Microbiota of spontaneously fermented game meat sausages

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DOCTORAL THESIS

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Statement of originality

I, Ana Žgomba Maksimović, hereby declare that I have independently authored doctoral dissertation under the title:

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Information about supervisor

Mirna Mrkonjić Fuka was born in Zagreb in 1976. She graduated from the Faculty of Science and Mathematics in Zagreb in 2002 and received her PhD at Technical University Munich (TUM) in 2007 with the thesis on structural and functional diversity of proteolytic bacteria in soil. Since 2007 she has worked at the Department of Microbiology at the Faculty of Agriculture, University of Zagreb, where she was appointed Associate Professor in 2016. Her main scientific interests are the analysis of functional and structural diversity of bacterial communities in complex ecosystems, molecular-biological identification of lactic acid bacteria and selection of starter and bioprotective cultures. Mirna Mrkonjić Fuka teaches at the Faculty of Agriculture of the University of Zagreb, where she coordinates seven courses at undergraduate and graduate level. She was a mentor of seven bachelor and eighteen master theses and mentor of two student work rewarded with a Rector's Award. Mirna Mrkonjić Fuka has obtained several awards and acknowledgments (DAAD, FEMS, ERASMUS, CEEPUS, Society of University Teachers and Other Scientists in Zagreb) for her scientific and academic work. She has participated in several international (Tempus IV Program (2015-2017), See-Era. Net Plus (2010-2012), European Regional Development Fund (2006-2007), DFG-German Research Foundation (2002-2005)) and national projects (MZOS 2007-2013) of which she was the leader of two bilateral projects with Germany (2012-2013), one with Austria (2014-2015), one with Slovenia (2016- 2017) and one with China (2018-2019) as well as the leader of the project founded by Croatian Science Foundation (2014-2017). Since 2007 Mirna Mrkonjić Fuka has spent more than fifteen months at renowned foreign universities and scientific research institutions (BOKU, Austria; TUM, Germany; Helmholtz Zentrum Munich, Germany; Vitoria University, Wellington, New Zealand; Huazhong University, Wuhan, NR China). Mirna Mrkonjić Fuka is a member of the Croatian Microbiological Society, a member of the Board for the Use of GMOs at the Croatian Ministry of Health, and was a member of the Steering committee of the Institute for Agriculture and Tourism in Poreč. She has held more than 20 lectures at renowned foreign scientific research institutes and congresses and has published more than 30 scientific papers quoted 275 times according to WOS.

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Summary

In the present study, the microbiota of three wild boar (WB1, WB2, WB3) and three deer meat (DS1, DS2, DS3) sausage types was characterised, with focus on lactic acid bacteria. The sausage samples were collected in triplicates (*n*=105) from each of the sausage producers at day zero (0), after 4, 7, 10, 20 days and at the end of the ripening period (20 or 40 days). Dominant LAB isolates collected from sausages were identified as *Le. mesenteroides* (*n*=259), *Lb. sakei* (*n*=190) and *E. casseliflavus* (*n*=106). However, high throughput amplicon sequencing revealed lactobacilli, mainly *Lb. sakei* and *Lb. curvatus*, as the predominant species, with 20.55 % of sequences at the beginning of fermentation and 70.48 % in mature sausages. It seems that the isolation of LAB on selective media favored the growth of *Le. mesenteroides,* since its predominance was also observed in the denaturing gradient gel electrophoresis (DGGE) analysis of bulk colonies collected from De Man, Rogosa and Sharpe agar (MRS) plates, indicating insufficient selectivity of the used media. On the other hand, HTS can also be biased due to DNA isolation (cell lysis) and polymerase chain reaction (PCR) analysis, which can favour certain microbial population. Regarding bacterial diversity during sausage production, the number of operational taxonomic units (OTUs) was highest at day 0 and day 4, followed by a decrease toward the later phases of fermentation and ripening, which might be explained by the decrease of pH and available water for microbial growth (a_w). Measured alpha diversity parameters (Shannon's index and Pielou evenness) were significantly higher in wild boar meat sausages compared to deer meat at day 4 (p<0.01) and day 7 (p<0.05), which might be linked to a different lifestyles, type of diet and feeding habits of wild boar and deer. With fermentation time, the abundance of undesired and potentially harmful microbiota decreased indicating the microbial stability and safety of final products. However, 33 % of ready-to-eat sausages can be considered as inappropriate for human consumption, due to the elevated cell counts of *Enterobacteriaceae* (DS2, WB3) as well as *E. coli*, coliforms and presumptive *B. cereus* (WB3). To select strains suitable to be used as indigenous starter cultures, 1326 LAB isolates were collected from sausages. Isolates were grouped based on their rep-PCR patterns, and 57 representatives were selected and screened for safety issues and technological potential, as well as probiotic potential. Around half of the tested representatives (56.14 %) was safe for application in food, of which 53.12 % showed good acidification activity (pH<4) and variable other technological traits. Based on the results of safety and technological characterization, 2 *Lb. sakei* strains (MRS_296 and C_7d_13) were selected and applied simultaneously in meat batter as indigenous starter cultures. Although respective strains were originally grouped in the same cluster, they exhibited different technological traits. The majority (86.49 %) of isolates recovered from inoculated batch had rep-PCR patterns that corresponded to the patterns of respective starter cultures, suggesting that applied strains were able to establish themselves from day zero and prevailed through the production. However, considering that rep-PCR patterns of MRS_296 and C_7d_13 were not possible to differentiate, it is not possible to say whether both strains survived, or only a single one, and if, which one. It seems that the inoculation of native starter cultures suppressed the growth of *Enterobacteriaceae*, coliforms and *L. monocyogenes.* The pH values were significantly lower throughout the production in the inoculated batch (p<0.05) than in control. The tyramine content was 67.18 % lower in the ready-to-eat inoculated batch compared to the control. Such results suggest the efficiency of applied starter cultures as well as highlighting their potential of its use in future.

Keywords: lactic acid bacteria, game meat sausages, rep-PCR, next-generation sequencing, microbial diversity, technological characterisation, starter cultures, bioprotective cultures, probiotics, food safety

Prošireni sažetak

Mikrobiota spontano fermentiranih kobasica od mesa divljači

Trajne, spontano fermentirane kobasice od mesa divljači se proizvode na malim obiteljskim gospodarstvima, prateći tradicionalne recepture i postupke. Zbog toga su prepoznate i cijenjene kao autohtoni, odnosno, tradicionalni proizvodi specifičnih senzornih osobina. Međutim, budući da se proizvode bez upotrebe starter kultura i to često u varijabilnim uvjetima, fermentacija ovisi o metaboličkoj aktivnosti prirodno prisutnih populacija bakterija mliječne kiseline (BMK) te stoga mikrobiološka sigurnost krajnjeg proizvoda može biti kompromitirana. Također, na mikrobiološku (zdravstvenu) sigurnost gotovih kobasica od mesa divljači utječu mnogi faktori povezani s načinom odstrijela divljači i daljnje obrade mesa. Da bi se osigurala potrebna kvaliteta gotovih proizvoda, potrebno je u potpunosti kontrolirati čitav proizvodni proces, a jedan od načina je upotreba detaljno karakteriziranih starter kultura u njihovoj proizvodnji. Budući da komercijalne starter kulture nisu jednako efikasne u svim tipovima proizvoda, tendencija je aplikacija autohtonih startera, a upravo spontano fermentirani proizvodi koji nisu termički obrađeni predstavljaju izvor velikog broja različitih bakterijskih sojeva, naročito BMK. Dok je mikrobiota mnogih drugih tradicionalnih fermentiranih proizvoda detaljno istražena i karakterizirana, dostupna literatura o takvim saznanjima o kobasicama od mesa divljači je vrlo limitirana. Stoga je prvi korak ovog istraživanja bio karakterizirati prirodno prisutnu mikrobiotu ovakvih kobasica, te nakon toga prikupiti i detaljno karakterizirati sojeve BMK s ciljem selekcije onih bakterijskih sojeva prikladnih za daljnje korištenje kao starter kultura.

Mikrobna raznolikost tri tipa kobasica od mesa divlje svinja (WB1, WB2, WB3) i tri od mesa jelena (DS1, DS2, DS3) je istražena koristeći metode ovisne i neovisne o uzgoju. Uzorci kobasica su prikupljeni u triplikatima tijekom različitih faza proizvodnje (n=105). Izdvajanjem BMK na selektivnim mikrobiološkim medijima tijekom različitih faza zrenja I fermentacije (0, 4, 7, 10, 20 ili/i 40 dana) te molekularno-biološkom identifikacijom prikupljenih izolata, *Leuconostoc mesenteroides* je prepoznat kao dominanta vrsta BMK (28,24 %), a sljedili su ga *Lactobacillus sakei* (20,72 %) i *Enterococcus casseliflavus* (11,55 %). *Le. mesenteroides* je izoliran i identificiran u velikoj mjeri na LamVab (52,31 %) i De Man, Rogosa i Sharpe agaru (MRS) (40,89 %) mikrobiološkim podlogama, od kojih se naročito LamVab smatra selektivnima za laktobacile, što upućuje na zaključak da su navedeni mediji favorizirali rast leukonostoka. Ovaj zaključak podupiru rezultati analize DNA u denaturirajućem gradijentnom gelu poliakrilamida (DGGE) konzorcija prikupljenih sa MRS medija, budući da su potvrdili dominaciju *Le. mesenteroides* na ovom mediju. Osim za izdvajanje i prikupljanje BMK, različiti selektivni mediji su korišteni za detekciju i određivanje vijabilnog broja (cfu/g) ostalih ciljanih mikrobnih skupina, uključujući mikrobiotu kvarenja i potecijalne patogene. Ovakav je pristup omogućio procjenu mikrobiološke ispravnosti gotovih kobasica, budući da su propisani referentni intervali temeljeni na uzgoju i određivanju broja naraslih kolonija. Utvrđeno je da ukupno 33,33 % kobasica predstavljaju rizik za konzumaciju zbog povećanog broja bakterija porodice *Enterobacteriaceae* (DS2, WB3), kao i *E. coli,* koliformnih bakterija i hemolitičkih bakterija iz grupe *B. cereus* (WB3). S druge strane, analizom podataka dobivenih sekvenciranjem sljedeće generacije (engl. *next generation sequencing*; NGS) koristeći Miseq platformu (Illumina), dobivena je šira slika o zastupljenosti različitih mikobnih zajednica, budući da je izdvojena i analizirana ukupna bakterijska DNA prisutna u kobasicama, dakle, analiza nije ograničena samo na ciljane mikrobne skupine. Općenito, najveća mikrobna raznolikost je zabilježena u samom početku proizvodnje kobasica (0. i 4. dan) nakon čega opada prema kasnijim fazama fermentacije i zrenja. Zabilježeni pad raznolikosti se može objasniti kao rezultat pada pH vrijednosti, smanjenja dostupne vode za rast bakterija (a_w) i antagonizmom prirodno prisutnih BMK. Iako je općenito raznolikost ostala ujednačena kod oba tipa kobasica kada su uspoređeni isti vremenski intervali, Shannonov index i ujednačenost po Pielou su ukazali na veću razinu raznolikosti bakterijskih populacija u 4. i

7. danu kod kobasica od mesa divlje svinje (WB) nego kod kobasica od mesa jelena (DS), što je vjerojatno povezano s različitim načinom života i prehrane ovih životinja. Zajedno s trendom pada ukupne bakterijske raznolikosti tijekom fermentacije i zrenja, zabilježen je i pad neželjenih mikroorganizama, s iznimkom bakterija iz roda *Stenotrophomonas i Pseudomonas* čiji pad nije zabilježen. Ova je metodologija također omogućila detaljniji uvid u strukturu prisutnih zajednica BMK, pri čemu su primijećena neslaganja s rezultatima dobivenima klasičnim metodama (ovisnima o uzgoju). Analizom NGS podataka vrste roda *Lactobacillus* su identificirane kao dominantne, i to vrste *Lb. sakei* (40,32 %), *Lb. curvatus* (40,29 %) i *Lb. plantarum* (1,31 %), dok su ostale BMK detektirane u malom broju. Iako se sekvenciranje sljedeće generacije pokazalo kao pouzdan pristup za *in situ* karakterizaciju mikrobiote prisutne u trajnim kobasicama od divljači, izolacija i prikupljanje BMK na selektivnim podlogama je nužna za prikupljanje kolekcije vijabilnih sojeva. U ovom istraživanju je prikupljeno ukupno 917 izolata na mikrobiološkim medijima selektivnima za BMK. Međutim, BMK su također prikupljene i iz drugih trajnih tradicionalnih kobasica od mesa divlje svinje i jelena te su uključene u ovo istraživanje, tako da je ukupno prikupljeno 1326 izolata. Svi izolati su identificirani koristeći molekularno-biološke metode (rod i vrsta specifični PCR, sekvenciranje po Sangeru) te su genotipizirani pomoću rep-PCR metode koristeći GTG_5 početnicu. Obrasci dobiveni rep-PCR metodom su pokazali visoku razlučivost između sojeva i omogućili njihovo grupiranje. Izabrano je 57 reprezentativnih sojeva koji su uključeni u daljnje analize s ciljem selekcije odgovarajućih starter kultura. Prvenstveno, istražena je sigurnost njihove upotrebe u hrani (rezistencija na antibiotike, prisutstvo gena koji kodiraju za biogene amine i virulente faktore, hemolitička aktivnost) te su istražene njihove tehnološke karakteristike (sposobnost brze acidifikacije, lipolitička, proteolitička i peptidazna aktivnost) kao i antimikrobna aktivnost. Među reprezentativnim predstavnicima, izabrano je 19 bakterijskih sojeva za koje je istražena sposobnost preživljavanja u simuliranim uvjetima gastrointestinalnog (GI) trakta. Rezultati su pokazali da je približno polovica reprezentativnih predstavnika (56,14 %) sigurna za primjenu u hrani, od kojih je 53,12 % pokazalo dobru sposobnost acidifikacije, odnosno, snizili su pH mesnog medija s početnih pH=5.8 na pH< 4 nakon 24 sata inkubacije. Velika većina reprezentativnih predstavnika koji su smatrani sigurnima je pokazala lipoličku (96.87 %) i proteolitičku aktivnost na mediju s obranim mlijekom (93.75 %), međutim samo je njih 9.37 % pokazalo sposobnost razgradnje sarkoplazmatskih proteina. Njihova je peptidazna aktivnost varirala između 203.50±0.00 i 5942.20±0.14 µM *p*NA. Iako je većina reprezentativnih predstavnika (*n*=57) u vijabilnom obliku pokazala antagonističku (antimikrobnu) aktivnost prema testiranim indikatorskim bakterijama, kada je testiranje provedeno korištenjem njihovih neutraliziranih supernatanata (slobodnih od bakterijskih stanica), antimikrobna aktivnost je zabilježena za 4 soja i to prema *Listeria innocua* ATCC 33090. Ovakav rezultat indicira da spomenuti sojevi mogu imati sposobnost produkcije bakteriocina. Jedan je soj (*E. durans* A_4d_1) pokazao sposobnost preživljavanja u simuliranih uvjetima GI trakta, naročito u uvjetima tankog crijeva koji su bili pogubni za ostale sojeve, zbog čega se može smatrati potencijalnim probiotičkim sojem. Temeljem rezultata sigurnosne i tehnološke karakterizacije, 2 soja *Lb. sakei* (MRS_296 and C_7d_13) su odabrana za primjenu u kobasicama kao starter kulture. Ovi sojevi se smatraju sigurnima za primjenu u hrani, budući da nisu bili hemolitički aktivni, nisu pokazali rezistenciju prema testiranim antibioticima te nije detektirano prisutstvo gena koji kodiraju za produkciju biogenih amina. Iako su ova dva soja originalno bila grupirana u isti klaster, pokazali su različite tehnološke karakteristike. MRS_296 je pokazao brzu sposobnost acidifikacije mesnog supstrata, što je najvažniji preduvjet svake starter kulture. Iako niti jedan od izabranih sojeva nije pokazao sposobnost razgradnje sarkoplazmatskih proteina, kod MRS_296 je detektirana proteolitička aktivnost na mediju s obranim mlijekom. Soj C_7d_13, nije pokazao dobru sposobnost acidifikacije, ali je pokazao zadovoljavajuću lipolitičku i peptidaznu aktivnost, svojstva koja su nedostajala kod MRS_296. Oba soja su pokazala antimikrobno djelovanje prema velikom broju testiranih bakterija. Izabrane autohtone starter kulture su dodane u mesnu smjesu za pripremu kobasica (15 kg) s brojem vijabilnih stanica od 9,34

log cfu/g (MRS_296) i 9,30 log cfu/g (C_7d_13) dok je neinokulirana smjesa služila kao kontrola. Kako bi se pratilo preživljavanje dodanih startera u kobasicama, izolati su izdvojeni na medijima selektivnima za BMK, prikupljeni i genotipizirani pomoću rep-PCR reakcije. Rep-PCR metodom je bilo moguće usporediti obrasce dodanih startera s obrascima prikupljenih sojeva, odnosno, bilo je moguće na ovaj način pratiti preživljavanje starter kultura. Velika većina sojeva (86,49 %) izoliranih iz kobasica s dodanim starterima je imala rep-PCR obrasce koji odgovaraju obrascima dodanih startera, što pokazuje da su dodani starteri preživjeli tijekom čitavog procesa proizvodnje kobasica. Međutim, budući da nije moguće razlikovati rep-PCR obrasce između MRS_296 i C_7d_13, nije moguće zaključiti da li su oba soja preživjela. Rezultati potpune mikrobiološke analize ukazuju na efikasnost primjenjenih starter kultura, odnosno, pokazuju da je primjenom startera kontroliran rast enterobakterija, koliformnih bakterija i *L. monocyogenes* kod inokulirane grupe (šarže), u kojoj su pH vrijednost i koncentracija tiramina bili značajno (p<0,05) niži, u odnosu na neinokuliranu kontrolu. Ovo istraživanje pruža uvid u *in situ* raznolikost i brojnost prirodno prisutnih mikrobnih populacija kobasica od mesa divljači, što doprinosi kompletnijem razumijevanju sastava i dinamike mikroorganizama kod ovakvog tipa proizvoda. Kao jedan od ključnih koraka za NGS, optimizirana je izolacija DNA iz uzoraka kobasica te je etablirana zbirka definiranih sojeva koji imaju potencijal za buduću uporabu kao starter i/ili bioprotektivne kulture. Također, kroz ovo istraživanje dobivena je efikasna starter kultura koja se sastoji od dva autohtona soja *Lb. sakei*, a koja se može primijeniti za proizvodnju visoko kvalitetnih kobasica od mesa divljači u tradicionalnoj ili industrijskoj proizvodnji.

Ključne riječi: bakterije mliječne kiseline, kobasice od mesa divljači, rep-PCR, sekvenciranje sljedeće generacije, mikrobna raznolikost, tehnološka karakterizacija, starter kulture, bioprotektivne kulture, probiotici

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List of abbreviations

ADP	Adenosine diphosphate
AR	Antibiotic resistance
ATP	Adenosine triphosphate
a_w	Water activity
B.	*Bacillus*
BA	Biogenic amines
C.	*Carnobacterium*
cfu	Collony forming unit
CNS	Coagulase negative staphylococci
CO_2	Carbon dioxide
DNA	Deoxiribonucleid acid
E.	*Enterococcus*
EMP	Embden–Meyerhof–Parnas pathway
EU	European Union
GI	Gastrointestinal
GRAS	Generally recognized as safe
G+C	Guanine-cytosine content in a DNA or RNA molecule
HGT	Horizontal gene transfer
HTS	High throughput sequencing
L.	*Listeria*
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*

Lc.	*Lactococcus*
Le.	*Leuconostoc*
MALDI-TOF MS	Matrix-assisted laser desorption／ionization time-of-flight mass spectrometry
NAD+; NADH	Nicotinamide adenine dinucleotide
OTU	Operational taxonomic unit; refers to a cluster of microorganisms grouped by DNA sequence similarity
P.	*Pseudomonas*
PCR	Polymerase chain reaction
Pe.	*Pediococcus*
PFGE	Pulse-field gel electrophoresis
QPS	Qualified Presumption of Safety
PCR	Polimerase chain reaction
rDNA	Ribosomal deoxiribonucleid acid; a DNA sequence that codes for ribosomal ribonucleid acid (rRNA)
RFLP	Restriction fragment length polymorphism
RNA	Ribosomal ribonucleic acid
rRNA gene	Ribosomal ribonucleiud acid gene; RNA component of the ribosome
S.	*Staphylococcus*
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Strept.	*Streptococcus*
spp.	Species
USFDA	United States Food and Drug Administration
V1, V2	Regions of 16S

W.	*Weissella*
x *g*	Relative centrifugal force
16S rRNA	16S ribosomal RNA; a component of the 30S small subunit of a prokaryotic ribosome

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identified as a=*Lactococcus garviae*, b=*Leuconostoc mesenteroides,* c=*Lactococcus garviae,* d=*Leuconostoc* spp., e=*Weissella* spp., f=*Lactobacillus* spp.

Figure 4.6. PCR products obtained by amplification of DNA from strains used as positive controls. Lane M: 1 kb DNA Ladder, lane MSB11: positive control for *hdc* (367 bp) and *tdc* (924 bp) genes, MSB425: positive control for *odc* gene (1446 bp), DSMZ 20346: positive control for *ldc* gene (1185 bp).

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Figure 4.12. Cluster analysis of all strains isolated on LamVab media from spontaneously fermented control batch using rep-PCR (90.41 % cut-off value). Production time points are indicated next to each isolate (0d, 7d and end) and replicas with A, B and C. Isolate code is numerated with capital numbers while sequenced isolates are shown with exponential enumeration. Labels marked with the numbers $^{1\text{-}7}$ (GeneBank No. MH197045-51), $^{12\text{-}20}$ (GeneBank No. MH197056-64) identify with the 99 % similarity *to Lb. sakei* and numbers $^{9\text{-}11}$ (GeneBank No. MH197053-55) identify with the 99 % similarity to *Le. mesenteroides*, while number ^{8} (GeneBank No. MH197052) identifies to *W. viridescens* with the 99 % similarity.

Figure 4.13. Cluster analysis of all strains isolated on LamVab media from the batch inoculated with two *Lb. sakei* strains (MRS_296, C_7d_13) using rep-PCR (90.41 %

cut-off value). Production time points are indicated next to each isolate (0d, 7d and end) and replicas with A, B and C. Isolate code is numerated with capital numbers while sequenced isolates are shown with exponential enumeration. Indicated sequenced isolates [1-3] (GeneBank No. MH197065-67) identify with the 99 % similarity to *Lb. sakei* and numbers [4-6] (GeneBank No. MH231452-54) identify with the 100 % similarity to *Le. mesenteroides*.

1. INTRODUCTION

Dry fermented game meat sausages are produced by local artisans that follow traditional recipes and therefore are recognised and appreciated as authentic or traditional products of characteristic sensory traits. Considering that sausages are produced without adding starter cultures, their organoleptic properties and microbiological stability depend on the metabolic activity of the naturally occurring microbiota, mainly lactic acid bacteria (LAB) and coagulase-negative staphylococci, as well as meat enzymes. The central role of the LAB in fermentation is rapid acidification of meat batter. Metabolic activity of LAB leads to accumulation of lactic acid and consequently the decrease in pH, which may suppress or inhibit the growth of spoilage and pathogenic species and at the same time provide favorable environment for certain chemical, physical and microbiological reactions relevant for the development of the taste, color and texture of sausages to occur (Kröckel 2013). Hovewer, since fermentation is dependent upon the metabolic activity of indigenus LAB, and the sausages are often manufactured in variable conditions with seasonal character and different choices of raw materials, the microbiological safety of artisan sausages can be compromised. While the indigenous microbiota of many traditional fermented products have been investigated and characterized (Comi et al., 2005; Skelin et al., 2012; Fontana et al., 2016; Mrkonjic Fuka et al., 2017), the data regarding game meat sausages is very limited (Markov et al., 2013; Marty et al., 2012). The microbiota of game meat carcasses and consequently of game meat sausages can be variable, since it is influenced by the numberous factors such as microorganisms present at the animal skin, anatomical location of shooting and evisceration (Gill 2007). The microbiological criteria for ready-to-eat foods are regulated at the EU level by Commission regulation (EC 2005) 2073/2005. The regulation demands the absence of *L. monocytogenes* in 25 g of a sample for products before they had left the production unit, or limits it's number to 2 log cfu/g for products placed on the market during their shelf-life. *Salmonella* spp. should be absent in 25 g of food samples at all time. According to the national Giudance on microbiological food criteria (2011), it is recommended to investigate the presence of *Enterobacteriaceae*, sulfite-reducing clostridia, coagulase-positive cocci and *Staphylococcus aures*, and the results are considered to be satisfactory if in 25 g of samples their number stays below 1 log cfu/g. In their study, Markov et al., (2013) microbiologically characterized sausages manufactured in Croatia from different types of game meat. *Salmonella* spp., sulfite-reducing clostridia and *L. monocytogenes* were not detected in any sausage type, but in some sausages, the cell counts of *Enterobacteriaceae* exceeded 1 log cfu/g. However, none of the sausages met the

requirements from the Giudance, since the cell counts of *S. aureus* were ≥ 1 log cfu/g. In a similar study, Marty et al., (2012) have pointed out that 9 out of 14 traditional sausages manufactured in Switzerland from the meat of game or domestic animals pose a health risk due to high cell counts of *S. aureus* and *Enterobacteriaceae*. Such results indicate the poor microbiological quality of traditional dry fermented sausages manufactured from game meat, and highlight the need for detailed characterization of indigeneous benefitial, as well as pathogenic and spoilage microbiota in order to evaluate the microbiological status of such products. Although molecular approaches based on culture-independed methods have gained much importance in analyzing microbial community structure in food matrixes, and are considered as methods of choice due to not introducing cultivation biases, traditional cultivation methods are still essential for obtaining and handling bacterial cultures. Microbial diversity of LAB represent a valuable pool of wild strains, which dominate during fermentation and suppress the proliferation of pathogenic and spoilage species (Leroy et al., 2006). Indigenous strains isolated from traditional fermented products often express higher metabolic activity compared to comercial starter cultures. Likewise, commercial starters are not equally efficient in all sausage types. Due to their ability to survive during production and to compete with naturally present microbiota, for successful fermentation of traditional fermeted game meat sausages the most promising are the strains isolated from that particular type of sausages (Dalla Santa et al., 2014; Frece et al., 2014; Palavecino Prpich et al., 2015). Having all that in mind, the need for a selection of appropriate indigenous strains and their application as starter cultures is very pronounced. Besides starter cultures, LAB strains isolated from different sausages have exhibited a good potential for their use as probiotic cultures, due to their tolerance to harsh environments present during fermentation and in gastrointestinal (GI) tract, as well as to functional aspects associated with promoting health (Ruiz-Moyano et al., 2008; Frece et al., 2010; Nogueira Ruiz et al., 2014). Contemporary demands for a high level of food safety and quality imply the conservation of the microbial diversity in artisan food products and the complete control of the whole production processes, possibly through the steering of microbiota present during the fermentation.

1.1. Aims and hypotheses

The main two aims of this research were to characterise the microbiota of artisanal game meat sausages and to select defined lactic acid bacteria (LAB) strains, which are going to be used as microbial cultures for standardisation of game meat sausage production.

We set several hypotheses:

1. Different raw materials and conditions during manufacturing are going to influence the microbial counts and composition of microbial communities in sausages.
2. The microbiota in sausages is going to be different between the stages of production, and the predominant are going to be LAB.
3. Some of the selected LAB strains are going to exhibit good technological potential for their application as indigenous microbial cultures in the manufacturing of a microbiologically safe and stable product of recognisable quality.

2. OVERVIEW OF THE LITERATURE

2.1. The significance of game meat and game meat sausages

Game meat is gaining importance during the recent years due to its characteristic strong taste, a small percentage of fat and cholesterol, high amount of proteins and a good ratio between unsaturated and saturated fatty acids. Compared with the meat of domestic animals, the nutritional composition of game meat is most similar to chicken (USDA Food Composition Databases). It is easily digested, dark red in colour, sharp in taste and tough, reflecting the fact that wild animals are in the constant move while searching for food (García Ruiz et al., 2007). Game meat is often processed into sausages, and such sausages hold an important place in Croatian tradition, where they are not only characterized by distinctive aroma and flavour, but by extended shelf life as well. Most commonly used are wild boar and deer meat. A positive attitude of customers towards traditional products is the result of considering traditional food as a part of local or regional cultural heritage and considering hunting a recreational activity that forms a part of personal identity. To provide high-quality products and possibly expand the production of traditional game meat sausages, high food safety standards have to be met. The possibilities are being investigated intensively, and frequently involve the use of starter cultures (Aymerich et al., 2006; Frece et al., 2010; Gao et al., 2014; Xie et al., 2015). Various other possibilities of producing nutritionally balanced sausages are also being investigated. For instance, reducing the percent of fat added during sausage production. According to WHO (2018), fat content should not exceed 30 % of total energy intake, and it is recommended to substitute saturated with unsaturated fatty acids so that the content of saturated fats do not exceed 10 % of total energy intake. Animal fat can be added to a lesser amount in meat batter used for sausage production, but it can be reduced only to a certain degree, otherwise sensory and technological properties of sausage could be affected. Healthier unsaturated oils can replace animal fat, but problems with incorporation with meat batter can occur, and also a higher amount of unsaturated fatty acids can lead to lipid oxidation and reduced shelf life (Bolger et al., 2017). The oxidative effect can be suppressed by emulsification or encapsulation of oil and by addition of antioxidants. A Norwegian product “Super Salami” has a reduced fat amount, of which 10 % is encapsulated vegetable oil (Holck et al., 2017). Besides fat, fermented sausages are rich in salt, which contributes to their microbiological safety, prolonged shelf life and sensory trait. Considering the harmful effect of excessive salt intake on the cardiovascular system, the possibilities of manufacturing sausages with reduced salt concentration are

being considered. One of the possible approaches implies partial replacement of Na^+ ions derived from NaCl with salts such as KCl and $CaCl_2$ (Holck et al., 2017). This line of research highlights the need for the production of high-quality food by applying the latest scientific knowledge, wherein the use of LAB strains, independently or in combination with other (bio)technological solutions, shows great potential.

2.2. The primary microbiota of animal carcasses and its influence on the microbiological quality of sausages

Microbiological quality of game meat sausages is greatly influenced by the quality of raw materials used for its production. The microbiota of raw game meat is usually comprised of Gram-negative, mostly psychotropic bacteria. Contrary to farm animals, which are slaughtered in a controlled environment, the diverse factors associated with hunting can greatly influence the microbiota of carcass. Some of those factors include the animals' skin, muscular and gut microbiota, anatomical location of the shooting, evisceration and further meat handling (Gill 2007; Kegalj 2012). For instance, Deutz et al. (2006) have demonstrated that the number of aerobic bacteria on the carcass of animals shoot in the chest was 4.6 cfu/cm^2 , while on the carcass of animals shot in abdomen their number was 5.6 cfu/cm^2 . Similarly, Avagnina et al. (2012) found higher numbers of aerobic bacteria on carcasses of animals shot in the abdomen, compared to animals shoot in other body parts. Immediate handling of killed animals is a crucial requirement for meat preservation, especially in summer months. Some of the most important measures include evisceration and cooling. The microbiological criteria for foodstuffs are defined at the EU level by the Commision regulation (EC 2005) 2073/2005, under which the criteria for handling the carcasses of farmed animals are considered, while the standards for handling game meat are not separately regulated. Recent years, studies have been performed in order to define microbial diversity in game meat and to define acceptable limits of certain microorganisms. Paulsen (2011) suggests that microbiological standards of game meat should be similar to the ones for farmed animals, *i.e.* that cell counts of total aerobic bacteria do not exceed the limit of 6 log cfu/cm^2 and *E. coli* 2 log cfu/cm^2.

2.3. The role and diversity of the LAB in spontaneously fermented sausages

LAB have a central role in meat fermentation. Their main role is to convert fermentable sugars in the sausage batter to organic acids, mainly lactic acid. The low pH provides a hostile environment for the growth of the undesirable microbiota and consequently prevents them from producing toxins. The production of lactic acid also has a direct

impact on sensory product quality by providing a mildly acidic taste, and by supporting the drying process which requires a sufficient decline in pH (Kröckel 2013). Also, acid environment enables various chemical, physical and microbiological reactions to take place, so the acidification plays a central role not only in the control of undesirable microorganisms, but also in the development of flavour, colour and texture of sausages during fermentation. LAB usually predominate in sausages after a week of fermentation. In spontaneously fermented sausages, the LAB cell counts amounts to 3.2 to 5.3 log cfu/g at the beginning (Drosinos et al., 2005; Fontana et al., 2005; Comi et al., 2005), then increses to 7-9 log cfu/g in the first days (Comi et al., 2005) and remains constant during ripening (Cocolin et al., 2001). The adaptability of the LAB to various food matrixes can be explained by genomic analyses revealing species-to-species variation in the number of genes directing metabolic ability and nutrient uptake (Holzapfel and Wood 2014). Furthermore, it appears that certain niche-specific genes have been acquired with a location on plasmids or adjacent to prophages (Schroeter and Klaenhammer 2009).

The prevalence of certain microbial populations in traditional artisanal sausages can exhibit strong variations even within a single country or region (Milicevic et al., 2014). Within the LAB, lactobacilli are predominant in dry spontaneously fermented sausages, although enterococci, leuconostoc, weissella, lactococci, pediococci, and carnobacteria can be found (Milicevic et al., 2014). The study of Papamanoli et al. (2003) showed that 90 % of the LAB isolated from naturally fermented dry sausages belonged to the genus *Lactobacillus*. Among lactobacilli, the most common species are *Lactobacillus sakei*, *Lactobacillus plantarum* and *Lactobacillus curvatus* (Milicevic et al., 2014). *Lb. sakei* appears to be the most competitive, probably due to their ability to grow and proliferate on low temperatures and in the presence of high concentrations of NaCl (Hüfner et al., 2007; Milicevic et al., 2014). Enterococci are found in relevant numbers in sausages and are believed to contribute to the characteristic taste of sausages (Kröckel 2013). Hovewer, the presence of enterococci in fermented food is debatable, especially if present at high numbers, since some strains carry antibiotic resistances and virulence determinants relevant in medicine. Leuconostocs and weissellas are associated with spoilage of meat and sausages but are present at low numbers in sausages. Carnobacteria are also associated with spoilage, but are more commonly found in meats with elevated pH, and rarely in sausages. Some strains of all noted genera (*Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Weissella*, *Lactococcus*, *Pediococcus* and *Carnobacterium*) are able to produce bacteriocins, but their contribution to the reduction of the undesired microbiota depends upon their abundance in sausages.

2.4. Taxonomy and phenotypic description of the LAB genera commonly associated with meat fermentation

LAB are a very diverse group of microorganisms that share common metabolic and physiological characteristics. Their diversity is reflected by their association with diverse habitats, including niches with extreme conditions ranging from relatively low and high temperatures (0-50 °C), and also with examples of growth at high salt concentrations (up to 25 % NaCl), low pH (around pH=3.9) and physiological bile salt concentrations (Holzapfel and Wood 2014). The first classification of the LAB was provided by Orla-Jensen (1919) defining them as Gram-positive, non-motile, non-spore forming, rod-or coccus-shaped organisms that ferment carbohydrates and higher alcohols to form mainly lactic acid. LAB were divided into six genera: *Betabacterium*, *Streptobacterium*, *Thermobacterium*, *Betacoccus*, *Streptococcus* and *Tetracoccus*. Since then, the description of their morphological and physiological characteristics had not changed much, but thanks to the advances in molecular biology and available molecular tools, LAB underwent several taxonomical reclassifications. Of the original six genera, only *Streptococcus* remained in contemporary classification, although modified. Holzapfel and Wood (2014) have reviewed some milestones in the reclassification of the LAB through time. For instance, data regarding the mol % G+C in the DNA and the interpeptide bridge of the cell wall peptidoglycan was used to define the taxa. The authors point out the importance of DNA:DNA and DNA:rDNA hybridization studies, used for identification of new genera and species within defined groups of taxa. This lead for the genus *Streptococcus* to be recognized as heterogeneous resulting in the separation of genus *Enterococcus*, *Lactococcus* and *Lactobacillus*. The authors also highlight the importance of the polyphasic approach and the use of a DNA reassociation value of ≥ 70 % DNA similarity, which was shown to correlate with ≥ 97 % sequence identity of the highly conserved 16S rRNA gene, which considerably improved the delineation and definition of the species as a taxonomic unit.

Nowadays, the typical LAB are defined as Gram-positive, not spore-forming, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, nutritionally fastidious, acid-tolerant and strictly fermentative, with lactic acid as the major end-product of sugar fermentation (Franz and Holzapfel 2011; Holzapfel and Wood 2014). LAB are classified within the bacterial phylum *Firmicutes* with low (≤ 55 mol %) G+C in the DNA. The *Firmicutes* are distinguished from the other Gram-positive phylum, the *Actinobacteria*, with high mol % G+C (≥ 55 mol %) in the DNA. *Firmicutes* can be subdivided into several classes, including *Clostridia*, *Mollicutes, Bacilli* and *Erysipelotrichia*. Bacilli can further be

divided into two orders: *Bacillales* and *Lactobacillales* (lactic acid bacteria). A schematic overview of the LAB taxonomy is shown in Table 2.1., with the data borrowed from Holzapfel and Wood (2014), Vandamme et al. (2014) and the LPSN (List of prokaryotic names with standing in nomenclature: http://www.bacterio.net/).

Table 2.1. The schematic overview of the LAB taxonomy. The data is borrowed from Holzapfel and Wood (2014), Vandamme et al. (2014) and the LPSN (http://www.bacterio.net/). The number of the validly recognised species within each genus until 2018, are listed in brackets.

Phylum	*Firmicutes*					
Class	*Bacilli*					
Order	*Lactobacillales* (lactic acid bacteria; LAB)					
Family	*Aerococcaceae*	*Carnobacteriaceae*	*Enterococcaceae*	*Lactobacillaceae*	*Leuconostoccaceae*	*Streptococcaceae*
Genus	*Abiotrophia* (1) *Aerococcus* (8) *Dolosicoccus* (1) *Eremococcus* (1) *Facklamia* (6) *Globicatella* (2) *Ignavigranum* (1)	*Alkalibacterium* (10) *Allofustis* (1) *Alloiococcus* (1) *Atopobacter* (1) *Atopococcus* (1) *Atopostipes* (1) *Bavariicoccus* (1) *Carnobacterium* (11) *Desemzia* (1) *Dolosigranulum* (1) *Granulicatella* (3) *Isobaculum* (1) *Lacticigenium* (1) *Marinilactibacillus* (2) *Trichococcus* (6)	*Catellicoccus* (1) *Enterococcus* (51) *Melissococcus* (1) *Pilibacter* (1) *Tetragenococcus* (6) *Vagococcus* (10)	*Lactobacillus* (188) *Pediococcus* (11)	*Fructobacillus* (5) *Leuconostoc* (15) *Oenococcus* (3) *Weissella* (21)	*Lactococcus* (11) *Lactovum* (1) *Streptococcus* (101)

2.4.1. The genus *Lactobacillus*

Lactobacilli are not spore-forming, mostly non-motile and generally rod-shaped, although coccobacilli can be observed. Cells are often organized in chains. The optimal growth temperature is mostly between 30 and 40 °C, although growth temperature can range from 2 to 53 °C. Lactobacilli can grow in an environment with a pH range from 3 to 8. They generally tolerate oxygen but grow well under anaerobic conditions. Their ability to produce lactic acid as the main fermentation product from sugars was the basis of their early taxonomy of the genus. Orla-Jensen (1919) distinguished three subgenera: *Thermobacterium* as obligately homofermentative, *Streptobacterium* as facultatively heterofermentative and *Betabacterium* as obligately heterofermentative bacteria. Since then, the genus *Lactobacillus* has undergone numerous taxonomic changes. At present, 188 species form a major and diverse phylogenetic cluster. The heterogenicity of this genus is reflected by the many phylogenetic groups in which they are divided, such as *Lb. delbrueckii*, *Lb. salivarius, Lb. reuteri*, *Lb. vaccinostercus*, *Lb. sakei*, *Lb. alimentarius*, *Lb. plantarum*, *Lb. brevis*, *Lb. collinoides*, *Lb. casei*, *Lb. kunkeei* and *Lb. ozensis* group, *Lb. fructivorans*, *Lb. buchneri*, *Lb. coryniformis* and *Lb. perolens*. Some of them are well-defined phylogenetic clusters, while some are unstable and are expected to be further reclassified.

2.4.2. The genus *Lactococcus*

Lactococci are characterised by ovoid cells that occur individually, in pairs or chains. They are facultatively anaerobic, mesophilic and moderately halophilic bacteria that usually grow in 4 % NaCl. Lactococccci can grow well at neutral pH, but fail to grow at about pH 4.5. Lister (1873) isolated *Bacterium lactis* (an earlier synonym of *Lactococcus lactis)* and used it as a model organism to show the causes of infectious disease in humans. *Bacterium lactis* was renamed to *Streptococcus lactis,* and later, Lancefield (1933) classified the lactic streptococci to group N, which separated them from the pathogenic streptococci (groups A, B and C) and enterococci (group D). Schleifer et al. (1985) established the new genus named *Lactococcus*. Currently, the genus *Lactococcus* has 11 species: *Lc. chungangensis, Lc. formosensis, Lc. fujiensis, Lc. garviae, Lc. lactis, Lc. laudensis, Lc. nasutitermitis, Lc. piscium, Lc. plantarum, Lc. raffinolactis* and *Lc. taiwanensis* (LPSN).

2.4.3. The genus *Enterococcus*

Enterococci are Gram-positive ovoid cocci that occur separately, in pairs, in short chains or in groups. Cells are often elongated in the direction of chains. They do not produce endospores. Enterococci are not mobile, only in *E. gallinarum* and *E. casseliflavus*

positive mobility was reported. They are catalase-negative but can exhibit pseudocatalase activity. Enterococci are homofermentative and they grow at temperatures between 10 and 45 °C, with an optimal temperature of 37 °C. Thiercelin (1899) first introduced the term “entérocoque” to name a group of intestinal Gram-positive diplococci. The first attempt to establish the genus *Enterococcus* was published by Thiercelin and Jouhaud (1903). Later, the enterococci were renamed to *Streptococcus faecalis* based on their morphology (ability to form short chains) and were considered to be a part of the genus *Streptococcus*. Thanks to the DNA-DNA and DNA-RNA hybridisation methods they were reclassified to the genus *Enterococcus*. At present, 51 species are recognised within the genus (LPSN), and *E. faecalis* is the type species.

2.4.4. The genus *Leuconostoc*

The genus *Leuconostoc* is phylogenetically closely related to the genera *Lactobacillus* and *Pediococcus*. The cells are Gram-positive, coccoid, non-motile, asporogenous and catalase-negative. The optimal growth temperatures are between 20 and 30 °C, and they cease to grow above 40 °C. The species are usually non-acidophilic and prefer an initial pH between 6 and 7. They are obligately heterofermentative. Leuconostocs are closely related to *Fructobacillus*, *Oenococcus* and *Weissella*, and together they are commonly known as the *Leuconostoc* group of the LAB. Originally, the LAB included in the *Leuconostoc* group were all classified as *Leuconostoc* species but later, the group was subjected to several taxonomic revisions. At the time of writing, genus *Leuconostoc* is comprised of 15 recognised species, with *Le. mesenteroides* being the type species.

2.4.5. The genus *Weissella*

Weissella species are Gram-positive, catalase-negative, asporogenous short rods with rounded, tapered ends or ovoid cells. They occur in pairs or short chains, and some species tend to pleomorphism. They are facultatively anaerobic and heterofermentative. The genus *Weissella* was proposed by Collins et al. (1993), after investigating an unknown bacteria, similar to leuconostocs (the so-called Leuconostoc-like microorganisms) isolated from fermented Greek salami.

2.4.6. The genus *Pediococcus*

Pediococci are Gram-positive, catalase-negative and oxidase-negative, homofermentative bacteria that grow under facultatively aerobic to microaerophilic conditions. Some strains may display pseudocatalase activity. The cells are spherical and occur in characteristical tetrade formations. They are non-motile and do not form spores or capsules. The optimum growth temperature is 25–35 °C, but this is species-dependent. Balcke in 1884 first used

the name *Pediococcus cerevisiae* and suggested that this species belongs to a new genus *Pediococcus*. However, this was not validly published since he did not isolate any bacteria, rather he observed tetrade formation under the microscope. So far, 11 species are recognised (LPSN).

2.4.7. The genus *Carnobacterium*

The cells are Gram-positive, non-spore-forming straight rods, motile or not, occurring singly, in pairs, or short/long irregularly curved chains. They are facultatively anaerobic and heterofermentative. They are mesophilic and psychrotolerant, growing at 0 °C and neutral pH. *Carnobacterium* species grow at low temperatures, and most of them were isolated from refrigerated food. Some species tolerate up to 5–6 % NaCl. The genus *Carnobacterium* was proposed by Collins et al. (1987), which subsequently lead to the reclassification of *Lactobacillus divergens* and *Lactobacillus piscicola* into a separate lineage on the species level of the newly created genus *Carnobacterium* along with its new species *C. mobile* and *C. gallinarum*.

2.5. Biochemical changes during meat fermentation with the role of LAB

2.5.1. Carbohydrate metabolism

Carbohydrate metabolism in sausages mainly depends upon bacterial activity, especially the LAB. The breakdown of carbohydrates and accumulation of lactic acid, which is the main product of LAB carbohydrate metabolism, marks the beginning of sausage fermentation. LAB usually produce D (−) and L (+) lactic acid. The pH decline due to lactic acid accumulation prevents the growth of the undesirable microbiota and contributes to the taste (Kröckel 2013). Also, it contributes to aroma development through the formation of metabolites and protein coagulation as pH approaches the isoelectric point of meat proteins.

Based on their metabolic pathways used for glucose and pentose fermentation, LAB are divided into 3 groups. The members of the first group are defined as obligately homofermentative and ferment glucose to exclusively lactic acid via the glycolytic pathway, also known as EMP (Embden–Meyerhof–Parnas) pathway but cannot ferment pentoses and related compounds (Endo and Dicks, 2014). In this pathway, 1 molecule of glucose is converted to 2 molecules of lactic acid and 2 molecules of ATP are generated (Figure 2.1.). Only some species of the genus *Lactobacillus* are included in this group.

Facultatively heterofermentative LAB belong to the second group. They ferment glucose to exclusively lactic acid by EMP pathway and ferment pentoses and related compounds via the phosphoketolase (6-phosphogluconate) pathway (Endo and Dicks, 2014). Members of the genera *Enterococcus*, *Lactococcus*, *Lactovum*, *Paralactobacillus*, *Pediococcus*, *Streptococcus*, *Vagococcus* as well as some *Lactobacillus* spp. belong to this group.

The third group is comprised of the heterofermentative LAB, which metabolizes glucose, pentoses and related compounds via the phosphoketolase pathway (Endo and Dicks, 2014). In phosphoketolase pathway, glucose is converted to 1 molecule of lactic acid, 1 molecule of CO_2 and 1 molecule of ethanol, with generating 1 molecule of ATP (Figure 2.2.). The production of ethanol from acetyl phosphate is important for oxidation of NADH to NAD+ in this pathway, and this reaction is linked with the rate at which glucose is fermented and subsequently to the growth rate of the heterofermentative LAB (Maicas et al., 2002). The availability of electron acceptors (oxygen, pyruvate, fructose, citrate, malate) can influence the composition and ratio of fermentation byproducts such as ethanol, acetate, H_20, H_20_2 and CO_2, as well as the amount of generated ATPs. *Leuconostoc*, *Oenococcus*, *Weissella* and some *Lactobacillus* spp. belong to this group.

Many LAB are also able to ferment fructose, galactose and mannose as well as pentoses such as arabinose, ribose and xylose and related carbohydrates such as gluconate. Some LAB metabolises disaccharides such as cellobiose, lactose, maltose, melibiose and sucrose.

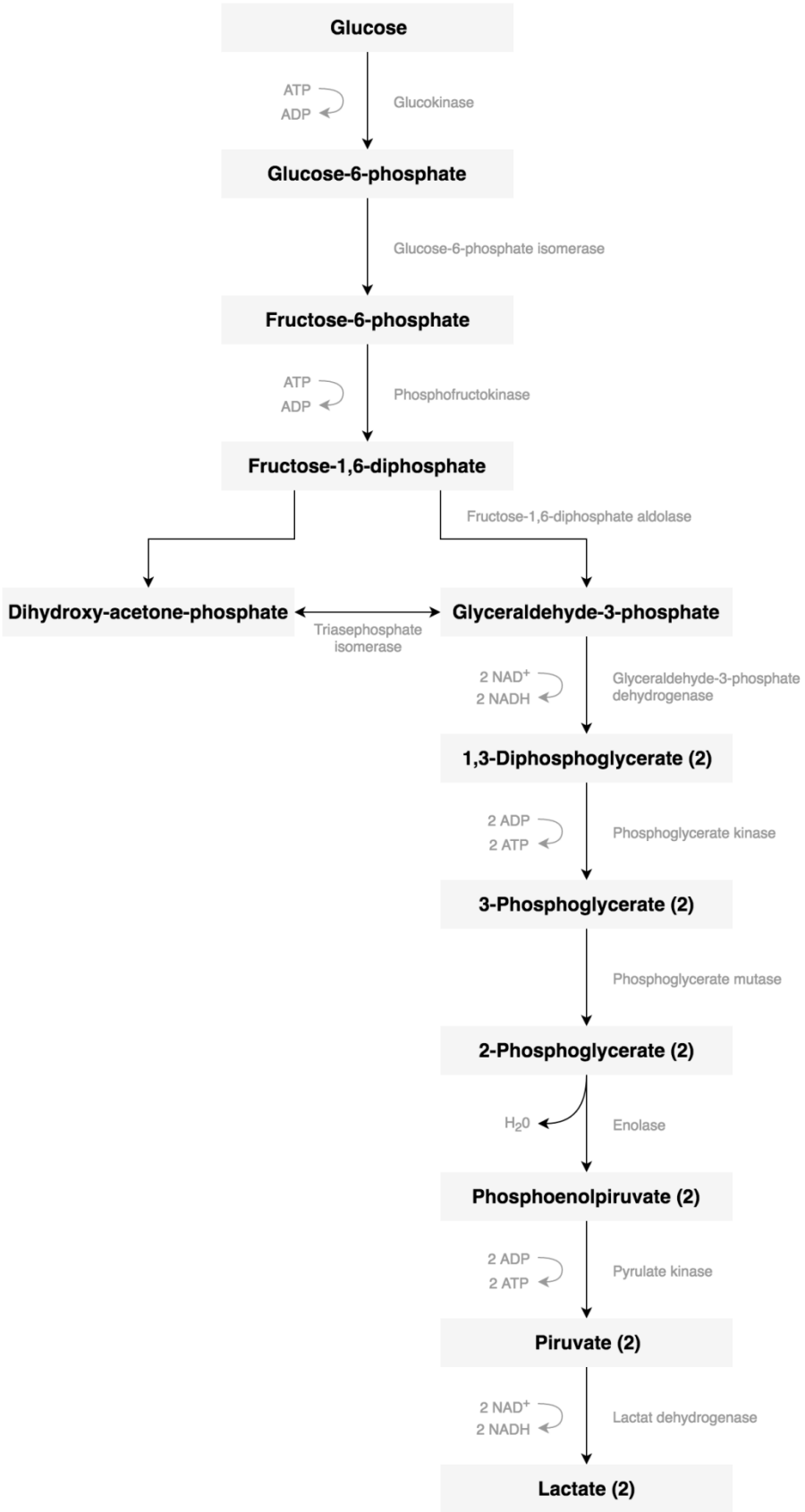

Figure 2.1. Homolactic fermentation of glucose (EMP pathway). The schematic overview is created according to Endo and Dicks (2014).

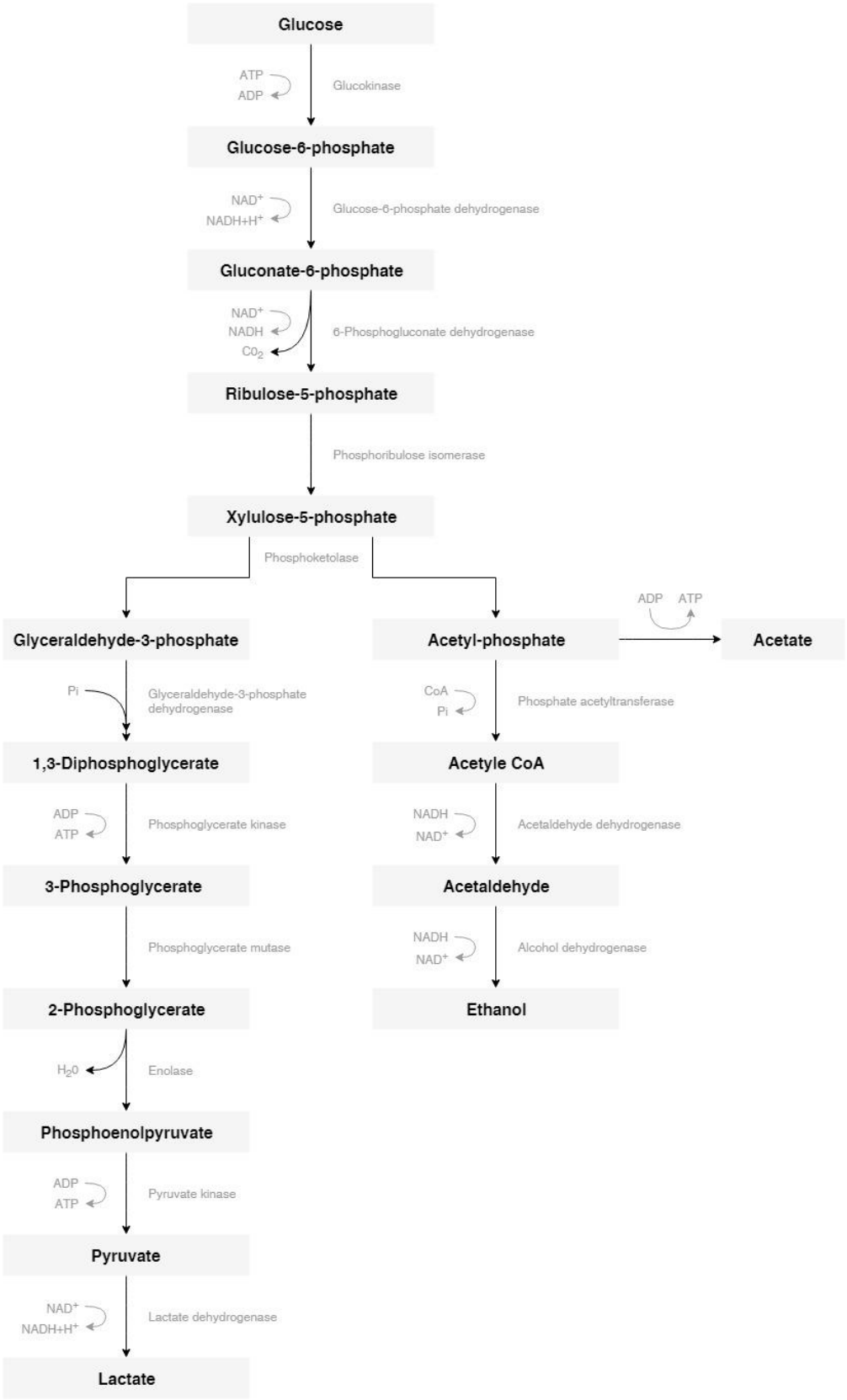

Figure 2.2. Heterolactic fermentation of glucose (phosphoketolase pathway). The schematic overview is created according to Endo and Dicks (2014).

2.5.2. Proteolysis

The content of meat proteins can vary depending on the type of animal and the feed it consumes. Degradation of meat proteins, mainly myofibrillar and sarcoplasmic, to peptides and amino acids contributes greatly to the characteristic taste of dry fermented sausages. The main role in these processes have endogenous meat enzymes. Generally, endopeptidases are responsible for protein breakdown, tri- and dipeptylpeptidases have a role in the generation of small tri- and dipeptides, while aminopeptidases and carboxypeptidases release free amino acids (Toldrá et al., 1992). The endopeptidases such as cathepsin B, D and L have shown to be active through the fermentation of sausages and have a considerable role in the proteolysis, particularly cathepsin D (Hierro et al., 1999; Molly et al., 1997). The activity of other muscle endopeptidases, like calpains, is inhibited by the low pH of sausages. Meat exopeptidases contribute to the conversion of peptides to amino acids. Although to a lesser extent than meat enzymes, microbial enzymes can also contribute to the texture, taste and aroma of fermented products. Proteolysis is mainly related to proteases of coagulase-negative staphylococci (CNS) (Hammes and Hertel 2009), although LAB also have a role in the proteolytic process (McSweeney and Sousa 2000), thanks to their complex proteolytic system capable of hydrolysing food proteins. The proteolytic system of LAB consists of cell wall proteases which convert proteins to oligopeptides. The generated peptides are then transported into the cell and are further degraded by different peptidases to small peptides and amino acids. Microbial enzymes hold greater significance in affecting oligopeptides that are formed during the later stages of ripening (Hughes et al., 2002), than in the initial breakdown of proteins. Within the clade of the LAB, proteolytically important species include *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactobacillus acidophilus* (Toledano et al., 2011). Processing conditions such as temperature, ripening time and salt concentration can influence both meat and microbial enzyme activity. Under certain conditions, excess proteolysis may result in bitter and metallic off-flavour due to the presence of bitter peptides. Microbial decarboxylation of released amino acids may produce biogenic amines, which, if accumulated in an excessive amount, may raise health concerns.

2.5.3. Lypolysis and esterase activity

The release of free fatty acids in the breakdown of lipids is primarily caused by lipases found in meat (Kenneally et al., 1998; Galgano et al., 2003) and, to a lesser extent, by the enzymatic activity of indigenous microbiota. Fatty acids may contribute to the taste directly, or indirectly, by the generation of aroma compounds through oxidative reactions.

Some of the most important lipases include the lysosomal acid lipase and acid phospholipase, located in muscles, as well as monoacylglycerol lipase, located in adipose tissue. The environmental conditions in sausages such as low pH and high salt concentration favour their activity. Alkenes, alkanes, alcohols, aldehydes, ketones and furans are the main products of the oxidative degradation of released fatty acids and are considered to be responsible for the characteristic aroma and flavour of sausages (Viallon et al., 1996; Chizzolini et al., 1998). Even though LAB possesses a range of esterolytic and lipolytic enzymes capable of hydrolysing esters of free fatty acids, tri-, di- and monoacylglyceride substrates, they are generally considered to be weakly lipolytic in comparison to species of genera such as *Pseudomonas*, *Acinetobacter* and *Flavobacterium* (Fox et al., 1993). Lipases/esterases of LAB appear to be exclusively intracellular (Chich et al., 1997). In some cases, excess oxidation of generated fatty acids can lead to off-flavours.

2.6. Safety issues associated with LAB

2.6.1. Meat spoilage and pathogens

Although most members of the LAB have GRAS (generally recognised as safe) status, some LAB can act as spoilage or/and can be harmful to human health. Enterococci represent one of the most controversial LAB genus. They are considered to be a part of the natural microbiota of many fermented products, where they have beneficial roles in flavour development and enhancing food safety. However, they are associated with certain health hazards and can be regarded as indicators of faecal contamination, especially if present in high numbers. Particularly, *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* are considered to be of faecal origin (Godfree et al., 1997), and can easily contaminate the meat at the time of slaughter/hunting. Enterococci are typical opportunistic pathogens which are known to cause infections, especially in immunocompromised patients. They are often associated with nosocomial (hospital) infections and cause bacteriemia, endocarditis and urinary tract infections. Majority of the enterococcal infections are caused by *E. faecalis* and *E. faecium* (Top et al., 2008). Other enterococcal species are rarely associated with human disease, but strains of *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. mundtii* and *E. raffinosus* have been reported in association with infections (Top et al., 2008). To cause infection, enterococci must have virulence factors that enable the infecting strain to colonise and invade host tissue and to translocate through epithelial cells and evade the host's immune response. Furthermore, such virulent strains must produce pathological changes either directly by toxin production or indirectly by inflammation. Virulence factors that occur in medical

isolates can also be found in enterococci isolated from food. Some of those virulence factors include the aggregation substance (*AS*), which has a role in binding to eukaryotic cells, cell aggregation and conjugation. The enterococcal surface protein produced by *E. faecalis* (*Espfs*) or *E. faecium* (*Espfm*) also functions as adhesion and is involved in immune evasion. The endocarditis antigens found in *E. faecalis* (*EfaAfs*) and *E. faecium* (*EfaAfm*) have a role as cell wall adhesins. Cytolysin is a cellular toxin that enhances virulence, while *cylA* has a role in activation of cytolysin, *cylM* in posttranslational modification of cytolysin and *cylB* in the transport of cytolysin. Gelatinase (*GelE*) is a toxin (extracellular metalloendopeptidase) that hydrolyses a variety of bioactive compounds (gelatin, collagen, hemoglobin). *GelE* can damage host tissue and allow bacterial migration and spread. Enterococci are noted for their capacity to exchange genetic information by conjugation, and virulence genes are known to be associated with some highly transmissible plasmids. Sex pheromones such as *cpd* and *cob* (virulence factors) are involved in facilitating conjugation. The process of conjugation used by plasmids requires a potential recipient cell to secrete small peptide signal molecules, known as sex pheromones. These pheromones induce genes leading to the production of aggregation substance in potential donor cells, resulting in a cell clumping phenotype. This process enhances the transfer frequency of the sex pheromone plasmid into plasmid-free recipient cells. Therefore, there is a risk that strains that do not possess virulence factors may acquire such genes by conjugation (Eaton and Gasson 2001).

Streptococci are also able to cause infections and are well known human pathogens. Their success as pathogens is related to the diverse array of virulence factors that have been attained by horizontal gene transfer, which enables their dominance in causing severe infections. For instance, S*trept. pyogenes* causes infections in the respiratory tract, skin, soft tissue and can cause secondary reactions due to the production of toxins. *Strept. pneumoniae* is a well-known human pathogen, causing pneumonia and bacterial meningitis. *Strept. mutans* are the main bacteria associated with dental caries, while *Strept. agalactiae* is one of the major colonisers of the intestinal and genital tracts and is today regarded as the main cause of neonatal meningitis and sepsis, which is spread from the vagina at birth. However, streptococci are rarely found in dry fermented sausages, and if any, they are present at very low numbers, so the risk of contracting the infection through food is very low.

Leuconostoc and *Weissella* are mainly associated with meat spoilage. The typical signs of leuconostoc spoilage include the formation of dextran (slime), bloating due to the CO_2 formation and acidic or buttery off-odours (Ammor and Mayo 2007). Some *Leuconostoc*

spp. have also caused infections, but mainly in immunocompromised patients. These bacteria are not a risk for healthy individuals and are considered as GRAS organisms. Weissella may be associated with bacteremia in humans, but as in the case of *Leuconostoc* infections, *Weissella* infections result mainly due to underlying diseases or immunosuppression of the host. *W. viridescens* may cause spoilage of meat and sausages due to green discolouration (Dušková et al., 2013). Some strains of pediococci have been associated with infections in humans and may be considered opportunistic pathogens. They may cause infections in individuals debilitated as a result of trauma or underlying disease.

Carnobacterium are not pathogenic. Several species have a considerable negative economic impact on the spoilage of chilled meat (beef and pork products) and fish. Members of the genus *Lactococcus* are mainly food associated and are considered GRAS organisms, although *Lc. lacts* and *Lc. garviae* have been known to cause infections (Lin et al., 2010; Chan et al., 2011). *Lactobacillus* infections are rare and occurs opportunistically, mostly causing endocarditis and bacteremia in immunocompromised individuals. Among them, *Lb. rhamnosus* and *Lb. casei* were recognised as the most common causes of infection (Salminen et al., 2004; Cannon et al., 2005).

The virulence determinants have been detected and well studied in enterococci and streptococci, but the data regarding other LAB is scarce. Several virulence factors were reported in lactobacilli isolated from food, mainly related to adhesion of collagen, aggregation substance and sex pheromones (Todorov et al., 2014; Dimitrov Todorov et al., 2017). The reported virulence factors were detected at a lower frequency in lactobacilli isolated from food, compared to food associated enterococci and clinical isolates. In their study, Dimitrov Todorov et al. (2017) have shown by phenotypic tests that none of the virulence determinants detected in investigated *Lactobacillus* strain were expressed. Many *Lactobacillus* spp. have been granted GRAS status and are considered as safe for human and other animal applications.

It can be concluded that in dry spontaneously fermented sausages LAB do not present a legitimate threat for human health, with the exception of enterococci. The LAB that constitute a part of the natural sausage's microbiota are not pathogenic, while pathogenic LAB, such as streptococci, are usually detected in negligible numbers. Enterococci remain the disputed genera within the LAB, since are associated with various safety issues and can be commonly found in sausages in considerable numbers.

2.6.2. Antibiotic resistance

The intrinsic resistance to antimicrobials is a trait evolved in bacteria long before the worldwide use of antibiotics. Intrinsic resistance is natural (inherent) characteristic of a species or genus. It is spread only by clonal dissemination, therefore pose no risk for non-pathogenic bacteria (Mathur and Singh 2005). For example, enterococci are intrinsically resistant to cephalosporins, low levels of β-lactams, sulphonamides, and low levels of clindamycin and aminoglycosides. Lactobacilli, pediococci and leuconostoc are intrinsically resistant to vancomycin. Some lactobacilli have a high natural resistance to antibiotics such as cefoxitin, kanamycin, gentamicin, streptomycin, teicoplanin (Mathur and Singh 2005). In addition to intrinsic resistance, bacteria possess a remarkable ability to acquire new resistances to antibiotics. Acquired antibiotic resistance (AR) determinants may be transferred between different taxa via horizontal gene transfer (HGT). Although in some bacteria low-level drug resistance leads to the development of high-level resistance, the majority of genes responsible for acquired AR are encoded on mobile genetic elements, *e.g.* conjugative plasmids and transposons. Therefore, mobile genetic elements are accepted as the main sources for dissemination of genes encoding for AR and are the most responsible for the emergence of antibiotic-resistant bacteria (Verraes et al., 2013). Genes can be horizontally transferred between different taxa via transformation (DNA from the environment is taken up in bacterial cells), transduction (bacteriophage-mediated transfer process) or conjugation. Conjugation is the most frequent mechanism involved in HGT among bacterial populations, especially via conjugative plasmids, while transposons are less common (Verraes et al., 2013). In Gram-positive bacteria, two types of conjugative plasmids are known: pheromone-responsive plasmids, exhibiting a narrow host range and mainly described in *E. faecalis*, and pheromone-independent plasmids, which have been found in all bacterial communities and are transferable to a wide host range.

Although the increasing prevalence of genes encoding for AR have usually been associated with clinical isolates, commensal bacteria are acquiring resistances as well, thereby forming a reservoir of AR genes (van den Bogaard and Stobberingh 2000). For instance, fermented food that is not heat treated, such as dry fermented sausages, may act as a reservoir of viable bacteria (and AR genes) and represent a direct route of introduction of those bacteria into human GI tract. In the GI tract, these AR genes can be horizontally transferred from commensal bacteria to pathogens. Among LAB, enterococci are particularly noted to contain multiple antibiotic resistance. Enterococci have acquired resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of β-lactams, fluoroquinolones and

glycopeptides such as vancomycin (Mathur and Singh 2005). Vancomycin resistance is of special concern because this antibiotic was considered as a last resort for treatment of multiply resistant enterococcal infections. Recently, new antibiotics (linezolid, oxazolidinone, daptomycin) have been developed and successfully used for the treatment of vancomycin-resistant enterococci, however, the development of new antibiotics in followed with the occurrence of strains resistant to those antibiotics. Acquired AR has also frequently been detected among lactobacilli isolated from sausages, among which resistance to tetracycline and erythromycin seems to be the most common (Holck et al., 2017). Antimicrobial resistance is a worldwide problem and one of the major challenges in modern medicine. Although AR confers a fitness cost in bacteria, the loss of ABR plasmids is usually very low (Hegstad et al., 2010), even in the absence of antibiotic-mediated selection pressure and AR determinants are often maintained in nature.

2.6.3. Production of biogenic amines

Dry fermented sausages are a protein-rich environment. Breakdown of proteins during fermentation and ripening results in a release of free amino acids. Fermented sausages contain viable bacteria in high numbers, some of which may transform amino acids to biogenic amines in the process of decarboxylation. Biogenic amines are basic, nonvolatile low-molecular-weight, nitrogenous compounds, naturally present in living organisms and are responsible for many essential functions. However, if taken by food in considerable amounts, they may impair health and are related to difficulties with nervous and GI system, as well as allergies (Ruiz-Capillas and Jiménez-Colmenero 2004). In fermented sausages, the most commonly accumulated biogenic amines include tyramine, histamine, cadaverine and putrescine. Of these, histamine and tyramine are the most toxic. Considerable variations in the type and concentration of accumulated amines have been detected in the same product types. Such fluctuations are attributed to many factors, like the diversity and abundance of sausage's microbiota, chemical and physical variables and the availability of precursors. Many undesirable microorganisms present in sausages (spoilage and pathogens) can produce biogenic amines, but also LAB and CNS may substantially contribute to biogenic amines (BA) accumulation thanks to their abundance of sausages. In their review, Holck et al., (2017) have considered health and safety implications of fermented sausages and have indicated Gram-negative contaminant enterobacteria present in the raw material as the most important BA producers before the onset of the fermentation by the LAB. Hence, good microbiological quality of raw meat and a rapid pH reduction in the initial stage of sausage production are essential for inhibition and control of BA production by these contaminants. The authors have indicated *Lb. curvatus* and many enterococci strains as the prominent tyramine producers among

LAB relevant for sausage fermentation. Histamine producers are very rare among sausage LAB and histamine, when present in sausage, is considered to be produced by mainly contaminant enterobacteria. However, the authors have noted that some specific strains, for example, strains belonging to *Lb. buchneri* and *Lb. parabuchneri*, harbour the histidine decarboxylase enzyme, but those species are not dominant in sausage fermentation. Other LAB relevant for sausage fermentation, such as *Lb. sakei* and *Lb. plantarum*, are generally non aminogenic.

The most implicated products in foodborne BA intoxication are fish and cheese, while no cases of BA poisoning have implicated fermented sausages as the cause, although measured amounts of BAs have in some instances reached similar levels as in fish related outbreaks (Holck et al., 2017). The concentration of histamine in fish is regulated at the EU level (Commission Regulation EC 1441/2007), but at the moment, the concentrations of histamine nor tyramine are regulated for dry fermented sausages. In some countries, the concentration of histamine up to 100-200 mg/kg is considered acceptable (Karovičová and Kohajdová 2005). Suzzi and Gardini (2003) have reviewed the concentration of tyramine in various fermented sausages and have reported concentrations of 14.00-332.10 mg/kg. In Croatian traditional dry sausage (Slavonian kulen), histamine and tyramine have been detected at a concentration of 330.8 mg/kg±126.3 and 233.9 mg/kg±124.7, respectively (Karolyi, 2011). The maximal tolerable level of tyramine in food is approximated to 100-800 mg/kg, while the concentration of 1,080 mg/kg is toxic for people (Ercan et al., 2013). Latorre-Moratalla et al. (2017) have estimated the risks of histamine and tyramine intake, related to the consumption of dry fermented sausages by the Spanish population. The authors have pointed out that the mean dietary exposure to tyramine and histamine was 6.2 and 1.39 mg/ meal, respectively. For tyramine, the safe threshold considered for the healthy population was 600 mg/meal/person, while for histamine it was 25 mg/meal/person. Therefore, the authors have concluded that the risk of intoxication by a healthy population due to their intake exclusively from dry fermented sausages, can be considered negligible.

2.7. The diversity and role of beneficial non-LAB microorganisms in sausage production

Members of the family *Micrococcaceae*, such as coagulase-negative staphylococci (CNS), *Micrococcus* spp. and *Kocuria* spp. participate in desireable reactions during sausage fermentation. Compared to the facultative anaerobic CNS, the activity of aerobic *Micrococcus* spp. and aerobic/facultative anaerobic *Kocuria* spp. is limited to the initial stages of fermentation and outer parts of sausages.They are all poorly competitive in the

presence of actively growing aciduric bacteria such as LAB and they rarely grow to more than 1 log cfu/g during ripening (Leroy et al., 2006). They can reduce nitrates to nitrites and are catalase-positive; thus they contribute to the stabilisation of colour and taste of sausages. Particularly, CNS have a major role in the development of sensory properties of fermented sausages due to their proteolytic and lipolytic activity (Leroy et al., 2006). Among them, *S. xylosus* and *S. carnosus* dominate the indigenous microbiota of sausages, although *S. eqourum*, *S. saprophyticus S. equorum, S. succinuss, S. warneri, S. vitulinus, S. pasteuri, S. epidermidis, S. lentus, S. haemolyticus, S. intermedius, S. saprophyticus, S. hominis, S. auricularis* etc. can also be isolated from fermented sausages, but with lower frequency (Milicevic et al., 2014). CNS associated with sausage production are mostly nonpathogenic and do not represent a hazard to consumers' health. They grow in the presence of high concentration of salts and lower water activity (a_w), the typical conditions present in sausages, but are sensitive to acids and have the tendency to die at lower pH values during and after the process of fermentation. So, they are active and contribute to sensory traits of sausages before fermentation, *i.e.* before LAB become the dominant microbiota (Cukon et al., 2012).

The activity of yeasts and moulds affects the development of the characteristic taste of the sausages, as well as their characteristic appearance. At the beginning of production, yeasts prevail on the surface of the sausages, after 2 weeks moulds and yeasts are equally present, and yeasts prevail again towards the end (4-8 weeks) (Cukon et al., 2012). Yeasts are present in lower numbers with respect to *Micrococcaceae,* particularly CNS, but can reach counts up to 6 log cfu/g at low ripening temperatures (Cocolin et al., 2006). They contribute to sensory traits of sausages by increasing the quantity of ammonia, decreasing the quantity of lactic acid and through proteolytic and catalase activity (Cukon et al., 2012; Milicevic et al., 2014). *Debaryomyces hansenii* represents the species most often isolated from fermented sausages (Cocolin and Rantsiou 2012). Moulds have an antioxidative effect, protect from rancidity and influence the development and maintaining the colour through catalase activity. As being strictly aerobic organisms, they grow exclusively on the surface of sausages where they prevent spontaneous colonisation of unwanted microorganism, they exhibit lipolytic and proteolytic activity as well as give sausages a typical appearance (Cukon et al., 2012). Due to the utilisation of acids and ammonia release as a consequence of protein degradation, the pH of such mould-ripened products is higher than of those without a microbiota (Cook 1995). Moulds can also negatively impact the sausages, by causing of green, brown or black spots which are unacceptable to most consumers, and some moulds can have a negative influence to taste and odour (Cukon et al., 2012). The most dangerous effect of some moulds is a

synthesis of mycotoxins which are toxic to humans and animals in small concentrations (Cukon et al., 2012). The most common species in the fermented sausages belong to the genera *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium* (Milicevic et al., 2014). Ripening period has s considerable effect on the development of the sausages' microbiota. Moulds are associated with the sausages with longer ripening period. In such sausages, CNS and yeast are also found in higher numbers, which leads to accumulation of different volatile compounds by their activity, whereas the sausages with a short period of ripening have more lactobacilli and a souer taste, especially if the ripening period is shorter than two weeks.

2.8. Spoilage and pathogenic microbiota associated with fermented sausages

Although meat is a favourable substrate for bacterial growth, the harsh conditions during fermentation of sausages, such as low pH and a_w values alongside with high salt concentration, disrupts the growth of many pathogenic bacteria. In fully ripened sausages, usually, the pH values vary between 5.2 and 5.8, a_w between 0.85 and 0.91 (Vignolo et al., 2010), and salt concentration between 2-4 % of the product. According to the United States Food and Drug Administration (USFDA, 2001), the pH value of 4.2 inhibits the growth of vegetative pathogens, while 4.6 inhibits sporulating pathogens. Majority of the pathogenic species are inhibited by a_w 0.86 or less (USFDA, 2001). *S. aureus* is an exception since they can grow at pH=4.0, at a_w 0.83 and produce toxins at a_w 0.88 (NSW Food Authority, 2008). Additionally, they tolerate salt concentrations of above 10 %. Elevated numbers of *S. aureus* have been reported in ready-to-eat game meat sausages (Marty et al., 2012; Markov et al., 2013), and staphylococcal food poisoning outbreak has been linked to smoked sausages (Huang et al., 2006). Staphylococci are most commonly introduced to meat batter or sausages by humans during meat processing and sausage production. Infected individuals experience the symptoms typical for food poisoning (vomiting, nausea, stomach cramps, diarrhoea). *S. aureus* and *E. coli* are the most resilient bacteria to conditions present during sausage fermentation and are considered as the most hazardous. Usually, meat contamination with *E. coli* occurs from the GI tract during slaughter or hunting and further processing. Some strains can grow at pH=4.4–9.0 and the a_w value 0.95. Shigatoxin producing *E. coli* are of major concern, especially in undercooked or non heat-treated products, because they may cause severe bloody diarrhoea or hemolytic uremic syndrome.

Listeria monocytogenes can cause infections called listeriosis. Healthy adults and children rarely become seriously ill from *Listeria* and develop mild symptoms typical for food

poisoning, while in immunocompromised individuals or embryos it can invade the central nervous system and cause meningitis. *L. monocytogens* can grow even at refrigeration temperatures, in an environment with high salt concentration (25 %), within a wide pH range (4.40–9.40), but they require an a_w value of 0.92 for growth. They are usually not present in dry fermented sausages after 3 weeks of fermentation (Työppönen et al., 2003).

Several *Salmonella* spp. outbreaks and infections via sausage consumption have been reported (Ethelberg et al., 2009; Bremer et al., 2004; Gossner et al., 2012). The main habitat of *Salmonella* spp. is the GI tract of animals, and meat is also considered to be a source of this bacteria. They grow at pH range from 3.8–9.5, but a minimum required a_w value is 0.93. The severity of the infection depends upon the serotype and the immune state of the individual.

Bacillus cereus belongs to a group of closely related aerobic spore-forming species, which is being referred to as the *B. cereus* group (Rajkovic et al., 2008). They are ubiquitous in the environment and abundant in soil, so they can contaminate the meat of wild animals at the time of hunting or evisceration when the meat is in close contact with soil. *B. cereus* can cause two types of food poisoning: emetic and diarrhoeal. The emetic type is caused by a heat stable toxin (cereulide) and it has been associated with life-threatening conditions such as fulminating liver failure and rhabdomyolysis (Rajkovic et al., 2008). A more mildly, the diarrhoeal type is caused by heat-unstable enterotoxins produced by vegetative cells, and this type is often associated with meat and meat products (Rajkovic et al., 2008). Bacilli can produce the diarrhoeal enterotoxins in the temperature range of 10-43 °C (Kramer and Gilbert 1989) and at a pH range between pH=5.5-10 (Sutherland and Limond 1993). This bacteria can tolerate up to 7.5 % salt, which enables it to grow in dry fermented sausages.

Clostridia spores are also ubiquitously present in the soil, as well as in the intestines of the animals from where they may contaminate various foods including meat products. While several *Clostridium* species can cause meat spoilage, toxin-producing species *C. botulinum and C. perfringens* can cause mild-to-fatal food poisoning. However, the conditions present during the ripening and storage of sausages do not support the growth of clostridia. *C. botulinum, C. perfringens* and other clostridia are controlled by the pH and water activity and do not represent a hazard for fermented sausages (Lücke 2000)

Campylobacter spp. are common enteric pathogens with *C. jejuni* and *C. coli* being most frequent in foods. The most common symptom of *Campylobacter* infection is diarrhoea,

which is often bloody. Complications from *Campylobacter* food poisoning are unlikely to occur. Although the meat of different animals is prone to contamination, the presence of *Campylobacter* in sausages is rare (NSW Food Authority 2008). Their optimum growth temperature ranges from 37 to 42 °C, the pH=4.9-8.0, but are sensitive to high concentrations of salt and drying, and require an a_w value above 0.91, which inhibits their growth in dry fermented sausages.

Especially if produced traditionally, game meat sausages can often have elevated numbers of pathogenic bacteria, particularly, *S. aureus* and intestinal bacteria, and therefore may pose a health hazard (Marty et al., 2012; Markov et al., 2013).

2.9. Functional meat starter cultures

According to Hammes et al. (1990) meat starter cultures are preparations that contain active or dormant microorganisms of known taxonomic identity that develop the desired metabolic activity in meat and cause changes in the sensory properties of food. Majority of commercially available starter cultures are comprised of a mixture of the LAB, usually *Lactobacillus* spp. and *Pediococcus* spp. (Demeyer and Toldrá 2004) with staphylococci and/or micrococci. Some moulds, such as *Penicillium nalgiovense*, *Penicillium chrysogenum*, *Penicillium camemberti* and *Penicillium gladioli*, as well as the yeast *Debaryomyces hansenni*, are commonly used as starter cultures (Milicevic et al., 2014). The main role of LAB strains as starter cultures is the rapid acidification of meat batter, while other components of the starter culture are usually more involved in other processes leading to the development of sensory traits and/or enhancing food safety. Due to rapid acidification, classic starter cultures can also be considered as bioprotective cultures, especially with respect to acid-sensitive microbiota. However, some strains may provide additional protective action, *e.g.* by producing bacteriocins, or may exhibit an additional probiotic or other functionality, so strains combining these traits have been defined as functional starter cultures (Kröckel 2013). Ammor and Mayo (2007) have rewieved and proposed the selection criteria for the LAB to be used as functional starter cultures in dry sausage production. According to their work, starter cultures have to be homofermentative and able to rapidly produce lactic acid at an amount sufficient to decrese pH < 5,1. The strains have to be competitive with natural raw meat microbiota, *i.e.* have to be able to survive and maintain metabolic activity in an anaerobic atmosphere, high salt concentration (2 – 10 %), low temperatures (2 – 24 °C) and low pH (4.2 – 6). Starter cultures have to show good catalase, proteolytic and lipolytic activity, they have to reduce nitrates to nitrites and tolerate other microbial components of the starter culture. Also, they must not be pathogenic nor show toxic activity, must not have amino decarboxylase

activity to avoid accumulation of biogenic amines and must not posess genes encoding for antibiotic resistance to avoid the horizontal gene transfer. Microbial diversity of traditional products represents the preferred source of wild strains that are prevalent in spontaneous fermentation and which, in comparison with commercial starters, usually have a higher metabolic activity and are more competitive (Leroy et al., 2006). For instance, Frece et al., (2014) have compared the effects of indigenous strains isolated from traditional sausages and the effects of commercial starters on the quality of industrial sausages. Results of this research have not only shown that indigenous strains have the ability to survive even during industrial production, but, compared with commercial starter, they have also displayed better results in the development of sensory properties, stability and microbiological safety of sausages. In addition, bearing in mind that commercial starters are not equally efficient in all types of sausages, there is a need for selection of appropriate strains that can maintain the necessary abundance and metabolic activity in a certain mixture of meat and for certain method of fermentation.

2.10. Bioprotective cultures

Vogel et al. (2011) have defined bioprotective cultures as "preparations consisting of live microorganisms (pure cultures or culture concentrates) that are added to foods with the aim of reducing risks by pathogenic or toxigenic microorganisms". They express their protective effect *via* metabolic pathways, as starter cultures in the classical sense do as well, but their primary purpose is improving food safety (Vogel et al., 2011). Such cultures can have bioprotective effect by competition for nutrients and binding sites with other bacteria and by the formation of antagonistically active substances, such as organic acids, ethanol, H_2O_2, CO_2, bacteriocins, antibiotics, or other antimicrobial effective substances (Vogel et al., 2011). Certain strains of LAB involved in sausage fermentation can produce bacteriocins, the antibacterial compounds that constitute a heterologous subgroup of ribosomally synthesised antimicrobial peptides. Most of the LAB strains used for biopreservation of sausages are selected due to their antilisterial activity, but other targeted microorganisms include *Enterobacteriaceae*, *Salmonella* spp., *S. aureus*, *Clostridium* spp., *E. coli* and *Yersinia enterolitica* (Oliveira et al., 2018). In some cases, the bacteriocin-producing strains are not able to survive during sausage production, so alternatively, bacteriocins can be extracted and added. In EU the use of bacteriocins in food products must be labelled as additives. Although the use of bacteriocin preparations in food have been extensively explored as the alternative of using bacteriocin-producing strains, the only commercially produced bacteriocins to date are nisin, produced by *Lactoccocus lactis*, and pediocin PA-1, produced by *Pe. acidilactici* (Oliveira et al., 2018).

2.11. Probiotics in fermented sausages

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (WHO/FAO, 2002). The main prerequisite for bacteria to be used as a probiotic culture is the ability to survive the passage and thrive in the host GI tract, where they positively impact health in terms of improving the microbial balance. Of special importance for probiotic strains are bile and acid tolerance, as they have to survive the passage through the stomach, where very acidic conditions prevail and the bile concentrations at the beginning of the small intestine to reach the human intestine. Most of the microorganisms used as probiotics belong to the *Lactobacillus* and *Bifidobacterium* genera, although the probiotic traits of other microorganisms with the QPS status (Qualified Presumption of Safety), such as *Lactococcus*, *Enterococcus*, *Sacharomyces*, *Propionibacterium*, *Bacillus* has also been reported (EFSA, 2007). Even though until recently probiotics were primarily associated with dairy products, it has been shown that the sausage matrix represents a suitable environment for the transmission of the probiotic strains (Rubio et al., 2014). Many studies have therefore focused on testing of the survival ability of the potential probiotic strains during sausage fermentation and in the host GI tract, as well as their functional characteristics and safety aspects (Ruiz-Moyano et al., 2008; Nogueira Ruiz et al., 2014). For example, Frece et al. (2010) have shown in an *in vivo* study that the *Lb. plantarum* 1K isolated from Slavonian kulen was able to establish the disturbed balance of the intestinal microbiota of a mice and therefore had a potential to be used as a probiotic.

2.12. Methods used for identification of the LAB

2.12.1. Culture-dependent methods

2.12.1.1. Phenotypic and biochemical methods

The conventional microbiological methods for identification of bacteria are based on morphological and physiological characteristics such as Gram staining, cell shape, spore formation, enzyme production, the fermentation of different carbohydrates *etc*. Such tests are usually easy to perform, and the results are obtained quickly. However, many LAB have similar nutritional and growth requirements, so phenotypic and biochemical methods are often not sufficient for their accurate and reliable identification. The most commonly used biochemical tests include the API system (BioMerieux, Marcy l'Etoile, France) and Biolog (Biolog, Hayward, USA). Both systems are based on the fermentation patterns of the microorganisms. The API used for LAB identification provides the fermentation patterns of 49 carbohydrates and esculin hydrolysis, whereas the Biolog is a unique plate

used for Gram-positive and Gram-negative bacteria and it analyses the fermentation of 96 carbohydrates. Although both systems can identify microorganisms with high certainty, they are often performed alongside with molecular-based methods for conclusive identification. Nowadays, molecular methods are considered to be the most reliable for identification of microorganism, whereas conventional microbiological methods are more used for characterisation of isolates, rather than for their identification.

2.12.1.2. Molecular-based methods

The development of molecular-based methods represents the driving force to constant taxonomical revisions in microbiology. Particularly, the DNA-DNA hybridisation, molecular technique that measures the degree of genetic similarity between pools of DNA sequences, has been used extensively in phylogeny and taxonomy. Although it is to be expected that whole genome sequencing will be the standard method in the future (Holzapfel and Wood 2014), DNA-DNA hybridisation is currently the gold standard to distinguish between bacterial species. A similarity value greater than 70 % indicates that the compared isolates belong to the same species, which corresponds to the 97 % similarity obtained by Sanger sequencing of 16S rRNA gene, a method routinely used for bacterial identification. The 16S rRNA gene, a well-conserved universal marker with constant and highly constrained functions that are relatively unaffected by environmental pressures, is the most commonly used gene for identification (Mohania et al., 2008) and it is the basis of current taxonomy. However, since 16S rRNA gene is so well conserved, sometimes the species or genus-specific PCR assays based solely on amplification of that gene can have limited discriminatory power. Different species of the LAB have been found to share nearly identical 16S rRNA gene sequences. Therefore, sequencing of several other genes has been evaluated for the identification of LAB. For instance, identification of enterococci using the D-alanine:D-alanine ligases (*ddl*) partial gene sequences were described (Ozawa et al., 2000). Similarly, RNA polymerase alpha subunit (*rpoA*) and phenylalanine-tRNA synthase (*pheS*) gene sequence analyses have been shown as reliable identification tools (Naser et al., 2005), as well as the manganese-dependent superoxide dismutase (*sodA*) gene (Poyart et al., 2000; Jackson et al., 2004).

Analysis of whole-cell protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) also represents a suitable identification tool. The method is a variant of polyacrylamide gel electrophoresis, for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate molecules to help identify and isolate protein molecules. It provides species-

specific fingerprints corresponding to DNA-DNA hybridisation results, which enables direct identification and differentiation of LAB.

Ribotyping refers to the use of nucleic acid probes to recognise ribosomal genes (Mohania et al., 2008). It is a rapid and specific method widely used in the analysis of microbial communities in food. Bacterial chromosomal DNA is subjected to enzymatic restriction and obtained DNA fragments are then separated in agarose gel electrophoresis, followed by Southern blotting, where the DNA is transferred to a membrane and hybridised with 23S and/or 16S rRNA probes. The discriminatory power of this method depends upon the size of the probe and upon the enzyme selection (restriction endonuclease). Although the method was proven to be useful for differentiation of certain LAB at the strain level (Giraffa and Neviani 2000), ribotyping shows high discriminatory power at the species level rather than on the strain level (Mohania et al., 2008).

Matrix-assisted laser desorption／ionization time-of-flight mass spectrometry (MALDI-TOF MS) enables the identification of microorganisms *via* the generation of fingerprints of highly abundant proteins, including many ribosomal proteins, which are assumed to be characteristic for each bacterial species. The fingerprints are compared to the reference spectra in a database. MALDI-TOF MS was mainly used for the identification of food associated pathogens but had recently emerged as a powerful, fast, reliable and cost-effective method for the identification of LAB associated with the production of fermented food (Doan et al., 2012). Multiple aspects of the applicability of MALDI-TOF to food microbiology have been reported, ranging from the classification of the LAB in fermented meat and the surveillance of probiotics in yoghurt, to strain identification and characterisation of biogenic amine-producing bacteria (Pavlovic et al., 2013).

While identification of LAB at the species level is crucial in almost every food microbiology analysis, the performance of certain LAB, for example, as a starter or probiotic culture is strain-dependent. Therefore, it is essential to differentiate LAB strains. Some of the most notable techniques to differentiate LAB at the subspecies or strain level include pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), repetitive extragenic palindromic PCR (rep-PCR) and random amplification of polymorphic DNA (RAPD). Although not extensively used with LAB, in clinical microbiology PFGE is considered as the gold standard for bacterial typing and epidemiology. In a PFGE analysis, genomic DNA is subjected to enzymatic restriction, and the fragments are separated in an alternating field of electrophoresis, with increasing pulse times throughout the run. PFGE has been shown to differentiate strains belonging to the same LAB

species, to group strains within a species, to distinguish between strains of different LAB species, and to place isolates in specific *Lactobacillus* species (Mohania et al., 2008). PCR based techniques are broadly used for genotyping of LAB isolated from food, where the target sequence can be a repetitive sequence motif (rep-PCR), or a random sequence (RAPD). Therefore, primers used in a rep-PCR reaction must be specific for the repetitive extragenic sequences present in multiple copies across the bacterial genome. In a RAPD analysis, primers are arbitrarily selected, and the segments of DNA that are amplified are random. These methods have been extensively used for characterization of LAB communities isolated from artisan sausages (Baruzzi et al., 2006; Tran et al., 2011) and artisan cheeses (Mrkonjic Fuka et al., 2017; Van Hoorde et al., 2008) as well as other fermented products such as vegetables (Kingston et al., 2010). RAPD and rep-PCR have also been used to follow the progress and competitiveness of LAB strains applied as starter cultures in various fermented foods (Ravyts et al., 2008; Plengvidhya et al., 2004). A disadvantage of those methods is their variable reproducibility.

Molecular-based techniques provide a reliable tool for the identification of LAB isolated from various complex environments, including fermented foods.

2.12.2. Culture-independent methods

The limitations resulting from cultivation biased methods can be overcome with new approaches in culture-independent molecular methods. Such methods are based on a direct extraction of a bacterial DNA or RNA from foodstuffs, without the need for enrichment and isolation. Individual members of a complex microbial population can be identified, and quantitative methods can provide a reliable evaluation of microbial abundance. Some of the most relevant and long-established culture-independent methods used in food microbiology include denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qRT PCR), whereas next-generation sequencing (NGS) provides a novel and fast tool for the direct sequencing of foodstuffs.

The general principle of DGGE is the separation of individual rRNA genes based on differences in their melting temperature. The bacterial DNA is first amplified using bacterial primers specific for a variable region of the rRNA gene, resulting in PCR products of the same length but with different, species-specific sequences. Polyacrylamide gels used in a DGGE analysis consists of a linear denaturing gradient formed by urea and formamide, which allows the separation of obtained PCR products and produces a fingerprint of the bacterial species. DGGE is highly sensitive, and it allows the detection of low abundant populations which may be lost during traditional analysis. The analysis was used for identification and monitoring the bacterial population dynamics

in various fermented sausages (Fontana et al., 2005; Cocolin et al., 2001). DGGE can also serve as a culture-dependent method if used for the analysis of bulk colonies (consortia) cultivated on microbiological media (Skelin et al., 2012).

Quantitative real-time PCR (qRT-PCR) detects fluorescence emitted by certain dye-containing molecules that bind to double-stranded DNA, such as SyberGreen. The DNA can also be labelled with fluorescent oligonucleotides (*e.g.Taq*Man fluorescence probes). The level of measured fluorescence corresponds to the level of produced PCR products and initial DNA concentration. In food microbiology, qRT-PCR is commonly used for the detection and quantification of opportunistic pathogens (Martínez et al., 2011). Its applications also include monitoring of the presence of antibiotic resistance genes in food, identification of GMOs, the detection of allergens and food ingredients (Martínez et al., 2011). The method provides an important tool for determination of the proportion of meat fractions in meat products (Köppel and Rentsch 2011), which is important to check if any alternations of the original food composition have been made. Besides the economic impact on the food industries, the substitutions of food components may have medical, ethical, religious and cultural consequences. In recent years qRT-PCR have also been used for quantitative detection of the LAB in food, including meat and meat products (Martín et al., 2006; Elizaquível et al., 2008; Mrkonjić Fuka et al., 2013).

NGS techniques have high-throughputs and produce thousands or even millions of sequences at the same time. These sequences provide detailed information about the presence and quantity of microorganisms. NGS technologies can be used for sequencing of the total microbial nucleic acids (shotgun sequencing), which can provide information about the number and potential function of genes within the community. Another approach is gene-specific sequencing (targeted sequencing), where segments of highly conserved DNA or cDNA sequences are first amplified by PCR using universal or group-specific primers. Several NGS platforms have been developed, with different engineering processes, sequencing chemistry, output (length of reads, number of sequences), accuracy and cost (Mayo et al., 2014). Current commercial platforms include the 454 (Roche), Illumina (Illumina), SOLiD and Ion Torrent (Life Technologies), and PacBio (Pacific Biosciences) systems (Mayo et al., 2014). The Illumina NGS workflows are described by Anonymus (Illumina, 2017). Workflows include four basic steps: library preparation, cluster generation, sequencing and data analysis. The sequencing library is prepared by random fragmentation of the DNA or cDNA sample, followed by 5′ and 3′ adapter ligation. Adapter-ligated fragments are then amplified in a PCR reaction and purified. For cluster generation, the library is loaded into a flow cell where fragments are

captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification. When cluster generation is complete, the templates are ready for sequencing. Sequencing is performed by a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands. During data analysis and alignment, the newly identified sequence reads are aligned to a reference genome. Following alignment, many variations of analysis are possible, such as single nucleotide polymorphism or insertion-deletion identification, read counting for RNA methods, phylogenetic or metagenomic analysis *etc*. The NGS analysis of nucleic acids from food samples requires optimization of each of the previously noted workflow steps, as well as nucleic acid extraction and purification. Foodstuffs vary in composition and associated microbiota, so the extraction protocols need to be carefully adapted for each food matrix. Inadequate extraction can provide unrealistic views of a food microbial community; also if food components, such as lipids and proteins have not been removed they can inhibit PCR reactions (Mayo et al., 2014).

NGS technologies have found a broad application in analyses of food, especially fermented foods. Various studies used NGS to describe microbial diversity in fermented meat products. For example, Połka et al., (2015) described the bacterial diversity in Italian salami at different ripening stages by 16S rRNA amplicon high throughput sequencing (HTS). In a study performed by Fontana et al. (2005), results obtained by HTS of 16S rRNA amplicons showed that the production technologies (artisanal vs pilot-plant scale) influenced the development of microbial communities in naturally fermented llama sausages, with a different composition through the whole fermentation process. NGS techniques could help to fill the knowledge on relationships between microbial diversity and sensorial and safety properties of food (Mayo et al., 2014). Subdominant and minority populations, which have been shown to vary widely between producers and batches, could contribute to generating, specific key components of aroma and taste (Mayo et al., 2014). Also, the isolation of strains belonging to newly discovered taxa will ultimately allow their functional characterisation and possibly their future use as starters (Mayo et al., 2014).

3. MATERIALS AND METHODS

3.1. Sausage production and sampling

Three types of wild boar meat sausages (WB) and three types of deer meat sausages (DS) were produced by five independent local sausages producers (Table 3.1.) in the continental part of Croatia. The sausage samples were collected in triplicates from each of the sausage producers (*n*=105) at day zero (0), after 4, 7, 10, 20 days and at the end of the ripening period (20 or 40 days). The sausages were produced as a mixture of wild boar (*Sus scrofa*) or deer (*Cervus elaphus*) meat and domestic pig (*Sus scrofa domesticus*) meat in the 1:1 ratio, without the application of starter cultures and nitrites. The lean meat and back fat (from domestic pig) ratio was approximately 70:30. The sausages were made with the addition of ingredients in varying amounts: salt (2.0–2.2), white wine (1 %), ground fresh garlic (0.3–0.4 %), ground dry red chili peppers (0.3 %), ground dry black peppers (0.1–0.2 %) and ground dry red sweet peppers (0.1 %). The meat was minced to a particle size of 8 mm and fat to 6 mm. After mixing of ground meat and fat with other ingredients, the mixture was filled into artificial collagen casings with a diameter of 35 mm (WB1, WB2, DS2, DS3) or in natural casings of 38 mm (WB3, DS1). Air temperature and relative humidity were monitored during production using data logger Log 32 (Dostmann electronic GmbH, Reicholzheim, Germany) with a measurement interval of 1 h, and the results were expressed as a daily average. The sausages were ripened in a drying chambers in a traditional way of production under varying relative humidity from 52 to 92 % (higher at the beginning) and temperature from −3 to 15 °C (lower at the beginning) by simultaneously smoking with cold smoke in breaks during the first two weeks.

Table 3.1. Overview of the meat recipe, production site, type of sausage and casings used in this study.

Meat mixture used for sausage production	Production site	Type of sausage	Origin and diameter (mm) of used casings
Wild boar and domestic pig meat, 1:1 ratio	A	WB1	Artificial, 35
	B	WB2	Artificial, 35
	C	WB3	Natural, 38
Deer and domestic pig meat, 1:1 ratio	C	DS1	Natural, 38
	D	DS2	Artificial, 35
	E	DS3	Artificial, 35

3.2. Physicochemical analyses

Water activity (a_w) and pH were measured at different time points (0, 4, 7, 10, 20 and 40 days). The water activity was measured using Rotronic HygroPalm HP23-AW-A (Bassersdorf, Switzerland) and the pH using a pHmeter IQ 150 (IQ Scientific Instruments, Carlsbad, USA) equipped with the spear type electrode BlueLine 21 pH (Schott AG, Mainz, Germany) inserted directly into the sausage samples. Mean values and standard deviations of three independent measurements were calculated for each sample.

3.3. Histamine and tyramine determination

Histamine and tyramine content in final products were determined by the application of high-performance liquid chromatography (HPLC). The analysis was performed at the Andrija Stampar Teaching Institute of public health, Division of Food and Consumer Goods Safety and Quality. Extraction and derivatisation of biogenic amines from the sausages were performed in triplicates as described by (Eerola et al. 1993). An HPLC (1100 Agilent Technologies) equipped with a binary pump, an auto sampler and diode array detector was used. Mobile phases consist of (A) 0.1 M ammonium acetate and (B) acetonitrile. Gradient elution was applied at flow rate 0.8 mL/min. Separation of biogenic amines was carried out using a Luna C-18 (Phenomenex, Torrance, USA) column (250 mm × 4.6 mm, 5 µm particle size) with a Phenyl guard column (4.0 × 3.0). The column was thermostated at 40 °C. The injection volume was 20 µL. Compounds were detected and quantified at 254 nm.

3.4. Microbiological analyses

Samples of each sausage (10 g) were taken (without casing) and homogenised for 1.5 min with 90 mL of sterile peptone water (0.1 %) using a Stomacher Lab-Blender 400 (Seward Medical, Worthing, UK). Serial dilutions were performed, and appropriate dilutions of homogenized samples were inoculated in growth media, in duplicates, for the detection of particular microbial groups. The detailed media preparation instructions are shown in Appendix 7.1. For the determination of aerobic plate counts, *Staphylococcus aureus*, *Bacillus cereus*, yeasts and moulds, 100 µL of appropriate dilutions were transferred and spread to selective microbiological media incubated under aerobic conditions. Aerobic plate counts were estimated on Plate Count Agar (PCA; Merck, Darmstadt, Germany), after incubation at 30 °C for 3 days. *Staphylococcus aureus* was determined on Baird-Parker Agar (BP; Labo-Life Sàrl, Pully, Switzerland) after incubation at 37 °C for 48h. *Bacillus cereus* group was determined on Mannitol Egg Yolk Polymyxin Agar (MYP; Biolife, Milano, Italy) after incubation at 30 °C for 48 h. Presumptive colonies were confirmed as *B. cereus* group after observed hemolytic activity on Columbia Blood Agar (Biolife). Yeasts and moulds were enumerated on Dichloran Rose-Bengal Chloramphenicol Agar (DRBC; Biolife) after incubation at 25 °C for 5 days according to ISO 21527-2:2008. For the enumeration of *Enterobacteriaceae*, *E.coli* and coliforms, 1 mL of appropriate dilutions were transferred to sterile Petri dishes and to each dish 25 mL of sterile media cooled down to 50 °C was added. *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (VRBG; Biolife) after incubation at 37 °C for 24 h according to ISO 21528–2:2004. From each plate, 10-20 colonies were randomly selected and subcultured on Nutrient Agar (Merck) to obtain pure cultures. Subsequently, each of the selected colonies was inoculated in 10 ml of Purple Glucose Agar and incubated at 37 °C for 24 h to screen for glucose fermentation. Colonies were confirmed as *Enterobacteriaceae* if they were able to ferment glucose, which was seen as a change of media colour from purple to yellow. According to ISO 4832:2006, blue colonies grew on Chromocult Coliform ES Agar (CCA; Biolife) after incubation at 37 °C for 24 h were enumerated as *E. coli*, while pink/red colonies were enumerated as coliforms. From each plate, 10 colonies were randomly selected and inoculated in 10 ml of Lauryl sulfate broth (Merck) containing Durham tubes and supplemented with fluorogenic compound MUG (Merck), followed by an incubation at 37 °C for 24 h. Gas formation and turbidity indicated growth of coliforms, while for *E. coli* fluorescence under UV light was additionally observed. Only plates with the number of grown colonies within counting limits were taken into consideration, and bacterial counts were revised accordingly to the confirmation test results. Generally, counting limits were between 10 and 300 grown colonies per plate,

except for *B. cereus* and yeasts, where the limits were between 10 and 150. The bacterial counts were expressed as log cfu/g. All grown colonies were counted on PCA plates, while on other plates only colonies characteristic for a particular microbial group were considered.

The presence of *Listeria monocytogenes* and *Salmonella* spp. in sausage samples was investigated according to ISO 11290 1:1996 + A1:2004 and ISO 6579:2002 + A1:2007, respectively. For the screening of *L. monocytogenes*, 25 g of sample was homogenised for 60 seconds with 225 ml of Listeria Fraser Broth Half Concentration (Biolife) and incubated at 30 °C for 24 h. Using inoculation loop, homogenised samples were streaked onto Oxoid Chromogenic Listeria Agar (OCLA; Oxoid, Wesel, Germany) and PALCAM (Biolife) plates in triplicates, while 0.1 ml was transferred to 10 ml of Listeria Fraser broth (second enrichment) (Biolife). Inoculated media were incubated at 37 °C for 48h. After incubation, a Fraser broth inoculum was streaked on OCLA and PALCAM plates (37 °C for 48 h). For screening of *Salmonella* spp., 25 g of sample was homogenised in 225 ml of peptone water. After incubation at 37 °C for 24h, 0.1 ml was transferred to 10 ml of Rappaport Vassiliadis Broth (RVS; Biolife), and 1 ml was transferred to 10 ml of Muller-Kauffmann Tetrathionate Novobiocin Broth (MKTTn; Biolab, Budapest, Hungary). RVS broth was incubated at 41.5 °C for 24 h and MKTTn broth at 37 °C for 24 h. Both RVS and MKTTn inocula were streaked onto Xylose Lysine Deoxycholate Agar (XLD; Biolab) and Salmonella Detection and Identification Agar (SMID; BioMérieux, Marcy-l'Étoile, France), incubated at 37 °C for 24h. Only characteristic colonies were considered.

3.5. Isolation and enumeration of LAB

Appropriate dilutions of homogenised samples (100 µL) were transferred and spread in duplicates to microbiological media selective for lactic acid bacteria (LAB). The media preparation instructions are shown in Appendix 7.1. Mayeux, Sandine, Elliker Agar (MSE; Biolife) was used for the growth of *Leuconostoc* species, while for the growth of lactobacilli De Man Rogosa Sharpe Agar (MRS; Sigma-Aldrich, Darmstadt, Germany) and LamVab media (Hartemink et al., 1997) were applied. Inoculated media were incubated under anaerobic conditions for 72 h at 30 °C. Enterococci were enumerated on Kanamycin Esculin Azide Agar (KAA; Biolife) incubated at 37 °C for 48 h. Only characteristic colonies were counted, and only plates with between 10 and 300 grown colonies were considered. After the enumeration of the plates, 10–15 colonies were randomly selected from each media and purified. Collected isolates were screened by Gram-staining and catalase-test (3 % H2O2), and stored as liquid cultures with glycerol (20 %) as cryoprotectant at -80 °C.

Additionally, MRS plates were washed with a sterile saline solution to collect consortia (bulk colonies), as described in section 3.12.1., which was used in a DGGE analysis.

3.6. Template DNA extraction

From collected LAB isolates and bulk colonies, template DNA was isolated by Wizard Genomic DNA Purification Kit (Promega, Madison, USA), following the manufacturer's instructions.

3.7. Fingerprinting and molecular identification of LAB

3.7.1. Fingerprinting of LAB by rep-PCR

All collected LAB isolates were straind by the repetitive (rep)–PCR with the $(GTG)_5$ primer (Švec et al., 2005) as described by Domig et al. (2014). Reactions were performed in a final volume of 25 µL, and all used reagents are shown in Table 3.2. Primer sequence was as follows: 5'GTGGTGGTGGTGGTG'3. PCR was performed at 95 °C for 7 min, 30 cycles of 90 °C for 30 s, 40 °C for 1 min, 65 °C for 8 min, and a final elongation step at 65 °C for 16 min. PCR reactions were carried out in a ProFlex PCR system (Applied Biosystems, Foster City, USA).

Table 3.2. Reagents used in a rep-PCR reaction.

Reagents	Initial volume (µL)	Initial concentration /	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	2.5	10 X	1 X
dNTP	1	10 mM	0.4 mM
Primer $(GTG)_5$	1	50 pmol/µL	2 pmol/µL
MQ water	18.5	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	1	2 U/µL	0.08 U/µL
DNA	1	n.a.	n.a.

n.a.=not applicable

Obtained rep-PCR products were separated by horizontal gel electrophoresis on a 2 % agarose gels. Wells were loaded with 4 µL of PCR products, mixed with 1 µL 6 X Loading Dye (Invitrogen, Waltham, USA). To the first, last and central well, 5 µL of DNA Ladder 100 bp plus (AppliChem, Darmstadt, Germany) was loaded. Electrophoresis was run for 1h and 50 min at 80 V. The gels were stained for 20 min in ethidium bromide and destained for 10 minutes in 1 X TAE buffer. Staining and destaining solutions were always prepared fresh and were discarded after 2 uses (2 gels). Gels were photographed under

UV illumination. The obtained rep-PCR patterns were analyzed using BioNumerics 7.6.1. Software (Applied Maths, Sint-Martens-Latem, Belgium). Dice coefficient was used for calculating the genetic similarity of the isolates and the Unweight Paired Group Arithmetic Average (UPGMA) method was used for clustering. The tolerance level of 1.0 % and optimization of 0.5 % were chosen for creating all dendrograms. Based on the cluster analysis, representatives of each cluster group were selected and further identified.

3.7.2. Molecular identification of LAB

All primers used for molecular identification of isolates collected from media selective for the LAB are shown in Table 3.3. Reagents used in these PCR reactions are listed in tables 3.4 to 3.9 and temperature profiles of each PCR reaction are described. All PCR reactions were carried out in a ProFlex PCR system (Applied Biosystems). All PCR products were separated by horizontal gel electrophoresis. PCR products (3 µL) were mixed with 2 µL of 6 X Loading dye and loaded onto agarose gels (1.5 %). Electrophoresis was run in 1 X TAE buffer for 90 min at 100 V. Gels were stained with ethidium bromide for 20 minutes and visualized under UV illumination..

Table 3.3. List of primers used for fingerprinting and molecular identification of LAB.

Primer name	Sekvence (5'-3')	Target gene	Amplicon size (bp)	References
DU1	CCTACTGATATTAAGACAGCG	*sodA*/ *Enterococcus durans*	295	Jackson et al. (2004)
DU2	TAATCCTAAGATAGGTGTTTG			
FL1	ACTTATGTGACTAACTTAACC	*sodA*/ *Enterococcus faecalis*	360	
FL2	TAATGGTGAATCTTGGTTTGG			
FM1	GAAAAAACAATAGAAGAATTAT	*sodA*/ *Enterococcus faecium*	215	
FM2	TGCTTTTTTGAATTCTTCTTTA			
CA1	TCCTGAATTAGGTGAAAAAAC	*sodA*/ *Enterococcus casseliflavus*	288	
CA2	GCTAGTTTACCGTCTTTAACG			
GA1	TTACTTGCTGATTTTGATTCG	*sodA*/ *Enterococcus gallinarum*	173	
GA2	TGAATTCTTCTTTGAAATCAG			
16S rRNA_Fw	GCTGGATCACCTCCTTTC	16S rRNA/ *Lactobacillus sakei*	220	Berthier & Ehrlich (1998)
LsakeR	ATGAAACTATTAAATTGGTAC	16S/23S rRNA ITS region/ *Lactobacillus sakei*		Berthier & Ehrlich (1999)

A1c_fw	GGAGGGTGTTCAGGAC	16S/23S rRNA ITS region/ *Lactobacillus curvatus*	260	Berthier & Ehrlich (1999)
A1c_rev	GGAGGGTGTTGATAGG			
L_mes_r	AGTCGAGTTACAGACTACAA	16S rRNA/ *Leuconostoc mesenteroides*	1150	Lee et al. (2000)
L_mes_f	AACTTAGTGTCGCATGAC			
Weissgrp_F	GATGGTTCTGCTACCACTAAG	16S rRNA/ genus *Weissella*	1200	Schillinger et al. (2008)
Weissgrp_R	GGNTACCTTGTTACGACTTC			
R16-1	CTTGTACACACCGCCCGTCA	16S rRNA/ genus *Lactobacillus*	250	Nakagawa et al. (1994)
LbLMA1	CTCAAAACTAAACAAAGTTTC	16S/23S rRNA ITS region/ *Lactobacillus sakei*		Dubernet et al. (2002)
E1	TCAACCGGGGAGGGT	16S rRNA/genus Enterococcus	733	Deasy et al. (2000)
E2	ATTACTAGCGATTCCGG			
bak4	AGGAGGTGATCCARCCGCA	16S rRNA/ Bacteria	1508	Dasen et al. (1998)
bak11	AGTTTGATCMTGGCTCAG			Greisen et al. (1994)

Representative isolates that were selected based on rep-PCR analysis were identified by 16S rRNA gene sequencing using bak4 and bak11 primers. PCR reactions were performed in a final volume of 30 µL, and all used reagents are shown in Table 3.4. Temperature profiles were set as follows: initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min. Final extension was performed at 72 °C for 7 min. PCR products were sent to sequencing (Eurofins MWG Operon and Macrogen) and received sequences were analysed with the BLASTn tool (http://blast.ncbi.nlm.nih.gov). A minimum sequence identity of 98 % was chosen as a criterion for species or genus identification.

Table 3.4. Reagents used in a PCR reaction for 16S rRNA gene amplification and sequencing of representative strains.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	3	10 X	1 X
dNTP	1	10 mM	0.33 mM
Primer bak4	1.2	10 pmol/µL	0.4 pmol/µL
Primer bak11	1.2	10 pmol/µL	0.4 pmol/µL
MQ water	22.3	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.3	2 U/µL	0.02 U/µL
DNA	1	n.a.	n.a.

n.a.=not applicable

After the representative isolates were identified, PCR tools were used to confirm the affiliation of all the other cluster members at the species level. If this was not possible, a genus-specific PCR was carried out. For identification of enterococci, modified PCR protocol of Jackson et al., (2004) was followed. Two different master mixes were prepared; the first one containing the primer sets for the identification of *E. faecalis*, *E. faecium* and *E. durans* and the second one for *E. casseliflavus* and *E. gallinarum*. Each master mix was prepared in a final volume of 25 µl (Tables 3.5. and 3.6.). After an initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min.

Table 3.5. Reagents used for the identification of *E. faecalis*, *E. faecium* and *E. durans* in a multiplex PCR reaction.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
Primer FL1	0.5	10 pmol/µL	0.2 pmol/µL
Primer FL2	0.5	10 pmol/µL	0.2 pmol/µL
Primer FM1	0.5	10 pmol/µL	0.2 pmol/µL
Primer FM2	0.5	10 pmol/µL	0.2 pmol/µL
Primer DU1	0.5	10 pmol/µL	0.2 pmol/µL
Primer DU2	0.5	10 pmol/µL	0.2 pmol/µL
MQ water	16.75	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.25	2 U/µL	0.02 U/µL
DNA	2	n.a.	n.a.

n.a.=not applicable

Table 3.6. Reagents used for the identification of *E. casseliflavus* and *E. gallinarum* in a multiplex PCR reaction.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
Primer CA1	0.5	10 pmol/µL	0.2 pmol/µL
Primer CA2	0.5	10 pmol/µL	0.2 pmol/µL
Primer GA1	0.5	10 pmol/µL	0.2 pmol/µL
Primer GA2	0.5	10 pmol/µL	0.2 pmol/µL
MQ water	17.75	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.25	2 U/µL	0.02 U/µL
DNA	2	n.a.	n.a.

n.a.=not applicable

To determine the affiliation to the *Enterococcus* genus, PCR assay was carried out with E1 and E2 primers as described by Deasy et al. (2000), in a final volume of 25 µL (Table 3.7.). Thermocycler was programmed as follows: initial denaturation at 94 °C for 5, 25 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Table 3.7. Reagents used for the identification of genus *Enterococcus* used in a PCR reaction.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
Primer E1	0.5	10 pmol/µL	0.2 pmol/µL
Primer E2	0.5	10 pmol/µL	0.2 pmol/µL
MQ water	18.7	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.3	2 U/µL	0.024 U/µL
DNA	2	n.a.	n.a.

Reagents (master mix) used for the identification of *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and genus *Lactobacillus* are shown in Table 3.8. Each master mix was performed in a total volume of 25 µL. For the identification of *Lb.sakei*, 16S rRNA_Fw and LsakeR primers were used (Berthier and Ehrlich, 1998; Berthier and Ehrlich, 1999). The thermal cycler was programmed to 95 °C for 3 min for initial denaturation, followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 2 min. Final extension was programmed at 72 °C for 7 min. For identification of *Lb. curvatus*, primers A1c_fw and A1c_rev were used (Berthier and Ehrlich 1999). Temperature profiles were as follows: 94 °C for 5 min, 30 repetitions of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 7 min. *Le. mesenteroides* was identified by using a primer set of L_mes_f and L_mes_r, according to Lee et al. (2000). The thermal cycler was programmed as follows: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Genus *Lactobacillus* was identified by R16-1 (Nakagawa et al., 1994) and LbLMA1 (Dubernet et al., 2002) primers, by using following temperature profile: denaturation at 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final elongation at 72 °C for 7 min.

Table 3.8. Reagents used for the identification of *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and genus *Lactobacillus* in a PCR reaction.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
Primer_forward*	1	10 pmol/µL	0.4 pmol/µL
Primer_reverse*	1	10 pmol/µL	0.4 pmol/µL
MQ water	17.75	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.25	2 U/µL	0.02 U/µL
DNA	2	n.a.	n.a.

*16S rRNA_Fw and LsakeR were used for the identification of *Lb. sakei*; A1c_fw and A1c_rev for *Lb. curvatus*; L_mes_f and L_mes_r for *Le. mesenteroides*; R16-1 and LbLMA1 for genus *Lactobacillus*.

Genus *Weissella* was identified with Weissgrp_F and Weissgrp_R primers, according to Schillinger et al. (2008). A master mix was done in a final volume of 50 µL, and used reagents are shown in Table 3.9. The temperature profile was programmed at 94 °C for 2 min, 33 cycles of 94 °C for 1 min, 50 °C for 1 min, and extension at 72 °C for 1.5 min.

Table 3.9. Reagents used for the identification of genus *Weissella* in a PCR reaction.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (10 mMTris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, pH=8.8)	5	10 X	1
dNTP	1	10 mM	0.2 mM
Weissgrp_F	2.5	10 pmol/µL	0.5 pmol/µL
Weissgrp_R	2.5	10 pmol/µL	0.5 pmol/µL
MQ water	36.7	n.a.	n.a.
Dynazyme DNA polymerase (Thermo Scientific)	0.3	2 U/µL	0.012 U/µL
DNA	2	n.a.	n.a.

3.8. Selection of strains

In the scope of the present study, a total of 917 isolates was collected from 3 wild boar and 3 deer meat sausages on media selective for the LAB (MRS, LamVab, MSE, KAA). However, LAB isolates were additionally collected from other artisanal dry fermented wild boar and deer meat sausages and were included in the present study. Therefore, a total of 1326 strains was collected. All strains were collected, identified and straind (grouped) as described in previous sections. Altogether 57 representative strains, corresponding to *E. durans*, *Lb. sakei*, *Lb. curvatus and Le. mesenteroides,* were selected for further safety and technological analyses. Duplicates were excluded for the clusters containing less than 20 isolates; however, for the clusters containing more than 20 isolates at least two representatives were selected. Clusters containing 5 or fewer strains were not considered. A dendrogram comprised of selected representative strains (*n*=57) is shown in Appendix 7.3.

3.9. Profiling of bulk colonies (consortia) by PCR-DGGE analysis to check the selectivity of media used for isolation of LAB

3.9.1. PCR amplification of the V3 region

To additionally check the selectivity of media applied for LAB isolation, profiling of bulk colonies (consortia) harvested from MRS plates was performed by DGGE as described by Skelin et al. (2012). The MRS plates were washed with 2 mL of sterile saline solution (0.85 %), and the suspension was transferred to sterile 2 mL tubes. Tubes were centrifuged (Eppendorf 5415R, Eppendorf, Hamburg, Germany) for 5 min at 5 000 x *g* and the DNA was extracted (see 3.5.) from obtained pellets. In a PCR reaction, a V3 region of the 16S rRNA gene was amplified by using universal bacterial primers F338-GC and 518r (Table 3.10.). Concentrations of all used reagents are shown in Table 3.11. All PCR reactions were performed in a final volume of 50 µL, in triplicates, and pooled after amplification. To increase the specificity of the amplification, a 'touchdown' PCR was performed and programmed as follows: after initial denaturation of DNA at 94 °C for 5 min, 30 cycles of 95 °C for 1 min, *55 °C for 1 min (*annealing temperature was gradually decreased from 65 to 55 °C by 2 °C each two PCR cycles) and 72 °C for 3 min were performed. Final extension was carried out at 72 °C for 10 min. PCR reactions were carried out in a ProFlex PCR system (Applied Biosystems).

Table 3.10. Primers used for amplification of the V3 region of 16S rRNA in the PCR-DGGE analysis.

Primer name	Sekvence (5'-3')	Target gene	Amplicon size(bp)	References
338f*	ACTCCTACGGGAGGCAGCAG	V3 region of 16S rRNA	236	Lane (1991)
518r	ATTACCGCGGCTGCTGG			(Muyzer et al., 1993)

*GC clamp attached to 338f primer (F338-GC):
5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG3'

Table 3.11. Reagents used for amplification of the V3 region of 16S rRNA in a PCR-DGGE analysis.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (200 mM Tris HCl, 500 mM KCl, pH=8.4)	5	10 X	1 X
$MgCl_2$	1.5	50 mM	1.5 mM
dNTP	5	2 mM	0.2 mM
primer (F338-GC)	1.25	10 pmol/µL	0.25 pmol/µL
primer (518r)	1.25	10 pmol/µL	0.25 pmol/µL
MQ water	34.25	n.a.	n.a.
Polymerase (Taq, Invitrogen)	0.25	5 U/µL	0.025 U/µL
DNA	1.5	n.a.	n.a.

n.a.=not applicable

3.9.2. DNA Ladder preparation

For the preparation of DNA Ladder, following cultures were used: *Leuconostoc mesenteroides* (DSM 20343), *Lactobacillus sakei* (LMG 9468), *Enterococcus faecalis* (DSM 20478), *Lactobacillus casei* (collection of microorganisms deposited at the Department of Microbiology University of Zagreb, Faculty of Agriculture) and *Lactobacillus gasseri* (collection of microorganisms deposited at the Department of Microbiology University of Zagreb, Faculty of Agriculture). From noted cultures, DNA was extracted as described in section 3.5. and amplified as described in the previous section (3.7.1.). PCR products were purified (see 3.7.3.) and pooled at equal concentrations. To 5 µL of pooled PCR products, 5 µL of 6 X Loading Dye (Invitrogen) and 20 µL of distilled water was added.

3.9.3. Purification and concentration of PCR products

PCR products were purified by Macherey-Nagel NucleoSpin purification kit (Macherey-Nagel, Düren, Germany) and their concentration was checked by agarose gel electrophoresis. PCR products (4 µL) were mixed with 1 µL 6X LD (Invitrogen) and loaded onto agarose gels. To the first and last well of each gel, 4 µL of Low DNA Mass Ladder (Invitrogen) was loaded. Electrophoresis was run in TAE buffer (1 X) for 70 minutes at 100 V. Gels were stained with ethidium bromide and visualised under UV illumination. The concentration of purified PCR products was determined by comparing band intensity with bands obtained from Low DNA Mass Ladder.

3.9.4. Preparation of polyacrylamide gels and DGGE analysis

For the preparation of polyacrylamide gels, 2 stock solutions were used. The first stock solution was prepared by dissolving 20 mL PAA (acrylamide 40 %, 37.5:1; Biorad, Berkeley, USA) and 2 mL TAE (50 X) buffer in distilled water filled up to 100 mL mark. The second stock solution was prepared by dissolving 20 mL PAA, 40 mL formamide (40 %; Biorad), 42 g urea (7 M; Sigma-Aldrich) and 2 mL TAE (50 X) buffer in distilled water filled up to 100 mL mark. For optimal separation of PCR products polyacrylamide gels (8 %) containing a 30 to 60 % urea-formamide denaturing gradient was used. Urea-formamide denaturing gradient (30 %) was prepared by mixing 16.8 mL of first stock solution, 7.2 mL of second stock solution, 10 µL of TEMED (N,N,N′,N′-Tetramethylethylenediamine; Sigma-Aldrich) and 100 µL of ammonium persulfate solution (APS 10 %; Sigma-Aldrich). Urea- formamide denaturing gradient (60 %) was prepared by mixing 9.6 mL of first stock solution, 14.4 mL of second stock solution, 10 µL of TEMED and 100 µL of APS solution (10 %). APS solution (10 %) was always prepared fresh, by dissolving 0.1 g of ammonium persulfate (Sigma-Aldrich) in 1 mL of distilled water. Finally, polyacrylamide gels were prepared by mixing 30 and 60 % denaturing gradients in DGGE apparatus. Gels were overlayed with ethanol (≥ 99.5 %; Sigma-Aldrich) until polymerisation. When gels polymerased, ethanol was removed and a mixture without denaturing agents, comprised of first stock solution (5 mL), TEMED (5 µL) and APS (50 µL) was added on the top and the combs were inserted. After complete polymerisation, gels were placed in DGGE apparatus containing TAE buffer (1 X) preheated to 60 °C. Combes was carefully removed, and wells were washed with TAE buffer (1 X) using a syringe. Purified PCR products (3 µL) were mixed with 3 µL 6 X Loading Dye (Invitrogen) and loaded in wells. To the first and the last well of each gel, 5 µL of DNA Ladder was added. Electrophoresis was run for 6 h at 120 V and 60 °C. The gels were stained for 30

min in 500 mL TAE buffer (1 X) containing 50 µL SYBR Green (Sigma-Aldrich) and photographed under UV illumination.

3.9.5. Identification of obtained DGGE bands

DGGE bands with different migration profiles were selected and excised from the gel with a sterile scalpel. Excised bands were processed following the protocol of Cocolin et al. (2007); they were put in a sterile 2 mL tubes containing 50 µL of sterile MQ water and stored overnight at 4 °C for DNA to diffuse. DNA was reamplified by using the same primer set, without GC clamp (Table 3.2.). A master mix was prepared as shown in Table 3.3., but with modified volumes of water (30.75 µL) and DNA (5 µL). A 'touchdown' PCR was performed under conditions described in section 3.6.1. Reamplified DNA was sent to sequencing (Eurofins MWG Operon and Macrogen). The received sequences were analyzed with the BLASTn tool (http://blast.ncbi.nlm.nih.gov), and a minimum sequence identity of 98 % was chosen as a criterion for species identification.

3.10. Safety aspects of selected representative LAB strains

3.10.1. Susceptibility to antibiotics

To determine the susceptibility of representative strains (*n*=57) to clinically important antibiotics, standardized agar disc diffusion test using BBL™ Sensi-Disc™ antimicrobial susceptibility test discs (Becton, Dickinson and Company, Le Pont de Claix, France) was performed. All strains were subcultured from glycerol (20 %) by streaking on BHI agar (Biolife) plates. Enterococci were grown overnight at 37 °C under aerobic conditions, while lactobacilli and *Leuconostoc mesenteroides* were grown for 3 days at 30 °C in an anaerobic atmosphere. Freshly grown cultures were taken from BHI plates with a sterile loop and transferred to 10 mL of sterile saline solution (0.85 %) at a concentration equal to 1 McFarland standard. Using a sterile cotton swab, such prepared bacterial suspensions were inoculated on Mueller-Hinton (MH; Merck) agar plates (enterococci) or LAB susceptibility test agar (LSM; Klare et al., 2005) plates (non enterococcal LAB strains). To inoculated plates, discs impregnated with specific concentrations of antibiotics were added. The plates were incubated for 24 h at 37 °C under aerobic conditions (enterococci) or for 72 h at 30 °C under anaerobic conditions (non enterococcal LAB strains). Enterococci were screened for their susceptibility to ampicillin (2 and 10 µg), penicillin (10 µg), tetracycline (10 µg), erythromycin (15 µg), vancomycin (5 µg), and chloramphenicol (30 µg). Other LAB were screened against ampicillin (2 µg), gentamicin (10 µg), erythromycin (2 µg), tetracycline (5 µg), kanamycin (30 µg), clindamycin (2 µg) and chloramphenicol (30 µg). As a control, *E.coli* ATCC 25922 was used.

3.10.2. PCR detection of genes encoding for the production of biogenic amines

All representatives were screened for the presence of genes encoding for the production of biogenic amines. A PCR assay was carried out to detect the genes encoding for the production of histamine (*hdc*), putrescine (*odc*) and tyramine (*tdc*) as described by de las Rivas et al. (2005), while detection of cadaverine (*ldc*) was performed in a separate PCR reaction following the protocol of de las Rivas et al. (2006). The sequences of used primers are listed in Table 3.12. Both reactions were prepared in a final volume of 25 µL, and used reagents are shown in Table 3.13. and Table 3.14. As a positive control for *hdc* and *tdc* genes, the DNA of MSB11 strain was used (collection of microorganisms deposited at the Department of Microbiology University of Zagreb, Faculty of Agriculture). As a positive control of *odc* gene, the DNA of strain MSB 425 was used (collection of microorganisms deposited at the Department of Microbiology University of Zagreb, Faculty of Agriculture). The DNA of *Leuconostoc mesenteroides* subsp. *cremoris* (DSMZ 20346) was used as a positive control for *ldc* gene. Master mix with no added DNA was used as a negative control. For a multiplex PCR reaction, thermocycler was programmed as follows: 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min and final elongation at 72 °C for 10 min. For amplification of cadaverine (*ldc*), temperature profiles were set as follows: 94 °C for 10 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min. PCR reactions were carried out in a ProFlex PCR system (Applied Biosystems).

Table 3.12. Primers used for PCR detection of genes encoding for the production of biogenic amines.

Primer name	Sekvence (5'-3')	Target gene	Amplicon size (bp)	References
JV16HC	AGATGGTATTGTTTCTTATG	*Hdc*	367	de las Rivas et al. (2005)
JV17HC	AGACCATACACCATAACCTT			
3F	GTNTTYAAYGCNGAYAARACNTAYTTYGT	*Tdc*	924	
16R	TACRCARAATACTCCNGGNGGRTANGG			
P1-rev	CCRTARTCNGGNATAGCRAARTCNGTRTG	*Odc*	1446	
P1-for	GAYATNATNGGNATNGGNYTNGAYCARG			
CAD2-R	CAYRTNCCNGGNCAYAA	*Ldc*	1185	de las Rivas et al. (2006)
CAD2-F	GGDATNCCNGGNGGRTA			

Table 3.13. Reagents used in a multiplex PCR reaction to detected genes encoding for the production of histamine (*hdc*), putrescine (*odc*) and tyramine (*tdc*).

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (200 mM Tris HCl, 500 mM KCl, pH=8.4)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
JV16HC	0.75	10 pmol/µL	0.3 pmol/µL
JV17HC	0.75	10 pmol/µL	0.3 pmol/µL
3F	2.5	10 pmol/µL	1 pmol/µL
16R	2.5	10 pmol/µL	1 pmol/µL
P1-rev	5	10 pmol/µL	2 pmol/µL
P2-for	5	10 pmol/µL	2 pmol/µL
$MgCl_2$	1.25	50 mM	2.5 mM
MQ water	2.95	n.a.	n.a.
Polimerase (Taq, Invitrogen)	0.3	5 U/µL	0.06 U/µL
DNA	1	n.a.	n.a.

Table 3.14. Reagents used in a PCR reaction to detected genes encoding for the production of cadaverine (*ldc*).

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (200 mM Tris HCl, 500 mM KCl, pH=8.4)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
CAD2-F	2.5	10 pmol/µL	1 pmol/µL
CAD2-R	2.5	10 pmol/µL	1 pmol/µL
$MgCl_2$	1.25	50 mM	2.5 mM
MQ water	14.45	n.a.	n.a.
Polimerase (Taq, Invitrogen)	0.3	5 U/µL	0.06 U/µL
DNA	1	n.a.	n.a.

Each obtained PCR product (3 µL) was mixed with 2 µL 6 X Loading dye and loaded onto agarose gels (1.5 %). Electrophoresis was run in 1 X TAE buffer for 90 min at 100 V. Gels were stained with ethidium bromide for 20 minutes and visualized under UV illumination.

3.10.3. PCR detection of genes encoding for the production of virulence factors

Isolates that were identified as enterococci were additionally screened for the presence of following virulence factors: aggregation substance (*agg*), gelatinase (*gelE*), cytolysin (*cylM*, *cylB*), cytolysin activator (*cylA*), enterococcal surface protein (*esp*) and sex pheromones (*cpd* and *cob*). List of used primers is shown in Table 3.15. Each reaction was prepared in a final volume of 25 µL, and all used reagents are shown in Table 3.16. PCR amplification was performed as previously described by Eaton and Gasson (2001), except for cob and agg genes, whose elongation cycles were prolonged to 45 s as described by Majhenič Čanžek et al. (2005) (Table 3.17.). As a positive control, the DNA from several strains from the collection of microorganisms deposited at the Department of Microbiology University of Zagreb, Faculty of Agriculture was used.

Table 3.15. List of primers used for PCR detection of genes encoding for the production of virulence factors.

Primer name	Sekvence (5'-3')	Target gene	Amplicon size (bp)	References
TE3	AAGAAAAAGTAGACCAAC	*agg*	1553	Eaton and Gasson (2001)
TE4	AACGGCAAGACAAGTAAATA			
TE9	ACCCCGTATCATTGGTTT	*gelE*	419	
TE10	ACGCATTGCTTTTCCATC			
TE13	CTGATGGAAAGAAGATAGTAT	*cylM*	742	
TE14	TGAGTTGGTCTGATTACATTT			
TE15	ATTCCTACCTATGTTCTGTTA	*cylB*	843	
TE16	AATAAACTCTTCTTTTCCAAC			
TE17	TGGATGATAGTGATAGGAAGT	*cylA*	517	
TE18	TCTACAGTAAATCTTTCGTCA			
TE34	TTGCTAATGCTAGTCCACGACC	*esp*	933	
TE36	GCGTCAACACTTGCATTGCCGAA			
TE51	TGGTGGGTTATTTTTCAATTC	*cpd*	782	
TE52	TACGGCTCTGGCTTACTA			
TE49	AACATTCAGCAAACAAAGC	*cob*	1405	
TE50	TTGTCATAAAGAGTGGTCAT			

Table 3.16. Reagents used in a PCR reaction for the screening of virulence factors among enterococci.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
Buffer (200 mM Tris HCl, 500 mM KCl, pH=8.4)	2.5	10 X	1
dNTP	0.5	10 mM	0.2 mM
Primer-forward*	0.5	100 pmol/µL	2 pmol/µL
Primer-reverse*	0.5	100 pmol/µL	2 pmol/µL
$MgCl_2$	0.75	50 mM	1.5 mM
MQ water	14.45	n.a.	n.a.
Polimerase (Taq, Invitrogen)	0.25	5 U/µL	0.05 U/µL
DNA	2	n.a.	n.a.

*different primers sets used for screening of virulence factors are shown in Table 3.15.

Table 3.17. The temperature profile of a PCR reaction for the screening of virulence factors. Elongation was performed at 72 °C for 45 s for *cob* and *agg* genes, while for all other genes it was performed for 15 s.

Parts of the PCR reaction	Temperature (ºC)	Duration
2 cycles of amplification		
• Denaturation	94	2 min
• Annealing	X*	2 min
• Elongation	72	2 min
29 cyles of amplification:		
• Denaturation	94	15 s
• Annealing	X*	15 s
• Elongation	72	15 s / 45 s
Final elongation	72	10 min

X=annealing temperatures: 49ºC for TE3/TE4, TE13/TE14, TE17/TE18; 47 ºC for TE5/6, TE49/50; 45 ºC for TE37/TE38; 55 ºC for TE34/TE36, TE15/TE16, TE51/TE52; 52 ºC for TE9/TE10.

PCR products (3 µL) were mixed with 2 µL 6 X Loading dye and loaded onto agarose gels (1.5 %). Electrophoresis was run in 1 X TAE buffer for 90 min at 100 V. Gels were stained with ethidium bromide for 20 minutes and visualised under UV illumination.

3.10.4. Hemolytic activity

Representative strains were subcultured from glycerol (20 %) by streaking on BHI agar plates. Enterococci were incubated in the aerobic atmosphere at 37 °C for 24 h, while other LAB strains were incubated in anaerobic conditions at 30 °C for 72 h. From freshly grown cultures, 1 colony was taken with a sterile loop and streaked on Columbia Blood Agar plates (Biolife). Again, enterococci were incubated at 37 °C for 48 h and other LAB at 30 °C for 72 h. The plates were observed for the formation of any clean (β-hemolysis) or greenish (α-hemolysis) hemolytic zones, or no such zone (γ-hemolysis) around the colonies grown on Columbia Blood Agar. As a positive control, *Bacillus cereus* DSM 6791 was used.

3.11. Technologically important traits and bioprotective role of selected representatives

3.11.1. Acidifying activity

The representatives were subcultured from glycerol by streaking on BHI agar plates. Enterococci were incubated at 37 °C for 48 h in an aerobic atmosphere, while other LAB were incubated anaerobically at 37 °C for 72 h. From freshly grown cultures, 1 colony was taken with a sterile loop and inoculated in 10 mL of sterile Lyophilized Pork Meat Media (LP; Baruzzi et al., 2006), in duplicates. Inoculated media was incubated at 37 °C (enterococci) or 30 °C (other LAB). The acidifying activity was measured after 24 hours and 7 days of incubation. Combined pH electrodes (InPro® 3030; Metter Toledo, Greifensee, Switzerland) were used and disinfected after each run using a 3 % HCl solution.

3.11.2. Lipolytic activity

The representative strains were subcultured from glycerol by streaking on BHI agar plates. Enterococci were incubated at 37 °C for 48 h in an aerobic atmosphere, while other strains were incubated anaerobically at 37 °C for 72 h. From freshly grown cultures, several colonies were taken with a sterile loop and inoculated in 10 mL of sterile saline solution (0.85 %) at a cell concentration corresponding to 0.5 McFarland standard. 10 µL of such prepared bacterial suspensions were added on sterile cellulose discs (Biorad) previously placed on Tributyrin Agar (Sigma-Aldrich; disc diffusion method). Simultaneously, 2 µL of prepared bacterial suspension were inserted directly into Tributyrin Agar (spot method). All plates were incubated at 30 °C for 72h, in aerobic (enterococci) or anaerobic (other LAB) conditions. The diameter of clear zones formed around the spotting point (spot method) and around the discs (disc diffusion method), were measured and results were expressed in mm, including the disc diameter. Each strain was tested twice. As a positive control, *Pseudomonas fluorescens* WCS 417r was used.

3.11.3. Proteolytic and peptidase activity

3.11.3.1. Proteolytic activity measures on plates with sarcoplasmic proteins

Proteolytic activity of the representative strains was screened on plates containing sarcoplasmic proteins. Proteins were extracted from lean pork meat, following the protocol that ensures the development of LAB, previously described by Fadda et al. (2010). Lean

pork meat (20 g) was homogenised for 8 min in Stomacher Lab-Blender 400 (Seward Medical) with 200 mL of sterile phosphate buffer (20 mM, pH=7.4). The protein solution was centrifuged at 11180 x *g* for 20 min at 4 °C to precipitate the insolubilized proteins and connective tissue. The pH of supernatant containing the sarcoplasmic proteins was adjusted to 6.5 with 1 M NaOH, complemented with 1 % glucose (w/v) and filtered through Whatman paper. Just before use, the sarcoplasmic system was supplemented with 0.01 % of sterile Tween-80. Sarcoplasmic protein media (see: 7.1.26) was prepared by adding extracted proteins at a final concentration of 1 mg/mL in a sterile media containing tryptone, yeast extract, glucose and agar (Mauriello et al., 2002). To each Petri dish, 25 mL of media was poured. After polymerization, using sterile pipette tips, 6 mm wells were poked out. Bacterial strains were cultured overnight in 2 mL BHI broth, and 70 µL of culture was taken and added to the wells in 2 replicates. The plates were incubated for 48 h, at 37 °C in the aerobic atmosphere (enterococci) or at 30 °C in anaerobic conditions (other LAB). After incubation, agar media was pulled out from the Petri dishes and stained for 5 min in a solution containing 50 mL of Brilliant Blue R dissolved in methanol (0.05 %), 10 mL of acetic acid and 40 mL of distilled water. Gels were destained in a solution containing 25 mL of methanol, 5 mL of acetic acid and 70 mL of water. The diameter of clear zones formed around the wells was evaluated. Each strain was tested twice. As a positive control, *Pseudomonas fluorescens* WCS 417r was used.

3.11.3.2. Proteolytic activity measured on skim milk plates

The proteolytic activity of all representatives was additionally screened on BHI agar supplemented with skimmed milk (Biolife; 1.5 %). Testing was performed as described for lipolytic activity (see: 3.9.2.), but instead of Tributyrin agar, skim milk plates were used. As a positive control, *Pseudomonas fluorescens* WCS 417r was used.

3.11.3.3. Peptidase activity measured by the chromogenic method

The peptidase activity of all representatives was tested by the chromogenic method as described by Savoy de Giory and Hébert (2001). Microorganisms were grown in 100 mL of MRS media supplemented with $CaCl_2 \cdot 2H2O$ (1.47 g/L) at 37 °C (enterococci) or 30 °C (other LAB) until the optical density (OD) of 1.5 at 600 nm was achieved. Cells were harvested by centrifugation at 10 000 g for 10 min at 4°C. Pellets were then washed twice with a $CaCl_2$ saline solution (10 mM) and resuspended in 5 mL Tris buffer (50 mM, pH=7.8). 200 µL of resuspended cells (enzyme solution) was taken and transferred to sterile 2 mL tubes, and following reagents were added: 287.5 µL of phosphate buffer (0.2M, pH=7.0), 225 µL 5M NaCl, and 37.5 µL S-Ala. The tubes were mixed gently and incubated at 37 °C (enterococci) or 37 °C (other LAB) for 30 min. The reaction was

stopped by the addition of 175 µL of 80 % (v/v) acetic acid. The tubes were centrifuged for 5 min at 13 000 x *g*. The release of *p*-nitroanilide (*p*NA) was measured at 410 nm. Each strain was tested twice.The concentration of *p*NA released was calculated according to the formula:

$$\mu M\ pNA = \varepsilon \Delta A_{41}\, F \cdot 10^{3}$$

3.11.4. Antagonistic (antimicrobial) activity

Antagonistic activity of representative strains was tested against 7 indicator bacteria: *Salmonella enterica* subsp. *enterica* (DSM 14221), *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* subsp. *aureus* (DSM 20231), *Brochotrix thermospachta* (LMG 17208), *Weissella viridescens* (DSM 20410), *Bacillus cereus* (DSM 6791). Testing was performed following the protocol of Domig et al. (2014). LAB cultures were grown overnight in 10 mL BHI broth media at 37 °C (enterococci) or 30 °C (other LAB). The cultures were streaked with a sterile cotton swab, in two parallel lines about 20 mm apart, on the center of the BHI agar plate. The plates were then incubated for 24 h, at 37 °C under aerobic conditions (enterococci) or at 30 °C under anaerobic conditions (other LAB). Overnight cultures of indicator bacteria were grown in BHI broth, of which 5 µl was taken and inserted directly into agar between the two pre-incubated LAB streaks. As a growth control, overnight cultures of indicator bacteria were also inserted into the agar at the margin of the BHI plate. Such prepared plates were incubated aerobically, at 30 °C (*Brochotrix thermospachta*, *Weissella viridescens*, *Bacillus cereus*) or 37 °C (*Salmonella enterica* subsp. *enterica, Listeria innocua*, *Escherichia coli*, *Staphylococcus aureus* subsp*. aureus*). Indicator colonies grown between two lines of lactobacilli were measured and compared with colonies grown on the margin of the plate. Each strain was tested twice. LAB strains which exhibited antagonistic activity were selected and then the inhibitory effect of their cell-free supernatants was determined by the agar well diffusion, as described by (Kim and Rajagopal 2001). All selected strains were grown in BHI broth media at 37 °C for 24 h, after which the cells were removed by centrifugation at 10 000 x *g* for 10 minutes. The centrifugation step was repeated twice. The supernatants were adjusted to pH=6.5 with 1 M NaOH and sterilized by filtering through a 0.45 µm membrane filter (Merck). BHI agar media was prepared, and 10 mL was poured to each of the Petri dishes. After polymerisation, the prepoured plates were overlayed with 10 mL of soft BHI agar inoculated with 0.3 mL of 1:10 dilution of an overnight culture of the indicator bacteria. When the media polymerised, a 6 mm diameter wells were poked out with sterile pipette tips, and 100 µL of the cell-free supernatant was added into each well. Such prepared plates were incubated aerobically, at 30 °C

(*Brochotrix thermospachta*, *Weissella viridescens*, *Bacillus cereus*) or 37 °C (*Salmonella enterica* subsp. *enterica, Listeria innocua*, *Escherichia coli*, *Staphylococcus aureus* subsp*. aureus*). After incubation, the zones of inhibition were examined.

3.12. Autoaggregation ability and survival of the LAB in simulated gastrointestinal (GI) conditions

3.12.1. Autoaggregation assay

Autoaggregation assay was performed according to Del Re et al. (2000). Overnight cultures were grown in 6 mL of BHI broth, from which 2 mL was taken and centrifuged at 15 min/10 000 x *g*/4 ºC. Supernatants were discarded while pellets were washed and resuspended in 1 X PBS buffer, which corresponded to a viable count of approx. 10^8 cfu/mL. The cell suspensions were vortexed for 10 s. The absorbance (A) was measured at 610 nm after 3 and 5 h of incubation at room temperature. Each strain was tested three times. The aggregation percentage was expressed as:

$$\text{Aggregation (\%)} = 1-(A_t/A_0)\cdot 100$$

where A_t represents the absorbance measured at 610 nm at time t=3 and 5 h, and A_0 the absorbance at t=0.

3.12.2. Survival in simulated conditions of the oral cavity

To simulate the possible hydrolysis of bacteria in the oral cavity, testing was performed as described by Morandi et al. (2013). Cultures were grown overnight in skimmed milk (10 %) and diluted 1:10 in phosphate buffer (PBS, 1 X, pH=7.2). Series of dilution (10^{-1}-10^{-5}) were performed, and 100 µL of culture was inoculated on BHI plates, in duplicates, to determine the initial viable counts. To overnight cultures diluted in PBS buffer, lysozyme (Sigma-Aldrich) was added to a final concentration of 100 mg/L and incubated for 5 min at 37 °C. Series of dilution (10^{-1}-10^{-5}) was performed again, and 100 µL was inoculated on BHI plates, in duplicates, to determine the viable bacterial counts. Enterococci were incubated at 37 °C for 48 h, while other LAB was incubated at 30 °C for 72 h. Each strain was tested three times. Bacterial counts were expressed as cfu/g, and survival rates were calculated as follows:

$$\text{Survival rate (\%)} = (\text{bacterial count after lysozyme digestion})/(\text{initial bacterial count})\cdot 100$$

3.12.3. Survival in simulated gastric and duodenum conditions

To estimate the cell survival rate in simulated gastric and intestinal conditions, testing was performed following the protocol of Doleyres et al. (2004), with slight modifications. Cultures were grown overnight in 6 mL of BHI broth. 1 mL of overnight culture was harvested by centrifugation (Eppendorf) at 10 000 x *g* for 10 min at room temperature. The pellets were washed twice with 0.1 % peptone water, resuspended in 100 µL of 0.1 % peptone and stored on ice until use. Series of dilution (10^{-1}-10^{-5}) were performed, and 100 µL of culture was inoculated on BHI plates, in duplicates, to determine the initial viable counts. To simulate the gastric digestion, a solution containing 0.5 % NaCl and 0.3 % pepsin was used, and the pH of cell suspension was adjusted to pH=2.5 with1 M HCl. The solution containing 270 µL of simulated gastric juices and 30 µL of cell suspension was then mixed and incubated at 37 °C for 30 min. For cell survival in simulated intestinal conditions, 270 µL of 0.4 % bile salts and 0.2 % pancreatin solution (Sigma-Aldrich) were mixed with 30 µL of cell suspension and incubated for 30 min at 37 °C. After digestion, series of dilution (10^{-1}-10^{-5}) was performed again, and 100 µL was inoculated on BHI plates, in duplicates, to determine the viable bacterial counts. Enterococci were incubated at 37 °C for 48 h, while other LAB was incubated at 30 °C for 72 h. Each strain was tested three times. Bacterial counts were expressed as cfu/g, and survival rates were calculated as follows:

Survival rate (%)=(bacterial count after digestion)/(initial bacterial counts)·100

3.13. Analysis of the structure of bacterial communities and the changes in microbial diversity during ripening by culture-independent methods (next-generation sequencing)

3.13.1. DNA extraction

DNA was extracted from the sausage samples following a modified Matrix Lysis protocol (Rossmanith et al., 2007; Rossmanith and Wagner 2010). Sausage samples were defrosted from -80 °C, after which 6 g was taken, blended and transferred to a sterile Stomacher bags to which 10 mL of solution A was added. After 30 minutes of homogenization, the homogenised materials were transferred to a Falcon tube by pressing through the Stomacher bag filters. Phosphate buffered saline (PBS; Oxoid) was added to a final volume of 40 mL. The tubes were incubated at 45 °C for 30 min in a horizontal position in a water bath with constant shaking at 200 rpm and centrifuged at 3200 x *g* for 30 min. Supernatants were discarded, and Lysis buffer was added up to the

35 mL mark. Tubes were again incubated at 45 °C for 30 min in a horizontal position in a water bath with constant shaking at 200 rpm and centrifuged at 3200 x *g* for 30 min. Supernatants were discarded, and Wash buffer was added up to the 35 mL mark. Tubes were again incubated and centrifuged as described in the previous step. In case any particles were left visible, the tubes were additionally centrifuged at 1000 x *g* for 5 min, and supernatants were discarded. Then the tubes were centrifuged at 3200 x *g* for 30 min, and supernatants were discarded. Remaining pellets were resuspended in 1 mL PBS solution, transferred to 1.5 mL tubes and centrifuged at 8000 x *g* for 5 min. This step was repeated twice. Finally, the obtained pellets were resuspended in 50 µL 1 X PBS and stored at -20 °C. From this point, after the cells were pelleted (4000 x *g* for 10 minutes), the extraction proceeded following the protocol of the peqGOLD Bacterial DNA Mini Kit (Peqlab, VWR, Germany). DNA extracts were stored at −20 °C until further analysis.

3.13.2. Measuring the DNA purity and concentration

To check the purity of the extracted DNA, the 260/280 and 260/230 ratios were measured on Nanodrop (ND-1000 spectrophotometer; ThermoScientific, Wilmington, USA). The DNA concentration was measured with Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions, on Gemini EM Microplate Reader (Molecular Devices, San Jose, USA).

3.13.3. 16S rRNA gene amplification

The V1-V2 region of the 16S rRNA gene was amplified using S-D-Bact-0008-a-S-16 (5'-AGAGTTTGATCMTGGC-3') and S-D-Bact-0343-a-A-15 (5'-CTG CTG CCT YCC GTA-3') primers (Muyzer et al., 1993; Klindworth et al., 2013). Used primers contained overhanging sequences at the 5' ends compatible to Nextera XT indices (Illumina, San Diego, USA). Each sample was amplified in triplicate in a 25 µl reaction mixture (Table 3.18).

Table 3.18. Reagents used in a PCR reaction for 16S rRNA gene amplification of the V1-V2 region.

Reagents	Initial volume (µL)	Initial concentration	Final concentration
NEBNext High-Fidelity Master Mix (New England Biolabs)	12.5	2 X	1 X
S-D-Bact-0008-a-S-16	0.5	10 pmol/µL	0.2
S-D-Bact-0343-a-A-15	0.5	10 pmol/µL	0.2
DEPC water	10.5	n.a.	n.a.
DNA (1-10 ng)	1	n.a.	n.a.

n.a. = not applicable

Thermocycler was programmed as follows: denaturation at 98 °C for 5 min, followed by 20 PCR cycles (98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s) and a final extension step at 72 °C for 5 min. Obtained PCR products were pooled and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), according to the manufacturer's instructions, with a slight modification: before use, NTI buffer was diluted in water at 1:4 ratio. The amplicons size and quality were assessed using an Agilent Bioanalyzer 2100 DNA Chip 7500 (Agilent Technologies, Santa Clara, USA) and quantified using the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific). Each PCR product was diluted to 10 ng.

3.13.4. Indexing PCR

Amplicons were combined with the sequencing adapters and dual indices using the Nextera XT v2 Indeks Kit (set A; Illumina), forming the multiplexed paired-end libraries. The schematic outline of the indexing PCR is shown in Figure 3.1. Indexing PCR mixture contained NEBNext High-fidelity 2x PCR Master Mix (New England Biolabs, Ipswich, USA). 5 µl of each primer and 10 ng of purified amplicons. The amplification conditions were as follows: denaturation at 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s and final elongation at 72 °C for 5 min. The library size and quality were checked on the Agilent Bioanalyzer 2100 DNA Chip 7500 (Agilent Technologies) and quantified using the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific). Individual libraries were diluted to 4 nM with DEPC water, denaturated with 0.2 N NaOH and spiked with 2.5 % (v/v) PhiX. Paired-end sequencing was performed on the MiSeq platform, using MiSeq Reagent Kit v3 2 x 300 cycle (Illumina), following the standard Illumina sequencing protocol.

Table 3.19. The schematic outline of indexing PCR performed to obtain the multiplexed paired-end libraries.

	N701	N702	N703	N704	N705	N706	N707	N710	N711
S502	WB1_0d	WB2_20d	DS1_4d	WB1_0d	WB2_20d	DS1_4d	WB1_0d	WB2_20d	DS1_4d
S503	WB1_4d	WB2_40d	DS1_7d	WB1_4d	WB2_40d	DS1_7d	WB1_4d	WB2_40d	DS1_7d
S505	WB1_7d	WB3_0d	DS1_20d	WB1_7d	WB3_0d	DS1_20d	WB1_7d	WB3_0d	DS1_20d
S506	WB1_20d	WB3_4d	DS1_40d	WB1_20d	WB3_4d	DS1_40d	WB1_20d	WB3_4d	DS1_40d
S507	WB1_40d	WB3_7d	DS2_0d	WB1_40d	WB3_7d	DS2_0d	WB1_40d	WB3_7d	DS2_0d
S508	WB2_0d	WB3_20d	DS2_4d	WB2_0d	WB3_20d	DS2_4d	WB2_0d	WB3_20d	DS2_4d
S510	WB2_4d	WB3_40d	DS2_7d	WB2_4d	WB3_40d	DS2_7d	WB2_4d	WB3_40d	DS2_7d
S511	WB2_7d	DS1_0d	DS2_40d	WB2_7d	DS1_0d	DS2_40d	WB2_7d	DS1_0d	DS2_40d
	Replicate-a			Replicate-b			Replicate-c		

3.13.5. Sequence data pre-processing and OTU picking

Several pre-processing steps were done using Adapter Removal v2 (Schubert et al., 2016) including removal of any remnant adaptor sequences; trimming of bases at 5' and 3' end with Phred score < 15; removal of reads shorter than 50 bp after adaptor removal and trimming; merging of overlapping paired-end reads in contigs, and removal of unmerged reads. Further processing included removal of PhiX reads with DeconSeq standalone (version 0.4.3) (Schmieder and Edwards 2011) using 90 % coverage and 94 % filtering options. The merged reads were further processed using QIIME 1.9.1 (Caporaso et al., 2010b). An additional quality filtering step was performed, and all reads that were below an average Phred score of 20 were discarded. To check for chimeric sequences the usearch61 algorithm (Edgar 2010; Edgar et al., 2011) implemented in QIIME 1.9.1 and sequences were assigned against the Greengenes 16S rRNA database (version 13_8) (McDonald et al., 2012). To facilitate the OTU picking a two-step open-reference OTU picking was performed. First, quality filtered reads were binned into OTUs based on 97 % sequence similarity. In this step, reads were clustered against Greengenes 16S rRNA database (McDonald et al., 2012) at 97 % sequence identity. The reads that did not group with any sequences in the reference database were clustered at 97 % similarity with respect to each other (*de novo* approach). Representative sequences were chosen for each *de novo* OTU and aligned against a Greengenes core set (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a). OTUs that appeared only once across all the samples (singletons) were removed from the downstream analyses. Taxonomy was assigned to the representative sequences of each OTU based on Ribosomal Database Project (RDP) Näive Bayesian Classifier v.2.2 (Wang et al., 2007). In addition, to improve taxonomic assignment resolution representative sequences of each OTU were blasted against RDP (16S rRNA training set 16) (Wang et al., 2007), SILVA (SSU Ref NR 128, September 2016) (Quast et al., 2013) and NCBI 16S ribosomal RNA (Bacteria and Archaea) (Altschul et al., 1990).

3.14. Application of selected strains in sausage production

3.14.1. Preparation of starter cultures

Based on the safety aspects and metabolic profiles of all representatives, two *Lb. sakei* strains (C_7d_13 and MRS_296) were selected for further testing of their efficiency as starter cultures when applied in meat batter. Strains were separately inoculated in MRS broth (100 mL) and grown at 37 °C until reaching optical density (OD) of 2.00, measured at 600 nm. Cultures were harvested by centrifugation at 8000 x *g* for 5 min, cell pellets

were pooled and resuspended in 100 mL of sterile skimmed milk solution (1.5 %) and added to the meat batter.

3.14.2. Sausage formulation and sampling

Two formulations of fermented sausages were prepared. In the first formulation, two *Lb. sakei* strains were applied as starter cultures (C_7d_13 and MRS_296), while the second formulation served as a spontaneously fermented control. Each treatment batch was prepared with 15 kg of meat batter. Both batches were made as a mixture of domestic pig (*Sus scrofa domesticus*) meat (60 %) and wild boar (*Sus scrofa*) meat (40 %), seasoned with following ingredients: salt (1.91 %), ground red chili peppers (0.51 %), ground red sweet peppers (0.2 %), ground onion (0.31 %), sugar (0.2 %) and ground black peppers (0.1 %). Meat batter was filled in natural casings with 38 mm diameter, and the sausages were ripened in a drying chamber under varying relative humidity and temperature. Sausage samples were taken in triplicates, at different time points during production, fermentation and ripening (0, 4, 7, 16, 20 and 40 days), except for complete microbiological analysis where only samples after 0, 7 and 40 days were analysed.

3.14.3. Physicochemical analysis

pH and water activity (a_w) were measured as previously described in section 3.2.

3.14.4. Histamine and tyramine content

The content of histamine and tyramine in final products were determined as described in section 3.3.

3.14.5. Microbiological analysis

Yeasts, moulds, *Enterobacteriaceae*, *E. coli*, coliforms, *Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes* were determined as previously described in section 3.3, while *Enterococcus* spp. were detected as described in section 3.4. Presumptive *L. monocytogenes* colonies were purified, and the DNA was extracted by Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. Isolates were identified by 16S rRNA gene sequencing (Eurofins MWG Operon and Macrogen) with bak4 and bak11 primers, as described in section 3.7.2. The received sequences were analyzed with the BLASTn tool (http://blast.ncbi.nlm.nih.gov), and a minimum sequence identity of 98 % was chosen as a criterion for species identification.

3.14.6. Isolation, enumeration and fingerprinting of lactobacilli

From both batches (the one inoculated with two *Lb. sakei* strains and the spontaneously fermented control), LAB were isolated and enumerated on LamVab media, as described in section 3.9. The DNA from purified cultures was extracted by Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. All collected isolates were straind by the repetitive (rep)–PCR with the $(GTG)_5$ primer and clustered, as described in section 3.11.1. Based on the cluster analysis, several strains were selected and sequenced as described in section 3.11.2.

3.15. Nucleotide sequence accession numbers

The sequences were deposited in the GeneBank database using the BankIt, web-based data submission tool (http://www.ncbi.nlm.nih. gov/BankIt/). All sequences of strains isolated from spontaneously fermented wild boar and deer meat sausages are deposited under accession numbers KY575875-KY575954. The sequence data obtained from NGS analysis was submitted to NCBI SRA (Sequence Read Archive) and is available under accession number PRJNA428753. Sequences of representative isolates collected from the inoculated batch as well as the control batch are deposited under accession numbers MH197045-51; MH197053-67 and MH231452-54.

3.16. Data processing and statistical analyses

For comparison of pH values, autoaggregation ability, survival in simulated conditions of GI tract and technological chraracterization of isolates, a two-way ANOVA was performed in SAS Studio University Edition 3.4 (SAS Institute, 2015), followed by Tukey's honest significance test (HSD). T-test assuming unequal variances was used for comparison of pH, water activity, microbial count and tyramine content between the two batches.

Statistical analyses of the data obtained by high throughput sequencing (NGS) were performed on the randomly subsampled dataset and computed using the R environment version 3.0.2 (R Core Team 2013). To correct for uneven sequencing depth reads from each sample were rarefied to the lowest number of reads per sample by using the rarefy function from GUniFrac package (Chen 2012). Rarefaction analysis for assessment of community coverage was performed using the vegan package (Oksanen et al., 2017) using 1000 rarefactions for each sampling step. Alpha diversity was estimated by calculating Shannon's diversity index, Pielou's evenness measure and observed species number using the vegan package. Statistically significant differences for alpha diversity were tested in R by calculating unpaired Wilcoxon rank-sum test using package dplyr (Wickham 2015). To identify significantly different taxa across fermentation stages of

different sausage, a two-way ANOVA was performed, followed by Tukey's honest significance test (HSD). If assumption of normality, tested for each taxon by Shapiro-Wilk test, and homogeneity, tested by Levene's test, were not met, permutation tests were performed instead.

4. RESULTS

4.1. Physicochemical analysis of spontaneously fermented game meat sausages

The weight of sausages filled in collagen casings was around 650 g, while for those filled in natural casings was around 550 g, and the production ended when weight loss reached 35-40 %. Relative humidity and temperature measured at different time points of sausage production are shown in Figure 4.1. Except DS3 sausage, relative humidity was roughly between 65 and 90 % during all measured time points. Relative humidity during the production of DS3 sausage was roughly between 50-65 %, and its production ended after 20 days, while the production of other sausages ended after 40 days. Compared to other sausages, the production of WB1 and WB2 was characterised by lower temperatures during the first 2 weeks (Figure 4.1.). The results of the pH and water activity (a_w), measured during different time points of production, are shown in Table 4.1. In respect to the pH, WB1 and WB2 sausages differed from all the sausages, having a constant pH or a very slight pH fall during all stages of production. In other sausages, the pH decreased up to day 7 (DS3) or day 10 (WB3, DS1, DS2), after which it stabilised and started increasing again. At the end of ripening, the lowest pH was measured in DS2 sausage (5.04), while the highest pH values were detected in WB1 and WB2 sausages, being 5.53 and 5.49, respectively. The water activity (a_w) values decreased below 0.90 after 20 days of ripening, varying between 0.83 and 0.87 at the end.

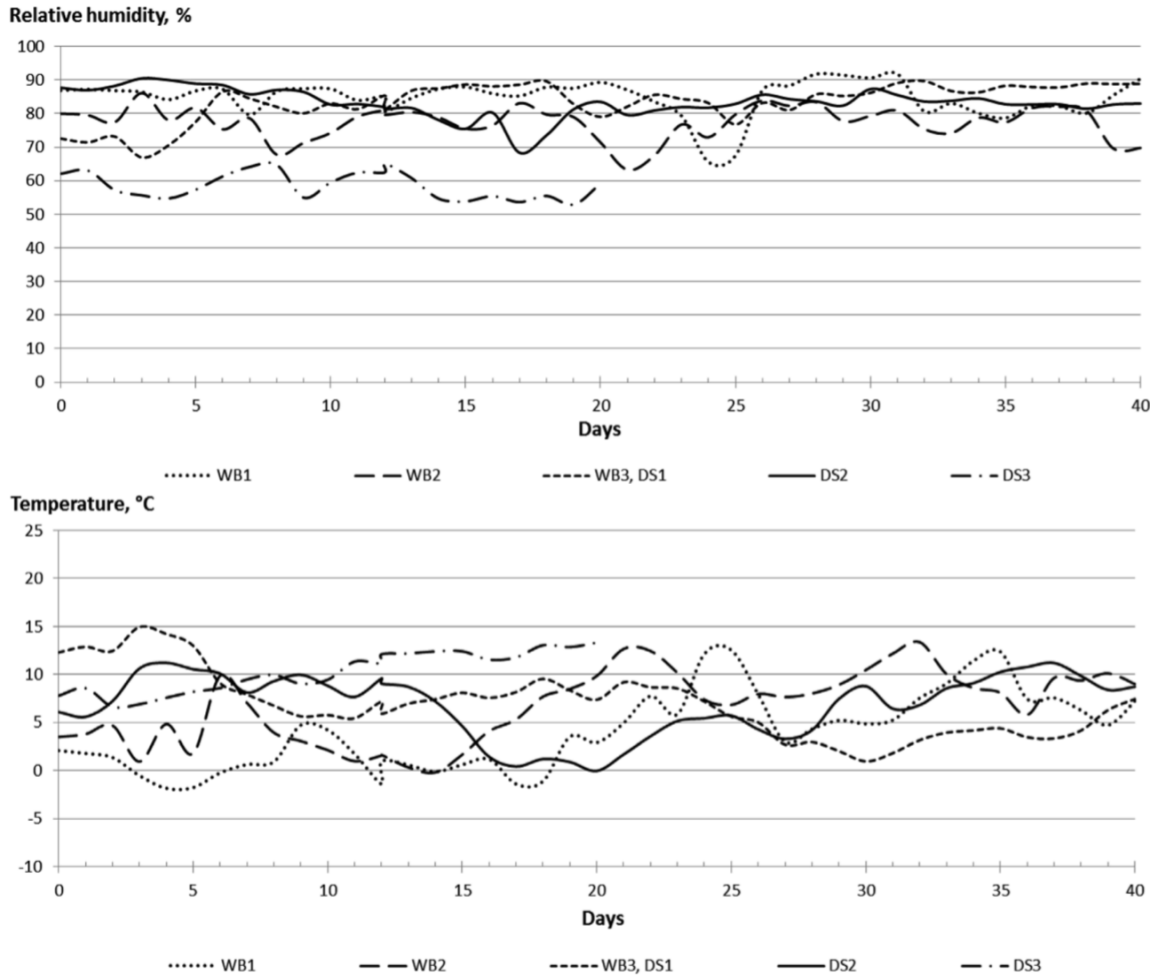

Figure 4.1. Relative humidity (%) and temperature (°C) measured at different time points of wild boar (WB1, WB2, WB3) and deer meat (DS1, DS2, DS3) sausage production. The sausages were produced at five different production units (WB1, WB2, WB3/D1, DS2, DS3).

Table 4.1. pH and water activity (a_w) measured in samples of wild boar (WB1, WB2, WB3) and deer meat (DS1, DS2, DS3) sausages at different time points of their production (0, 4, 7, 10, 20 and 40 days).

Sausage type	Production time points (days)	pH±st.dev	Water activity (a_w)±st.dev
WB1	0	5.54±0.03	n.p.
	4	5.55±0.03	0.94±0.00
	7	5.54±0.01	0.95±0.00
	10	5.55±0.00	0.94±0.00
	20	5.51±0.04	0.93±0.00
	end (40)	5.53±0.00	0.86±0.00
WB2	0	5.55±0.00	n.p.
	4	5.43±0.04	0.95±0.00
	7	5.39±0.03	0.94±0.00
	10	5.45±0.04	0.93±0.00
	20	5.42±0.02	0.93±0.00
	end (40)	5.49±0.01	0.86±0.00
WB3	0	5.74±0.03	0.98±0.00
	4	5.60±0.00	0.93±0.00
	7	5.15±0.05	0.93±0.00
	10	5.09±0.03	0.92±0.00
	20	5.11±0.09	0.97±0.00
	end (40)	5.27±0.03	0.87±0.00
DS1	0	5.59±0.00	0.98±0.00
	4	5.41±0.01	0.95±0.00
	7	5.03±0.05	0.92±0.00
	10	4.98±0.02	0.92±0.00
	20	5.00±0.02	0.90±0.00
	end (40)	5.11±0.03	0.84±0.00
DS2	0	5.55±0.05	0.95±0.00
	4	5.48±0.08	0.94±0.00
	7	4.99±0.03	0.93±0.00
	10	4.88±0.02	0.92±0.00
	20	4.89±0.02	0.91±0.00
	end (40)	5.04±0.01	0.87±0.00
DS3	0	5.61±0.03	0.97±0.00
	4	5.55±0.04	0.94±0.00
	7	5.19±0.03	0.92±0.00
	10	5.23±0.02	0.90±0.00
	end (20)	5.28±0.06	0.83±0.00

4.2. Histamine and tyramine content

In this study, the histamine content was below 5.0 mg/kg for all final products. The content of tyramine, however, varied between sausage samples (Table 4.2.) and it was between 47.30±6.60 mg/kg (DS3) and 219.00±13.00 mg/kg (DS2).

Table 4.2. The content of tyramine measured in ready-to-eat wild boar (WB) and deer meat (DS) sausages.

Sausage type	Level of detected tyramine (mg/kg)
WB1	81.10±11.40
WB2	62.60±8.80
WB3	99.80±14.00
DS1	91.30±12.80
DS2	219.00±31.00
DS3	47.30±6.60

4.3. Microbiological analysis of spontaneously fermented game meat sausages

The results of the microbiological analysis of wild boar and deer meat sausages are reported in Table 4.3. The appearances of characteristic colonies grown on media used for microbiological analysis are shown in Figure 4.2. In general, the number of yeasts increased during fermentation and ripening, while the undesirable microbiota decreased. No moulds were detected. The total number of aerobic bacteria (PCA) in WB1 and WB2 stayed at much lower count during production than in other sausages, probably due to the low temperatures present at that time (Figure 4.1.). *S. aureus* was sporadically present up to 20 days, but it was absent in all ready-to-eat sausages. *Salmonella* spp. were not detected at all and *L. monocytogenes* was only detected during fermentation and ripening of WB3 sausage (<2 log cfu/g) but it was absent in final products. However, in the ready-to-eat WB3 sausage, the detected number of *E. coli* and *Enterobacteriaceae* (4.07 and 3.87 log cfu/g, respectively) exceeded the limits set by the German Society for Hygiene and Microbiology (DGHM, 2004). In the same sausage, the high number of coliforms and hemolytic *Bacillus cereus* group was noticed (3.81 and 5.68 log cfu/g, respectively). Beside WB3 sausage, DS2 sausage was also unfit for human consumption due to the number of *Enterobacteriaceae* (4.52 log cfu/g) which exceeds the limit of 3 log cfu/g set by the German Society for Hygiene and Microbiology (DGHM, 2004).

Table 4.3. Microbiological analysis of wild boar (WB1, WB2, WB3) and deer meat (DS1, DS2, DS3) sausages at different time points of production (0, 4, 7, 10, 20 and 40 days). Values are expressed as log cfu/g.

Time point (days)	Sausage	PCA (total aerobic count)	DRBC (yeasts)	CCA (*E. coli*)	CCA (coliforms)	VRBG (*Enterobacteriaceae*)	BP (*Staphylococcus aureus*)	MYP (*Bacillus cereus* group)
0	WB1	5.19	4.24	< 1.00	4.38	4.41	3.26	4.32
	WB2	5.22	4.23	< 1.00	< 1.00	< 1.00	3.32	< 1.00
	WB3	4.98	3.17	4.49	4.51	4.47	< 1.00	4.72
	DS1	4.97	3.71	2.74	< 1.00	3.69	< 1.00	3.78
	DS2	4.64	4.51	< 1.00	2.98	4.31	3.40	3.40
	DS3	5.97	< 1.00	< 1.00	2.70	2.68	3.58	< 1.00
4	WB1	6.04	4.72	< 1.00	4.18	4.00	< 1.00	4.62
	WB2	4.98	3.34	< 1.00	< 1.00	< 1.00	4.41	< 1.00
	WB3	4.92	2.75	4.73	5.23	5.69	< 1.00	4.54
	DS1	4.67	< 1.00	2.97	< 1.00	3.24	< 1.00	4.24
	DS2	4.55	4.52	< 1.00	2.95	5.05	< 1.00	< 1.00
	DS3	4.56	< 1.00	< 1.00	2.73	2.19	3.58	< 1.00
7	WB1	5.38	4.72	3.15	3.45	3.53	3.41	4.71
	WB2	8.51	5.18	< 1.00	< 1.00	< 1.00	3.66	< 1.00
	WB3	8.77	< 1.00	4.43	4.44	4.68	< 1.00	6.11
	DS1	7.62	5.18	3.64	< 1.00	2.42	< 1.00	3.85
	DS2	8.89	4.25	< 1.00	2.43	5.41	< 1.00	< 1.00
	DS3	8.78	< 1.00	< 1.00	1.81	2.00	3.43	< 1.00
10	WB1	5.25	5.20	< 1.00	3.45	3.11	3.50	3.30
	WB2	6.73	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
	WB3	9.04	< 1.00	4.92	4.97	4.35	< 1.00	5.80
	DS1	8.29	4.62	2.61	< 1.00	3.86	< 1.00	3.77
	DS2	8.89	5.42	< 1.00	3.47	5.00	< 1.00	< 1.00
	DS3	8.22	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00

20	WB1	5.84	5.76	2.08	3.00	2.68	< 1.00	4.50
	WB2	7.57	4.73	< 1.00	< 1.00	< 1.00	3.85	< 1.00
	WB3	9.16	5.13	4.69	4.33	4.58	< 1.00	5.72
	DS1	8.46	4.59	2.58	< 1.00	2.38	< 1.00	< 1.00
	DS2	8.67	5.04	< 1.00	< 1.00	4.69	< 1.00	< 1.00
	DS3 (end)	8.70	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00
40	WB1	5.28	6.04	< 1.00	< 1.00	< 1.00	< 1.00	4.41
	WB2	7.24	5.51	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00
	WB3	8.43	6.92	4.07	3.81	3.87	< 1.00	5.68
	DS1	7.96	5.44	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00
	DS2	7.34	4.08	< 1.00	< 1.00	4.52	< 1.00	< 1.00

n.p.=not performed

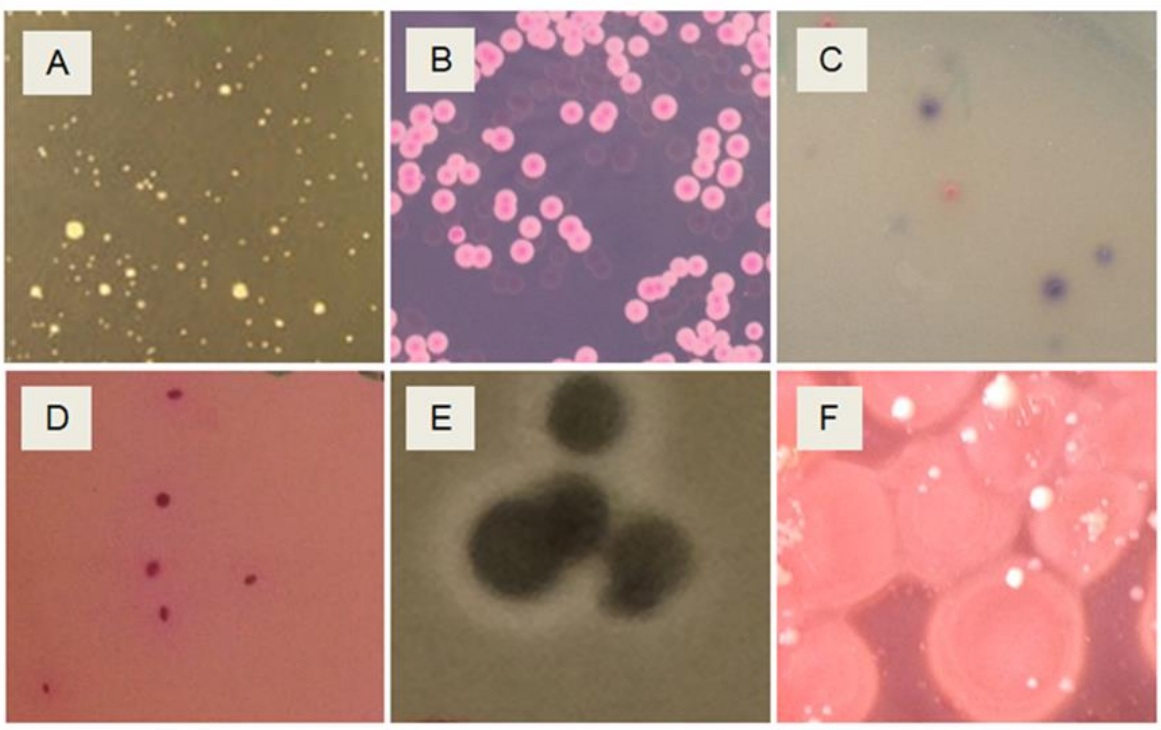

Figure 4.2. The appearances of characteristic colonies grown on media used for microbiological analysis. All colonies grown on PCA plates (A) were counted. Pink colonies without mycelium are characteristic for yeasts grown on DRBC agar (B). Dark blue to purple colonies grown on CCA agar are characteristic for *E. coli* (C) and pink to red colonies are characteristic for coliforms (C). *Enterobacteriaceae* grown on VRBG agar (D) are typically pink to red or purple with or without precipitation haloes. *S. aureus* grown on BP agar (E) appears as black, shiny, convex colony with 1-1.5 mm diameter and clear or opaque haloes. *Bacillus cereus* colonies grown on MYP agar (F) are rough, with a bright pink background of egg yolk precipitate.

4.4. Isolation and enumeration of LAB

To cover the total diversity of indigenous LAB during fermentation and ripening of wild boar and deer meat sausages, isolation on different selective media was carried out. Due to the observed insufficient selectivity of MRS media, LamVab was additionally introduced for the isolation of *Lactobacillus* spp. from deer meat sausages. The maximum number of the LAB (8.99 log cfu/g) was detected on MRS media in DS1 sausage after 7 days of production. In general, the number of LAB increased during fermentation and remained stable over the ripening, except KAA media (Table 4.4.). The cell counts on KAA media either decreased below the detection limit (<1 log cfu/g) after 10 days of production (WB1, WB2, DS1) or were not detected at any stage of production (DS2, DS3). Only in WB3

sausage, it remained stable. Also, in WB3 sausage the highest number of cell counts on KAA media was detected, reaching 4.09 log cfu/g.

Table 4.4. Number of LAB (log cfu/g±st.dev) in samples of wild boar (WB1, WB2, WB3) and deer meat (DS1, DS2, DS3) sausages at different time points of their production (0, 4, 7, 10, 20 and 40 days).

Farm	Production time points (days)	Different media used in this study (log cfu/g±st.dev.)			
		KAA media (*Enterococcus*)	MRS (*Lactobacillus*)	LamVab (*Lactobacillus*)	MSE (*Leuconostoc*)
WB1	0	< 1.00	< 1.00	n.p.	3.59 ± 0.05
	4	3.52 ± 0.07	< 1.00	n.p.	5.22 ± 0.17
	7	3.04 ± 0.00	2.89 ± 0.04	n.p.	3.68 ± 0.04
	10	3.17 ± 0.04	3.18 ± 0.02	n.p.	3.31 ± 0.04
	20	< 1.00	3.89 ± 0.07	n.p.	3.36 ± 0.05
	end (40)	< 1.00	5.96 ± 0.05	n.p.	3.87 ± 0.03
WB2	0	3.97 ± 0.05	4.87 ± 0.12	n.p.	4.27 ± 0.06
	4	3.80 ± 0.11	4.87 ± 0.08	n.p.	3.71 ± 0.08
	7	3.89 ± 0.02	4.79 ± 0.03	n.p.	3.64 ± 0.02
	10	3.54 ± 0.24	4.15 ± 0.07	n.p.	3.58 ± 0.03
	20	< 1.00	4.88 ± 0.05	n.p.	3.34 ± 0.11
	end (40)	< 1.00	4.36 ± 0.01	n.p.	3.87 ± 0.10
WB3	0	3.40 ± 0.08	4.69 ± 0.02	n.p.	4.23 ± 0.05
	4	4.06 ± 0.11	7.71 ± 0.00	n.p.	5.31 ± 0.12
	7	4.09 ± 0.13	8.47 ± 0.00	n.p.	6.59 ± 0.04
	10	4.08 ± 0.12	7.48 ± 0.00	n.p.	6.77 ± 0.02
	20	3.96 ± 0.07	8.83 ± 0.03	n.p.	6.15 ± 0.02
	end (40)	3.77 ± 0.03	8.17 ± 0.05	n.p.	6.85 ± 0.22
DS1	0	3.8 ± 0.19	< 1.00	< 1.00	3.79 ± 0.41
	4	3.34 ± 0.03	6.74 ± 0.01	6.73 ± 0.03	4.51 ± 0.03
	7	3.80 ± 0.17	8.99 ± 0.01	9.14 ± 0.09	6.37 ± 0.06
	10	3.17 ± 0.16	7.90 ± 0.03	8.23 ± 0.07	7.63 ± 0.03
	20	< 1.00	7.68 ± 0.06	8.30 ± 0.00	7.68 ± 0.07
	end (40)	< 1.00	7.68 ± 0.02	6.70 ± 0.06	4.93 ± 0.12
DS2	0	< 1.00	3.95 ± 0.05	< 1.00	3.09 ± 0.02
	4	< 1.00	6.04 ± 0.05	6.22 ± 0.02	5.24 ± 0.12
	7	< 1.00	8.85 ± 0.11	7.98 ± 0.06	6.23 ± 0.04
	10	< 1.00	7.13 ± 0.01	8.34 ± 0.01	7.25 ± 0.02
	20	< 1.00	8.32 ± 0.02	8.30 ± 0.03	7.13 ± 0.01
	end (40)	< 1.00	8.69 ± 0.10	8.07 ± 0.05	7.56 ± 0.07
DS3	0	< 1.00	3.23 ± 0.00	4.17 ± 0.00	3.06 ± 0.08
	4	< 1.00	3.23 ± 0.00	3.04 ± 0.00	3.06 ± 0.07
	7	< 1.00	7.06 ± 0.04	7.72 ± 0.02	6.12 ± 0.08
	10	< 1.00	6.29 ± 0.02	6.38 ± 0.04	7.85 ± 0.05
	end (20)	< 1.00	7.09 ± 0.05	7.14 ± 0.07	7.56 ± 0.07

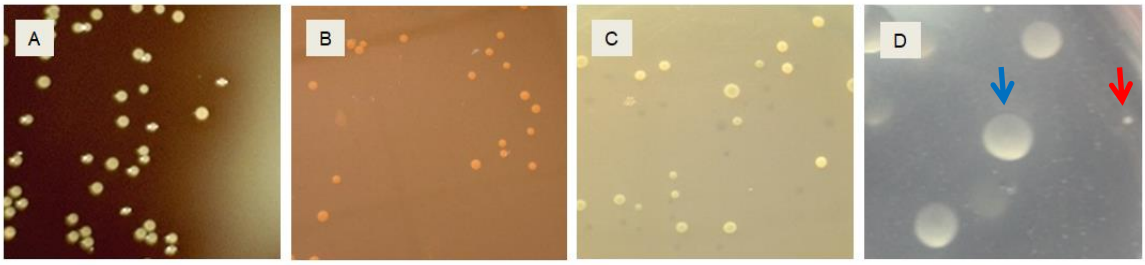

Figure 4.3. The appearances of characteristic colonies grown on media used for isolation and enumeration of the LAB. Enterococci grown on KAA media (A) appear as white, grey or black colonies surrounded by black zones of aesculin degradation. Lactobacilli grown on MRS agar (B) are typically compact or feathery and are small, opaque and white. Lactobacilli grown on LamVab media (C) are typically white or green small colonies. Leuconostoc on MSE media (D) appear as very small (approx. 1mm in diameter), smooth, round colonies (red arrow) and are often slime-forming (blue arrow).

4.5. Fingerprinting, identification and distribution profile of LAB

All isolates collected from wild boar and deer meat sausages at different time points of their production (*n*=917) on media selective for LAB, were straind by the repetitive (rep)–PCR and then molecular tools were used for their identification. In cluster analysis, discrimination was only considered when strains were less than 90 % similar, and all strains were clustered in 283 groups. *Le. mesenteroides* was identified as the most frequently isolated species (28.24 %), followed by *Lb. sakei* (20.72 %) and *E. casseliflavus* (11.55 %). In this study, strong differences were observed in the distribution profiles of dominant LAB among the investigated sausages (Table 4.5.). *Le. mesenteroides* was the predominant species found in WB1, WB2, DS1 and DS2 sausages, while *Lb. sakei* was the most frequently isolated from WB3 and DS3 sausages. *E. casseliflavus* (WB1, WB2, DS3) and *E. durans* (WB3, DS1, DS2) were detected as predominant enterococci, while *E. faecium* and *E. faecalis* were detected only sporadically. Other LAB members, such as *Weissella*, *Carnobacterium*, *Lactococcus* and *Pediococcus* were detected sporadically and at low frequencies (2.51, 0.87, 1.09 and 0.22 % of total LAB microbiota, respectively).

Table 4.5. Identification of isolates collected from wild boar (WB1, WB2, WB3) and deer meat (DS1, DS2, DS3) sausages at different time points of their production (0, 4, 7, 10, 20 and 40 days). Isolation media are listed in brackets.

Sausage	Identification of isolates collected from different media (KAA, MRS, MSE or LamVab)	Number of detected strains at the different time point of sausage production (days)						Total
		0	4	7	10	20	40	
WB1 (*n*=151)	*Leuconostoc mesenteroides* (MRS, MSE)	2	23	7	15	14	9	70
	Enterococcus casseliflavus (KAA, MRS)	4	16	6	7	5	5	43
	Enterococcus spp. (MSE, KAA)	0	2	7	2	2	4	17
	Enterococccus faecalis (MSE, MRS)	2	2	2	3	0	0	9
	Weissella viridescens (MRS)	2	0	0	0	0	1	3
	Lactobacillus sakei (MSE, MRS)	0	0	0	0	0	3	3
	Enterococccus gallinarum (KAA)	0	2	0	0	0	0	2
	Micrococcus spp. (MSE)	0	1	0	1	0	0	2
	Enterococcus faecium (KAA)	0	0	1	0	0	0	1
	Streptococcus spp. (MRS)	0	0	1	0	0	0	1
WB2 (*n*=123)	*Leuconostoc mesenteroides* (MRS, MSE)	1	16	12	6	10	6	51
	Enterococcus casseliflavus (KAA)	0	13	1	0	4	2	20
	Weissella viridescens (MRS)	3	9	0	0	0	1	13
	Micrococcus spp. (MSE)	1	4	0	4	1	0	10
	Lactococcus garviae (KAA, MSE)	1	1	5	0	0	3	10
	Lactobacillus sakei (MSE, MRS)	1	0	2	0	0	4	7
	Enterococcus faecalis (KAA, MSE)	0	0	4	0	0	0	4
	Enterococcus spp. (MSE)	1	1	0	0	1	0	3
	Streptococcus spp. (KAA)	1	0	1	0	0	0	2
	Weissella spp. (MSE)	0	0	0	0	1	1	2
	Enterococcus durans (KAA)	0	1	0	0	0	0	1
WB3 (*n*=208)	*Lactobacillus sakei* (MRS, MSE)	3	25	10	14	12	16	80
	Enterococcus durans (KAA)	0	7	12	6	5	2	32
	Leuconostoc mesenteroides (MRS, MSE)	1	9	3	4	7	1	25
	Enterococcus faecium (KAA)	0	9	0	3	7	5	24
	Enterococcus casseliflavus (KAA)	10	3	0	0	0	0	13
	Streptococcus gallolyticus (MRS, MSE)	11	0	0	0	0	0	11
	Enterococcus spp. (MSE, KAA)	3	2	0	0	2	1	8
	Enterobacteriaceae (MRS)	0	6	1	0	0	0	7
	Lactobacillus spp. (MSE)	0	0	3	0	0	0	3
	Enterococcus faecium / faecalis (KAA)	0	0	0	0	0	2	2
	Enterococcus hirae (KAA)	0	0	0	1	1	0	2
	Leuconostoc spp. (MSE)	0	0	1	0	0	0	1
DS1 (*n*=186)	*Leuconostoc mesenteroides* (LamVab, MRS, MSE)	4	10	10	10	2	0	36
	Enterococcus durans (KAA, MSE, LamVab)	1	7	10	8	3	6	35
	Lactobacillus spp. (LamVab, MRS, MSE)	2	0	0	0	1	16	19

	Enterococcus casseliflavus (KAA, MSE, MRS)	14	4	4	1	1	2	26
	Bacillus subtilis (MRS, MSE)	1	6	3	0	6	0	16
	Lactobacillus curvatus (MRS)	2	0	4	5	3	1	15
	Enterococcus spp. (LamVab, MSE)	1	2	0	3	2	1	9
	Staphylococcus spp. (MRS)	1	0	1	0	2	2	6
	Enterococcus faecium (MSE)	0	0	3	3	0	0	6
	Lactobacillus sakei (MSE, MRS)	0	1	0	0	0	4	5
	Carnobacterium maltaromaticum (MSE)	0	0	0	3	0	0	3
	Bacillus thuringiensis (KAA, MSE)	0	2	0	0	0	1	3
	Enterococcu faecium / hirae (KAA)	0	3	0	0	0	0	3
	Bacillus spp. (KAA, MSE)	0	0	2	0	0	0	2
	Streptococcus spp. (MSE)	0	0	1	0	0	0	1
	Carnobacterium divergens	0	0	0	1	0	0	
DS2 (*n*=126)	*Leuconostoc mesenteroides* (LamVab, MSE, MRS)	15	9	11	16	12	1	64
	Lactobacillus sakei (MRS, LamVab, MSE)	3	6	1	5	7	9	31
	Enterococcus durans (KAA)	0	1	4	1	0	0	6
	Weissella spp. (MRS, LamVab)	3	0	1	0	0	0	4
	Micrococcus luteus (MRS)	2	1	0	0	0	0	3
	Carnobacterium maltaromaticum (MSE)	3	0	0	0	0	0	3
	Lactobacillus spp. (MRS. MSE)	1	0	1	0	0	0	2
	Staphylococcus spp. (MRS)	0	1	0	0	0	1	2
	Pediococcus pentosaceus (LamVab)	0	0	0	1	0	1	2
	Enterococcus faecium / faecalis/ hirae (KAA)	0	0	0	2	0	0	2
	Enterococcus spp. (MSE)	0	0	0	2	0	0	2
	Enterobacteriaceae (MRS)	1	0	0	0	0	0	1
	Bacillus spp. (MSE)	0	0	0	0	0	2	2
	Bacillus thuringiensis (MSE)	0	0	0	0	0	1	1
	Weissella helenica (LamVab)	0	0	0	0	0	1	1
DS3 (*n*=123)	*Lactobacillus sakei* (MRS, LamVab)	12	13	10	17	12		64
	Lactobacillus spp. (MSE, LamVab)	4	5	4	1	1		15
	Enterococcus spp. (MSE)	0	0	4	7	4		15
	Leuconostoc mesenteroides (LamVab, MRS)	5	4	1	1	2		13
	Enterococcus casseliflavus (MSE)	0	3	1	0	0		4
	Enterobacteriaceae (LamVab)	0	0	3	0	0		3
	Staphylococcus epidermis (LamVab, MSE)	1	0	0	2	0		3
	Micrococcus spp. (MSE, LamVab)	0	1	1	0	0		2
	Bacillus thuringiensis (MSE)	0	0	1	1	0		2
	Bacillus spp. (MSE)	0	0	1	0	0		1
	Carnobacterium maltaromaticum (MSE)	1	0	0	0	0		1

4.6. The intraspecies diversity of dominant LAB

Cluster analysis of the most frequently isolated species; *Le. mesenteroides*, *Lb. sakei* and *E. casseliflavus* was performed based on their rep-PCR patterns and discrimination was only considered when strains were less than 90 % similar. All collected *Le. mesenteroides* isolates (*n*=259) were clustered in 42 groups, of which 20 were unique strains (Appendix 7.4.1.). For *Lb. sakei* (*n*=190), a total of 59 clusters were obtained, among which 33 containing single isolates (Appendix 7.4.2.) and for *E. casseliflavus* 47 clusters of which 21 containing single isolates, were noticed (Appendix 7.4.3.). Therefore, when *n* is taken into consideration, *E. casseliflavus* isolates exhibited the highest degree of heterogeneity in comparison to *Le. mesenteroides* and *Lb. sakei*. Cluster analysis of *Le. mesenteroides* (Appendix 7.5.1.) revealed a dominant strain that was able to establish and proliferate through the whole production process in two sausages (WB1 and WB2), while *for Lb. sakei* (Appendix 7.5.2.) it was evident only for one sausage (WB3). For *E. casseliflavus* (Appendix 7.5.3.), no dominant strain that was able to persist during the whole production process was noticed. Most of the strains exhibited the tendency of sausage-specific clustering, implying that the raw meat used for sausage production was the main source of bacterial strains, rather than the production environment. To confirm this, cluster analysis of *Le. mesenteroides*, *E. casseliflavus*, *E. durans* and *Lactobacillus* spp. strains obtained from WB3 and DS1 sausages (produced at the same production unit) was performed, as this species was isolated at fairly equal frequencies from both sausages. *Le. mesenteroides* strains showed the tendency of clustering according to the sausage type, but only 1 of the *Le. mesenteroides* clusters were completely formed by strains obtained from a particular sausage, while *E. casseliflavus*, *E. durans* and *Lactobacillus* spp. strains exhibited strict sausage-specific clustering (Table 4.6.) sharing only one strain (cluster number 16 for *E. casseliflavus*) of 67 in total.

Table 4.6. Cluster analysis of strains isolated from WB3 and DS1 sausages, both produced at the same production unit.

Identification	Cluster	Similarity (%)	No of isolates	Sausage type	
				WB3	DS1
Leuconostoc mesenteroides	1	-	1	0	1
	2	-	1	0	1
	3	-	1	0	1
	4	-	1	1	0
	5	-	1	1	0
	6	-	1	1	0
	7	-	1	0	1
	8	96	7	4	3
	9	90	11	10	1
	10	-	1	0	1
	11	100	2	0	2
	12	-	1	0	1
	13	-	1	0	1
	14	93	15	2	13
	15	-	1	0	1
	16	-	1	0	1
	17	100	2	1	1
	18	91	12	5	7
Enterococcus casseliflavus	1	-	1	0	1
	2	-	1	0	1
	3	100	2	0	2
	4	93	3	0	3
	5	-	1	1	0
	6	100	2	0	2
	7	96	3	0	3
	8	95	3	3	0
	9	-	1	0	1
	10	-	1	1	0
	11	92	2	0	2
	12	100	3	0	3
	13	97	5	0	5
	14	100	2	2	0
	15	100	2	0	2
	16	90	7	6	1
Enterococcus durans	1	-	1	0	1
	2	100	2	0	2
	3	-	1	0	1
	4	-	1	0	1

	5	100	2	0	2
	6	-	1	0	1
	7	100	26	0	26
	8	-	1	0	1
	9	90.00	20	20	0
	10	-	1	1	0
	11	-	1	1	0
	12	100	2	2	0
	13	92.86	8	8	0
	14	-	0	0	0
	15	-	0	0	0
Lactobacillus spp.	1	100	3	0	3
	2	-	1	0	1
	3	95.65	5	0	5
	4	95.44	15	0	15
	5	90.91	4	0	4
	6	-	1	1	0
	7	100	3	3	0
	8	-	1	1	0
	9	100	2	0	2
	10	-	1	0	1
	11	-	1	1	0
	12	91.43	4	4	0
	13	100	2	2	0
	14	-	1	1	0
	15	91.89	20	20	0
	16	-	1	0	1
	17	91.66	3	0	3
	18	-	1	1	0
	19	-	1	1	0
	20	100	8	8	0
	21	100	5	5	0
	22	-	1	0	1
	23	91.45	35	35	0
	24	-	1	0	1
	25	-	1	0	1
	26	-	1	0	1

4.7. Selectivity of media used for isolation of LAB

4.7.1. Identification of isolates collected from media selective for LAB

Bacterial taxa detected at each of the media used for isolation of LAB are shown in Figure 4.4. KAA media was highly selective for *Enterococcus* spp., with 95.57 % of the isolates confirmed as enterococci. MSE media was the least selective one, with the highest diversity of genera detected. The majority of isolates collected from MSE were identified as *Enterococcus* spp. (32.13 %), followed by *Lactobacillus* spp. and (26.51 %) and *Leuconostoc* spp. (22.09 %). On MRS media supplemented with cycloheximide and LamVab, mostly *Lactobacillus* spp. and *Leuconostoc* spp. were isolated, although other genera of the LAB were also detected, as well as non-LAB isolates. Out of all 917 isolates collected from media selective for the LAB, a total of 7.20 % did not belong to the LAB, rather belonging to *Enterobacteriaceae*, *Bacillus* spp., *Staphylococcus* spp. and *Micrococcus* spp.

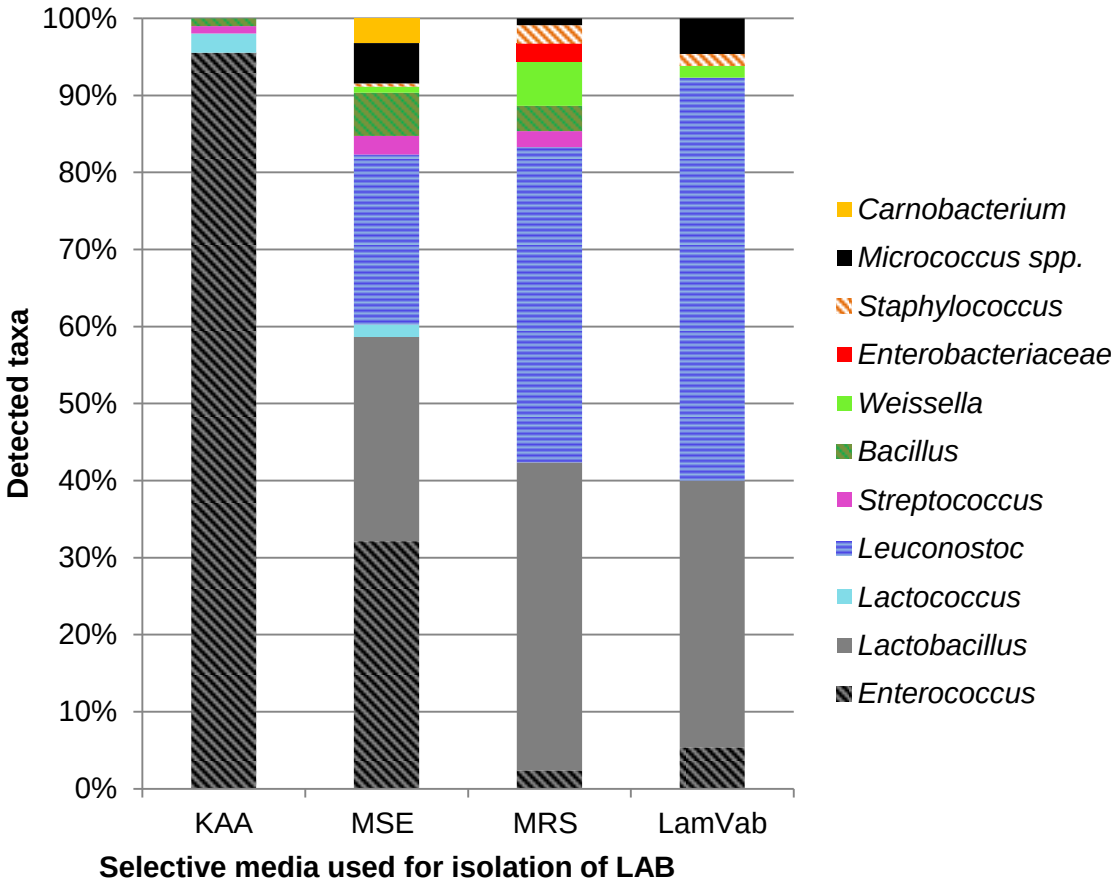

Figure 4.4. Bacterial taxa detected at media selective for the LAB.

4.7.2. PCR-DGGE analysis

PCR-DGGE analysis of total bulk consortia harvested from MRS media was applied to additionally check the selectivity of the media. The concentration of all purified PCR products used in DGGE was approximately 30 ng/µL. After DGGE, sequencing of the excised bands with different migration profiles revealed *Leuconostoc mesenteroides, Lactococcus garviae, Lactobacillus* spp. and *Weissella* spp. as dominant bacterial groups isolated on respective media (Figure 4.5.). The prevalence of bacterial groups detected in DGGE analysis is comparable to the frequency of genera appearances when single isolates collected from MRS media were identified (Figure 4.4.). *Lactococcus garviae* is an exception; although single colonies were not collected from MRS media, it seems that *Lc. garviae* was able to grow and proliferate on respective media.

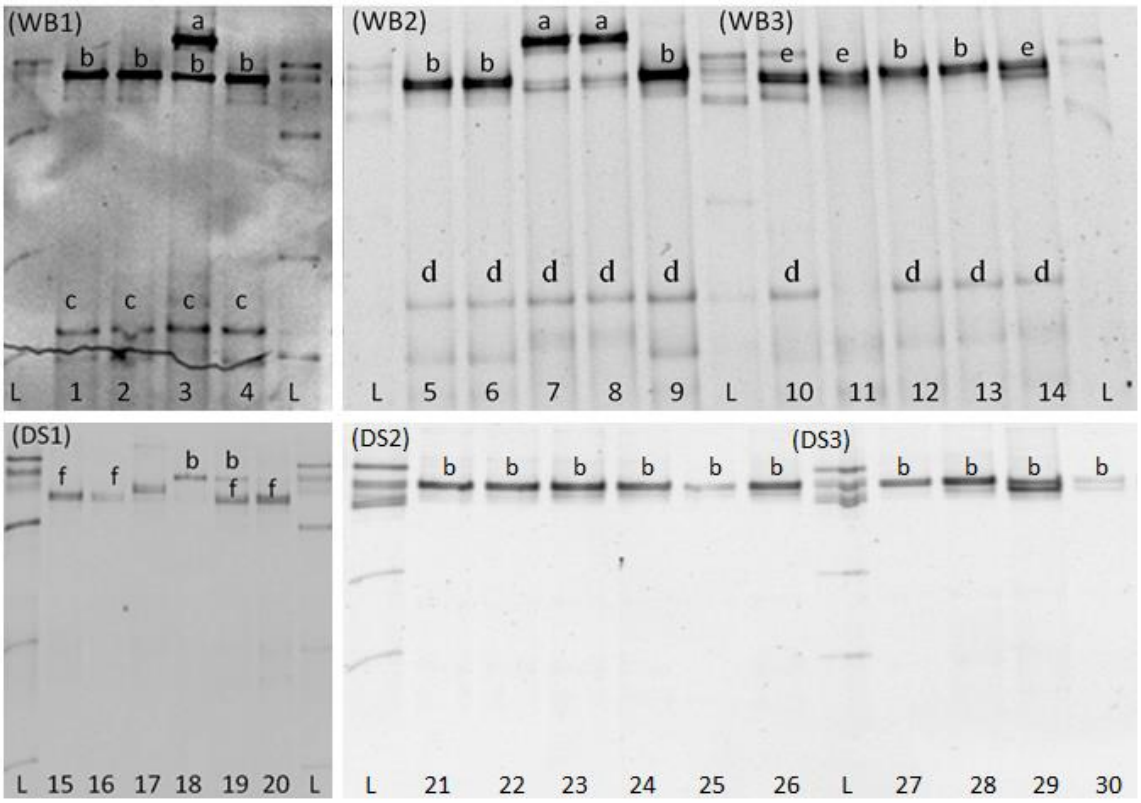

Figure 4.5. DGGE fingerprinting profiles of amplified V3 region 16S rRNA gene, of bulk colonies (consortia), collected from different MRS agar plates. Lanes 1-4: WB1 (2, 4, 7, 10 days), lanes 5-9: WB2 (0, 2, 4, 7, 10 days), lanes 10-14: WB3 (2, 4, 10, 20, 40 days), lanes 15-20: DS1 (0, 4, 7, 10, 20, 40 days), lanes 21-26: DS2 (0, 4, 7, 10, 20, 40 days), lanes 27-30: DS3 (0, 4, 7, 20 days). Lanes L: DNA Ladder consisting of *Lactobacillus gasseri, Leuconostoc mesenteroides* (DSM 20343), *Lactobacillus sakei* (LMG 9468), *Enterococcus faecalis* (DSM 20478) and *Lactobacillus casei*. Bands were identified as

a=*Lactococcus garviae*, b=*Leuconostoc mesenteroides,* c=*Lactococcus garviae,* d=*Leuconostoc* spp., e=*Weissella* spp., f=*Lactobacillus* spp.

4.8. Safety aspects of selected representative LAB strains

4.8.1. Susceptibility to antibiotics

The susceptibility of representative strains to antibiotics used in this study are shown in Table 4.7. All enterococcal (100.00 %) and a vast majority of *Le. mesenteroides* strains (94.74 %) were non-resistant to any of the tested antibiotics. Only one *Le. mesenteroides* strain showed resistance to antibiotics; B_15d_4 to erythromycin (2 µg) and clindamycin (2 µg). Compared to them, lactobacilli were more prone to exhibit resistance to a single antibiotic (24.14 %) or multiple (≥2) antibiotics (17.24 %). They were most commonly resistant to kanamycin (30 µg; 24.14 %) and gentamicin (10 µg; 17.24 %), followed by erythromycin (2 µg; 6.90 %), tetracycline (5 µg; 6.90 %) clindamycin (2 µg; 6.90 %) and ampicillin (2 µg; 3.45 %). None of the 57 representatives showed resistance to chloramphenicol (30 µg).

Table 4.7. Susceptibility of 57 representative strains to antibiotics using the disc diffusion method.

Identification	Strain code	Antibiotics used for *Enterococcus durans*						
		A (2)	A (10)	P (10)	TE (10)	E (15)	VA (5)	C (30)
Enterococcus durans (*n*=9)	A_0d_1	S	S	S	S	S	S	S
	A_0d_2	S	S	S	S	S	S	S
	A_0d_4	S	S	S	S	S	S	S
	A_4d_1	S	S	S	S	S	S	S
	A_7d_3	S	S	S	S	S	S	S
	A_7d_4	S	S	S	S	S	S	S
	A_40d_2	S	S	S	S	S	S	S
	A_40d_3	S	S	S	S	S	S	S
	A_40d_4	S	S	S	S	S	S	S
Identification	Strain code	Antibiotics used for *Lactobacillus* spp. and *Leuconostoc mesenteroides*						
		A (2)	GEN (10)	E (2)	TE (5)	K (30)	CLI (2)	C (30)
Lactobacillus sakei (*n*=27)	C_7d_13	S	I	S	S	S	S	S
	C_7d_14	S	I	S	R	I	S	S
	C_15d_13	S	S	S	S	S	S	S
	C_end_1	S	S	S	S	I	S	S
	MSE_24	S	R	I	S	R	R	S
	MSE_44	S	R	S	S	R	S	S
	MRS_280	S	S	S	R	R	R	S
	LamVab_106	S	S	S	S	S	S	S
	LamVab_74	S	R	S	S	S	S	S

	MRS_641	S	S	S	S	S	S	S
	MRS_604	S	S	R	S	S	S	S
	MRS_579	S	S	S	S	S	S	S
	MRS_567	S	I	S	S	I	S	S
	C_end_2	S	S	S	S	I	S	S
	MSE_40	S	R	S	S	S	S	I
	MSE_45	S	S	S	S	R	S	S
	MSE_38	S	S	S	I	I	S	S
	MSE_43	S	I	S	S	I	S	S
	MSE_104	S	S	S	I	S	S	S
	MSE_100	R	S	R	S	S	S	S
	MSE_23	S	I	S	S	R	S	S
	MSE_22	S	I	S	S	I	S	S
	MSE_26	S	I	S	S	I	S	S
	MSE_13	S	S	S	S	I	S	S
	MRS_267	S	I	S	S	I	S	S
	MRS_296	I	I	S	S	I	S	S
	MRS_331	I	I	S	S	I	S	S
Lactobacillus curvatus (*n*=2)	MRS_520	S	S	S	S	R	S	S
	MRS_526	I	R	S	S	R	S	S
Leuconostoc mesenteroides (*n*=19)	MRS_532	I	S	S	S	S	S	S
	C_15d_2	S	S	S	S	S	S	S
	C_15d_3	S	S	S	S	S	S	S
	C_15d_8	S	S	S	S	I	S	S
	B_15d_4	S	S	R	S	S	R	S
	MSE_92	S	S	S	S	I	S	S
	MSE_102	I	S	S	S	I	S	S
	MRS_120	S	S	S	S	I	S	S
	MRS_104	S	S	S	S	I	S	S
	MSE_359	S	S	S	S	I	S	S
	MSE_349	S	S	S	S	I	S	S
	MSE_317	I	S	S	S	S	S	S
	LamVab_116	S	I	S	S	S	S	S
	LamVab_96	S	S	S	S	S	S	S
	LamVab_27	S	S	S	S	S	S	S
	LamVab_2	S	I	S	S	I	S	S
	MRS_602	S	S	S	S	I	S	S
	MRS_506	S	S	S	S	I	S	S
	MRS_605	S	S	S	S	I	S	S

S=susceptible, I=intermediate, R=resistant

A=ampicillin (2 and 10 µg), P=penicillin (10 µg), TE=tetracycline (5 and 10 µg), E=erythromycin (2 and 15 µg), V=vancomycin (5 µg), and C=chloramphenicol (30 µg), GEN=gentamicin (10 µg), K=kanamycin (30 µg), CLI=clindamycin (2 µg).

4.8.2. PCR detection of genes encoding for the production of biogenic amines

A PCR assay was performed to detect the genes encoding for the production of histamine (*hdc*), putrescine (*odc*), tyramine (*tdc*) and cadaverine (*ldc*) in representative LAB strains. The presence of *hdc*, *odc* and *ldc* genes was not confirmed in any of the representatives, and only *tdc* gene, encoding for tyrosine decarboxylase, was determined (Table 4.8.). The frequency of *tdc* gene was 26.31 % in representatives (*n*=57), with the highest rate confirmed in *E. durans* (55.55 %; *n*=9) followed by *Le. mesenteroides* (31.58 %; *n*=19) and *Lb. sakei* (14.81 %, *n*=27).

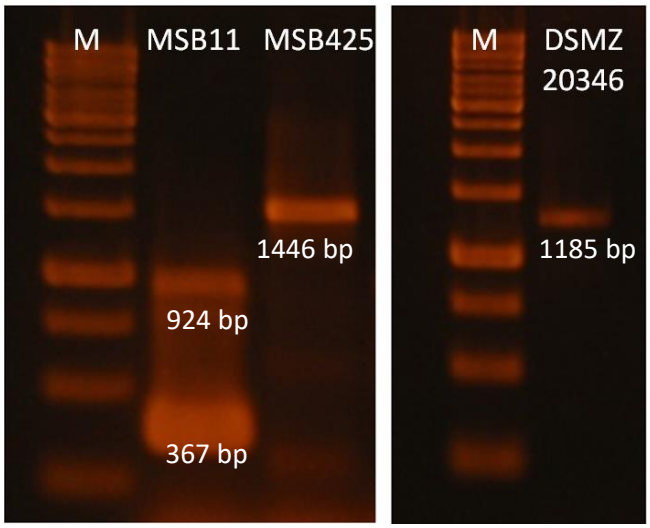

Figure 4.6. PCR products obtained by amplification of DNA from strains used as positive controls. Lane M: 1 kb DNA Ladder, lane MSB11: positive control for *hdc* (367 bp) and *tdc* (924 bp) genes, MSB425: positive control for *odc* gene (1446 bp), DSMZ 20346: positive control for *ldc* gene (1185 bp).

Table 4.8. List of all representatives with detected *tdc* gene, encoding for tyrosine decarboxylase.

Identification	Strains with detected *tdc* gene, encoding for tyrosine decarboxylase
Enterococcus durans	A_0d_1 A_7d_4 A_40d_2 A_40d_3 A_40d_4
Lactobacillus sakei	MRS_280 LamVab_106 LamVab_74 MRS_641
Leuconostoc mesenteroides	C_15d_2 C_15d_3 C_15d_8 B_15d_4 MSE_102 MSE_359

4.8.3. PCR detection of virulence factors in enterococcal strains

The presence of 10 genes encoding for virulence factors and potential pathogenicity of enterococcal strains was evaluated by PCR. In the tested strains (*n*=9), the presence of none of the virulence factors was confirmed.

4.8.4. Hemolytic activity

Although some LAB can induce a hemolytic reaction, none of the 57 tested strains in this study were found to cause hemolysis.

4.9. Technologically important traits and bioprotective role of selected representatives

The results of all technologically important traits measured in this study for selected representatives are shown in Table 4.9. To evaluate their possible bioprotective role, antagonistic activity of the representatives was tested against 7 indicator bacteria (Table 4.10).

4.9.1. Acidification

The initial pH of LP media was 5.8. Approximately half of the strains (45.61 %) were able to decrease pH below 4.00 after 24 hours of incubation, and a vast majority was able to

do so after 7 days of incubation (92.98 %). Enterococcal and leuconostoc strains were comparable (p>0.05) regarding their capacity to lower pH of LP media, with average pH=3.90 and pH=4.01 after 24 h, *i.e.* 3.44 and 3.47 after 7 days, respectively. Compared to them, lactobacilli exhibited lower acidification ability (p<0.05), with average pH=5.00 (24h) and 3.63 (7 days).

4.9.2. Lipolytic activity

Out of all 57 tested strains, only one strain (MRS_104) did not exhibit any lipolytic activity (Table 4.9.). No statistical differences (p>0.05) were found between enterococci, lactobacili and leuconostoc regarding their capacity to hydrolyze tributyrin. However, differences were noticed between strains, varying from 0 to 9.75 mm (disk diffusion) and 0 to 7.5 mm (spot method). The example of lipolysis on Tributyrin agar is shown in Figure 4.7.

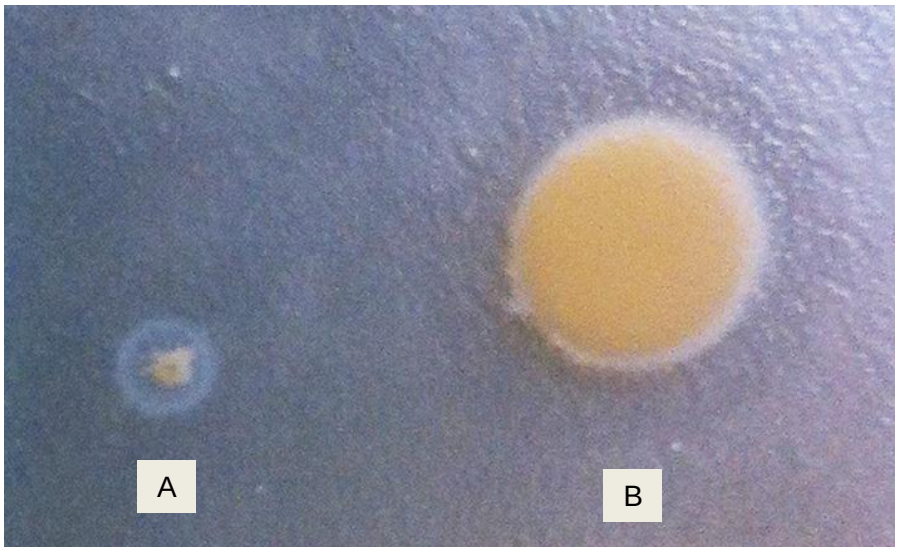

Figure 4.7. Lipolytic activity on Tributyrin agar using spot (A) and disc diffusion method (B).

4.9.3. Proteolytic and peptidase activity

4.9.3.1. Proteolytic activity on plates with sarcoplasmic proteins

Majority of strains (84.21 %) did not exhibit any form of proteolytic activity on plates with added sarcoplasmic proteins, while 9 strains (15.79 %) exhibited weak proteolytic activity. Only one strain (C_7d_14) showed strong capacity to degrade sarcoplasmic proteins (Table 4.9.), comparable to *Pseudomonas fluorescens* WCS 417r used as a positive control (Figure 4.8.).

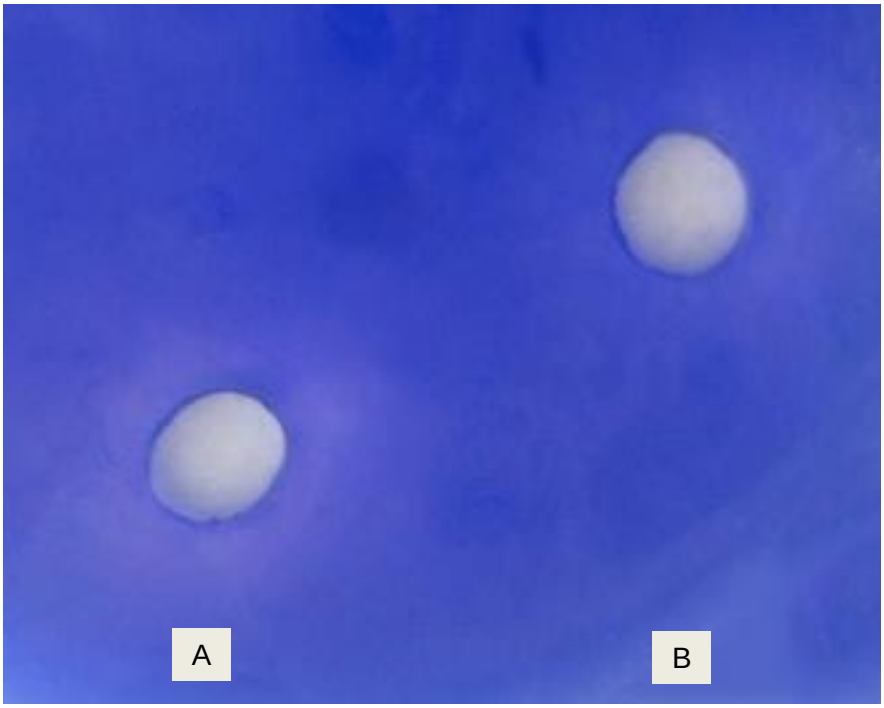

Figure 4.8. Proteolytic activity of strain C_7d_14 (A) and *Pseudomonas fluorescens* WCS 417r used as a positive control (B).

4.9.3.2. Proteolytic activity on plates with added skim milk

The majority of strains (96.49 %) were able to hydrolyse casein on plates with added skim milk (Table 4.9.). When measured by disc diffusion method, enterococci showed stronger proteolytic activity (p<0.05) than lactobacilli. Spot method seemed to be more sensitive since it revealed significant differences between all three groups of bacteria. The stronger proteolytic activity of enterococci compared to lactobacilli was confirmed (p<0.01), but also compared to leuconostoc (p<0.05), while leuconostoc were better able to hydrolize casein than lactobacilli. However, strong differences were also detected between strains of the same species (Table 4.9.).

4.9.3.3. Peptidase activity measured by the Chromogenic method

With regard to peptidase activity, as a result of measuring the amount of *p*NA released from the chromogenic peptide (S-Ala), leuconostoc strains had significantly higher (p<0.01) activity than lactobacilli. Although not statistically significant, leuconostoc strains had higher activity compared to enterococci, with average releases of 2454.85 and 1467.46 µM *p*NA, respectively. The peptidase activity of all representatives is shown in Table 4.9. It is important to note that strong differences were detected between strains of the same species, similarly to other technologically important traits.

Table 4.9. Technologically important traits of selected representatives measured in this study. Results are expressed as an average value of three independent measurements ± standard deviation.

Identification	Strain code	Acidification		Lipolytic activity		Proteolytic activity on sarcoplasmic proteins	Proteolytic activity on skim milk media		Peptidase activity (chromogenic method; µM *p*NA)
		pH (24 h)	pH (7d)	Disck diffusion (mm) ±st.dev.	Spot method (mm) ±st.dev.		Disck diffusion (mm) ± st.dev.	Spot method (mm) ± st.dev.	
Enterococcus durans (*n*=9)	A_0d_1	3.92±0.13	3.47±0.08	8.25±0.35	3.75±0.35	+	10.00±0.00	7.00±0.00	3479.85±0.01
	A_0d_2	3.89±0.13	3.46±0.05	8.75±0.35	3.50±0.00	+	9.50±0.71	6.00±0.00	1424.50±0.00
	A_0d_4	3.50±0.42	3.45±0.01	8.25±0.35	3.00±0.00	-	10.00±0.00	7.25±0.35	732.60±0.01
	A_4d_1	3.90±0.16	3.47±0.06	8.75±0.35	3.50±0.00	-	12.00±0.00	10.50±0.71	1058.20±0.01
	A_7d_3	3.83±0.13	3.45±0.07	8.00±0.00	3.75±0.35	-	12.00±0.00	7.50±0.71	1851.85±0.03
	A_7d_4	4.87±1.31	3.43±0.10	8.50±0.71	3.00±0.00	+	12.00±0.00	10.00±0.00	61.05±0.00
	A_40d_2	3.77±0.04	3.38±0.05	8.75±0.35	3.50±0.71	-	11.50±0.71	8.50±0.71	956.45±0.01
	A_40d_3	3.70±0.03	3.40±0.01	8.00±0.00	3.00±0.00	-	9.50±0.71	7.25±0.35	1750.10±0.01
	A_40d_4	3.70±0.01	3.41±0.01	8.25±0.35	3.50±0.00	-	10.75±1.06	7.00±0.00	1892.55±0.00
Average values (*E. durans*)		3.90[a]	3.44[a]	8.39[a]	3.39[a]	n.a.	10.80[a]	7.89[a]	1467.46[ab]
Lactobacillus sakei (*n=27*)	C_7d_13	5.34±0.56	3.53±0.24	9.50±0.00	3.00±0.00	-	0.00±0.00	0.00±0.00	2503.05±0.00
	C_7d_14	4.99±1.14	3.53±0.08	8.25±0.35	3.50±0.00	++	7.25±0.35	3.50±0.71	1607.65±0.00
	C_15d_13	3.76±0.04	3.40±0.03	9.00±0.00	4.00±0.00	-	7.00±0.00	4.50±0.71	1566.95±0.00
	C_end_1	5.70±0.08	3.45±0.08	9.75±0.35	3.50±0.00	-	9.50±0.71	3.50±0.71	997.15±0.01
	MSE_24	4.41±0.33	3.58±0.09	8.50±0.71	3.00±0.35	-	11.50±0.71	6.75±0.35	1790.80±0.01
	MSE_44	5.33±0.03	4.15±0.57	9.00±0.00	7.50±0.71	-	12.00±0.00	10.00±0.00	1180.30±0.01
	MRS_280	3.76±0.04	3.38±0.03	8.75±0.35	3.25±0.35	-	11.00±1.41	9.50±0.71	691.90±0.00
	LamVab_106	5.77±0.01	3.39±0.00	8.00±0.00	2.50±0.00	+	7.75±0.35	4.00±0.00	834.35±0.00
	LamVab_74	4.93±1.19	3.42±0.11	8.00±0.00	3.25±0.35	-	7.75±0.35	4.50±0.71	1200.65±0.01
	MRS_641	4.64±0.06	3.65±0.08	7.25±0.35	2.75±0.35	-	8.00±0.00	5.75±1.06	569.80±0.01

	MRS_604	3.78±0.01	3.37±0.02	7.75±0.35	2.25±0.35	-	0.00±0.00	2.75±0.35	2462.35±0.01
	MRS_579	5.34±0.60	3.40±0.06	8.00±0.00	2.00±0.00	-	7.25±0.35	3.50±0.71	447.70±0.00
	MRS_567	5.32±0.06	3.44±0.01	7.50±0.00	3.00±0.00	-	6.75±0.35	4.50±0.71	1505.90±0.01
	C_end_2	4.10±0.02	3.59±0.04	8.75±0.35	3.25±0.35	-	8.00±0.00	4.25±0.35	345.95±0.01
	MSE_40	5.79±0.08	3.43±0.13	6.75±0.35	2.00±0.00	-	10.50±0.71	6.00±0.00	1892.55±0.00
	MSE_45	5.69±0.07	3.79±0.05	5.75±1.06	1.00±1.41	-	12.00±0.00	5.50±0.71	630.85±0.00
	MSE_38	5.78±0.01	3.63±0.01	6.50±0.00	2.00±0.00	-	11.50±0.71	5.50±0.71	3357.75±0.01
	MSE_43	5.14±0.07	4.45±0.06	7.50±0.71	0.00±0.00	-	11.00±1.41	5.00±0.00	508.75±0.01
	MSE_104	5.87±0.06	3.68±0.11	6.75±0.35	1.00±1.41	-	6.50±0.71	3.50±0.71	1261.70±0.01
	MSE_100	4.63±0.10	3.81±0.08	7.50±0.71	2.00±0.00	-	6.50±0.71	3.00±0.00	1485.55±0.00
	MSE_23	5.20±0.08	3.73±0.02	6.75±0.35	2.25±0.35	-	7.00±0.00	4.50±0.71	1241.35±0.01
	MSE_22	5.71±0.04	4.01±0.15	7.25±0.35	2.25±0.35	-	8.50±0.71	4.50±0.71	793.65±0.01
	MSE_26	5.75±0.04	4.08±0.25	9.50±0.71	3.50±2.12	-	12.50±0.71	7.00±0.00	2035.00±0.01
	MSE_13	5.89±0.06	3.53±0.06	8.50±0.71	5.50±0.71	-	8.00±0.00	4.50±0.71	203.50±0.00
	MRS_267	3.83±0.04	3.58±0.12	6.25±0.35	2.50±0.71	-	10.00±0.00	5.00±0.00	854.70±0.00
	MRS_296	3.88±0.03	3.56±0.06	5.50±0.71	2.75±0.35	-	9.50±0.71	5.00±0.00	630.85±0.00
	MRS_331	3.97±0.06	3.53±0.06	5.75±0.35	2.50±0.07	-	10.50±0.71	6.00±0.00	1282.05±0.00
Lactobacillus curvatus (n=2)	MRS_520	5.09±1.02	3.44±0.06	9.00±0.00	6.00±0.00	-	8.50±0.71	5.50±0.00	284.90±0.00
	MRS_526	5.77±0.02	3.67±0.39	7.25±0.35	2.00±0.00	-	7.00±0.00	5.50±0.00	142.45±0.00
Average values (*Lactobacillus* spp.)		5.00[b]	3.63[b]	7.76[a]	2.95[a]	n.a.	8.35[b]	4.86[b]	1152.49[b]
Leuconostoc mesenteroides (n=19)	MRS_532	3.74±0.02	3.37±0.01	8.25±1.06	4.50±0.71	-	7.25±0.35	2.75±0.35	264.55±0.00
	C_15d_2	4.19±0.37	3.75±0.04	8.25±0.35	3.25±0.35	-	10.00±2.83	8.75±0.35	5535.20±0.02
	C_15d_3	4.36±0.48	3.58±0.11	8.00±0.00	7.00±0.00	+	7.75±0.35	6.00±0.00	8343.50±0.05
	C_15d_8	3.98±0.08	3.52±0.08	8.00±0.00	2.75±0.35	-	7.00±0.00	5.50±0.71	3398.45±0.08
	B_15d_4	3.91±0.12	3.58±0.00	9.50±0.71	3.75±0.35	-	11.00±1.41	7.00±0.00	691.90±0.01
	MSE_92	3.87±0.05	3.39±0.04	7.75±0.35	2.25±0.35	-	13.50±0.71	7.00±0.00	5942.20±0.14
	MSE_102	3.79±0.03	3.36±0.02	8.00±0.00	2.25±0.35	-	11.50±0.71	7.50±0.71	1180.30±0.01
	MRS_120	4.33±0.54	3.52±0.17	8.50±0.00	3.50±0.00	-	7.00±0.00	5.50±0.71	936.10±0.01

	MRS_104	4.09±0.25	3.48±0.21	0.00±0.00	0.00±0.00	-	7.25±0.35	5.50±0.71	2197.80±0.00
	MSE_359	4.19±0.45	3.66±0.21	8.50±0.00	3.00±0.00	+	12.50±0.71	10.00±0.00	2849.00±0.00
	MSE_349	3.62±0.31	3.41±0.01	8.00±0.00	3.00±0.00	-	13.50±0.71	8.00±0.00	2442.00±0.00
	MSE_317	4.22±0.49	3.65±0.21	8.25±0.35	3.00±0.00	+	15.00±0.00	8.50±0.71	427.35±0.00
	LamVab_116	5.31±0.29	3.38±0.24	7.25±0.35	3.00±0.00	-	7.75±0.35	5.00±0.00	5535.20±0.01
	LamVab_96	3.60±0.04	3.37±0.01	8.25±0.35	3.00±0.00	+	8.25±0.35	5.50±0.71	549.45±0.00
	LamVab_27	3.99±0.37	3.50±0.21	8.00±0.00	3.25±0.35	-	7.00±0.00	4.50±0.71	2747.25±0.01
	LamVab_2	3.97±0.48	3.48±0.28	8.00±0.00	3.25±0.35	-	8.00±0.00	6.00±0.00	284.90±0.00
	MRS_602	3.67±0.21	3.25±0.11	8.00±0.00	2.50±0.71	-	7.25±0.35	5.50±0.71	223.85±0.00
	MRS_506	3.72±0.10	3.36±0.06	7.75±0.35	2.75±0.35	-	12.00±0.00	6.50±0.71	2523.40±0.00
	MRS_605	3.74±0.01	3.34±0.05	8.00±0.00	2.75±0.35	-	0.00±0.00	0.00±0.00	569.80±0.00
Average values (*Le. mesenteroides*)		4.01^{a}	3.47^{a}	7.67^{a}	3.01^{a}	n.a.	9.24^{ab}	6.24^{c}	2576.54^{a}

- = no proteolytic activity detected, + =weak proteolytic activity, ++ = strong proteolytic activity, comparable to control strain *P. fluorescens* WCS 417r. n.a.=not applicable. a-c=values in the same column not followed by a common letter are significantly different (p<0.05), differences between bacterial genera (*Enterococcus*, *Lactobacillus*, *Leuconostoc*).

4.9.4. Antagonistic activity

Representatives were the most active against *L. innocua*, where approximately half of them (52.63 %) displayed antagonistic activity. Against *Salmonella enterica* and *E.coli,* equal activity was detected (42.10 %), followed by *Bacillus cereus* (36.84 %), *Weissella viridescens* (35.09 %), *Brochotrix thermospachta* (31.58 %) and *Staphylococcus aureus* (29.82 %). When the inhibitory effect of neutralised cell-free supernatants was tested, a total of 4 strains was able to inhibit the growth of *L. innocua*. The example of the different degree of antagonistic activity, as well as the inhibitory effect of neutralised cell-free supernatants, are shown in Figure 4.9. and Figure 4.10., respectively. The results of the antagonistic activity of all representatives are shown in Table 4.10.

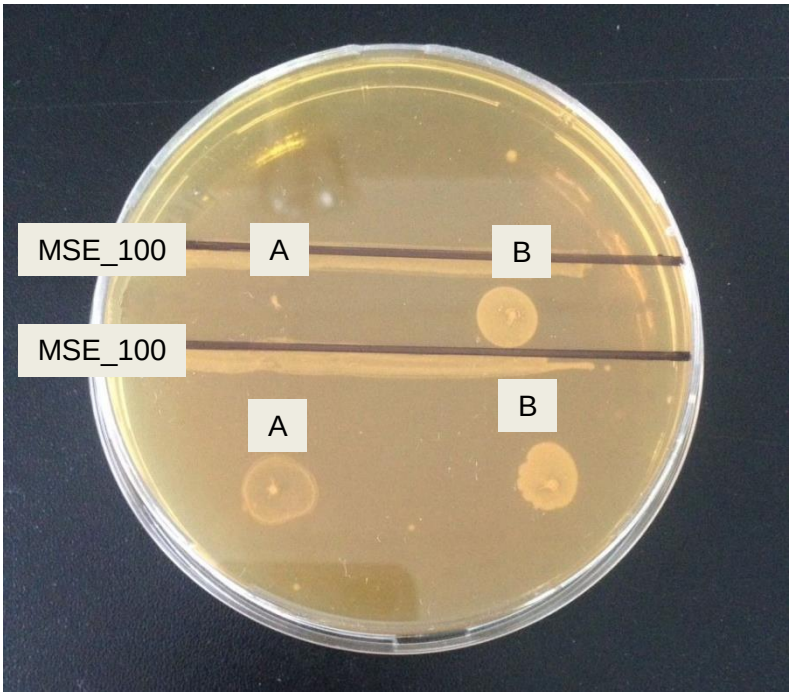

Figure 4.9. Antagonistic activity of *Lb. sakei* strain (MSE_100) against *Listeria innocua* (A; ATCC 33090) and *Salmonella enterica* subsp. enterica (B; DSM 14221). The complete inhibition of *Listeria innocua* is displayed by no growth in the area between the two lines of *Lb. sakei*, while the growth of *Salmonella enterica* was not affected (no inhibition). The corresponding growth controls are suited near the margin of the agar plate.

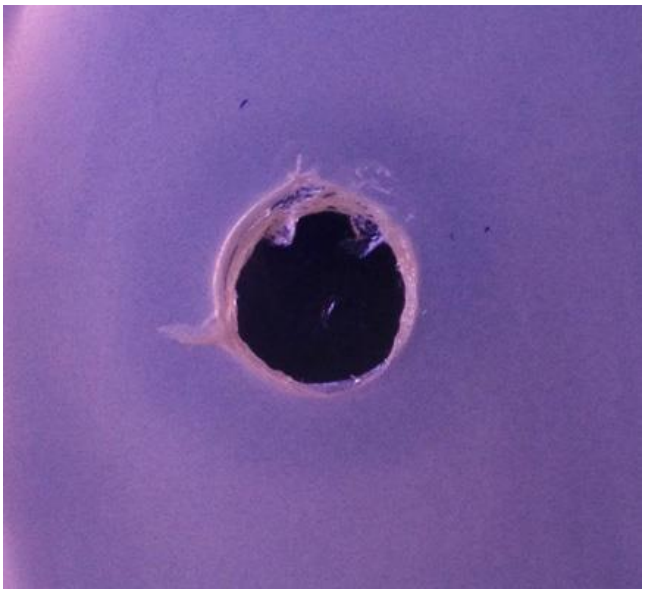

Figure 4.10. Inhibition of *Listeria innocua* (ATCC 33090) by neutralised cell-free supernatant of *Lb. sakei* strain C_end_1.

Table 4.10. Antagonistic activity of representatives against selected indicator bacteria. Indicator colonies grown between two lines of lactobacilli were measured and compared with colonies grown on the margin of the plate. Results are expressed as the difference in measured diameter, where - = no inhibition (the difference in their diameter is < 1 mm); + = weak inhibition (grown colonies are 1-2 mm smaller compared to the growth control); ++ = pronounced inhibition (grown colonies are > 2 up to 4 mm smaller compared to the growth control); +++ = very strong inhibition (grown colonies are > 4 mm smaller compared to the growth control), c.i = complete inhibition (no colonies growing in-between the test lines). Asterisks (*) indicate antagonistic activity of strains against *Listeria monocytogenes* confirmed by cell-free supernatants.

Identification	Strain code	Indicator bacteria used in this study						
		Salmonella enterica subsp. *enterica* (DSM 14221)	*Listeria innocua* (ATCC 33090)	*Escherichia coli* (ATCC 25922)	*Staphylococcus aureus* subsp. *aureus* (DSM 20231)	*Brochotrix thermospachta* (LMG 17208)	*Weissella viridescens* (DSM 20410)	*Bacillus cereus* (DSM 6791)
Enterococcus durans (*n*=9)	A_0d_1	++	++ *	+	-	-	-	-
	A_0d_2	-	-	-	-	-	-	-
	A_0d_4	++	+	++	-	+	c.i.	-
	A_4d_1	-	-	-	-	-	-	-
	A_7d_3	-	+	-	-	-	-	-
	A_7d_4	-	-	-	-	-	-	-
	A_40d_2	++	++	-	-	-	-	-
	A_40d_3	++	++	-	-	++	++	-
	A_40d_4	++	-	++	-	++	+	-
Lactobacillus sakei (*n*=27)	C_7d_13	c.i.	+	+	-	+	c.i.	c.i.
	C_7d_14	-	-	-	-	-	-	-
	C_15d_13	-	-	-	-	-	-	-
	C_end_1	+	+ *	+	-	-	-	-
	MSE_24	c.i.	c.i.	+	c.i.	c.i.	c.i.	c.i.
	MSE_44	+	+	+	+	-	+	c.i.
	MRS_280	+	+ *	+	+	+	+	c.i.
	LamVab_106	+	c.i.	+	+	-	-	-

	LamVab_74	-	-	-	-	-	+	c.i.
	MRS_641	c.i.	+	c.i.	c.i.	c.i.	c.i.	c.i.
	MRS_604	-	-	-	-	+	+	+
	MRS_579	-	+	-	-	-	-	-
	MRS_567	-	-	-	-	-	-	-
	C_end_2	-	-	-	-	-	-	-
	MSE_40	+	+	c.i.	c.i.	c.i.	c.i.	c.i.
	MSE_45	+	+	+	+	+	+	+
	MSE_38	-	+	-	-	-	-	-
	MSE_43	+++	+	-	-	-	+	-
	MSE_104	-	-	-	-	-	-	-
	MSE_100	-	c.i.	c.i.	c.i.	c.i.	-	+
	MSE_23	c.i.	c.i.	c.i.	c.i.	c.i.	c.i.	c.i.
	MSE_22	-	-	-	-	-	-	-
	MSE_26	-	-	-	-	-	-	-
	MSE_13	c.i.	c.i.	c.i.	c.i.	c.i.	c.i.	c.i.
	MRS_267	+	+	c.i.	c.i.	+	-	+
	MRS_296	c.i.	+	+	c.i.	+	-	c.i.
	MRS_331	-	+ *	+	+	+	-	+
Lactobacillus curvatus (*n*=2)	MRS_520	-	-	-	-	-	-	-
	MRS_526	-	-	-	-	-	-	-
Leuconostoc mesenteroides (*n*=19)	MRS_532	-	-	c.i.	c.i.	-	+	+
	C_15d_2	-	-	-	-	-	-	-
	C_15d_3	-	+	-	-	-	-	-
	C_15d_8	-	-	-	-	-	-	-
	B_15d_4	-	+	-	-	-	-	-
	MSE_92	-	+	-	-	-	-	-
	MSE_102	-	-	-	-	+	+	c.i.
	MRS_120	+	+	+	+	-	-	-
	MRS_104	-	-	-	-	-	-	-
	MSE_359	-	-	-	-	-	-	-
	MSE_349	-	-	-	-	-	-	-
	MSE_317	-	-	-	-	-	-	-

	LamVab_116	-	-	-	-	-	-	-
	LamVab_96	-	++	-	-	-	-	-
	LamVab_27	+	-	+	-	-	-	c.i.
	LamVab_2	+	+	+	-	-	+	c.i.
	MRS_602	+	-	-	-	-	-	-
	MRS_506	-	-	+	+	-	+	+
	MRS_605	+	+	+	+	+	+	+

4.10. Autoaggregation ability and survival of the LAB in simulated GI conditions

4.10.1. Autoaggregation ability

Representative enterococci and lactobacilli that were not resistant to antibiotics and possessed no genes encoding for the production of biogenic amines were selected (*n*=19) and their ability to aggregate and to survive in simulated GI tract conditions, as a prerequisite for their potential probiotic activity, was evaluated. *Lb. sakei* exhibited significantly higher autoaggregation rates after 3 ($p<0.001$) and 5 ($p<0.001$) hours of incubation, compared to *E. durans*. However, both *E. durans* and *Lb. sakei* exhibited poor capacity to autoaggregate, with average values of 12.02 % and 23.35 % after 5 h of incubation, respectively (Table 4.11).

4.10.2. Survival of LAB in simulated gastrointestinal (GI) conditions

The results of the survival rates of the tested LAB are shown in Table 4.11. Strains were best able to tolerate the simulated conditions of the oral cavity with an average survival rate of 89.08 %, while in the simulated environment of the stomach the viability of bacteria significantly ($p<0.001$) decreased to an average of 17.95 %. At the simulated conditions of the small intestine, strains exhibited the lowest average survival rates (2.27 %). Although in such environment enterococci exhibited significantly higher ($p<0.05$) survival rates than lactobacilli; only one enterococcal strain (A_4d_1) differentiated with the survival rate of 30.86 %. The survival rate of lactobacilli did not surpass 2.07 %.

Table 4.11. Autoaggregation ability and the survival rates in simulated conditions of GI tract for *E. durans* and *Lb. sakei* strains that were designated as safe for their potential application in food.

Identification	Strain	Autoaggregation ability (%)		Survival in simulated GI tract conditions		
		t=3h	t=5h	Oral cavity (%)	Gastric (%)	Intestinal (%)
E. durans (*n*=4)	A_0d_2	5.98	9.14	37.78	23.55	7.27
	A_0d_4	13.03	18.35	67.94	30.52	1.63
	A_4d_1	4.15	10.29	99.02	51.23	30.86
	A_7d_3	8.52	10.30	93.22	36.58	0.00
Average (all *E. durans*)		7.92^{a}	12.02^{a}	74.49^{a1}	35.47^{a2}	9.94^{a3}
Lb. sakei (*n*=15)	C_7d_13	15.41	25.68	94.12	0.00	0.00
	C_15d_13	13.18	14.97	98.59	0.12	2.07
	C_end_1	14.48	17.24	98.77	1.56	0.00
	MRS_579	13.43	16.81	95.11	1.33	0.00
	MRS_567	16.77	19.77	96.06	0.13	0.00
	C_end_2	20.49	27.05	99.54	0.67	0.00
	MSE_38	16.83	25.02	98.33	14.78	0.00
	MSE_43	11.46	30.65	95.65	0.77	0.01
	MSE_104	25.36	27.79	87.76	0.00	0.00
	MSE_22	11.38	26.71	92.06	0.96	0.21
	MSE_26	21.35	25.99	95.42	1.05	0.19
	MSE_13	7.98	20.69	94.61	13.32	0.01
	MRS_267	14.21	22.98	55.37	0.13	0.10
	MRS_296	12.15	17.78	94.03	85.58	0.59
	MRS_331	21.40	31.15	99.20	78.82	0.23
Average (*Lb. sakei*)		15.72^{b}	23.35^{b}	88.35^{a1}	13.28^{a2}	0.23^{b3}

a-b=values in the same column not followed by a common letter are significantly different (p<0.05), differences between bacterial species (*E. durans and Lb. sakei).* 1-2=values in the same row not followed by a common number are significantly different (p<0.05), differences between survival rate in different simulated environments of GI tract (oral, gastric, intestinal).

4.11. The structure of bacterial communities and the changes in microbial diversity during ripening observed by culture-independent methods

4.11.1. The purity and concentration of DNA extracted from sausage samples

When measured at 260/280 and 260/30 nm, the DNA ratios were approximately 1.8 and 2.0, respectively, indicating the adequate purity of the extracted DNA. DNA concentrations varied between 1.25 and 107.93 ng/µL (Table 4.12.).

4.11.2. Library size and quality

The library size was approximately 500 pb with no relevant unspecific peeks detected. After indexing PCR, library concentration measured with PicoGreen varied between 4.06 and 19.38 ng//µL.

Table 4.12. The concentration of DNA extracted from wild boar (WB) and deer meat (DS) sausages at different time-points of production; and the concentration after indexing PCR (library quantification). The concentrations were measured with PicoGreen.

Sausage type	Production time points (days)	The concentration of extracted DNA (ng//µL)	Library quantification (ng/µL)
WB1	0	36.79	4.06
	4	44.75	12.85
	7	30.05	12.20
	20	36.58	13.99
	40	35.97	19.38
WB2	0	6.26	10.03
	4	2.19	14.17
	7	2.69	14.36
	20	1.25	11.22
	40	2.27	13.69
WB3	0	56.81	12.86
	4	68.36	13.36
	7	93.42	13.94
	20	90.48	13.83
	40	24.80	14.98
DS1	0	26.54	16.53
	4	40.47	15.84
	7	48.56	13.00
	20	48.74	14.39

	40	55.41	14.13
DS2	0	107.93	13.98
	4	80.78	10.13
	7	60.58	13.33
	40	40.35	15.02

4.11.3. Bacterial diversity during fermentation and ripening

Partial 16S rRNA gene sequencing resulted in a total of 7,750,330 raw reads. After trimming, quality filtering, merging of reads and removal of contaminant sequences, a total of 3,662,689 high-quality sequences remained for analyses. OTU picking, removal of singletons and taxonomic annotation was followed by a further decline in numbers of sequences to 3,213,232 reads which could be binned into 644 unique OTUs. In total, 11.60 % of reads were left unassigned. The smallest number of reads was obtained for the replicate WB 40 b (4,604). Subsampling at this depth did not provide satisfactory coverage. Prior to alpha diversity analysis all samples were rarefied to the next smallest number of reads per sample (15,203), to correct for uneven sequencing depth. Using this sampling depth rarefaction analysis showed good coverage of diversity for all analysed samples. Due to this, replicate WB 40 b was omitted from further analyses. The number of OTUs was highest at day 0 (264±38) and day 4 (248±68), and decreased toward the later phases of fermentation and ripening (day 7, 200±38, to day 40, 219±100). The observed differences were significant for day 0 and day 7 (p=<0.05) and day 4 and day 7 (p<0.05). Shannon-Wiener diversity index and Pielou evenness followed the same pattern, with the highest values obtained at day 0 (Shannon's-Wiener index: 3.26±0.32, Pielou evenness:0.58±0.05) and day 4 (Shannon's-Wiener index: 2.96±0.51, Pielou evenness: 0.54±0.08), followed by decrease towards day 7 (Shannon's-Wiener index: 2.22±1.18, Pielou evenness: 0.41±0.19) and day 40 (Shannon's-Wiener index: 2.00±0.95, Pielou evenness: 0.37±0.16). Shannon's-Wiener diversity was significantly different for all investigated time points (p<0.05). When the same time points for both sausage types were compared, overall diversity patterns were consistent.

4.11.4. Identification of bacterial communities present during fermentation and ripening

Using the GreenGenes database, all OTUs could be assigned to the class level, 42 % of the OTUs to the order level, 26 % to genus and 27 % species level. Representative sequences of identified OTUs were aligned against other databases, including SILVA, RDP and NCBI. The comparison of taxonomic classification with different databases showed that the taxonomic assignment of all databases was consistent up to family and

genus level. Discrepancies were noted only for members of *Enterobacteriaceae* at the genus level. Since 95.71 % of the assigned OTUs could be classified to the genus level when the RDP database was performed, this database was chosen for further annotation of reads. The majority of OTUs (*n*=538) appeared in both wild boar and deer meat sausages, whereas 77 OTUs were specific for wild boar and 29 OTUs for deer meat sausages (Figure 4.11.). Common OTUs included 97.37 % of total sequences, whereas OTUs specific for wild boar and for deer meat comprised 0.84 % and 1.79 % of total sequences, respectively. Similarly, 542 OTUs were present at all time points of fermentation indicating that a stable microbiome has developed. 4 OTUs were specific for day 0, 15 OTUs were shared between day 0 and 4, 48 OTUs between day 0, 4 and 7, 16 OTUs between day 4, 7 and 40, and 4 OTUs between day 7 and 40 (Figure 4.11.). OTUs common for all ripening times included 98.69 % of total sequences, whereas 1.31 % were shared between different ripening times. High abundant OTUs which were shared between wild boar and deer meat sausages mostly belong to the LAB, bacteria associated with food spoilage and *Bacillus*. LAB could be classified as members of the order Lactobacillales (106 OTUs), the family *Lactobacillaceae* (12 OTUs), and the genera *Weissella* (32 OTUs), *Lactobacillus* (14 OTUs), *Leuconostoc* (13 OTUs), *Enterococcus* (12 OTUs) and *Lactococcus* (11 OTUs). OTUs associated with the genus *Staphylococcus* (39 OTUs in total) included predominantly coagulase-negative staphylococci (38 OTUs) and only one OTU associated with the pathogenic species *S. aureus*. Bacteria associated with food spoilage, such as *Brochothrix* (33 OTUs) and *Pseudomonas* (16 OTUs) were also present among common OTUs. 64 OTUs were associated with *Bacillus*, while other potentially pathogenic bacteria included genera such as *Bacillus* (64 OTUs), *Clostridium* (1 OTU), *Listeria* (1 OTU), *Yersinia* (1 OTU) and *Erysipelothrix* (2 OTUs). Interestingly, OTUs associated with different taxa were present in different types of sausages. For example, OTUs associated with the genus *Bacillus* were predominantly associated with wild boar, whereas genera *Brochothrix* and *Carnobacterium* with deer meat sausages.

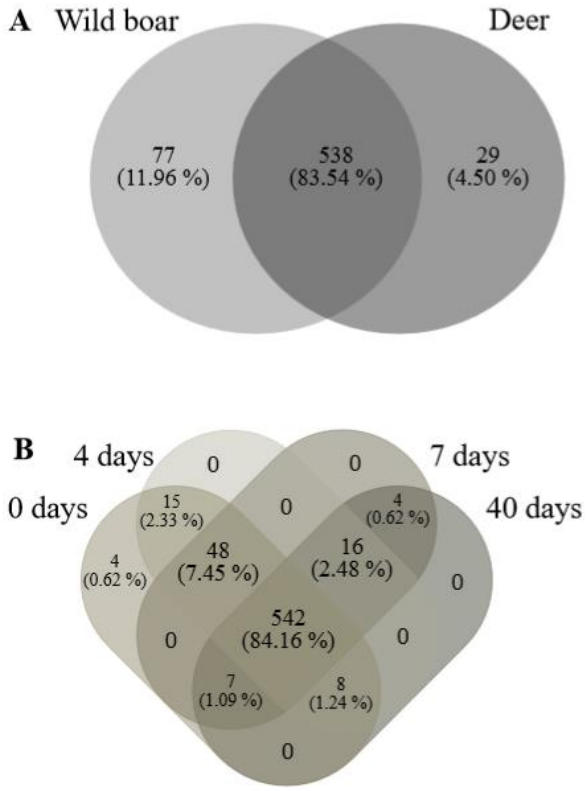

Figure 4.11. The OTUs associated with the wild boar and deer meat (A) and different ripening phases (B). The values in brackets depict the relative abundance of shared and specific OTUs.

4.11.5. Bacterial community structure response to the ripening process and the meat origin

The dominant taxa present in wild boar and deer meat sausages are shown in Table 4.13. *Firmicutes* and *Proteobacteria* were the dominant phyla, with 81.85 % and 15.24 % of all assigned sequences respectively, followed by *Actinobacteria* (3.12 %) and *Bacteroidetes* (0.05 %). In wild boar meat sausages, *Firmicutes* (71.11 %) and *Proteobacteria* (23.95 %) were also the dominant phyla. The most abundant families were *Lactobacillaceae* (29.36 %), *Bacillaceae* (17.53 %), *Staphylococcaceae* (6.39 %), *Leuconostocaceae* (6.15 %), and *Enterococcaceae* (3.38 %) for *Firmicutes*, and the *Xanthomonadaceae* (15.09 %) and *Pseudomonadaceae* (4.53 %) for *Proteobacteria*. For deer meat sausages, the relative abundance of *Firmicutes* was significantly higher (96.84 %) compared to wild boar and dominated by the families *Lactobacillaceae* (60.18 %), *Listeriaceae* (12.22 %), *Leuconostocaceae* (9.14 %), *Carnobacteriaceae* (5.28 %), *Bacillaceae* (3.73 %) and *Enterococcaceae* (1.42 %), followed by *Proteobacteria* (2.53 %) which included *Xanthomonadaceae* (0.35 %) and *Pseudomonadaceae* (0.70 %). In wild boar meat sausages, the abundance of *Lactobacillus* increased during fermentation from 10.86 % to

58.38 %. In contrast, the abundance of other genera belonging to LAB remained almost constant or decreased with production time. In deer meat sausages, the abundance of *Lactobacillus* also increased with the fermentation time. The largest increase in abundance was detected between day 4 and day 7 (from 37.21 to 90.62 %) followed by a decrease to 82.58 % at the end of ripening. For other LAB only *Leuconostoc* ($p<0.05$), which was present in low abundance at the start of the production process, increased in abundance, whereas enterococcal ($p<0.05$) and lactococcal ($p<0.01$) abundance decreased. Another important group of fermentative microorganisms identified in both types of sausages was *Staphylococcus* ($p<0.01$) and *Kocuria* ($p<0.01$). According to the NCBI, coagulase-negative *S. saprophyticus*, *S. sciuri*, *S. succinus* and *S. equorum* were present. In contrast to the wild boar meat sausages where the abundance of staphylococci decreased, in deer meat sausages their abundance increased. The abundance of *Kocuria* species exceeded 0.3 % only in wild boar sausages at day 0, and during ripening, its abundance decreased. Regarding lactobacilli, a majority of them was identified as *Lb. sakei* (40.32 %), *Lb. curvatus* (40.29 %) and *Lb. plantarum* (1.31 %). Although the initial abundance of *Lactobacillus* species was significantly higher ($p<0.05$) in deer meat sausages compared to wild boar meat sausages, their numbers started increasing later in the fermentation and ripening. Even though WB3 and DS1 sausages were produced by the same manufacturer, WB3 sausage showed the same initial low abundance of *Lactobacillus* species as the rest of the wild boar game meat sausages.

Among spoilage and pathogenic microbiota, *Bacillus* spp. and *Stenotrophomonas* spp. were most abundant in the wild boar meat sausages, while in deer meat sausages OTUs linked to *Brochothrix, Carnobacterium* and *Weissella* were dominant. Although more prevalent in wild boar sausages, *Bacillus* was identified at both sausage types during all investigated time points. At the beginning of production (0d), *Bacillus* was identified at a relative abundance of 27.32 % in wild boar and 5.35 % in deer meat sausages. Its abundance decreased during the fermentation and ripening and was identified at a relative abundance of 3.40 % (WB) and 2.17 % (DS) at the end of the production. *Stenotrophomonas* was found in high relative abundance at all investigated time points in WB sausages, from 12.27 % at the beginning (0 d) to 17.96 % at the end (40 d). In DS sausages, *Stenotrophomonas* was detected exclusively at the beginning of production (0 and 4 d) at a relative abundance <1 %. Despite their overall prevalence in DS sausages, *Brochotrix* and *Weissella* were found at a high relative abundance at the beginning of fermentation, but their number rapidly decreased at day 7. Similarly, *Carnobacterium* was identified at a relative abundance of 13.66 % at day 0, and 7.29 % at day 4, but was not detected in any further ripening stages. Regarding WB sausages, *Carnobacterium* was

not detected, *Brochotrix* was detected sporadically, while *Weissella* was detected through the production, but at a maximal relative abundance of 7.08 % at day 0. *Kurthia* and *Pseudomonas* were only sporadically detected in DS sausages, while in WB sausages *Pseudomonas* was detected through the production and in higher relative abundance. Potentially pathogenic genera such as *Listeria*, *Yersinia* and *Erysipelothrix* were identified in both types of sausages at relative abundance <0.3 % at the initial phase of the production process only. Genera *Clostridium* and *Corynebacterium* were found only in WB sausages at the beginning of production at an abundance <1 %, and their relative abundance also decreased to the point of extinction during the ripening process.

Table 4.13. The most abundant taxa (>0.3 %) present in the wild boar and deer sausages at different time points of sausage production (0, 4, 7 and 40 days).

0 days				4 days			
WB		DS		WB		DS	
Taxon	RA [%]	Taxon	RA [%]	Taxon	RA [%]	Taxon	RA [%]
Class							
						Bacilli	0.31
Order							
Actinomycetales	2.10	Lactobacillales	1.55	Actinomycetales	1.75	Lactobacillales	1.00
Bacillales	0.94			Bacillales	0.98		
Actinomycetales	0.58			Lactobacillales	0.77		
Enterobacteriales	0.48			Rhizobiales	0.36		
Family							
		Enterobacteriaceae	0.53	*Enterobacteriaceae*	0.75	*Enterobacteriaceae*	0.47
				Microbacteriaceae	0.67		
Genus							
Bacillus	27.32	*Lactobacillus*	30.25	*Lactobacillus*	26.58	*Lactobacillus*	37.21
Stenotrophomonas	12.27	*Brochothrix*	23.43	*Bacillus*	19.54	*Brochothrix*	22.85
Lactobacillus	10.86	*Carnobacterium*	13.66	*Stenotrophomonas*	16.86	*Weissella*	14.27
Staphylococcus	8.98	*Weissella*	11.00	*Staphylococcus*	5.97	*Carnobacterium*	7.29
Lactococcus	8.97	*Bacillus*	5.35	*Lactococcus*	4.00	*Bacillus*	6.93
Weissella	7.08	*Pseudomonas*	2.15	*Pseudomonas*	3.63	*Kurthia*	2.38
Enterococcus	3.76	*Acinetobacter*	1.66	*Enterococcus*	3.03	*Enterococcus*	2.29
Pseudomonas	3.42	*Psychrobacter*	1.34	*Weissella*	3.02	*Lactococcus*	0.84
Kurthia	1.38	*Enterococcus*	1.32	*Bradyrhizobium*	1.95	*Leuconostoc*	0.74
Bradyrhizobium	1.29	*Kurthia*	1.29	*Methylobacterium*	1.45	*Stenotrophomonas*	0.43
Microbacterium	0.94	*Lactococcus*	0.92	*Microbacterium*	0.79	*Vagococcus*	0.33
Vagococcus	0.87	*Stenotrophomonas*	0.71	*Vagococcus*	0.69		
Kocuria	0.85	*Vagococcus*	0.54	*Brevibacterium*	0.69		
Streptococcus	0.77	*Macrococcus*	0.41	*Kurthia*	0.67		
Brachybacterium	0.75	*Leuconostoc*	0.38	*Leuconostoc*	0.60		

Brevibacterium	0.72	*Staphylococcus*	0.32	*Corynebacterium*	0.58		
Corynebacterium	0.69			*Brachybacterium*	0.45		
Methylobacterium	0.67			*Clostridium*	0.40		
Clostridium	0.54			*Streptococcus*	0.35		
Leuconostoc	0.41						
Other RA < 0.3 %	3.38		3.17		3.49		2.65
7 days				40 days			
WB		DS		WB		DS	
Taxon	RA [%]	Taxon	RA [%]	Taxon	RA [%]	Taxon	RA [%]
Class							
		Bacilli	0.46	Bacilli	0.34	Bacilli	0.50
Order							
Actinomycetales	1.16	Lactobacillales	2.04	Lactobacillales	0.86	Lactobacillales	1.98
Lactobacillales	0.80					Actinomycetales	0.32
Bacillales	0.73						
Rhizobiales	0.34						
Family							
Microbacteriaceae	0.51						
Sphingomonadaceae	0.32						
Genus							
Lactobacillus	26.26	*Lactobacillus*	90.62	*Lactobacillus*	58.38	*Lactobacillus*	82.58
Stenotrophomonas	18.55	*Weissella*	3.80	*Stenotrophomonas*	17.69	*Weissella*	4.36
Bacillus	13.62	*Leuconostoc*	1.11	*Weissella*	3.46	*Bacillus*	2.17
Pseudomonas	9.20	*Brochothrix*	0.63	*Bacillus*	3.40	*Staphylococcus*	2.11
Staphylococcus	7.57	*Bacillus*	0.36	*Staphylococcus*	3.06	*Brochothrix*	1.85
Leuconostoc	3.59			*Pseudomonas*	2.02	*Leuconostoc*	0.91
Enterococcus	3.05			*Leuconostoc*	1.90	*Enterococcus*	0.82
Lactococcus	3.03			*Lactococcus*	1.49	*Kurthia*	0.40
Bradyrhizobium	1.89			*Bradyrhizobium*	1.43	*Pseudomonas*	0.33
Weissella	1.67			*Methylobacterium*	0.97		
Methylobacterium	0.84			*Enterococcus*	0.81		
Vagococcus	0.58			*Brochothrix*	0.51		

Microbacterium	0.57		*Kurthia*	0.49	
Brachybacterium	0.42		*Serratia*	0.34	
Corynebacterium	0.41				
Brevibacterium	0.41				
Brochothrix	0.33				
Propionibacterium	0.32				
Kurthia	0.31				
Other					
RA < 0.3 %	3.53	0.98		2.86	1.67

WB=wild boar sausages, DS=deer sausages, RA=relative abundance.

4.12. Survival rates and efficiency of selected strains applied as starter cultures in sausage production

4.12.1. Physicochemical analysis and growth of lactobacilli

Indigenous *Lb. sakei* strains MRS_296 and C_7d_13 were inoculated in meat batter at cell counts of 9.34 and 9.30 log cfu/g, respectively. Although strains MRS_296 and C_7d_13 were previously clustered to the same group, they were both selected and applied as starter cultures due to exhibited different technological traits. In both batches, the one inoculated with *Lb. sakei* strains and the non-inoculated control, the growth pattern of lactobacilli followed similar tendency; lower cell counts at the beginning followed by an increase during fermentation and ripening and stabilisation at the end (Table 4.14.). Cell counts of lactobacilli were significantly higher (p<0.05) in inoculated batch at all measured time points than in control, with the exception of day 16 where their numbers were comparable. At the initial stages of sausage production, the number of lactobacilli was high, with 6.96 log cfu/g (0d) increasing to 8.73 log cfu/g (4d), whereas in spontaneously fermented batch their numbers approximated 8 log cfu/g after 16 days. Regarding pH, the values between batches were comparable at day zero but decreased significantly (p<0.05) at day 4 in inoculated batch and remained lower through fermentation and ripening (Table 4.13). Ready-to-eat sausages resulted with pH values from 5.11 (inoculated batch) and 5.43 (control). Water activity (a_w) gradually decreased during the manufacturing process to between 0.87 and 0.89 at the end of ripening, with no significant differences observed between batches.

Table 4.14. pH, water activity (a_w) and abundance of lactobacilli in batch inoculated with two *Lb. sakei* strains (MRS_296 and C_7d_13) and in non inoculated control batch. Results are expressed as a mean value ± standard deviation of the values from three replicates.

Batch	Production time points (days)	pH±st.dev.	Water activity (a_w)±st.dev.	Cell counts of lactobacilli detected on LamVab media (log cfu/g ±st.dev.)
Inoculated batch (*Lb.sakei* MRS_296 and C_7d_13)	0	5.58±0.03	0.96±0.00	6.93±0.07[a]
	4	5.09±0.03[a]	0.95±0.01	8.73±0.04[a]
	7	5.07±0.01[a]	0.95±0.00	8.96±0.18[a]
	16	5.11±0.01[a]	0.93±0.00	8.78±0.21
	20	5.10±0.07[a]	0.92±0.00	9.05±0.08[a]
	end (40)	5.11±0.03[a]	0.88±0.02	8.46±0.19[a]
Non inoculated control	0	5.59±0.04	0.96±0.00	3.78±0.44[b]
	4	5.62±0.01[b]	0.95±0.00	4.54±0.45[b]
	7	5.50±0.02[b]	0.95±0.00	7.82±0.01[b]
	16	5.44±0.06[b]	0.93±0.00	8.09±0.06
	20	5.47±0.06[b]	0.93±0.00	8.12±0.16[b]
	end (40)	5.43±0.03[b]	0.87±0.02	7.75±0.01[b]

a-b=values in the same column and day of ripening not followed by a common letter are significantly different (p<0.05), differences between batches.

4.12.2. Histamine and tyramine content in final products

The level of histamine was below 5.00 mg/kg in both batches. However, tyramine was detected at a level of 97.2±13.6mg/kg in spontaneously fermented control, while it was significantly lower (31.9±4.5; p<0.05) in the inoculated batch (Table 4.15).

Table 4.15. Histamine and tyramine content in batch inoculated with two *Lb. sakei* strains (MRS_296 and C_7d_13) and in non inoculated control batch, at the end od production (40 d).

Batch	Level of detected tyramine (mg/kg)±st.dev	Level of detected histamine (mg/kg)±st.dev
Inoculated batch (*Lb. sakei* MRS_296 and C_7d_13)	31.9±4.5[a]	<5.00
Non inoculated control	97.2±13.6[b]	<5.00

a-b=values in the same column not followed by a common letter are significantly different (p<0.05), differences between batches.

4.12.3. Survival rates of native starter cultures

Considering *Lb. sakei* strains MRS_296 and C_7d_13 were selected from the same cluster (regarded as the same strain), their rep-PCR patterns were not possible to differentiate. When clustered by UPGMA, all rep-PCR profiles of respective strains yielded a similarity level of 90.41 %. Those levels were considered as a cutoff value since levels above cutoff value cannot distinguish between strains. From batch inoculated with MRS_296 and C_7d_13, *Lb. sakei* strains with rep-PCR patterns corresponding to the patterns of respective starter cultures were recovered, indicating that a single or both strains were able to survive and establish themselves through the manufacturing, fermentation and ripening processes. Out of 37 strains collected from the inoculated batch, 32 (86.49 %) were clustered to the same group as applied starters. It is important to point out that since rep-PCR patterns of MRS_296 and C_7d_13 were not possible to differentiate, it is not possible to say whether both strains established themselves, or only a single one and if, which one. From spontaneously fermented control, no strains with rep-PCR patterns that corresponded to the patterns of applied starters were obtained. Control batch was characterised by higher diversity at a strain level with 27 clusters (Figure 4.12.), while in inoculated batch 2 cluster were recognised (Figure 4.13.).

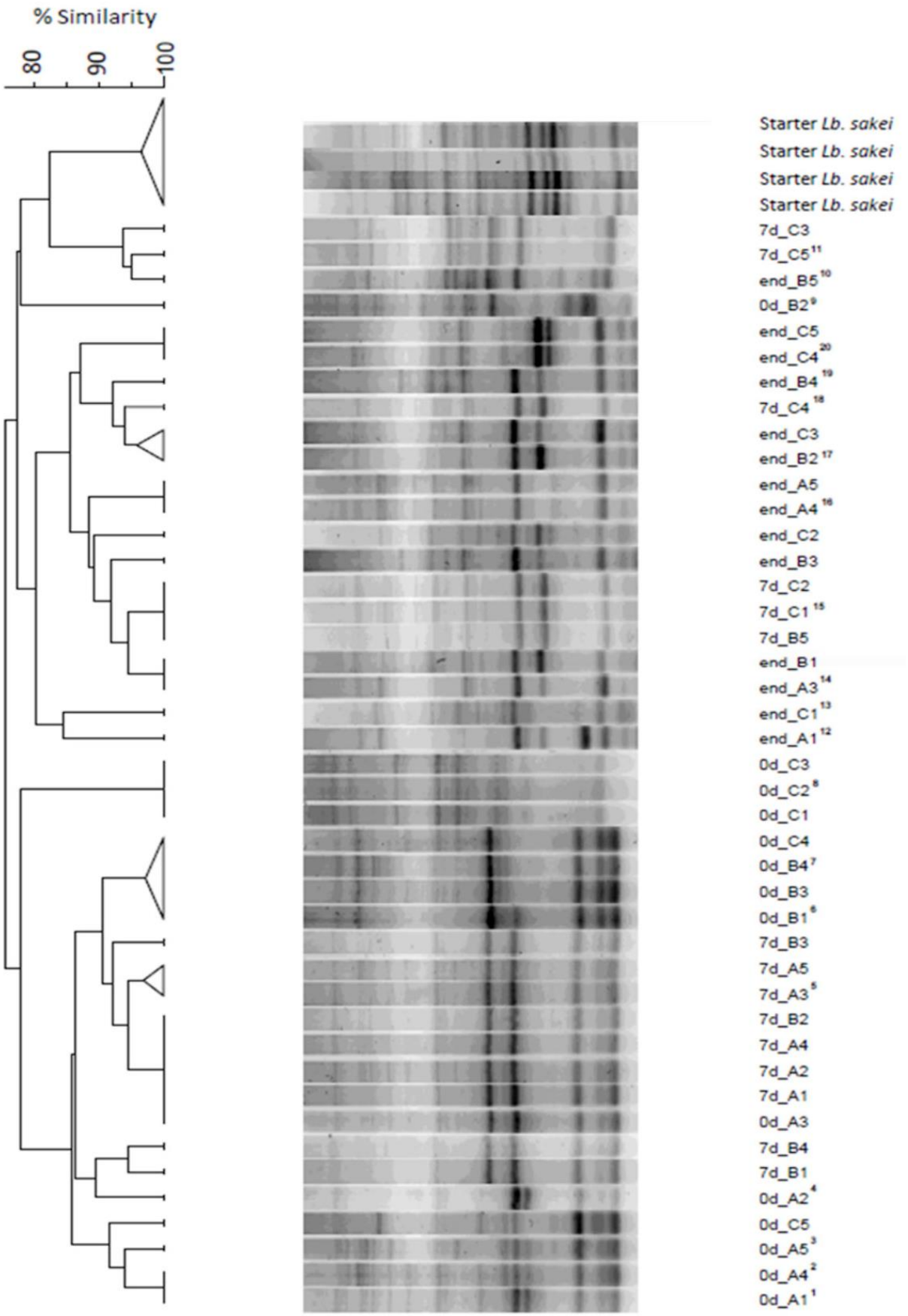
% Similarity
80
90
100
Starter Lb. sakei
Starter Lb. sakei
Starter Lb. sakei
Starter Lb. sakei
7d_C3
7d_C5 11
end_B5 10
0d_B2 9
end_C5
end_C4 20
end_B4 19
7d_C4 18
end_C3
end_B2 17
end_A5
end_A4 16
end_C2
end_B3
7d_C2
7d_C1 15
7d_B5
end_B1
end_A3 14
end_C1 13
end_A1 12
0d_C3
0d_C2 8
0d_C1
0d_C4
0d_B4 7
0d_B3
0d_B1 6
7d_B3
7d_A5
7d_A3 5
7d_B2
7d_A4
7d_A2
7d_A1
0d_A3
7d_B4
7d_B1
0d_A2 4
0d_C5
0d_A5 3
0d_A4 2
0d_A1 1

Figure 4.12. Cluster analysis of all strains isolated on LamVab media from spontaneously fermented control batch using rep-PCR (90.41 % cut-off value). Production time points are indicated next to each isolate (0d, 7d and end), and replicas with A, B and C. Isolate code is numerated with capital numbers while sequenced isolates are shown with exponential enumeration. Labels marked with the numbers [1-7] (GeneBank No. MH197045-51), [12-20] (GeneBank No. MH197056-64) identify with the 99 % similarity *to Lb. sakei* and numbers [9-11] (GeneBank No. MH197053-55) identify with the 99 % similarity to *Le. mesenteroides*, while number [8] (GeneBank No. MH197052) identifies to *W. viridescens* with the 99 % similarity.

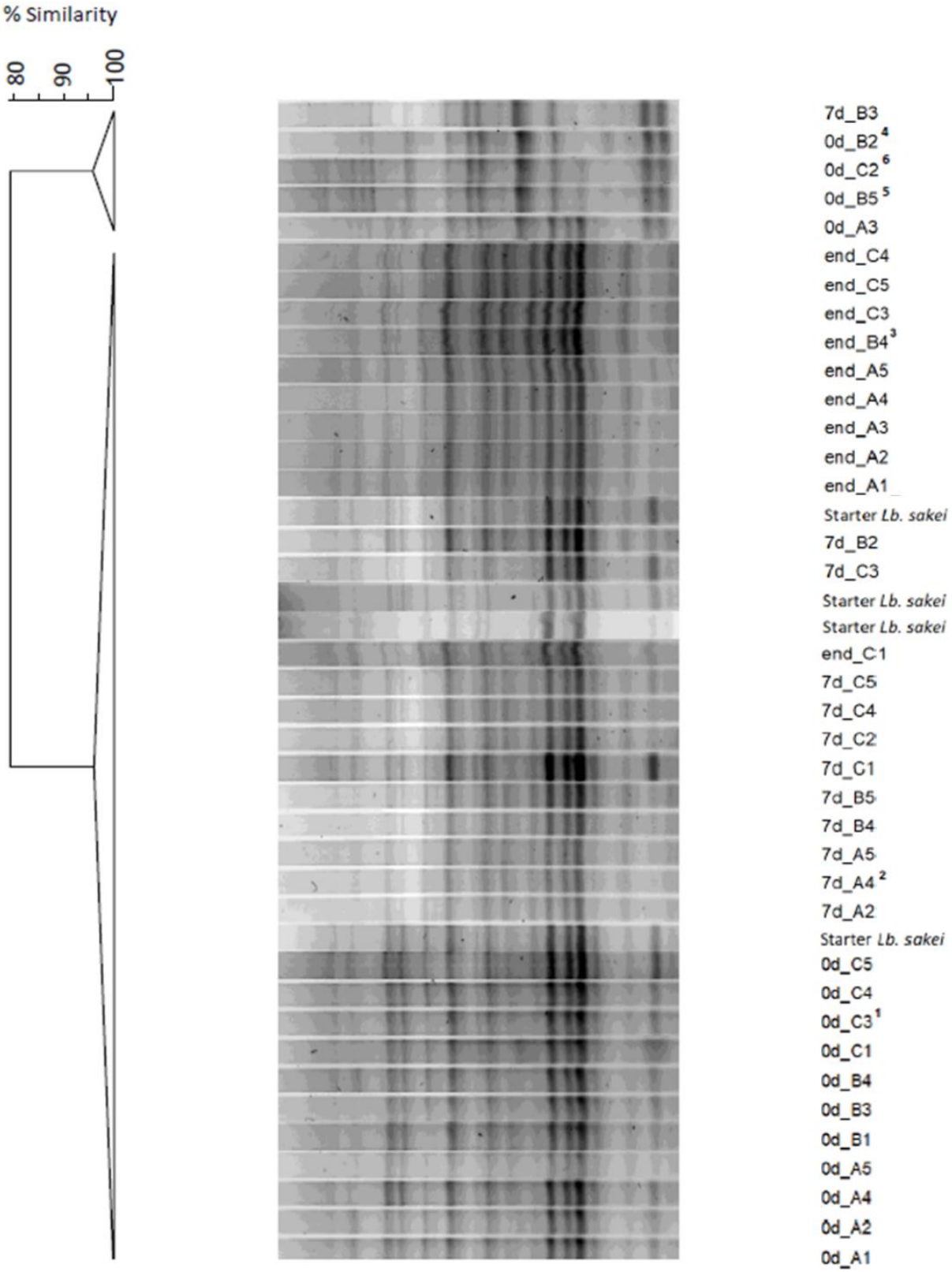
% Similarity
80
90
100
7d_B3
0d_B2 4
0d_C2 6
0d_B5 5
0d_A3
end_C4
end_C5
end_C3
end_B4 3
end_A5
end_A4
end_A3
end_A2
end_A1
Starter Lb. sakei
7d_B2
7d_C3
Starter Lb. sakei
Starter Lb. sakei
end_C1
7d_C5
7d_C4
7d_C2
7d_C1
7d_B5
7d_B4
7d_A5
7d_A4 2
7d_A2
Starter Lb. sakei
0d_C5
0d_C4
0d_C3 1
0d_C1
0d_B4
0d_B3
0d_B1
0d_A5
0d_A4
0d_A2
0d_A1

Figure 4.13. Cluster analysis of all strains isolated on LamVab media from the batch inoculated with two *Lb. sakei* strains (MRS_296, C_7d_13) using rep-PCR (90.41 % cut-off value). Production time points are indicated next to each isolate (0d, 7d and end), and replicas with A, B and C. Isolate code is numerated with capital numbers while sequenced isolates are shown with exponential enumeration. Indicated sequenced isolates [1-3] (GeneBank No. MH197065-67) identify with the 99 % similarity to *Lb. sakei* and numbers [4-6] (GeneBank No. MH231452-54) identify with the 100 % similarity to *Le. mesenteroides*.

4.12.4. Microbiological quality

The results of the complete microbiological analysis of both batches are reported in Table 4.16. In general, the number of yeasts increased during fermentation and ripening, while the undesirable microbiota decreased. Cell counts of enterococci decreased from 4.33 and 4.17 log cfu/g (0d) to 3.22 and 3.68 log cfu/g (end stage) in inoculated and control batch, respectively. In ready-to-eat spontaneously fermented control, the numbers of *Enterobacteriaceae* and coliforms exceeded the limits of 2 log cfu/g to be considered satisfactory (Health Protection Agency (HPA) 2009; Health Canada, 2010). *L. monocytogenes* was detected in control batch. *Staphylococcus aureus* was not detected in any of the batches at any time point, and the number of *E. coli* decreased below the detection limit (< 1.00 log cfu/g) in both batches at the end stage. *Salmonella* spp. was not detected in any end product.

Table 4.16. Results of the microbiological analysis of the batch inoculated with two *Lb. sakei* strains (MRS_296, C_7d_13) and spontaneously fermented control.

Batch	Production time points (days)	Selective media used in this study (log cfu/g±st.dev)					
		KAA (*Enterococcus* spp.)	DRBC (yeasts)	VRBG (*Enterobacteria-ceae*)	CCA (*Escherichia coli*)	CCA (coliforms)	BP (*Staphylococcus aureus*)
Inoculated batch	0	4.33±0.08	3.12±0.04	3.95±0.12	3.76±0.08	4.15±0.12	< 1.00
	7	4.19±0.07	< 1.00	3.39±0.10	< 1.00	2.19±0.02	< 1.00
	end (40)	3.22±0.02	5.31±0.05	< 1.00	< 1.00	< 1.00	< 1.00
Non inoculated control batch	0	4.17±0.07	4.22±0.09	4.77±0.20	3.59±0.21	3.90±0.01	< 1.00
	7	4.26±0.05	3.28±0.03	3.97±0.03	2.04±0.06	2.91±0.05	< 1.00
	end (40)	3.68±0.03	5.25±0.08	2.68±0.03	< 1.00	2.42±0.02	< 1.00

5. DISCUSSION

In this study, the microbiota of three wild boar (WB1, WB2, WB3) and three deer meat (DS1, DS2, DS3) sausages was characterised by culture-dependent and culture-independent methods. In all investigated sausages LAB were recognised as the most abundant group of bacteria, and their abundance increased during the fermentation process, as previously observed by many authors (Comi et al., 2005; Cocolin et al., 2009; Połka et al., 2015). The high cell counts of LAB, as well as their high relative abundance detected by high throughput amplicon sequencing indicates their main role in the fermentation processes, which is in an agreement with previous studies (Fontana et al., 2005, 2016; Bonomo et al., 2008; Cocolin et al., 2009; Połka et al., 2015). However, discrepancies were noted between the two approaches with regard to the structure of LAB communities. The predominant LAB isolated from sausages were identified as *Le. mesenteroides* (WB1, WB2, DS1) and *Lb. sakei* (WB3, DS3). While some studies also showed the domination of *Le. mesenteroides* (Babić et al., 2011; Danilović et al., 2011), usually, the predominant species isolated from dry fermented sausages are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Milicevic et al., 2014). Contrary to *Lb. sakei*, which beneficial effects in food fermentation are well known, the presence of heterofermentative *Le. mesenteroides* is controversial, especially when present in high numbers. *Le. mesenteroides* is generally either absent or present in low numbers in dry fermented sausages (Milicevic et al., 2014). However, analysis of the NGS data has identified members of the genus *Lactobacillus* as the most abundant species and most probably as a main driver of fermentation in both wild boar and deer meat sausages. A majority of them was identified as *Lb. sakei* (40.32 %), *Lb. curvatus* (40.29 %) and *Lb. plantarum* (1.31 %). LAB other than genus *Lactobacillus*, as well as coagulase-negative staphylococci (CNS) and *Kocuria* spp., were present in low relative abundance. It seems that isolation of LAB on selective media favoured the growth of *Le. mesenteroides*, considering that 52.31 %, 40.89 % and 22.09 % of isolates obtained from LamVab, MRS and MSE media, respectively, were identified as *Le. mesenteroides*. The overgrowth of *Le. mesenteroides* was also confirmed by DGGE analysis of the bulk colonies (consortia) collected from MRS plates. Such results suggest a low selectivity of the media used for the isolation of particular LAB genera from complex microbial ecosystems and the need for careful interpretation of the results of the culture-dependent analysis. Differences in detected bacterial taxa from various foods by culture-dependent and culture-independent methods were previously reported by other authors. For instance, in the work of Jackson et al. (2013) pyrosequencing allowed for the identification of low abundance bacteria in

leaf salad vegetables not detected by culture-dependent methods. Nguyen et al. (2013) applied PCR-DGGE as a culture-independent method for the identification of LAB associated with a Vietnamese fermented meat product (Nem Chua) and detected species of which no isolates were recovered. Likewise, not all isolated bacteria were detected by PCR-DGGE. Although the molecular barcoding approach used in the current study to describe bacterial community structure is a good tool to analyse food associated microbiomes in a high throughput manner, without introducing cultivation biases, the initial presence of unassigned sequences in the dataset implied the presence of unknown species and further stressed the importance of traditional cultivation methods for improving the taxonomic resolution. Also, comparison of BLAST results from different databases has indicated that a choice of the database can significantly amend the taxonomic resolution, underlining the importance of choosing an appropriate database in dependence to the type of analysed samples. Therefore, the combination of culture-dependent and culture-independent methods is often necessary for reliable and detailed investigation of LAB communities in fermented food products (Kesmen et al., 2012). Regarding bacterial diversity during sausage production, the number of OTUs was highest at day 0 and day 4, followed by a decrease in the later phases of fermentation and ripening. This reduced diversity pattern might be explained by significant changes in the physicochemical conditions of the sausages during the fermentation process including a reduction of pH induced by the naturally occurring LAB (Francesca et al., 2013). In addition, the reduction of available water for microbial growth (lower a_w values) and antagonistic effect of the naturally occurring LAB and CNS on other microbiota could explain the decrease of diversity over time (Ducic et al., 2016). Ready-to-eat dry fermented sausages generally have pH values between 5.2 and 5.8, and a_w values between 0.85 and 0.91 (Vignolo et al., 2010). In this study, wild boar sausages exhibited an average pH=5.43 and a_w 0.86, while deer meat sausages had an average pH=5.14 and a_w 0.85, which is in accordance with other similar products.

Comparing the same time points for both types of sausages, overall diversity patterns were consistent. However, all measured alpha diversity parameters (Shannon's index and Pielou evenness) were significantly higher in wild boar meat sausages compared to deer meat at day 4 ($p<0.01$) and day 7 ($p<0.05$). This might be linked to different lifestyle, type of diet and feeding habits of wild boar and deer (Gebert and Verheyden-Tixier 2001; Schley and Roper 2003). Since the meat fermentation is a complex microbiological process highly dependent on the presence and interaction of co-existing species that greatly influences its physicochemical environment, the differences in the LAB growth and fermentation dynamics between wild boar and deer meat sausages could be an indication

of the different bacterial composition of the raw starting material. In addition, cluster analysis of the most frequently isolated LAB (*Le. mesenteroides*, *E. casseliflavus*, *E. durans*, *Lactobacillus* spp.) from WB3 and DS1 sausages, both produced at the same production unit, revealed the tendency of sausage-specific clustering, implying that the raw meat used for sausage production was the main source of bacterial strains, rather than the production environment. This claim is supported by Talon et al. (2007) who found no evidence of cross-contamination between environment and meat, such assuming that the bacterial diversity of the meat batter was mainly due to the microbiota of the raw materials.

The abundance of bacteria associated with food spoilage and opportunistic pathogens was low at the beginning of the fermentation process, with the exception of OTUs related to *Bacillus and Stenotrophomonas* in wild boar and *Brochothrix, Carnobacterium* and *Weissella* in deer meat sausages. With fermentation time, the abundance of undesired and potentially harmful microbiota decreased indicating the microbial stability and safety of the final product. Only for OTUs linked to *Stenotrophomonas* in WB*,* no decrease was observed, and members of this genera were present at a relative abundance of 17.69 % in ready-to-eat WB sausages. However, 33.33 % ready-to-eat sausages were unsafe for consumption, due to the elevated cell counts of *Enterobacteriaceae* (DS2, WB3) as well as *E. coli*, coliforms and presumptive *B. cereus* (WB3). In all final products the number of enterococci was below the detection limit (<1.00 log cfu/g), with the exception of WB3 sausage, where their number was 3.77 log cfu/g. Elevated numbers of *Enterobacteriaceae, E. coli* and/or coliforms, as well as high cell counts of enterococci, can indicate faecal contamination suggesting poor raw meat hygiene, most probably due to the contamination at shooting or evisceration. However, enterococci in food are not always of fecal origin and can also be flavor contributors. Their numbers in dry fermented sausages can be variable and mostly depend upon the hygienic quality of the raw material (Kozačinski et al., 2006). The legislation in force (Commission Regulation (EC 2007) 1441/2007) does not set limits for enterococci in food. Usually, *E. faecalis* and *E. faecium* are the most frequently encountered species of enterococci in fermented sausages (Correia Santos et al., 2017), however, in this study *E. casseliflavus* (WB1, WB2, DS3) and *E. durans* (WB3, DS1, DS2) were detected as the predominant species, while *E. faecium* and *E. faecalis* were detected only sporadically, similar to the results of Danilović et al. (2011).

B. cereus is widespread in nature, readily found in soil and has been recovered from a wide range of food types, including raw and processed meats (Eglezos et al., 2010). The

presence of *B. cereus* is often associated with contaminated raw materials and the subsequent resistance of spores to manufacturing processes, during which spores may germinate, enabling *B. cereus* to multiply and/or produce high levels of toxins. The most cases of foodborne outbreaks caused by the *B. cereus* group have been associated with levels above log 5 cfu/g, but cases of both emetic and diarrheal illness involved lower levels of *B. cereus* (EFSA Panel on Biological Hazards, 2016). It is possible that high numbers of *B. cereus* detected in this study arose from game meat contaminated with soil bacteria during hunting and/or evisceration. Thus, more hygienic conditions during hunting and especially evisceration, with minimalised exposure of game meat to soil bacteria could prevent such high counts of this potential pathogen. The microbiological safety of sausages ripened for a shorter period (*e.g.* 20 days) is even more compromised which emphasises the importance of ripening time to reach the microbiological quality that falls within the safety parameters.

Spontaneously fermented game meat sausages can represent a microbial pool that could serve as a source of technologically important strains relevant for standardisation of sausage production. Based on the cluster analysis of the rep-PCR patterns of the 1326 collected isolates, 57 representative LAB strains were selected and screened for possible safety issues, technological potential and bioprotective role. The selected representatives belong to the following species: *Lactobacillus sakei* (*n*=27), *Leuconostoc mesenteroides* (*n*=19), *Enterococcus durans* (*n*=9) and *Lactobacillus curvatus* (*n*=2). Although LAB is technologically fundamental and has a long history of safe use in food fermentation (Kröckel 2013), before any application in the food they need to be characterized in details and designated as safe. Among LAB, enterococci raise a considerable safety concerns, since they have been known to harbour different virulence factors, suggesting that fermented food could act as an important environmental reservoir for human infections. Many of virulence genes, *e.g.* aggregation substances, cytolysin and gelatinase genes are known to have a silent state, making their genotypic study more reliable than phenotyping one because they might remain undetected under the *in vitro* conditions but be expressed *in vivo*, so the pathogenic potential of such strains might be underestimated (Morandi et al., 2013). The PCR screening of virulence determinants among enterococcal (*E. durans*) strains in this study did not reveil the presence of any such genes, in accordance to the results of Eaton and Gasson (2001). Generally, the occurrence of virulence determinants appears to be higher in *E. faecalis* than in *E. faecium* and *E. durans* strains (Eaton and Gasson 2001; Cebrián et al., 2012). Another safety concern associated with LAB is their ability to produce a considerable amount of bioactive compounds related to acute adverse health effects, such as biogenic amines (EFSA Panel on Biological Hazards, 2011),

especially tyramine. In the current study, *tdc* gene encoding for tyrosine decarboxylase was detected among 26.31 % of the representatives. The gene was detected at different frequencies between *E. durans* (55.55 %)*, Le. mesenteroides* (31.58 %) and *Lb. sakei* (14.81 %) strains, which emphasizes the distinct potential of the indigenous LAB to contribute to biogenic amines formation in spontaneously fermented game meat sausages.

The content of histamine measured in final products by HPLC analysis did not surpass 5.00 mg/kg, therefore representing no risk of intoxication. The content of tyramine, however, varied between sausage samples and it was between 47.3 ± 6.6 mg/kg (DS3) and 219.0 ± 13.0 mg/kg (DS2). Such variations are in accordance with the results of other studies (Suzzi and Gardini 2003). With the exception of DS2 sausage, the concentration of tyramine in all final products was below the 100 mg/kg. Since the maximal tolerable level of tyramine in food is not clearly defined, rather approximated to a broad range between 100-800 mg/kg (Ercan et al., 2013), it is hard to assess the risk associated with tyramine content in DS2 sausage. However, considering that tyramine was detected in DS2 sausage in a far less concentration then 1,080 mg/kg, which is toxic for people (Ercan et al., 2013) and that no cases of BA poisoning have implicated fermented sausages as the cause, DS2 sausage can be assumed as safe for consumption, considering the BA content. Although enterococci have previously been recognised as the main tyramine producers among LAB (Holck et al., 2017), they were not detected in DS2 sausage at any investigated time-point. So, the accumulation of tyramine can probably be assigned to *Le. mesenteroides,* considering their abundance and the presence of *tdc* gene among collected isolates. *Enterobacteriaceae,* which are commonly associated with the production of cadaverine and putrescin in sausages, are also known tyramine producers (Ruiz-Capillas and Jiménez-Colmenero 2004) and may have contributed to its accumulation since were abundant during all stages of sausage production.

Regarding the antibiotic resistance, the tested representatives were mainly non-resistant. None of the enterococcal (*E. durans*) strains showed resistance to any of the tested antibiotics. Among enterococci, acquired resistance to vancomycin is of special concern since vancomycin-resistant strains are very difficult to treat. The frequency of vancomycin-resistant enterococci in food is mostly low and vary between 0–25 % (Wang et al., 2014). Although enterococci from food have been reported to exhibit resistance to a broad spectrum of antibiotics and are prone to obtain resistance to multiple antibiotics (Giraffa 2002), due to their abundance in food and clinical significance, the scientific focus had been on describing AR profiles of *E. faecalis* and *E. faecium* (Peters et al., 2003).

Therefore the available data mostly refers to the respective species, while the data regarding AR profiles of *E. durans* is scarce. *E. faecium* is recognised as the enterococcal species most prone to acquire resistance to multiple antibiotics (Marothi et al., 2005). *Le. mesenteroides* strains were also mainly (94.74 %) non-resistant to the tested antibiotics, while lactobacilli were more prone to show resistance to a single or to multiple antibiotics. They were most commonly resistant to kanamycin (30 µg; 24.14 %) and gentamicin (10 µg; 17.24 %), which seems to indicate intrinsic resistance. It seems that lactobacilli investigated in this study acquired resistance to erythromycin (2 µg; 6.90 %) and tetracycline (5 µg; 6.90 %). Strains that are suspected of acquiring transmisable genes encoding for AR must not be used as starter cultures, nor applied in food for any other purposes (*eg*. probiotic and bioprotective cultures). Resistance to clindamycin (2 µg) was exhibited by 6.90 % of lactobacilli, while 3.45 % of them was resistant to ampicillin (2 µg). Resistance to multiple antibiotics have been reported for lactobacilli and leuconostocs isolated from different types of food. Aymerich et al. (2006) have collected LAB isolates (*Lb. sakei*, *Lb. curvatus* and *Le. mesenteroides*) from slightly fermented sausages, all of which were resistant to at least two antibiotics. About half of the isolates (48 %) were only resistant to vancomycin and gentamicin, whereas 42.8 % were resistant towards 3-4 antibiotics. Of all isolates 8.8 % (mainly *Lb. sakei*) displayed resistance to 5 or 6 of the antibiotics tested, including vancomycin, gentamicin, ampicillin and penicillin. In the work of Belén Flórez et al., (2016) dairy leuconostocs exhibited multiple resistance: *Le. mesenteroides* subsp. *mesenteroides* LbE16 to kanamycin, streptomycin, tetracycline and virginiamycin, and *Le. mesenteroides* subsp. *dextranicum* LbE15 to erythromycin and clindamycin. Some LAB may induce a hemolytic reaction, but in accordance with other studies (Baruzzi et al., 2006; Domingos-Lopes et al., 2017), none of the tested strains were found to cause haemolysis.

To be considered as a part of the starter culture, strains must, besides posing no threat to human health and well being, exhibit desirable technological traits. The most important trait of any starter culture is the ability of rapid acidification of the raw material. Enterococci and leuconostocs were equally efficient at lowering the pH of pork meat media, while lactobacilli exhibited lower acidification ability. Compared to the study of Baruzzi et al. (2006), where lactobacilli isolated from sausages were able to decrease the pH values between pH=4.6 and pH=5.9 after 24h of incubation in pork meat media, lactobacilli in the current study exhibited somewhat better acidification ability by lowering the pH between pH=3.76 and pH=5.89 after 24h of incubation. Notable differences in the acidification ability were also observed between different strains of the same species, indicating that this trait is strain-specific. This claim is supported by Bonomo et al. (2008),

who found that LAB acidifying ability diversified among species and also within each species. Other technological traits, like lipolytic, proteolytic and peptidase activity are also believed to be strain-specific, which is reflected in the results of this study, considering statistical differences in the noted activities within species. In disagreement with the results of the present study, where all but one strain displayed lipolytic activity on Tributyrin agar, Domingos-Lopes et al. (2017) detected lipolytic activity in 30 % of isolates (leuconostocs, lactobacilli, enterococci) collected from artisanal cheese. However, authors of other studies reported a higher prevalence of lipolytic activity among LAB isolates from food (Dinçer and Kivanç 2018). In addition to lipolytic activity, a small number of strains (15.79 %) showed the ability to hydrolyse sarcoplasmic proteins, while the majority of them (96.49 %) were able to hydrolize skim milk proteins, particularly caseins. Similarly, Bonomo et al. (2008) observed the ability to hydrolyse sarcoplasmic proteins in 22 % of LAB isolated from traditional fermented sausages. Proteolysis due to microbial activity in sausages is mainly related to CNS proteases (Hammes and Hertel 2009), and their ability to hydrolyze sarcoplasmic proteins have been reported (Mauriello et al., 2002). Even when muscle enzymes are greatly involved in meat protein degradation, bacterial proteolytic activity leads to a richer composition of small peptides and amino acids which contribute to the ripening process either as direct flavour enhancers or as precursors of other flavour compounds (Fadda et al., 2010). Fadda et al. (2010) reported that peptides of lower molecular mass (21 and 18 kDa) were absent in fresh meat, but were present in samples of fermented sausages, possibly a consequence of their solubilization on high ionic strengths or due to protein hydrolysis during the ripening process. With regard to peptidase activity, as a result of measuring the amount of *p*NA released from the chromogenic peptide (S-Ala), strains tested in the present study showed high variability, ranging between 61.05 and 8343.50 µM *p*NA.

Conventional starter cultures have a bioprotective role as a result of acidification. Accumulation of lactic acid, the major metabolic end product of LAB carbohydrate fermentation, is a powerful mechanism for inhibition of growth of many spoilage and pathogenic species. According to the conclusions of the American Food and Drug Administration Panel of Experts on food security and stability (USFDA, 2001), the pH value of 4.6 inhibits the growth of spore-forming pathogens, whereas such value for the vegetative pathogens is lower and amounts to 4.2. Strains that show low acidification capacity and are not appropriate candidates for use as starters may be used as bioprotective cultures if they produce antimicrobial substances, such as bacteriocins. Antagonistic activity of food isolates belonging to the LAB, against various pathogenic and spoilage bacteria frequently present in meat and sausages have been well documented

(Oliveira et al., 2018). In the present study, representative strains displayed antagonistic activity mainly against *L. innocua,* and to a lesser extent against *Salmonella enterica*, *E.coli, Bacillus cereus*, *Weissella viridescens*, *Brochotrix thermospachta* and *Staphylococcus aureus.* However, when the testing was performed using neutralised cell-free supernatants, antagonistic activity was confirmed for 4 strains against *L. innocua.* It seems that the antagonistic effect shown by majority of representatives was due to metabolic production of lactic acid and some other antagonistic molecules such as ethanol, H_2O_2, CO_2 and other, rather then as a result of bacteriocin production, whereas it is possible that cell-free supernatants were detrimental to *L. innocua* due to bacteriocins excreted by the respective strains. Out of 4 possible bacteriocin producing strains, 2 *Lb. sakei* strains (C_end_1 and MRS_331) have the potential to be used in food production. The other 2 strains are unfit for food application due to detected *tdc* gene responsible for tyramine production (*E. durans*) or due to the exhibited resistance to several antibiotics (*Lb. sakei*), but alternatively, bacteriocins may be extracted and then applied in sausage production. Results of the numerous research have shown that the application of bacteriocin-producing LAB strains, as well as extracted bacteriocins, could control the growth of *L. monocytogens* and other undesirable microorganisms (Ravyts et al., 2008; Gao et al., 2014; Oliveira et al., 2018).

Representative *E. durans* and *Lb. sakei* that were designated as safe (not resistant to antibiotics and possessed no genes encoding for the production of biogenic amines) were selected (n=19) and their ability to aggregate and to survive in simulated GI tract conditions, as a prerequisite for their potential probiotic activity, were evaluated. All strains showed the low capacity to autoaggregate, with average values of 12.02 % after 3 h of incubation, *i.e.* 23.35 % after 5 hours of incubation. Strains with autoaggregation capacity exceeding 80 % are considered as strongly autoaggregating (Del Re et al., 2000). Regarding survival rates in simulated GI tract conditions, all strains were best able to tolerate the conditions of the oral cavity with average survival rates of 89.08 %. In the simulated environment of the stomach, where low pH (pH=2) and pepsin were present, the viability of bacteria significantly (p<0.001) decreased to an average of 17.95 %. In the third barrier (simulated conditions of the small intestine) pH is more favourable for bacterial survival, but it seems that the presence of bile salts and pancreatin had a detrimental effect, with an average survival rate of 2.27 %. Aside from survival during the passage through GI tract, probiotic bacteria must persist in the intestine at a minimum level of between 6 and 8 log cfu/g to be effective (Marteau and Rambaud 1993). *E. durans* A_4d_1 showed the survival rate of 30.86 % in the simulated conditions of the small intestine, which corresponds to a cell count of 7.40 log cfu/g and can, therefore, be

considered as a potential probiotic strain which should be further examined (Banić et al., 2018).

Based on the results of safety characterisation and technological traits of representative strains, *Lb. sakei* strains MRS_296 and C_7d_13 were selected and inoculated in meat batter at cell counts of 9.34 and 9.30 log cfu/g, respectively. The cell counts of lactobacilli were higher, and the pH decreased more rapidly in the inoculated batch compared to the spontaneously fermented control batch. Such findings are in accordance with the results of other studies (Pragalaki et al., 2013; Ba et al., 2016).

The vast majority (86.49 %) of isolates recovered from inoculated batch had rep-PCR patterns that corresponded to the patterns of respective starter cultures, suggesting that strains applied as starter cultures were able to establish themselves from day zero and prevailed through the production. Also, spontaneously fermented control showed greater microbial diversity, at least at a strain level. However, considering that rep-PCR patterns of MRS_296 and C_7d_13 were not possible to differentiate, it is not possible to say whether both strains established themselves, or only a single one, and if, which one.

Although histamine content was found to be below 5 mg/kg in all ripened sausages, HPLC analysis revealed significantly (p<0.05) lower tyramine content in the batch inoculated with the indigenous decarboxylase negative *Lb. sakei* strains (31.9 mg/kg) compared to spontaneously fermented control (97.2 mg/kg). Such results highlight the importance of detailed screening and selection of strains that do not possess genes encoding for production of biogenic amines. The inoculation of competitive and decarboxylase-negative starter culture has been shown to inhibit indigenous aminogenic microbiota and thus considerably reduce aminogenesis (Bover-Cid et al., 2000; Xie et al., 2015). Furthermore, the inoculation with non-amine forming starters constrains buildup of biogenic amines in ready-to-eat fermented sausages during their storage time (Kongkiattikajorn 2013). The results of the complete microbiological analysis may suggest that the inoculation of indigenous *Lb. sakei* starter cultures suppressed the growth of *Enterobacteriaceae*, coliforms and *L. monocyogenes.* The inhibition of undesirable microbiota due to *Lb. sakei* inoculation had been previously demonstrated. For instance, Wang et al. (2013) have reported the inhibition of *E. coli* and *Enterobacteriaceae* in Chines fermented sausages, while Pragalaki et al. (2013) showed that *Lb. sakei* inhibited the growth of *L. monocytogenes* and *E.coli* in traditional Greek sausages. Frece et al. (2014) showed that strains isolated from traditional sausages have the ability to survive during industrial production, and compared with commercial starters, they have also displayed better results in the development of sensory properties, stability and microbiological safety of

sausages. The antagonistic activity of starter cultures is most probably due to their proliferation and rapid acidification during the first days of production, ensuring an unfavourable environment for undesirable microbiota to develop and consequently preventing them to participate in the formation of biogenic amines (Latorre-Moratalla et al., 2017). The decrease of gram-negative bacteria due to lactic acid accumulation was previously reported (Latorre-Moratalla et al., 2007). Low pH at the beginning of fermentation in the inoculated batch contributed not only to the inhibition of undesirable microbiota and consequently preventing the accumulation of biogenic amines, but it may have as well provided a suitable environment for the activity of enzymes involved in biochemical processes associated with defining sensory traits of final products.

Therefore, it may be concluded that the applied starter cultures were efficient in ensuring a microbiologically safe end products, and the starters could be used in future for standardisation of sausage production.

6. CONCLUSIONS

By implementing ISO standards, successful isolation and enumeration of undesirable and potentially harmful microbial groups on various selective media were performed. Monitoring of such microorganisms through the production was enabled, as well as the evaluation of the microbial status of ready-to-eat sausages.

Approximately one third (33.33 %) of ready-to-eat game meat sausages were unsafe for consumption, due to the elevated cell counts of several opportunistic pathogens (*E. coli*, *Enterobacteriaceae*, coliforms, *B. cereus*).

Microbiological media used for isolation of LAB exhibited insufficient selectivity. LamVab and MRS media which is believed to be more selective for *Lactobacillus* spp. favored the growth of *Le. mesenteroides*. On MSE media, considered to be selective for *Le. mesenteroides*, various LAB were detected. Results of the DGGE analysis of bulk colonies collected from MRS media confirmed the prevalence of *Le. mesenteroides* on respective media, supporting previously observed insufficient media selectivity.

Conventional microbiological methods that rely on cultivating bacteria and used microbiological media supported the isolation and collection of 1326 LAB isolates from sausages, of which 917 isolates were collected from 3 wild boar and 3 deer meat sausages within the scope of this study.

The rep-PCR method with GTG_5 primer proved to be a valuable tool for distinguishing between LAB strains and enabled their clustering. The method was also proven as rapid, reliable and sensitive for monitoring the survival rates of inoculated starter cultures.

Total of 57 representative strains was selected based on their rep-PCR profiles and characterized to select the appropriate strains as a starter and/or bioprotective cultures. They were mainly non-resistant to the tested antibiotics. Notable differences were observed among strains of the same species with regard to exhibited technological traits, such as acidification, lipolytic, proteolytic and peptidase activity, featuring those traits as strain-specific and highlighting the need for selection of suitable strains. Antilisterial activity was detected for cell-free supernatants of 4 strains, indicating possible bacteriocin production. The bacterial culture collection of in details characterised strains was formed, for their possible future application.

One strain (*E. durans* A_4d_1) was able to survive in the simulated conditions of the small intestine at a cell count of 7.40 log cfu/g and can, therefore, be considered as a potential probiotic strain which should be further examined.

Raw meat used for sausage production was identified as the probable source of bacterial diversity, at least at a strain level, rather than the production environment.

As a key step for NGS analysis, DNA isolation from the matrix of sausages was successfully optimized.

Next-generation sequencing, as a culture-independent method, was proven to be a reliable tool for describing naturally present microbiota of game meat sausages. High throughput amplicon sequencing revealed members of the genus *Lactobacillus,* among which *Lb. sakei* (40.32 %) and *Lb. curvatus* (40.29 %) as the most abundant species and most probably as the main drivers of fermentation. Bacterial diversity was highest at the beginning of production (day 0 and 4), after which a decrease was followed toward the later phases of fermentation and ripening.

Inoculation of well-defined indigenous starter cultures resulted with more rapid acidification and lower pH values in inoculated batch, compared to the control. The applied starters were able to establish and survive throughout the sausage production and were efficient in suppressing the growth of undesirable microorganisms.

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8. ABOUT THE AUTHOR

Ana Žgomba Maksimović was born 17th September 1988 in Pula. After elementary school, finished nursing school in Pula. She received a Bachelor's Degree in 2011 after graduating at the University of Zagreb, Faculty of Agriculture (Agroecology). Her thesis ˝Isolation and characterization of the indigenous enterococcal community from Cheese in a sack˝ was performed at the Department of Microbiology, under the supervision of Assoc. Prof. Mirna Mrkonjić Fuka, Ph.D. The same year she enroled in the Graduate study programme (Microbial biotechnology in agriculture) and was awarded the title Master od Science in 2014. The thesis ˝Technological potential of *E. faecalis* strains isolated from Istrian cheese˝ was performed at the same department, also under the supervision of Assist. Prof. Mirna Mrkonjić Fuka, Ph.D. The following year (2015) she was employed as an expert associate at the Department of Microbiology, Faculty of Agriculture and enrolled in the Postgraduate doctoral (Ph.D.) study.

As a collaborator on three modules (Food Microbiology, Microbiology in animal production and Molecular methods in microbial agroecology), she provided practical courses for undergraduate and graduate students. As a laboratory supervisor, she participated in performing two graduate theses. She is a member of Croatian Microbiological Society.

Supported by the Erasmus+ grant, she stayed at the German Research Center for Environmental Health (Helmholtz Zentrum) in Munich at the duration of three months, and within the CEEPUS scholarship, financed by the Austrian Federal Ministry of Science, Research and Economics she stayed at the University of Natural Resources and Life Sciences(BOKU) in Vienna for the duration of one month.

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9. APPENDIXES

9.1. List of all microbiological media used in this study with preparation instructions (in alphabetic order)

9.1.1. Baird-Parker Agar (BP)

BP agar (Labo-Life Sàrl, Pully, Switzerland) was prepared by dissolving 58 g of media in 1 L of distilled water. Media was sterilised at 121 °C for 15 min, cooled to 50 °C and supplemented with an egg yolk tellurite emulsion (20%; VWR International, Switzerland).

9.1.2. Brain Heart Infusion Agar

BHI broth (Biolife, Milano, Italy) was prepared by dissolving 52 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH=7.4±0.2.

9.1.3. Brain Heart Infusion Agar supplemented with skimmed milk (1.5 %)

BHI agar (Biolife, Milano, Italy) was prepared by dissolving 52 g of media in 900 mL of distilled water and sterilised at 121 °C for 15 min. In a separate bottle, 15 g of skim milk (Biolife, Milano, Italy) was dissolved in 100 mL of distilled water and sterilised at 110 °C for 15 min. After sterilisation, the skim milk solution was added to the BHI agar and mixed well. 25 mL of media was poured into each Petri dish.

9.1.4. Brain Heart Infusion Broth

BHI broth (Biolife, Milano, Italy) was prepared by dissolving 37 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH= 7.4±0.2.

9.1.5. Chromogenic Coliform ES Agar (CCA)

CCA agar (Biolife, Milano, Italy) was prepared by dissolving 27.1 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH= 6.8 ± 0.2.

9.1.6. Columbia Blood Agar

Ready to use plates were purchased (Biolife, Milano, Italy).

9.1.7. De Man Rogosa Sharpe Agar (MRS)

MRS agar (Biolife, Milano, Italy) was prepared by dissolving 70.2 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Media was supplemented with cycloheximide (0.1 g/L; Sigma-Aldrich, Darmstadt, Germany). Final pH=6.4±0.2.

9.1.8. Dichloran Rose-Bengal Chloramphenicol Agar (DRBC)

DRBC agar (Biolife, Milano, Italy) was prepared by dissolving 15.5 g of media in 500 mL of distilled water and sterilised at 121 °C for 15 min. Media was supplemented with 1 vial of Chloramphenicol Antimicrobic Supplement. Final pH=5.6±0.2.

9.1.9. Kanamycin Esculin Azide Agar (KAA)

KAA agar (Biolife, Milano, Italy) was prepared by dissolving 21.3 g of media in 500 mL of distilled water, sterilised at 121 °C for 15 min and cooled to 50 °C. Kanamycin Selective supplement (1 vial) was reconstituted with 5 mL of sterile distilled water and added to media. Final pH=7.0±0.2.

9.1.10. LAB susceptibility test agar (LSM)

LSM media was prepared as described by Klare et al. (2005), with slight modifications. 21.06 g of ISO-sensitest broth (IST; Oxoid, Wesel, Germany), 5.52 g of De Man Rogosa Sharpe Agar (MRS; Biolife, Milano, Italy) and 15 g of agar (Biolife, Milano, Italy) were dissolved in 1 L of distilled water. pH was adjusted to pH=6.7. The media was sterilized at 121 °C for 15 min. 20 mL of sterile media was poured into each Petri dish.

9.1.11. LamVab

LamVab was prepared as described by Hartemink et al. (1997). The media consists of 3 different solutions. Solution A was prepared by dissolving 110.4 g of MRS Broth (Biolife, Milano, Italy), 0.5 g cysteine-HCl (Sigma-Aldrich, Darmstadt, Germany) and 0.05 Bromocresol Green (Sigma-Aldrich, Darmstadt, Germany) in 1 L of distilled water. The pH was adjusted to 5.0±0.1 before autoclaving. Solution B was prepared by dissolving 40 g of Agar Technical (Biolife, Milano, Italy) in 1 L of distilled water. Solution C was prepared by dissolving 40 mg of Vancomycin hydrochloride in 20 mL of water. Solution A and B were sterilised at 121 °C for 15 min, while solution C was sterilised by filtration using a 0.2 µm Millipore filter (Merck, Darmstadt, Germany). Solution B was cooled down in a water bath to 50 °C and added to solution A. Finally, 20 mL of solution C was added to the media. Final vancomycin concentration was 20 mg/L.

9.1.12. Lauryl Sulfate Broth

Lauryl Sulfate broth (Merck, Darmstadt, Germany) was prepared by dissolving 35.5 g of media in 1 L of distilled water. Media was supplemented with 1 vial of MUG reagent (50 mg 4-methylumbelliferyl-ß-D-glucuronide; Thermo Fisher Scientific, Waltham, USA) and sterilised at 121 °C for 15 min. Final pH=6.8 (approx.).

9.1.13. Listeria Fraser Broth Half Concentration

Listeria Fraser Broth Half Concentration (Biolife, Milano, Italy) was prepared by dissolving 27.4 g of media in 500 mL of distilled water and sterilised at 121 °C for 15 min. Sterile media was cooled to 50 °C, and 1 vial of Ferric Ammonium Citrate Supplement was added, previously reconstituted in 5 ml of sterile distilled water. Final pH=7.2±0.2.

9.1.14. Listeria Fraser Broth

Listeria Fraser Broth (Biolife, Milano, Italy) was prepared by dissolving 27.4 g of media in 500 mL of distilled water and sterilised at 121 °C for 15 min. Media was supplemented with 1 vial of Ferric Ammonium Citrate. Final pH=7.2±0.02.

9.1.15. Lyophilized Pork Meat Media (LP)

LP media was prepared as described by Baruzzi et al. (2006). The media was prepared by mixing 10 g glucose (Biolife, Milano, Italy), 11.5 g lyophilised pork meat and 3.5 g starch (Biolife, Milano, Italy) with 1 L of distilled water. pH=5.8.

9.1.16. Mannitol Egg Yolk Polymyxin Agar (MYP)

MYP agar (Biolife, Milano, Italy) was prepared by dissolving 21.5 g of media in 450 mL and sterilised at 121 °C for 15 min. Media was supplemented with 1 vial of *Bacillus Cereus* Antimicrobic Supplement (Biolife, Milano, Italy). Final pH=7.2±0.2.

9.1.17. Mayeux, Sandine, Elliker Agar (MSE)

MSE agar (Biolife, Milano, Italy) was prepared by dissolving 136.5 g of media in 1 L of distilled water. Media was heated to boiling and sterilised at 110 °C for 15 min. Final pH=6.9±0.2.

9.1.18. Mueller-Hinton agar (MH)

Mueller-Hinton agar (MH; Merck, Darmstadt, Germany) was prepared by dissolving 17 g of media in 1 L of distilled water. Media was sterilised at 121 °C for 15 min. pH=7.3±0.2.

9.1.19. Muller-Kauffmann Tetrathionate Novobiocin Broth (MKTTn)

MKTTn (Biolab, Budapest, Hungary) was prepared by dissolving 45 g of media in 480 mL of distilled water and sterilised at 121 °C for 15 min. Media was cooled to 50 °C, and 1 vial of Novobiocin (20 mg) reconstituted with 4 ml of distilled water and 5 ml of Brilliant Green Solution were added. Media was mixed and additionally supplemented with 1 vial of Tetrathionate Iodine-Iodide selective supplement reconstituted with 10 mL of distilled water.

9.1.20. Nutrient Agar

Nutrient agar (Merck, Darmstadt, Germany) was prepared by dissolving 20 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH=7.0+0.2.

9.1.21. Oxoid Chromogenic Listeria Agar (OCLA)

OCLA (Oxoid, Wesel, Germany) was prepared by dissolving 34.5 g of media base in 480 mL of distilled water and sterilised at 121 °C for 15 min. Media was cooled to 46 °C, and 1 vial of OCLA selective supplement and 1 vial of OCLA differential supplement were added. Final pH=7.2±0.2.

9.1.22. PALCAM

PALCAM (Biolife, Milano, Italy) was prepared by dissolving 35.4 g of media in 500 mL of distilled water and sterilised at 121 °C for 15 min. Listeria PALCAM antimicrobic supplement (1 vial) reconstituted with 5 mL of sterile distilled water was added. Final pH=7.2±0.2.

9.1.23. Plate Count Agar (PCA)

PCA agar (Merck, Darmstadt, Germany) was prepared by dissolving 22 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH=7.0 (approx.).

9.1.24. Purple Glucose Agar

Purple Glucose agar was prepared by dissolving 10 g of Enzymatic Digest of Casein (Biolife, Milano, Italy), 1.5 g of yeast extract (Biolife, Milano, Italy), 10 g of glucose (Biolife, Milano, Italy), 5 g of sodium chloride (Sigma-Aldrich, Darmstadt, Germany), 0.015 g of Bromcresol Purple (Sigma-Aldrich, Darmstadt, Germany) and 12.2 g of agar technical (Biolife, Milano, Italy) in 1 L of distilled water. Media was sterilised at 121 °C for 15 min, and 10 mL of media was poured into each of the sterile test tubes. Final =7.0 (approx.).

9.1.25. Rappaport Vassiliadis Broth (RVS)

RVS (Biolife, Milano, Italy) was prepared by dissolving 26.5 g of media in 1 L of distilled water and sterilised at 115 °C for 15 min. Final pH=5.2±0.2.

9.1.26. Sarcoplasmic protein media

Sarcoplasmic protein media was prepared as described by Mauriello et al. (2002). In 500 mL of distilled water, 5 g of tryptone (Biolife, Milano, Italy), 2.5 g of yeast extract (Biolife, Milano, Italy), 1 g of glucose (Biolife, Milano, Italy) and 1.5 g of agar (Biolife, Milano, Italy) was dissolved. Media was sterilised at 121 °C for 15 min, cooled down to 50 °C and 500 mL of extracted sarcoplasmic proteins (concentration~2mg/mL) were added. The final concentration of sarcoplasmic proteins in media was ~1 mg/mL. pH was 6.9. 25 mL of

media was poured into each Petri dish. Before use, sterility control was performed in a way that two non-inoculated plates were incubated for 72 h, aerobically at 37 °C and anaerobically at 30 °C. The plates were considered as sterile if no colonies were grown.

9.1.27. Skimmed milk solution (1.5 %)

Skimmed milk solution (1.5 %) was prepared by dissolving 15 g of skim milk powder (Biolife, Milano, Italy) in 1 L of distilled water. The solution was sterilized at 110 °C for 15 min

9.1.28. Skimmed milk solution (10 %)

Skimmed milk solution (10 %) was prepared by dissolving 100 g of skim milk powder (Biolife, Milano, Italy) in 1 L of distilled water. The solution was sterilized at 110 °C for 15 min

9.1.29. SMID

Ready to use plates were purchased (BioMérieux, Marcy-l'Étoile, France).

9.1.30. Soft BHI agar

This media was prepared by dissolving 52 g of BHI broth (Biolife, Milano, Italy) and 7.5 g Agar technical (Biolife, Milano, Italy) in 1 L of distilled water and sterilised at 121 °C for 15 min.

9.1.31. Tributyrin Agar

Tributyrin agar (Sigma Aldrich, Darmstadt, Germany) was prepared by dissolving 23 g of Tributyrin agar base in 990 mL of distilled water. The media was sterilised at 121 °C for 15 min, cooled down to 80 °C and 10 mL of tributyrin was added. The media was homogenised with ultrasound at 2 min on 20 V. 20 mL of media was distributed to each Petri dish.

9.1.32. Violet Red Bile Glucose Agar (VRBG)

VRBG (Biolife, Milano, Italy) was prepared by dissolving 41.5 g of media in 1 L of distilled water. Media was not sterilised in an autoclave; it was heated until boiling with frequent agitation until all particles dissolved completely. Final pH=7.4±0.2.

9.1.33. Xylose Lysine Deoxycholate Agar (XLD)

XLD (Biolab, Budapest, Hungary) was prepared by dissolving 57 g of media in 1 L of distilled water and sterilised at 121 °C for 15 min. Final pH=7.4 (approx.).

9.2. List of all buffers and solutions used in this study with preparation instructions (in alphabetic order)

9.2.1. $CaCl_2$ saline solution (10 mM)

The solution was prepared by dissolving 4 g of NaCl (Sigma-Aldrich, Darmstadt, Germany) and 0.73 g of $CaCl_2 \cdot 2H_20$ (Sigma-Aldrich, Darmstadt, Germany) in 500 mL of distilled water and sterilised at 121 °C for 15 min.

9.2.2. Ethidium bromide gel staining solution

EtBr gel staining solution was prepared by adding 50 µL of Ethidium bromide solution (10 mg/ml; Biorad, Berkeley, USA) to 1L of distilled water.

9.2.3. Lysis buffer (8 M urea, 1 % SDS)

The buffer was prepared by dissolving 480,8 g urea (Sigma-Aldrich, Darmstadt, Germany) and 10 g sodium dodecyl sulfate (SDS; Sigma-Aldrich, Darmstadt, Germany) in MQ water. The water was gradually added until all particles dissolved and PBS buffer (see: 7.2.10) was added up to 1 L. The buffer was stored at room temperature.

9.2.4. McFarland standard (0.5 and 1)

A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175 % barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$; Sigma-Aldrich, Darmstadt, Germany), with 9.95 mL of 1 % sulfuric acid (H_2SO_4; Sigma-Aldrich, Darmstadt, Germany). That approximates to a cell density of $1.5 \cdot 10^8$ cfu/mL. A 1 McFarland standard was prepared by mixing 0.1 mL of barium chloride dihydrate, with 9.9 mL of sulfuric acid, which corresponds to 3·108 cfu/mL.

9.2.5. NaCl (5 M)

146.25 g NaCl (Sigma-Aldrich, Darmstadt, Germany) was dissolved in 500 mL dH2O and sterilised at 121 °C for 15 min.

9.2.6. Phosphate buffer (PBS; 0.1 M, pH 7.0).

PBS was prepared by mixing two solutions. For solution A (0.2 M Na_2PO_4), 27.6 g NaH2PO4·H2O (Sigma-Aldrich, Darmstadt, Germany) was weighted, and distilled water was added up to 100 mL. For solution B (0.2 M Na_2HPO_4), 53.05 g $Na_2HPO_4 \cdot 7H_2O$ (Sigma-Aldrich, Darmstadt, Germany) was weighed, and distilled water was added up to 100 mL. To prepare 1 L 0.1 M phosphate buffer (pH=7.0), 195 mL of solution A and 305 mL of solution B were mixed, and distilled water was added up to 1 L. Buffer was sterilised at 121 °C for 15 min.

9.2.7. Phosphate buffer (PBS; 0.2 M, pH=7.0)

For the buffer preparation, 27.6 g NaH2PO4·H2O (Sigma-Aldrich, Darmstadt, Germany) and 53.05 g of Na2HPO4·7H2O (Sigma-Aldrich, Darmstadt, Germany) were weighted, and water was added up to 200 mL.

9.2.8. Phosphate buffer (PBS; 1X, pH=7.2)

This buffer was prepared by dissolving 1.6 g NaCl (Sigma-Aldrich, Darmstadt, Germany), 0.04 g KCl (Sigma-Aldrich, Darmstadt, Germany), 0.28 g Na_2HPO_4 (Sigma-Aldrich, Darmstadt, Germany) and 0.04 g KH_2PO_4 (Sigma-Aldrich, Darmstadt, Germany) in 160 mL distilled water. pH was adjusted to pH=7.2 and water was added up to 200 mL. The buffer was sterilised at 121 °C for 15 min.

9.2.9. Phosphate buffer (PBS; 20 mM, pH=7.4)

The buffer was prepared by dissolving 3.27 g Na_2HPO_4-$7H_2O$ and 0.94 g NaH_2PO_4. The pH value was adjusted to 7.4, and distilled water was added up to 1 L. The buffer was sterilised at 121 °C for 15 min.

9.2.10. Phosphate buffer used for DNA extraction

The buffer was prepared by dissolving 10 PBS tablets (Oxoid, Wesel, Germany) in 1 L MQ water and sterilised at a 115 °C for 10 min.

9.2.11. Solution A used for DNA extraction

The solution was prepared by dissolving 21.39 g sucrose (Sigma-Aldrich, Darmstadt, Germany), 0.09 g EDTA (Sigma-Aldrich, Darmstadt, Germany), 12.5 mL Tris-buffer (50 X) and 0.25 g savinase (Novozymes, International) in 250 mL distilled water. The solution was left to mix overnight at a magnetic stirrer. Then, the solution was poured to 50 mL tubes and centrifuged at 3220 x g for 30 min. Supernatants were carefully transferred to new tubes and stored at 4 °C.

9.2.12. Substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (S-Ala)

S-Ala (Sigma-Aldrich, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Darmstadt, Germany) at the concentration of 20 mM (25 mg S-Ala in 2 mL DMSO). The solution was stored at -20 °C.

9.2.13. TAE buffer (50 X; 1X)

TAE buffer (50 X) was prepared by dissolving 242 g Tris free base (Sigma-Aldrich, Darmstadt, Germany) and 18.61 g disodium EDTA (Sigma-Aldrich, Darmstadt, Germany) in approximately 700 mL of distilled water. Then, 57.1 mL Glacial Acetic acid (Sigma-Aldrich, Darmstadt, Germany) was added, and the volume was adjusted to 1 L with distilled water. TAE buffer (1x) was prepared by diluting 20 mL TAE buffer (50 X) with 1 L distilled water.

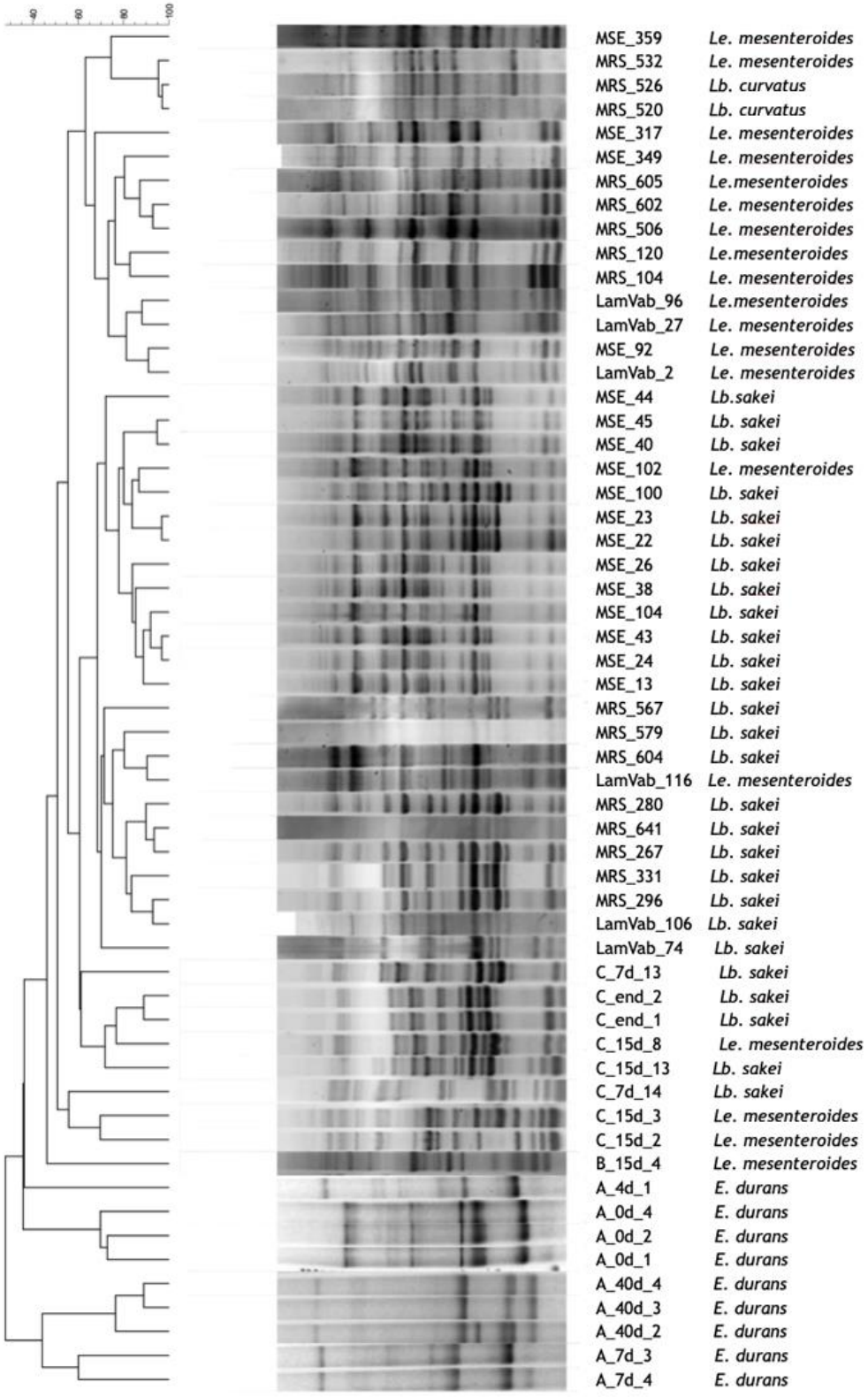
9.2.14. Tris buffer (50 mM, pH=7.8)

The buffer (50 mM, pH=7.8) was prepared by dissolving 1.21 g Tris (Sigma-Aldrich, Darmstadt, Germany) in 50 mL distilled water. pH was adjusted to pH=7.8 with HCl and water was added up to 200 mL.

9.2.15. Wash buffer

The buffer was prepared by mixing 3.5 mL PBS buffer (see: 7.2.10) and 3.5 mL Lutensol (BASF SE, Germany). The buffer was stored at room temperature.

9.3. Dendrogram based on rep-PCR patterns of selected 57 representative strains



9.4. Analysis based on rep-PCR patterns of species most frequently isolated from wild boar (WB1, WB2, WB3) and deer meat sausages (DS1, DS2, DS3) and clustered according to the sausage types

9.4.1. Cluster analysis of *Leuconostoc mesenteroides*

Cluster	Similarity (%)	No. of isolates in cluster	Sausage type					
			WB1	WB2	WB3	DS1	DS2	DS3
1	-	1	0	0	0	0	1	0
2	100	3	0	0	0	0	3	0
3	-	1	0	0	0	0	0	1
4	-	1	0	0	0	0	0	1
5	-	1	0	0	0	0	1	0
6	-	1	0	0	0	1	0	0
7	-	1	0	0	0	0	0	1
8	-	1	0	0	0	0	1	0
9	-	1	0	0	0	0	1	0
10	100	3	0	0	0	0	3	0
11	-	1	0	0	0	0	1	0
12	91	3	0	0	0	1	2	0
13	-	1	0	0	0	0	1	0
14	93	3	0	0	0	0	3	0
15	100	5	0	0	0	0	5	0
16	-	1	0	0	0	1	0	0
17	-	1	0	0	0	0	1	0
18	100	3	0	0	1	1	1	0
19	-	1	0	0	0	1	0	0
20	100	3	0	0	0	2	0	1
21	-	1	0	0	0	1	0	0
22	-	1	0	0	0	1	0	0
23	-	1	0	0	0	0	1	0
24	91	2	0	0	0	1	1	0
25	-	1	0	0	0	0	0	1
26	90	5	0	0	0	0	5	0
27	-	1	0	0	1	0	0	0
28	100	4	0	0	0	0	4	0
29	94	12	5	6	0	0	1	0
30	100	5	1	4	0	0	0	0
31	90	11	0	0	10	1	0	0
32	100	2	0	0	0	1	0	1
33	-	1	0	0	1	0	0	0
34	93	65	63	2	0	0	0	0
35	92	8	0	0	0	0	8	0
36	90	6	0	0	0	0	2	4
37	95	55	0	38	7	3	5	2
38	-	1	0	0	0	1	0	0
39	92	6	0	0	0	0	5	1

40	100	13	0	0	0	13	0	0
41	95	16	1	1	5	1	8	0
42	97	6	0	0	0	6	0	0
Number of collected isolates		259	70	51	25	36	64	13

9.4.2. Cluster analysis of *Lactobacillus sakei*

Cluster	Similarity (%)	No of isolates in cluster	Sausage type					
			WB1	WB2	WB3	DS1	DS2	DS3
1	-	1	0	1	0	0	0	0
2	-	1	0	0	0	0	0	1
3	-	1	0	0	0	0	0	1
4	-	1	0	0	0	0	0	1
5	94	20	0	0	0	0	6	14
6	91	5	0	0	0	0	0	5
7	-	1	0	0	0	0	0	1
8	93	5	0	0	0	0	0	5
9	-	1	0	0	0	0	0	1
10	93	5	0	0	0	0	0	5
11	-	1	0	0	0	0	1	0
12	100	3	0	0	0	0	0	3
13	100	2	0	2	0	0	0	0
14	-	1	0	0	0	0	1	0
15	-	1	1	0	0	0	0	0
16	-	1	0	0	1	0	0	0
17	92	2	0	2	0	0	0	0
18	-	1	0	0	1	0	0	0
19	92	20	0	0	20	0	0	0
20	92	4	0	0	4	0	0	0
21	100	2	0	0	2	0	0	0
22	94	2	0	0	0	0	2	0
23	-	1	0	0	0	0	1	0
24	-	1	0	0	1	0	0	0
25	98	5	0	0	0	0	0	5
26	100	2	0	0	0	0	0	2
27	-	1	0	0	0	0	0	1
28	93	7	0	0	0	0	0	7
29	92	3	0	0	0	3	0	0
30	-	1	0	0	1	0	0	0
31	-	1	0	0	0	0	1	0
32	100	2	0	0	0	0	2	0
33	-	1	0	0	1	0	0	0
34	-	1	0	0	0	0	0	1
35	-	1	0	0	0	0	1	0

36	-	1	0	0	0	0	1	0
37	100	2	0	0	0	0	1	1
38	-	1	0	0	0	0	1	0
39	-	1	0	0	0	1	0	0
40	-	1	0	0	0	0	0	1
41	94	3	0	0	0	0	0	3
42	94	2	0	2	0	0	0	0
43	100	8	0	0	8	0	0	0
44	100	2	2	0	0	0	0	0
45	97	3	0	0	0	0	3	0
46	-	1	0	0	0	0	1	0
47	100	5	0	0	5	0	0	0
48	91	39	0	0	35	0	0	4
49	-	1	0	0	0	0	1	0
50	-	1	0	0	0	1	0	0
51	-	1	0	0	0	0	1	0
52	-	1	0	0	0	0	0	1
53	-	1	0	0	0	0	1	0
54	-	1	0	0	0	0	1	0
55	-	1	0	0	0	0	1	0
56	-	1	0	0	0	0	1	0
57	-	1	0	0	0	0	1	0
58	97	2	0	0	0	0	1	1
59	93	2	0	0	1	0	1	0
Number of collected isolates		190	3	7	80	5	31	64

9.4.3. Cluster analysis of *Enterococcus casseliflavus*

Cluster	Similarity (%)	No of isolates in cluster	Sausage type					
			WB1	WB2	WB3	DS1	DS2	DS3
1	-	1	0	0	0	1	0	0
2	100	5	0	0	0	1	0	4
3	-	1	1	0	0	0	0	0
4	100	2	2	0	0	0	0	0
5	-	1	1	0	0	0	0	0
6	-	1	1	0	0	0	0	0
7	-	1	1	0	0	0	0	0
8	100	2	0	0	0	2	0	0
9	100	2	0	0	0	2	0	0
10	-	1	1	0	0	0	0	0
11	93	3	0	0	0	3	0	0
12	96	3	0	0	0	3	0	0
13	98	6	0	6	0	0	0	0
14	95	3	0	0	3	0	0	0
15	-	1	0	0	0	1	0	0

16	-	1	0	0	1	0	0	0
17	-	1	0	1	0	0	0	0
18	-	1	0	1	0	0	0	0
19	97	5	0	0	0	5	0	0
20	100	2	0	0	2	0	0	0
21	100	2	0	0	0	2	0	0
22	-	1	1	0	0	0	0	0
23	90	7	0	0	6	1	0	0
24	-	1	0	1	0	0	0	0
25	-	1	1	0	0	0	0	0
26	91	3	1	0	0	2	0	0
27	100	2	2	0	0	0	0	0
28	100	3	0	0	0	3	0	0
29	-	1	0	1	0	0	0	0
30	90	2	0	2	0	0	0	0
31	-	1	1	0	0	0	0	0
32	-	1	0	0	1	0	0	0
33	-	1	0	1	0	0	0	0
34	100	3	3	0	0	0	0	0
35	-	1	1	0	0	0	0	0
36	-	1	1	0	0	0	0	0
37	92	6	6	0	0	0	0	0
38	95	2	2	0	0	0	0	0
39	91	2	2	0	0	0	0	0
40	100	5	2	3	0	0	0	0
41	100	2	2	0	0	0	0	0
42	90	3	3	0	0	0	0	0
43	-	1	1	0	0	0	0	0
44	92	4	4	0	0	0	0	0
45	-	1	1	0	0	0	0	0
46	96	2	2	0	0	0	0	0
47	95	4	0	4	0	0	0	0
Number of collected isolates		106	43	20	13	26	0	4

9.5. Analysis based on rep-PCR patterns of species most frequently isolated from wild boar (WB1, WB2, WB3) and deer meat sausages (DS1, DS2, DS3) and clustered according to the time points of their isolation

9.5.1. Cluster analysis of *Leuconostoc mesenteroides*

Sausage type	Cluster	Similarity (%)	Number of isolates	The time point of isolation (days)					
				0	4	7	10	20	40
WB1 (*n*=70)	1	-	1	0	1	0	0	0	0
	2	91.58	6	2	2	0	0	0	2
	3	92.74	63	0	20	7	15	14	7
WB2 (*n*=51)	1	-	1	0	0	0	0	1	0
	2	91.83	38	0	11	10	6	7	4
	3	96.55	6	0	1	1	0	2	2
	4	100	4	0	3	1	0	0	0
	5	94.44	2	1	1	0	0	0	0
WB3 (*n*=25)	1	-	1	0	0	1	0	0	0
	2	100	5	0	4	0	0	0	1
	3	100	2	0	0	0	0	2	0
	4	96.30	4	0	2	2	0	0	0
	5	-	1	0	0	0	0	1	0
	6	96.01	10	0	2	0	4	4	0
	7	-	1	1	0	0	0	0	0
	8	-	1	0	1	0	0	0	0
DS1 (*n*=36)	1	-	1	0	0	0	0	1	0
	2	-	1	0	0	0	1	0	0
	3	90.32	4	3	0	0	1	0	0
	4	-	1	0	0	0	1	0	0
	5	-	1	0	1	0	0	0	0
	6	100	2	0	0	2	0	0	0
	7	-	1	0	1	0	0	0	0
	8	-	1	0	0	0	1	0	0
	9	-	1	0	0	0	0	1	0
	10	100	13	0	0	8	5	0	0
	11	-	1	0	1	0	0	0	0
	12	-	1	0	0	0	1	0	0
	13	-	1	0	1	0	0	0	0
	14	92.33	7	1	6	0	0	0	0
DS2 (*n*=64)	1	-	1	0	0	0	1	0	0
	2	100	3	1	2	0	0	0	0
	3	-	1	1	0	0	0	0	0
	4	-	1	0	0	0	1	0	0

	5	92.51	3	0	0	0	3	0	0
	6	-	1	0	0	1	0	0	0
	7	-	1	0	0	0	0	1	0
	8	96.00	2	0	0	0	0	2	0
	9	-	1	1	0	0	0	0	0
	10	-	1	1	0	0	0	0	0
	11	-	1	1	0	0	0	0	0
	12	100	3	3	0	0	0	0	0
	13	-	1	0	0	0	1	0	0
	14	-	1	0	0	0	1	0	0
	15	-	1	0	1	0	0	0	0
	16	90.47	5	0	0	0	5	0	0
	17	100	5	0	0	0	3	2	0
	18	95.93	8	0	0	5	1	2	0
	19	100	5	0	0	0	0	4	1
	20	100	4	0	0	4	0	0	0
	21	90.32	3	3	0	0	0	0	0
	22	-	1	0	0	0	0	1	0
	23	90.78	11	4	6	1	0	0	0
DS3 (*n*=13)	1	-	1	0	0	0	0	1	
	2	-	1	0	0	0	1	0	
	3	-	1	0	0	0	0	1	
	4	-	1	1	0	0	0	0	
	5	-	1	0	0	1	0	0	
	6	-	1	1	0	0	0	0	
	7	-	1	1	0	0	0	0	
	8	92.41	5	1	4	0	0	0	
	9	-	1	1	0	0	0	0	
Number of collected isolates			259	28	71	44	52	47	17

9.5.2. Cluster analysis of *Lactobacillus sakei*

Sausage type	Cluster	Similarity (%)	No of isolates	Time point of isolation (days)					
				0	4	7	10	20	40
WB1 (*n*=3)	1	100	2	0	0	0	0	0	2
	2	0	1	0	0	0	0	0	1
WB2 (*n*=7)	1	92.31	2	0	0	2	0	0	0
	2	-	1	1	0	0	0	0	0
	3	94.12	2	0	0	0	0	0	2
	4	100	2	0	0	0	0	0	2
WB3 (*n*=80)	1	-	1	0	0	1	0	0	0
	2	-	1	0	0	0	1	0	0
	3	-	1	0	0	1	0	0	0
	4	91.43	4	0	3	0	0	1	0
	5	100	2	0	2	0	0	0	0

	6	-	1	0	1	0	0	0	0
	7	91.89	20	0	9	0	8	0	3
	8	-	0	0	0	0	0	0	0
	9	-	1	0	0	1	0	0	0
	10	100	8	0	2	6	0	0	0
	11	-	1	0	0	0	0	0	1
	12	100	5	0	0	1	0	0	4
	13	91.45	35	3	8	0	5	11	8
DS1 (*n*=5)	1	-	1	0	0	0	0	0	1
	2	91.66	3	0	0	0	0	0	3
	3	-	1	0	1	0	0	0	0
DS2 (*n*=31)	1	100	6	0	0	0	3	3	0
	2	-	1	1	0	0	0	0	0
	3	-	1	0	1	0	0	0	0
	4	-	1	1	0	0	0	0	0
	5	-	1	0	0	0	0	0	1
	6	-	1	0	0	0	1	0	0
	7	-	1	0	0	0	0	1	0
	8	-	1	0	0	1	0	0	0
	9	-	1	0	0	0	0	0	1
	10	100	2	0	0	0	0	2	0
	11	-	1	0	1	0	0	0	0
	12	-	1	1	0	0	0	0	0
	13	-	1	0	0	0	0	0	1
	14	94.12	2	0	0	0	0	0	2
	15	96.78	3	0	0	0	0	1	2
	16	-	1	0	0	0	1	0	0
	17	-	1	0	1	0	0	0	0
	18	-	1	0	1	0	0	0	0
	19	-	1	0	1	0	0	0	0
	20	-	1	0	0	0	0	0	1
	21	-	1	0	0	0	0	0	1
	22	-	1	0	1	0	0	0	0
DS3 (*n*=64)	1	-	1	0	0	0	0	1	
	2	100	3	0	0	1	2	0	
	3	97.56	5	0	5	0	0	0	
	4	100	2	1	1	0	0	0	
	5	-	1	1	0	0	0	0	
	6	93.33	7	3	4	0	0	0	
	7	-	1	0	0	0	0	1	
	8	-	1	0	0	0	1	0	
	9	96.55	4	0	0	2	0	2	
	10	-	1	0	0	0	0	1	
	11	94.15	3	1	0	1	0	1	
	12	-	1	0	0	0	0	1	

	13	-	1	0	0	0	0	1	
	14	-	1	0	0	0	0	1	
	15	93.53	14	0	0	4	7	3	
	16	90.84	5	3	0	0	2	0	
	17	-	1	1	0	0	0	0	
	18	93.31	5	0	0	0	5	0	
	19	-	1	0	0	1	0	0	
	20	-	1	1	0	0	0	0	
	21	92.60	5	1	3	1	0	0	
Number of collected isolates			190	19	45	23	36	31	36

9.5.3. Cluster analysis of *Enterococcus casseliflavus*

Sausage type	Cluster	Similarity (%)	No of isolates	Time point of isolation (days)					
				0	4	7	10	20	40
WB1 (*n*=43)	1	-	1	0	0	0	0	1	0
	2	100	2	0	0	0	2	0	0
	3	-	1	0	0	0	1	0	0
	4	-	1	0	0	1	0	0	0
	5	91.30	2	2	0	0	0	0	0
	6	100	2	2	0	0	0	0	0
	7	-	1	0	0	0	0	0	1
	8	100	3	0	3	0	0	0	0
	9	-	1	0	0	0	0	0	1
	10	-	1	0	0	1	0	0	0
	11	-	4	0	2	2	0	0	0
	12	-	1	0	0	0	0	0	1
	13	96.00	2	0	1	0	1	0	0
	14	100	2	0	0	0	1	0	1
	15	-	1	0	0	0	0	0	1
	16	90.00	3	0	0	1	0	2	0
	17	-	1	0	1	0	0	0	0
	18	-	1	0	1	0	0	0	0
	19	-	1	0	0	0	0	1	0
	20	90.90	2	0	2	0	0	0	0
	21	100	2	0	1	0	0	1	0
	22	92.35	6	0	5	0	1	0	0
	23	95.24	2	0	0	1	1	0	0
WB2 (*n*=20)	1	98.33	6	0	2	0	0	4	0
	2	-	1	0	1	0	0	0	0
	3	-	1	0	1	0	0	0	0
	4	100	3	0	0	1	0	0	2
	5	-	1	0	1	0	0	0	0
	6	90.32	2	0	2	0	0	0	0
	7	-	1	0	1	0	0	0	0

	8	-	1	0	1	0	0	0	0
	9	95.34	4	0	4	0	0	0	0
WB3 (*n*=13)	1	-	1	0	1	0	0	0	0
	2	94.94	3	3	0	0	0	0	0
	3	-	1	1	0	0	0	0	0
	4	100	2	2	0	0	0	0	0
	5	92.58	6	4	2	0	0	0	0
DS1 (*n*=26)	1	-	1	0	0	0	1	0	0
	2	-	1	0	0	1	0	0	0
	3	100	2	0	2	0	0	0	0
	4	92.31	2	0	0	1	0	1	0
	5	100	3	1	0	2	0	0	0
	6	100	2	0	1	0	0	0	1
	7	96.78	5	5	0	0	0	0	0
	8	-	1	0	1	0	0	0	0
	9	-	1	0	0	0	0	0	1
	10	100	2	2	0	0	0	0	0
	11	96.30	3	3	0	0	0	0	0
	12	93.33	3	3	0	0	0	0	0
DS2 (*n*=0)									
DS3 (*n*=4)	1	100	4	0	3	1	0	0	
Number of collected isolates			106	28	39	12	8	10	9