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Assessment of microbial communities on freshly killed wild boar meat by MALDI-TOF MS and 16S rRNA amplicon sequencing

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ABSTRACT

Wild boars (*Sus scrofa*) are the most widely distributed large mammals and recent increase in consumption of wild boar meat urges the need of microbiological quality criteria. The aim of the study was to characterize the initial bacterial contamination on freshly-killed wild boar meat using a culture-dependent approach with ISO-methods combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification and 16S rRNA amplicon sequencing. Moreover, the presence of foodborne pathogens was examined using Real-Time-PCR and confirmed by classical isolation. Analysing 22 unrelated wild boar meat samples showed a higher bacterial contamination level compared to pork, with *Salmonella* present in almost one third of the samples. A great variability of the microbial contamination between the samples was recorded, as well as complementary results between culturing and 16S rRNA amplicon sequencing as frequently isolated genera were not always detected, and vice versa. Furthermore, the foodborne pathogen *Salmonella* was never detected with 16S rRNA amplicon sequencing, demonstrating the necessity for a cautious approach in the implementation of new analysis techniques in food safety. The present work determines that attention should be paid to the trade of non-inspected meat directly to retail or consumers.

1. Introduction

Wild boar (*Sus scrofa*) is currently considered one of the most destructive and invasive mammal species in the world (Lowe et al., 2000). Wild boar are present on all continents, except for Antarctica (Sales et al., 2017), and thrive in almost any condition, climate or ecosystem, including urban areas (Keuling et al., 2018). In 2012, based on the hunting bag study, a total of 2.2 million wild boars were hunted in 18 European countries (Massei et al., 2015). However, the number of free-range wild boars is increasing and an accurate estimation of the population density seems to be a difficult task (Keuling et al., 2018). Wild hogs can cause significant economic losses as they may cause damage to crops and natural vegetation (Massei et al., 2015). Moreover, they can spread diseases to both livestock and humans, including African Swine fever, *Salmonella* spp., Shiga toxin-producing *Escherichia* (*E.*) *coli* and *Campylobacter* (Keuling et al., 2018; Ruiz-Fons, 2017). The main causes of natural mortality are starvation due to extreme weather conditions, diseases and predation by wolves, but nowadays, hunting has the highest impact on the wild population density (Massei et al., 2015; Nores et al., 2008; Okarma et al., 1995; Toïgo et al., 2008).

The meat of wild hogs eaten only by hunters and their families does not require any inspection whatsoever. According to Regulation (EC) No. 853/2004, large wild game hunted with the intention to sell on the market must be eviscerated (stomach and intestines) as soon as possible after killing and, if necessary, be bled. The temperature of the carcasses must be brought down to a maximum of 7 °C and within a reasonable time frame. Moreover, the carcass and viscera must be examined on the spot by a "trained person" in order to identify potential health risks. This trained person must have sufficient knowledge of the anatomy, physiology, behaviour, pathology and further processing of wild game. Furthermore, no meat can be sold without passing veterinary inspection by a competent authority in a game-handling establishment. However, depending on national legislation, the supply of a small number of wild game or small quantities of meat directly to the final consumer or to

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local retail establishments does not require official post-mortem inspection.

Nowadays, wild boar meat is considered as healthy and delicious food for its high nutritional value and special sensory proprieties and is available throughout the year (Atanassova et al., 2008; Naya et al., 2003; Strazdina et al., 2014). In Italy, consumption of wild boar increased in recent years to approximately 0.2 kg per capita/year (Pedrazzoli et al., 2017). Data on the consumption of game meat at a European level are still limited. Furthermore, within the European Union, there are no specific microbiological criteria for wild boar meat products. Therefore, microbiological criteria for pork, included in Regulation (EC) No. 1441/2007 of 5 December 2007 amending Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs, are commonly applied to assess microbiological quality (Atanassova et al., 2008). Microbial contamination of pork has already been studied extensively (Koo et al., 2016; Mann et al., 2016; Tian et al., 2017). *Pseudomonas* spp., *Enterobacteriaceae*, *Brochothrix thermosphacta* and lactic acid bacteria have been identified as the dominant bacterial taxa present, contributing to meat flavouring but spoilage as well. In contrast to domesticated pigs, wild boars roam free and their diet is uncontrolled. Furthermore, when animals are shot, bled and eviscerated in the environment, there is no access to hygienic conditions like those present in modern slaughterhouse facilities. The microbiological contamination therefore depends on the circumstances in which the animals are killed (e.g. different hunting methods), dressed and further handled from the collection to the chilling point. Various studies have explored the microbiological quality of game meat, (Atanassova et al., 2008; Avagnina et al., 2012; Gill, 2007; Mirceta et al., 2015) but still little is known regarding the microbial communities present in wild boar meat.

Conventional bacteriological isolation methods and identification using biochemical tests are laborious and biased by specific culture and laboratory conditions. Detection and identification of microorganisms using culture-independent approaches, such as 16S rRNA amplicon sequencing, are now commonly used to assess the microbial ecology of different foods like cheeses, seafood, meat and meat products (Gori et al., 2013; Roh et al., 2009; Tian et al., 2017). The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (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 (Höll et al., 2016; Hilgarth et al., 2018; Lagier et al., 2016). The application of MALDI-TOF MS in food microbiology is rather preliminary due to the still clinical-oriented database. Nevertheless the combination of MALDI–TOF MS and 16S RNA sequencing has proven a promising approach in studying microbial populations in food (Lagier et al., 2016; Peruzy et al., 2019).

Considering the increasing consumption of wild boar meat and the related public health risk for zoonotic pathogens, the aim of the present study was to assess the microbial communities on hunted wild boar meat using both a culture dependent and independent approach resulting in more complete picture of initial microbial contamination.

2. Materials and methods

2.1. Sampling

From October to December 2017, meat samples of 22 wild boars (W1 to W22), 10 males and 12 females, aged between 1 and 6 years, were collected in the province Campania, southern Italy (Table 1). On different occasions, the animals were shot by official hunters and immediately bled in the field. They were brought to collection places where the evisceration and skinning were performed. Subsequently, approx. 100 cm^2 meat was aseptically cut out the shoulder area (at least 100 g each) and individually placed in sterile stomacher blender bags. This area was selected as it has been reported as more frequently contaminated due to the inverted position of the pig carcass at slaughter (Baer et al., 2013). Samples were transported to the lab at +2 °C, and examined within a maximum of 24 h after collection.

2.2. Culture-dependent bacteriological examination

Ten grams of meat and 90 ml (1:10 (W/W)) of sterilized Peptone Water (PW, CM0009, OXOID, Basingstoke, UK) were placed in a sterile stomacher bag and homogenized for 3 min at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France). Subsequently, ten-fold serial dilutions of each homogenate were prepared in PW, followed by quantitative bacterial isolation in duplicate for: a) total aerobic bacterial (TAB) counts performed according to ISO 4833-2:2013 on Plate Count Agar (PCA; CM0325, Oxoid), incubated at 30 °C for 48 to72-h; b) psychotropic aerobic bacterial counts on PCA incubated at 7 °C for 10 days (Ercolini et al., 2009); c) total anaerobic bacterial counts (TANAB) on PCA by pour plating and incubation in an anaerobic atmosphere (anaerobic GasPak jar system, Oxoid) at 30 °C for 48/72-h; d) lactic acid bacteria (LAB) according to ISO 15214:1998 on De man, Rogosa and Sharpe agar (MRS, CM0361, Oxoid) incubated aerobically at 30 °C for 72-h; e) presumptive *Pseudomonas* spp. according to ISO 13720:2010 on Cephalothin-Sodium Fusidate-Cetrimide Agar with Modified CFC Selective Supplement (CFC, CM0559B with SR0103E, Oxoid) incubated aerobically at 25 °C for 48-h; f) *E. coli* according to ISO 16649-2:2001 selectively isolated on Tryptone Bile X-Glucuronide (TBX, CM0945, Oxoid) incubated at 44 °C for 24/48-h, and g) total *Enterobacteriaceae* (EB) according to ISO 21528-2:2017 selectively isolated on Violet Red Bile Glucose Agar (VRBG, CM1082, Oxoid) incubated at 37 °C for 24-h.

After incubation, all colonies were counted and subsequently all picked from the agar plates with bacterial growth between 30 and 300 CFU/plate on PCA, and between 15 and 150 CFU/plate from all others (Bernadin et al., 2018; Sutton et al., 2011). All harvested colonies were subsequently subcultured on Tryptic Soy Agar (TSA, CM0131, Oxoid) or MRS and incubated in the appropriate conditions as described above.

For the detection of relevant foodborne pathogens, 25 grams portions of each sample were homogenized once in 225 ml (1:10 (W/W)) buffer peptone water (BPW, CM0509, Oxoid) and incubated at 37 °C for 24-h for the detection of *Salmonella*, in 225 ml Half Fraser broth (HF, CM1053, Oxoid) and incubated at 30 °C for 24-h for the detection of *Listeria* (*L.*) *monocytogenes*, and once in 225 ml Peptone Sorbitol Bile Broth (PSB, 17192, Sigma-Aldrich) for the detection of *Yersinia* (*Y.*) *enterocolitica*. The “iQ-Check Real-Time PCR Kits” were applied for the detection of *Salmonella* (*S.*) (BR3578123, Bio-Rad, Hercules, CA, USA) and *L. monocytogenes* (BR3578124, Bio-Rad, Hercules, CA, USA), following manufacturer's recommendations. For the detection of *Y. enterocolitica*, DNA was extracted using the Chelex-100-resin method (1422822, Bio-Rad, Hercules, CA, USA) whereby two ml of each incubated homogenate was transferred into a two 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, a SYBR green PCR-assay was conducted, with the gene *ystA* as target for the pathogenic biotype (Peruzy et al., 2017). Therefore, 3 µl of DNA extract was added to 22 µl of PCR mix. The mastermix contained 12.5 µl of Qiagen QuantiTect SYBR Green PCR Kit (1x), 100 nM of both primers ystA-F (5′-ATCGACACCAATAACCGCTGAG-3′) and ystA-R (5′-CCAATCACTACTGACTTCGGCT-3′). To evaluate the presence of the biotype 1A, the presence of the target gene *ystB* gene was examined (Peruzy et al., 2017). Three µl of DNA extract was added to 22 µl of PCR mastermix containing 12.5 µl of Qiagen QuantiTect SYBR Green PCR Kit (1x), 150 nM of both primers ystB-F (5′-GTACATTAGGCCAAGAGACG-3′) and ystB-R (5′-GCAACATACCTCACAACACC-3′). The fluorescence of SYBR Green and the melting

Table 1

Gender, weight (kg) and age (years) of the shooted animals, total bacterial counts (log CFU/g) in twenty two wild boar meat samples (W1–W22) on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7 °C), anaerobic bacteria (TANAB 30 °C), *E.coli* on TBX, *Enterobacterales* on VRBG, presumptive *Pseudomonas* spp. on CFC and Lactic Acid Bacteria (LAB) on MRS and number of isolates per sample picked from the different agar plates and subsequently analysed by MALDI-TOF.

	Gender	Weight (kg)	Age (years)	TAB 30 °C (log CFU/g)	TAB 7 °C (log CFU/g)	TANAB 30 °C (log CFU/g)	*E. coli* (log CFU/g)	*Enterobacterales* spp. (log CFU/g)	*Pseudomonas* spp. (log CFU/g)	LAB (log CFU/g)	Isolates (n.)
W1	M	40	1	4.69	4.69	5.10	2.46	3.49	4.66	3.93	231
W2	M	25	1	6.05	3.76	4.24	1.96	2.57	3.56	0.00	163
W3	M	70	3	6.01	5.12	5.75	4.56	5.34	3.82	4.64	363
W4	F	60	2	3.61	2.56	4.46	3.67	2.86	0.00	2.66	74
W5	M	60	2	3.26	1.96	3.63	2.13	2.36	2.56	3.62	162
W6	F	30	1	5.55	3.12	5.36	4.26	5.44	2.80	3.41	176
W7	F	108	5	6.72	6.69	5.98	2.10	5.67	6.62	5.28	209
W8	M	125	5	5.44	4.75	4.37	2.11	3.92	2.59	2.66	182
W9	M	70	3	5.59	3.88	4.76	4.36	4.61	4.37	3.55	166
W10	F	60	2	6.03	5.54	4.67	2.41	4.69	5.16	3.07	164
W11	F	60	3	4.32	4.80	5.28	2.96	4.45	3.89	4.37	184
W12	F	40	1	6.51	5.34	5.72	3.49	5.57	4.88	5.61	156
W13	M	60	3	5.50	4.56	5.46	4.44	4.86	3.66	4.98	169
W14	F	65	4	4.08	3.06	3.03	2.96	3.41	3.04	3.36	130
W15	M	125	6	5.51	5.36	4.87	2.30	4.62	4.39	4.76	164
W16	F	30	1	5.74	5.05	5.34	2.21	4.93	3.75	5.28	211
W17	F	30	1	6.95	4.99	3.44	2.26	4.48	4.69	3.62	248
W18	F	53	2	5.73	3.37	5.10	1.66	0.00	2.28	0.00	74
W19	M	70	3	6.65	3.74	5.71	2.96	4.98	4.24	4.00	106
W20	F	40	1	5.85	4.04	3.88	1.26	3.64	3.26	3.04	166
W21	F	40	1	4.66	4.55	3.44	1.91	3.82	4.13	4.26	162
W22	M	40	1	6.67	6.58	5.21	3.47	5.05	5.74	4.14	129

curve were generated using the CFX96 system (Bio-Rad). A specific melting temperature (T_m) of 78.5 ± 1 °C indicated a positive result. While awaiting the RT-PCR results, the enrichment broths were stored at 4 °C. RT-PCR positive results for *Salmonella* spp., *L. monocytogenes* or *Y. enterocolitica* were confirmed using the corresponding normalized microbiological isolation methods ISO 6579-1:2017, 11290-1:2017, and 10273:2017.

Salmonella 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).

2.3. Isolate identification strategy

All isolates, except those on MRS plates, were analysed by MALDI-TOF MS first 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 (BTS Bruker Daltonics) was used as mass calibration and reference standard in each series of MALDI measurements. Mass spectra were generated with the Microflex™ LT MALDI-TOF mass spectrometer, equipped with a nitrogen laser (l1/4337 nm) and Flex Control 3.4 software using recommended settings in a linear positive ion detection mode (Bruker Daltonics). Identifications were obtained by comparing the mass spectra to the Bruker MSP database (MBT Compass Library, 5989 entries) using the Bruker Compass software at default settings. Identification score criteria were classified according to Jeong et al. (2016): a score of ≥2.3 indicated highly probable species identification, between 2.0 and 2.3 secure genus and probable species identification, a score between 1.7 and 1.99 probable genus and < 1.7 non-reliable identification. Isolates for which a score of < 2.0 was obtained with the “direct colony method” and all isolates from the MRS agar plates (Alatoom et al., 2011) were analysed using the ethanol/formic acid extraction procedure from Bruker Daltonics (03.04.2006). Therefore, colonies were suspended in 800 μl of TSB or MRS broth and incubated at 28 °C for 24 h. Subsequently, samples were centrifuged (1533*g* at 4 °C) for 10 min, the supernatants discarded and the pellets washed twice in 500 μl of Milli-Q water and centrifuged (1533*g* at 4 °C) for 10 min. After the second centrifugation, the supernatants were removed and the pellets suspended in 100 μl of Milli-Q water. Next, 50 μl of formic acid and 50 μl of acetonitrile were added and mixed thoroughly by pipetting followed by centrifugation (1533*g* at 4 °C) for 10 min. One microliter of each sample was spotted onto a 96-spot plate and allowed to dry at room temperature. Afterwards, 1 μl of matrix solution was added. The analysis was repeated when the spots resulted in ‘no peaks found’. Isolates for which 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 heated for 15 min at 95 °C. After a short spin, 180 μl of Milli-Q water was added. Subsequently, the suspension was centrifuged for 5 min at 10,000 × *g* at 4 °C. To amplify part of the 16S rRNA gene, the oligonucleotide primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 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 a 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 assessed using the nucleotide BLAST algorithm as implemented within the NCBI web service (https://www.ncbi.nlm.nih.gov).

Table 2

Presence of foodborne bacteria in wild boar meats detected using RT-PCR and *Salmonella* serotypes identified by using the Kaufmann-White scheme.

Samples	*Salmonella* spp.	*Salmonella* (*S.*) serotypes	*L. monocytogenes*	*Y. enterocolitica*	
				ystA	*ystB*
W1	N.D.		N.D.	N.D.	78.5[b]
W2	N.D.		N.D.	N.D.	N.D.
W3	N.D.		N.D.	N.D.	N.D.
W4	N.D.		N.D.	N.D.	N.D.
W5	N.D.		N.D.	N.D.	N.D.
W6	N.D.		N.D.	N.D.	N.D.
W7	N.D.		N.D.	N.D.	N.D.
W8	26.25[a]	*Monophasic S. Typhimurium*	N.D.	N.D.	N.D.
W9	N.D.		N.D.	N.D.	78.5[b]
W10	N.D.		N.D.	N.D.	78.5[b]
W11	N.D.		N.D.	N.D.	N.D.
W12	29.25[a]	*S. kasenyi*	N.D.	N.D.	N.D.
W13	N.D.		N.D.	N.D.	N.D.
W14	33.78[a]	*S. stanleyville*	N.D.	N.D.	N.D.
W15	31.59[a]	*S. stanleyville*	N.D.	N.D.	N.D.
W16	N.D.		N.D.	N.D.	N.D.
W17	26.11[a]	*S. kasenyi*	N.D.	N.D.	N.D.
W18	N.D.		N.D.	N.D.	N.D.
W19	37.86[a]	NI	N.D.	N.D.	N.D.
W20	N.D.		N.D.	N.D.	N.D.
W21	25.78[a]	*S. kasenyi*	N.D.	N.D.	N.D.
W22	N.D.		N.D.	N.D.	N.D.

N.D. Not detected.

N.I. Not isolated.

[a] Ct: threshold cycle.

[b] Tm: melting temperature.

2.4. Culture independent community profiling by 16S rRNA amplicon sequencing

To independently culture and identify microorganisms present in wild boar meat, DNA was extracted from 1.8 ml of each initial stomachered sample 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 purity, the ratio of absorbance at 260 nm and 280 nm was evaluated with a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA extracts of ≥100 ng, concentration ≥ 5 ng/μl and volume ≥ 20 μl, (OD260/280 = 1.8–2.0) were submitted to Novogene (HK) Company Limited for 16S rRNA amplicon sequencing (https://en.novogene.com). DNA was used to construct the library in which V3-V4 amplicons were amplified with primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). Truseq-DNA-PCR-free-library-prep kit was used to construct the DNA libraries of paired ends with 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 244 bases of both forward and reverse reads were truncated. After denoising the data using the dada2 denoise-paired method, representative sequences of each sample were retained. Before assigned to taxa, bacterial representative sequences were sifted out by against with Greengene 99% based on 97% identity using method vsearch. Then, both original representative sequences and filtered sequencing were 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. Statistical analysis

To compare the bacterial counts, a one-way analysis of variance (ANOVA) was calculated using PAST software package (https://folk.uio.no/ohammer/past/). PAST software was also used to calculate the richness expressed by the Chao1 index and diversity indices of community information obtained from 16S rRNA amplicon sequencing. A probability value of < 0.05 ($p < 0.05$) was defined as statistically significant.

3. Results

3.1. General bacterial indicators

Counts of the different bacterial parameters are shown in Table 1. Total aerobic bacteria determined at 30 °C ranged from 3.26 to 6.95 log CFU/g (mean ± SE = 5.5 ± 0.22 log CFU/g) and from 1.96 to 6.69 log CFU/g (mean ± SE = 4.43 ± 0.25 log CFU/g) for the determination at 7 °C. Within each sample, the viable aerobic count determined at 30 °C and 7 °C showed significant difference ($p < 0.5$). Total anaerobic bacteria ranged from 3.03 to 5.98 log CFU/g (mean ± SE = 4.76 ± 0.18 log CFU/g), and the mean number of presumptive *Pseudomonas* spp. and lactic acid bacteria were 3.82 ± 0.29 and 3.65 ± 0.31 log CFU/g respectively. The count of typical blue *E. coli* colonies on TBX and the purple/pink *Enterobacterales* colonies on VRBG showed a mean of 2.81 ± 0.21 and 4.12 ± 0.28 log CFU/g respectively. No significant correlation was observed between the bacterial count and the gender, age and weight of the animals.

3.2. Presence of foodborne bacteria

Salmonella was detected in 7 samples (31.82%) and all, except sample W19, were confirmed using the reference isolation method (Table 2). Three serovars were identified (*monophasic S.* Typhimurium, *S.* Stanleyville and *S.* Kasenyi). Regarding *Y. enterocolitica*, the gene *ystA* was not detected in the samples, though *ystB* was present in W1, W9 and W10. Also *L. monocytogenes* was never detected (Table 2).

3.3. Community profiling using isolation and MALDI-TOF MS identification

A total of 3789 bacterial isolates were analysed by MALDI-TOF MS (Table 1), of which 956 were picked from the PCA plates incubated at 30 °C, 526 from PCA at 7 °C, 240 from PCA incubated anaerobically, 701 from VRBG plates, 582 from CFC plates, 452 from TBX and 332 from the MRS agar plates. With the “direct colony identification method” applied on 3457 colonies (=all except isolates on MRS), 19.79% (n = 684) were identified at a highly probable species level, 36.71% (n = 1269) at secure genus and probable species level, 18.48% (n = 639) at genus level and 25.02% (n = 865) did not yield any identification. Colonies with a score value < 2 (n = 1504) were re-examined using “the extraction method” of which 7.65% (n = 115) were additionally identified at species level, 32.85% (n = 494) at genus level and 36.37% (n = 547) at probable genus level but 23.14% (n = 348) remained unidentified. With this second extraction analysis method, about 67.91% of the isolates (n = 1022) obtained a higher MALDI-TOF MS score value then by the direct colony identification method but 11.23% (n = 169) scored values lower than with the previous direct colony identification method. The same score value was obtained with both methods in 20.80% (n = 313) of the isolates. Interestingly, the identification outcome at species level of 182 isolates (179 *Pseudomonas*, two *Raoultella*, one *Staphylococcus*) differed between the two analysis methods.

Analysis of the isolates on MRS agar plates (n = 332) was performed only with the “extraction method” and resulted in the identification at species level, secure genus and probable genus level in 27.11% (n = 90), 28.01% (n = 93), and 28.31% (n = 94) respectively.

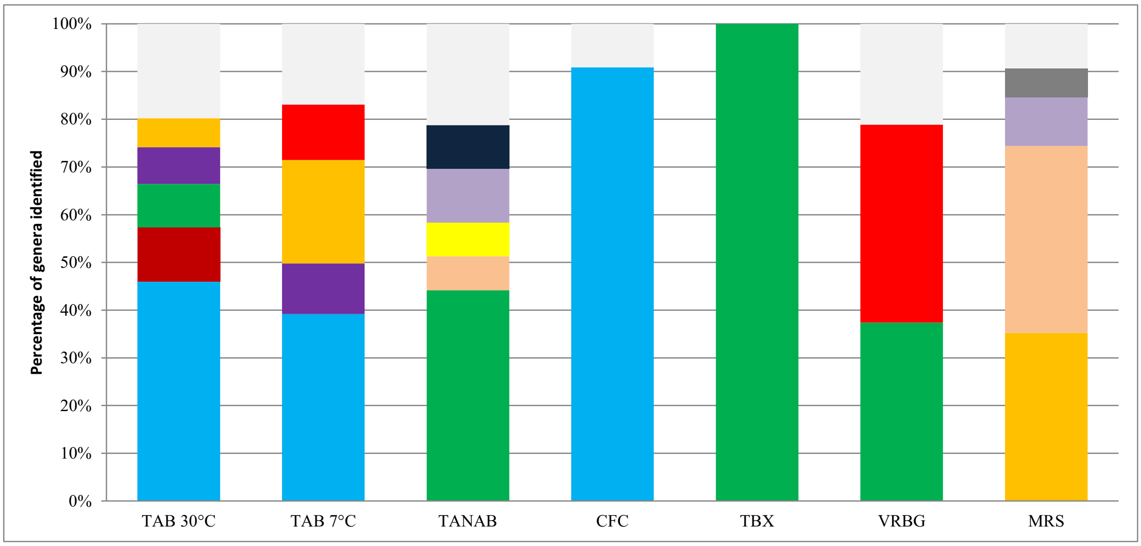

Fig. 1. Most abundant genera identified by MALDI-TOF (≥5%) and 16S gene sequencing on different agars: mesophilic bacteria on PCA (TAB 30 °C), psychotropic bacteria on PCA (TAB 7 °C), anaerobic bacteria (TANAB), *E.coli* on TBX, *Enterobacterales* on VRBG, presumptive *Pseudomonas* spp. on CFC and Lactic Acid Bacteria (LAB) on MRS.

🟦 *Pseudomonas* spp., 🟥 *Macrococcus* spp., 🟩 *Escherichia* spp., 🟪 *Acinetobacter* spp.,

🟧 *Candida* spp., 🟥 *Pantoea* spp., 🟫 *Leuconostoc* spp., 🟨 *Carnobacterium* spp.,

🟪 *Enterococcus* spp., ⬛ *Lactobacillus* spp., ⬛ *Lactococcus* spp., ⬜ Other (<5%).

No identification was obtained for 55 isolates (16.57%).

Isolates without a reliable MALDI-TOF MS identification were analysed using 16S rRNA gene sequencing (n = 118 isolates) and two families (*Comamonadaceae* and *Deinococcaceae*) and nine genera (*Acidovorax*, *Deinococcus*, *Exiguobacterium*, *Frigoribacterium*, *Gibbsiella*, *Micrococcus*, *Okibacterium*, *Psychrobacter* and *Rothia*) were additionally identified.

The dominant genera (with a percentage ≥ 5%) isolated on the different media is shown in Fig. 1. In general, bacteria identified by MALDI-TOF MS could be assigned to 28 families and 44 genera. Moreover, the yeasts *Candida* spp., *Cryptococcus* spp. *Rhodotorula* spp. and *Yarrowia* were also isolated. Members of genera *Acinetobacter*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Lactococcus*, *Lelliottia*, *Leuconostoc*, *Macrococcus*, *Pantoea*, *Pseudomonas* and *Rahnella* were isolated in at least 50% of the samples (Supplementary material 1). In particular, *Pseudomonas* was present in 17 samples (77.27%) among the mesophilic and psychrotrophic population on PCA plates. At 30 °C, *Macrococcus* and *Acinetobacter* were also frequently isolated and, in particular, they were present in 54.55% and 45.45% of the samples respectively. When incubation at 7 °C was applied, *Pantoea* was frequently isolated (72.73% of the samples). Except for one *Propionibacterium* isolate in sample W10, anaerobic incubation showed no additional contribution as all bacteria present anaerobically turned out to be facultative anaerobic and were also isolated in aerobic condition. In particular, on PCA plates incubated anaerobically, the genus *Escherichia* was isolated in 59.09% of the samples (13 samples).

Concerning the specificity of selective isolation media on CFC plates, besides the dominant presence of *Pseudomonas* spp., other genera were isolated or detected by 16S rRNA gene sequencing (Supplementary material 2) but except for the genus *Shewanella*, all of them were oxidase negative. On the TBX plates all typical blue colonies were confirmed as *E. coli*. On the VRBG plates, 98.72% of the purple/pink colonies were confirmed as members of the order *Enterobacterales*, with *Escherichia* present in 72.73% and *Patoea* in 63.64% of the samples. Members of the genera *Pseudomonas* and the yeast *Rhodotorula* were however also isolated and displayed the same phenotypic features. On the MRS plates, *Leuconostoc* and Candida were the genera most frequently isolated (59.09%) followed by the Gram positive *Lactococcus* (36.36%).

3.4. Culture-independent community analysis

Using 16S rRNA amplicon sequencing, a total of 1,209,441 reads were obtained. After filtering out all non-bacterial and non-archaea sequences, using the quality-control plugin in QIIME2, 535,513 reads were taken into account as bacterial reads for further analysis. Only exact amplicon sequence variants (OTUs) accounting for > 0.5% of the total reads of each sample were further retained. These sequences were clustered into 112 genera encompassing 64 families (Supplementary material 3). However, groups of bacteria were identified only at family or even at a higher taxonomic level and 3.5% of the sequences were identified only at kingdom level. The relative abundance of each OTU varied among the 22 samples (Fig. 2). Richness expressed by the Chao1 index showed sample W10 as that with the lowest richness and sample W5 as the richest sample. The equitability (evenness) index showed that no genera clearly dominate (Table 3) the bacterial communities on wild boar meat, though the genera *Acinetobacter* and *Pseudomonas* were detected in all samples. *Macrococcus* and *Propionibacterium* were present in 90.91%, *Pediococcus* and *Enterobacter* in 86.36%, *Escherichia* in 77.28%, *Faecalibacterium* and *Oscillospira* both at 72.73%, *Bacteroides*, *Prevotella* and *Roseburia* in 68.18%, *Janthinobacterium* and *Rahnella* in 63.64%, *Brochothrix* and *Streptococcus* in 59.09%, *Pseudobutyrivibrio* and *Citrobacter* in 54.55% and *Fusobacterium* and an uncharacterized member of the *Dethiosulfovibrionaceae* family in 50% of the samples.

Comparing both analytical approaches, among the 22 samples *Pseudomonas* was always detected and isolated. However, it resulted as a dominant genus with the culture-dependent approach but not with culture-independent one. *Acinetobacter* was detected and isolated in 81.82% of the samples and only detected in 5 (W9, W12, W16, W18 and W21). 86 genera were only detected using 16S amplicon sequencing

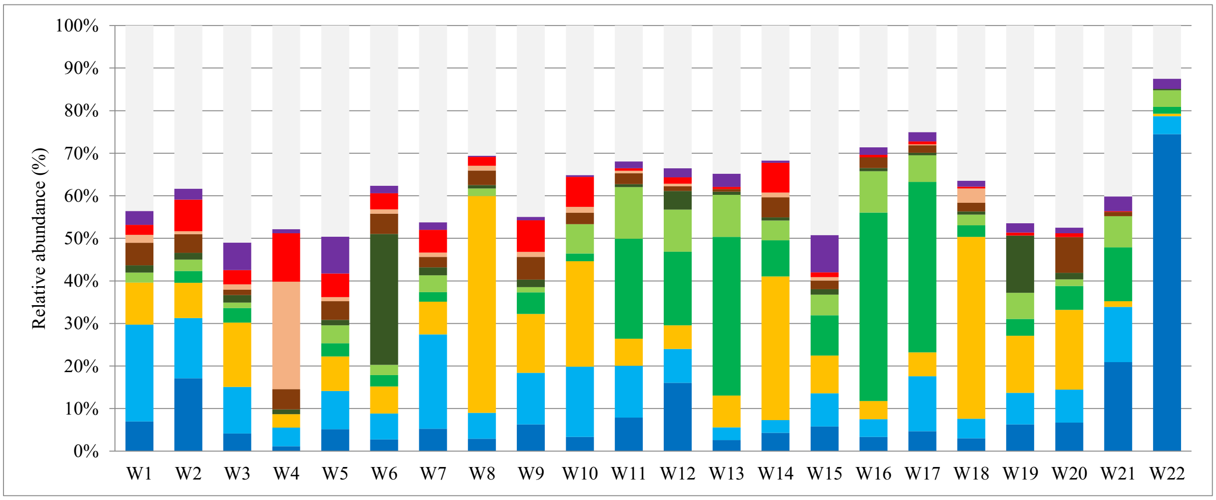

Fig. 2. Top 10 abundant microbial communities identified in wild boar meat samples by 16S rRNA amplicon sequencing.
The relative proportion of the most abundant microbial communities identified upon the highest taxonomic level (o: order; f: family; g: genus.)

Proteobacteria

🟦 g__*Acinetobacter*

🟦 g__*Pseudomonas*

🟩 g__*Enterobacter*

🟩 f__Enterobacteriaceae

🟩 g__*Escherichia*

Firmicutes

🟫 f__Lachnospiraceae

🟧 g__*Pseudobutyrivibrio*

🟨 o__Lactobacillales

Bacteroidetes

🟥 g__*Bacteroides*

Actinobacteria

🟪 o__Actinomycetales

⬜ Others

(Table 4). In contrast, 28 genera were only isolated and not detected (Table 4). Even when OTUs with < 0.5% of total reads were taken into account, only 6 genera (*Arthrobacter*, *Erwinia*, *Exiguobacterium*, *Leuconostoc*, *Serratia* and *Weissella*) could be additionally obtained.

4. Discussion

In the present study, though not completely comparable due to the larger region that has to be sampled on pork carcasses, levels of the total aerobic bacteria and *Enterobacteriaceae*, both indicators commonly applied in hygiene evaluation, were respectively higher in 16 and 18 wild boar meat samples than the EU microbiological criteria for pork (Aerobic colony count: m < 4.0 log CFU/cm^2 and M < 5.0 log CFU/cm^2; *Enterobacteriaceae* m < 2.0 log CFU/cm^2 and M < 3.0 log CFU/cm^2) (Regulation 1441/07). These higher contamination levels are not totally surprising as the animals were not slaughtered and further processed in modern slaughter facilities. Total aerobic bacteria levels were in line with those previously reported for wild boar meat (Mirceta et al., 2015), but levels of *Enterobacterales*, and *E. coli* in particular, were higher than in other studies (Membré et al., 2011; Mirceta et al., 2015). This identifies a faecal-meat or faecal-skin-meat transmission during processing and an intrinsic risk for public health. This is also underpinned by the high presence (31.82%) of *Salmonella* in the samples. The correlation of lower levels of *Enterobacteriaceae* resulting in less *Salmonella* positive wild boar meat samples was demonstrated in the studies of Mirceta et al. (2017) where *Salmonella* was detected in only 1.9% of the samples, in 2.15% positive Japanese wild boar meat samples at retail (Kanai et al., 1997) and in the studies of Atanassova et al. (2008) and Paulsen and Winkelmayer (2004) where *Salmonella* was not detected in any of the 127 and 50 meat samples respectively. However,

Table 3
Richness (expressed by Chao1 index) and diversity (expressed by Shannon and Evenness indexes) of the bacterial communities identified by 16 S amplicon sequencing.

	Chao-1	Shannon	Evenness
W1	239	7.42478	0.939742
W2	247	7.570253	0.952429
W3	199	7.111292	0.931209
W4	342	7.271982	0.863876
W5	348	8.027176	0.950756
W6	212	6.710971	0.868406
W7	285	7.738421	0.948938
W8	228	5.740676	0.732894
W9	146	6.573831	0.914324
W10	115	6.257811	0.914151
W11	286	7.534417	0.92335
W12	302	7.691718	0.933642
W13	231	6.892995	0.877893
W14	282	6.768272	0.831529
W15	360	8.087605	0.952396
W16	270	6.826319	0.845175
W17	299	6.672652	0.811363
W18	254	6.223689	0.779063
W19	189	6.945976	0.918507
W20	134	6.693117	0.947217
W21	250	7.449607	0.935201
W22	186	4.862044	0.644905

wild hogs acting as a carrier of *Salmonella* have been demonstrated by its isolation from 24.8% of wild boar intestinal samples, 10.8% of wild boar faeces and from the surfaces and the deep tissues of 4.2% samples in three previous Italian studies (Chiari et al., 2013; Zottola et al., 2013; Decastelli et al., 1995). Besides slaughter procedure, these differences may be related to the fact that *Salmonella* prevalence can vary between regions and the time of the year (Gill, 2007). Furthermore, Chiari et al. (2013) showed that wild boars can host of a variety of *Salmonella* serovars. In the present work, three serovars were isolated: *monophasic S.* Typhimurium, *S.* Stanleyville and *S.* Kasenyi. *Monophasic S.* Typhimurium represents one of the five most reported serovars in human salmonellosis (EFSA, 2017). Serovar *Salmonella* Kasenyi has not been reported from wild boar meat yet.

Pigs are considered a major reservoir for pathogenic *Y. enterocolitica*, but this foodborne pathogen was not isolated in the present study. Only the gene *ystB* was detected in 13.63% of the samples, indicating the presence of non-pathogenic strains (biotype 1A) (Peruzy et al., 2017). This finding is consistent with previous wild boar meat studies in which only non-pathogenic *Y. enterocolitica* strains were recovered (Gill (2007), Avagnina et al. (2012)). Also the absence of *L. monocytogenes* is in accordance with other studies (Membré et al., 2011; Paulsen and Winkelmayer, 2004).

For the identification of the different bacterial colonies with MALDI-TOF MS, the "direct identification colony method" and the "extraction method" were used. With the latter method, the individual score value increased for almost 40% of the isolates. However, for almost 90% of the isolates, the identification obtained with the extraction method did not differ, not even at species level.

Combining the two methods, 89.93% of the isolates were identified at genus level of which 23.11% were identified at species level. This effect has also been reported by Alatoom et al. (2011) showing that "extraction" resulted in higher level identifications than with the "direct colony method". However, identification of some microorganisms, in particular *Pseudomonas* species, remains unreliable since different species identification was obtained after using the direct identification colony method and extraction method in 182 isolates. Rapid and reliable identification of *Pseudomonas* isolates remains however a challenge in many studies due the high number of species and variable taxonomy (Mulet et al., 2012). Analysis of the isolates present on MRS was performed only with the extraction method as it has been reported that this method is more efficient for this group of bacteria (Alatoom et al., 2011).

Among the isolates without a reliable MALDI-TOF MS identification

Table 4
Genera only detected using 16S amplicon sequencing (Culture independent method) and genera only and identified with MALDI TOF-MS and 16S gene sequencing (Culture dependent methods).

Culture independent	Culture dependent		
Actinobacillus	*Geobacillus*	*Rhodoplanes*	*Aerococcus*
Agrobacterium	*Gluconacetobacter*	*Roseburia*	*Aeromonas*
Akkermansia	*Granulicatella*	*Rubellimicrobium*	*Agrococcus*
Anaerolinea	*HA73*	*Rubrobacter*	*Arthrobacter*
Anaerovorax	*Haemophilus*	*Ruegeria*	*Buttiauxella*
Asticcacaulis	*Inquilinus*	*Ruminococcus* (*Lachnospiraceae*)	*Cronobacter*
Bacteroides	*Kocuria*	*Ruminococcus* (*Ruminococcaceae*)	*Curtobacterium*
Balneimonas	*Lachnospira*	*Rummeliibacillus*	*Erwinia*
Bifidobacterium	*Luteimonas*	*Shuttleworthia*	*Exiguobacterium*
Blautia	*Massilia*	*SMB53*	*Filobasidium*
Bradyrhizobium	*Megamonas*	*Sphingobium*	*Frigoribacterium*
Burkholderia	*Megasphaera*	*Sphingomonas*	*Gibbsiella*
Caldicellulosiruptor	*Methylobacterium*	*Succinivibrio*	*Hafnia*
Campylobacter	*Methyloversatilis*	*Sutterella*	*Kluyvera*
Candidatus Solibacter	*Morganella*	*Syntrophomonas*	*Kurthia*
Catenibacterium	*Nocardioides*	*Syntrophus*	*Leclercia*
CF231	*Oscillospira*	*T78*	*Lelliottia*
Clostridium (*Clostridiaceae*)	*Paenibacillus*	*Tepidimonas*	*Leucobacter*
Clostridium (*Lachnospiraceae*)	*Parabacteroides*	*Treponema*	*Leuconostoc*
Clostridium (*Ruminococcaceae*)	*Paracoccus*	*Turicibacter*	*Micrococcus*
Comamonas	*Pediococcus*	*vadinHB04*	*Okibacterium*
Coprococcus	*Pedobacter*	*Vagococcus*	*Pantoea*
Corynebacterium	*Phascolarctobacterium*	*Veillonella*	*Pseudoclavibacter*
Dermabacter	*Phenylobacterium*	*Vibrio*	*Raoultella*
Devosia	*Photobacterium*	*Wohlfahrtiimonas*	*Rhodotorula*
Dietzia	*Prevotella*	*YRC22*	*Rothia*
Dorea	*Propionicimonas*		*Serratia*
Enhydrobacter	*Providencia*		*Weissella*
Faecalibacterium	*Pseudobutyrivibrio*		
Fusobacterium	*Pseudonocardia*		

and further analysed with 16S gene sequencing, three genera were still not present in the Bruker database (*Frigoribacterium*, *Gibbsiella* and *Okibacterium*). Six others were already included but were not identified. An explanation for the latter can be that spectra generated for certain species can differ between isolates due to media and growth conditions (Williams et al., 2003).

Applying culture independent 16S amplicon sequencing, the relative abundance of each OTU varied among the samples and there were no same genera or even same families dominating the different samples.

Pseudomonas spp. and *Acinetobacter* spp. were detected in all samples and are indicative of a soil or water contamination (Chaillou et al., 2015). Members of the genera occur commonly on fresh meat and were also abundant in the Japanese study of Asakura et al. (2017). *Pseudomonas* present on meat stored under refrigerated conditions often becomes the dominant bacterial flora and causes spoilage (Doulgeraki et al., 2012), while *Acinetobacter* contributes more to the breakdown of food components and may produce off-odours (Rawat, 2015).

Macrococcus and *Propionibacterium* were also frequently detected with 16S amplicon sequencing (90.9%) but while *Macrococcus* was isolated from 14 samples of which 12 samples on plates incubated at 30 °C and in other two samples on the plates incubated anaerobically, *Propionibacterium* was only isolated from one sample. The latter is more associated with dairy products, silages and vegetables, ruminal content and faeces, human stools and faeces from poultry and pigs (Freitas et al., 2015). Instead, macrococci are typically isolated from animal skin and food of animal origin (Baba et al., 2009). *Pediococcus*, a lactic acid bacteria commonly present in the mammalian gut (Araya et al., 2002), was detected in 19 samples but never isolated, probably because the MRS plates were incubated aerobically and without the addition of inhibiting antibiotics (Simpson et al., 2006).

The high rate of detection of members of the *Enterobacterales* order (*Enterobacter*, *Escherichia* and to a slightly lesser extent, *Rahnella* and *Citrobacter*) provide again, along with the high contamination levels described above, evidence of a faecal contamination of the samples. Moreover they are bacterial groups that contain human pathogens (Leight et al., 2018). However, even if in the present study a selective media for the enumeration of *Enterobacterales* was used, the isolation rate of *Enterobacter Citrobacter* and *Rahnella* was low.

In almost 70% of the wild boar meat samples two genera belonging to the family of *Ruminococcaceae* (*Faecalibacterium* and *Ocillospira*) were present. Particularly *Ruminococcaceae* is one of the most abundant families from the order *Clostridiales* present in the mammalian gut environment and has been associated with the maintenance of gut health (Biddle et al., 2013).

Two genera belonging to the order of *Bacteroidales* (*Bacteroides* (*Bacteroidaceae* family) and *Prevotella* (*Prevotellaceae* family)) and one genus belonging to the order of *Clostridiales* (*Roseburia* (*Lachnospiraceae* family)) were detected in 15 samples applying 16S amplicon sequencing. None of the three were retrieved by cultivation. *Bacteroides* and *Roseburia* have been detected in the faeces of a variety food animals (Park et al., 2014; Young Ko et al., 2018) and *Prevotella* has been found in the gut microbiota of ruminants and chimpanzees (Holman et al., 2017). *Janthinobacterium*, *Brochothrix* and *Streptococcus* were detected in around 60% of the samples. *Janthinobacterium* was once identified in water, soil and spoiled raw milk (Chaillou et al., 2015; Liu et al., 2018; Molin, 2000) but, the role of this genus in the spoilage process is still unknown (Liu et al., 2018). Instead, the species *Brochothrix thermosphacta*, known as common meat contaminant, is considered one of the major cause of spoilage (Rouger et al., 2017). However, *Janthinobacterium* and *Brochothrix* were isolated on PCA plates incubated at 7 °C only in one and three samples respectively. *Streptococcus* was isolated from four samples on PCA incubated at 7 °C and also in anaerobic conditions and MRS incubated aerobically. This genus contains several species that are part of the normal flora of human and animals but some are also pathogenic (Lu et al., 2014; Lun et al., 2007).

Bacteroides, *Faecalibacterium*, *Fusobacterium*, *Oscillospira*, *Roseburia*, *Prevotella* and *Pseudobutyrivibrio*, bacteria frequently detected with 16S amplicon sequencing but not isolated, were all obligate anaerobic, indicating that the current commonly applied anaerobic culture method using Gaspak did not support bacterial recovery.

Other discording result between culturomics and 16S amplicon sequencing occurred for the genus *Pantoea* belonging to the *Erwiniaceae* family that was frequently isolated but never detected. Moreover, also the percentage of isolation and detection of the genus *Pseudomonas* also differs between the two approaches. It could be explained by the fact that organisms with high growth rates, even if they are present with a smaller percentage in an ecosystem may overlay on organisms present with a higher number but with low growth rates (Großkopf et al., 1998). It is certain that culture-dependent methods are biased due to the media and the condition used but the direct 16S rRNA analysis also seems biased according to the primer used, variable region selection, amplicon size and the number of PCR cycles (Fuks et al., 2018; Knight et al., 2018). Also *Salmonella*, detected by RT- PCR and confirmed by isolation, was not detected using 16S amplicon sequencing applied on the first meat homogenate. It is certain that the portions analysed with RT-PCR have not been the same portions as for the other analysis and moreover, an enrichment step was used for the pathogen detection. However it has been previously reported that these bacteria usually are present at a very low concentration and only deep Next Generation Sequencing seems to overcome this problem (Rouger et al., 2017). Although the aim of this study was to characterize for the first time the microbial diversity and richness in fresh wild boar meat samples, taxonomic resolution could be improved in follow up studies by using, for example, full length 16S amplicon sequencing (Martínez-porchas et al., 2016). Nevertheless, even when applying improved methods for taxonomic resolution, it is important to keep the impact of the DNA extraction protocol on the results in mind (Yu et al., 2019). Moreover, in the present research, it is confirmed again that culture-dependent and independent approaches are complementary in the study of the complex relationship between microorganisms in a food as already reported by Lagier et al. (2018) and Peruzy et al. (2019).

In conclusion, in the present study, the initial bacterial contamination on wild boar meat was studied, and showed to be a greater public health risk compared to pork. The need for better slaughter hygiene was demonstrated and the non-inspected distribution of meat directly to consumers is not recommended. Although often claimed as such, culture-independent analysis proved not to be a reliable alternative as it fails to detect the foodborne pathogen *Salmonella*. Only in the study of bacterial communities, a combination of both approaches might be relevant.

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