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Determination of the microbiological contamination in minced pork by culture dependent and 16S amplicon sequencing analysis

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ABSTRACT

Routine evaluation of bacterial contamination in minced pork is still mainly performed by the enumeration of indicator bacteria, including total aerobic colony count and *E. coli*, using standardized isolation methods. However, the bacterial community structure as well as the effect of the storage time and temperature on the aerobic plate count are largely unknown for this matrix. The aim of the study was to characterize the microbial community in minced pork by 16S rRNA amplicon sequencing compared to classical isolation methods combined with identification by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS) and 16S rRNA gene sequencing. Analysis of 14 unrelated samples showed that total aerobic counts determined at 30 °C and 7 °C showed no significant difference, but the richness was higher on PCA at 30 °C for 7 samples, equal in 5, and higher at 7 °C for 2 samples. Members of the genus *Pseudomonas,* along with the genera *Brochothrix* and *Carnobacterium* were commonly identified among both the mesophilic and psychrotrophic population. Comparing to 16S rRNA amplicon sequencing, some contrasting data were obtained. Except for *Brochothrix* spp. and *Pseudomonas* spp., that were abundant and always detected, genera obtained with the two methods in the same sample were not always the same. Comparison of different sample preparation techniques and DNA extraction methods demonstrated also in this matrix that different results on the microbial composition and complexity are obtained. Present data illustrate that the combined isolation and identification of isolates using MALDI TOF MS and 16S gene sequencing and overall community profiling using 16S rRNA amplicon sequencing provides complementary results and yields important insights into the complex relationship between microorganisms in a food.

1. Introduction

Pork products are widely consumed all over the world with a mean consumption of 12,3 kg per capita per year (OECD, 2018). These products, especially minced meat, have a short shelf life and have been identified as potential sources of foodborne pathogens (Koutsoumanis and Sofos, 2004). In many countries, minced pork is the basis for different meat preparations (e.g. sausages, rollè, meatballs, burgers), but also consumed raw. Contamination of ground meat with microorganisms can occur during the whole processing, though in particular in meat cutting plants or at retail when the meat is cut or minced with more surfaces exposed (Ejeta et al., 2004). For food safety, hurdle technology can be applied, including cooling or heating, addition of additives as nitrate and nitrite, pH lowering by fermentation, but in many cases due to specific meat product characteristics cannot be implemented or is insufficient to reduce or eliminate pathogens completely.

According to EU regulation 853/2004, minced meat must be chilled to a core temperature of no > 2 °C immediately after production. Although low temperature controls growth of many microorganisms, some pathogens as well as a range of food spoilage organisms are not completely inhibited nor killed. In meat, *Pseudomonas* species are known to cause spoilage at low temperatures, but *Brochothrix thermosphacta* and specific lactic acid bacteria are also common spoilage causers, though rather more involved in souring then putrefaction (Nychas et al., 2008). Spoilage by members of the family Enterobacteriaceae at the other hand is rare (Nychas et al., 2008).

Setting microbiological parameters for the determination and control of food hazards from processing till consumption remains problematic due to the variety of products and applications (Koutsoumanis

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and Sofos, 2004). General microbiological parameters as total aerobic colony count (TAB) at 30 °C and enumeration of *E. coli* are generally applied in food hygiene monitoring (EU 1441/2007). However, while *E. coli* acts as an indicator of faecal contamination, determination of aerobic microorganisms at 30 °C provides only indication about the level of culturable bacteria present in or on food, without additional information at taxonomic level, bacterial diversity or potential pathogenicity. Additionally, current food hygiene criteria do not focus on specific spoilage bacteria, though their level could be used as a freshness indicator (Bruckner et al., 2012). In fact, meat spoilage is mainly caused by psychrotrophic bacteria which do not or only slowly grow at 30 °C. The current use of mesophilic counts as parameter for food hygiene could therefore be biased (Ercolini et al., 2009; Jay, 2002).

Methods to enumerate hygiene associated microbes are based on quantitative isolation which is not only labour intensive, but also influenced by specific isolation media and conditions. Furthermore, microbial levels are often underestimated due to the stressed condition the target microbes resident at the time of sampling are subject to. Moreover, as no or only some colonies are picked for further identification, knowledge on the microbial diversity of the counted microorganisms is lacking. Therefore, more recently, culture-independent techniques have been introduced as new approaches to identify bacterial communities in different ecosystems (Cocolin et al., 2004, 2013) with currently high throughput analysis by 16S rRNA amplicon sequencing as the preferred method. However, disadvantages are the generation of yet not assignable sequences (Lagier et al., 2016), and still the unavoidably time consuming steps of DNA isolation, amplification and data management. These limitations are among the main reasons why information on the comparison of the application of culture dependent versus culture independent methods on fresh pork is largerly lacking.

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). The application of MALDI-TOF MS in food microbiology is still preliminary as databases are still oriented towards clinical relevant microorganisms. Nevertheless, MALDI TOF MS could be a promising technique in food microbiology analysis as well, especially when a timely identification or confirmation of the isolates is the target (Carannante et al., 2015; Nicolaou et al., 2012; Pavlovic et al., 2013).

The aim of the present study was to assess the contamination level and diversity among mesophilic and psychrophilic bacterial communities of minced pork, using both classical isolation combined with MALDI TOF MS analysis, and 16S rRNA amplicon sequencing. Additionally, the impact of different sample preparation and DNA extraction methods on the identification accuracy and community profiling was evaluated, for 16S rRNA amplicon sequencing.

2. Materials and methods

2.1. Sampling

From January to September 2017, a total of 14 unrelated minced pork meat samples (M1 to M14), each between 200 and 300 g, were bought 1–2 days before expiration day at different supermarkets in "Campania region" in south Italy. Samples were transported at 4 °C to the laboratory and analysed within 1 h after purchase.

2.2. Bacterial isolation

Ten grams of each sample 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 stomachered homogenate (called "SH 1 to 14") were prepared in PW, followed by quantitative bacterial isolation in duplicate for: a) total aerobic bacteria (TAB) counts performed according to ISO 4833-2:2013 on Plate Count Agar (PCA; CM0325, Oxoid) was incubated at 30 °C for 48 to72 h; b) psychotropic bacteria counts on PCA incubated at 7 °C for 10 days (Ercolini et al., 2009); c) 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; d) 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; e) *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. The colonies were counted and subsequently all picked from the plates with a bacterial growth between 30 and 300 CFU/plate on PCA, and between 15 and 150 CFU/plate from all others. Harvested colonies were subcultured on Tryptic Soy Agar (TSA, CM0131, Oxoid) or MRS and incubated at the temperature and atmosphere as described above.

2.3. MALDI-TOF MS identification and data analysis

Colonies isolated from samples M1 to M6 were analysed using the "direct colony identification method" (Alatoom et al., 2011). In brief, bacterial growth was smeared in duplicate onto a MALDI-TOF MS target plate (Bruker Daltonics, Bremen, Germany). After air-drying, the sample was covered with 1 µl matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoracetic acid (50:47.5:2.5, [vol/vol/vol]).

To assess the impact of the bacterial preparation technique on the MALDI-TOF MS identification performance, for samples M7 to M14, "bacterial suspensions" were prepared based on the protocol of Williams et al. (2003). In brief, one colony was suspended in 800 µl of TSB broth and incubated at 28 °C for 24 h. Subsequently, the incubated broth was centrifuged (1533*g* at 4 °C) for 10 min. The supernatant was discarded and the pellet was washed twice with 500 µl of Milli-Q water and centrifuged (1533*g* at 4 °C) for 10 min. After the second centrifugation, the supernatant was removed, the pellet suspended in 100 µl of Milli-Q water, and 3 µl of each bacterial suspension was spotted in duplicate on a MALDI target plate. After air-drying, the sample was covered with 1 µl matrix solution as described above.

As previously reported, lactic acid bacteria require a different approach (Alatoom et al., 2011), therefore identification of the isolates on MRS agar plates (samples M1 to M14) was performed with the more laborious "extraction method" based on a modified 'Microorganism profiling "Ethanol/Formic Acid extraction" procedure' (03.04.2006) from Bruker Daltonics. Individual colonies were suspended in 800 µl of MRS broth and incubated at 28 °C for 24 h. Bacterial suspensions were prepared as described above and centrifuged (1533*g* at 4 °C) for 10 min. Next, 50 µl formic acid and 50 µl of acetonitrile were added to the pellet and thoroughly mixed by pipetting, followed by centrifugation (1533*g* at 4 °C) for 10 min. One microliter of the supernatant was spotted in duplicate onto a 96-spot plate and allowed to dry at room temperature. Afterward, 1 µl of matrix solution was added.

Each series of MALDI measurements was preceded by a calibration step with the bacterial test standard (BTS 155 255,343; Bruker Daltonics) in order to validate the run. Mass spectra were generated by 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 score of ≥2.3 indicated 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 between 1,7 and 2,0 or < 1,7 was obtained with the "direct colony identification method", were analysed a second time using the extraction method.

The analysis was repeated when both spots of the same colony resulted in 'no peaks found' or when different outcomes where obtained. Isolates for which spectra were obtained with the extraction or suspension method but with a score below 1,7 were imported into the BioNumerics 7.2.6 software (Applied Maths, Sint-Martens-Latem, Belgium) to first cluster and then visually select representatives. 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. For this, 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). Subsequently, the suspension was centrifuged for 5 min at 10,000 ×*g* at 4 °C. To amplify the partial 16S rRNA gene sequencing, the oligonucleotide primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used. The PCR mixture (final volume, 25 µl) contained 2,5 µl template DNA, 0,25 µl of each primer at concentration of 10 µM, 2,5 µl of each deoxynucleoside triphosphate at a concentration of 2 µM each, 0,5 µl AmpliTaq DNA polymerase (1 U/µl) and 16,5 µl of Milli Q water. PCR conditions consisted of 30 cycles. Amplicons were collected and submitted for Sanger sequencing (Eurofins). Subsequently, to obtain the identification, the sequences were blast towards the gene bank database (https://www.ncbi.nlm.nih.gov).

2.4. Community identification by 16S rRNA amplicon sequencing

To characterize the microbial communities in eight minced meat samples (M7 to M14), two DNA extraction methods, FastDNA® SPIN Kit for Soil (MP Biomedicals) and PowerFood Microbial DNA Isolation kit (Qiagen, Germany) (Quigley et al., 2012) were applied. DNA was extracted following manufacturer's recommendations:

1) directly from 500 mg of minced meat (named: MS for the samples extracted with FastDNA® SPIN Kit for Soil and MP for the samples extracted with PowerFood Microbial DNA Isolation kit),
2) from a pellet obtained from 1,8 ml of SH (named: MSA for the samples extracted with FastDNA® SPIN Kit for Soil and MPA for the samples extracted with PowerFood Microbial DNA Isolation kit) and
3) from 1,8 ml of meat homogenate prepared by stomaching 20 g minced meat with 20 ml (1:2) of peptone water (named: MSB for the samples extracted with FastDNA® SPIN Kit for Soil and MPB for the samples extracted with PowerFood Microbial DNA Isolation kit).

DNA quantity was measured using the dual-channel Quantus™ Fluorometer (Promega USA), and for the 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). DNA extracts with an amount ≥ 100 ng, concentration ≥ 5 ng/µl, volume ≥ 20 µl, OD 260/280 = 1.8–2.0 were sent 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-PCRr-free-library-prep kit was used to construct the DNA libraries of paired ends with single index. Amplicons from different samples were mixed in equimolar amounts and sequenced on the Illumina2500 platform with Sequencing strategy PE250. Qiime2 (version 2017.10) software pipeline (https://qiime2.org) was used for data analysis. Reads were demultiplexed with q2-demux (https://github.com/qiime2/q2-demux). Then q2-dada2 plugin was implemented 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. Statistic 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/).The differences between the three different bacterial preparation methods for MALDI TOF MS analysis were assessed by chi square test. A probability value of < 0.05 ($p < 0.05$) was defined as statistically significant. Richness expressed by the Chao1 index and diversity indices of community information obtained from MALDI-TOF MS identification and richness expressed by the Chao1 index and standard deviation of community information obtained from 16S rRNA amplicon sequencing were calculated by PAST software, as well as the rarefaction curves (Hammer et al., 2001).

3. Results

3.1. Bacterial isolation

Bacterial counts for the 14 samples are shown in Fig. 1. Depending on the incubation temperature, total aerobic counts on PCA ranged from 6,2 to 7,55 log CFU/g (mean ± SD = 7,08 ± 0,4 log CFU/g) for mesophilic bacteria and from 6,37 to 8,13 log CFU/g (mean ± SD = 7,28 ± 0,5 log CFU/g) for psychrotrophic bacteria. However, the viable counts determined at 30 °C and 7 °C showed no significant difference ($p > 0.05$). The mean number of bacterial colonies present on CFC and MRS agar plates were 7,28 ± 0,66 and 5,2 ± 0,68 log CFU/g, respectively. The count of typical blue *E. coli* colonies on TBX showed always to be < 1 log CFU/g, except for samples M3 and M13 where the levels were equal to 1,26 log.

3.2. Bacterial identification with MALDI-TOF MS

In the present study, 2800 bacterial isolates from the 14 minced meat samples were harvested and further analysed by MALDI-TOF MS. Using the "direct colony identification method" on 1055 isolates from the samples M1 to M6, almost 57% were identified at genus level, of which almost 10% at species level (score values ≥ 2,3) (Table 1). The remaining 43,22% (n = 456) were subsequently tested by "the extraction method" of which 70 were additionally identified at species level, 169 had secure genus and probable species identification, 137 had a probable genus identification, and for 80 of them, a score below 1,69 was generated. For samples M7 to M14 (n = 989), the use of the "bacterial suspension method" yielded, in comparison with the other two methods, the highest amount (42,57%) of organisms without an identification (score < 1,69; $p < 0.05$).

The analysis of the isolates on the MRS agar plates (n = 756) performed with the "extraction method" for all samples (M1-M14), resulted in no identification at genus level for almost 40% (n = 273) of the colonies (Table 1).

In general, bacteria identified by MALDI-TOF MS could be assigned to 15 families and 18 genera (Table 2). Moreover, *Debaryomyces* spp. and *Cutaneotrichosporon* spp., belonging to the fungi kingdom, were also isolated: *Cutaneotrichosporon* was isolated only in sample M10, in contrast to *Debaryomyces* which was isolated from all samples except for samples M6 and M14.

Among the isolates without MALDI TOF MS identification (originating from M1-M14) but analysed with 16S rRNA gene sequencing

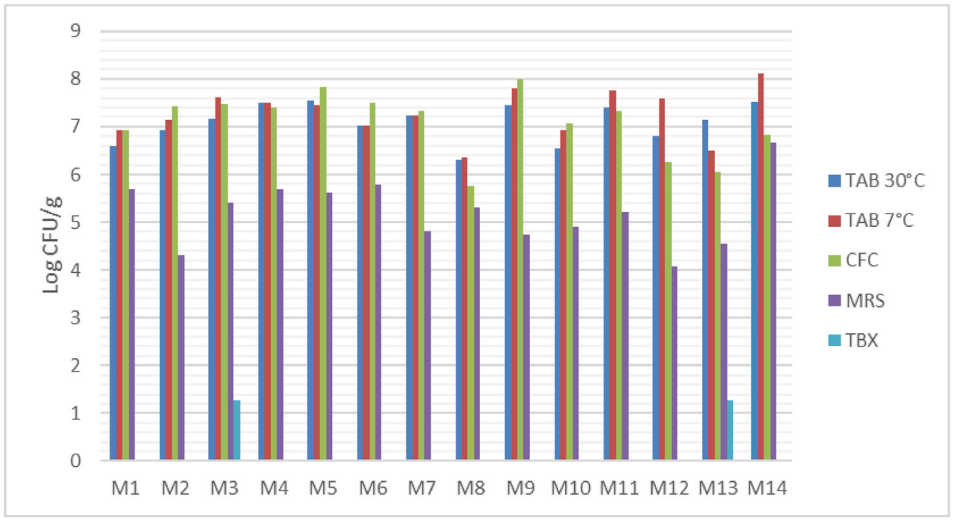

Fig. 1. Total bacterial counts in fourteen pork minced meat samples (M1–M14) on different media: mesophilic bacteria on PCA (TAB 30 °C), psychotropic bacteria on PCA (TAB 7 °C), presumptive *Pseudomonas* spp. on CFC, Lactic Acid Bacteria on MRS and *E. coli* on TBX.

Table 1
Number and percentage of isolates with a highly probable species identification (score value: ≥2,3), secure genus and probable species identification (score value: between 2,0 and 2,3), probable genus identification (score value: between 1,7 and 2,0) and non-reliable identification (score value: < 1,7) with MALDI-TOF MS through direct colony identification and subsequent extraction method for the samples from M1 to M6, suspension method for the samples from M7 to M14, and extraction method for the identification of the bacteria isolated on MRS (presumptive Lactic acid Bacteria) for the samples from M1 to M14.

	N.	%	N.	%
Direct colony identification (M1–M6)			Extraction (M1–M6)	
< 1,69	301	28,53	80	17,54
1,70–1,99	155	14,69	137	30,04
2,0–2,2	497	47,11	169	37,06
> 2,3	102	9,67	70	15,35
Suspension (M7–M14)				
< 1,69	421	42,57		
1,70–1,99	268	27,10		
2,0–2,2	273	27,60		
> 2,3	27	2,73		
Extraction (LAB) (M1–M14)				
< 1,69	273	36,11		
1,70–1,99	173	22,88		
2,0–2,2	266	35,19		
> 2,3	44	5,82		

Table 2
Number (n.) and percentage (%) of isolates identify at genus level with MALDI-TOF MS and 16S rRNA gene sequencing.

MALDI-TOF MS			16S rRNA gene sequencing		
Microorganisms	n.	%	Microorganisms	n.	%
Genera (Bacteria)			Genera (Bacteria)		
Brevundimonas spp.	2	0,07	*Acinetobacter* spp.	3	2,10
Brochothrix spp.	240	8,57	*Brevundimonas* spp.	1	0,70
Carnobacterium spp.	20	0,71	*Brochothrix* spp.	23	16,08
Corynebacterium spp.	2	0,07	*Carnobacterium* spp.	46	32,17
Enterococcus spp.	1	0,04	*Kocuria* spp.	2	1,40
Escherichia spp.	42	1,50	*Lactobacillus* spp.	8	5,59
Kocuria spp.	3	0,11	*Lactococcus* spp.	18	12,59
Lactobacillus spp.	342	12,21	*Leuconostoc* spp.	19	13,29
Lactococcus spp.	68	2,43	*Pseudomonas* spp.	6	4,20
Leuconostoc spp.	69	2,46	*Psychrobacter* spp.	15	10,49
Microbacterium spp.	4	0,14	*Rahnella* spp.	1	0,70
Paracoccus spp.	1	0,04	*Serratia* spp.	1	0,70
Pediococcus spp.	1	0,04			
Pseudomonas spp.	1022	36,50			
Rahnella spp.	3	0,11			
Serratia spp.	13	0,46			
Shewanella spp.	1	0,04			
Staphylococcus spp.	2	0,07			
Genera (Fungi)					
Cutaneotrichosporon spp.	1	0,04			
Debaryomyces spp.	189	6,75			
Organisms without an identification	774	27,64			

(n = 143), the majority (32,17%) belonged to the genus *Carnobacterium,* and one family (Moraxellaceae) and two genera (*Acinetobacter* and *Psychrobacter*) were additionally identified (Table 2).

Members of the genus *Pseudomonas,* along with the genera *Brochothrix* and *Carnobacterium* were commonly identified among the mesophilic and psychrotrophic population on PCA (Fig. 2). *Lactococcus* were also frequently identified among the psychrotrophic. *Shewanella* was only isolated from PCA plates incubated at 7 °C aerobically. Among the isolates present on the MRS agar plates, *Lactobacillus* was the genus most frequently present, followed by the fungus *Debaryomyces* spp. On the CFC plates *Pseudomonas* was the dominant genus. Other genera isolated or detected by 16S rRNA amplicon sequencing on the CFC plates were the oxidase negative genera *Escherichia* spp., *Rahnella* spp. and Serratia spp. (Fig. 2). On the TBX plates, although other non-typical bacteria were present, all typical blue colonies were confirmed as *E. coli*.

Among the isolates with a MALDI-TOF score of ≥2,3, only 13 species were reliably identified: *Brochothrix thermosphacta, Enterobacter cloacae, Escherichia coli, Kocuria rhizophila, Lactobacillus sakei, Lactococcus lactis, Leuconostoc gelidum, Leuconostoc mesenteroides, Pseudomonas fragi, Pseudomonas galenii, Pseudomonas proteolytica, Serratia liquefaciens* and *Serratia proteamaculans.*

3.3. Bacterial identification with 16S rRNA amplicon sequencing

Using the 16S rRNA amplicon sequencing strategy, a total of 2.588.365 reads were obtained from samples M7-M14. Only exact amplicon sequence variants (OTUs) accounting for > 0,5% of total reads were retained in the analyses (n = 2.347.581 reads). A total of 11 genera encompassing 10 families were identified. However, in all samples groups of bacteria (mean = 13,37%) could only be identified at

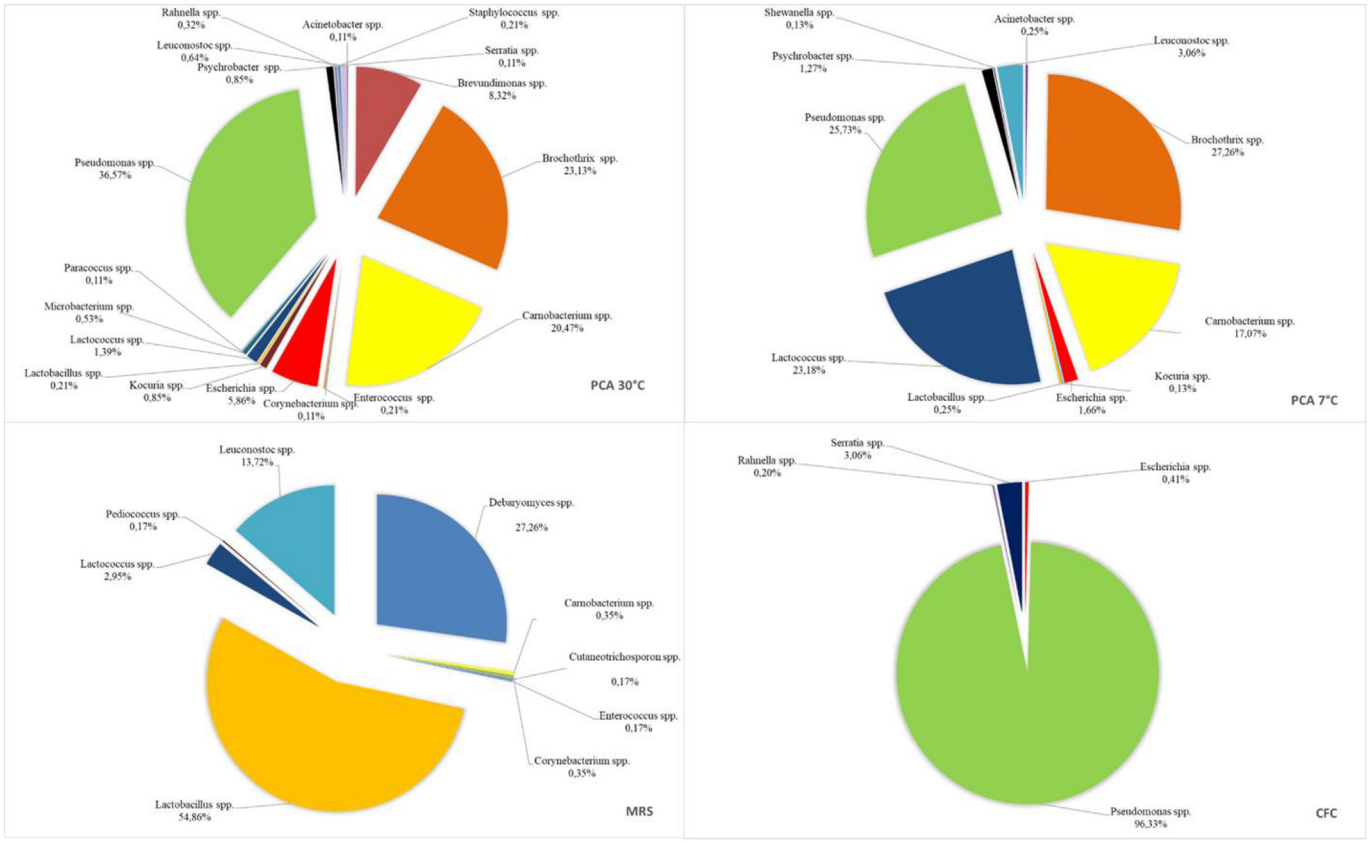

Fig. 2. Bacteria identified at genus level by MALDI-TOF, 16S gene sequencing and subsequent cluster analysis with BioNumerics 7.2.6 software, present on different agars: Plate Count Agar (PCA) at 30 °C, PCA at 7 °C, Cephalothin-Sodium Fusidate-Cetrimide Agar with Modified CFC Selective Supplement (CFC) and De man, Rogosa and Sharpe agar (MRS).

family or higher taxonomic level, and were attributed to undetermined Enterobacteriaceae and Lachnospiraceae or to undetermined Bacilli and Gamma-Proteobacteria.

Comparing the culture independent method (16S rRNA amplicon sequencing) with the identification upon culturing (MALDI TOF and 16 rRNA gene sequence), among the 8 samples (M7-M14), *Pseudomonas* and *Brochothrix* were always detected. However, *Escherichia, Rahnella, Microbacterium* and *Paracoccus* were identified only with the culture dependent methods and *Marinomonas, Staphylococcus* and *Photobacterium* were detected only using the culture independent 16S rRNA amplicon sequencing (Table 3).

3.4. Effect of sample preparation and DNA extraction

The relative abundances of each OTUs varied according to the sample preparation and DNA extraction kit applied (Fig. 3). *Marinomonas* spp. was never detected when using pellets of 1:2 dilutions (MSB and MPB), nor when using the pellet of 1:10 in combination with the FastDNA® SPIN Kit for Soil extraction method (MSA). *Shewanella* spp. was never detected when FastDNA® SPIN Kit for Soil extraction method was used (MSA and MSB), nor when using the pellet of 1:2 in combination with the PowerFood Microbial DNA Isolation kit extraction method (MPB), and *Leuconostoc* spp. was detected only when using pellets of 1:2 dilutions in combination with the FastDNA® SPIN Kit for Soil extraction method (MSB).

Nevertheless, regardless of the sample preparation and extraction method used, *Photobacterium, Pseudomonas* and *Brochothrix* were the most abundant genera, accounting for > 50% of the total microbial community observed (Fig. 3).

3.5. Statistical analysis

The microbial diversity on the PCA plates incubated at 30 °C and 7 °C was compared for all 14 samples (Table 4). The richness, expressed by Chao1 index, was higher on PCA at 30 °C for 7 samples (M3, M5, M6, M8, M11, M12 and M13), was equal among the two conditions in 5 samples (M1, M2, M4, M7 and M9), and in 2 samples (M10 and M14) higher on PCA at 7 °C. Samples M8 and M13 showed the lowest equitability (evenness) index, as they were dominated by *Carnobacterium* and *Brevundimonas* respectively.

The richness (expressed by the Chao1 index) of the microbial communities extracted directly from the meat using PowerFood Kit (MP) (mean ± SD = 316 ± 128,64) was higher than with the other methods (Table 5).

4. Discussion

In the present study, the levels of total lactic acid bacteria and *E. coli* in 14 unrelated minced pork samples were similar to those reported in other studies (Andritsos et al., 2012; Han et al., 2014). However, the average total aerobic bacterial counts, both at 7° and 30 °C, as well as the number of presumptive *Pseudomonas* species (Fig. 1), were higher (Andritsos et al., 2012; Koo et al., 2016). Moreover, the European food hygiene criterion for total aerobic bacteria at 30 °C was exceeded in 11 samples (EU 1441/2007). Statistical analysis showed that the temperature of incubation of the PCA plates (30° or 7 °C) did not have a significant impact on the bacterial colony counts ($p > 0.05$). A possible explanation for this may be that around 70% of the bacteria grown on PCA in both conditions belonged to *Pseudomonas*, *Brochothrix* and *Carnobacterium*, which are able to grow both at mesophilic as well as at psychrotrophic temperatures. They represent the primary cause of

Table 3
Comparison of the genera identified via culture dependent (MALDI TOF and 16S gene sequencing) and independent (16S rRNA amplicon sequencing) methods in pork meat samples (M7 to M14).

Family	Genus	M7		M8		M9		M10		M11		M12		M13		M14	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Carnobacteriaceae	*Carnobacterium*		x	x	x		x		x	x	x	x	x	x	x	x	x
Caulobacteraceae	*Brevundimonas*													x			
Corynebacteriaceae	*Corynebacterium*			x					x								
Enterobacteriaceae	*Escherichia*					x		x		x		x		x		x	
	Rahnella									x		x				x	x
	Serratia		x	x					x	x		x	x	x	x	x	
Enterococcaceae	*Enterococcus*		x	x									x		x		x
Lactobacillaceae	*Lactobacillus*		x	x	x	x		x	x	x		x	x	x	x	x	x
Leuconostocaceae	*Leuconostoc*		x	x	x			x	x	x		x	x	x		x	
Listeriaceae	*Brochothrix*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Microbacteriaceae	*Microbacterium*													x			
Micrococcaceae	*Kocuria*			x													
Moraxellaceae	*Acinetobacter*		x		x		x		x	x	x		x		x		x
	Psychrobacter		x		x		x	x	x	x	x		x	x	x		x
Oceanospirillaceae	*Marinomonas*												x		x		x
Pseudomonadaceae	*Pseudomonas*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Rhodobacteraceae	*Paracoccus*													x			
Shewanellaceae	*Shewanella*		x		x		x		x	x	x		x		x		x
Staphylococcaceae	*Staphylococcus*		x		x				x				x		x		
Streptococcaceae	*Lactococcus*		x	x	x		x	x	x	x	x	x	x		x	x	x
Vibrionaceae	*Photobacterium*		x		x		x		x		x		x		x		x

A: genera identified via MALDI TOF and 16S rRNA gene sequencing.
B: genera identified via 16S rRNA amplicon sequencing (OTUs accounting for > 0.5% of total reads).

reduced shelf life and spoilage in fresh meat (Kilcher et al., 2010; Kameník, 2013; Mills et al., 2018). Additionally, although the meat was bought before expiration day, and changes in colour at the moment of purchase were not observed, it has been demonstrated that the mean level of *Pseudomonas* (7,28 log CFU/g) present on the samples, is close to the level at which signs of spoilage become visible (7.5 log CFU/g) (Kameník, 2013). Explanations for these high levels could be the initial contamination on the carcasses surfaces due to slaughter, or the non hygienic handling during deboning and cutting of the meat (EFSA, 2016). Moreover, though specific temperature requirements for transport, cutting and storage are stipulated in EC legislation (EU 853/2004), temperatures above 10 °C during transportation and storage at retail have been reported (Koutsoumanis et al., 2006). For the present study, storage at the supermarkets fulfilled the temperature requirement, but temperature data from the slaughterhouses and meat-cutting plants were not available.

Bacterial colonies from the different isolation plates were analysed applying three different bacterial preparation methods for MALDI TOF MS analysis. The "suspension method" resulted in more low-level identifications than the "direct colony identification" and "extraction" methods. Moreover, compared with the direct colony identification method, the suspension method requires additional processing time. The suspension method was originally introduced as a method for directly identifying bacteria from "whole cells" and not only from the proteins (Williams et al., 2003). However, as reported by Williams et al. (2003) the final concentration of the cells in water, not tested in the present study, can influence the spectra and therefore also the identification obtained. In the present study, the "direct colony identification method", although not recommended as first choice method, yielded the best results with a minimum of effort: 57% of the isolates were validly identified at genus or species level. This is lower than reported then in the study of Bizzini et al. (2010) where 75% of the isolates were

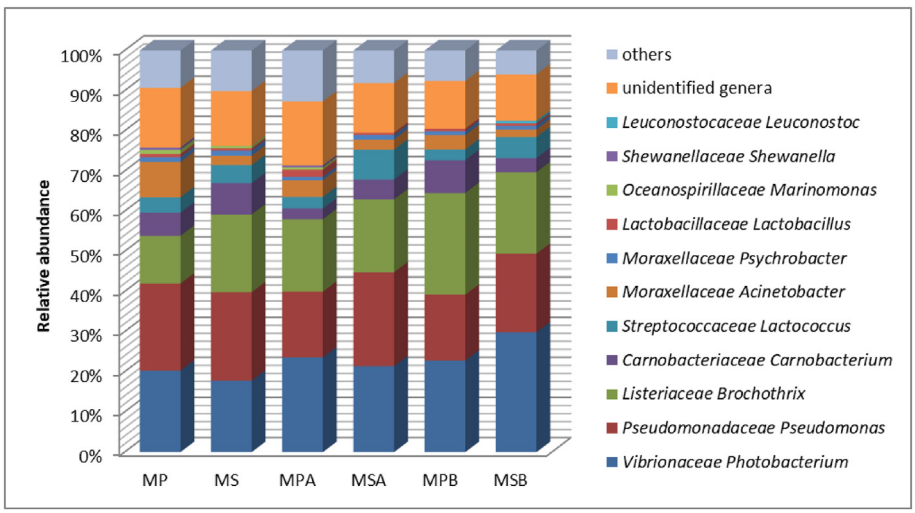

Fig. 3. Average microbial communities identified by 16S rRNA amplicon sequencing in eight minced meat samples (M7 to M14). Relative abundances of each OTUs relating with the extraction kit or extraction protocol. Genera with < 0.5% relative abundance were summed up and denoted as others.

Table 4
Richness (expressed by Chao1 index) and diversity (expressed by Shannon and Evenness indexes) of the bacterial communities identified by MALDI-TOF and 16S gene sequencing between the 14 minced meat samples both on PCA at 30 °C and on PCA at 7 °C.

	30 °C	7 °C	30 °C	7 °C	30 °C	7 °C	30 °C	7 °C	30 °C	7 °C	30 °C	7 °C	30 °C	7 °C
	M1		M2		M3		M4		M5		M6		M7	
Chao1	5,33	5	3	3	5	4	2	2	3	2	5,5	3	2	2
Shannon	1,52	1,24	0,97	0,72	1,12	1,28	0,27	0,69	0,85	0,5	0,85	0,63	0,53	0,49
Evenness	0,92	0,69	0,88	0,68	0,61	0,9	0,66	0,99	0,78	0,82	0,47	0,63	0,85	0,81
	M8		M9		M10		M11		M12		M13		M14	
Chao1	7,5	5	3	3	3	7	7,5	6	7	5	10	5	4,5	6
Shannon	0,96	1,22	0,69	0,68	0,77	1,12	1,3	1,06	1,26	1,34	0,88	1,25	0,64	1,04
Evenness	0,37	0,68	0,66	0,66	0,72	0,51	0,53	0,58	0,59	0,77	0,35	0,7	0,48	0,47

Table 5
Richness, expressed by the Chao1 index, of the microbial communities identified by 16S rRNA amplicon sequencing issued from the pork meat samples (M7 to M14) related to the extraction protocol used.

	Richness (mean ± SD)
MP	316 ± 128,64
MPA	291,14 ± 117,63
MPB	249,75 ± 128,164
MS	243,25 ± 115,34
MSA	219,625 ± 77,99
MSB	208 ± 76,13

MP: DNA extracted from 500 mg of the minced meat using PowerFood Microbial DNA Isolation kit; MPA: DNA extracted from a pellet obtained from 1,8 ml of SH using PowerFood Microbial DNA Isolation kit; MPB: DNA extracted from a pellet obtained from 1,8 ml of meat homogenate in Peptone water with a ratio of 1:2 using PowerFood Microbial DNA Isolation kit; MS: DNA extracted from 500 mg of the minced meat using FastDNA® SPIN Kit for Soil; MSA: DNA extracted from a pellet obtained from 1,8 ml of SH using FastDNA® SPIN Kit for Soil; MSB: DNA extracted from a pellet obtained from 1,8 ml of meat homogenate in Peptone water with a ratio of 1:2 using FastDNA® SPIN Kit for Soil.

identified, but in the latter, cut-off values of 2,0 were applied. The "direct colony identification method" results in fast (about 1 h to prepare and examine 96 samples) identification at minimal cost and, based on our results, can be recommended as a as first screening tool when large amounts of isolates have to be examined.

When the "direct colony identification method" fails to achieve a reliable identification due to low scoring or absence of spectrum, the extraction method is the subsequent analysis strategy proposed. The extraction method is generally recognized to be the best method for the identification of bacterial isolates by MALDI TOF, since it usually generates high-quality spectra (Alatoom et al., 2011). Moreover, isolates present on MRS plates were only analysed with it, as it has previously been demonstrated that the use of other methods with most of Gram-positives is not sufficient to disrupt the cell wall (Alatoom et al., 2011). However, the extraction method is more time consuming and expensive. Furthermore, even though it increases the level of identification, we observed that the majority of the Gram-positive bacteria (more than 80%) were only identified after 16S gene amplification. Since most of these bacteria were already included in the Bruker database, this indicates that the lack of identification was not due to the lack of reference strains, as also previously mentioned by Veloo et al. (2018).

The analysis of bacterial communities among the 14 samples between PCA at 7 °C and 30° showed that the microbial diversity was only higher in two samples in the plates incubated in psychotropic conditions. (Table 4). Plate count agar is a non-selective medium suggested in ISO 4833 for the colony count at 30 °C (reference method, EU 1441/07) but also commonly used for the cultivation of psychrotrophic bacteria from food (Ercolini et al., 2009). Ercolini et al. (2009) and Jay (2002) reported that psychotropic bacteria are inhibited at 30 °C, however, in the present study, except for *Shewanella,* (e.g. Gram-negative and proteolytic bacteria with some psychrophilic species (Vogel et al., 2005)), all bacteria present on PCA incubated at 7 °C also grew at 30 °C. This suggests that incubation at 30 °C does not cause a significant loss of information and saves at least 5 days of analysis time.

The dominant bacterial communities present on the MRS plates belong to the genera *Lactobacillus* (54,86%) and *Leuconostoc* (13,72%). *Leuconostoc* spp. were also present on PCA plates both at 30 °C and 7 °C but, unlike described by Geeraerts et al. (2017) who predominantly isolated it on PCA and less on MRS, in the present study, the opposite was observed. On MRS plates, the genus *Carnobacterium* was also isolated but to a lesser extent than on PCA at 7 °C. This result can be attributed to acetate that is present in MRS and can inhibit the growth of this important spoilage bacteria (Geeraerts et al., 2017). *Brochotrix* spp. that was dominant on PCA, was not present on MRS in contrast to the study of Geeraerts et al. (2017).

Concerning the specificity of selective media, on TBX, the screening based on the blue-green colour of the colonies is shown to be 100% reliable to identify *E. coli* among the other bacteria. On the CFC plates, < 4% of the colonies were not *Pseudomonas,* but all were oxidase negative, a phenotypic test included for confirmation. This result is in contrast with the study of Tryfinopoulou et al. (2001) where non-*Pseudomonas*, oxidase positive bacteria (36,5%) were isolated from fish samples. Instead, MALDI TOF can be used as an effective, rapid and cheap test to differentiate the genera. However, also with MALDI TOF, as for the conventional phenotypic tests and 16S rRNA gene sequence analysis, reliable identification of *Pseudomonas* species remains questionable (Mulet et al., 2012).

To characterize the microbial communities, two different DNA extraction methods, and three sample preparations were evaluated. It has already been demonstrated that for human faecal samples, DNA extraction method has an important impact on the outcome of molecular based analysis (Costea et al., 2017). In the present study, we assessed two comparable methods, both combining bead beating with chemical lysis of cells. The PowerFood™ Microbial DNA isolation Kit, already reported as a suitable kit to obtain a very pure DNA extract (Quigley et al., 2012), allowed to detect the highest number of genera. The dilution and pelleting of samples before extraction, which is commonly reported in literature, resulted in a loss of microbial diversity. When assessing the specific differences in microbial composition comparing the extraction methods and sample preparation combined, it appears that pelleting of samples might already rupture the cell walls of specific Gram-negative bacteria, leading to destruction of the DNA during the actual extraction process and the absence of these bacteria in the 16S rRNA amplicon profile. Although this is speculative, the observation that these species seem less sensitive to 1:10 dilutions as compared to 1:2 dilutions, with more material present and thus more physical stress exerted in the latter situation, seem to substantiate this reasoning. If the

disappearance of species would simply result from the dilution itself, there would be less chance to observe them in the more diluted samples. Likewise, the FastDNA® SPIN Kit for Soil extraction method appears to be more performant towards extracting DNA from specific Gram-positive at the expense of probably destroying DNA of Gram-negative species. Extraction of the DNA directly from the meat, regardless the kit used, resulted in the highest richness of the microbial diversity. However, although the relative abundances of each OTU varied according to the DNA extraction kit applied, *Photobacterium* spp., *Brochothrix* spp. and *Pseudomonas* spp. were always the dominant genera with all the methods.

Comparing the genera detected by 16S rRNA amplicon sequencing with the ones identified by MALDI TOF and 16S gene sequencing, contrasting data were obtained. It has already previously been reported by Nieminen et al. (2012) that culture independent methods may lead to different results than those obtained using an included culturing step. In the present study, except for *Brochothrix* spp. and *Pseudomonas* spp., which were abundant and always detected, genera obtained with the two methods in the same sample were not always the same. It is certain that both strategies are biased by different factors. Culture-dependent methods are related with the media and the condition used, but the direct 16S rRNA analysis seems also biased by genomic size, number of rRNA operons, number of PCR cycles, the primers used (Farrelly et al., 1995; Wilson and Blitchington, 1996). In the past few years, culturing bacteria has been neglected in favour of metagenomics techniques. Lagier et al. (2016) who studied the human gut microbiota, introduced the concept of microbial culturomics as high-throughput method that uses several culture conditions and MALDI TOF and 16S rRNA analysis for the identification of the organisms. They were able to identify 197 potentially new species and showed that culturomics can be used to identify prokaryotes corresponding to sequences previously not assigned.

In conclusion MALDI TOF MS is a promising platform for the identification of isolates. Using an extensive database, MALDI TOF MS analysis combined with the enumeration of total microbiota will provide more valuable information. However, currently it cannot be used as a tool to study the microbial diversity in a complex matrix as minced meat because of the high number of unidentified bacteria in combination with the low number of isolates reliably identified at species level. The present comparison of different sample preparation techniques and DNA extraction methods demonstrated also in this matrix that different results on the microbial composition and complexity are obtained, depending on the methods used. When applying culture independent techniques, it is thus highly important to tailor the DNA extraction method and sample preparation to the research questions asked. Present data illustrates that the identification of bacterial strains using MALDI TOF MS and 16S gene sequencing and overall community profiling using 16S rRNA sequencing technique are complementary and yield important insights in the complex relationship between microorganisms in a food.

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