analysis was done with a temperature range of 65 °C to 95 °C for 1 cycle, acquiring data at each degree. At least one positive, one negative and one blank were included in each run. The fluorescence of SYBR Green and the melting curve were generated using the CFX96 system (Bio-Rad). A specific melting temperature (T_m) of 78.5 ± 1 °C indicated a positive result. qPCR positive results, were confirmed using the corresponding reference analytical microbiological methods ISO 10273:2017.

2.6. Statistical analysis and visualization

To compare the bacterial counts one-way analysis of variance (ANOVA) was calculated by PAST software package (https://folk.uio.no/ohammer/past/). A probability value of less than 0.05 ($p < 0.05$) was defined as statistically significant. PAST was also used to calculate the richness expressed by the Chao1 index and diversity indices of community information obtained from 16 rRNA amplicon sequencing. Rstudio 1.0.153 (https://www.rstudio.com) was used to run beta diversity and dissimilarity analysis. Phyloseq was applied for visualization of these analyses.

3. Results

3.1. Bacterial isolation

Bacterial counts for the 8 carcasses (C1-C8), per sampling point and per slaughterhouse are shown in Table 2. The mean (± SD) of the total aerobic counts on PCA ranged from 3 ± 0.45 (C4) to 5.36 ± 0.05 (C2) log CFU/cm^2, from 1.44 ± 1.68 (C2) to 3.38 ± 0.52 (C3) log CFU/cm^2 and from 2.54 ± 0.63 (C7) to 3.84 ± 0.80 (C8) log CFU/cm^2 for mesophilic, psychrotrophic and anaerobic bacteria, respectively. The count of typical purple/pink *Enterobacteriaceae* colonies on VRBG and the blue *E. coli* colonies on TBX ranged from 0.89 ± 1.02 (C4) to 3.28 ± 0.62 (C3) log CFU/cm^2 and from 0.63 ± 0.77 (C5) to 3.50 ± 0.35 (C3) log CFU/cm^2, respectively. The mean (± SD) number of bacterial colonies present on CFC and MRS agar plates ranged from 1.47 ± 1.78 (C7) to 3.61 ± 0.09 (C2) log CFU/cm^2 and from < 2 log (C1, C4, C7 and C8) to 2.75 ± 1.18 (C5) log CFU/cm^2, respectively. However, the contamination levels on the 8 carcasses did not show a significant difference ($p > 0.05$).

Of the four investigated areas, Back and Jowl presented respectively the lowest and the highest contamination but observed differences were not significant ($p > 0.05$).

Slaughterhouse A showed a higher level of contamination with TAB 30 °C, *Enterobacteriaceae*, *E. coli*, *Pseudomonas* spp., and LAB compared to slaughterhouse B. However, the bacterial count of the carcasses (C1-C4) sampled at SA and of carcasses (C5-C8) sampled at SB did not show a significant difference ($p > 0.05$).

Table 2
Bacterial counts per cultivation condition: per pork carcasses (C1-C8) (i), per the sampling area (ii) and per the slaughterhouse (SA, SB) (iii). Bacterial enumeration on different media: mesophilic bacteria on PCA (TAB 30 °C), psychotropic bacteria on PCA (TAB 7 °C), anaerobic bacteria (TANAB), *Enterobacteriaceae* (EB) on VRBG, *E. coli* on TBX, presumptive *Pseudomonas* spp. on CFC and Lactic acid bacteria (LAB) on MRS.

	i	ii		iii	
	Bacterial counts in 8 carcasses (C1-C8)[a]	Bacterial counts depending on the sampling point		Bacterial counts depending the slaughterhouse	
	Mean ± SD log CFU/cm^2	Minimum	Maximum	SA	SB
		Mean ± SD log CFU/cm^2 (s.p.[b])		Mean ± SD log CFU/cm^2	
TAB 30 °C	3.87 ± 0.78	3.6 ± 0.77 (B)	4.14 ± 0.86 (J)	4.01 ± 0.93	3.74 ± 0.60
TAB 7 °C	2.84 ± 0.94	2.53 ± 0.53 (B)	3.07 ± 0.37 (Y)	2.64 ± 1.19	3.03 ± 0.58
TANAB	3 ± 0.83	2.57 ± 1.15 (H)	3.24 ± 0.67 (B)	2.90 ± 0.96	3.10 ± 0.70
EB	1.80 ± 1.09	1.52 ± 1.20 (B)	2.04 ± 0.41 (J)	2.15 ± 1.24	1.45 ± 0.82
E. coli	1.77 ± 1.38	1.30 ± 1.54 (B)	2.36 ± 1.04 (J)	2.50 ± 1.40	1.05 ± 0.92
Pseudomonas spp.	2.54 ± 1.03	1.92 ± 1.28 (B)	2.98 ± 0.56 (J)	3.04 ± 0.65	2.33 ± 0.82
LAB	1.2 ± 1.38	1.01 ± 1.40 (B)	1.53 ± 1.37 (Y)	2.51 ± 0.58	2.56 ± 0.84

[a] Considering the results of the investigated areas (H, B, J, and Y) pooled to one value.
[b] s.p.: sampling point (H: ham; B: back; J: jowl; Y: belly).

3.2. MALDI-TOF MS identification and data analysis (culture-based)

Colonies were all picked from agar plates with bacterial growth between 30 and 300 CFU/plate on PCA, and between 15 and 150 CFU for all other plates. A total of 2620 bacterial isolates from 4 areas (H, B, J, and Y) of 8 pork carcasses (C1-C8) were analyzed by MALDI-TOF MS. In particular, 739 of these were harvested from the hams, 451 from the backs, 709 from the jowls and 721 from the bellies. Using the "direct colony identification method" on 2567 colonies, namely all the isolates except those from MRS, 15% (n = 394) had a highly probable species identification, 32% (n = 816) secure genus and probable species, 19% (n = 488) were identified at genus level and 34% (n = 869) did not have any identification. The colonies with a score value < 2 (n = 1357) were repeated by "the protein extraction method" of which 9% (n = 119) were additionally identified at species level, 49% (n = 671) at genus level, 31% (n = 415) at probable genus level and 11% (n = 152) could still not be identified.

The analysis of the isolates on the MRS agar plates (n = 53) performed only with the "the protein extraction method" resulted in the identification at species level, at genus level and at probable genus of the 9.4% (n = 5), 45.3% (n = 24), and 45.3% (n = 24) of the colonies respectively.

In general, bacteria identified by MALDI-TOF MS could be assigned to 27 families and 39 genera, and among the isolates with a MALDI-TOF MS score value of ≥2.3, 36 species were reliably identified (Table 3).

Moreover, *Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Trichosporon* spp. and *Yarrowia* spp. belonging to the fungi kingdom were also isolated. *Candida* spp. was isolated in 13 samples (C2B, C2Y, C3B, C3H, C3J, C3Y, C5B, C5Y, C6J, C6Y), *Yarrowia* was isolated in C3J and C4J and *Cryptococcus*, *Rhodotorula* and *Trichosporon* in C6H, C5J and C3Y respectively. Among the isolates without MALDI TOF MS identification but analyzed with 16S rRNA gene sequencing (n = 50) two families (*Dermabacteraceae* and *Sanguibacteraceae*) and four genera (*Brachybacterium*, *Leucobacter*, *Psychrobacter* and *Sanguibacter*) were additionally identified.

In general, *Staphylococcus* was the dominant genus on PCA at 30 °C (46%), *Pseudomonas* was the dominant genus on PCA at 7 °C (70%) and on CFC (72%) and *Escherichia* was the dominant genus on PCA incubated in anaerobic conditions (63%), on VRBG (81%) and on TBX (100%). On MRS the fungus Candida was commonly identified (77%) (Fig. 2).

As far as the distribution of the microbiota on the carcasses between the four sampling points is concerned, 27, 21, 22 and 34 bacterial genera were isolated on ham, back, jowl and belly respectively using culture. Among the mesophilic population, *Pseudomonas* (36%) and *Rothia* (16%) were the dominant genera on ham and *Staphylococcus* was the dominant genus on the back (65.91%), on the jowl (45%) and on the belly (55%). Among the psychrotrophic population *Pseudomonas* was the dominant genus in all four sampling areas (H = 85%, B = 50%, J = 55% and Y = 61%). On PCA incubated anaerobically (TANAB) *Escherichia* was the dominant genus on ham (90%) and on the back (95%). *Escherichia* (51%) and *Staphylococcus* (43%) were the dominant genera on jowl, while *Escherichia* (28%), along with the genera *Citrobacter* (24%) and *Enterobacter* (16%) was frequently isolated on the belly.

As far as the differences between the two slaughterhouses, a total of 30 and 31 bacterial genera were isolated on samples from slaughterhouse A and slaughterhouse B, respectively using culture. In particular, 12 bacterial genera were isolated only in carcasses sampled in slaughterhouse A and 13 in the carcasses belonging to slaughterhouse B. On the carcasses belonging to slaughterhouse A (C1 to C4), *Staphylococcus* (64%), *Pseudomonas* (81%) and *Escherichia* (65%) were the dominant genera on PCA at 30 °C, on PCA at 7 °C and on PCA incubated in an anaerobic condition, respectively. On the carcasses belonging to slaughterhouse B (C5 to C8), *Rothia* (31%) along with the genera *Acinetobacter* (19%) and *Microbacterium* (16%) were the dominant genera on PCA at 30 °C. *Pseudomonas* (46%) along with *Acinetobacter* (27%) and *Psychrobacter* (16%) were commonly isolated among the psychrotrophic population and *Escherichia* (56%) and *Staphylococcus* (32%) were the dominant genera on PCA at 30 °C, on PCA at 7 °C and on PCA incubated in an anaerobic condition, respectively.

3.3. Community identification by 16S rRNA amplicon sequencing (culture-independent method)

Using 16S rRNA amplicon sequencing, a total of 2,451,630 reads were obtained. Only amplicon sequence variants (OTUs) accounting for more than 0.5% (> 0.5% = 1,797,210 reads) of the total reads were taken in to account for the analyses. These reads could be assigned to 26 genera and 22 families. The top abundant genera, collectively representing more than 50% of the total microbial diversity observed, were *Brochothrix* (23%), *Pseudomonas* (8%), *Rothia* (4%), *Acinetobacter* (4%), *Psychrobacter* (4%), *Chryseobacterium* (2%), *Shewanella* (2%), *Bacteroides* (1%), *Corynebacterium* (1%), *Gluconacetobacter* (1%) and *Paracoccus* (1%) spp. Opposite to the contamination level, using sequencing the lowest bacterial richness was observed in a Jowl sample (C2J, Chao 1 index = 68), while the highest in Belly (C5B, Chao 1 index = 376). Diversity indices, expressed by evenness (mean ± SD = 0.87% ± 0.06) and Shannon (mean ± SD = 6.45% ± 0.97) indices, indicate that sample C4J and C2J showed the lowest evenness and Shannon indices as they were dominated by *Brochothrix* (85%) and *Psychrobacter* spp. (61%) respectively, while C5Y showed the highest diversity indices as no genera clearly dominate the sample.

Members of the *Brochothrix* genus, along with the genera *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, and *Rothia* spp., were the most abundant genera in each of the different sampling sites (ham, back, jowl and belly) (Fig. 3). Ham (Chao 1 index: mean ± SD = 154.74 ± 65.37; Shannon index: mean ± SD = 6.22 ± 0.73) and back (Chao 1 index: mean ± SD = 199 ± 105.13; Shannon index: mean ± SD = 6.66 ± 0.89) showed respectively the lowest and highest Chao1 and Shannon indices. While the back showed the highest evenness indices (mean ± SD = 0.89 ± 0.04), the ham (mean ± SD = 0.87 ± 0.04), jowl (mean ± SD = 0.87 ± 0.01) and belly (mean ± SD = 0.87 ± 0.06) showed the same.

The analysis of the genera obtained from all samples collecting in Slaughterhouse A showed a lower (mean ± SD = 111.19 ± 23.87) richness than slaughterhouse B (mean ± SD = 252.19 ± 71.37). Moreover, Slaughterhouse A showed the lowest evenness (mean ± SD = 0.84% ± 0.06) and Shannon (mean ± SD = 5.67% ± 0.54) indices, as it was dominated by *Brochothrix* spp. accounting for 53% of the total reads, whereas Slaughterhouse B showed the highest evenness (mean ± SD = 0.91% ± 0.03) and Shannon (mean ± SD = 7.24% ± 0.59) indices and, indeed, no clearly dominating taxa were observed in the samples processed in this slaughterhouse. Moreover, according to the beta diversity and dissimilarity analysis the composition of the detected microbial communities was highly distinct between both slaughterhouses (Fig. 4).

3.4. Detection of pathogenic bacteria

Using qPCR, *Salmonella* was isolated in 7 out of 8 carcasses. Particularly, the pathogen was isolated in 3 carcasses belonging to slaughterhouse A and in all the carcasses sampled in slaughterhouse B. *Salmonella* spp. were isolated in 50% (n = 4 carcasses) at ham, in 50% (n = 4 carcasses) at back, in 88% (n = 7 carcasses) at jowl and in 38% (n = 3 carcasses) at belly site (Table 4). After serotyping 7 *Monophasic S.* Typhimurium, 6 *S.* Brandenburg, 4 *S.* Derby and 1 *S.* Rissen were identified (Table 4). Interesting, the same serotype was always isolated at the different sampling sites from the couple of carcasses sampled the same day (C1-C2, C5-C6 and C7-C8). Concerning *Y. enterocolitica*, the gene *ystA* was never detected in the samples, though *ystB* was present on the ham of one carcass (C1).

Table 3
Number and percentage of isolates identified at probable genus level (1.7 ≥ Log score < 2.0), secure genus and probable species (2.0 ≥ Log score < 2.3) and highly probable species identification (Log score ≥ 2.3).

Genera (bacteria)	Species	Log score ≥ 1,7		≥2 Log score < 2,3		Log score ≥ 2,3	
		n.	%	n.	%	n.	%
Acinetobacter spp.		122	4,66				
	A. guillouiae			4	0,26	3	0,58
	A. haemolyticus			1	0,07		
	A. johnsonii			33	2,18	28	5,41
	A. junii					1	0,19
	A. lwoffii			28	1,85	2	0,39
Aerococcus spp.		5	0,19				
Aeromonas spp.		2	0,08				
	A. veronii			2	0,13		
Alcaligenes spp.		9	0,34				
	A. faecalis			1	0,07		
Arthrobacter spp.		3	0,11				
	A. ardleyensis			1	0,07		
	A. arilaitensis					1	0,19
	A. bergerei			1	0,07		
Brevibacterium spp.		4	0,15				
	B. casei			2	0,13		
Brevundimonas spp.		3	0,11				
	B. diminuta			2	0,13	1	0,19
Brochothrix spp.		11	0,42				
	B. thermosphacta			8	0,53	2	0,39
Buttiauxella spp.		7	0,27				
	B. agrestis			2	0,13		
	B. gaviniae			3	0,20		
Carnobacterium spp.		2	0,08				
	C. maltaromaticum					2	0,39
Chryseobacterium spp.		12	0,46				
	C. piscium			3	0,20		
	C. scophthalmum			5	0,33	1	0,19
Citrobacter spp.		8	0,31				
	C. braakii			7	0,46	1	0,19
Corynebacterium spp.		2	0,08				
Delftia spp.		1	0,04				
	D. acidovorans			1	0,07		
Enterobacter spp.		4	0,15				
	E. asburiae			4	0,26		
Enterococcus spp.		3	0,11				
	E. cecorum			1	0,07		
	E. faecalis			1	0,07		
	E. faecium			1	0,07		
Escherichia spp.		560	21,37				
	E. coli			214	14,16	341	65,83
	E. fergusonii					1	0,19
Janthinobacterium spp.		1	0,04				
Klebsiella spp.		11	0,42				
	K. pneumoniae			2	0,13	9	1,74
Kocuria spp.		67	2,56				
	K. palustris			4	0,26	1	0,19
	K. rhizophila			42	2,78	3	0,58
	K. salsicia			6	0,40	1	0,19
Lelliottia spp.		7	0,27				
	L. amnigena			2	0,13	5	0,97
Leuconostoc spp.		19	0,73				
	L. mesenteroides			9	0,60	9	1,74
Luteococcus spp.		1	0,04				
	L. japonicus			1	0,07		
Macrococcus spp.		19	0,73				
	M. caseolyticus			1	0,07		
Microbacterium spp.		147	5,61				
	M. foliorum			1	0,07		
	M. liquefaciens			56	3,71	14	2,70
	M. maritypicum			24	1,59	13	2,51
	M. mitrae			1	0,07		
	M. oxydans			12	0,79	5	0,97
	M. paraoxydans			1	0,07		
	M. phyllosphaerae			4	0,26	1	0,19
Micrococcus spp.		5	0,19				
	M. luteus			4	0,26		
Moraxella spp.		1	0,04				
	M. osloensis			1	0,07		

(*continued on next page*)

Table 3 (*continued*)

Genera (bacteria)	Species	Log score ≥ 1,7		≥2 Log score < 2,3		Log score ≥ 2,3	
		n.	%	n.	%	n.	%
Ochrobactrum spp.		1	0,04				
Pantoea spp.		12	0,46				
	P. agglomerans			11	0,73	1	0,19
Pseudochrobactrum spp.		3	0,11				
	P. asaccharolyticum			2	0,13	1	0,19
Pseudomonas spp.		464	17,71				
	P. aeruginosa			1	0,07	1	0,19
	P. brenneri			8	0,53		
	P. cedrina fulgida			1	0,07		
	P. extremorientalis			10	0,66		
	P. fluorescens			5	0,33		
	P. fragi			42	2,78	8	1,54
	P. fulva			5	0,33	1	0,19
	P. gessardii			4	0,26	2	0,39
	P. kilonensis			1	0,07		
	P. libanensis			82	5,43		
	P. lundensis			52	3,44		
	P. orientalis			2	0,13		
	P. poae			1	0,07		
	P. proteolytica			1	0,07		
	P. putida					1	0,19
	P. synxantha			66	4,37	10	1,93
	P. taetrolens			3	0,20		
	P. tolaasii			8	0,53		
	P. trivialis			1	0,07		
Rothia spp.		132	5,04				
	R. endophytica			83	5,49	21	4,05
	R. terrae					1	0,19
Shewanella spp.		14	0,53				
	S. baltica			14	0,93		
Sphingobacterium spp.		5	0,19				
	S. daejeonense			1	0,07		
	S. faecium			1	0,07		
Staphylococcus spp.		728	27,79				
	S. aureus			3	0,20	1	0,19
	S. capitis			1	0,07		
	S. chromogenes			2	0,13		
	S. epidermidis			14	0,93		
	S. haemolyticus			2	0,13		
	S. hominis			2	0,13		
	S. pasteuri			17	1,13		
	S. saprophyticus			2	0,13		
	S. simulans			4	0,26		
	S. warneri			25	1,65		
	S. xylosus			511	33,82	20	3,86
Stenotrophomonas spp.		4	0,15				
	S. maltophilia			2	0,13		
Streptococcus spp.		1	0,04				
Vagococcus spp.		2	0,08				
	V. fluvialis			1	0,07	1	0,19
Yersinia spp.		1	0,04				
	Y. massiliensis					1	0,19
Genera (fungi)	Species (Fungi)						
Candida spp.		48	1,83				
	C. parapsilosis			2	0,13		
	C. zeylanoides			21	1,39	3	0,58
Cryptococcus spp.		1	0,04				
Rhodotorula spp.		2	0,08				
Trichosporon spp.		1	0,04				
Yarrowia spp.		13	0,50	1	0,07		
Organism no identified		152	5,80				

4. Discussion

According to the EC Regulation No.1441/07, TAB 30 °C is supposed to be, along with the *Enterobacteriaceae*, an indicator of the slaughter hygiene process. In the present study, although the counts were slightly higher in slaughterhouse A, no significant differences were observed between the two slaughterhouses ($p > 0.05$). In order to enable comparison with other studies, when pooling the results of the investigated areas to one value for complete carcass evaluation, the mean of TAB 30 °C was within the satisfactory range (< 4.0 log CFU/cm^2) in 7 carcasses (87%) and it was unsatisfactory (> 5.0 log CFU/cm^2) in one carcass (13%) (C2, mean ± SD = 5.32 ± 0.05 log CFU/cm^2). According to microbial limits (m = minimum and M = maximum) imposed by the European legislation (EC Regulation No.1441/07), the *Enterobacteriaceae* count on VRBG agar plates was within the satisfactory range (m < 2.0 log CFU/cm^2) in 6 carcasses (75%), within the acceptable range (m < 2.0 log CFU/cm^2 and M < 3.0 log CFU/cm^2) in one carcass (13%) (C1, mean ± SD = 2.63 ± 0.60 log CFU/cm^2)

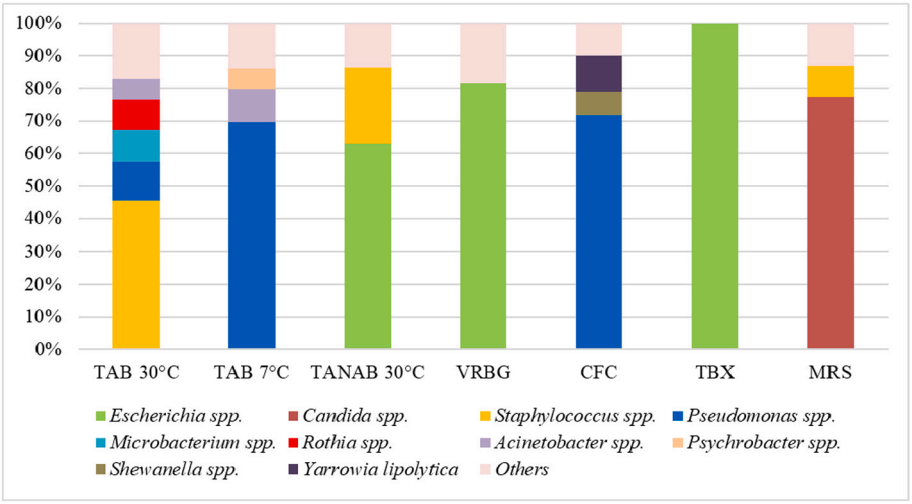

Fig. 2. Most abundant genera observed using cultivation on the 8 pork carcasses. Genera with a relative abundance ≥5% at genus level identified by MALDI-TOF MS and 16S gene sequencing on the 8 carcasses on different agar plates: plate count agar (PCA) at 30 °C (TAB 30 °C), PCA at 7 °C (TAB 7 °C), PCA under anaerobic conditions (TANAB 30 °C), *Enterobacteriaceae* on VRBG, *E. coli* on TBX, presumptive *Pseudomonas* spp. on CFC and Lactic acid bacteria on MRS. Genera with a relative abundance < 5% were summed and are denoted as 'others'.

and it was unsatisfactory (> 3.0 log CFU/cm^2) in one carcass (C3, mean ± SD = 3.28 ± 0.62 log CFU/cm^2). The present TAB results are in accordance with Lindblad et al. (2007), but they were higher compared to the results of Mrdovic et al. (2017). Moreover, the carcasses sampled in the present study showed a high level of fecal contamination and, indeed, the mean count of *Enterobacteriaceae* on VRBG and *E. coli* on TBX was higher than the results of the previously cited studies (Lindblad et al., 2007; Mrdovic et al., 2017). Statistical analysis showed that the counts on VRBG, on TBX and on PCA incubated under anaerobic conditions were not significantly different ($p > 0.05$) and indeed, more than 81% and more than 60% of the bacteria grown on VRBG and PCA incubated under anaerobic conditions, respectively, belonged to *E. coli*. A low level of bacteria on PCA incubated at 7 °C and on CFC was recorded, probably because the carcasses were sampled before the cooling process and the probable growth of psychrotrophic bacteria had not started yet.

In contrast to the study of Biasino et al. (2018) the different carcass areas did not show difference in the contamination level here; however, in line with Biasino et al. (2018) the back site was the least contaminated with TAB 30 °C, *Enterobacteriaceae*, and *E. coli*. This is not surprising because in the back area there is no contact with the digestive tract and compared to the other areas the handling by the food business operator and/or the contact of the carcass with the equipment and contaminated surfaces is less extensive. The jowl, on the contrary, resulted in being the area most highly contaminated with mesophilic bacteria, *Pseudomonas* spp. on CFC, *Enterobacteriaceae* and, *E. coli*. Moreover, the jowl also resulted as the area most highly contaminated with *Salmonella*. A possible explanation for this may be that during the evisceration process the gastrointestinal tract, before its complete removal, could be suspended inverted above the head and contaminate the jowl or it could be due to the proximity of this part to the wet floor (Wheatley et al., 2014).

In the present study, all the carcasses except one were contaminated by *Salmonella*. According to EC Regulation No.1441/07 research on this pathogen on carcasses is performed in order to evaluate the hygienic status of the slaughterhouse. This means when the carcasses are *Salmonella*-contaminated, sanitary authorities have to take measures to improve the process hygiene but they can be still placed on the market. High number of *Salmonella*-contaminated carcasses was also reported in Belgium by Biasino et al. (2018) (64%) but low *Salmonella* prevalence was previously observed in another study in Belgium (Ghafir and Daube, 2008) (8.9%), in Serbia (Choi et al., 2013) (1%) and in Korea (Mrdovic et al., 2017) (0,29%). After serotyping, *S.* Brandenburg, *S.* Derby, and Monophasic *S.* Typhimurium, were identified in three

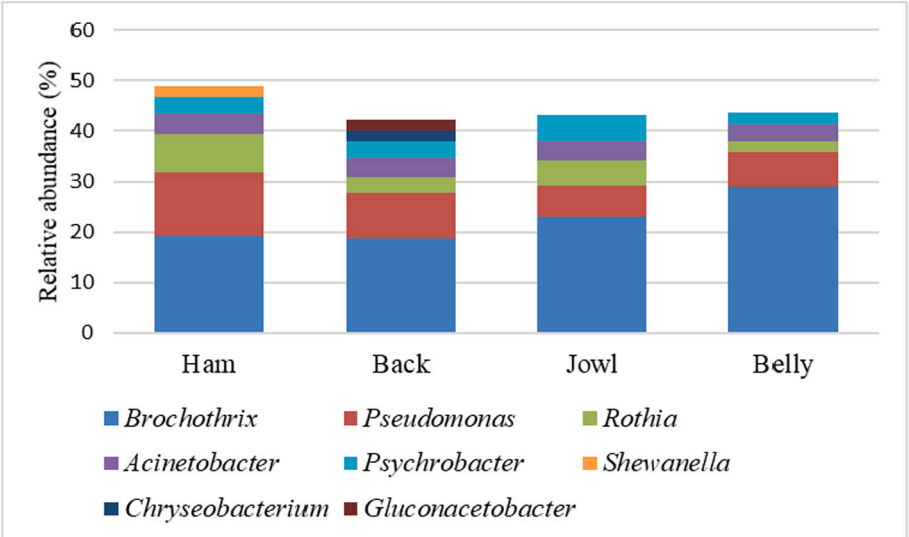

Fig. 3. Microbial composition on the pork carcasses (C1-C8) per sampling area (Ham, Back, Jowl and Belly) identified by 16S rRNA amplicon sequencing. Average microbial communities with a relative abundance ≥2% are shown.

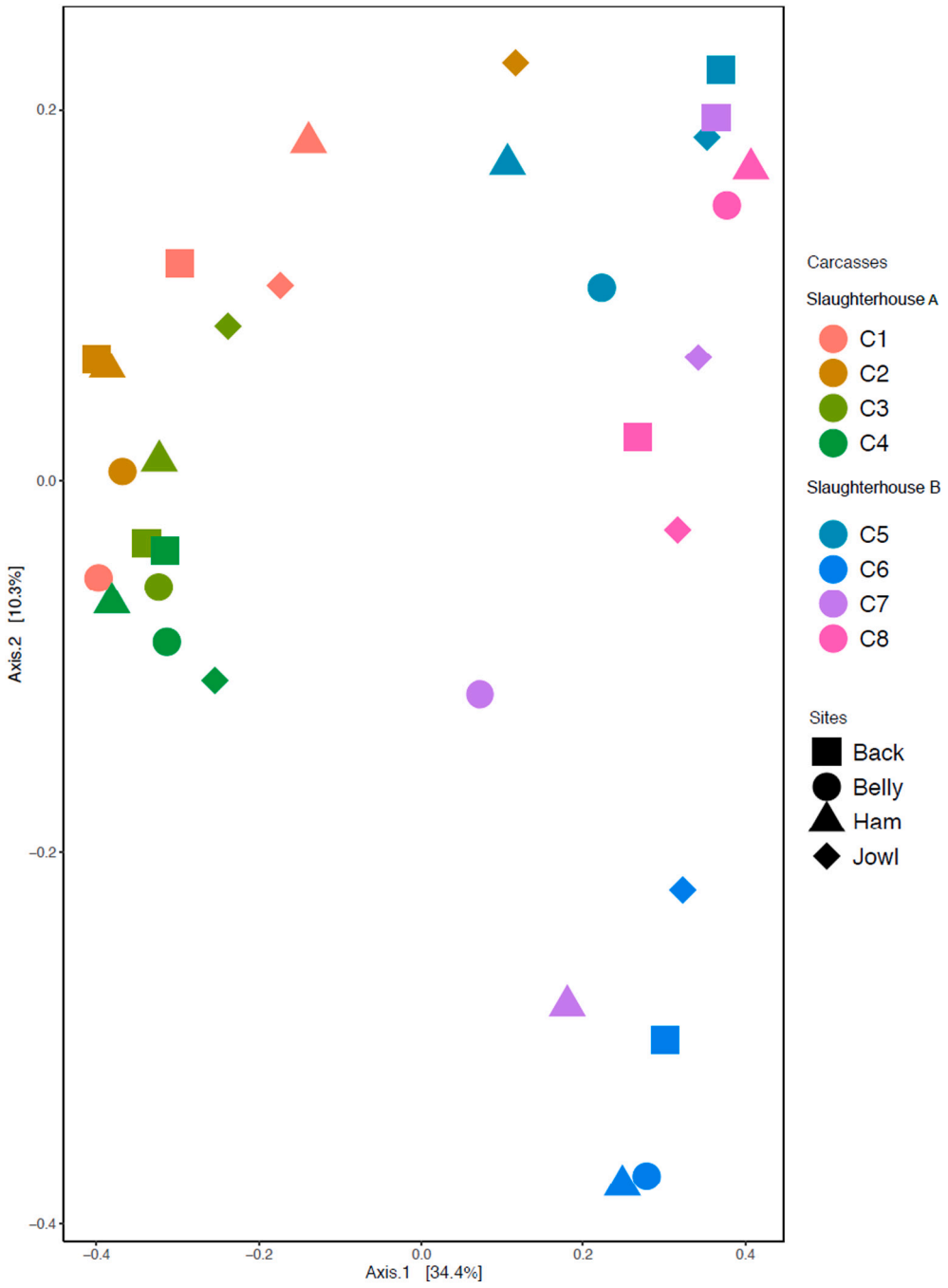

Fig. 4. Visualization of the beta diversity and dissimilarity analysis between the four sampling areas (Back, Belly, Ham and Jowl) of the eight pork carcasses treated in two slaughterhouses (A and B). Community diversity reveals clustering per carcass and per slaughterhouse rather than per sampling area for the different samples based on amplicon sequencing. Axis 1 indicates the principal factor affecting the dissimilarity between bacterial communities and axis 2 indicates the weaker one.

Table 4
Isolation and serotyping of *Salmonella* spp. on the different sampled areas (ham (H), back (B), jowl (J) and belly (Y)) of the 8 pork carcasses (C1-C8) through analytical microbiological methods ISO 6579-1:2017.

	Salmonella (*S.*) serotypes							
	C1	C2	C3	C4	C5	C6	C7	C8
Ham	*S.* Brandenburg	N.I.	N.I.	N.I.	N.I.	*S.* Derby	Monophasic *S.* Typhimurium	Monophasic *S.* Typhimurium
Back	*S.* Brandenburg	*S.* Brandenburg	N.I.	N.I.	N.I.	N.I.	Monophasic *S.* Typhimurium	Monophasic *S.* Typhimurium
Jowl	*S.* Brandenburg	*S.* Brandenburg	N.I.	*S.* Rissen	*S.* Derby	*S.* Derby	Monophasic *S.* Typhimurium	Monophasic *S.* Typhimurium
Belly	*S.* Brandenburg	N.I.	N.I.	N.I.	N.I.	*S.* Derby	N.I.	Monophasic *S.* Typhimurium

N.I.: not isolated.

couples of carcasses (respectively in C1-C2, C5-C6, and C7-C8). These serotypes, along with *S.* Rissen are commonly associated with pigs and pork meat (EFSA, 2016). Furthermore, Monophasic *S.* Typhimurium and *S.* Derby are the third and fifth most commonly reported *Salmonella* serovars in human cases acquired in Europe (EFSA, 2017). The research of *Yersinia enterocolitica* has been carried out since pork is considered one of the main reservoirs for this pathogen. However, the gene *ystB*, used as a target for the biotype 1A, was detected once on the ham. The low number of contaminated carcasses were already reported previously in the study of Choi et al. (2013) where in none of the selected sampled areas (rump, midline and brisket) was this pathogen isolated. However, according to Van Damme et al. (2015) *Y. enterocolitica* was isolated from the mandibula and sternal region, the pelvic duct and split surface near the sacral vertebrae, particularly in the Van Damme study, a high level of this pathogen was found also in the tonsils and in the faeces. Moreover, other studies suggested that *Yersinia* may spread from intestines and tonsils to carcasses (Fredriksson-Ahomaa et al., 2000), and cross-contaminate pluck sets (Laukkanen et al., 2008; Vilar et al., 2015).

In the present study the direct colony identification method was used as first choice as this is the fastest, cheapest and easiest way to perform MALDI TOF MS. However, those isolates that did not result in a confident identification score were analyzed again using the extraction method. Results of the present study illustrate that this way of working, first direct followed by protein extraction on a selection is efficient in terms of time and cost when a great number of isolates have to be examined because out of 2620 colonies, only 152 could not be identified ($< 6\%$). The use of MALDI-TOF MS in microbiology has revolutionized routine identification of a huge amount of isolates, allowing the exploration of abundances and relations in microbiota studies (Lagier et al., 2016). However, MALDI TOF MS cannot be used as a tool to study the microbial diversity in a food matrix because of the unreliable identification of the isolates at species level, in particular concerning the *Pseudomonas* genus. Nevertheless, the combination of MALDI–TOF MS and 16S RNA sequencing has proven a promising approach to study microbial populations in food (Lagier et al., 2016). After 16S rRNA gene sequencing of the isolates, all the genera identified, except for *Sanguibacter* spp. were already in the database of MALDI TOF MS. These results confirm again that the spectra generated from the same species can differ, and therefore, if it is not present in the database, identification is not always possible, not even at the genus level (Williams et al., 2003).

The dominant bacteria isolated from the eight carcasses belonged to *Staphylococcus*, *Pseudomonas* spp., and *E. coli*. Majority of species belonged to the *Staphylococcus* genus are usually harmless and normally reside on the epithelium and mucous membranes of humans and animals. However, some species, especially *S. aureus*, can be responsible for foodborne illness in humans (Baer et al., 2013). Among the isolates reliably identified at species level, *S. xylosus* and *S. aureus* were identified. However, if a MALDI TOF MS cut-off value of 2.0 is applied for the identification at secure genus and probable species level, *S. capitis*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. pasteuri*, *S. saprophyticus*, *S. simulans* and *S. warneri* were isolated. The occurrence of *S. xylosus* is common in small mammals and farm animals and it is frequently isolated from meat products (Fija et al., 2016; Leroy et al., 2017). However, other Staphylococci species reported in the present study (e.g. *S. epidermidis*, *S. hominis*) are non-indigenous bacteria of pigs and their presence on the skin of the pork carcasses indicate improper handling (Fija et al., 2016).

Pseudomonas genus is one of the major food spoilers in refrigerated meat stored in aerobic conditions, in particular, *P. fragi*, reliably identified at species level in the present study, is one of the most frequently found species in meat (Raposo et al., 2017). Moreover, also *P. aeruginosa* which is a human and animal pathogen was identified with a score value ≥ 2.3. However, even when the value score is high, the identification with MALDI TOF MS at species level of the *Pseudomonas* genus remains dubious (Mulet et al., 2012).

With 16S amplicon sequencing, *Brochothrix* was the most abundant one, immediately followed by *Pseudomonas*. In contrast with this latter genus, *Brochothrix* was not frequently isolated from the agar plates. *Brochothrix* and *Pseudomonas* are known to be an abundant member of the aerobic microbial population on fresh meat (Doulgeraki et al., 2012). These species are known to cause spoilage at low temperatures, but in contrast with *Pseudomonas*, *Brochothrix* is more involved in souring then putrefaction (Nychas et al., 2008).

Rothia and *Acinetobacter* were respectively the third and fourth most frequently detected genera. These genera were also isolated in the present study with the culture-dependent methods. *Rothia* is a common inhabitants in oral and intestinal microbiomes of humans, pigs, and rodents (Abidi et al., 2016; Gaiser et al., 2017) while *Acinetobacter* is commonly found in the environment, like in soil, water and food (Hrenovic et al., 2014). Some members of *Acinetobacter* have been also reported in relation of foodborne illnesses, however, the pathogenicity has to be confirmed (De Amorim and Nascimento, 2017).

Chryseobacterium, *Psychrobacter* and *Shewanella* also detected and isolated in the present study on the pork carcass surface, are all potential spoilage bacteria (Odeyemi et al., 2019; Rouger et al., 2018; Yuan et al., 2018). Moreover, among the top abundant genera detected, the genus *Corynebacterium* also isolated encompass some species that are recognized as pathogenic organisms which cause severe health problems; infection that may also result from the consumption of contaminated food products (Beauty Dlamini and Njie Ateba, 2014). Interestingly, *Staphylococcus* spp., which, as described above, was frequently isolated from the agar plates, was detected at a very low level (0.68%) with the culture-independent method.

Thus, as already reported by Lagier et al. (2016) for the human gut microbiota, culture-dependent and independent approaches are complementary in the study of the microbial populations in the foods.

Moreover, to our knowledge, this is the first study that provides a complete overview of the composition of the bacterial community on four areas of pig carcasses. Although some bacteria were detected or isolated (at low levels) in specific carcass areas, the present study showed, surprisingly, that the microbial population of the ham, back, jowl and belly were dominated by the same genera.

On the other hand, important differences have been observed between the two slaughterhouses. With 16S amplicon sequencing, Slaughterhouse A showed the lowest diversity index and, indeed, the microbiota on the carcasses sampled in this slaughterhouse was dominated by *Brochothrix* spp. On the contrary, Slaughterhouse B, with the highest Evenness and Shannon indices, did not exhibit a clear cut dominant genus. This difference was also observed when analyzing the bacterial population isolated on the different agar plates. The microbiota on the non-selective medium (PCA) incubated at 7°, 30°and under anaerobic conditions, isolated from the carcasses C1-C4 was dominated by one specific genus per temperature (TAB 30 °C = *Staphylococcus*; TAB 7 °C = *Pseudomonas*; TANAB = *E. coli*), whereas the microbial population isolated on the carcasses C5-C8 was composed of different genera. The differences between the two groups are clear in Fig. 4 which shows, through the beta diversity, the dissimilarity between the carcasses belonging to the different slaughterhouses is high. Likely, the recorded differences may be put in relation to the size and with the technological facilities of the two slaughterhouses. In particular, differences may arise from the use of different singeing method. Indeed, in slaughterhouse B in which this step was carried out manually, the higher bacterial diversity recorded was probably due to a not uniform or incorrectly singeing procedure.

5. Conclusions

In conclusion, in the small slaughterhouses studied, the bacterial

community of each carcass may depend mainly on the microbial population of the slaughterhouse to which it belongs rather than on the indigenous microbiota of the slaughtered animals. However, to confirm this hypothesis further studies on the environmental population of the slaughterhouse's facilities should be performed. Moreover, the results of the comparison of different sampling areas show the absence of clear and significative difference between ham, back, jowl and belly, both of the microbial count and of the composition of the microbial community. Thus, the sampling of only one area by official authorities may be appropriate for the evaluation of the hygiene of the slaughter process in slaughterhouse with a small daily production capacity.

Declaration of competing interest

The authors declare no potential conflict of interests.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108902.

References

Abidi, M.Z., Ledeboer, N., Banerjee, A., Hari, P., 2016. Morbidity and mortality attributable to Rothia bacteremia in neutropenic and nonneutropenic patients. Diagn. Microbiol. Infect. Dis. 85, 116–120. https://doi.org/10.1016/j.diagmicrobio.2016.01.005.

Alatoom, A.A., Cunningham, S.A., Ihde, S.M., Mandrekar, J., Patel, R., 2011. Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 49, 2868–2873. https://doi.org/10.1128/JCM.00506-11.

Baer, A.A., Miller, M.J., Dilger, A.C., 2013. Pathogens of interest to the pork industry: a review of research on interventions to assure food safety. Compr. Rev. Food Sci. Food Saf. 12, 183–217. https://doi.org/10.1111/1541-4337.12001.

Beauty Dlamini, S., Njie Ateba, C., 2014. Isolation of corynebacterium species from retail mutton and lamb in the North West Province, South Africa. J. Food Nutr. Res. 2, 377–382. doi:10.12691/jfnr-2-7-8.

Biasino, W., Zutter, L. De, Mattheus, W., Bertrand, S., Uyttendaele, M., Van Damme, I., 2018. Correlation between slaughter practices and the distribution of Salmonella and hygiene indicator bacteria on pig carcasses during slaughter. Food Microbiol. 70, 192–199. https://doi.org/10.1016/j.fm.2017.10.003.

Cherkaoui, A., Hibbs, J., Emonet, S., Tangomo, M., Girard, M., Francois, P., Schrenzel, J., 2010. Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J. Clin. Microbiol. 48, 1169–1175. https://doi.org/10.1128/JCM.01881-09.

Choi, Y.M., Park, H.J., Jang, H.I., Kim, S.A., Imm, J.Y., Hwang, I.G., Rhee, M.S., 2013. Changes in microbial contamination levels of porcine carcasses and fresh pork in slaughterhouses, processing lines, retail outlets, and local markets by commercial distribution. Res. Vet. Sci. 94, 413–418. https://doi.org/10.1016/j.rvsc.2012.11.015.

De Amorim, A., Nascimento, J., 2017. Review Acinetobacter: an underrated foodborne pathogen? J. Infect. Dev. Ctries. 11, 111–114. https://doi.org/10.3855/jidc.8418.

Doulgeraki, A.I., Ercolini, D., Villani, F., Nychas, G.J.E., 2012. Spoilage microbiota associated to the storage of raw meat in different conditions. Int. J. Food Microbiol. 157, 130–141. https://doi.org/10.1016/j.ijfoodmicro.2012.05.020.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA Journ al 2016;14(12):4634, 231.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA Journal 2017;15(12):5077, 228.

Ercolini, D., 2013. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl. Environ. Microbiol. 79, 3148–3155. https://doi.org/10.1128/AEM.00256-13.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., Villani, F., 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. Appl. Environ. Microbiol. 75, 1990–2001.

Fija, K., Peitler Fijałkowski, D., Karakulska, J., 2016. Staphylococci isolated from ready-to-eat meat – identification, antibiotic resistance and toxin gene profile. Int. J. Food Microbiol. 238, 113–120. https://doi.org/10.1016/j.ijfoodmicro.2016.09.001.

Fredriksson-Ahomaa, M., Korte, T., Korkeala, H., 2000. Contamination of carcasses, offals, and the environment with yadA-positive Yersinia enterocolitica in a pig slaughterhouse. J. Food Prot. 63, 31–35. https://doi.org/10.4315/0362-028X-63.1.31.

Gaiser, R., Medema, M.H., Kleerebezem, M., Van Baarlen, P., Wells, J.M., 2017. Draft genome sequence of a porcine commensal, Rothia nasimurium, encoding a non-ribosomal peptide synthetase predicted to produce the ionophore antibiotic valinomycin Rogier. Genome Announc. 5, 17–18.

Ghafir, Y., Daube, G., 2008. Comparison of swabbing and destructive methods for microbiological pig carcass sampling. Lett. Appl. Microbiol. 47 (4), 322–326.

Hrenovic, J., Durn, G., Goic-Barisic, I., Kovacic, A., 2014. Occurrence of an environmental Acinetobacter baumannii strain similar to a clinical isolate in paleosol from Croatia. Appl. Environ. Microbiol. 80, 2860–2866. https://doi.org/10.1128/AEM.00312-14.

Jeong, S., Hong, J.S., Kim, J.O., Kim, K.H., Lee, W., Bae, I.K., Lee, K., Jeong, S.H., 2016. Identification of acinetobacter species using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Ann. Lab. Med. 36, 325–334. https://doi.org/10.3343/alm.2016.36.4.325.

Knight, R., Navas, J., Quinn, R.A., Sanders, J.G., Zhu, Q., 2018. Best practices for analysing microbiomes. Nat. Rev. 16(7), 410-422. Microbiol. doi:https://doi.org/10.1038/s41579-018-0029-9.

Koutsoumanis, K., Sofos, J.N., 2004. Microbial contamination of carcasses and cuts. Meat Sci. 67 (8), 1624–1629. https://doi.org/10.1016/B0-12-464970-X/00070-2.

Lagier, J.C., Khelaifia, S., Alou, M.T., Ndongo, S., Dione, N., Hugon, P., Caputo, A., Cadoret, F., Traore, S.I., Seck, E.H., Dubourg, G., Durand, G., Mourembou, G., Guilhot, E., Togo, A., Bellali, S., Bachar, D., Cassir, N., Bittar, F., Delerce, J., Mailhe, M., Ricaboni, D., Bilen, M., Dangui Nieko, N.P.M., Dia Badiane, N.M., Valles, C., Mouelhi, D., Diop, K., Million, M., Musso, D., Abrahão, J., Azhar, E.I., Bibi, F., Yasir, M., Diallo, A., Sokhna, C., Djossou, F., Vitton, V., Robert, C., Rolain, J.M., La Scola, B., Fournier, P.E., Levasseur, A., Raoult, D., 2016. Culture of previously uncultured members of the human gut microbiota by culturomics. Nat. Microbiol. 1 (12), 1–8. https://doi.org/10.1038/nmicrobiol.2016.203.

Laukkanen, R., Martínez, P.O., Siekkinen, K.M., Ranta, J., Maijala, R., Korkeala, H., 2008. Transmission of yersinia pseudotuberculosis in the pork production chain from farm to slaughterhouse. Appl. Environ. Microbiol. 74, 5444–5450. https://doi.org/10.1128/AEM.02664-07.

Le Minor, L., Popoff, M.Y., 1987. Designation of Salmonella enterica. Int. J. Syst. Bacteriol. 37, 465–468.

Leroy, S., Vermassen, A., Ras, G., Talon, R., 2017. Insight into the genome of staphylococcus xylosus, a ubiquitous species well adapted to meat products. Microorganisms. 5, 52. doi:https://doi.org/10.3390/microorganisms5030052.

Lindblad, M., Lindmark, S., Thisted Lambertz, S., Lindqvist, R., 2007. Microbiological baseline study of swine carcasses at Swedish slaughterhouses. J. Food Prot. 70, 1790–1797.

Mann, E., Wetzels, S.U., Pinior, B., Metzler-Zebeli, B.U., Wagner, M., Schmitz-Esser, S., 2016. Psychrophile spoilers dominate the bacterial microbiome in musculature samples of slaughter pigs. Meat Sci. 117, 36–40. https://doi.org/10.1016/j.meatsci.2016.02.034.

Mrdovic, B., Raseta, M., Brankovic-Lazic, I., Milijasevic, M., Baltic, B., Ivan, N., 2017. Pigs and cattle slaughter process hygiene in a large - scale and a small - scale abattoir: a report from one county in Serbia. Scientific Journal "Meat Technology" 58 (2), 65–72.

Mulet, M., Gomila, M., Scotta, C., Sánchez, D., Lalucat, J., García-Valdés, E., 2012. Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus Pseudomonas. Syst. Appl. Microbiol. 35, 455–464. https://doi.org/10.1016/j.syapm.2012.08.007.

Nychas, G.J.E., Skandamis, P.N., Tassou, C.C., Koutsoumanis, K.P., 2008. Meat spoilage during distribution. Meat Sci. 78, 77–89. https://doi.org/10.1016/j.meatsci.2007.06.020.

Odeyemi, O.A., Burke, C.M., Bolch, C.C.J., Stanley, R., 2019. Spoilage microbial community profiling by 16S rRNA amplicon sequencing of modified atmosphere packaged live mussels stored at 4oC. Food Res. Int. 121, 568–576. https://doi.org/10.1016/j.foodres.2018.12.017.

Peruzy, M.F., Murru, N., Perugini, A.G., Capuano, F., Delibato, E., Mercogliano, R., Korkeala, H., Proroga, Y.T.R., 2017. Evaluation of virulence genes in Yersinia enterocolitica strains using SYBR green real-time PCR. Food Microbiol. 65, 231–235. https://doi.org/10.1016/j.fm.2017.03.004.

Peruzy, M.F., Murru, N., Yu, Z., Cnockaert, M., Joossens, M., Proroga, Y.T.R., Houf, K., 2019a Determination of the microbiological contamination in minced pork by culture dependent and 16S amplicon sequencing analysis Int. J. Food Microbiol. 290, 27–35. doi:https://doi.org/10.1016/j.ijfoodmicro.2018.09.025.

Peruzy, M.F., Murru, N., Yu, Z., Kerkhof, P., Neola, B., Joossens, M., Proroga, Y.T.R., Houf, K., 2019b. Assessment of microbial communities on freshly killed wild boar meat by MALDI-TOF MS and 16S rRNA amplicon sequencing. Int. J. Food Microbiol., 301, 51–60. doi:https://doi.org/10.1016/j.ijfoodmicro.2019.05.005.

Raposo, A., Pérez, E., de Faria, C.T., Ferrús, M.A., Carrascosa, C., 2017. Food spoilage by Pseudomonas spp.—an overview. Foodborne Pathog. Antibiot. Resist. First Ed. Ed. by Om V. Singh 3, 41–71.

Rouger, A., Moriceau, N., Prévost, H., Remenant, B., Zagorec, M., 2018. Diversity of bacterial communities in French chicken cuts stored under modified atmosphere packaging. Food Microbiol. 70, 7–16. https://doi.org/10.1016/j.fm.2017.08.013.

Sánchez-Rodríguez, J.A., Navas, L., Vinuesa, F.M., Castells, C., Martínez, M.A., López, A., Lindez, B., Cabrera-Vique, C., 2018. New insights on the risk factors associated with the presence of Salmonella on pig carcasses. Lessons from small slaughterhouses. Food Control 87, 46–52. https://doi.org/10.1016/j.foodcont.2017.12.016.

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 6, 1–16. https://doi.org/10.3389/fmicb.2015.00791.

Van Damme, I., Berkvens, D., Vanantwerpen, G., Baré, J., Houf, K., Wauters, G., Zutter, L. De, 2015. Contamination of freshly slaughtered pig carcasses with enteropathogenic Yersinia spp.: distribution, quanti fi cation and identification of risk factors. Int. J. Food Microbiol. 204, 33–40. doi:https://doi.org/10.1016/j.ijfoodmicro.2015.03.016.

Vilar, M.J., Virtanen, S., Korkeala, H., 2015. Bayesian modelling to identify the risk factors for Yersinia enterocolitica contamination of pork carcasses and pluck sets in slaughterhouses. Int. J. Food Microbiol. 197, 53–57. https://doi.org/10.1016/j.ijfoodmicro.2014.12.020.

Wheatley, P., Giotis, E.S., McKevitt, A.I., 2014. Effects of slaughtering operations on carcass contamination in an Irish pork production plant. Ir. Vet. J. 67, 1–6. https://doi.org/10.1186/2046-0481-67-1.

Williams, T.L., Andrzejewski, D., Lay, J.O., Musser, S.M., 2003. Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. J. Am. Soc. Mass Spectrom. 14, 342–351. https://doi.org/10.1016/S1044-0305(03)00065-5.

Yu, Z., Peruzy, M.F., Dumolin, C., Joossens, M., Houf, K., 2019. Assessment of food microbiological indicators applied on poultry carcasses by culture combined MALDI-TOF MS identification and 16S rRNA amplicon sequencing. Food Microbiol. 82, 53–61. https://doi.org/10.1016/j.fm.2019.01.018.

Yuan, L., Sadiq, F.A., Liu, T., Li, Y., Gu, J., Yang, H., He, G., 2018. Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. J Zhejiang Univ Sci B 19 (8), 630–642.