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PhD Thesis

"Assessment of the microbial contamination on pork and wild boar meat by a culture-dependent and independent approach"

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There is a driving force more powerful than steam,

electricity and nuclear power: the ***will***

Albert Einstein

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List of abbreviations

ail	attachment invasion locus
ANOVA	analysis of variance
API	analytical profile index
BPS	blown pack spoilage
B	back
CFC	cephalothin-Sodium Fusidate-Cetrimide Agar
CFU	colony forming units
CHCA	α-Cyano-4-hydroxycinnamic acid
DADA	divisive Amplicon Denoising Algorithm
DHB	2,5 dihydroxybenzoic acid
DNA	deoxyribonucleic acid
EB	Enterobacteriaceae
ERIC	enterobacterial repetitive intergenic consensus
GHE	game-handling establishment
H	ham
hreP	host-responsive element P
HTS	high-throughput sequencing
inv	Invasin
ISO	International Organization for Standardization
J	jowl
LAB	Lactic Acid Bacteria
LPS	lipopolysaccharide

MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometry
MP	DNA extracted from 500mg 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
MRS	De man, Rogosa and Sharpe agar
MRSA	methicillin-resistant Staphylococcus aureus
MS	DNA extracted from 500mg 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
MSM	mechanically separated meat
myf	mucoid Yersinia factor
NGS	next-generation sequencing
OTUs	operational Taxonomic Units
PCA	plate Count Agar
PCR	polymerase chain reaction
PMF	peptide mass fingerprint
PSB	peptone Sorbitol Bile Broth

List of abbreviations

PW	peptone Water
pYV	plasmid of *Yersinia* virulence
qPCR	quantitative polymerase chain reaction
REP	repetitive extragenic palindrome
SA	slaughterhouse A
SB	slaughterhouse B
SH	stomachered homogenate
TAB	total aerobic bacteria
TANAB	total anaerobic bacterial counts
TBX	tryptone Bile X-Glucuronide
TFA	trifluoro acetic acid
TSA	tryptone Soy Agar
TSB	tryptone soy broth
UPGMA	unweighted Pair Group Method with Arithmetic Mean
virF	virulence regulon transcriptional activator F
VRBG	violet Red Bile Glucose Agar
Y	belly
yadA	*Yersinia* adhesin A
ymoA	*Yersinia*-modulating protein A
yop	*Yersinia* outer membrane proteins

yst	*Yersinia* stable toxin

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Abstract

Domestic pigs and wild boars are members of the same species (*Sus scrofa)* and they are classified in the subspecies *domestica* and *scrofa* respectively. Pork meat, in particular, minced pork meat, is widely consumed in the world and the consumption of the wild boar meat, although is still low compared to pork, is increasing. The carcasses and the cuts of these animals may support the growth and serve as a source of various spoilage and pathogen microorganisms (e.g. *Salmonella*, *Yersinia enterocolitica* and *Listeria monocytogenes*) which may have important consequences for the quality and safety of the product.

In pork chain the food hygiene monitoring (1441/07) is based on the count of indicator microorganisms, however, knowledge of the microbial diversity on the agar plates of the counted bacteria is lacking. Moreover, the hygiene criteria for pork are commonly applied to assess the microbiological quality of wild boar meat since no microbial limits are settled for this animal.

For the past few years culture independent methods allowed to study different ecosystems overcoming the limits of the classic microbial cultivation and bacteria identification. Recently, Matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometry has been introduced in clinical microbiology for routine identification of clinical isolates. Moreover, MALDI TOF technology combined with 16S amplicon sequencing have resulted a promising approach to study the microbiota. The **general aim** of this thesis was to gain more insight in the **microbiological contamination of pork and wild boar meat with culture-dependent** based on MALDI TOF MS technique combined with 16S amplicon sequencing **and an independent method**.

Moreover, with the purpose of evaluating the contamination of the samples with *Y. enterocolitica* and with the necessity to select gene target for the real-time PCR, an initial study on the distribution of virulence genes in *Y. enterocolitica* strains has been conducted.

Therefore, in **Chapter 1** a total of 161 *Y. enterocolitica* strains grouped into four biotypes (1A, 2 (serotypes O3, O5 and O9); 3 (serotypes O3 and O9), and 4 (serotype O3)) and isolated in eight countries were analyzed in order to evaluate the distribution of *yadA*, *virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* virulence genes.

The results showed that not all the strains analyzed were positive to all the virulence genes tested. However, the most common virulence-associated gene in pathogenic *Y. enterocolitica* proved to be *ystA*, which can, therefore, be considered the best target gene to be amplified in order to evaluate the presence of pathogenic biotypes. While to identify *Y. enterocolitica* 1A strains, *ystB* can be proposed.

In **Chapter 2**, the microbial community of 14 unrelated pork minced meat samples was evaluated through a culture-independent method (16S 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 amplicon sequencing. Moreover, the impact of three sample preparation (direct colony identification, bacterial suspension and extraction method) for MALDI TOF analysis and different DNA extraction methods for the culture-independent method (16S amplicon sequencing) was evaluated. The results of the present study illustrate that the suspension method resulted in more low-level identifications, instead, the direct colony identification method can be recommended as a as first screening tool when large amounts of isolates have to be examined. Bacteria identified by MALDI-TOF MS and 16S amplicon sequencing were assigned to 16 families and 20 genera. Members of the genus *Pseudomonas*, along with the genera *Brochothrix* and *Carnobacterium* were commonly identified among the mesophilic and psychrotrophic population. Moreover, 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 ($p>0.05$) and the analysis of bacterial communities among the 14 samples between PCA at 7°C and 30°C showed that the microbial diversity was higher in mesophilic conditions. Comparing the results of the culture dependent methods 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. Moreover, different results were obtained among the DNA extraction methods used.

In **Chapter 3**, the microbial community on four areas (ham, back, jowl and belly) of 8 different pork carcasses belonging to two different slaughterhouses (Slaughterhouse A= C1, C2, C3 and C4; Slaughterhouse B= C5, C6, C7, C8) was evaluated through 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 amplicon sequencing. Moreover, the presence of *Salmonella* spp. and *Y. enterocolitica* was also evaluated using qPCR from the enrichment broths. *Salmonella* was isolated in 7 carcasses. The serotypes identified in the present work (monophasic *S.* Typhimurium, *S.* Brandenburg, *S.* derbyand *S.* Rissen) are commonly associated with pigs and pork meat. Concerning *Y. enterocolitica*, the gene *ystA* was never detected in the samples, though *ystB* was present on the ham of one carcass. The dominant bacterial communities isolated from the 8 carcasses belonged to *Staphylococcus*, *Pseudomonas*, and *E. coli*, while with 16S amplicon sequencing, the diversity indices showed that no genus clearly dominated the 8 carcasses. However, *Brochothrix* was the most abundant genus detected with 16S amplicon sequencing, immediately followed by *Pseudomonas.* Important differences have been observed between the two slaughterhouses. However, regardless of the method used (culture dependent and independent) the present data illustrate, that the microbial population of the ham, back, jowl and belly were dominated by the same genera.

In **Chapter 4**, the microbial community of 22 unrelated wild boar meat samples by a culture-dependent approach using ISO-methods combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification, and 16S rRNA amplicon sequencing was evaluated. Moreover, the presence of *Salmonella*, *Y. enterocolitica* and *L. monocytogenes* was examined by qPCR and confirmed by classical isolation. The samples resulted highly contaminated by mesophilic bacteria and Enterobacteriaceae. Moreover, 31.82% were positive to *Salmonella* spp.; three serovars were identified (monophasic *S.* Typhimurium, *S.* Stanleyville and *S.* Kasenyi) of which *Salmonella* Kasenyi has not been reported from wild boar meat yet. Concerning *Y. enterocolitica*, the gene *ystB* was present in three samples. Instead, *L. monocytogenes* was never detected. A great variability of the microbial contamination between

the samples was recorded, and moreover genera frequently isolated were not always detected with 16S amplicon sequencing, and *vice versa*.

As described in the general conclusion, this thesis demonstrated that the study of a microbial community in an ecosystem can be best achieved by using a combination of culturomics based on the application of MALDI TOF technology and 16S amplicon sequencing and culture independent methods. In fact, every time that in this thesis culture dependent and independent methods were compared, contrasting data were obtained. Concerning the three samples preparation of the MALDI TOF analysis the use first of "direct colony identification" and then "extraction method" on a selection is efficient in term of time and cost when a lot of isolates must be examined. Moreover, when applying culture independent techniques, it is important to adapt the DNA extraction method to the research questions asked. The data of the present thesis illustrate, furthermore, that the bacterial community of each carcass mainly depends on the microbial population of the slaughterhouse and the sampling of only one area for the evaluation of the hygienic status of the carcasses by the official authority may be sufficient. Moreover, for the first time, the bacterial population of wild boar meat has been in depth studied and the data showed that it can be a great public health risk. Thus, particular attention has to be paid to the non-inspected meat supplied directly to consumers.

Riassunto

I suini domestici e i cinghiali sono membri della stessa specie (*Sus scrofa*) e sono classificati rispettivamente nelle sottospecie *domestica* e *scrofa*. Il consumo di carni di maiale, comprese quelle macinate, presenta da anni un trend positivo sia in Italia che in Europa, mentre il consumo di carni di cinghiale, nonostante mostri un incremento del consumo, non si attesta a livelli di altre specie. Le carni di queste specie rappresentano un ottimo *pabulum* di crescita di microrganismi sia patogeni (ad es. *Salmonella* spp., *Y. enterocolitica e L. monocytogenes*) che alteranti (ad es. *Pseudomonas* spp. e *Brochothrix* spp.), con effetti sia sugli aspetti igienici che sanitari.

Il monitoraggio dell'igiene nella filiera delle carni suine, secondo quanto riportato dalla normativa comunitaria (Regolamento CE 1441/07 della commissione del 5 dicembre 2007 che modifica il Regolamento CE 2073/2005 sui criteri microbiologici applicabili ai prodotti alimentari), si basa sulla conta dei microrganismi indicatori; tuttavia il tipo di popolazione microbica che cresce sulle piastre di agar usate per la numerazione è perlopiù sconosciuto. Inoltre, i criteri di igiene definiti per le carni suine vengono comunemente applicati per la valutazione della qualità microbiologica delle carni di cinghiale in quanto non esistono riferimenti legislativi specifici per questa specie.

Negli ultimi anni i metodi coltura–indipendenti hanno permesso di studiare diversi ecosistemi superando i limiti della classica coltivazione microbica e dei test fenotipici per l'identificazione dei batteri. Recentemente per l'identificazione di vari isolati batterici è stato introdotto in microbiologia clinica una tecnica basata sul desorbimento/ionizzazione laser assistito da matrice, comunemente indicato con l'acronimo MALDI, combinata con un analizzatore di ioni a tempo di volo (TOF). La tecnologia MALDI TOF, associata al sequenziamento del gene 16S, si è rivelata come un approccio promettente per lo studio del microbiota.

Lo **scopo generale** di questa tesi è stato la **valutazione della contaminazione microbica di carni suine e di carni di cinghiale mediante metodi di cultura-dipendenti** (MALDI TOF MS e sequenziamento del gene 16S degli isolati batterici) e un **metodo di coltura–indipendente**.

Inoltre, allo scopo di valutare la naturale contaminazione dei campioni summenzionati da *Y. enterocolitica*, è stato condotto uno studio sulla distribuzione dei geni di virulenza in ceppi di *Y. enterocolitica* isolati da vari campioni vista la necessità di selezionare un gene target per la PCR real-time.

Pertanto nel **Capitolo 1** n. 161 ceppi di *Y. enterocolitica*, raggruppati in quattro biotipi (1A, 2 (sierotipi O3, O5 e O9), 3 (sierotipi O3 e O9) e 4 (sierotipo O3)) ed isolati in otto paesi, sono stati analizzati al fine di valutare la distribuzione dei seguenti geni di virulenza: *yadA*, *virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* e *ymoA*. I risultati hanno mostrato che non tutti i ceppi analizzati presentavano tutti i geni di virulenza testat. Tuttavia il gene associato alla virulenza più frequentemente rilevato nei ceppi di *Y. enterocolitica* patogena isolata è stato *ystA* che può, quindi, essere utilizzato come gene target per valutare la presenza di biotipi patogeni. Per l'identificazione di ceppi di *Y. enterocolitica* 1A, può essere proposto il gene *ystB*.

Nel **Capitolo 2** è stata valutata la comunità microbica di 14 campioni di carne macinata di maiale mediante sequenziamento del gene 16S direttamente dalla carne e/o dall'omogenato di carne in acqua peptonata e mediante metodi di isolamento classici (MALDI TOF MS e sequenziamento del gene 16S degli isolati batterici). Sono inoltre stati utilizzati tre differenti protocolli (identificazione diretta delle colonie, sospensione batterica e metodo di estrazione) al fine di valutare il metodo più efficace per l'identificazione degli isolati batterici con il MALDI TOF. È stato quindi valutato l'impatto di diversi metodi di estrazione del DNA batterico sul sequenziamento del gene 16S. I risultati del presente studio dimostrano che il "metodo di sospensione" e il "metodo di estrazione" comportano rispettivamente il più basso ed il più alto tasso di identificazione delle colonie; il "metodo di identificazione diretta delle colonie", tuttavia, può essere raccomandato come primo strumento di screening quando devono essere esaminati un numero cospicuo di isolati. I batteri identificati attraverso metodi di isolamento classici appartengono a 16 famiglie e 20 generi. Membri del genere *Pseudomonas*, insieme ai generi *Brochothrix* e *Carnobacterium*, sono stati comunemente identificati tra la popolazione mesofila e psicrofila. L'analisi statistica ha inoltre

mostrato che la temperatura di incubazione delle piastre di PCA (30° C o 7° C) non ha avuto un impatto significativo sul numero di colonie batteriche isolate ($p > 0.05$); attraverso lo studio degli indici di diversità si è inoltre evidenziato che a 30° C, piuttosto che a 7° C, la diversità microbica era maggiore.

Sono emerse delle differenze confrontando questi risultati con quelli ottenuti dal sequenziamento 16S direttamente dalla carne e/o dall'omogenato di carne in acqua peptonata: eccetto *Brochothrix* spp. e *Pseudomonas* spp., rilevati con entrambe le strategie, i generi ottenuti con i due metodi non erano sempre gli stessi e sono stati ottenuti risultati diversi tra i metodi di estrazione del DNA utilizzati.

Nel **Capitolo 3** si è proceduto alla valutazione del livello e del tipo di contaminazione microbica di quattro aree della carcassa (coscia, dorso, guancia e pancia) di 8 differenti maiali macellati in due diversi stabilimenti (Macello A = C1, C2, C3 e C4; Macello B = C5, C6, C7, C8) sia mediante sequenziamento diretto del gene 16S direttamente dall'omogenato della "*sponge*" in acqua peptonata che mediante MALDI TOF MS e sequenziamento del gene 16S previo isolamento delle colonie su piastra. È stata inoltre valutata la presenza di *Salmonella* spp. attraverso l'ISO 6579 -1:2017 e la presenza di *Y. enterocolitica* mediante Real-Time PCR da brodi di arricchimento. *Salmonella* spp. è stata isolata in 7 carcasse: i sierotipi identificati nel presente lavoro (variante monofasica *S.* Typhimurium, *S.* Brandenburg, *S.* derby e *S.* Rissen) sono comunemente associati al maiale ed alle sue carni. Per quanto riguarda *Y. enterocolitica*, in questi campioni non è mai stato rilevato il gene *ystA*: tuttavia il gene *ystB* è stato rilevato sulla coscia di una carcassa.

Le comunità batteriche dominanti, isolate dalle 8 carcasse, appartengono ai generi *Staphylococcus*, *Pseudomonas* ed *E. coli*, mentre con il sequenziamento diretto del gene 16S gli indici di diversità mostrano l'assenza di generi batterici dominanti. *Brochothrix* tuttavia, con il metodo coltura–indipendente, è risultato essere il genere maggiormente rilevato, immediatamente seguito da *Pseudomonas*. Importanti differenze sono state osservate tra i due macelli.

I dati mostrano che, indipendentemente dal metodo usato (coltura–dipendente o coltura–indipendente), la popolazione microbica dominante della coscia, del dorso, della guancia e della pancia appartiene ai medesimi generi.

Nel **Capitolo 4** è stata valutata la comunità microbica di 22 campioni di carne di cinghiale mediante un approccio coltura–dipendente (MALDI TOF MS associato al sequenziamento del gene 16S degli isolati batterici) e mediante il sequenziamento del gene 16S dall'omogenato di carne in acqua peptonata. Inoltre è stata ricercata *Salmonella* spp., *Y. enterocolitica* e *L. monocytogenes* mediante Real-Time PCR da brodi di arricchimento. I campioni sono risultati altamente contaminati da batteri mesofili ed Enterobacteriaceae ed il 31,82 % era positivo per *Salmonella* spp. Appartenenti a tre sierotipi (variate monofasica *S.* Typhimurium, *S.* Stanleyville e *S.* Kasenyi). La *S.* Kasenyi non era mai stata descritta nella carne di cinghiale. Per quanto riguarda *Y. enterocolitica*, il gene *ystB* era presente in tre campioni. *L. monocytogenes* non è mai stata rilevata. Tra i campioni è stata registrata una grande variabilità delle specie microbiche ed i generi isolati con le due tecniche spesso non sono risultati sovrapponibili.

Queste ricerche hanno dimostrato che lo studio di una comunità microbica in un ecosistema può essere raggiunto solo utilizzando una combinazione di metodi cultura-dipendenti, basati sull'applicazione della tecnologia MALDI TOF e sequenziamento del gene 16S, e metodi cultura-indipendenti: queste ricerche hanno infatti evidenziato differenze nei risultati confrontando le due strategie. L'allestimento della colonia da sottoporre a MALDI TOF è stato effettuato, come precedentemente descritto, con tre protocolli differenti consentendo così di valutare che l'approccio più efficiente, oltre che più vantaggioso economicamente, è quello di "identificazione diretta su tutte le colonie" seguito dal "metodo di estrazione su una selezione". I risultati di queste ricerche evidenziano che la comunità batterica di ciascuna carcassa è strettamente correlata all'ecologia microbica del macello, che l'area di prelievo non incide sul risultato e che quindi il sito di campionamento potrebbe essere a discrezione dell'autorità competente. È stata studiata per la prima volta in maniera approfondita la popolazione batterica della carne di cinghiale e i

dati hanno dimostrato una certa diversità rispetto al maiale: ulteriori studi sono necessari al fine di valutare alcune variabili (ad es. metodo di caccia, stagione di caccia, tecnica di eviscerazione, competenza e formazione dell'operatore) che nel presente lavoro non sono state tenute in considerazione.

Gedomesticeerde varkens en everzwijnen behoren tot dezelfde soort (*Sus scrofa*) en worden respectievelijk ingedeeld in de ondersoorten *domestica* en *scrofa*. Varkensvlees, en het bijzonder gehakt, wordt op vele plaatsen in de wereld rauw geconsumeerd en ook de consumptie van everzwijnvlees, hoewel nog steeds laag in vergelijking tot varkensvlees, neemt toe. Varkensvleesproducten zijn vaak een bron van verschillende pathogene micro-organismen (zoals *Salmonella*, *Y. enterocolitica*, *Campylobacter* en *L. monocytogenes*) en contaminatie van deze producten heeft belangrijke consequenties voor de voedselveiligheid en het imago van de sector.

De bewaking van de bacteriële levensmiddelenhygiëne is nog steeds gebaseerd op de bepaling van een aantal indicator micro-organismen, waarbij kennis van de microbiële identiteit en diversiteit van de isolaten eigenlijk ontbreekt. Door het ontbreken van specifieke microbiologische criteria voor everzwijn worden de hygiënecriteria voor varkensvlees vaak overgenomen, zonder dat dit eerst werd geëvalueerd.

Sinds enkele jaren wordt met behulp van cultuuronafhankelijke methoden verschillende microbiële ecosystemen bestudeerd, waarbij de grenzen van de klassieke kweek en identificatie van bacteriën worden overschrijden. Daarnaast is Matrix-geassisteerde laserdesorptie/ionisatie tijd-van-vlucht massaspectrometrie (MALDI-TOF MS) geïntroduceerd in klinische microbiologie voor routinematige identificatie van klinische isolaten. In dergelijke studies bleek ook de combinatie van MALDI-TOF MS met 16S-gensequencing een veelbelovende aanpak om de microbiota in meer detail te identificeren. Dergelijke gecombineerde studies op voeding waren echter bij de aanvang van dit doctoraatsonderzoek nagenoeg niet uitgevoerd.

Het **algemene doel** van dit proefschrift was om meer inzicht te krijgen in de **microbiologische contaminatie van varkensvlees en vlees van wilde everzwijnen**, en dit met **cultuur afhankelijke en cultuur onafhankelijke methoden**. Daarnaast werd eerst onderzoek gevoerd naar de identificatie en selectie van een specifiek gen voor gebruik in real-time PCR (qPCR), om zo de

contaminatie van de varkensvlees met pathogene *Yersinia enterocolitica* stammen snel te detecteren.

In **Hoofdstuk 1** werden in totaal 161 *Y. enterocolitica*-stammen, afkomstig uit acht landen, gegroepeerd in vier biotypes 1A, 2 (serotype O3, O5 en O9); 3 (serotype O3 en O9) en 4 (serotype O3) en het voorkomen van de virulentiegenen yadA, virF, inv, ystA, ystB, myfA, hreP en ymoA geëvalueerd. De resultaten toonden aan dat niet alle stammen alle virulentiegenen bevatten. Het meest voorkomende, met virulentie geassocieerde gen in pathogene *Y. enterocolitica* bleek ystA te zijn. Dit gen werd dan ook als doel gen geselecteerd bij de evaluatie van pathogene biotypen. Voor de *Y. enterocolitica* 1A-stammen wordt het gen ystB voorgesteld.

In **Hoofdstuk 2** werd de microbiële gemeenschap van 14 niet-gerelateerde varkensgehaktmonsters beoordeeld via 16S rRNA amplicon-sequencing in vergelijking met klassieke isolatiemethoden gecombineerd met identificatie door middel van MALDI-TOF MS en 16S rRNA gen sequentiebepaling. Bovendien werd de impact van de staalbereiding van de bacteriële monsters (directe kolonie-identificatie, bacteriesuspensie en extractiemethode) voor MALDI TOF-analyse, en verschillende DNA-extractiemethoden voor 16S rRNA amplicon-sequencing geëvalueerd. De resultaten van de studie illustreren dat de "suspensie extractie methode" resulteerde in een lager MALDI-identificatie score. Daarom wordt de "directe kolonie-identificatiemethode" aanbevolen als een eerste screening wanneer grote hoeveelheden isolaten moeten worden onderzocht. Bacteriën geïdentificeerd door MALDI-TOF MS en 16S-gensequencing werden toegewezen aan 16 families en 20 genera. Leden van het genus *Pseudomonas*, samen met de genera *Brochothrix* en *Carnobacterium* werden vaak geïdentificeerd in de mesofiele en psychrotrofe bacteriële populatie. Bovendien toonde statistische analyse aan dat de temperatuur van incubatie van de PCA-platen (30° of 7° C) geen significante invloed had op het aantal geïsoleerde bacteriën. Analyse van bacteriële gemeenschappen tussen PCA platen geïncubeerd bij 7° C en 30° toonde aan dat de microbiële diversiteit hoger was in mesofiele incubatie omstandigheden. Door de resultaten van de kweekafhankelijke methode te vergelijken met 16S rRNA amplicon-sequencing werden enkele contrasterende data

verkregen. Met uitzondering van *Brochothrix* spp. en *Pseudomonas* spp., die overvloedig aanwezig waren en altijd werden gedetecteerd, kwamen de identificaties op genus niveau van de andere isolaten, tussen beide methodes niet steeds overeen. Bovendien werden met amplicon-sequencing verschillende resultaten verkregen naargelang de gebruikte DNA-extractiemethoden.

In **Hoofdstuk 3** werd de microbiële gemeenschap op vier zones (ham, rug, schouder en buik) van 8 verschillende varkenskarkassen (uit twee slachthuizen, slachthuis A = C1, C2, C3 en C4; slachthuis B = C5, C6, C7, C8) bestudeerd door middel van 16S rRNA-amplicon-sequencing en klassieke isolatie gecombineerd met identificatie door middel van MALDI-TOF MS en 16S rRNA-gensequentie bepaling. Bovendien werd de aanwezigheid van *Salmonella* spp. en *Y. enterocolitica* geëvalueerd met behulp van qPCR. *Salmonella* werd geïsoleerd vanop 7 verschillende karkassen. Voor *Y. enterocolitica* werd het gen ystA nooit in de monsters gedetecteerd, maar het gen ystB was aanwezig op de ham van één karkas. De dominante bacteriële gemeenschappen op de verschillende karkassen behoorden tot *Staphylococcus*, *Pseudomonas* en *E. coli*, terwijl met 16S amplicon-sequencing de diversiteitsindexen aantoonden dat geen enkel geslacht de 8 karkassen duidelijk domineerde. *Brochothrix* was echter het meest voorkomende genus dat werd gedetecteerd met 16S amplicon-sequencing, onmiddellijk gevolgd door *Pseudomonas*. Belangrijke verschillen zijn waargenomen tussen de twee slachthuizen. Ongeacht de gebruikte methode (cultuurafhankelijk en -onafhankelijk) toonden de data dat de microbiële populatie van de ham, rug, schouder en buik gedomineerd werden door dezelfde genera, waardoor geen duidelijke plaats afhankelijkheid kon worden aangetoond.

In **Hoofdstuk 4** werd de microbiële gemeenschap van 22 niet gerelateerde vleesmonsters van wilde everzwijnen door middel van een cultuurafhankelijke benadering (isolatie met ISO-methoden en identificatie door MALDI-TOF MS) en 16S rRNA amplicon-sequencing geëvalueerd. Bovendien werd terug de aanwezigheid van *Salmonella*, *Y. enterocolitica* en *L. monocytogenes* onderzocht met qPCR met bevestiging door klassieke isolatie. Analyse toonde een hoge contaminatie met mesofiele bacteriën en meer specifiek

met Enterobacteriaceae aan. Bovendien bleken bijna 32% van de stalen positief voor de aanwezigheid van *Salmonella*. Er werden drie serovars geïdentificeerd (monofasisch S. Typhimurium, S. Stanleyville en S. Kasenyi) waarbij S. Kasenyi nog niet eerder werd gerapporteerd uit vlees van wilde zwijnen. Voor *Y. enterocolitica* werd enkel het gen ystB gedetecteerd in drie monsters. *L. monocytogenes* werd nooit gedetecteerd. Een grote variabiliteit van de microbiële contaminatie tussen de monsters werd waargenomen en bovendien werden genera die vaak werden geïsoleerd niet altijd gedetecteerd met 16S-ampliconsequentie bepaling, en omgekeerd.

Zoals beschreven in de algemene conclusie heeft dit proefschrift aangetoond dat de studie van een microbiële gemeenschap in een ecosysteem best kan worden uitgevoerd door een combinatie van culturomics, gecombineerd met identificatie door middel van MALDI-TOF aangevuld met 16S-rRNA gen sequencing wanneer nodig, en een cultuuronafhankelijke benadering. Er werden evenwel vaak andere identificaties met beide benaderingen bekomen, en geen enkele methode op zich heeft het potentieel om de volledige microbiële belasting weer te geven.

Met betrekking tot de voorbereiding van de drie monsters van de MALDI TOF-analyse is het gebruik van eerst "directe kolonie-identificatie" en vervolgens "extractiemethode" op een selectie van isolaten efficiënter wanneer veel isolaten moeten worden onderzocht. Bovendien is het bij het toepassen van cultuuronafhankelijke technieken belangrijk om de DNA-extractiemethode aan te passen aan de gestelde onderzoeksvragen. De resultaten bekomen in dit proefschrift suggereren dat de bacteriële gemeenschap op een varkenskarkas wordt beïnvloed door de microbiële populatie van het slachthuis maar ook dat de bemonstering van slechts één gebied voor de evaluatie van de hygiënische status van de karkassen door de officiële autoriteit voldoende kan zijn. Bovendien is voor het eerst de bacteriepopulatie op vlees van wilde everzwijnen uitvoerig bestudeerd en uit de gegevens blijkt dat dit consumptie van het vlees een risico voor de volksgezondheid kan vormen. Daarom moet bijzondere aandacht worden besteed aan het niet-geïnspecteerde vlees dat rechtstreeks aan de consument wordt geleverd.

1. Introduction

1.1 General overview

Domestic pigs and wild boars are members of the same species (*Sus scrofa)* but they are classified into the sub species *domestica* and *scrofa* respectively (Grossi et al., 2006).

Domestic swine were domesticated about 10,000 years ago (Grossi et al., 2006) and nowadays, pork represents the most widely eaten meat in the world with a mean consumption of 12,3 Kg per capita per year (OECD, 2018). Particularly, in Europe in 2017, 23 311 tonnes of pigs were slaughtered (EUROSTAT), the mean consumption was of 32,5 (Kg/capita). Among the pork products, minced meat, which can be consumed fresh or can be used as ingredient in further processing, has the highest market penetration levels (Verbeke et al., 2010).

Wild boars are the most widely distributed large mammals in the world (Massei et al., 2015) and moreover, they occupy a wide variety of habitats including urban areas. They are characterized by the highest reproductive rate among the ungulates and thus, in recent years the wild boar population has dramatically increased in size (Lombardini et al., 2016). However, a real estimation of the population density is a difficult task (EFSA, 2018). Economic interest for these animals is related to the damage of crops and husbandry and the possibility of transmitting disease to livestock and humans (Massei et al., 2015). Starvation due to extreme weather conditions, diseases, and predation by wolves are the main cause of natural mortality, though hunting for sport and or food currently makes the greatest contribution to wild boar mortality (Massei et al., 2015; Nores et al., 2008; Okarma et al., 1995). A century ago the meat was eaten only by hunters and their families, but nowadays the meat is perceived as healthy and it is sold all year round (Atanassova et al., 2008; Naya et al., 2003). Compared to pork its consumption is still low but is increasing in Europe. Usually the meat is eaten cooked, though some wild boar meat products are not heat treated but only dry cured, cold smoked and dried (Mirceta et al., 2017).

1.1.1 From the slaughter process to meat production in pork

Specific hygiene rules for the slaughter are laid down in the European regulation 853/04 and the principal steps in the slaughter process for pigs are the following (Ninios, 2014):

Stunning: Stunning cause the lack of consciousness and sensibility before the animals are killed. A list of stunning methods is reported in Regulation 1099/09. In particular, pigs usually are stunned by the use of electrical or gas methods.

Bleeding: Immediately after the stunning, sticking is performed. In pigs, the incisions of the main blood vessels are made in the center of the neck, in front of the breastbone and the draining of the blood results in the death of the animals from cerebral anoxia.

Scalding: After that the blood flow becomes negligible, the pigs are immersed in a scalding tank of water (60-62°C).

Dehairing: A rotating scraper is used to remove the epidermis from the skin surface and pull the hairs out of the follicles.

Singeing: A flame is used to removes any remaining hairs, shrinks and sets the skin.

Evisceration: This step starts with tying off the rectum. Subsequently, the skin is cut along the middle line from the anal opening to the neck and the bladder, sexual organs, abdominal and thoracic viscera are removed. The kidneys are usually removed after meat inspection and the head at this stage is left on.

Splitting: The carcasses of pigs are cut into half-carcasses with a manual or mechanical saw.

Cooling: The temperature of the carcasses after the dressing stage can reach 43°C in the leg, loin and shoulders (Ninios et al.,2014).

The chilling in the slaughterhouse has to ensure a temperature of the meat of no more than 7°C (853/04). Usually the carcasses are placed overnight in chill rooms where the ventilation and the humidity of the air are controlled.

In the slaughterhouse, the carcass can only be cut into half-carcasses or quarters, and half carcasses into no more than three wholesale cuts; all the further cutting is carried out in the cutting plant.

Regulation 853/04, moreover, lays down specific hygiene rules for the production of minced meat, meat preparations or mechanically separated meat (MSM). Particularly, minced pork meat that is consumed globally (Baer et al., 2013), as defined by the regulation as *"boned meat that has been minced into fragments and contains less than 1% salt"* and immediately after production it has to be wrapped or packaged and chilled to an internal temperature of no more than 2°C.

1.1.2 Wild boars: From the field to the table

A common method of hunting is the drive hunt where hunting teamsare driven by dogs and they kill the animals using shotguns. According to the Regulation 853/04, at least one person of a hunting team (trained person) in order to undertake an initial examination of wild boars on the spot, must have sufficient knowledge of the anatomy, physiology, behavior and pathology of the animals and of hygiene rules during handling after killing and the production of the meat. After killing, stomachs and intestines must be removed has soon as possible and if necessary the animal has to be bled and the trained person must carry out an examination of the viscera and the body. The animals can be eviscerated in the field, lying on the ground at a collection point or in the game-handling establishment (GHE). Subsequently, if the animals are killed with the purpose of placing the meat on the market the body of the animals must be transported to a GHE and they must be presented to a competent authority for inspection. If no abnormal characteristics are found during the examination by the trained person, the viscera, except for the diaphragm, may not accompany the body. Instead, head and diaphragm, and viscera with abnormal characteristics or that belonging to animals with abnormal behaviour must accompany the body (Reg 853/04). Unlike pigs, wild boars are usually skinned, however, for the following steps (evisceration, splitting and cooling) the techniques adopted are the same. The post-mortem inspection consists in the visual examination of the organs and the carcasses and in palpation where appropriate (Reg.854/04). Lastly, the meat must achieve an internal temperature of no more than 7°C (Reg 853/04). However, for private domestic consumption and for

the supply of small quantities of meat hunted game does not undergo any official regulation except for a declaration of the sex and estimated age.

1.2 Microbial contamination and foodborne pathogens of pork carcasses and cuts

Pork carcasses and pork cuts may support the growth and serve as a source of various spoilage and pathogens microorganisms which may have important consequences for the quality and safety of the product (Koutsoumanis and Sofos, 2004).

1.2.1 Spoilage Microbial contamination in pork

Food spoilage is a metabolic process that causes an evident change in sensory characteristics that are undesirable or unacceptable for human consumption (Rawat, 2015). The spoilage microorganisms on fresh carcasses usually mainly consist of *Pseudomonas spp.*, *Micrococcus spp*., *Brochotrix thermosphacta*, *Serratia* and *Staphylococcus spp*. but others Gram-negative (*Acinetobacter*, *Alcaligenes*, *Moraxella*, Enterobacteriaceae) and Gram-positive (Lactic acid bacteria) bacteria can be present even if in a small number (Koutsoumanis and Sofos, 2004). Only a small fraction of the initial microbial population present on the carcasses is the cause of spoilage (Koutsoumanis and Sofos, 2004) and in refrigerated meat stored in aerobic condition *Pseudomonas* is one of the most dominant genera, followed by Enterobacteriaceae, especially cold-tolerant species (e.g., *Halfia alvei*, *Serratia liquefaciens*, *Enterobacter agglomerans*) even though the latter rarely contribute to food spoilage. When O2 is limited or when CO_2 is present in meat stored under anaerobic conditions (eg. vacuum-packed or modified atmosphere) the growth of Gram-negative is restricted promoting the growth of Gram-positive bacteria (Del Blanco et al., 2017) and the main bacteria responsible for meat spoilage are *Brochotrix thermosphacta*, Lactic acid bacteria, and *Clostridium spp*.

1.2.1.1 Characteristics of the main spoilage bacteria

Pseudomonas genus is one of the most ecologically diverse groups of bacteria; it has been found in all types of environment (e.g. soil, water, plants and animal surfaces) (Singh, 2017).Three species of *Pseudomonas* (*P. flourescens, P. fragi*,and *P. ludensis*) are the main species responsible for organoleptic change to the meat caused by the utilization of food nutrients and the production of both volatile

and non-volatile metabolites. Particularly, when they reach the level of 10^7-10^8 CFU/cm^2 they produce lipases and proteases and when the glucose and lactate are exhausted, the metabolization of the nitrogenous compound (such as amino acids) results in off-odours (sulphides, esters, amines) and in the formation of biofilm (slime) (Nychas et al., 2008; Rawat, 2015).

Brochotrix thermosphacta and Lactic acid bacteria cause souring when they reach a number of 10^8 CFU/cm^2. Both of them use meat glucose as the main substrate and the main end products from glucose degradation are lactate and acetate for LAB and lactate and acetoin with a concentration depending on glucose concentration and atmosphere composition (Del Blanco et al., 2017).

The spoilage caused by *Clostridium spp*. is known as "blown pack spoilage" (BPS) characterized by a putrid smell (H_2S) and a metallic sheen on the meat (Cavill et al., 2011). *Clostridium* competes with LAB and *B. thermoshacta* for glucose utilization. However, the production of lactic acid by those that lead to a drop in pH may inhibit the growth of Clostridia. Psychrotrophic clostridia in the form of spores may contaminate the slaughterhouse environment through the soil. Moreover, they are primary cause agents of deep tissue spoilage ("bone taint") (Kamenik, 2013).

1.2.2 Pathogens in pork

Carcasses and pork cuts can carry pathogenic bacteria that may be responsible for human illness, hospitalizations and even death (Baer et al., 2013). The most important pathogenic bacteria associated with pork include *Salmonella, Campylobacter, Staphylococcus aureus, Yersinia enterocolitica* and *Listeria monocytogenes* (Choi et al., 2013). The infection in pigs by *Salmonella* is acquired through the ingestion or the inhalation of the pathogen from the feed or environment and it is excreted from the animals for several weeks or even months (Rönnqvist et al., 2018). In 2016, 3.5% of pigs, 1.9% pigs' carcasses, 2.38% of fresh pig meat and 1.93% of RTE minced pork meat, pork meat preparation and pork meat products were *Salmonella* positive (EFSA 2017). *Salmonella Typhimurium* was the most commonly reported serovar from pigs and pig meat and along with *S. Enteritidis* it was the most frequently isolated

species in confirmed human cases in the EU (EFSA, 2017) and worldwide (Hendriksen et al., 2011).

Among the *Campylobacter* genus the main species identified in pigs and pork meat is *Campylobacter coli*, which is present in normal gut microbiota and pigs become colonized less than 1 week after birth. In finishing swine *Campylobacter* was isolated in 71%, 51.5% and 24.7% of the the digestive carriage, gastric content, and tonsils respectively (Fosse et al., 2009). On a retail level, *Campylobacter* was isolated even if the levels were low.

Staphylococcus is a Gram-positive bacterium present in the skin and upper respiratory tract of animals, birds, and humans. Generally, the contamination occurs during the handling of meat product because it is not able to compete against other bacteria in the food system. In human medicine a particularly concern is for methicillin-resistant *Staphylococcus aureus* (MRSA) which is a multidrug-resistant bacteria whose prevalence in pigs in the last decades is increasing (Ivbule and Valdovska, 2017). *S. aureus* and MRSA were found with a prevalence that varies between different slaughterhouses from 52.0% to 89.0% for *S. aureus* and from 8.0% to 88.6% for MRSA (Ivbule and Valdovska, 2017). In a study conducted by Choi et al., (2013) *S. aureus* was isolated from carcasses (8%) and from fresh pork (2%). In Chile *S. aureus* was detected in 23.3% of the pork carcasses (Velasco et al., 2018).

Listeria monocytogenes is a Gram-positive bacterium which is ubiquitously persistent in the environment. Feed represents the main source of *Listeria* for pigs but contamination from the environment is also possible (Fosse et al., 2009). At the slaughterhouse, the carcasses can be contaminated by *Listeria* already present in a previously infected live animal or by cross-contamination from the environment (Baer et al., 2013). *L. monocytogenes* was found in 27% of pork carcasses (Choi et al., 2013) and in a study conducted by Li et al., (2018) 15% of samples (raw pork, retail environments,and insects in a retail market) were positive to *L. monocytogenes*.

As far as *Y. enterocolitica*is concerned, characteristics of the bacterium and prevalence in human, animals, and foods are reported in detail in paragraph 1.4.

1.2.3 Source of contamination in carcasses and cuts

All animals carry a very large number of bacteria in their stomachs and intestines, which are excreted in their feces. Moreover, the hide of live animals can carry a high number of bacteria (10^{10} cm^{-2}). However, there is no evidence of a correlation between the visible cleanliness of hides and the microbiological condition of the carcasses (Ninios et al., 2014). The contamination of the carcasses by pathogenic and non-pathogenic bacteria occurs mainly during the dressing, chilling and cutting of the meat. Pork carcasses are dressed with the skin on, which represents one of the main sources of microbial contamination (Ninios et al.,2014). Moreover, if an incomplete bleeding occurs the bacteria can proliferate in the blood which is an ideal medium for their growth. The use of hot water (60°C) after bleedings reduces the microbial population on the carcass surface but fecal material can spread on the carcasses surface by recirculating water in the scalding tank (Stopforth et al., 2003) and moreover, bacteria present on dehairing equipment may subsequently recontaminate the carcasses (Gill and Bryant, 1992). However, the singeing process also has a significant effect on the decrease of microbial load. Scalding, singeing and washing can reduce the level of bacteria to a range of 10^{2}-10^{4} CFU/cm^{2} (Ninios et al., 2014). Contamination may also occur during the operation of evisceration by enteric bacteria present in the gastrointestinal tract. The bacteria can spread on the carcass surface by direct contact with material from the digestive tract or by contact with a knife or be hand contaminated (Koutsoumanis and Sofos, 2004). Moreover, the carcasses can be contaminated by the bacteria present on the head since the pigs are dressed, most of the time, before the head is removed. During the subsequent dressing operations, further contamination may occur through the contact of the carcasses with equipment, contaminated surfaces or the hands of the workers (Choi et al., 2013).

The microbial population on the carcasses after the dressing stage is composed of a mixture of mesophilic and psychrotrophic bacteria. According to 853/04 the temperature of the carcasses must be lower than 7°C, which is recognized as the temperature limit below which most pathogens do not grow (Koutsoumanis and Sofos, 2004) even if some pathogens, as well as a range of food spoilage organisms, are not completely inhibited nor killed. Microbial population on the

carcasses may decrease during the first stage of cooling if the meat surface becomes dry through the control of the ventilation and the air humidity.

During boning and cutting operations one of the main source of contamination is the incoming meat and the microbiological proliferation or contamination is caused by the contact of the meat with working surfaces, hands, and equipment which may carry a high number of bacteria (Choi et al., 2013). When the meat is cut into pieces more microorganisms are added to the surface of the exposed tissue, so even the presence of low microbiological load or small number of pathogens on the carcasses can lead to heavy contamination of the meat, especially minced meat (Del Blanco et al., 2017). Temperature is the most important factor that influences the quality and the safety of the meat (Nychas et al., 2008). During cutting, boning, trimming, slicing, dicing, wrapping, packaging, storing and transport the meat must be kept at a temperature of no more than 7ºC (843/04). On the other hand, immediately after production, minced meat must chill to an internal temperature of no more than 2ºC. However, it is recognized that transportation and storage are the weakest points in the actual chill chain allowing spoilage or pathogenic bacteria to grow.

1.2.4 Microbiological parameters and sampling methods according to European legislation

According to Regulation 852/04 for the control the hygiene of foodstuffs, it is necessary to establish microbiological criteria (Art.1, letter F). Microbiological testing is essential to evaluate the presence of a hazard or a risk and to determine hygiene during the production process. The microbiological criteria for certain micro-organisms are indicated by Regulation 2073/2005 and any subsequent amendments. This regulation establishes the following definitions:

microbiological criterion: a criterion defining the acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number of micro-organisms, and/or on the quantity of their toxins/ metabolites, per unit(s) of mass, volume, area or batch.

food safety criterion: a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market

process hygiene criterion: criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market. It sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law.

The evaluation of food safety criteria is done by testing the food for the presence of pathogenic bacteria (e.g. *Salmonella*, *Listeria*). If the results are unsatisfactory the food has to be removed from or not placed on the market. As far as process hygiene criterion is concerned, this is conducted by testing for the count of indicator bacteria (e.g. mesophilic bacteria, Enterobacteriaceae, *E. coli*, *Salmonella*) which are categories or groups of bacteria that, besides being used to evaluate the hygiene of the production process of the food, also act as pathogen control (Koutsoumanis and Sofos, 2004). If the results exceed the limits only corrective action must be initiated. While the enumeration of the Enterobacteriaceae or/and *E.coli* act as indicators of faecal contamination, mesophilic bacteria load is an indicator of overall hygiene and provides only an indication for the level of culturable bacteria present in or on food. Criteria for carcasses and minced meat and the specific methods that must be used for the enumeration/isolation of the microorganisms according to Regulation 2073/05 are listed in Table 1.1. As far as the detection and enumeration of microorganism on the surface of carcasses is concerned, the sampling sites should be chosen with the purpose of examining the point with the highest level of contamination (e.g. back, ham, jowl and belly) (Figure 1.1.) and the reference method is the ISO 17604/2015 which specifies the sampling method that can be used: the excision method or the swabbing method. 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). In both case, the results are expressed in colony form units (CFU) per cm^2.

Table 1.1 Food safety and process hygiene criteria of minced meat and pork carcass according to EU Regulation 2073/05 and any subsequent amendments.

Food safety criteria						
Food catergory	Micro-organisms	Sampling-plan		Limits	Analytical reference method	Stage where the criterion applies
		n	c			
Minced meat intended to be eaten raw	*Salmonella*	5	0	Absence in 25 g	EN/ISO 6579	Products placed on the market during their shelf-life
Minced meat made from intended to be eaten cooked	*Salmonella*			Absence in 10 g		

Process hygiene criteria								
Food catergory	Micro-organisms	Sampling-plan		Limits		Analytical reference	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
Carcases of pigs	Aerobic colony count			4,0 log CFU/cm^2	5,0 log CFU/cm^2	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			2,0 log CFU/cm^2	3,0 log CFU/cm^2	ISO 21528-2		
	Salmonella	50	5	Absence in the area tested per carcase		EN/ISO 6579		Improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin
Minced Meat	Aerobic colony count	5	2	5x10^5 CFU/g	5x10^6 CFU/g	ISO 4833	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
	E. coli	5	2	50 CFU/g	500 CFU/g	ISO 16649-1/2		

*n= number of units comprising the sample; c = number of sample units giving values between m and M

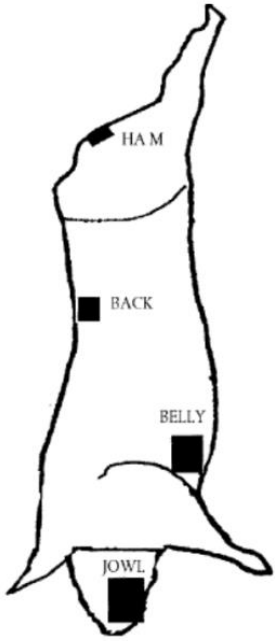

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Figure 1.1 Examples of sampling sites on Pork carcass.

1.3 Microbial contamination and foodborne pathogens of wild boar carcasses

Meat from wild boars is frequently subject to spoilage and defects (e.g. slime, browning, colour modifications, unpleasant smell) which may depend on the intrinsic characteristics of the meat or errors during processing (Casoli et al., 2005). Various studies have explored the hygienic quality of game meat (Atanassova et al., 2008; Avagnina et al., 2012; Gill, 2007) showing that the microbiological status can vary greatly between meat samples belonging to different wild boars (Paulsen et al., 2011). In contrast to domesticated pigs, wild boars roam free and their diet is uncontrolled, thus the natural microbial population on the skin and in the digestive tract can significantly differ between the animals. In fact, the microbiological load can differ depending on the circumstance in which the animals are killed (e.g. different hunting methods). For example, the use of a shotgun slug, made of lead, may cause a wound without killing the animal that can keep on running producing an increase of the contamination level (Atanassova et al., 2008). Similarly, the location of the shot has a great impact on the microbiological contamination. Besides the fact that the bullet itself may carry various bacteria, carcasses belonging to animals shot in sites posterior to the diaphragm have a higher risk of contamination than those shot in the heart, head and neck and spine (Avagnina et al., 2012). The respective level of mesophilic bacteria and Enterobacteriaceae was on average higher in wild boars shot in abdomen (3.9 $\log_{10}$ CFU/cm^2 and 2.4 $\log_{10}$ CFU/cm^2) as compared to levels retrieved after a correct shot placement (3 $\log_{10}$ CFU/cm^2 and 1.8 $\log_{10}$ CFU/cm^2) (Atanassova et al., 2008).

Moreover, the condition under which the animals are dressed and handled from the collection to the chilling point can influence the final microbiological quality of the meat. Usually, they are killed, eviscerated and bled in the wild where it is difficult to follow proper hygienic procedures. Reasonably, a higher level of mesophilic bacteria and Enterobacteriaceae are present on the carcasses eviscerated lying on the ground at collection point compared to carcasses eviscerated at GHE (Mirceta et al., 2017).

Within the European Union, there are no specific microbiological criteria settled for the evaluation of the carcasses or the meat and, for this reason, the microbiological limits for pigs are commonly used as a reference to assess quality hygiene of the wild boar meat. However, even when good hygiene practices are respected the microbiological load of the carcasses results as being higher than the limit set for domestic animals (Mirceta et al., 2017).

The diverse bacterial community on pork meat has been studied quite extensively (Koo et al., 2016; Mann et al., 2016; Tian et al., 2017), instead, still little is known regarding the microbial contamination of wild boar meat. On a retail level in Japan, the microbial population of game meats was mainly composed by *Pseudomonas*, *Acinetobacter*, and *Arthrobacter* (Asakura et al., 2017).

Wild boars share the common pathogens with domestic pigs. Some of these pathogens (*e.g. Salmonella spp.*, *Y. enterocolitica*, and *L. monocytogenes*) can cause foodborne illness in humans, who may be infected by consuming contaminated wild boar meat. *Salmonella spp*. frequency varies across the population (Gill, 2007), in fact, this pathogen was not detected in faecal samples in Sweden, on carcasses hunted in Austria, Germany and Italy (Atanassova et al., 2008; Avagnina et al., 2012; Paulsen and Winkelmayer, 2004) and in meat retailing in Japan (Naya et al., 2003). However, in another Italian study conducted in 1995 (Decastelli et al., 1995) *Salmonella spp*. were detected from the muscles of wild boars either skinned in the field or at the dressing facilities and in another Japan study in 1997 (Kanai et al., 1997) *Salmonella* were detected in few samples on a retail level. *Y. enterocolitica* was frequently detected both in the faeces and meat of wild boars even if the *Yersinia* were not pathogenic (Avagnina et al., 2012; Gill, 2007). On the contrary, the detection of *Listeria* was extremely low, but the recovered Listerias include pathogenic strains (Gill, 2007).

Introduction

1.4 The genus *Yersinia*

The genus *Yersinia* belongs to the family of Enterobacteriaceae and comprises 18 species (Guern et al., 2016). Three species of *Yersinia*, *Y. enterocolitica*, *Y. pestis* and *Y.pseudotubercolosis*, are known to be pathogenic for mammals (Ackers et al., 2000: Sharma et al.,2003) and one, *Y. ruckerii*, for fish (Ewing et al., 1978). The 14 other species are: *Yersinia aldovae*, *Yersinia aleksicia*e, *Yersinia bercovieri*, *Yersinia entomophaga*, *Yersinia frederiksenii*, *Yersinia intermedia*, *Yersinia kristensenii*, *Yersinia massiliensis*, *Yersinia mollaretii*, *Yersinia nurmii*, *Yersinia pekkanenii*, *Yersinia rohdei*, *Yersinia similis* and *Yersinia wautersii*.

Yersinia is a gram-negative, non spore-forming, facultative anaerobic bacteria that grows at between 0 and 45 °C(Zadernowska et al., 2014); thus, albeit slowly, it is able to grow at refrigerator temperature both on industrial and consumer levels (Gupta 2015).

1.4.1 Yersinia enterocolitica

Y. enterocolitica is the species most commonly isolated from human cases, foods and animals (EFSA 2017). Based on biochemical characteristics and lipopolysaccharide (LPS) O-antigens, *Y. enterocolitica* can be divided into six biotypes 1A, 1B, 2, 3, 4, 5 and more than 57 different serotypes (Robbins-Browne, 2007). The strains belonging to the biotype 1B, 2, 3, 4, 5 are pathogenic and exhibit an approximately 70-kb plasmid called pYV (plasmid for *Yersinia* virulence). Biotype 1A was to be considered "non-pathogenic" or "environmental" since they do not have pYV plasmid (Tennant et al., 2003). However, in 2015, *Yersinia enterocolitica* biotype 1A was the causative pathogen in 48% of the human cases in Denmark (ECDC 2018).

The virulence of pathogenic biotypes depends on the presence of both chromosomal and plasmid-borne genes (Bottone, 1999; Cornelis et al., 1998). Chromosomal virulence genes include: (a) attachment invasion locus (*ail)* which encodes for a surface protein responsible for serum resistance and adhesion to cells and extracellular matrix; (b) invasion (*inv*) encoding an outer membrane protein that, binding the B_1 integrins, mediates the translocation through the M-cells of the Peyer's patches (Nelson et al., 2001); (c) mucoid *Yersinia* factor (*myf*) that encodes for proteins responsible

of the expression and assemblage of a fibrillar structure (Iriarte and Cornelis, 1995); (d) host responsive element P (*hreP)* codes for a protease that is expressed specifically during infection as a proprotein, specific for *Y. enterocolitica* , as it cannot be found in other *Yersinia* species (Wagner et al., 2009); (e) *yersinia* stable toxin (*yst*) which can be divided into three subtypes (*ystA*, *ystB* and *ystC*) encoding the heat-stable enterotoxin that plays a role in diarrhea during infection (Young & Miller, 1997). Moreover, an important chromosomal gene is *ymoA* encoding for YmoA (*Yersinia* modulating protein A) protein which negatively regulates the expression of various genes; particularly it inhibits the expression of *inv* and *ystA* (Platt-Samoraj et al., 2006). Moreover, there are several virulence plasmid genes (pYV), among them adhesin A (*yadA*), whose product is involved in autoagglutination, serum resistance and adhesion (Skurnik and Wolf-Watz, 1989) and transcriptional regulator (*virF*), which encodes transcriptional activators of the *Yersinia* outer membrane proteins (yop regulon) (Cornelis et al., 1998) and is, therefore, fundamental for the type III secretion system. Strains of biotype 1A were considered to be non-pathogenic since they do not have pYV plasmid and some chromosomal virulence genes, i.e. *ystA* and *myfA* (Tennant et al., 2003) and even *inv* is present, it seems to be non-functional in most 1A strains (Pierson and Falkow, 1990). Nevertheless, the strains carry other virulence genes such as *ystB* and *hreP* and some biogroup 1A strains carry *ail* gene (Bancerz-kisiel, 2018; Stachelska, 2018).

1.4.2 Y. enterocolitica in humans

Yersiniosis is the third most commonly reported zoonosis in the EU where 6,861 confirmed cases were reported in 2016 and *Y. enterocolitica* was the most widely reported species (EFSA,2017). The biotype distribution of *Y. enterocolitica* is different worldwide. In Europe in 2016 the most common serotype in human infection were O:3, O:9 and O:8 and the most commonly reported biotypes were biotype 4, 2 and 3 (EFSA,2017). The bioserotype 4/O:3 is also dominant in North America, while 3/O:3 is the most prevalent bioserotype in China (Duan et al.,2017). However, a reliable incidence rate is not available because the notification of Yersiniosis is not mandatory in all the EU states like Belgium, France, Italy and Luxembourg where the notification system is voluntary (EFSA,2017). Usually humans are infected through the ingestion of contaminated food or water; particularly the consumption of raw pork products, where *Y. enterocolitica* is frequently isolated (EFSA, 2016), is considered to be the most important risk factor for *Y. enterocolitica* infection (Rosner BM et al., 2012 and Boqvist et al., 2009). Based on collected data the highest rates of Yersiniosis is found in 0–4-year-old children both in males and females (ECDC 2018) where it is associated with a wide range of clinical and immunological manifestations, including diarrhea, at times bloody, fever, mesenteric lymphadenitis and terminal ileitis. Symptoms (abdominal pain and fever) may be confused with appendicitis (EFSA 2013). *Y. enterocolitica* may also cause extra-intestinal infections (e.g. pharyngitis, abscess formation in liver and spleen, pneumonia, osteomyelitis, encephalitis) and even if usually Yersiniosis is self-limiting, some complications may appear like arthritis and erythema nodosum (Miller et al., 1989).

1.4.3 Y. enterocolitica in animals and foods

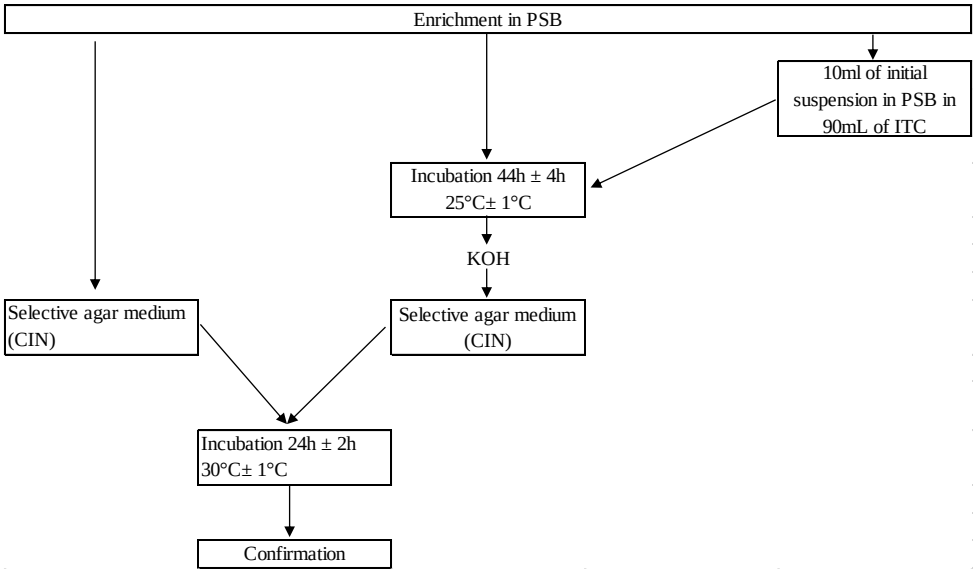
Very little data on the incidence of *Y. enterocolitica* in foods and animals is available from EU states probably because according to the Zoonoses Directive 2003/99/EC, reports of *Yersinia* occurrence or prevalence are not mandatory (EFSA,2017). Pigs are considered the major reservoir where the bacterium have been commonly isolated from the tongue and gastro-intestinal tract and the contamination of the meat result from improper slaughter and evisceration techniques and refrigeration temperatures, unlike the other Enterobacteriacea are not able to inhibit the growth of *Y.*

enterocolitica (Fredriksson-ahomaa et al., 2006; Zadernowska et al., 2014). Moreover, a new, underestimated reservoir of *Y. enterocolitica* appears to be the wild boar population where it was isolated in 26,5% rectal swabs of wild boars hunted in Poland (Bancerz-kisiel and Szweda, 2015; Morka et al., 2018). However, *Y. enterocolitica* can also contaminate a wide variety of other foods like bovine and sheep meat, milk, dairy products and vegetable derived foods (Gupta et al., 2015).

1.4.4 Detection of Y. enterocolitica

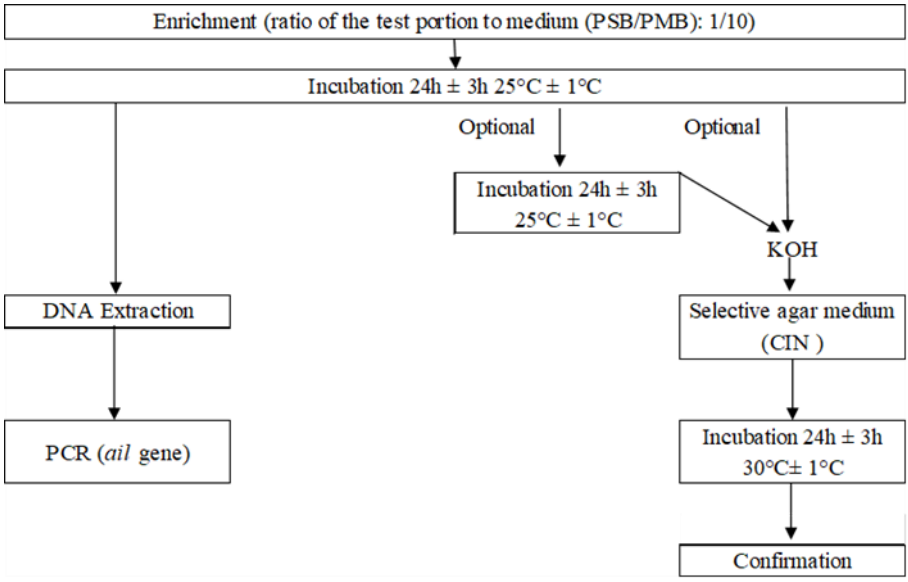
The number of contaminated foods is probably underestimated as currently, culture-dependent methods have several limitations. The official monitoring of *Y. enterocolitica* from food samples with culture-dependent methods is usually performed following the ISO 10273:2017 (Table 1.2).

Table 1.2. Schematic overview of the ISO 10273:2017



However this method is time-consuming and ineffective (Damme et al., 2013) (Fredriksson-Ahomaa et al., 2008). Over the last few years several methods for the isolation of the colony have been developed. However, currently a perfect procedure able to recover pathogenic *Y. enterocolitica* from foods does not seem to exist. The main procedures involve the use of enrichment broth followed by plating onto selective media. The major disadvantages are the presence of the abundant natural microbial population in the samples and the low concentration of pathogenic strains. Up till now CIN agar has been considered the best medium for the isolation of *Y. enterocolitica* colonies. However, the indigenous bacteria of the samples can over growth and cover the colonies of *Y. enterocolitica* (Ravangnan and Chiesa, 1995 and Toora et al., 1994). Moreover, most of the bacteria show the same morphology of the *Y. enterocolitica* colonies on CIN agar. Exploiting the psychrotrophic nature of *Y. enterocolitica*, the use of cold enrichment methods can improve the recovery from food samples since the refrigeration temperature can suppress the growth of the background microbial population. However, this procedure requires more than fourteen days of incubation (Petsios et al., 2016). Although the isolation of the colony is a step that cannot be avoided, molecular methods can improve the detection of this pathogen as they are reliable and require less time compared to culture dependent methods. The reference method for the detection of pathogenic *Y. enterocolitica* is the ISO 18867:2015 (Table 1.3.) that indicatesthe *ail* gene as the target gene for the PCR. However, the pathogenicity of *Y. enterocolitica* cannot be reliably determined based on *ail* detection in simple PCR because sequences homologous to the *ail* gene are now more often detected in the 1A biotype (Bancerz-kisiel, 2018). Thus studies aimed at investigating the presence and distribution of the different genes in *Y. enterocolitica* strains are necessary in order to develop probe-specific real-time PCR to determine the presence of pathogenic *Y. enterocolitica* in food samples.

Table 1.3. Schematic overview of the ISO 18867:2015



1.5 Cultivation-dependent methods

1.5.1 Traditional methods for isolation and identification of bacteria

The identification of microorganisms means the assignment of a species to an isolate, as previously described. However, in the world more than hundreds of millions of species exist and around 10000 are validly described (Welker, 2011).

For a long time microbiological species have been described by their morphology and metabolic activities and currently conventional methods use these aspects to identify the isolates. Thus, conventional methods based on the culture on microbiological media and identification by the biochemical test are still used in the daily routine in food microbiology laboratories. Particularly, these methods are sensitive and inexpensive, but they are long and time-consuming. Biochemical screening usually involves the use of kits as analytical profile index (API) that encompass a series of 10-20 biochemical tests. After a period of incubation (usually 24 hours) the results of the reactions are converted to numerical biochemical profiles which are identified by using a computer software (Mandal et al., 2011). However, the database of the API test is limited and requires the use of different kits to identify several colonies. Therefore, a complete series of tests is often required before any identification can be confirmed (Mandal et al., 2011).

Nowadays, species description is based on nucleic acid sequences and the sequence of 16S ribosomal RNA gene is considered the "gold standard" method for prokaryotes for the identification of sample isolate. However, the identification of the isolates with the 16S gene sequences is expensive, it is not suitable for routine testing in food microbiology laboratories and it is not adequate for a more refined typing for epidemiological studies (Singhal et al., 2015; Welker, 2011).

The analysis of proteins can be considered a method with an intermediary position between phenotypic- genotypic dichotomy as the genome encodes the sequences of each protein that is expressed in cells (Welker, 2011). In recent years, 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).

1.5.2 Matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometry (MALDI-TOF MS)

Mass spectronomy, discovered in the early 1900s, is a technique where chemical compounds are ionized and the mass to charge (m/z) of the charged molecules is measured (Singhal et al., 2015). However, itsuses inthe beginning were limited to the chemical sciences. The use of MS in biology increased with the development in 1980 of the Matrix-Assisted Laser Desorption/Ionization.

MALDI is based on the "soft ionization" method where peptides are converted into ions by either the addition or loss of one or more than one proton with a laser beam without a significant loss of sample integrity. Once in the gas phase, the ions, accelerated in an electric field, are separated from each other by their mass-to-charge ratio and then detected using different type of mass analyzers (e.g. quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers) (Singhal et al., 2015) In microbiology, most of the time TOF mass analyzersare is used. Since ions have the same kinetic energy but different speeds depending on their mass, after leaving the electric field, TOF analyzers measures the m/z ratio of the ions by determining the time required for them to pass through a field-free section (the fight tube) (Figure 1.2.) (Pavlovic et al., 2013). Thus small ions take less time to traverse the flight tube than large ions. The identification of the microorganism is done by comparing the peptide mass fingerprint (PMF), created on the basis of the TOF information, with a reference spectrum in a database. Mass spectra are generated after a defined number of laser pulse cycles, generally hundreds, to yield a representative average mass spectrum. In particular, a raw spectrum is processed to produce the PMF which contains information about the peak apex m/z values (Welker, 2011). For species-level identification of the microbes, a mass range of 2000-20000Da is selected. Most of the recorded peaks can be assigned to some ribosomal proteins that are abundant in cells. In a mass range lower than 20kDa, the function of a large share of proteins is still unknown, while with a higher mass range only a limited number of mass signals are recorded (Welker, 2011). The pre-conditions for a species identification is necessary:

1) PMF of different species has to be different;
2) PMF of the strains of a single species need to be similar
3) PMF of the strains of a single species has not to be identical to allow the typing.

The MALDI-TOF MS profile, however, can change depending on the use of different culturing conditions that can affect the microbial physiology and protein expression profile (Welker, 2011).

The sample for the analysis is mixed or covered with a matrix solution and placed on a stainless steel target plate. The most useful matrix for microbiological applications are: α-Cyano-4-hydroxycinnamic acid (CHCA) 2,5 dihydroxybenzoic acid (DHB) and 3,5-dimethoxy-4-hydroxycinnamic acid (Singhal et al.,2015). A strong acid like Trifluoro acetic acid (TFA) and acetonitrileis used to dissolve the matrix. The solvents ensure the solubility of the matrix and penetrate the cell wall of microorganism assuring the extraction of the intracellular proteins. The evaporation of the solvent results in a rapid co-crystallization of protein molecules and cellular compounds (Welker, 2011). In this way, the radiation is absorbed by the organic compounds and then transferred to the sample and the proteins become ionized without degradation.

For the identification two methods are described for the sample preparation, the isolates can be either applied directly onto a steel plate (direct colony identification method) (Saffert et al.,2011) or can be lysed by using chemicals (e.g. acetonitrile, formic acid) or enzymes to obtain proteins (extraction method). While the use of the direct colony method for the Gram-negative bacteria may be sufficient, for the presence of abundant peptidoglycan in the wall of Gram-positive bacteria (Alatoom et al., 2011) the use of the extraction method for their identification is suggested. Moreover, using the direct colony identification method the crystallization process can be compromised by the presence of metabolites, pigment and /or agar material (Du et al., 2002). However, the last method is fast, simple and cheaper so when the number of samples is high the direct colony identification method is preferred.

The first MALDI-TOF capable of a microbial identification was the MALDI Biotyper developed by Bruker Daltonics. Mass spectra are 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.

Using a biostatistical algorithm, the fingerprint is compared to Main spectra as reference spectra in the reference database obtained from multiple spectra of a single species and a log (score) value is calculated. For the MALDI-Biotyper^{tm}, a log value higher than 2.0 give a species identification, while a score between 1.7 and 2.0 give a identification at the genus level. The identification is not possible if the score is under 1.7.

Disadvantages of the use of the MALDI-TOF are the following (Cherkaoui et al., 2010; Singhal et al., 2015; Welker, 2011):

1) High initial cost of the machine,

2) High amount of cell material (10^4-10^5 cells),

3) Difficulties in discriminating certain bacterial species (e.g. *E.coli* and *Shigella spp*.),

4) Limited database especially in food microbiology as databases are still oriented towards clinical relevant microorganisms.

Nevertheless, MALDI TOF MS is a fast technique that allows the identification in less than 5 min per samples, it does not require specialized laboratory personnel as the protocol is easier to learn and it has low marginal cost. Thus, MALDI TOF MS could be a promising technique in food microbiology analysis as well, especially when time and identification or confirmation of the isolates are important targets (Carannante et al., 2015; Nicolaou et al., 2012; Pavlovic et al., 2013).

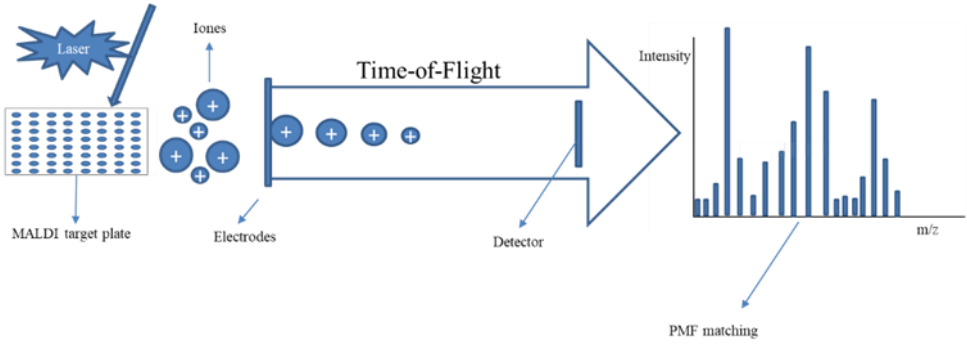


Figure 1.2 Schematic diagram of MALDI-TOF(modified according to Pavlovic et al., (2013) and Singhal et al., (2015)

1.6. Culture-independent techniques as approaches to identifying bacterial communities

In recent years microbial culture techniques have been neglected because of their limitations (Cocolin et al., 2013; Lagier et al., 2016; Olofsson et al., 2007):

1) Labour intensive,
2) Influenced by specific isolation media and conditions,
3) Levels are often underestimated due to the stressful condition wherein the target microbes reside at the time of sampling,
4) Presence of viable but non-cultivable cells (VNC),
5) Numerically less present population can be masked on the plates and not detected.

Thus, cultivation-independent methods have been used to study the microbial communities of the different matrices. It is estimated that in nature >99% of microorganism cannot be cultivated using the standard techniques (Cocolin et al., 2013) and therefore, nowadays culture-independent techniques are seen as the only way to study entire microbial communities. Particularly, bacterial communities are studyied using the high-throughput sequencing (HTS) approach and several next-generation sequencings (NGS) have been developed (Ercolini, 2013). With the NGS (also known as massively parallel sequencing) millions of sequencing reactions happen in parallel and, therefore, it can be used to sequence entire genomes or small numbers of individual genes in a short time (Behjati and Tarpey, 2013). The microbial analysis should be reproducible, However, “gold standard” protocols from the nucleic acid extraction until the analysis of the data have not yet been established (Chistoserdova, 2010).

Most of the culture-independent techniques are PCR-based (Table 4) and they require efficient DNA extraction. In the case of food matrices, the amplification of DNA can be compromised by the presence of inhibitors like fats, proteins,and calcium (Ercolini, 2013). The steps used to extract DNA are (Quigley et al., 2012):

1) Mechanical homogenization which allows the release of the microorganism into suspension,

2) Mechanical lysis: microbial cells are lysed through the vibration of a microbead that consents the opening of bacterial cells and the release of the DNA,

3) Treatment with buffers, enzymes and/or detergents to break cell walls and help remove non-DNA organic and inorganic material,

4) Organic extraction of the DNA.

Commonly DNA is extracted using commercial kits using DNA-binding matrices (e.g. silica membrane-based) or magnetic solid-phase supports (using magnetic microparticles) (Quigley et al., 2012). It has been reported that the DNA extraction from a complex matrix, such as faecal or food samples, can have a large effect on the outcome of the further analysis (Costea et al., 2017; Quigley et al., 2012).

In order to study the microbial communities, different technologies (Table1.4) and different approaches can be used (Knight et al., 2018):

- Whole metagenomic analysis: Sequence all the DNA present in a sample, including viral and eukaryotic DNA, giving a taxonomic resolution at species and strain level,
- Metatranscriptome analysis: Profile transcription in microbiomes giving information on gene expression and thus, on the functional activities of microbial communities
- Marker gene analysis: Study of microbial communities using a specif region of a gene of taxonomic interest (e.g.16S rRNA for bacteria).

,

Table 1.4. Culture-independent techniques commonly used to study microbial population in different ecosystems (modified according to Reis-filho, 2009)

Method	Amplification	Base pairs of the reads	Sequence reaction
454 FLX Roche	Emulsion PCR	~400	Pyrosequencing
Illumina (Solexa) Genome Analyzer	Bridge PCR	36 to 175	Reverse terminator
ABI SOLiD	Emulsion PCR	~50	Ligation sequencing
Helicos Heliscope	None	30 to 35	Single molecule sequence by synthesis
Pacific Biosciences	None	>1,000	Single molecule real-time DNA sequencing

1.6.1 Illumina Genome Analyzer

The sequencing by synthesis on a glass solid phase surface, commonly referred to as “the Solexa”(Shendure and Ji, 2008), has its origin in work by Fedurco et al., (2006) and Turcatti et al., (2008).

The construction of a library involves random fragmentation of template DNA and the ligation with adapter sequences to the 5' and 3' extremities of nucleic acids. This strategy involves the use of a bridge amplification process (Fedurco et al., 2006) where forward and reverse primers, with the complementary to the adaptor, are tethered to a glass surface by a flexible linker. The adaptor flanked DNA fragments are subsequently hybridized to the oligonucleotides which are immobilized onto the glass flow cell. The new synthesized strands are hybridized with the complementary oligonucleotides through a Bst DNA polymerase, causing the formation of double-stranded DNA bridge structures resulting, after denaturation with formamide, into the formation of two separate DNA fragments (Cao et al., 2017). Amplicons, produced from a single DNA fragment after multiple rounds of the amplification process, will be clustered to a single physical location on an array and each cluster will consist of ≈ 1000 clonal amplicons. After cluster generation the sequencing primer is hybridized to an universal sequence flanking the region of interest (Shendure and Ji, 2008). Cycles of sequencing are performed through a single- base extension with a modified DNA polymerase and four nucleotides. A chemically cleavable moiety at the 3’hydroxyl position allows the incorporation of a single base at each cycle. Moreover, each nucleotide is labeled with a fluorescent reporter, also chemically cleavable so that each base can be identified (Shendure and Ji, 2008). Each cycle extends a single base followed by the acquisition of the image performed in four channels. This is followed by chemical cleavage of both fluorescent labels and terminating moiety. Due to a short read-length, bacterial classification using 16S rRNA can often be performed only at genus level(Cao et al., 2017).

1.6.2 16s rRNA amplicon sequences

While whole metagenomic and metatranscriptome analysis are expensive, laborious and require qualified personnel because the samples preparation and the analysis of the data are complex, the 16S amplicon sequencing is the most common HTS because it is quick, simple and cost-effective. Moreover, the preferential choice of the HTS of the 16S rRNA, compared to the others approaches, is also owed to the large existing database and to the possibility of discriminating the microbial DNA from the host DNA (Knight et al., 2018).

16S rRNA that codes for the 16S rRNA which is on the 30S ribosomal subunit is used as the gene target for bacteria because: i) it is present in all bacteria, ii) It has conserved region that can serve as binding sites for PCR primers, iii) it has 9 variable regions for which differentiation between bacteria is possible (Cocolin et al., 2013; Knight et al., 2018).

However, technical factors such as the choice of the primers, variable regions selection, amplicons size and PCR cycles may be sources of bias. Moreover, the further analysis of the data can greatly influence the outcome of the results. Different analysis packages (e.g. QIIME, Mothur, USEARCH, LotuS) are available for processing 16S amplicon HTS data (Mysara et al., 2017). Currently, sequencing errors are solved through quality filtering and cluster (usually with a 97% similarity threshold) in Operational Taxonomic Units (OTUs) (Callahan et al., 2016). However, with the use of this approach, real biological sequence variations are misinterpreted as errors and consolidated into a single OTU. Algorithms such as the Divisive Amplicon Denoising Algorithm (DADA) can correct amplicon errors creating a table of DNA sequences and counting the different sequences per sample (Knight et al., 2018).

A further important step is the assignation of taxonomic names to the sequences that can be achieved using analysis packages such as QIIME and Mothur (Knight et al., 2018) that match the sequences with reference database (e.g.Greengenes, RDP and Silva). However, due to the short reads obtained using 16S rRNA amplicon sequencing the identification is often only at genus level (Cao et al.,2017). Moreover, the analysis packages still produce a large

number of unassigned sequences. Thus, recently a culturing approach based on combined identification of bacterial isolates using MALDI TOF MS and 16S amplicon sequencing has allow the culture of organism corresponding to sequences previously not assigned (Lagier et al., 2016).

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2. Aims

Aims

Domestic pigs and wild boars are members of the same species (*Sus scrofa)* and they are classified in the subspecies *domestica* and *scrofa* respectively. Pork meat is widely consumed in the world and minced meat, among the pork products, has the highest penetration level, whereas the consumption of wild boar meat is still low, although it is increasing.

Concerning pork meat production, routine evaluation of the slaughter process is usually performed by the enumeration on different sampling sites on the pork carcasses of the aerobic colony count, Enterobacteriaceae, *E. coli* and *Salmonella* spp.. Aerobic colony count and the enumeration of *E. coli* are, instead, applied in food hygiene monitoring for minced meat. According to EU regulation 1441/07, methods for the evaluation of process hygiene criteria are based only on the quantitative count of hygiene associate microbes and on the isolation of few colonies for the further identification, however, knowledge on the microbial diversity of the counted microorganism is lacking.

Within the European Union, there are no specific microbiological criteria for wild boar carcass and/or meat and still little is known regarding the microbial communities present on wild boar meat.

High throughput analysis by 16S rRNA amplicon sequencing is the preferred method to identify bacterial communities in different ecosystems, but, recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as rapid and accurate method for routine of clinical isolates.

Moreover, domestic pigs and wild boars share common pathogens (e.g. *Salmonella spp.*, *Yersinia enterocolitica*, and *Listeria monocytogenes*) that may be responsible for human illness. It has been demonstrated that healthy pigs are the principal reservoir of *Y. enterocolitica*, but this pathogen seem to be present also in the wild boar population. However, current methods for the isolation of pathogenic *Y. enterocolitica* bacteria in food samples are time-consuming and inefficient. Real-Time- PCR with specific primers can be used to detect rapidly and with high specificity and sensitivity this pathogen from foods; however, in order to develop a probe-specific real-time PCR, studies aimed to investigate the

presence and distribution of the different genes in *Y. enterocolitica* strains are needed.

The **general aim** of this thesis was to gain more insight in the **microbiological contamination of pork and wild boar meat with culture-dependent and independent methods**. Moreover, with the purpose of evaluating the contamination of the samples with *Y. enterocolitica* and with the necessity to select gene target for the real-time PCR, a study on the distribution of virulence gene in *Y. enterocolitica* strains has been conducted (Chapter 1). During this thesis 14 minced meat samples (Chapter 2), four (ham, back, jowl and belly) sampling areas of 8 eight pork carcasses (Chapter 3), and 22 wild boars meat samples (Chapter 4) were evaluated through the use of a culture-independent method (16S amplicon sequencing) compared to classical isolation methods combined with the identification by MALDI-TOF MS and 16S amplicon sequencing. Moreover, three sample preparations for the identification with MALDI-TOF MS and six DNA extraction protocols for the further 16S rRNA amplicon sequencing were performed (Chapter 2).

3. Chapter 1

Evaluation of virulence genes in *Yersinia enterocolitica* strains using SYBR Green real-time PCR

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

Abstract

Yersinia enterocolitica comprises six biotypes 1A, 1B, 2, 3, 4, and 5. The virulence of the strains belonging to biotypes 1B and 2–5 depends on the presence of both chromosomal and plasmid-borne genes. Strains belonging to biotype 1A do not carry the virulence plasmid pYV. However, they carry other virulence genes, such as *ystB* and *hreP*. The aim of this study was to investigate the distribution of *yadA, virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* virulence genes in *Y. enterocolitica* strains in order to select the target genes that could be used for the development of a probe-specific real-time PCR to determine the presence of *Y. enterocolitica* in food samples. A total of 161 *Y. enterocolitica* strains isolated in eight countries and grouped into biotypes 1A, 2 (serotypes O3, O5 and O9), 3 (serotypes O3 and O9) and 4 (serotype O3) were examined for virulence genes. The most common virulence-associated gene in pathogenic *Y. enterocolitica* proved to be *ystA*, which can therefore be considered the best target gene to be amplified in order to evaluate the presence of pathogenic biotypes. By contrast, to identify *Y. enterocolitica* 1A strains, *ystB*, which codes for the enterotoxin YstB, can be proposed. This has been found in all non-pathogenic biotypes studied, but never in pathogenic biotypes.

Keywords *Y. enterocolitica*, Sybr green RT-PCR, *ystA* gene, *ystB* gene

3.1 Introduction

Yersinia enterocolitica is a gram-negative bacteria belonging to the genus *Yersinia* and to the family of Enterobacteriaceae. Within the genus *Yersinia*, two species, in addition to *Y. enterocolitica*, are pathogenic for mammals, *Y. pestis* and *Y. pseudotuberculosis*, and one, *Y. ruckerii*, for fish (Ewing et al., 1978). Strains of *Y. enterocolitica* can be classified into six biotypes 1A, 1B, 2, 3, 4, 5 (Gupta et al., 2015) and at least 57 different serotypes (Robbins-Browne, 2007). The bio/serotypes mainly associated with human infection are 4/O:3, 2/O:9, 1B/O:8, 2/O:5,27, 1B/O:20, 1B/O:13a and 1B/O:13b (Bottone, 1999; Kwaga et al., 1992). Most yersiniosis cases caused by *Y. enterocolitica* are reported as sporadic cases.

The biotype distribution of *Y. enterocolitica* is different worldwide. According to the EFSA (2015), 4/O:3 and 2/O:9 are the primary bio/serotypes in humans in Europe. In the USA, most human pathogenic strains belong to serotype O:8, followed by O:3, O:5,27, O:13a, 13b, O:20, and O:9 (Kwaga et al., 1992). In China, serotypes O:3, O:9 and O:8 are the most frequent (Wang et al., 2008).

The virulence of pathogenic biotypes depends on the presence of both chromosomal and plasmid-borne genes (Bottone, 1999, Cornelis et al., 1998). Chromosomal virulence genes include: Attachment and invasion locus (*ail*), Invasin (*inv*), Mucoid *Yersinia* factor (*myf*), Host-responsive element P (*hreP*) and *Yersinia* stable toxin (*yst*) (Young & Miller, 1997). Moreover, an important chromosomal gene is *ymoA* (*Yersinia*-modulating protein) encoding for the YmoA protein, which negatively regulates the expression of various genes. It inhibits particularly the expression of *inv* and *ystA* (Platt-Samoraj et al., 2006). Moreover, there are several virulence plasmid genes (pYV). These include Adhesin A (*yadA*), whose product is involved in autoagglutination, serum resistance and adhesion (Skurnik and Wolf-Watz, 1989), and Transcriptional regulator (*virF*), which encodes transcriptional activators of the yop regulon (Cornelis et al., 1998) and is therefore fundamental for the type-III secretion system. To date, strains of biotype 1A have been considered to be non-pathogenic, since they do not have pYV plasmid and some chromosomal virulence genes, e.g. *ystA* and *myfA* (Tennant et al., 2003). Although *inv* is present, it seems to be non-functional in most 1A strains (Pierson and Falkow, 1990).

Nevertheless, the 1A strains carry other virulence genes, such as *ystB* and *hreP*, and some biotype 1A strains have been isolated from humans with gastrointestinal infections (Baghat and Virdi, 2006).

Although the source of infection and the transmission routes are still not fully known, it has been demonstrated that healthy pigs are the principal reservoir of *Y. entercolitica* (Fredriksson-Ahomaa et al., 2006; Nesbakken, 1985) and contaminated raw pork is suspected to be the principal source of human yersiniosis cases (EFSA, 2015). Moreover, *Y. enterocolitica* can grow at 0°C, and hence in foods stored at refrigerator temperatures without sign of spoilage.

Yersiniosis occurs mostly in young children and is associated with a wide range of clinical and immunological manifestations, including diarrhea, at times bloody, fever, mesenteric lymphadenitis and terminal ileitis. Abdominal pain and fever can often be confused with appendicitis (EFSA, 2013). The disease is usually self-limiting. However, some intestinal and extra-intestinal complications, such as reactive arthritis or infected aneurysm may appear (Miller et al., 1989). Yersiniosis is the third most common bacterial enteric disease in Europe. In 2013, 6,471 confirmed cases were reported and *Y. enterocolitica* was the predominant species among human cases. Unfortunately, no reliable incidence rates of yersiniosis are available in several countries (i.e. Belgium, France, Italy, Luxembourg and Spain) where notification is not compulsory, and no surveillance system exists in Greece, the Netherlands and Portugal. Therefore, the number of cases is probably underestimated. Moreover, the availability of reports on the prevalence of *Y. enterocolitica* in food and animals is also limited in EU member states (EFSA, 2015).

Current methods for the detection of pathogenic *Y. enterocolitica* bacteria in food samples are time-consuming and inefficient (Fredriksson-Ahomaa et al., 2008, Van Damme et al., 2013). Although the routine laboratory detection of bacteria is still commonly based upon traditional microbiological methods, alternative methods based on nucleic acid detection are now being increasingly adopted (Palomino-Camargo and Gonzalez-Muñoz, 2014). By means of PCR, pathogenic *Y. enterocolitica* in samples can be detected rapidly and with high specificity and sensitivity (Fredriksson-Ahomaa and Korkeala, 2003).

The aim of this study was to evaluate the presence of virulence genes in *Y. enterocolitica* strains originating from eight different countries by using SYBR Green real-time PCR, including Tm analysis, to select target genes for the development of a probe-specific real-time PCR for the detection of pathogenic *Y. enterocolitica* biotypes in food samples.

3.2 Materials and Methods

3.2.1 Strains

A total of 161 *Y. enterocolitica* strains isolated from 2003 to 2007 in eight countries were analyzed. *Y. enterocolitica* strains were grouped into four biotypes:

1) biotypes 1A (9 strains);
2) biotypes 2:
   - serotypes O3 (5 strains);
   - serotypes O5 (5 strains);
   - serotypes O9 (9 strains).
3) biotypes 3:
   - serotypes O3 (2 strains);
   - serotypes O9 (1 strain).
4) biotypes 4:
   - serotype O3 (130 strains).

The samples were isolated from pig tonsils (134), human feces (19), pig pluck sets (5), pig feces (2) and lamb feces (1). The strains were grown in tryptone soy broth (Oxoid LTD, Basingstoke, Hampshire, United Kingdom) at 30°C for 24 h.

3.2.2 Real-Time PCR-based protocol

One mL of each broth culture was transferred into a clean microcentrifuge tube, and centrifuged for 10 min at 10,000 ×g at 4°C. The supernatant was carefully discarded and the pellet was re-suspended in 200 µL of 6% Chelex 100 (Biorad, Hercules, CA, USA) by vortexing, and incubated for 20 min at 56°C and then 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.

Two µL of the DNA extracted (DNA concentration = 15 ±3.36 ng/µL) was used as a template for *Y. enterocolitica* Real-Time PCR evaluation assay (Josefsen et al., 2007) and added to 18 µl of mastermix (iQ^{TM} SYBR Green Supermix; Bio-Rad, USA). The mastermix contains: 10 µl of iQ^{TM} SYBR Green Supermix (2x) to 2 µl of forward and reverse primers and 6 µl of H_2O. The fluorescence intensity of SYBR Green and the melting curve analysis were studied by means of the CFX96 system (Bio-Rad). A threshold cycle (C_t) under 35 and a specific melting temperature

(T_m) indicated a positive result. Eight genes were investigated: two plasmid-borne genes, *yadA* and *virF*, and six virulence genes *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* located in the chromosome. The primers used in this study are presented in Table 3.1.

Table 3.1. Primers used for PCR.

Gene	Primer sequence (5'--> 3')	Amplicon size	Reference
inv	TGCCTTGGTATGACTCTGCTTCA	1144	Bhagat &Virdi (2006)
	AGCGCACCATTACTGGTGGTTAT		
myfA	CAGATACACCTGCCTTCCATCT	272	Kot & Trafny (2004)
	CTCGACATATTCCTCAACACGC		
ystA	ATCGACACCAATAACCGCTGAG	79	Thoerner, et al. (2003)
	CCAATCACTACTGACTTCGGCT		
ystB	GTACATTAGGCCAAGAGACG	146	Thoerner, et al. (2003)
	GCAACATACCTCACAACACC		
ymoA	GACTTTTCTCAGGGGAATAC	330	Grant, et al. (1998)
	GCTCAACGTTGTGTGTCT		
hreP	GCCGCTATGGTGCCTCTGGTGTG	757	Bhagat & Virdi (2006)
	CCCGCATTGACTCGCCCGTATC		
yadA	TAAGATCAGTGTCTCTGCGGC	747	Lucero Estrada, et al. (2012)
	TAGTTATTTGCGATCCCTAGCAC		
virF	GGCAGAACAGCAGTCAGACATA	591	Bhaduri,et al. (1997)
	GGTGAGCATAGAGAATACGTCG		

3.3 Results

All 161 strains tested for the *ymoA* gene were positive (100%). However, the most common virulence-associated gene in pathogenic serotypes was *ystA*. The yersinia stable toxin A gene was found in 99% of 4/O:3 strains tested and in all (100%) the strains belonging to the other pathogenic biotypes analyzed. By contrast, *ystB* was present in all non-pathogenic biotype 1A strains, but was never detected in isolates belonging to pathogenic bio/serotypes (Table 3.2).

Although belonging to the same plasmid, the single plasmid-genes displayed different prevalence. Moreover, in 4/O:3 strains, the *yadA* gene amplicon was found in a higher prevalence than that of *virF* (p value < 0.05). Differences were observed in the prevalence of the *inv* and *hreP* genes in 4/O:3 isolates from the eight countries (Table 3.3.).

The invasin gene (*inv*) was found in all Spanish, Finnish and Nigerian strains, and in 97% of Italian, 93% of Belgian, 91% of Russian and English, and 87% of Estonian strains. *hreP* was found in all Spanish, Russian, Estonian, Nigerian and Belgian strains; 97% of Italian samples, 96% of Finnish strains and 91% of English strains were positive to *hreP* gene amplification. The presence of virulence-associated genes in 4/O:3 isolates from different sources is reported in Table 3.4. Although the number of the samples was limited, the same virulence-associated gene pattern was found in pluck sets and feces of pigs. Strains isolated from human feces were all positive to *ystA*, *myfA*, *inv*, *hreP* and *ymoA*, whereas *yadA* and *virF* genes were absent in six and five strains, respectively.

.

Table 3.2. The presence of different virulence genes in Yersinia enterocolitica strains grouped according to biotype

Bio/serotypes (n)	*ystA*	*ystB*	*myfA*	*inv*	*hreP*	*yadA*	*virF*	*ymoA*
4/O:3 (130)	99%	0	98%	94.61%	97.69%	90%	80%	100%
2/O:9 (9)	100%	0	100%	100%	100%	55.55%	66.66%	100%
2/O:5(5)	100%	0	100%	100%	100%	100%	100%	100%
2/O:3(5)	100%	0	100%	100%	100%	80%	100%	100%
3/O:3 (2)	100%	0	100%	50%	50%	100%	100%	100%
3/O:9 (1)	100%	0	100%	100%	100%	100%	100%	100%
1A (9)	0	100%	22.22%	100%	88.88%	0	0	100%

Table 3.3. The presence of different virulence genes in Yersinia enterocolitica 4/O:3 strains,grouped according to origin.

Country (n)	*ystA*	*ystB*	*myfA*	*inv*	*hreP*	*yadA*	*virF*	*ymoA*
Italy (33)	100%	0	93.93%	96.96%	96.96%	87.87%	78.78%	100%
Finland (24)	100%	0	100%	100%	95.83%	95.83%	91.66%	100%
Russia (22)	100%	0	100%	90.9%	100%	100%	81.81%	100%
Estonia (15)	100%	0	100%	86.66%	100%	93.33%	80%	100%
Belgium (14)	100%	0	100%	92.85%	100%	85.71%	71.42%	100%
England (11)	100%	0	100%	90.90%	90.90%	90.90%	90.90%	100%
Spain (10)	90%	0	100%	100%	100%	70%	60%	100%
Nigeria(1)	100%	0	100%	100%	100%	0	0	100%

Table 3.4. The presence of different virulence genes in Yersinia enterocolitica 4/O:3 strains, grouped according tosource.

Source (n.)	*ystA*	*ystB*	*myfA*	*inv*	*hreP*	*yadA*	*virF*	*ymoA*
Pig tonsils (133)	99.24%	0	98.49%	93.98%	96.99%	92%	82%	100%
Human feces (11)	100%	0	100%	100%	100%	45.45%	54.54%	100%
Pig pluck sets(5)	100%	0	100%	100%	100%	100%	100%	100%
Pig feces (2)	100%	0	100%	100%	100%	100%	100%	100%

3.4 Discussion

The results showed that not all of the strains analyzed were positive to all the virulence genes tested. It is possible that not all of these genes are necessary for the virulence of *Yersinia enterocolitica*. Alternatively, other, as yet unknown, markers may exist that could play an important role in the pathogenesis of pathogenic *Y. enterocolitica* (Zheng et al., 2008).

The *ymoA* gene was present in all the *Y. enterocolitica* isolates studied, as reported by Bancerz-Kisiel et al. (2012). The percentage of *ystA* detection was higher than those reported by Tadesse (2013, 62%) and Ramamurthy et al. (1997, 78%), but similar to those recorded by Momtaz et al. (2013, 93%) and Bancerz-Kisiel et al. (2012, 100%). *Inv* gene prevalence was lower than in Momtaz et al. (2013, 100%), while Schneeberger et al. (2015) reported a 100% prevalence of the *myfA* gene. Thus, we hypothesized that there was no association between the occurrence of the *ymoA* and *ystA* genes, as reported by Bancerz-Kisiel et al. (2012), and no association was identified between the *ymoA* and *inv* genes.

The prevalence of the plasmid-borne genes *yadA* and *virF* seems to differ somewhat among the countries; however, this result may arise from the fact that the plasmids are lost during laboratory cultivation (Farmer et al., 1992). Moreover, as reported in other studies, there is a conflicting occurrence of *yadA* and *virF*; while both belong to the pYV plasmid, they are not always are present together in the same bacteria (Momtaz, et al., 2013; Schneeberger et al., 2015; Zheng et al., 2008). It is possible that the plasmids are lost and that beneficial genes are integrated into the bacterial chromosome (Harrison and Brockhurst, 2012); another possibility is the deletion of sections of the plasmid genome, and hence the loss of some genes. Indeed, selection may involve genes encoding core functions (Heuer et al., 2007).

The distribution of a small group of virulence-associated genes in isolated strains from eight countries revealed that *inv* and *hreP*, which are importantin the infection of *Y. enterocolitica*, show different prevalence among the countries. Few studies have described the prevalence of *hreP* in the *Y. enterocolitica* genome; *hreP* is a species-specific gene for *Y. enterocolitica*, and has never

been reported in other *Yersinia* pathogens (Young and Miller, 1997). The bacterial subtilisin /Kexin-like protease codified by this gene is required for the full virulence of *Y. enterocolitica*, as observed in a mouse model of infection; indeed, *hreP* mutant strains display a reduction in both 50% lethal dose and *in vivo* survival assay (Young and Miller,1997).

The role of *hreP* and *myfA* in the virulence of the non-pathogenic *Y. enterocolitica* strains is not fully understood (Bhagat and Virdi, 2011). Campioni and Falcão (2013) described a correlation between *hreP* and *ystB* genes, and Bhagat and Virdi (2006) found a correlation between *myfA* and *ystB*. In the present study, however, no association between the occurrence of these genes was observed. The prevalence of the *myfA* gene in the study by Bhagat and Virdi (44%) was higher than that observed by us. The percentage of *ystB* detection in the present study was similar to those reported by Schneeberger et al., (2015) and Bonardi et al. (2014). However, this gene was detected in 26% of strains analyzed by Tadesse et al. (2013) and in 5% of strains in a study by Zheng et al. (2008). Moreover, more recently than Bhagat and Virdi (2006), Campioni and Falcão (2013) also demonstrated that *myfA*, *hreP and ystB* were not present in all 1A isolates, but only in strains belonging to a specific clonal group, identified by repetitive extragenic palindrome (REP) and enterobacterial repetitive intergenic consensus (ERIC).

The detection rates in the strains isolated from pig tonsils 94% of *inv* positive and 100% of *ymoA* positive is in line with the observations made by Schneeberger et al. (2015). However, in the 4/O:3 strains, they reported a prevalence of 100% for the *ystA* and *myfA* genes. These results conflict with the findings of our study, in which *ystA* and *myfA* were found in 99% and 98% of the strains, respectively.

In conclusion, *Y. enterocolitica* isolation is currently difficult and requires long incubation. The detection of this bacteria by means of molecular biology tools can be rapid, with high specificity and sensitivity; thus, a real-time PCR technique able to evaluate the presence of pathogenic *Y. enterocolitica* in foods in a rapid and sensitive way is needed. Not all pathogenic *Y. enterocolitica* carry all the traditional virulence genes. On the basis of the isolated strains analyzed, *ystA*, one of the important virulence markers, can

be considered the best target gene to amplify in order to evaluate the presence of pathogenic serotypes in food, water or clinical material. If this gene alone were tested, 99 % of the pathogenic strains would be characterized correctly. To identify the biotype 1A strains, the *ystB* gene, which codes for the enterotoxin YstB, can be proposed, as it is widely distributed in this biotype. Indeed, it has been found in all non-pathogenic strains (100%), but never in pathogenic serotype strains analyzed. Unfortunately, epidemiological data on the prevalence of *Y. enterocolitica* in foods, animals and the environment are still lacking in many countries. The development of more sensitive methods for the detection of *Y. enterocolitica* is important in order to obtain more information and to prevent the spread of this bacteria. Additional research is clearly needed in order to investigate the loss or the lack of chromosomal virulence genes.

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4. Chapter 2

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

Peruzy, M. F., Murru, N., Yu, Z., Cnockaert, M., Joossens, M., & Houf, K. (2018). Determination of the microbiological contamination in minced pork by culture dependent and 16S amplicon sequencing analysis. *International Journal of Food Microbiology* In press

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 through the use of a culture-independent method (16S 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 amplicon 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 the culture-independent method, 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 amplicon sequencing and overall community profiling using a culture-independent method provides complementary results and yields important insights into the complex relationship between microorganisms in a food.

Keywords: Pork, isolation, MALDI-TOF MS, 16S rRNA gene sequencing, 16S rRNA amplicon sequencing

4.1 Introduction

Pork products are widely consumed all over the world with a mean consumption of 12,3 Kg per capita per year (OECD,2017). 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 more than 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 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 a culture-

independent method (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.

4.2 Materials and Methods

4.2.1 Sampling

From January to September 2017, a total of 14 unrelated minced pork meat samples (M1 to M14), each between 200 and 300 grams, 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 one hour after purchase.

4.2.2 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 three minutes 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 30-300 CFU/plate on PCA, and between 15-150 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.

4.2.3 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 500µl 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 100µl of Milli-Q water, and 3µl of each bacterial suspension was spotted in duplicate on a MALDI target plate. After air-drying, the sample was covered with 1µl matrix solution as described above.

As previously reported, lactic acidbacteria 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 (1533g 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, 1µl of matrix solution was added.

Each series of MALDI measurements was preceded by a calibration step with the bacterial test standard (BTS 155 255343; 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 and colleagues (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 less than 1,7 was obtained with the "direct colony identification method", were analyzed 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 amplicon sequencing. For this, DNA was extracted using alkaline lyses where one colony was suspended into 20 µl of lysis buffer (2,5ml 10%SDS, 5ml1N NaOH and 92,5ml 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, 25µL) 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 16,5µl 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).

4.2.4 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:

a) directly from 500mg 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),
b) 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 Isolationkit) and
c) 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 quantitywas measured usingthe 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/).

4.2.5 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 bychi square test. A probability value of less than 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).

4.3 Results

4.3.1 Bacterial isolation

Bacterial counts for the 14 samples are shown in Figure 4.1. 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 less than 1 log CFU/g, except for samples M3 and M13 where the levels were equal to 1,26 log.

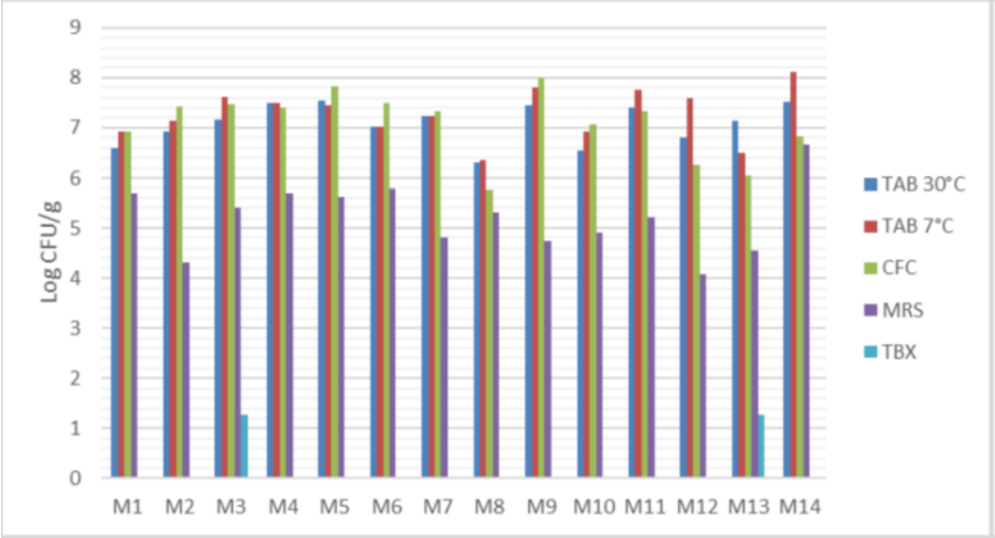

Figure 4.1 Total bacterial counts in fourteen pork minced meat samples (M1-M14) on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), presumptive Pseudomonas spp. on CFC, Lactic Acid Bacteria on MRS and E. coli on TBX.

4.3.2 Bacterial identification with MALDI-TOF MS

In the present study, 2800 bacterial isolates from the 14 minced meat samples were harvested and further analyzed 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 4.1). 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 4.1).

Table 4.1. 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.

	Direct colony identification (M1-M6)		Extraction (M1-M6)	
	N.	%	N.	%
< 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)	
	N.	%
< 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)	
	N.	%
< 1,69	273	36,11
1,70- 1,99	173	22,88
2,0 - 2,2	266	35,19
> 2,3	44	5,82

In general, bacteria identified by MALDI-TOF MS could be assigned to 15 families and 18 genera (Table 4.2). 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 amplicon sequencing (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 4.2).

Table 4.2. 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			

Members of the genus *Pseudomonas,* along with the genera *Brochothrix* and *Carnobacterium* were commonly identified among the mesophilic and psychrotrophic population on PCA (Figure 4.2). *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. (Figure 4.2). 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.*

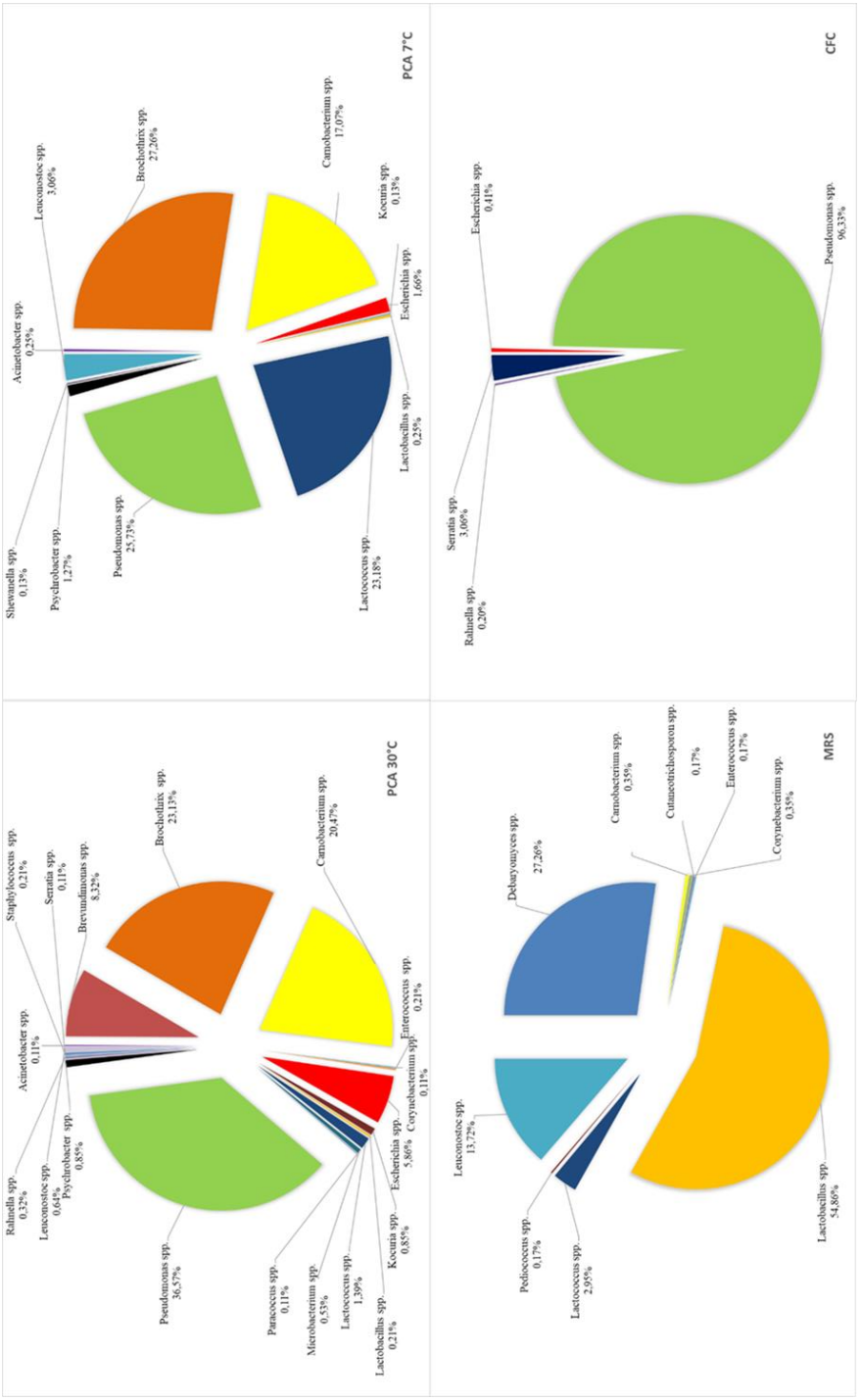
PCA 7°C
Shewanella spp. 0,13%
Acinetobacter spp. 0,25%
Leuconostoc spp. 3,06%
Psychrobacter spp. 1,27%
Brochothrix spp. 27,26%
Pseudomonas spp. 25,73%
Carnobacterium spp. 17,07%
Lactococcus spp. 21,18%
Kocuria spp. 0,13%
Escherichia spp. 1,66%
Lactobacillus spp. 0,25%
CFC
Escherichia spp. 0,41%
Serratia spp. 3,06%
Rahnella spp. 0,20%
Pseudomonas spp. 96,33%
PCA 30°C
Rahnella spp. 0,32%
Staphylococcus spp. 0,21%
Acinetobacter spp. 0,11%
Serratia spp. 0,11%
Leuconostoc spp. 0,64%
Psychrobacter spp. 0,85%
Brevundimonas spp. 8,32%
Brochothrix spp. 23,17%
Pseudomonas spp. 36,57%
Carnobacterium spp. 20,47%
Paracoccus spp. 0,11%
Enterococcus spp. 0,21%
Microbacterium spp. 0,53%
Corynebacterium spp. 0,11%
Lactococcus spp. 1,39%
Escherichia spp. 5,86%
Lactobacillus spp. 0,21%
Kocuria spp. 0,85%
MRS
Leuconostoc spp. 13,72%
Debaryomyces spp. 27,26%
Carnobacterium spp. 0,35%
Cutaneotrichosporon spp. 0,17%
Enterococcus spp. 0,17%
Corynebacterium spp. 0,35%
Pediococcus spp. 0,17%
Lactococcus spp. 2,95%
Lactobacillus spp. 54,86%

Figure 4.2. Bacteria identified at genus level by MALDI-TOF,16S amplicon 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).

4.3.3 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 more than 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 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 amplicon sequence), among the 8 samples (M7-M14), *Pseudomonas* and *Brochothrix* were always detected. However, *Escherichia, Rahnella, Microbacterium* and *Paracoccu*s 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 4.3).

Table 4.3. Comparison of the genera identified via culture dependent (MALDI TOF and 16S amplicon sequencing) and independent (16S rRNA amplicon sequencing) methods in pork meat samples (M7 to M14).

		M7		M8		M9		M10		M11		M12		M13		M14	
Family	Genus	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 more than 0.5% of total reads)

4.3.4 Effect of sample preparation and DNA extraction

The relative abundances of each OTUs varied according to the sample preparation and DNA extraction kit applied (Figure 4.3). *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 more than 50% of the total microbial community observed (Figure 4.3).

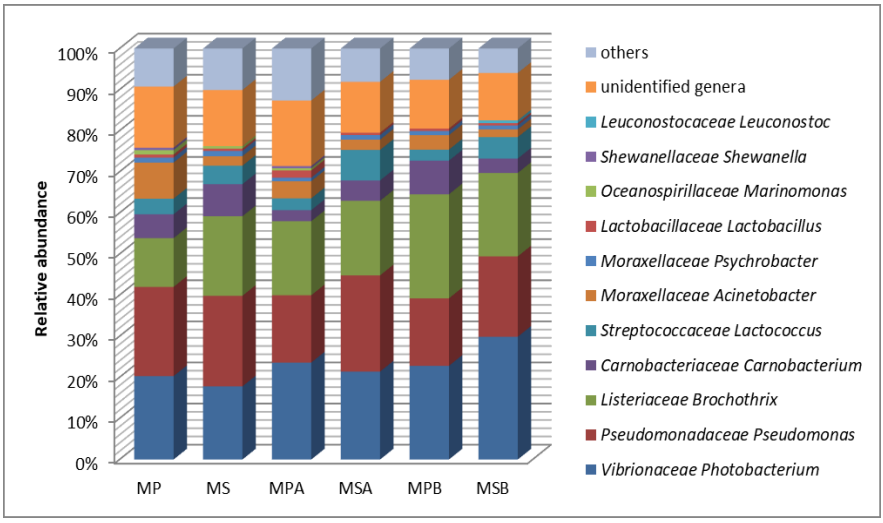

Figure 4.3. Average microbial communities identified by 16S rRNA amplicon sequencing in eight minced meat samples (M7 to M14). Relative abundances of each OTUs relating with the extraction kit or extraction protocol. Genera with <0.5% relative abundance were summed up and denoted as others.

MP: DNA extracted from 500mg 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 500mg 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

4.3.5 Statistical analysis

The microbial diversity on the PCA plates incubated at 30°C and 7°C was compared for all 14 samples (Table 4.4). 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 4.5).

Table 4.4. Richness (expressed by Chao1 index) and diversity (expressed by Shannon and Evenness indexes) of the bacterial communities identified by MALDI-TOF and 16S amplicon sequencing between the 14 minced meat samples both on PCA at 30°C and on PCA at 7°C.

	M1		M2		M3		M4		M5		M6		M7	
	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
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	
	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
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 4.5. 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 500mg 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 ofmeat homogenate in Peptone water with a ratio of 1:2 using PowerFood Microbial DNA Isolation kit; MS: DNA extracted from 500mg 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 ofmeat homogenate in Peptone water with a ratio of 1:2 using FastDNA® SPIN Kit for Soil.

4.4 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 (Figure 4.1), 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 reduced shelf life and spoilage in fresh meat (Kilcher et al., 2010; Kamenik, 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) (Kamenik, 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 identified, but in the latter, cut-off values of 2,0 were applied. The “direct colony identification method” results in fast (about one hour 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 than80%) were only identified after 16S amplicon sequencing. 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 4.4). Plate count agar is a non-selective medium suggested in ISO 4833 for thecolony 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, less than 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 amplicon 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^{tm} 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 amplicon 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 amplicon 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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5. Chapter 3

Microbial contamination of different pork carcass areas through culture-dependent and independent methods

Abstract

Routine evaluation of the slaughter process is performed by the enumeration on the carcass of the aerobic colony count, Enterobacteriaceae and *Salmonella* spp. through destructive or non-destructive methods. With non-destructive methods the bacteria are recovered from a minimum area of 100 cm^2 of different sampling sites on the pork carcasses using absorbent material, and the results of investigated areas are pooled to one value for the complete carcass evaluation (a total of 400 cm^2). However, the type of bacterial community present on the different sampling areas is 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 amplicon sequencing) and independent (16S rRNA amplicon sequencing) methods. Moreover, the presence of *Salmonella* spp. and *Y. enterocolitica* was also assessed. The dominant bacterial communities isolated from the 8 carcasses belonged to *Staphylococcus*, *Pseudomonas*, and *E. coli*, however, with 16S rRNA amplicon sequencing no genus clearly dominated the 8 carcasses. The microbial population of the ham, back, jowl and belly were dominated by the same genera, but important differences between the two slaughterhouses were observed. Thus, present data illustrate that the bacterial community of each carcass depends mainly on the microbial population of the slaughterhouse in which it is found rather than the indigenous microbiota of animals and the sampling of more than one area for the assessment of the hygienic status of the carcasses by the official authorities may be useless.

Keyword: Pork carcass, carcass areas, microbiological community, MALDI TOF MS, 16S amplicon sequencing.

5.1 Introduction

In Europe in 2017, 23,311 tonnes of pigs were slaughtered (EUROSTAT) and the mean consumption was of 32.5 (Kg/capita). The microbial load of meat depends on the spread of microorganisms during the slaughtering process (stunning,bleeding, scalding,dehairing,singeing,evisceration, splitting and cooling) (Mann et al., 2016). It has been shown that microbes in raw meat originate from the porcine itself and from the pig's environment (Koutsoumanis and Sofos, 2004); in particular, contamination of the carcasses by pathogenic and not pathogenic bacteria may occur from the hide, equipment, contact surfaces, worker and from the gastrointestinal tract (Mrdovic et al., 2013). On the pork carcasses *Aeromonas*, *Brochothrix*, *Serratia*, and *Pseudomonas* represent the most abundant genera (Mann et al., 2016). *Brochothrix*, *Serratia* and *Pseudomonas* are known to be an abundant member of the aerobic spoilage microbial population on fresh meat (Doulgeraki et al., 2012). However, if Good Manufacturing Practice (GMP) procedures are not respected during the evisceration after which no longer treatment phase is present,enteric bacteria can spread and contaminate the carcass and the environment (Mrdovic et al., 2013). These bacteria are of great concern as important foodborne pathogens associated with pork meat are of enteric origin (*Salmonella*, *Campylobacter*, and *Yersinia*) (Choi et al., 2013). According to EU regulation 853/2004 after the dressing stage, carcasses must be cooled down to a temperature of no more than 7°C which is recognized as the limit temperature below which most pathogens do not growth (Koutsoumanis and Sofos, 2004). However, some pathogens, as well as a range of food spoilage organisms, are not completely inhibited nor killed. To evaluate the hygiene of the slaughter process, the EU regulation 1441/07 require the obligatory control through the excision (destructive) and swabbing (non-destructive), of total aerobic colony count (TAB) at 30°C, of the Enterobacteriaceae and of *Salmonella* spp. on different sampling sites on the pork carcasses that should be chosen with the purpose of examining the point with the highest level of contamination (e.g. back, ham, jowl, and belly) (ISO 176604/2015). TAB is considered as an indicator of the overall hygiene in the slaughterhouse while Enterobacteriaceae and *Salmonella* evaluate the level of fecal contamination on the carcasses. However,

additional taxonomic information about the bacterial diversity between the different sampling site is missing. Culture-independent techniques are commonly used to study the entire microbial communities. Particularly, bacterial communities are being studied using high-throughput sequencing (HTS) approach and several next-generation sequencings (NGS) have been developed (Ercolini, 2013).To study the microbial communities different approaches can be used (Knight et al., 2018), but the 16S amplicon sequencing is the most common HTS because it is quick, simple and cost-effective. However, it is subject to amplification bias and a large number of unknown taxa are produced (Knight et al., 2018). Recently, in studying human gut microbioma a culturing approach based on the combined identification of bacterial isolates using MALDI TOF MS and 16S amplicon sequencing has allowed the culture of organism corresponding to sequences previously not assigned (Lagier et al., 2016). 16S amplicon sequencing is considered the "gold standard" method for the identification of the isolates. However, it is not suitable for routine in food microbiology laboratory because it is expensive and 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 is still limited to the clinical relevant microorganism.

Combined identification of bacterial strains using MALDI TOF MS and 16S amplicon sequencing with overall community profiling using a culture-independent method (16S rRNA sequencing technique) is complementary and yields important insights into the complex relationship between microorganisms in a food. The aim of the present study was to evaluate the microbial diversity present on four sampling sites of pork carcasses slaughtered in two different slaughterhouses.

5.2 Materials and Methods

5.2.1 Sampling

A total of 8 pork carcasses (C1 to C8) originating from Italian farms and slaughtered in two different abattoirs (SA(C1-C4) and SB (C5-C8)) in the Campania region of southern Italy were examined. The slaughterhouses were regularly inspected by the competent authority and the daily production capacity of SA and SB was of ≈ 150 and ≈ 48 carcasses, respectively. The layout of the slaughter processes were similar, only differing in the singeing step because in SA it was performed with a singeing machine while in SB it was manual. Each slaughterhouse was visited twice to collect swab samples. From each carcass, four (ham (H), back (B) (loin-near the split surface), jowl (J) and belly (Y) (near the split surface)) areas (100 cm^2 each) were swabbed using cellulose sponges (WPB01245WA, Sigma-Aldrich) prehydrated with 10ml sterilized peptone water (PW; CM0009, OXOID, Basingstoke, UK) (3 sponges for each areas). All samples were taken from one carcass half after evisceration and trimming of the carcass but before cooling. Samples were transported at 4°C to the laboratory and analyzed within one hour after sampling.

5.2.2 Bacterial isolation

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). 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) psychrotrophic 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 anaerobic atmosphere (anaerobic GasPak jar system) 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: g) Enterobacteriaceae (EB) according to the ISO 21528-2:2017 selectively isolated on Violet Red Bile Glucose Agar (VRBG, CM1082, Oxoid) incubated at 37°C for 24-h.

The colonies were counted and subsequently all picked from the agar plates with a bacterial growth between 30-300 CFU/plate on PCA, and between 15-150 CFU from all others. All harvested colonies were subcultured on Tryptic Soy Agar (TSA, CM0131, Oxoid) or MRS and incubated at the appropriate conditions as described above.

5.2.3 Detection of Pathogenic bacteria

The detection of *Salmonella spp*. was performed 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 (Popoff and Le Minor,1992).

The detection of *Yersinia enterocolitica* was performed through Real Time PCR with specific primers. In particular, the swabs were homogenized once in 90 ml (1:10 (W/W)) Peptone Sorbitol Bile Broth (PSB,17192, Sigma-Aldrich). DNA was extracted using the Chelex-100-resin method (1422822**,** Biorad, Hercules, CA, USA) where two ml of each homogenate was transferred into a 2ml 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 at 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 (1x) to 0.025 µl of forward ystA-F (5'-ATCGACACCAATAACCGCTGAG-3') and 0.025 µl reverse

primers ystA-R (5'-CCAATCACTACTGACTTCGGCT-3') and 9.45 µl of H_2O. To evaluate the presence of the biotype 1A, the *ystB* gene was used as target (Peruzy et al., 2017). Three µl of DNA extract were added to 22 µl of PCR mix. The mastermix contained: 12.5 µl of Qiagen QuantiTect SYBR Green PCR Kit (1x) to 0.0375 µl of forward ystB-F (5'-GTACATTAGGCCAAGAGACG-3') and 0.0375 µl reverse primers ystB-R (5'-GCAACATACCTCACAACACC-3') and 9.425 µl of H_2O. 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. While awaiting the qPCR results, the enrichment broths were always stored at 4°C. qPCR positive results, were confirmed using the corresponding reference analytical microbiological methods ISO 10273:2017.

5.2.4 MALDI-TOF MS identification and data analysis

Isolates were first analysed 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 and colleagues (2016) with a score 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. Individual colonies were suspended in 800 µl of TSB and onto MRS broth and incubated at 28°C for 24 hours. Subsequently, the sample was 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 (1533g 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 amplicon sequencing. DNA was extracted using alkaline lyses where one colony was suspended into 20 µl of lysis buffer (2.5ml 10%SDS, 5ml 1N NaOH and 92.5ml 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 part of 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 16.5µl 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).

5.2.5 Community identification by 16S rRNA amplicon sequencing

To characterize the microbial of the carcasses meat samples, 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 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 and volume ≥20 µl, (OD260/280=1.8-2.0) were submitted to Novogene (HK) Company Limitation for 16S rRNA amplicon sequencing (https://en.novogene.com). DNA was used to library construction in which V3-V4 amplicons were amplified with primer 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 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/).

5.2.6 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/). 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. Explicet was used for the Bray-Curtis analysis and for visualization of all OTUs detected.

5.3 Results

5.3.1Bacterial isolation

Overall, bacterial counts for the 8 carcasses (C1-C8) and the bacterial counts depending on the sampling point are shown in table 5.1.

Bacterial counts of the carcasses (C1-C4) sampled at SA and bacterial counts of carcasses (C5-C8) sampled at SB are shown in figure 5.1 and figure 5.2 respectively. The contamination level determined at SA and SB did not show a significant difference ($p > 0.05$). Considering the results of investigated areas pooled to one value for the complete carcass evaluation SA was the most contaminated with TAB 30°C (mean±SD= 4.1± 0.93 log CFU/cm^2), Enterobacteriaceae (mean±SD= 2.45± 1.24 log CFU/cm^2), *E. coli* (mean±SD= 2.49± 1.40 log CFU/cm^2) *Pseudomonas* spp. (mean±SD= 3.04± 0.65 log CFU/cm^2), and LAB (mean±SD= 1.25± 1.35 log CFU/cm^2) while SB was the most contaminated with TAB 7° (mean±SD= 3.03± 0.58 log CFU/cm^2) and TANAB (mean±SD= 3.10± 0.70 log CFU/cm^2).

Table 5.1 Total bacterial counts in 8 pork carcasses (C1-C8) and in the different sampling point on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), anaerobic bacteria (TANAB), Enterobacteriaceae on VRBG, E.coli on TBX, presumptive Pseudomonas spp. on CFC and Lactic acid bacteria on MRS

	Range of mean bacterial counts in 8 carcasses (C1-C8)		mean±SD log CFU/cm^2
	Minimum	Maximum	
	mean ±SD log CFU/cm^2 (s.*)	mean ±SD log CFU/cm^2 (s.*)	
TAB 30°C	3 ± 0.45 (C4)	5.36 ± 0.05 (C2)	3.87± 0.78
TAB 7°C	1.44± 1.68 (C2)	3.38± 0.52 (C3)	2.84± 0.94
TANAB	2.54± 0.63 (C7)	3.84± 0.80 (C8)	3± 0.83
VRBG	0.89 ± 1.02 (C4)	3.28± 0.62 (C3)	1.80± 1.09
TBX	0.63± 0.77 (C5)	3.50± 0.35 (C3)	1.77± 1.38
CFC	1.47± 1.78 (C7)	3.61± 0.09 (C2)	2.54± 1.03
MRS	<2 log (C1, C4, C7 and C8)	2.75± 1.18 (C5)	1.2± 1.38
	Range of the mean bacterial counts depending on the sampling point		
	Minimum	Maximum	
	mean ±SD log CFU/cm^2 (s.p.**)	mean ± SD log CFU/cm^2 (s.p.**)	
TAB 30°C	3.6± 0.77 (B)	4.14± 0.86 (J)	
TAB 7°C	2.53± 0.53 (B)	3.07± 0.37 (Y)	
TANAB	2.57± 1.15 (H)	3.24± 0.67 (B)	
VRBG	1.52± 1.20 (B)	2.04 ± 0.41 (J)	
TBX	1.30 ± 1.54 (B)	2.36± 1.04 (J)	
CFC	1.92± 1.28 (B)	2.98± 0.56 (J)	
MRS	1.01± 1.40 (B)	1.53± 1.37 (Y)	

*s.: Sample

**s.p.: Sampling point (H: ham; B: back; J: jowl; Y: belly)

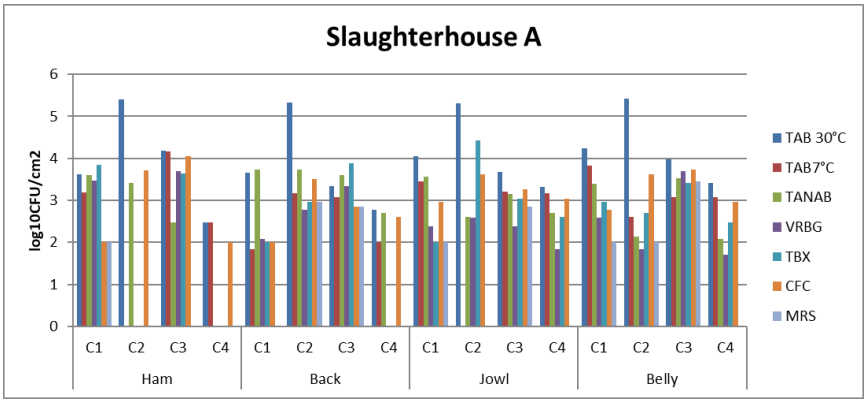


Figure 5.1. Total bacterial counts in 4 pork carcasses (C1-C4) sampled at slaughterhouse A on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), anaerobic bacteria (TANAB), Enterobacteriaceae on VRBG, E. coli on TBX, presumptive Pseudomonas spp. on CFC and Lactic acid bacteria on MRS.

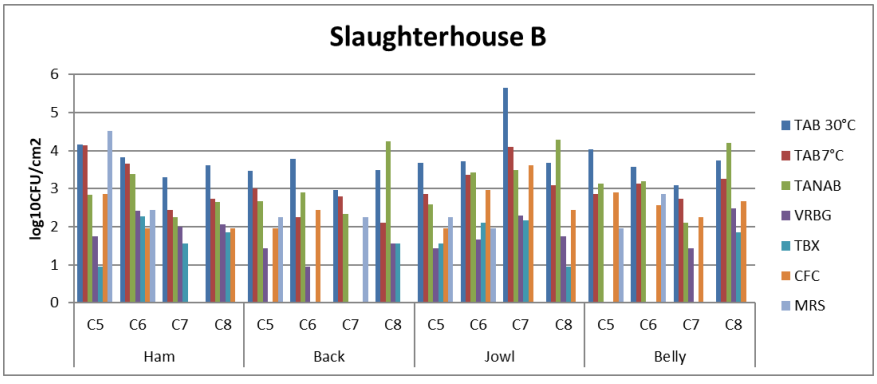


Figure 5.2. Total bacterial counts in 4 pork carcasses (C5-C8) sampled at slaughterhouse B on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), anaerobic bacteria (TANAB), Enterobacteriaceae on VRBG, E. coli on TBX, presumptive Pseudomonas spp. on CFC and Lactic acid bacteria on MRS.

5.3.2. Presence of foodborne bacteria

Using qPCR, *Salmonella* was isolated in 7 out 8 carcasses (87.5 %). Particularly, the pathogen was isolated in 3 carcasses belonging to slaughterhouse A (C1, C2, C4) and in all the carcasses sampled in slaughterhouse B (C5, C6, C7, C8). *Salmonella* spp. was isolated in 50% (n=4 carcasses) at ham, in 50% (n=4 carcasses) at back, in 87.5 % (n= 7 carcasses) at jowl and in 37.5% (n=3 carcasses) at belly level (Table 5.2). After serotyping 7 monophasic *S.* Typhimurium, 6*S.* Brandenburg, 4 *S.* derbyand 1 *S.* Rissenwere identified (Table 5.2). Always, the same serotype was isolated on the different sampling area belonging to the same carcass. Particularly, *S.* Brandenburg (C1 and C2) and *S.* Rissen (C4) were isolated on the carcasses belonging to the slaughterhouse A and *S.* Derby (C5 and C6) and *Monophasic S.* Typhimurium (C7 and C8) belonging to the slaughterhouse B. Concerning *Y. enterocolitica*, the gene *ystA* was never detected in the samples, though *ystB* was present on the ham carcasses 1 (C1H) (Table 5.2).

Table 5.2. Isolation and serotyping of Salmonella spp. and detection of Y. enterocolitica 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 and qPCR, respectively.

	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	
	Y. enterocolitica															
	C1		C2		C3		C4		C5		C6		C7		C8	
	ystA	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*	*ystA*	*ystB*
Ham	N.D.	78,5*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Back	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Jowl	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Belly	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.I.: Not isolated

*Tm: melting temperatur

5.3.3 Bacterial identification

In the present study, 2620 bacterial isolates from 4 areas (H, B, J, and J) of 8 pork carcasses (C1-C8) were analyzed by MALDI-TOF MS. 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 (= all except isolates on MRS), 15.35% (n=394) had a highly probable species identification, 31.79 % (n=816) secure genus and probable species, 19.01% (n= 488) were identified at genus level and 33.85% (n= 869) did not have any identification. The colonies with a score value < 2 (n= 1357) were repeated by "the extraction method" of which 8.77% (n= 119) were additionally identified at species level, 49.45% (n= 671) at genus level, 30.58% (n= 415) at probable genus level and 11.2% (n= 152) did not have again an identification. The analysis of the isolates on the MRS agar plates (n=53) performed only with the "extraction method" resulted in the identification at species level, at secure genus and probable genus of the 9.43% (n=5), 45.28% (n=24), and 45.28% (n=24) of the colonies respectively. Interestingly, the identification outcome of isolates 96 (92 identified as *Pseudomonas*, one as *Acinetobacter*, one as *Microbacterium*, one as *Escherichia* and as *Chryseobacterium*) was different after analysis with the "direct colony identification method" compared to the "extraction method" giving a different identification at species level, but not at genus level.

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 5.3).

Table 5.3. 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		≥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		

Genera (Bacteria)	Species	Log score ≥ 1,7		≥2 Log score <2,3		≥2,3	
		n.	%	n.	%	n.	%
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		
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

Genera (Bacteria)	Species	Log score ≥ 1,7		≥2 Log score <2,3		≥2,3	
		n.	%	n.	%	n.	%
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				

Moreover, *Candida spp.*, *Cryptococcus* spp., *Rhodotorula spp. Trichosporon* spp. and *Yarrowia* spp. belonging to the fungi kingdom were also isolated (Table 5.3.). *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* only in C6H, C5J and C3Y respectively. Among the isolates without MALDI TOF MS identification but analysed with 16S rRNA amplicon 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 (45.51%), *Pseudomonas* was the dominant genus on PCA at 7°C (69.64%) and on CFC (71.71%) and *Escherichia* was the dominant genus on PCA incubated in anaerobic conditions (62.96%), on VRBG (81.48%) and on TBX (100%). On MRS the fungus *Candida* was commonly identified (77.36 %) (Figure 5.3).

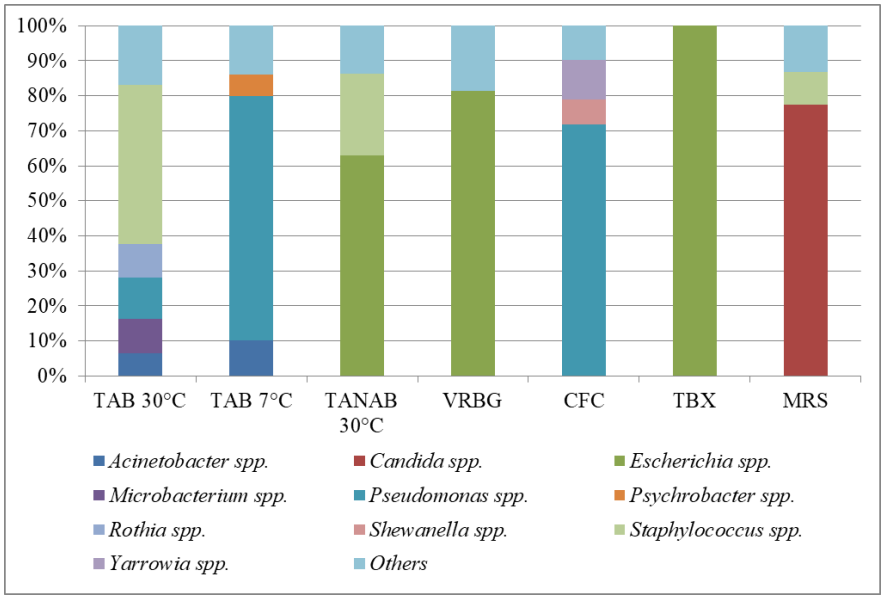


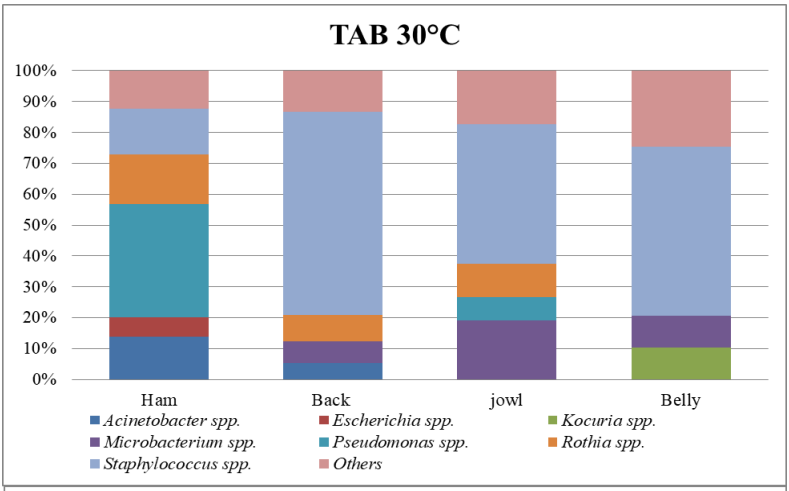
Figure 5.3. Genera identified with a percentage ≥ 5% at genus level by MALDI-TOF MS and 16S amplicon sequencing on the 8 carcasses on different agar plates: Plate count agar (PCA) at 30°C (TAB 30°C), PCA at 7°C (TAB 7°C), PCA under anaerobic conditions (TANAB 30°C), Enterobacteriaceae on VRBG, E.coli on TBX, presumptive Pseudomonas spp. on CFC and Lactic acid bacteria on MRS.

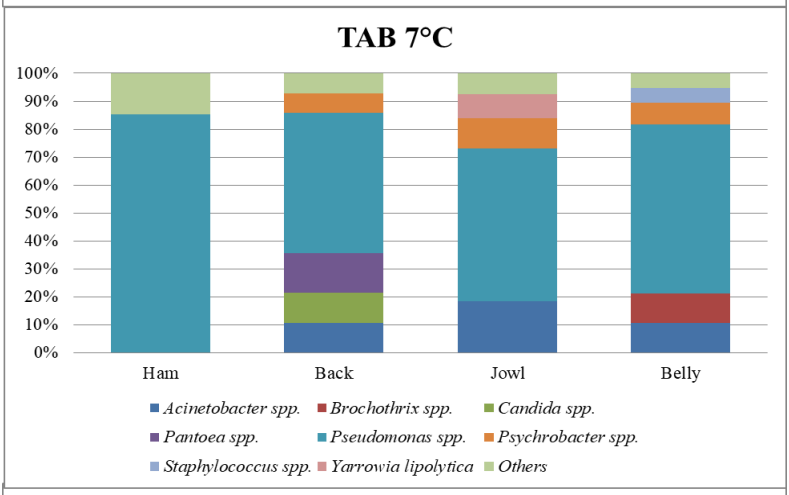
As far as the distribution of the microbial population on the carcasses between the four sampling points is concerned, 27, 21, 22 and 34 bacterial genera were isolated on ham, back, jowl and belly respectively. In particular, *Buttiauxella* and *Yersinia* were isolated only on ham, *Aeromonas* was isolated only on the back, *Janthinobacterium* was isolated only on jowl and, *Brachybacterium*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Lelliottia*, *Luteococcus*, *Ochrobactrum*, *Streptococcus*, and *Vagococcus*,were isolated only on the belly (Table 5.4).

Table 5.4. Bacteria identified at genus level by MALDI-TOF MS and 16S amplicon sequencing on the different sampled areas (Ham (H), Back (B), Jowl (J) and Belly (Y)) of the 8 pork carcasses (C1-C8).

Family	Genus	Ham	Back	Jowl	Belly
Aerococcaceae	*Aerococcus spp.*		x	x	x
Aeromonadaceae	*Aeromonas spp.*		x		
Alcaligenaceae	*Alcaligenes spp.*	x			x
Brevibacteriaceae	*Brevibacterium spp.*	x	x	x	
Brucellaceae	*Ochrobactrum spp.*				x
	Pseudochrobactrum spp.			x	x
Carnobacteriaceae	*Carnobacterium spp.*		x		x
Caulobacteraceae	*Brevundimonas spp.*	x	x	x	
Comamonadaceae	*Delftia spp.*	x			
Corynebacteriaceae	*Corynebacterium spp.*	x	x		x
Dermabacteraceae	*Brachybacterium spp.*				x
Enterobacteriaceae	*Buttiauxella spp.*	x			
	Citrobacter spp.	x			x
	Enterobacter spp.				x
	Escherichia spp.	x	x	x	x
	Klebsiella spp.		x	x	x
	Lelliottia spp.				x
Enterococcaceae	*Enterococcus spp.*	x			x
	Vagococcus spp.				x
Erwiniaceae	*Pantoea spp.*	x	x		x
Flavobacteriaceae	*Chryseobacterium spp.*	x	x		x
Leuconostocaceae	*Leuconostoc spp.*	x	x	x	x
Listeriaceae	*Brochothrix spp.*	x	x		x
Microbacteriaceae	*Leucobacter spp.*	x			x
	Microbacterium spp.	x	x	x	x
Micrococcaceae	*Arthrobacter spp.*	x		x	
	Kocuria spp.	x	x	x	x
	Micrococcus spp.	x		x	x
	Rothia spp.	x	x	x	x
Moraxellaceae	*Acinetobacter spp.*	x	x	x	x
	Moraxella spp.				
	Psychrobacter spp.	x	x	x	x
Oxalobacteraceae	*Janthinobacterium spp.*			x	
Propionibacteriaceae	*Luteococcus spp.*				x
Pseudomonadaceae	*Pseudomonas spp.*	x	x	x	x
Sanguibacteraceae	*Sanguibacter spp.*			x	x
Shewanellaceae	*Shewanella spp.*	x		x	x
Sphingobacteriaceae	*Sphingobacterium spp.*	x	x	x	x
Staphylococcaceae	*Macrococcus spp.*	x	x	x	x
	Staphylococcus spp.	x	x	x	x
Streptococcaceae	*Streptococcus spp.*				x
Xanthomonadaceae	*Stenotrophomonas spp.*			x	x
Yersiniaceae	*Yersinia spp.*	x			

Among the mesophilic population, *Pseudomonas* (36.47%) and *Rothia* (16.24%) were the dominant genera on ham and *Staphylococcus* was the dominant genus on the back (65.91%), on the jowl (45.14%) and on the belly (54.84%). Among the psychrotrophic population *Pseudomonas* was the dominant genus in all four-sampling point (H= 85.42%, B= 50%, J= 54.84% and Y= 60.53%) (Figure 5.4). On PCA incubated anaerobically (TANAB) *Escherichia* was the dominant genus on ham (90.48%) and on the back (94.74%). *Escherichia* (51.31%) and *Staphylococcus* (43.42%) were the dominant genera on jowl, while *Escherichia* (28%), along with the genera *Citrobacter* (24%) and *Enterobacter* (16%) was frequently isolated on the belly (Figure 5.4).


TAB 30°C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ham
Back
jowl
Belly
Acinetobacter spp.
Escherichia spp.
Kocuria spp.
Microbacterium spp.
Pseudomonas spp.
Rothia spp.
Staphylococcus spp.
Others


TAB 7°C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ham
Back
Jowl
Belly
Acinetobacter spp.
Brochothrix spp.
Candida spp.
Pantoea spp.
Pseudomonas spp.
Psychrobacter spp.
Staphylococcus spp.
Yarrowia lipolytica
Others

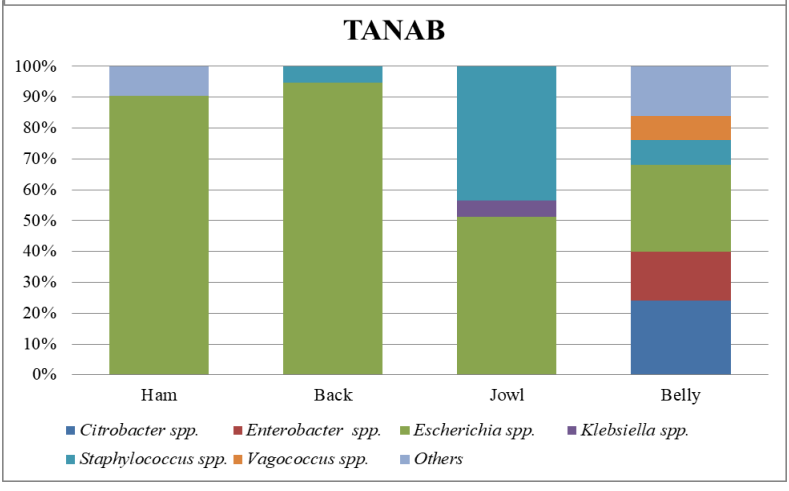

TANAB
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ham
Back
Jowl
Belly
Citrobacter spp.
Enterobacter spp.
Escherichia spp.
Klebsiella spp.
Staphylococcus spp.
Vagococcus spp.
Others

Figure 5.4. Genera identified with a percentage ≥ 5% at genus level by MALDI-TOF MS and 16S amplicon sequencing on the different sampled areas (Ham, Back, Jowl and Belly) of the 8 pork carcasses (C1-C8) on non-selective 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).

As far as the differences between the two slaughterhouses is concerned, 30 and 31 bacterial genera were isolated on slaughterhouse A and slaughterhouse B, respectively. 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* (80.97%) and *Escherichia* (64.84%) 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* (30.55%) along with the genera *Acinetobacter* (19.12%) and *Microbacterium* (16.04%) were the dominant genera on PCA at 30°C. *Pseudomonas* (45.92%) along with *Acinetobacter* (26.53%) and *Psychrobacter* (16.33%) were commonly isolated among the psychrotrophic population and *Escherichia* (55.88%) and *Staphylococcus* (32.36%) were the dominant genera on PCA at 30°C, on PCA at 7°C and on PCA incubated in an anaerobic condition, respectively (Figure 5.5).

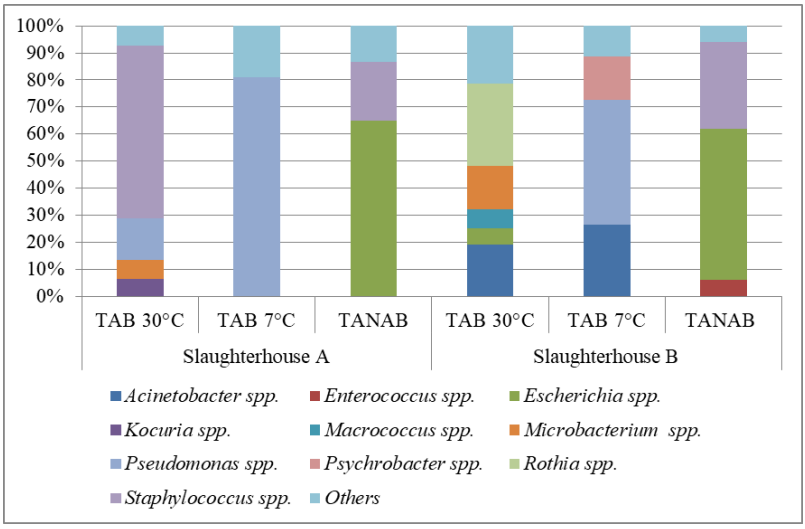


Figure 5.5. Genera identified with a percentage ≥ 5% at genus level by MALDI-TOF MS and 16S amplicon sequencing on the carcasses sampled at slaughterhouse A (C1-C4) and on the carcasses sampled at slaughterhouse B (C5-C8) on the non-selective agar plates: Plate count agar (PCA) at 30°C (TAB 30°C), PCA at 7°C (TAB 7°C), PCA under anaerobic conditions (TANAB 30°C).

5.3.4 Community identification

Using the 16S rRNA amplicon sequencing, a total of 2,451,630 reads were obtained. Only exact amplicon sequence variants (OTUs) accounting for more than 0.5% of the total reads were taken into account for the final results.

Concerning all the samples (C1 to C8) the sequences (>0.5% = 1797210 reads) were clustered into 26 genera encompassing 22 families. However, groups of bacteria, (mean= 12.62%) could only be identified at family or higher taxonomic level. More than 50% of the total microbial community observed was represented by *Brochothrix* (22.83%), *Pseudomonas* (8.36%), *Rothia* (4.32%), *Acinetobacter* (3.81%), *Psychrobacter* (3.58%), *Chryseobacterium* (1.60%), *Shewanella* (1.51%), *Bacteroides* (1.25%), *Corynebacterium* (1.14%), *Gluconacetobacter* (1.11%) *and Paracoccus* (1.07%).

Members of the *Brochothrix* genus, along with the genera *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, and *Rothia*, were the

most abundant genera in each of the different sampling points (ham, back, jowl and belly) (Figure 5.6). However, *Anoxybacillus*, *Pelomonas*, *Ruminococcus* were only detected on the ham; *Facklamia*, *Deinococcus*, *Rahnella*, *Brevibacillus*, *Clostridium*, and *Anaerococcus* were only detected on the back; *Comamonas*, *Moraxella*, *Actinobacillus*, *Sphingobacterium* and *Stenotrophomonas* were only detected on the jowl and *Lachnospira*, *Roseburia* and *Faecalibacterium* were only detected on the belly.

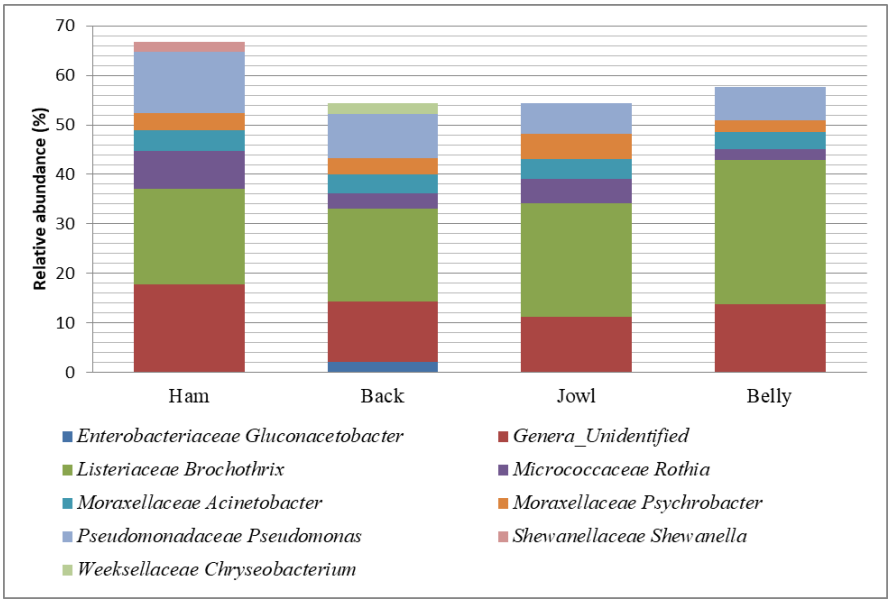


Figure 5.6. Average microbial communities identified by 16S rRNA amplicon sequencing in 8 carcasses (C1-C8) with a relative abundance ≥ 2% on the different sampled areas (Ham, Back, Jowl and Belly).

The microbial community in samples belonging to slaughterhouse A was dominated by *Brochothrix* accounting for 53.27% of the total reads, while no genus or genera clearly dominated the samples belonging to slaughterhouse B (Figure 5.7).

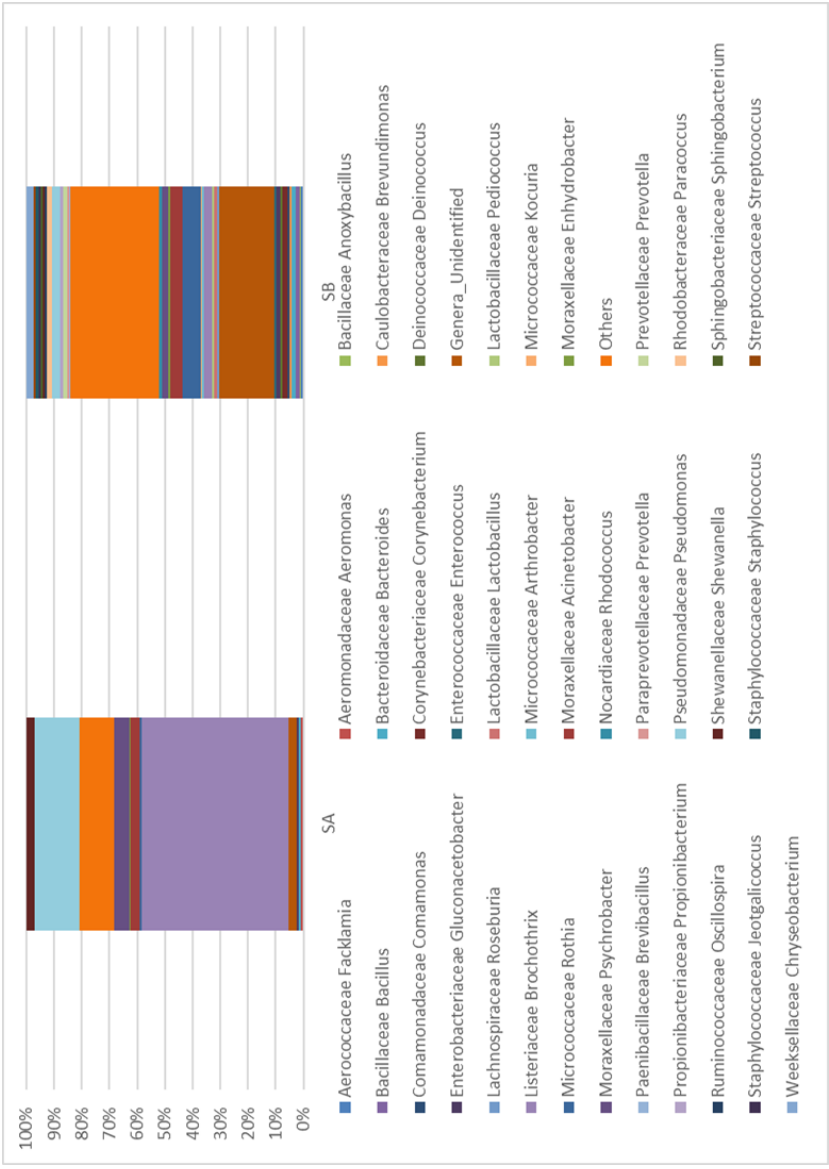


Figure 5.7. Average microbial communities identified by 16S rRNA amplicon sequencing in the 4 carcasses sampled at slaughterhouse A (C1-C4) and in the 4 carcasses sampled at slaughterhouse B (C5-C8). Genera with <0.5% relative abundance were summed up and denoted as others.

5.3.5 Statistical analysis

The viable counts determined in the different agar plates showed no significant difference (p>0.005). The analysis of the data obtained with16S amplicon sequencing showed that the highest richness expressed by Chao1 index was found in C5B (376) and lowest in C2J (68). Ham (mean ± SD = 154.74± 65.37) and back (mean ± SD = 199 ± 105.13) showed the lowest and highest Chao1 indices. Moreover, slaughterhouse A showed a lower (mean ± SD = 111.19 ± 23.87) richness than slaughterhouse B (mean ± SD = 252.19 ± 71.37).

Diversity indices, expressed by evenness (mean ± SD = 0.87% ± 0.06) and Shannon (mean ± SD = 6.45% ± 0.97) indices, indicate that no genera clearly dominate in each samples. In particular, sample C4J (0.69) and C5Y (0.94) showed the lowest and highest evenness indices, respectively. While sample C2J (4.59) and C5Y (7.93) showed the lowest and highest Shannon indices, respectively. In regards to the differences between the four-sampling points, the back showed the highest evenness indices (mean ± SD = 0.89± 0.04). 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. Ham (mean ± SD = 6.22± 0.73) and back (mean ± SD = 6.66± 0.89) showed the lowest and highest Shannon indices, respectively. Slaughterhouse A showed the lowest evenness (mean ± SD = 0.84% ± 0.06) and Shannon (mean ± SD = 5.67% ± 0.54) indices whereas Slaughterhouse B showed the highest ones (evenness (mean ± SD = 0.91% ± 0.03) and Shannon (mean ± SD = 7.24% ± 0.59)). According to Bray-Curtis analysis highly distinct OUT patterns were found among the two slaughterhouses (Figure 5.8).

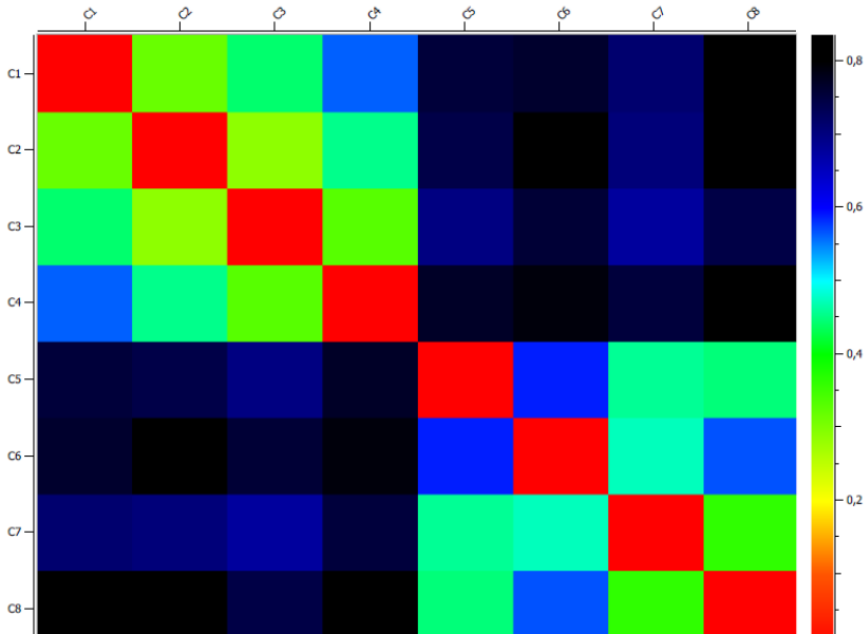


Figure 5.8. Bray-Curtis dissimilarity (1= 100% dissimilar, 0= 0% dissimilar) between the 8 carcasses.

5.4 Discussion

According to the EC Regulation No.1441/07, aerobic colony count (TAB 30°C) is considered, along with the Enterobacteriaceae, to be an indicator of the slaughter process. In the present study, although the counts were slightly higher in slaughterhouse A, no significant differences were observed between the two slaughterhouses ($p > 0.05$). Considering the results of investigated areas pooled to one value for the complete carcass evaluation, the mean of TAB 30°C was within the satisfactory range (< 4.0 log CFU /cm^2) in 7 (87.5%) carcasses and it was unsatisfactory (> 5.0 log CFU /cm^2) in one (12.5%) carcass (C2,mean±SD = 5.32± 0.05 log CFU /cm^2). The Enterobacteriaceae count on VRBG agar plates was within the satisfactory range (m < 2.0 log CFU /cm^2) in 6 (75%) carcasses, within the acceptable range (m < 2.0 log CFU /cm^2 and M< 3.0 log CFU /cm^2) in 1 (12.5%) carcass (C1, mean±SD =2.63±0.60log CFU /cm^2) 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 results of the aerobic colony count are in accordance with Lindblad (2007) (mean±SD = 3.48±0.67 log log CFU/ cm^2) but they are higher compared to the results of Mrdovic et al., (2013) where the mean (±SD) of the count was 1.99±1.01 log CFU /cm^{-1}and 1.86±1.10 log CFU /cm^{-1} in the carcasses sampled in a large and small scale abattoir, respectively. Moreover, the carcasses sampled in the present study showed a high level of fecal contamination and, indeed, the mean count of Enterobacteriaceaeon VRBG and *E. coli* on TBX was higher than both of the results of the previously cited studies (Lindblad, 2007; Mrdovic et al., 2013). Moreover, more than 81.48% and more than 60% of the bacteria grown on VRBG and PCA incubated under anaerobic conditions, respectively, belonged to *E. coli*. In fact, statistical analysis showed that the counts on VRBG, on TBX and on PCA incubated under anaerobic conditions were not significantly different ($p>0.05$). Contrarily to these results, a low level of bacteria on PCA incubated at 7°C and *Pseudomonas* spp. on CFC were recorded, probably because the carcasses were sampled before the cooling process and the probable growth of psychrotrophic bacteria had not started yet. Indeed, these latter bacteria take over the mesophilic population even after only few days (4/5 days) of storage of the meat at refrigerated temperatures (psychrotrophic bacteria: mean±SD = 7.28 ± 0.5 log

CFU/g; *Pseudomonas* spp. on CFC: mean±SD = 7.28 ± 0.66 log CFU/g) (Peruzy et al., 2018).

Contrarily to the study of Biasino et al., (2018) the different carcass areas no showed diversity in the contamination level; however, the number of the carcasses examined in the present study was much lower. The back site was the least contaminated with TAB 7°C, *Pseudomonas* spp., LAB and as already reported by Biasino et al., (2018), 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., 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.

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 the carcasses is performed in order to evaluate the hygienic status of the slaughterhouse. This means that even when the carcasses are *Salmonella*-positive they can be placed on the market. High levels of *Salmonella* were also reported in Belgium by Biasino et al., (2018) but low Salmonella prevalence was observed in other older studies in Belgium (Botteldoorn et al., 2003; Ghafir et al., 2008), in Serbia (Choi et al., 2013) and in Korea (Mrdovic et al., 2013). After serotyping, Monophasic *S.* Typhimurium, *S.* Derby, and *S.* Brandenburg were identified in three different couples of carcasses. These serotypes, along with *S.* Rissenare commonly associated with pigs and pork meat (EFSA,2016). Furthermore, monophasic *S.* Typhimuriumand *S.* Derby are the third and fifth most commonly reported *Salmonella* serovars in human cases acquired in Europe (EFSA, 2017). In regards to *Y. enterocolitica* the gene *ystB*, used as a target for the biotype 1A, was detected once on the ham; low levels of contamination were already reported previously in the study of Choi et al., (2013) whereas in none of the selected sampled areas (rump,

midline and brisket) was this pathogen isolated. However, in a study conducted by 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.

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. The results of the present study illustrate that this way of working (first direct then 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 did not have an identification. However, MALDI TOF MS cannot be used anymore 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. Moreover, after 16S rRNA amplicon sequencing, all the genera identified except for *Sanguibacter* were present 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 bacterial communities isolated from the 8 carcasses belonged to *Staphylococcus*, *Pseudomonas*, and *E. coli*. 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 species of Staphylococci

reported in the present study (e.g. *S. epidermidis, S. hominis)* are not indigenous bacterial contaminant of animals and the their presence on the skin of the pork carcasses can be associated with 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 (Singh, 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 the culture-independent method (16S amplicon sequencing), the diversity indices showed that no genus clearly dominated the 8 carcasses. However, *Brochothrix* another well-known spoilage bacterium, was the most abundant one, immediately followed by *Pseudomonas.* In contrast with this latter genus, *Brochothrix* was not frequently isolated from the agar plates. *Rothia,* common inhabitants of the human oral bacterial population and the respiratory cavity (Abidi et al., 2016), and *Acinetobacter*, described on healthy human skin (Rouger et al., 2017), were respectively the third and fourth most frequently detected genera. These genera were also isolated in the present study with the culture-dependent methods. Present species can be responsible for human illness, however, and the pathogenicity of isolates of these bacteria obtained from products of animal origin has to be investigated (Abidi et al., 2016; Máximo et al., 2017). Moreover, *Acinetobacter*, along with *Chryseobacterium*, also detected and isolated in the present study, is a potential spoilage bacterium for its production in vitro of lipases and proteases enzymes (spoilage enzymes) (Yuan et al., 2018). Within the microbial community of the carcasses *Psychrobacter* already reported in different foods (Lasa and Romalde, 2017), *Shewanella* of which some species can be associated with food spoilage (Rouger et al., 2017) and *Corynebacterium* a putative zoonotic bacteria (Burkovski, 2015) have also been both detected and isolated in the present work. Interestingly, *Staphylococcus*, which, as described above, was frequently isolated from the agar plates, was detected at a very low level (0.68%) with 16S amplicon sequencing.

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 the culture-independent method (16S amplicon sequencing), Slaughterhouse A showed the lowest diversity index and, indeed, the microbial population on the carcasses sampled in this slaughterhouse was dominated by *Brochothrix*. On the contrary, Slaughterhouse B, with the highest Evenness and Shannon indices, did not exhibit a clear cut dominant genus. This difference was also shown when analyzing the bacterial population isolated on the different agar plates. The microbial population on the non-selective medium (PCA) incubated at 7°, 30°C and under anaerobic conditions, isolated from the carcasses C1-C4 was dominated by one different genus (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 Figure 5.8 which shows, through the Bray-Curtis analysis, that the dissimilarity among the samples belonging to the same group is low, whereas it is high between the carcasses belonging to the different slaughterhouses.

In conclusion, in the small slaughterhouses studied the bacterial community of each carcass depends mainly on the microbial population of the slaughterhouse to which it belongs rather than on the indigenous flora of animals. 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 more than one area for the evaluation of the hygienic status of the carcasses by official authorities may be useless.

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6. Chapter 4

Assessment of the microbial contamination on wild boar meat by MALDI-TOF MS and 16S rRNA amplicon sequencing

Abstract

Wild boars (*Sus scrofa*) are the most widely distributed large mammals, and recent increase in consumption urges the need of microbiological quality criteria. Aim of the study was to characterize the bacterial contamination on wild boar meat by a culture-dependent approach using 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 by 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 genera frequently isolated 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 underpins that attention should be paid to the trade of non-inspected meat directly to retail or consumers.

Keywords: Wild boar meat, MALDI-TOF MS, 16S rRNA amplicon sequencing, *Salmonella* spp.

6.1 Introduction

Wild boar (*Sus scrofa*) is currently considered one of the most destructive and invasive mammal species in the world (Lowe et al., 2000). It is present on all continents, except for Antarctica (Sales et al., 2017), and thrives 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 harvested in 18 European countries (Massei et al., 2015). However, the number of free-ranging 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 coli* and *Campylobacter* (Keuling et al., 2018; Ruiz-Fons, 2017). Main causes of natural mortality are starvation due to extreme weather conditions, diseases and predation by wolfs, 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).

Meat of wild hogs eaten only by hunters and their families, does not require any inspection what so ever. 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 this within a reasonable time. Moreover, 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, behavior, 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 local retail establishments does not require official post-mortem inspection.

Nowadays, wild boar meat is considered as healthy and delicious food, available throughout the year (Atanassova et al., 2008; Naya

et al., 2003). 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 an 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 on 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 bacteria 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 as 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 on 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 (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 to study microbial populations in food (Lagier et al., 2016; Peruzy et al., 2018). 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 expand current knowledge on the microbial contamination on hunted wild boar meat by applying both a culture-dependent and -independent approach.

6.2 Materials and Methods

6.2.1 Sampling

From October to December 2017, meat samples of 22 wild boars (W1 to W22), 10 males and 12 females, age between 1 and 6 years, were collected in the province Campania, southern Italy (Table 6.1). 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.

6.2.2 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 three minutes 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 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 30-300 CFU/plate on PCA, and between 15-150 CFU/plate from all others. All harvested colonies were subcultured on Tryptic Soy Agar (TSA, CM0131, Oxoid) or MRS, and incubated at the appropriate conditions as described above.

For the detection of relevant foodborne pathogens, 25 gram 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 monocytogenes*, and once in 225 ml Peptone Sorbitol Bile Broth (PSB,17192, Sigma-Aldrich) for the detection

of *Yersinia enterocolitica*. The "iQ-Check Real-Time PCR Kits" were applied for the detection of *Salmonella* (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 at 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), 0,025 µl each of primers ystA-F (5'-ATCGACACCAATAACCGCTGAG-3') and ystA-R (5'-CCAATCACTACTGACTTCGGCT-3') and 9,45 µl of H_2O. 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), 0,0375 µl each of primers ystB-F (5'-GTACATTAGGCCAAGAGACG-3') and ystB-R (5'-GCAACATACCTCACAACACC-3'), and 9,425 µl of H_2O. 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. While awaiting the qPCR results, the enrichment broths were stored at 4°C. qPCR 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 (Popoff and Le Minor,1992).

6.2.3 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 less than 2,0 was obtained with the "direct colony method", and all isolates from the MRS agar plates (Alatoom et al., 2011) were analyzed 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 (1,533 g at 4°C) for 10 min, the supernatants discarded, and pellets washed twice in 500 µl of Milli-Q water and centrifuged (1,533 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 formic acid and 50 µl acetonitrile were added and thoroughly mixed by pipetting, followed by centrifugation (1,533g at 4°C) for 10 min. One microliter of each sample was spotted onto 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 amplicon sequencing. DNA was extracted using alkaline lyses where one colony was suspended into 20 µl of lysis buffer (2,5ml 10%SDS, 5ml 1N NaOH and 92,5ml Milli-Q water) and heated for 15 min at 95°C. After a short spin, 180 µl Milli-Q water was added. Subsequently, the suspension was 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 25µl) 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 16,5µl 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).

6.2.4 Culture independent community profiling by 16S rRNA amplicon sequencing

To culture independently identify microorganisms present on 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 quantitywas 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 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 taxausing Naive Bayes classifiers pre-trained on Greengenes 13_8 99% OTUs full-length sequences. (https://docs.qiime2.org/2017.12/data-resources/)

6.2.5 Statistical analysis

To compare the bacterial counts one-way analysis of variance (ANOVA) was calculated by 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 less than 0.05 ($p<0.05$) was defined as statistically significant.

6.3 Results

6.3.1 General bacterial indicators

Counts of the different bacterial parameters are shown in Table 6.1. Total aerobic bacteria determined at 30°C ranged from 3,26 to 6,95 log CFU/g (mean±SD = 5,5 ± 1,02 log CFU/g), and from 1,96 to 6,69 log CFU/g (mean±SD = 4,43 ± 1,19 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±SD = 4,76 ± 0,85 log CFU/g), and the mean number of presumptive *Pseudomonas* spp. and lactic acid bacteria were 3,82 ± 1,36 and 3,65 ± 1,45 log CFU/g, respectively. The count of typical blue *E. coli* colonies on TBX and the purple/pink Enterobacteriaceae colonies on VRBG showed a mean of 2,81 ± 0,97 and 4,12 ± 1,32 log CFU/g, respectively. No significant correlation was observed between the bacterial count and the gender, age and weight of the animals.

Table 6.1. Gender, Weight (Kg) and Age (years) of the shooted animals and 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 (TANA 30°C), E.coli on TBX , Enterobacteriaceae on VRBG, presumptive Pseudomonas spp. on CFC and Lactic Acid Bacteria (LAB) on MRS.

	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)*	*Enterobacteriaceae* spp. (log CFU/g)	*Pseudomonas* spp. (log CFU/g)	LAB (log CFU/g)
W1	M	40	1	4,69	4,69	5,10	2,46	3,49	4,66	3,93
W2	M	25	1	6,05	3,76	4,24	1,96	2,57	3,56	0,00
W3	M	70	3	6,01	5,12	5,75	4,56	5,34	3,82	4,64
W4	F	60	2	3,61	2,56	4,46	3,67	2,86	0,00	2,66
W5	M	60	2	3,26	1,96	3,63	2,13	2,36	2,56	3,62
W6	F	30	1	5,55	3,12	5,36	4,26	5,44	2,80	3,41
W7	F	108	5	6,72	6,69	5,98	2,10	5,67	6,62	5,28
W8	M	125	5	5,44	4,75	4,37	2,11	3,92	2,59	2,66
W9	M	70	3	5,59	3,88	4,76	4,36	4,61	4,37	3,55
W10	F	60	2	6,03	5,54	4,67	2,41	4,69	5,16	3,07
W11	F	60	3	4,32	4,80	5,28	2,96	4,45	3,89	4,37
W12	F	40	1	6,51	5,34	5,72	3,49	5,57	4,88	5,61
W13	M	60	3	5,50	4,56	5,46	4,44	4,86	3,66	4,98
W14	F	65	4	4,08	3,06	3,03	2,96	3,41	3,04	3,36
W15	M	125	6	5,51	5,36	4,87	2,30	4,62	4,39	4,76
W16	F	30	1	5,74	5,05	5,34	2,21	4,93	3,75	5,28
W17	F	30	1	6,95	4,99	3,44	2,26	4,48	4,69	3,62
W18	F	53	2	5,73	3,37	5,10	1,66	0,00	2,28	0,00
W19	M	70	3	6,65	3,74	5,71	2,96	4,98	4,24	4,00
W20	F	40	1	5,85	4,04	3,88	1,26	3,64	3,26	3,04
W21	F	40	1	4,66	4,55	3,44	1,91	3,82	4,13	4,26
W22	M	40	1	6,67	6,58	5,21	3,47	5,05	5,74	4,14

6.3.2 Presence of foodborne bacteria

Salmonella was detected in 7 out 22 samples, and all, except sample W19, were confirmed using the reference isolation method. Three serovars were identified (*monophasic S.* Typhimurium, *S.* Stanleyville and *S.* Kasenyi). Concerning *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.

6.3.3 Community profiling using isolation and MALDI-TOF MS identification

A total of 3,789 bacterial isolates were analyzed by MALDI-TOF MS, 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 3,457 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, 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 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. Interesting, 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. No identification was obtained for 55 isolates (16,57%).

Isolates without a reliable MALDI-TOF MS identification were analysed using 16S rRNA amplicon 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 distribution of the dominant genera between the 22 samples on the different isolation media is shown in Figure 6.1. 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 50% (or more) of the samples. 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, 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 (Figure 6.1).

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 amplicon sequencing, but except for the genus *Shewanella*, all of them were oxidase negative (Figure 6.1). 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 family Enterobacteriaceae, 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%).

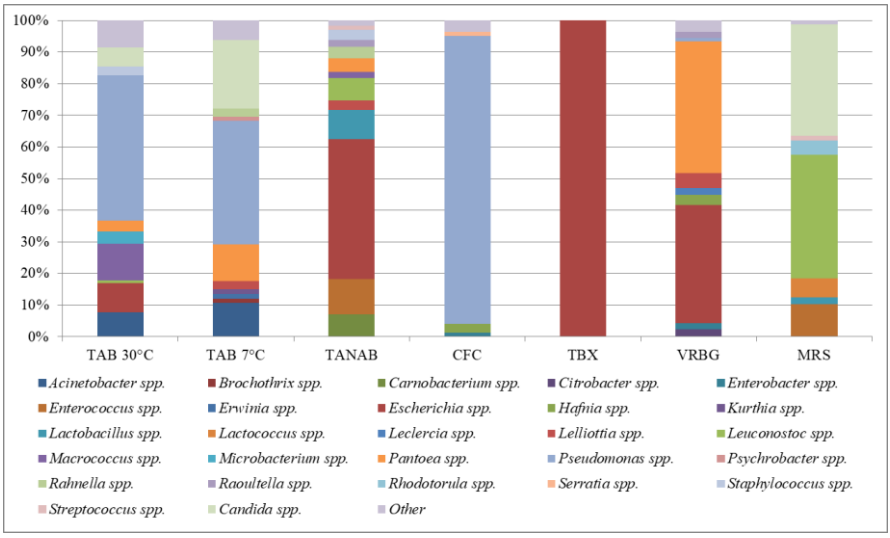

Figure 6.1 Genera identified with a percentage ≥1% by MALDI-TOF and 16S gene sequencing on different agars: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), anaerobic bacteria (TANAB), E.coli on TBX , Enterobacterales on VRBG, presumptive Pseudomonas spp. on CFC and Lactic Acid Bacteria (LAB) on MRS. Genera identified with a percentage <1% are denoted as other.

6.3.4 Culture-independent community analysis

Using 16S rRNA amplicon sequencing, a total of 1,209,441 reads were obtained. After filtering, 535,513 reads were taken into account as bacterial reads for further analysis. Only exact amplicon sequence variants (OTUs) accounting for more than 0,5% of the total reads were further retained. These sequences were clustered into 97 genera encompassing 58 families (Figure 6.2). However, groups of bacteria, from 8,45% (sample W22) to 42,52 (sample W16), were identified only at family level. On average, only 30,20 % of sequences could be attributed to a higher taxonomic level. The relative abundance of each OTU varied among the 22 samples (Figure 6.2).

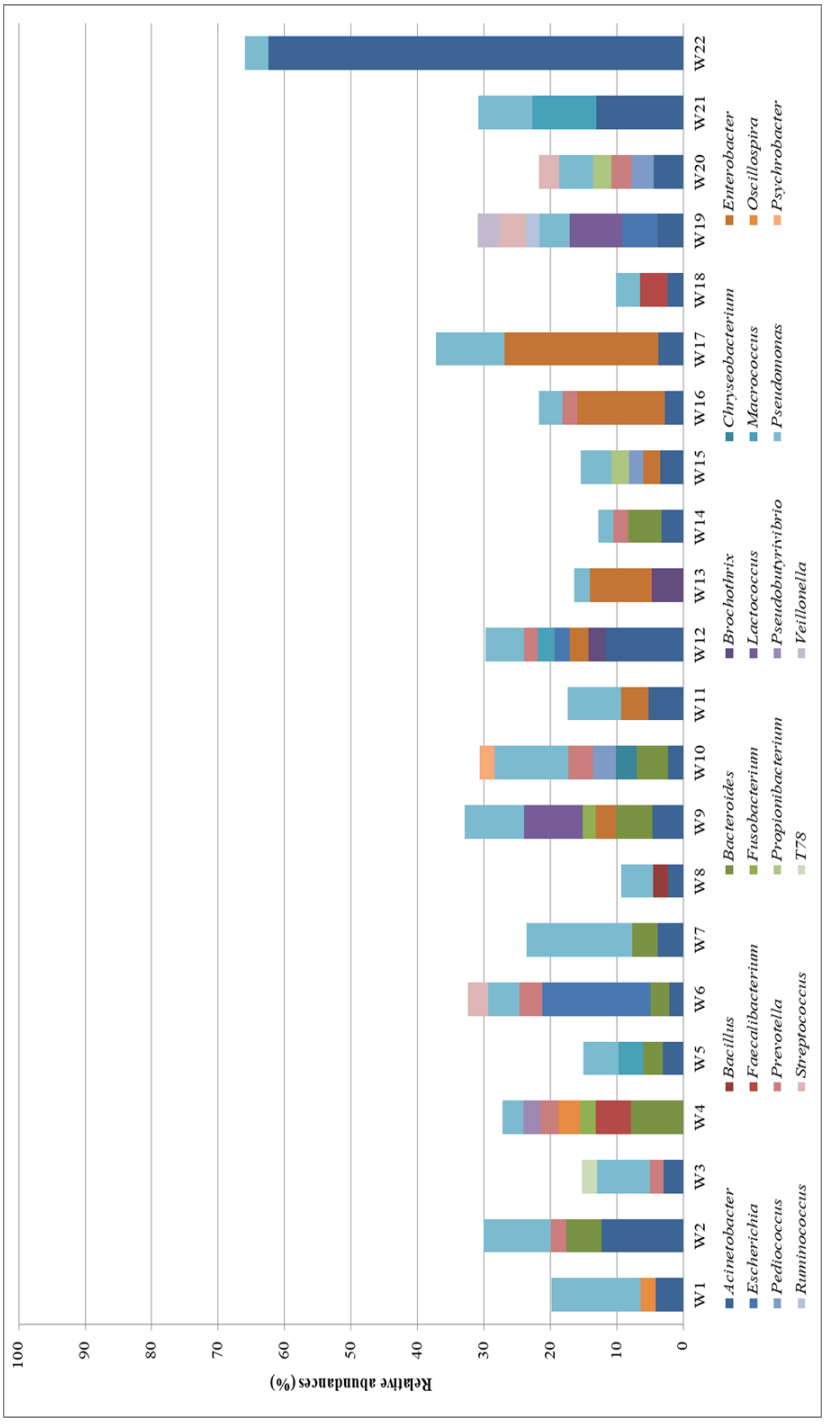
Relative abundances (%)
0
10
20
30
40
50
60
70
80
90
100
W1
W2
W3
W4
W5
W6
W7
W8
W9
W10
W11
W12
W13
W14
W15
W16
W17
W18
W19
W20
W21
W22
Acinetobacter
Escherichia
Pediococcus
Ruminococcus
Bacillus
Faecalibacterium
Prevotella
Streptococcus
Bacteroides
Fusobacterium
Propionibacterium
T78
Brochothrix
Lactococcus
Pseudobutyrivibrio
Veillonella
Chryseobacterium
Macrococcus
Pseudomonas
Enterobacter
Oscillospira
Psychrobacter

Figure 6.2. Microbial communities identified by 16S rRNA amplicon sequencing in wild boar meat samples. Relative abundances of each OTUs (genera with ≥2% relative abundance).

Richness expressed by the Chao1 index showed sample W10 as that with the lowest richness and sample W5 as the richest samples. The equitability (evenness) index showed that no genera clearly dominate (Table 6.2) 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* in 86,36%, *Enterobacter* in 77,28%, *Faecalibacterium* and *Oscillospira* both at 72,73%, *Bacteroides* and *Prevotella* in 68,18%, *Brochothrix* and *Streptococcus* in 59,09% and *Fusobacterium* and an uncharacterized member of the Dethiosulfovibrionaceae family in 50% of the samples.

Table 6.2. Richness (expressed by Chao1 index) and diversity (expressed by Shannon and Evenness indexes) of the bacterial communities identified by 16 S amplicon sequencing.

Sample	Chao-1	Shannon	Evenness
W1	239	7,42	0,94
W2	247	7,57	0,95
W3	199	7,11	0,93
W4	342	7,27	0,86
W5	348	8,03	0,95
W6	212	6,71	0,87
W7	285	7,74	0,95
W8	228	5,74	0,73
W9	146	6,57	0,91
W10	115	6,26	0,91
W11	286	7,53	0,92
W12	302	7,69	0,93
W13	231	6,89	0,88
W14	282	6,77	0,83
W15	360	8,09	0,95
W16	270	6,83	0,85
W17	299	6,67	0,81
W18	254	6,22	0,78
W19	189	6,95	0,92
W20	134	6,69	0,95
W21	250	7,45	0,94
W22	186	4,86	0,64

Comparing both analysis approaches, among the 22 samples, *Pseudomonas* was always detected and isolated. However, it resulted a dominant genus with culture-dependent approach but not with culture-independent one. *Acinetobacter* was detected and isolated in 81,82% of the samples, and only detected in 4 (W9, W16, W18 and W21) and 70 genera were only detected using 16S amplicon sequencing (Table 6.3). In contrast, 28 genera were only isolated and not detected (Table 6.3). Even when OTUs with less than 0,5% of total reads were taken into account, only 6 genera (*Arthrobacter, Erwinia, Exiguobacterium, Leuconostoc, Serratia and Weissella*) could be additionally obtained. .

Table 6.3. Genera only detected using 16S amplicon sequencing (Culture independent method) and genera only and identified with MALDI TOF-MS and 16S amplicon sequencing (Culture dependent methods).

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

6.4 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 in 15 and 18 wild boar meat samples higher than the EU microbiological criteria for pork (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 Enterobacteriaceae, 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, wild hogs acting as a as carrier of *Salmonella* has 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.* Stanleyvilleand *S.* Kasenyi. *monophasic S.* Typhimuriumrepresents one of the five most reported serovars in human salmonellosis (EFSA, 2017). Serovar *Salmonella* Kasenyihas not been reported from wild boar meat yet.

Pigs are considered as 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 none-pathogenic *Y. enterocolitica* strains were recovered (Gill (2007), Avagnina et al., (2012)). Also the absence of *L. monocytogenes* accords 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, even not at species level.

Combining the two methods, 89,93% of the isolates were identified at genus level of which 23,11% 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 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 and further analysed with 16S amplicon sequencing, three genera were not present yet 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 the culture-independent method (16S amplicon sequencing), no genera clearly dominated the bacterial contamination on wild boar meat.

Pseudomonas spp. and *Acinetobacter* spp.were detected in all samples, and are indicative for a contamination by soil or water (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 population and cause 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*, only isolated from one sample, is more associated with dairy products, silages and vegetables, ruminal content and faeces, human stool, 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 was incubated aerobically and without the addition of inhibiting antibiotics (Simpson et al., 2006).

In almost 70% of the wild boar meat samples, one specific genus belonging to the family of Enterobacteriaceae (*Enterobacter*) and 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). *Enterobacter*, commonly present in humans and animals, has also been found in water, plants and plant materials, insects, and dairy product (Eugene Sanders and Sanders, 1997).

Two genera belonging to the order of *Bacteroidale* were detected in 15 samples applying 16S amplicon sequencing: the genus *Bacteroide*s belonging to the family of Bacteroidaceae and the genus *Prevotella* belonging to Prevotellaceae. Both were not retrieved by cultivation. *Bacteroides* has been detected in a the faeces of a variety food animals (Young Ko et al., 2018), and *Prevotella* has been found in the gut microbiota of ruminants and chimpanzees (Holman et al., 2017). *Brochothrix* and *Streptococcus*, two Gram-positive bacteria were detected in almost 60% of the samples. The species *Brochothrix thermosphacta*, known as common meat contaminant, is considered one of the major cause of spoilage (Rouger et al., 2017). However, *Brochothrix* was isolated only in three samples, in particular on PCA plates incubated at 7°C. *Streptococcus* was isolated from four samples on PCA incubated at 7°C, but also in anaerobic condition and MRS incubated aerobically. This genus contains several species that are part of the normal microbial population of human and animals but some are also pathogenic (Lu et al., 2014; Lun et al., 2007).

Bacteroides, Faecalibacterium, Fusobacterium, Oscillospira and *Prevotella,* 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 results between culturomics and 16S amplicon sequencing occurred for the genera *Escherichia* and *Pantoea,* belonging to the family of Enterobacteriaceae. *Escherichia* was isolated from all samples and identified with MALDI-TOF MS, but only detected in 9 samples with 16S amplicon sequencing. *Pantoea,* frequently isolated was however never detected. Also *Salmonella,* detected by qPCR and confirmed by isolation, was not detected using 16S amplicon sequencing applied on the first meat homogenate. This has been previously reported, and explained by the fact 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).

In conclusion, in the present study, the bacterial contamination on wild boar meat was studied, and showed to be of great public health

risk and the non-inspected distribution of meat directly to consumers is not recommended. Although often claimed, culture-independent analysis showed to be no reliable alternative, as it fails to detect the foodborne pathogen *Salmonella*. Only in the study of the bacterial communities, combination of both approaches might be relevant.

6.5 References

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7. General discussion

7. General discussion

Currently, official methods for the evaluation of process hygiene criteria in the pork supply chain are only based on quantitative counts of hygiene-associated microbes (e.g. mesophilic bacteria, Enterobacteriaceae, E.coli) and since no specific microbiological criteria for the meat coming from hunting animals exists, these criteria are also often applied to evaluate the hygienic status of wild boar meat.

On starting this thesis information about the microbial diversity present on the counted plates was lacking. During the dissertation qualitative data were gathered using Matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometry (MALDI-TOF MS) an emerging technology for microbial identification and, 16S gene sequencing. Moreover, this approach based on the cultivation of bacteria on different agar plates was compared with 16S rRNA amplicon sequencing (culture independent method), commonly used to study bacterial communities in different ecosystems. Special attention was also paid to different sample preparations for the MALDI-TOF MS analysis and to different extraction protocols for the 16S rRNA amplicon sequencing (chapter 2).

Moreover, in order to gain data on the presence of *Yersinia enterocolitica* in pork and wild boar meats, a study on the distribution of virulence genes among *Y. enterocolitica* strains was performed (chapter 1).

7.1 Culture dependent methods

Recently, MALDI TOF MS has revolutionized the identification of bacteria in clinical microbiology (Singhal et al., 2015). In this thesis, the applicability of MALDI-TOF technology in food microbiology was evaluated. First, in chapter 2, the impact of three different sample preparations (direct colony identification, bacterial suspension and extraction method) was studied to establish a reliable method for the routine identification of cultured bacteria. Bacterial suspension, based on the analysis of the "whole cells", resulted in the lowest level of identification compared to the other two methods, while as also previously reported by Alatoom et al., (2011) for Gram +, bacteria extraction was clearly the superior method. However, when many isolates are to be examined, this method requires excessively long processing times. Definitively, the choice of examining all the isolates first with direct colony identification, which is an efficient screening tool in terms of time and cost, and then with the extraction method on a selection, results in being the best way to analyze a large number of colonies. This approach was, for the aforementioned reasons, chosen for the analysis of the isolates from pork carcasses (chapter 3) and from wilds boar meat (chapter 4). However, only ≈20% of isolates received identification at species level and moreover, identification with MALDI TOF MS of *Pseudomonas* species, as with the conventional phenotypic test and 16S gene sequencing remains questionable (Mulet et al., 2012). Furthermore, 11.2%, 17.54% and 23.14% of the colonies isolated respectively from pork carcasses, minced pork and wild boar meats did not receive any identification. The spectra of these isolates were imported into BioNumerics 7.2.6 software to first cluster and then visually select representatives for subsequent 16S amplicon sequencing. The latter still considered as the "gold standard" method for prokaryotes for the identification of sample isolate. This method is expensive and not suitable for routine identification; however, it allowed the identification of all

remaining colonies on the plates. Among the isolates without a reliable MALDI TOF MS identification and further analysed with 16S amplicon sequencing, four genera turned out not to not be present yet in the Bruker database (*Frigoribacterium*, *Gibbsiella*, *Okibacterium*, and *Sanguibacter*) though others (n=35) were already included (Table 7.1).

Table 7.1. Number and percentage of isolates in minced meat (Chapter 2), carcasses (Chapter 3) and in wild boar meat (Chapter 4) identified at genus level through 16S amplicon sequencing of the representative isolates, visually selected after cluster analysis with BioNumerics 7.2.6 software.

Minced Meat (Chapter 2)			Carcasses (Chapter 3)			Wild Boar Meat (Chapter 4)		
Genera	N.	%	Genera	N.	%	Genera	N.	%
Carnobacterium	46	32,17	*Psychrobacter*	12	24	*Pseudomonas*	25	21.19
Brochothrix	23	0,67	*Acinetobacter*	10	20	*Macrococcus*	19	16.10
Leuconostoc	19	0,56	*Pseudomonas*	9	18	*Acinetobacter*	11	9,32
Lactococcus	18	12,59	*Microbacterium*	4	8	*Pantoea*	11	9,32
Psychrobacter	15	10,49	*Brachybacterium*	2	4	*Microbacterium*	6	5,08
Lactobacillus	8	5,59	*Leucobacter*	2	4	*Sphingobacterium*	6	5,08
Pseudomonas	6	4,20	*Rothia*	2	4	*Erwinia*	5	4,24
Acinetobacter	3	2,10	*Sanguibacter*	2	4	*Enterobacter*	4	3,39
Kocuria	2	1,40	*Sphingobacterium*	2	4	*Klebsiella*	3	2,54
Brevundimonas	1	0,70	*Arthrobacter*	1	2	*Micrococcus*	3	2,54
Rahnella	1	0,70	*Brevibacterium*	1	2	*Psychrobacter*	3	2,54
Serratia	1	0,70	*Brevundimonas*	1	2	*Arthrobacter*	2	1,69
			Kocuria	1	2	*Chryseobacterium*	2	1,69
			Shewanella	1	2	*Curtobacterium*	2	1,69
						Enterococcus	2	1,69
						Stenotrophomonas	2	1,69
						Acidovorax	1	0,85
						Deinococcus	1	0,85
						Exiguobacterium	1	0,85
						Frigoribacterium	1	0,85
						Gibbsiella	1	0,85
						Janthinobacterium	1	0,85
						Lactococcus	1	0,85
						Leucobacter	1	0,85
						Leuconostoc	1	0,85
						Okibacterium	1	0,85
						Rothia	1	0,85
						Staphylococcus	1	0,85

An explanation could be that within same species, protein expression can vary between different strainsdue to media and growth conditions and, in the MALDI TOF MS database the number of the spectra for each species present is higher for the common bacteria compared to those less frequently isolated. Moreover, some bacteria, such as species of the *Rothia* genus, produce tiny or mucoid colonies that may lead to an insufficient amount of protein necessary for a good spectrum (Alatoom et al., 2011). At present MALDI TOF MS, due to the relative high number of non-identified bacteria and low number of bacteria reliably identified at species level, is not sufficient to study cultivable microbial diversity in a complex matrix. However, it will be a promising technique when the database becomes more extensive, or if own library are constructed.

7.2 16S amplicon sequencing

In recent years, culture independent methods have been used to study the microbial communities in different ecosystems (Cocolin et al., 2013, 2004). In this thesis, 16S amplicon sequencing technology was applied to study the microbial population in minced meat (chapter 2), on pork carcasses (chapter 3) and wild boar meat (chapter 4). It has already been previously reported that DNA extraction is a crucial step for the outcome of molecular-based analysis in faecal samples (Costea et al., 2017). In chapter 2, the impact of two different DNA extraction methods (FastDNA® SPIN Kit for Soil (MP Biomedicals) and PowerFood Microbial DNA Isolation kit (Qiagen, Germany)) and three sample preparations were evaluated. With the PowerFood Microbial DNA Isolation kit, already reported by Quigley et al., (2012) as a suitable kit for DNA extraction of bacteria in raw milk and cheese highest number of genera were detected. Therefore, it was subsequently chosen for the extraction of DNA in the pork carcasses and wild boar meats studies.

The most abundant genera present in minced meat (*Photobacterium*, *Pseudomonas*, and *Brochotrix*) were always detected, regardless of the extraction kit and/or the protocol used. However, according to the protocol used, differences in the results were observed with the less present bacteria.

Using 16S rRNA amplicon sequencing, a total of 2,588,365, 2,451,630 and 1,209,441 reads were obtained from minced, pork and wild boar meat, respectively. After assigning taxonomic names to microbial sequences, in all samples groups of bacteria (minced meat = 13.37%; pork carcass = 12.62%; wild boar meat = 30.20%) could only be identified at family or higher taxonomic level. In general, the short reads obtained from 16S amplicon sequencing protocols, especially with the Illumina platform used in the present thesis, led to an identification only at genus level. Moreover, the accuracy of the analyses depends heavily on the choice of primers and on the choice of the target region of the 16S gene (Cao et al., 2017; Klindworth et al., 2013).

7.3 Identification upon culturing (MALDI TOF MS and/or 16S amplicon sequencing) versus culture independent method (16S rRNA amplicon sequencing)

Comparing the genera identified by MALDI TOF MS and/or 16S amplicon sequencing to the ones detected with the culture-independent method (16S amplicon sequencing), discordant results have been obtained. In chapter 2 and 4, the genera dominating the samples were the same both with culture dependent and with culture independent methods (minced meat= *Brochothrix* and *Pseudomonas*; wild boar meat = *Pseudomonas* and *Acinetobacter*). In chapter 3, the dominant bacterial communities isolated from the 8 carcasses belonged to *Staphylococcus*, *Pseudomonas*, and *E. coli* while *Brochothrix*, not often isolated from the agar plates,was the genus most frequently detected with 16S amplicon sequencing.

Moreover, except for the most abundant bacteria, the genera obtained from the two methods were not always the same. As expected, the number of genera detected with 16S amplicon sequencing was higher than the ones isolated. Nevertheless not all bacteria present on the agar plates and subsequently identified by MALDI TOF and/or 16S gene sequencing were detected with the culture independent method. In the three studies, 4, 24 and 28 genera were identified only with culture dependent methods from minced meat, pork carcass and wild boar, respectively.

In the past few years microbial culture techniques have been neglected in favour of metagenomics (Lagier et al., 2016). However, in the present thesis it has been clearly shown that only the combination of both methods can yield important insights into the complex relationship between microorganisms in a food.

7.4 Genera isolated from pork carcasses, pork minced meat and wild boar meat on different agar plates, and identified by MALDI TOF MS and 16S amplicon sequencing

Different agar plates and/or different conditions were used in order to gain data about the microbial diversity of the counted microorganisms (Table 7.2). Plate count agar is a non-selective medium suggested in ISO 4833 for the aerobic colony count at 30°C (reference method, EU 1441/01). This agar plate was also incubated at 7°C in aerobic conditions and at 30°C in a GasPak jar system in order to evaluate the psychrotrophic and anaerobic population, respectively.

Statistical analysis performed in chapters 2, 3 and 4 showed that the temperature (30°C or 7°C) and the conditions (aerobic) of incubation of PCA plates did not have a significant impact on the bacterial colony count ($p>0.005$). Moreover, the microbial diversity was higher on the PCA plates incubated at 30°C. In the present thesis, except for *Exiguobacterium*, *Frigoribacterium* and *Janthinobacterium* (bacteria with some psychrophilic species (Da Costa et al., 2018; Kämpfer et al., 2000; Kim et al., 2018)) all bacteria present on PCA plates incubated at 7°C were also isolated under other conditions or on other media.

On PCA plates incubated under anaerobic conditions, all genera except for *Luteococcus*, *Propionibacterium* and *Vagococcus* were also isolated in aerobic conditions. This result, along with the fact that with 16S amplicon sequencing obligate anaerobic bacteria were frequently detected, indicates that the currently commonly applied anaerobic culture method using Gaspak not completely support bacterial recovery.

On MRS agar plates *Lactobacillus* and *Leuconostoc* were frequently isolated from minced pork. *Leuconostoc* was also present in a high percentage on MRS in wild boar. However, *Candida* was also frequently isolated and, moreover, represented the main genus identified on MRS from the carcasses.

On TBX agar plates all typical blue-green colonies isolated were identified as *E. coli* meaning that this media allows the reliable

identification of this species. On the VRBG plates, the main part of the purple/pink colonies was confirmed as members of the Enterobacteriaceae family. However, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae, Sporidiobolaceae, and Staphylococcaceae families were also identified.

On CFC plates, the dominant genus was *Pseudomonas* spp, but other genera were isolated or detected by 16S rRNA gene sequencing, some of which (*Aeromonas*, *Chryseobacterium*, *Kocuria*, and *Shewanella*) were oxidase positive. The use of this phenotypic test for the confirmation of *Pseudomonas* genus can lead to a overestimation.

Table 7.2 Genera identified in pork carcasses, pork minced meat and wild boar meat on different agar plates by MALDI TOF MS and 16S gene sequencing.

Genus	*TAB 30°C*	*TAB 7°C*	*TANAB*	*TBX*	*VRBG*	*CFC*	*MRS*
Acidovorax spp.	x						
Acinetobacter spp.	x	x	x		x	x	
Aerococcus spp.	x						
Aeromonas spp.					x	x	
Agrococcus spp.	x						
Alcaligenes spp.	x						
Arthrobacter spp.	x	x					
Bacillus spp.	x		x				
Brachybacterium spp.	x						
Brevibacterium spp.	x						
Brevundimonas spp.	x						
Brochothrix spp.	x	x					
Buttiauxella spp.		x			x		
Candida spp.	x	x			x		x
Carnobacterium spp.	x	x	x			x	x
Chryseobacterium spp.	x	x				x	
Citrobacter spp.		x	x		x	x	
Corynebacterium spp.	x						x
Cronobacter spp.	x						
Cryptococcus spp.	x	x					
Curtobacterium spp.	x						
Cutaneotrichosporon spp.							x
Deinococcus spp.	x						
Delftia spp.	x						
Enterobacter spp.	x	x	x		x	x	
Enterococcus spp.	x	x	x				x
Erwinia spp.	x	x			x	x	
Escherichia spp.	x	x	x	x	x	x	
Exiguobacterium spp.		x					
Filobasidium spp.							x
Frigoribacterium spp.		x					
Gibbsiella spp.	x						
Hafnia spp.	x	x			x	x	
Janthinobacterium spp.		x					
Klebsiella spp.	x		x		x		
Kluyvera spp.					x		
Kocuria spp.	x	x				x	
Lactobacillus spp.	x	x	x				x
Lactococcus spp.	x	x					x
Leclercia spp.	x				x		
Lelliottia spp.	x	x	x		x		

Genus	*TAB 30°C*	*TAB 7°C*	*TANAB*	*TBX*	*VRBG*	*CFC*	*MRS*
Leucobacter spp.	*x*						
Leuconostoc spp.	*x*	*x*	*x*				*x*
Luteococcus spp.			*x*				
Macrococcus spp.	*x*		*x*				
Microbacterium spp.	*x*	*x*					
Micrococcus spp.	*x*						
Moraxella spp.	*x*						
Ochrobactrum spp.	*x*						
Okibacterium spp.	*x*						
Pantoea spp.	*x*	*x*	*x*		*x*	*x*	
Paracoccus spp.	*x*						
Pediococcus spp.							*x*
Propionibacterium spp.			*x*				
Pseudochrobactrum spp.	*x*						
Pseudoclavibacter spp.	*x*						
Pseudomonas spp.	*x*	*x*	*x*		*x*	*x*	
Psychrobacter spp.	*x*	*x*					
Rahnella spp.	*x*	*x*	*x*		*x*	*x*	
Raoultella spp.		*x*	*x*		*x*		
Rhodotorula spp.					*x*		*x*
Rothia spp.	*x*						
Sanguibacter spp.	*x*						
Serratia spp.	*x*	*x*			*x*	*x*	
Shewanella spp.	*x*	*x*				*x*	
Sphingobacterium spp.	*x*	*x*					
Staphylococcus spp.	*x*	*x*	*x*		*x*		*x*
Stenotrophomonas spp.	*x*					*x*	
Streptococcus spp.		*x*	*x*				*x*
Trichosporon spp.						*x*	
Vagococcus spp.			*x*				
Weissella spp.							*x*
Yarrowia spp.	*x*	*x*				*x*	*x*
Yersinia spp.						*x*	

7.5 Microbial contamination in pork carcass and minced meat by culture dependent and 16S amplicon sequencing.

According to Regulation (EC) No.1441/07, aerobic colony count (TAB 30°C) is considered, along with Enterobacteriaceae for carcasses and *E. coli* for minced meat, as an indicator of process hygiene. In the present thesis, this criterion (TAB 30°C) was exceeded in one carcass (> 5,0 Log CFU /cm2) and in 11 minced meat (> 5x106 CFU/g) samples. As far as the indicators of faecal contamination are concerned, the count of Enterobacteriaceae was unsatisfactory in one carcass (> 3,0 Log CFU /cm2) while none of the minced meat samples exceeded the criterion for *E. coli* (< 500 CFU/g). The high levels of mesophilic bacteria in minced meat may be explained by non-hygienic handling during the deboning and cutting of the meat (Del Blanco et al., 2017) or by the lack of compliance with the temperatures stipulated in EC legislation during the transportation and storage (Koutsoumanis et al., 2006; EFSA,2016).

Although a real comparison between the counts on carcasses and in minced meat cannot be made because they were performed in different studies (chapter 2 and chapter 3), the levels of bacteria on PCA incubated at 7°C and *Pseudomonas* spp. on CFC were higher, as expected, in minced meat. This may be explained by the fact that the carcasses were sampled before the cooling process and the probable growth of psychrotrophic bacteria had not yet started, while in minced meat samples the chilling temperature allowed their proliferation and a reduction of mesophilic bacteria.

In chapter 3, two different slaughterhouses were sampled and no statistical differences in bacterial counts were recorded. Moreover, on each carcass ham, back, jowl and belly were examined, and the results showed the areas of sampling had no significant impact on bacteria contamination (p>0,005).

The microbial population on the carcasses at the end of the slaughter process identified with MALDI TOF MS and/or 16S gene sequencing was dominated by mesophilic bacteria like *Staphylococcus* spp.and *E. coli*. The psychrotrophic population was represented mainly by *Pseudomonas* spp. On the other hand, with

16S amplicon sequencing, even if there was not a real dominant genus, *Brochothrix*, was the most frequently detected genus on the carcasses (22.83%).

Chapter 3 shows, that the microbial population on the ham, back, jowl and belly were dominated by the same genera. Thus, sampling only one area on pork carcass may be sufficient to monitor the overall hygiene of the slaughter process.

It is also interesting that the microbial community present on each carcass seems to be linked to the bacterial population of the slaughterhouse as shown by the Bray-Curtis analysis where highly distinct OUT patterns were found among the two groups of animals belonging to the two slaughterhouses (chapter 3).

After the slaughter process according to 853/04, the temperature of the carcasses must be lower than 7°C during storage and transport (Koutsoumanis and Sofos, 2004). However, when minced meat is produced it must be chilled to a core temperature of no more than 2ºC. Temperatures should control the growth of many microorganisms, but a range of food spoilage associated organisms are not inhibited. In the present thesis, in fact, as already reported by Doulgeraki et al., (2012), the dominant bacterial flora isolated and detected on minced meat stored under refrigerated conditions were mainly composed of *Pseudomonas*, *Brochrotrix* and *Carnobacterium*, common spoilage causers.

7.6 Microbial contamination in wild boar meat by culture dependent and 16S amplicon sequencing

Within the European Union legislation, there are no microbiological criteria for wild game animals. Criteria for pork meat included in Regulation (EC) No. 1441/2007 are generally applied to evaluate the microbial quality of wild boar meat. If these microbiological criteria were taken into account, more than 60% of the wild meat sampled in the present thesis would exceed the limits regarding the total aerobic bacteria and Enterobacteriaceae given in the above mentioned regulation. However, compared to pigs, the conditions in which the wild boar are killed and subsequently processed are different. Indeed, the mesophilic count is in line with the study of Mirceta et al., (2015). However, the levels of Enterobacteriaceae and *E. coli* were particularly high, indicative of a probable faecal contamination of the carcasses.

Differences in the microbial population were reported with 16S amplicon sequencing between the different wild boar meat samples and was not possibly identify a genus or few genera dominating the samples. However, members of the *Pseudomonas* spp., *Acinetobacter* spp., *Macrococcus* and *Propionibacterium* genera were frequently detected.

In the present thesis the microbial population of wild boar meat has been studied for the first time. The results revealed a different distribution of genera among the samples and suggest the need for more focus on game meat and particularly to non-inspected meat provided directly to retail or consumers.

7.7 Differences in the microbial contamination between the pork carcass and wild boar meat

In the present thesis, wild boar resulted more contaminated than pork (Figure 7.1),though the data are not completely comparable due to the different sampling methods used (wild boar= excision method; pork carcass= swab method) and due to the larger region that has to be sampled on pork carcasses.

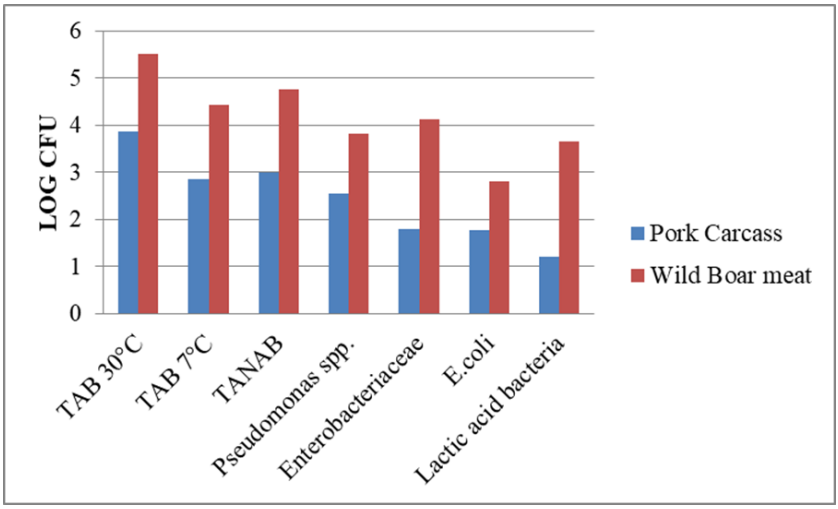

Figure 7.1. Mean of bacterial counts of the pork carcasses and wild boar meats on different media: mesophilic bacteria on PCA (TAB 30°C), psychotropic bacteria on PCA (TAB 7°C), anaerobic bacteria (TANAB), presumptive Pseudomonas spp. on CFC, Enterobacteriaceae on VRBG, E.coli on TBX, and Lactic acid bacteria on MRS.

High presence of *Salmonella* in the samples were reported both in pork (87,5%) and in wild boars (31,82 %). However, after serotyping *S.* Brandenburg, *S.* derbyand, *S.* Rissenwere identified only in the pork carcass samples and *S.* Stanleyville and *S.* Kasenyi only in wild boars samples. In regards to *Y. enterocolitica* low levels of contamination were detected in the two subspecies (*Sus scrofa domestica* and *Sus scrofa scrofa*).

Analysis with culture dependent and independent methods show that pork and wild boar share common bacteria, but as expected some genera could only be identified in pork or wild boar meat. What emerges from the present thesis that wild boars present an higher variability in the microbial contamination among the different animals compared with pork.

These difference in the contamination levels and in the microbial flora are not totally surprising as the animals are slaughtered and further processed in different way; in particular wild boars are shot, bled and eviscerated in different environment, where, moreover, there is no access to hygienic conditions as present in modern slaughterhouse facilities.

7.8 Detection of foodborne bacteria

In the present thesis the presence of *Salmonella* spp. and *Y. enterocolitica* on pork carcasses (chapter 3) and *Salmonella* spp., *Y. enterocolitica* and *L. monocytogenes* in wild boar meat (chapter 4)was evaluated with Real-Time-PCR with specific primers from enrichment broth after incubation.

Salmonella spp. were frequently detected and/or isolated both on pork carcasses (87.5 %) and wild boar meat (31.82 %). However, these data were discordant with those of other studies on pork carcasses (Choi et al., 2013; Mrdovic et al., 2013) and wild boar (Mirceta et al., 2017), where the prevalence of this pathogen was lower. These differences may be related to the fact that *Salmonella* can be prevalent in some regions (Gill, 2007). The serotypes monophasic *S.* Typhimurium, *S.* Brandenburg, *S.* derbyand *S.* Rissen found in the present work on pork carcasses are commonly associated with pigs and pork meat (EFSA,2016). Among the serovars identified in wild boar meat *Salmonella* Kasenyihas not yet been reported.

Interestingly, *Salmonell*a was not detected using 16S amplicon sequencing applied on the first meat homogenate. It is certain that the portions analysed with qPCR have not been the same portions as for the other analysis and moreover, an enrichment step was used for the pathogen detection. This could be explained by the fact that these bacteria are usually present at a very low concentration, and only deep Next Generation Sequencing is able to detect them (Rouger et al., 2017).

Pigs are considered a reservoir of *Y. enterocolitica* and recently more attention is being given to wild boars that are more often the subject of studies aiming at investigate the presence of this pathogen. In chapter 1, a preliminary study was performed in order to evaluate the distribution of different virulence genes among *Y. enterocolitica* strains. The results show that the gene *ystA* can be considered the best target gene in order to evaluate the presence of pathogenic biotypes whereas *ystB* can be proposed as a target gene for the biotype 1A. Thus these two genes were used in order to evaluate the presence of *Y. enterocolitica* in the samples.

Pathogenic *Y. enterocolitica* was never detected. However, the biotype1A was detected once on pork carcass on the ham and in three wild boar meat samples. The presence of *Y. enterocolitica* was not confirmed using the corresponding normalized microbiological isolation method (ISO 10273:2017). These low levels of contamination had already been reported previously by Avagnina et al., (2012) and Choi et al., (2013). Furthermore, the absence of *L. monocytogenes* confirms findings in other studies (Membré et al., 2011; Paulsen and Winkelmayer, 2004).

Data of the present thesis illustrates that particular attention should be paid in regards to the presence of *Salmonella* spp. both in pork and wild boar which represent important reservoirs of this pathogen. However, a continued monitoring of *Y. enterocolitica* and *L. monocytogenes* should be performed because, despite being detected in very few samples or not detected at all, they are a cause, along with *Salmonella*, of human illness and may represent a public health risk.

7.9 Future perspective

Results of the present thesis illustrate that the study of a microbial community in an ecosystem is best achieved by using a combination of culturomics based on the application of MALDI TOF technology and 16S gene sequencing and culture independent methods. In the present study, the microbial population of pork carcasses, pork minced meat, and wild boar meat was evaluated, but this new strategy can be proposed to study other ecosystems. However, with the prospect of identifying as many bacteria as possible, different agar plates and culture conditions could be used in future research.

It could be interesting to assess the diversity of the microbial population from the live animal to the final product in order to gain more information about the dynamics of the flora during all the production steps. In addition, in chapter 3 four areas from the pork carcasses were evaluated and no differences in the contamination levels and microbial diversity among them were noted; the study of the bacterial communities on other sampling sites would be useful to better address the official inspection of slaughterhouse hygiene by authorities. Moreover, the evaluation of carcasses found in slaughterhouses located in different countries would be useful for assessing if it could be possible to create traceability of the meat through microbial diversity.

Moreover, the evaluation of the microbial diversity combined with studies on the flavour in meat products with a protected designation of origin (PDO) and protected geographical indication (PGI), could be of interest in order to study whether the typical flavor of these products is linked to the flora present in the meat and/or to the environment from which it originates.

Findings in the present thesis indicate that wild boar may pose a public health risk. However, little is known about the wild boar products that are increasingly more widely consumed. Furthermore, the study of the microbial population could be extended to other game animals to find out more about the risks linked to their consumption.

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