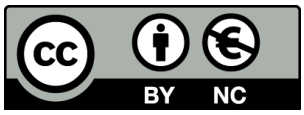
Universitat de Girona

GENOMICS AND FORMULATION OPTIMIZATION FOR ENHANCING THE MICROBIAL SAFETY OF DRY FERMENTED SAUSAGES

Núria Ferrer Bustins



IRTA
Institute
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Doctoral thesis

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2024

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GENOMICS AND FORMULATION OPTIMIZATION FOR ENHANCING THE MICROBIAL SAFETY OF DRY FERMENTED SAUSAGES

Núria Ferrer Bustins

2024

Doctoral Programme in Technology

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Presented to obtain the degree of PhD at the University of Girona

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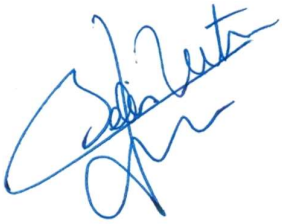
WE DECLARE:

That the thesis "GENOMICS AND FORMULATION OPTIMIZATION FOR ENHANCING THE MICROBIAL SAFETY OF DRY FERMENTED SAUSAGES", presented by **NÚRIA FERRER BUSTINS** to obtain a doctoral degree, has been carried out under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document:



Dr. Anna Jofré i Fradera Dr. Sara Bover i Cid Dr. Belén Martín Juárez

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Diuen que un només sap què és fer una tesi, quan ha passat per ella, o que és una carrera d'obstacles llarga on no saps què trobaràs darrere cada revolt. I sí, han estat més de quatre anys a IRTA corrent, esprintant, fent marxa i caminant, on, com a tota cursa, hi ha companys que es tornen amics i amics que es tornen família.

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List of publications

The PhD thesis has been written as a compendium of peer-reviewed articles in accordance with the regulations of the University of Girona. The articles that group this PhD project are as follow:

Article I

Ferrer-Bustins N, Martín B, Llauger M, Bou R, Bover-Cid S, Jofré A. Dynamics of Microbial Communities in Nitrite-Free and Nutritionally Improved Dry Fermented Sausages. *Fermentation*. 2023; 9(4):403. https://doi.org/10.3390/fermentation9040403

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Article II

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Article III

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Impact factor (2023): 3.2736, ranked 72/174 (Q2) in the category of *BIOTECHNOLOGY & APPLIED MICROBIOLOGY*.

List of abbreviations

ALDEx	ANOVA-Like differential expression
ANOVA	Analysis of the variance
ARG	Antimicrobial resistance genes
ASV	Amplicon sequence variant
AU	Arbitrary units
a_w	Water activity
BAC	Bacteriocin
bp	Base pair
CC	Clonal complex
CCD	Central composite design
CFU	Colony forming units
cg-	Core genome
CLR	Centre log ratio
DADA2	Divisive amplicon denoising algorithm2
ddNTPs	Di-deoxynucleotide triphosphates
DFS	Dry fermented sausage
DNA	Desoxyribonucleic acid
DNB	DNA nanoball
DNBSEQ	DNA nanoballs sequencing
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
EC	European commission
ECC	Embotits crus curats (dry-cured meat products)
ECDC	European centre for disease prevention and control
EEA	European economic area
EFSA	European food safety authority
EFSA BIOHAZ Panel	EFSA panel on biological hazards
ESBL	Extended-spectrum β-lactamases
EU	European Union
FAO	Food and agriculture organization
FBO	Food business operator/s
FDA	Food and drug administration
FSIS	Food safety and inspection service
GB	Giga byte
GCC+	Gram-positive catalase-positive cocci
GDL	Glucono-delta-lactone
GRAS	Generally recognised as safe
HACCP	Hazard analysis and critical control points
HTS	High throughput sequencing

ITS	Internal transcribed spacer
ISO	International Organization for Standardization
k_d	Inactivation rate constant
kDa	Kilo Daltons
Kbp	Kilo base pairs
LA	Lactic acid
LAB	Lactic acid bacteria
MAGs	Metagenome-assembled genomes
MGE	Mobile genetic elements
MLST	Multi locus sequence typing
MS	EU member states
N_{max}	Maximum population density
OECD	Organisation for economic co-operation and development
OMP	Outer membrane protein
OTU	Operational taxonomic unit
PacBio	Pacific Biosciences
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
QPS	Qualified presumption of safety
RH	Relative humidity
RNA	Ribonucleic acid
RSM	Response surface methodology
RTE	Ready-to-eat
SBS	Sequencing by synthesis
SGI	*Salmonella* genomic islands
SMRT	Single molecule sequencing at real-time
SMS	Single molecule sequencing
SNP	Single nucleotide polymorphism
SPI	*Salmonella* pathogenicity islands
SS	Shelf stable
ssDNA	Single stranded DNA
ST	Sequence type
Tn	Transposon
μ_{max}	Maximum growth rate
USA	United States of America
wg-	Whole genome
WGS	Whole genome sequencing
Y_{BAC}	Bacteriocin yield
Y_{LA}	Lactic acid yield

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Abstract

Dry fermented sausages (DFS) are traditional meat products with high production and consumption in Mediterranean countries. Microbial communities of DFS vary throughout the production process, providing characteristic organoleptic traits. Acidification and drying are two of the main processes contributing to the shelf-stability and food safety of DFS, while other antimicrobials (e.g., bacteriocins) can contribute to the safety. Currently, genomic technologies are changing the paradigm in food microbiology, covering the identification of microbial communities within a complex food or surface sample with culture-independent techniques and the genomic characterization of pathogenic isolates recovered from a contaminated sample. The present PhD thesis is composed of three scientific articles dealing with multidisciplinary approaches including classical and molecular microbiology, bioinformatics and mathematical modelling methodologies focused on the food safety and quality of low-acid DFS (mainly *fuet*-type). The aim of the thesis was to study the microbial communities of DFS, with innovative formulation and production processes, and to develop strategies to control *Salmonella* and *Listeria monocytogenes* as the main pathogens in this type of meat products.

The objective of the first study was to investigate the microbiota of DFS elaborated (i) without nitrifying salts and adding a pork liver auto-hydrolysate innovative ingredient, rich in zinc protoporphyrin as a colour-enhancement agent and, (ii) with a sodium reduction by replacing NaCl by KCl. It is noteworthy that both strategies respond to consumer demands in addition to valuing the reintroduction of pig liver, as a by-product of the meat industry, into a commercial product and thus contributing to the sector's bioeconomy. The bacterial communities were followed by the 16S rRNA gene metataxonomic approach. DFS formulated with no added nitrifying salts and submitted to low temperatures allowed the growth of spoilage-related bacteria, as those containing the liver auto-hydrolysate ingredient, which added putative probiotic species to the product, and showed high bacterial diversity. In contrast, less bacterial diversity was shown in nitrified DFS formulated with the *Latilactobacillus sakei* CTC494 starter culture, that led the fermentation process and was the dominant species.

The studies of DFS-related pathogens were tackled with different perspectives considering the nature of *Salmonella* and *L. monocytogenes*, as the main relevant pathogens of DFS. As *Salmonella* is present along the DFS production chain and the causative agent of recalls and outbreaks, the second study aim was to genomically characterize *Salmonella* isolates and search for phylogenomic relationships between industrial isolates, nationally and internationally. Within the ten different *Salmonella* serovars encountered in the genomic panel (N=173 *Salmonella* genomes), *S.* 1,4,[5],12:i:- was the most prevalent serovar in DFS while *S.* Derby was in pig carcasses (understood as the primary raw material for DFS production). Phylogenetic clusters were found within genomes with few allelic and single nucleotide polymorphisms differences in the core genome. Antimicrobial and biocide resistance genes, virulence genes and mobile genetic elements were found distinctly between the *Salmonella* serovars, highlighting the multidrug resistance gene profile in 91% of the *S.* 1,4,[5],12:i:- genomes and the extended-spectrum β-lactamase genes detection in Typhimurium and Derby serovars. The multidrug resistance genes identification and persistence of *Salmonella*, especially *S.* 1,4,[5],12:i:-, in DFS production chain is of importance by food safety managers and DFS producers, respectively.

Monitoring and genomic characterization of isolates are here presented as a first step for future strategies based on the hurdle technology.

DFS contamination by *L. monocytogenes* can occur either in raw materials or through cross-contamination with contaminated surfaces of the DFS producer. The aim of the third study was to optimise the bacteriostatic and bactericidal potential of *L. sakei* strains against *L. monocytogenes* in distinct formulation and process conditions of DFS production. The sakacin K producer *L. sakei* CTC494 and the non-bacteriocinogenic *L. sakei* 23K were cocultured with *L. monocytogenes* as well as in monoculture in meat simulation media at different formulation of NaCl (0–40 g/L), manganese (0.08–0.32 g/L) and glucose (0–40 g/L), and temperature (3–37 °C) conditions drawn in a central composite design in order to study the behaviour of the pathogen. Glucose was identified as the most influencing factor in the pH decrease and lactic acid production, and moderate temperatures (10 and 20 °C being the optimal) in the sakacin K production. Only when *L. sakei* strains entered the early stationary phase, *L. monocytogenes* growth was inhibited by the non-bacteriocinogenic 23K strain, while the pathogen was inactivated in coculture with the bioprotective CTC494 strain. A remarkable 5-log reduction of *L. monocytogenes* was achieved at 20 °C, 20 g/L NaCl, 0.20 g/L Mn and 40 g/L glucose. The use of bacteriocinogenic starter cultures with antilisterial potential is proposed as a control strategy for *L. monocytogenes* growth during the DFS production and the results here obtained emphasise the importance of product formulation and process optimal conditions for enhancing bioprotective starter cultures efficacy.

Resum

Els embotits crus curats (ECC) són productes carnis tradicionals amb una elevada producció i consum en els països mediterranis. Les comunitats microbianes dels ECC varien al llarg del procés de producció, proporcionant trets organolèptics característics. L'acidificació i l'assecat són dos dels principals processos que contribueixen en l'autoestabilitat i seguretat alimentària dels ECC mentre que altres antimicrobians (per exemple, les bacteriocines) poden contribuir en la seguretat. Actualment, les tecnologies genòmiques estan canviant el paradigma de la microbiologia dels aliments des de la identificació de les comunitats microbianes d'una mostra alimentària o de superfície complexa amb tècniques independents de cultiu fins a la caracterització genòmica de patògens aïllats d'una mostra contaminada. La present tesi doctoral està formada per tres articles científics multidisciplinaris que inclouen microbiologia clàssica i molecular, bioinformàtica i metodologies de modelització matemàtica focalitzats en la seguretat alimentària i la qualitat dels DFS poc àcids (principalment de tipus fuet). L'objectiu de la tesi va ser estudiar les comunitats microbianes dels ECC, amb processos innovadors de formulació i producció, i desenvolupar estratègies per controlar *Salmonella* i *Listeria monocytogenes* com a principals patògens relacionats amb aquest tipus de productes carnis.

L'objectiu del primer estudi va ser d'investigar la microbiota dels ECC elaborats (i) sense salts nitrificants i addicionant un ingredient innovador basat en un autohidrolitzat de fetge de porc, ric en protoporfirina de zinc com a agent que millora el color, i (ii) amb una reducció de de sodi, substituint NaCl per KCl. Cal destacar que ambdues estratègies responen a les demandes dels consumidors a més de valorar la reintroducció del fetge de porc, com a subproducte de la indústria càrnia, en un producte comercial i contribuir d'aquesta manera a la bioeconomia del sector. El seguiment de les comunitats bacterianes es va fer a través de l'enfocament metataxonòmic, seqüenciant el gen 16S rRNA. Els ECC formulats sense sals nitrificants i sotmesos a baixes temperatures va permetre el creixement de créixer bacteris alteradors, així com en els que contenien l'ingredient autohidrolitzat de fetge que va aportar espècies putatives probiòtiques al producte i van demostrar una alta diversitat bacteriana. Per altra banda, es va observar poca diversitat bacteriana en els ECC formulats amb agents nitrificants i el cultiu iniciador *Latilactobacillus sakei* CTC494, el qual va liderar el procés de fermentació i va ser identificada com a espècie dominant.

Els estudis dels patògens relacionats amb els ECC es va abordar des de diferents perspectives tenint en compte la naturalesa de *Salmonella* i *L. monocytogenes*, com a principals patògens dels ECC. La *Salmonella* està present al llarg de la cadena de producció de DFS i és l'agent causant de retirades i brots, en aquest sentit l'objectiu del segon l'estudi va ser caracteritzar genòmicament aïllats de *Salmonella* d'ambients industrials de producció i buscar relacions filogenòmiques, a nivell nacional i internacional. D'entre els deu serovars trobats al panell genòmic d'estudi (N=173 genomes), *S.* 1,4,[5],12:i:- va ser el serovar més freqüent en ECC, mentre que *S.* Derby va ser el més freqüent en canals de porc (enteses com a matèria primera primària per a la producció de ECC). Es van identificar agrupacions filogenòmiques entre genomes amb poques diferències al·lèliques i mutacions puntuals en la seqüència nucleotídica del genoma central i més conservat dins l'espècie. Els gens de resistència a antimicrobians i biocides, gens de virulència i els elements genètics mòbils, es van trobar en miscel·lània entre els serovars de *Salmonella*, destacant el perfil genètic de resistència a múltiples antimicrobians en el 91% dels

genomes de *S.* 1,4,[5],12:i:-, mentre que els gens de resistència a β-lactamases d'espectre estès es van trobar als serovars de Typhimurium i Derby. La identificació de gens de resistència a múltiples antimicrobians i la persistència de *Salmonella*, especialment *S.* 1,4,[5],12:i:-, a la cadena de producció de DFS és d'importància per als gestors de seguretat alimentària i els productors de DFS, respectivament. El seguiment i la caracterització genòmica dels aïllats es presenten aquí com un primer pas per a estratègies futures basades en la tecnologia d'obstacles.

La contaminació de *L. monocytogenes* en ECC pot provenir de l'ús de matèries primeres contaminades o per contaminació creuada amb superfícies contaminades durant l'elaboració. L'objectiu del tercer estudi va ser la optimització del potencial bacteriostactic i bacteriocida en front *L. monocytogenes* per part de soques de *L. sakei*. La soca productora de sakacina K *L. sakei* CTC494 i la no bacteriocinogènica *L. sakei* 23K es van fer créixer en monocultiu i cocultiu amb *L. monocytogenes* en un medi de simulació de carn en diferents condicions de formulació de NaCl (0–40 g/L), manganès (0,08–0,32 g/L) i glucosa (0–40 g/L), i temperatura (3–37 °C) definides a partir d'un disseny central compost per estudiar el comportament del patogen. La glucosa es va identificar com el factor més influent en la disminució del pH i la producció d'àcid làctic, i les temperatures moderades (10 i 20 °C sent les òptimes) en la producció de sakacina K. Només quan *L. sakei* va començar a entrar en fase estacionària, *L. monocytogenes* es va inhibir en el cocultiu amb la soca 23K i es va inactivar en el cocultiu amb la soca CTC494. Una notable reducció de 5 unitats logarítmiques de *L. monocytogenes* van ser a 20 °C, 20 g/L de NaCl, 0,20 g/L de Mn i 40 g/L de glucosa. Es proposa l'ús de cultius iniciadors bacteriocinògens amb potencial antilisteria com a estratègia de control del creixement de *L. monocytogenes* durant la producció dels ECC. Els presents resultats emfatitzen la importància de la formulació del producte i les condicions òptimes del procés per millorar l'eficàcia dels cultius iniciadors bioprotectors.

Resumen

Los embutidos crudo-curados (ECC) son productos cárnicos tradicionales de elevada producción y consumo en los países mediterráneos. Las comunidades microbianas de los ECC varían a lo largo del proceso de producción, proporcionando rasgos organolépticos característicos. La acidificación y el secado son dos de los principales procesos que contribuyen en la autoestabilidad y seguridad alimentarias de los ECC, mientras que otros antimicrobianos (por ejemplo, las bacteriocinas) pueden contribuir en la seguridad. Actualmente, las tecnologías genómicas están cambiando el paradigma de la microbiología de los alimentos desde la identificación de las comunidades microbianas de una muestra alimentaria o de superficie complexa con técnicas independientes de cultivo hasta la caracterización genómica de patógenos aislados de una muestra contaminada. La presente tesis doctoral está formada por tres artículos científicos multidisciplinares que incluyen microbiología clásica y molecular, bioinformática y metodologías de modelización matemática, focalizados en la seguridad y calidad alimentarias de los ECC poco ácidos (principalmente del tipo *fuet*). El objetivo fue estudiar las comunidades microbianas de los ECC, con procesos innovadores de formulación y producción, y desarrollar estrategias para controlar *Salmonella* y *Listeria monocytogenes* como principales patógenos relacionados con estos productos cárnicos.

El objetivo del primer estudio fue la investigación de la microbiota de los ECC elaborados (i) sin sales nitrificantes y adicionando un ingrediente innovador basado en un autohidrolizado de hígado de cerdo, rico en protoporfirina de zinc como agente de mejora del color, y (ii) con una reducción de sodio, sustituyendo el NaCl por KCl. Cabe destacar que ambas estrategias satisfacen las demandas de los consumidores además de valorar la reintroducción del hígado de cerdo como subproducto de la industria cárnica en un producto comercial y así contribuir en la bioeconomía del sector. El seguimiento de las comunidades bacterianas se hizo a través del enfoque metataxonómico, secuenciando el gen 16S rRNA. Los ECC formulados sin sales nitrificantes y sometidos a bajas temperaturas permitieron el crecimiento de bacterias alterantes, así como los que contenían el ingrediente de hígado, que aportó especies putativas probióticas al producto y demostraron una alta diversidad bacteriana. Por el contrario, se observó poca diversidad bacteriana en los ECC formulados con nitrificantes y con el cultivo iniciador *Latilactobacillus sakei* CTC494, quien lideró el proceso de fermentación y fue identificada como especie dominante.

Los estudios de los patógenos relacionados con los ECC se abordaron con distintas perspectivas teniendo en cuenta la naturaleza de *Salmonella* y *L. monocytogenes*, como principales patógenos de los ECC. *Salmonella* está presente a lo largo de la cadena de producción de los ECC y es el agente causante de retiradas y brotes, en este sentido el objetivo del segundo estudio fue la caracterización genómica de aislados de *Salmonella* de ambientes industriales de producción y buscar relaciones filogenómicas entre ellas, a nivel nacional e internacional. De entre los diez serovares hallados en el panel genómico (N=173 genomas), *S.* 1,4,[5],12:i:- fue el serovar más frecuente en ECC, mientras que *S.* Derby fue el más frecuente en canales de cerdo (entendidas como materia prima primaria para la producción de ECC). Se identificaron agrupaciones filogenómicas entre genomas con pocas diferencias alélicas y mutaciones puntuales en la secuencia nucleotídica del genoma central y más conservado en la especie. Los genes de resistencia a antimicrobianos y biocidas, genes de virulencia y los elementos genéticos móviles,

se encontraron en miscelánea entre los serovares de *Salmonella*, destacando el perfil genético de resistencia múltiples antimicrobianos en el 91% de los genomas de *S.* 1,4,[5],12:i:-, mientras que los genes de resistencia a β-lactamasas de espectro extendido solo se encontraron en los serovares Typhimurium y Derby. La identificación de genes de resistencia múltiples antimicrobianos y la persistencia de *Salmonella*, especialmente el serovar 1,4,[5],12:i:-, en la cadena de producción de ECC es de relevante importancia para los gestores de la seguridad alimentaria y los productores de ECC, respectivamente. El seguimiento y la caracterización genómica de *Salmonella* se presentan aquí como un primer paso para estrategias futuras basadas en la tecnología de los obstáculos.

La contaminación por *L. monocytogenes* en ECC puede proceder del uso de materias primas contaminadas o por contaminación cruzada con superficies contaminadas durante la elaboración. El objetivo del tercer estudio fue la optimización del potencial bacteriostático y bactericida frente *L. monocytogenes* por cepas de *L. sakei*. La cepa productora de sakacina K *L. sakei* CTC494 y la no bacteriocinogénica *L. sakei* 23K se hicieron crecer en monocultivo y cocultivo con *L. monocytogenes* en un medio de simulación de la carne en diferentes condiciones de formulación de NaCl (0–40 g/L), manganeso (Mn; 0,08–0,32 g/L) y glucosa (0–40 g/L), y temperatura (3– 37 °C), definidas a través de un diseño central compuesto, para estudiar el comportamiento del patógeno. La glucosa fue identificada como el factor más influyente en la disminución del pH y la producción de ácido láctico, y las temperaturas moderadas (10 y 20 °C siendo las óptimas) en la producción de sakacina K. Solo cuando *L. sakei* empezó a entrar en fase estacionaria, *L. monocytogenes* se inhibió en el cocultivo con la cepa 23K e inactivó en el cocultivo con la cepa CTC494. Una notable reducción de 5 unidades logarítmicas de *L. monocytogenes* fueron a 20 °C, 20 g/L de NaCl, 0,20 g/L de Mn y 40 g/L de glucosa. Se propone el uso de cultivos iniciadores bacteriocinógenos con potencial antilisteria como estrategia de control del crecimiento de *L. monocytogenes* durante la producción de los ECC. Los presentes resultados enfatizan la importancia de la formulación del producto y las condiciones del proceso óptimas para mejorar la eficacia de los cultivos iniciadores bioprotectores.

INTRODUCTION

1. Dry fermented sausages

Meat fermentation is a culture characteristic tradition dating back to ancient times whose objective was to preserve raw meat, after slaughtering, for further consumption (Zeuthen, 2007). In past decades, the preservation of meat occurred through processes of fermentation, salting and drying, which resulted in stable and microbiologically safe meat products. Nowadays meat availability in developed countries is a daily fact since meat production is a common commercial activity and meat can be refrigerated or frosted for preservation (Aste et al., 2017). Despite the improved refrigeration and packaging methodologies for raw meat preservation, fermented, salted and dried meat products are still produced and present in many diets due to their unique and much appreciated flavour and convenience, as in example fermented sausages.

Fermented sausages probably originated in Roman times in the Mediterranean countries, where winter climate with moderate temperatures and frequent rainfall was favourable for sausage ripening (Lücke, 1998). Due to its later production spread, nowadays dry fermented sausages (DFS) are produced worldwide, although Europe is still the major producer and consumer of a wide variety of DFS and represent an important economical income in specific regions (Oliveira et al., 2018; Toldrá & Hui, 2014). Table 1 shows the main differences of Northen and Southern European DFS and North America DFS, which originate from European countries. Due to differences in the availability of raw materials and climate, each region in the world developed and have evolved its particular type of products.

In general, DFS are composed by lean minced meat (mainly pork and beef) mixed with fat (Olivares et al., 2009; Wirth, 1988), spices, salt, sugar, sodium nitrite (sometimes nitrate), and in some cases starter culture (Holck et al., 2017; Wirth, 1988). The mix or meat batter can be stuffed into natural, cellulose or synthetic casings, which are sometimes surface inoculated with mould spores or smoked (Roseiro et al., 2011), and finally subjected to a fermentation and ripening process under controlled conditions of temperature and relative humidity. Nowadays, the worldwide range of dry fermented sausages differ in (i) formulation, (ii) manufacturing procedures and (iii) production process (fermentation and drying), factors that determine the safety and organoleptic properties of the final product (Table 1).

The basis of the meat batter in the Mediterranean and Northen Europe DFS is mainly pork and pork back fat seasoned with salt and dextrose, except for those elaborated in Arabic countries with Muslim religion (e.g., Morocco and Turkey) that use beef instead of pork. North America and Eastern Europe countries use pork, beef meat or a mixture of both. The meat batter seasoned with the characteristic ingredients of every region is stuffed into casings with different diameters, within a range between 40 to 120 mm in North America, between 22 to 80 mm in the Mediterranean countries and between 43 to 105 mm in Northen Europe (Talon et al., 2007; Toldrá et al., 2007). The length of the sausage is characteristic to the DFS type, thus classifying them as long ($\geq$ 45 cm) or short (< 45 cm), as is the hanging shape (*i.e.*, horse shape or straight).

Table 1. Summary of the main characteristics of DFS produced in Northen Europe, Southern Europe/Mediterranean countries, North America. Information retrieved from Talon et al. (2007) and Toldrá et al. (2007).

Region	Northen Europe	Southern Europe/Mediterranean countries	North America
Meat origin	Pork	Pork and beef	Pork and beef
Ingredients	Pepper, glutamate, nutmeg, ginger, paprika mild, ascorbic acid, garlic, coriander, caraway seed, …	Salt, sugars, pepper, paprika, cardamom, garlic, red wine, …	Salt, glucose, sucrose, black pepper, nutmeg, allspice, red pepper, doves, cinnamon, ginger, mustard, mace, ground cloves, …
Additives	No added nitrate	Sodium nitrate and nitrite	Sodium erythorbate, Sodium nitrite
Size: diameter (Ø) and length (l)	Ø = 43–75 mm l = up to 20 cm	Ø = 22–80 mm l = 30–60 cm	Ø = 40–120 mm l = up to 47 cm
Fermentation time and temperature	Fast (< 30h to reach pH = 5.3) 22–26 °C	Slow (> 40h to reach pH = 5.3) 18–24 °C	Very fast (< 15h to reach pH = 5.3) > 32 °C
Production time	< 3 weeks	≥ 3 weeks	2–3 weeks
Smoking	Yes	No	Often right after fermentation
Cooking	No	No	Often right after fermentation
Mould cover	No	Yes	No
Lowest pH	4.5-4.8	> 5.0	< 4.8
Product type and final a_w	Semidry a_w > 0.9	Dry a_w < 0.9	Semidry a_w > 0.9
Examples of DFS	German Mettwurst, Danish salami	French saucisson, Naples salami, Salame Milano, Italian pepperoni, Spanish Chorizo	American pepperoni, Summer sausage, Lebanon Bologna

Fermentation-drying and smoking (when applied) processes are usually consecutive to each other regardless of the order, as some sausages will first go through the smoking and then

fermentation (*e.g.*, Turkish *sucuk*), while others will first be fermented and then smoked (*e.g.*, Spanish *chorizo*). There are also DFS where these two processes occur at the same time.

The fermentation and drying process depends not only on the type of DFS but the producer as the parameters of the process will determine the sensory characteristics of the final product. Depending on the DFS type, the fermentation and drying processes duration and temperatures are diverse, e.g., fermentation form 2h to 4 days and 7 to 35 °C and drying from 14 to 40 days and 4 to 14 °C. Some products can include an initial stewing step to promote fermentation of, for instance, 2h at 35 °C or one day at 20 °C while other processes a preformed at a constant low (e.g. 7 °C) temperature. Drying can last from 13 days to more than 40 days (Gou et al., 1996; Ortiz et al., 2014; Zampouni et al., 2022, 2024).

Table 1 summarizes the main differences on DFS regarding its geographic location of manufacturing and production. In Northen and Eastern Europe DFS are mainly fast fermented, reaching a pH < 5.3 at temperatures from 22 to 26 °C for less than 30h. These are smoked but not cooked and classified as semidry and acid sausages as the final a_w values are above 0.90 and reaches low pH of 4.5. The production process time is less than 3 weeks, and typical Northen Europe DFS are German Mettwurst and Danish salami.

In Southern Europe and Mediterranean style DFS, the fermentation process is low, taking more than 40 hours to reach a pH of 5.3 in the product. Fermentation temperatures are mild, from 18 to 24 °C, and there is a soft acidification, reaching pH values of above 5.0. There is no smoking nor cooking processes, but production process lasts for 3 weeks or more due to the drying. These sausages are classified as dry and a_w of the final product are below 0.90. Typical examples are French saucisson, Naples *salami*, Salame Milano, Italian pepperoni, Spanish *chorizo* and Catalan *fuet* (Porto-Fett et al., 2022). Specifically, the production process of Catalan *fuet* (DFS object of this PhD thesis) includes a mild fermentation and a drying of low calibre sausages elaborated with pork lean meat, fat and ingredients/additives at temperatures ranging from 11 to 23 °C for periods of less than one month. In general, the final products usually have pH $\geq$ 5.3 and $a_w \leq 0.92$ (Serra-Castelló et al., 2021).

In North America, fast and high temperatures (i.e., > 32 °C) fermentation processes are usually performed (Leroy et al., 2006). In North America, DFS are smoked and fermented at temperatures *ca.* 30 °C, dried and acidified reaching values of pH below 5.0. Often after fermentation, DFS are smoked and cooked, then these sausages are dried for 2 to 3 weeks reaching final a_w values of *ca.* 0.90. In North America, typical examples of DFS are American pepperoni, Summer sausage, Lebanon Bologna.

Dry fermented sausages typical from Asia and Africa, apart from pork and beef, can contain meat from duck, buffalo, goat, or camel, and be seasoned with different species and cereals. Examples of DFS in these continents are Lap cheong (China) are Droewors (South Africa).

1.1. DFS production and consumer trends

1.1.1. Pork production within global, European Union and Spanish contexts

Pork accounts for the 33% of total meat consumption globally and Asia and Europe have been the main pork consumers and producers, at continent level, for the last 20 years. The last report release of the French Pig Institute (also called Institute du porc or IFIP), 2023-2024 (IFIP, 2024), indicates that at the beginning of the 21st century, about 87 million tonnes of pork were produced globally and an increase of the production was observed until 2014 reaching a top value of *ca.* 118 million tonnes, especially in Asia, producing, 58 to 70 million tonnes in a period of 14 years (2000−2014). From then on, the production in Asia slightly decreased and maintained until 2020, where a remarkable decrease was produced (52 million tonnes) and then the production recuperated its high pork production values (*ca.* 60 million tonnes in 2022). In the EU and America (including North, Central and South), the pork production has been slowly increasing from the beginning of the century on.

During the recent years, an increasing tendency on pork production globally has been observed. In 2021 (IFIP, 2024), the pig industry produced a total of 113.9 million tonnes of pork globally. The global pork production by continent, in a decreasing order range, Asia (57.2 million tonnes) leaded the ranking followed by Europe (29.7 million tonnes), North and Central America (16.2 million tonnes), South America (8.7 million tonnes), Africa (1.6 million tonnes) and Oceania (0.5 million tonnes) (Figure 1). At country[1] level, globally, China was the most pork producing country globally (44.0%) followed by the USA (11.7%), Spain (4.8%), Germany (4.4%), Brazil (4.1%) and Russia (3.4%). The "Pigmeat Dashboard" (Pigmeat Dashboard 2024) published the pig meat production values for 2022, where data from the main pig producer countries was collected. In 2022, 121.5 million tonnes of pork were produced globally, and 6 countries accounted for 83.6% of the total global production. Again, the top countries were China (46%), USA (10%), Brazil (4.3%), Spain (4.2%), Germany (3.7%) and Russia (3.4%).

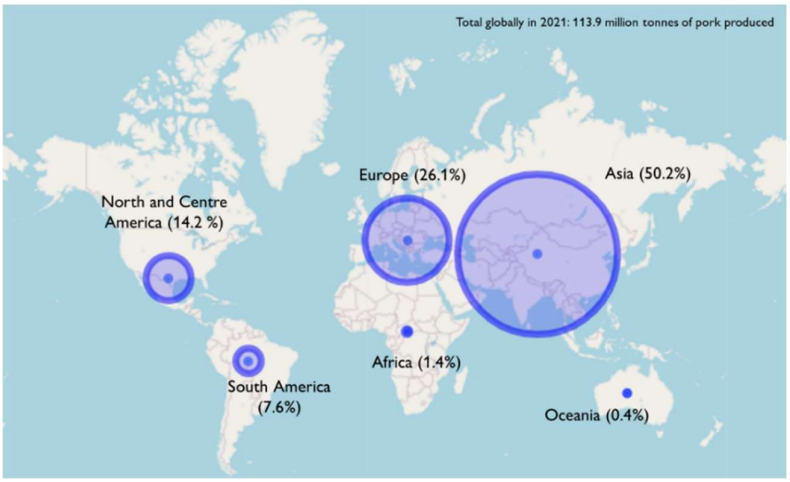

Figure 1. Pork production worldwide in 2021 per continent (data source: *Porc par les chiffres: données récentes de la filiére porcine* ((IFIP, 2024)).

[1]Only countries with a pork production > 3% are described.

The European Union (EU) pork production was 20.6 million tonnes in 2023 and, in 2022, 22.1 million tonnes (18% of the global) and 7 member states (MS) accounted for the 82% of the total EU production, and 22.1 million tonnes in 2021. In 2022, Spain was the most pork meat producer EU country (5.1 million tonnes) quoting the 23% of the EU pig production, followed by Germany (4.5 million tonnes), France (2.2 million tonnes), Polonia (1.8 million tonnes) and The Netherlands (1.7 million tonnes) with the 20%, 10%, 8% and 8%, respectively, of the total EU pork production (Figure 2). The evolution of the pork production in EU follows an increasing tendency since 2005, highlighting, as the two leading pork production countries in EU, an important increase in Spain from 2013 to 2021, from 3.4 to 5.2 million tonnes of pork (51%), and a constant decrease in Germany from 2013 to 2021, from 5.5 to 5.1 million tonnes of pork (-7%) due to the reduction of national pig production and multiple economic crisis. Overall, in the EU, the economic and animal health crisis lost millions of piglets and pigs for sacrifice in 2022 and therefore reduced the pork offer and production.

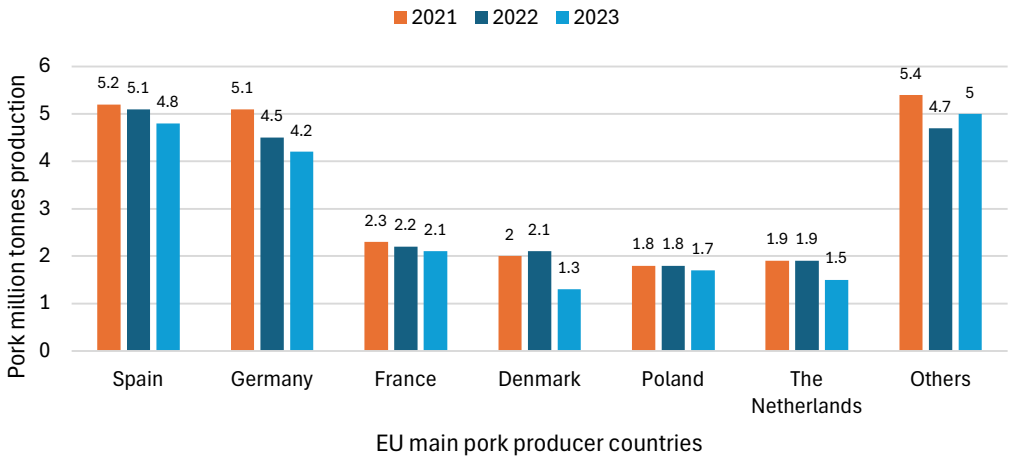

Figure 2. EU main pork producer countries for the last three years (2021, 2022 and 2023) (data source: *Porc par les chiffres: données récentes de la filiére porcine* (Institut du porc (IFIP), 2024)).

Within the classification of the top 5 regions with the highest pig production (pig head values) in the EU Aragon (Spain) and Catalonia (Spain) are in the third and fourth ranking place, which produced a total of 9,623 and 7,949 thousand of pig heads in 2022. In the meanwhile, that the IFIP predicts a clear diminution on the pork production in Northen Europe, the pork production in Spain will continue rising in the coming years (Jan Peter van Ferneij, 2021).

In Spain, Aragon gathered the leading position of pig production (28% of the total pig production in Spain), followed by Catalonia (23%), and these two regions grouped the 51% of the total pig census in 2022. However, there is a shift in the leading positions when focusing on the pork production in Spain, where Catalonia was the most pork productive region (40,2%) and Aragon the second (19.5%) in 2022, both regions accumulating the 59.7% of the total Spanish pork production. A total of 2.0 and 1.0 million tonnes of pork (pig carcasses after slaughtering) were produced in 2022 in Catalonia and Aragon, respectively. The evolution of the pork production in Spain from 2013 to 2022 has increased a 46.5%, from 3.4 to 5.1 million tonnes and that fact

has also been reflected in Catalonia and Aragon, increasing from 1.5 to 2.0 million tonnes (34.0%) and from 0.3 to 1.0 million tonnes (261.5%). As stated, an historical maximum of pork production was registered in 2021 (5.2 million tonnes of pork) and thus in Catalonia and Aragon (2.1 and 1.1 million tonnes of pork, respectively).

The production of DFS (i.e., the representative *chorizo*, *salchichon/salami* and *fuet/llonganissa*) was of 103 thousand tonnes in Spain in 2020, a production value that has maintained for the last 20 years, between 2004 to 2020 the average of DFS production in Spain was of 98 ± 5 thousand tonnes. Respecting the total pork meat produced in Spain in 2020, DFS production supposed the 2.6% ± 0.4. Focusing on each DFS product type, a decrease was observed in the production of chorizo and *salchichon/salami* DFS in Spain from 2004 to 2020 (decrease of 6% and 17%, respectively), in contrast *fuet/llonganissa* DFS production increased (increase of 38% in the production of the DFS classified as *fuet/llonganissa*) (Figure 3).

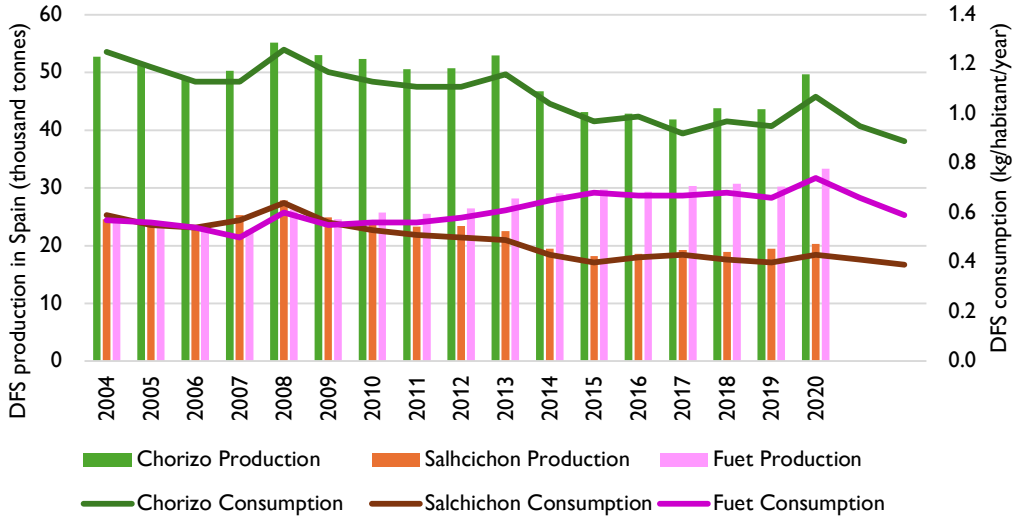

Figure 3. Historical production (thousand tonnes) and consumption (kg/habitant/year) of dry fermented sausages in Spain from 2004 to 2020 (data source: "Indicadores economicos sector porcino 2022" report, MAPA, 2022, and "Informe consumo 2022", MAPA, 2023).

1.1.2. Pork exportations and importations within global, European Union and Spanish contexts

World-wide pork exportations are led by the European Union (EU) with a total of 3.9 million tonnes of pig products exported in 2022, followed by USA with 2.3 million tonnes and Brazil with 1.1 million tonnes. Whereas global pork importations are led by China and Hong-Kong with a total of 2.5 million tonnes of pork imported in 2022, followed by Mexico (1.2 million tonnes) and Japan (1.0 million tonnes).

In Spain a total of 2.9 million tonnes of pork products were exported in 2022. For the last 20 years in Spain, it has been observed an increase (511%) on pork products exportation, starting from 1.4 million tonnes in 2001. The ranking of pork products exported in Spain in 2022 were

frozen pork (40.5%), fresh/in refrigeration pork (28.7%), pig offal/dressing (17.0%), bacon/lard/fat (4.7%), cured meat products (3.2%), salty/dried/smoked pig offal (2.4%), pigs for sacrifice (2.0%), meat preparations and preserves (1.5%).

Pork products importation in Spain has also increased for the last 20 years, in 2001 was of 50 thousand tonnes and in 2022 was of 307 thousand tonnes, thus supposing an increase of 514%. The main pork products imported in Spain in 2022 were fresh/in refrigeration pork (18.3%), pigs for sacrifice (17.1%) and bacon/lard/fat (15.7%).

Spanish pork has been exported globally, furthermore China and France were the most importing countries in 2022, clustering the 31% and 10% of the pork exportations in Spain. In contrast, the pork products imported in Spain mainly came from EU countries, thus were France (24%), The Netherlands (16%), Portugal (13%), Germany (12%) and Italy (11%).

In Catalonia a total of 1.6 million tonnes of pork products were exported in 2022. For the last 20 years in Catalonia, it has been observed an increase (540%) in pork products exportation, starting from 0.25 million tonnes in 2001. The ranking of pork products exported in Catalonia in 2022 were frozen pork (41.7%), fresh/in refrigeration pork (30.4%), pig offal/dressing (17.1%), bacon/lard/fat (3.9%), cured meat products (3.2%), salty/dried/smoked pig offal (1.9%), meat preparations and canned meat (1.7%) and pigs for sacrifice (0.01%).

1.1.3. Pork and pork thereof products consumption

Pork accounted for the 33% of total meat consumption globally in 2022 and Asia and Europe have been the main pork consumers, at continent level, for the last 20 years. The meat consumption projection made by the Organisation for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO) predicts an increase of the 11% in pigmeat consumption globally by 2032 (OECD/FAO, 2023), led by developing countries and China

Globally, pork consumption has increased for the last 15 years (19.2%), from 93.6 million tonnes in 2007 to 113.4 million tonnes in 2022. China is the pork most consumer country (50.6% of the total global consumption), registering a consumption of 57.4 million tonnes of pork consumed in 2022, followed by the EU (16.3%) and the EUA (2.4%), with 18.4 and 9.9 million tonnes consumed in 2022, respectively.

Nowadays in the EU, health, environmental and societal concerns should continue shifting consumer preferences to meat analogues and weigh negatively on EU pigmeat consumption. As a result, apparent EU pigmeat consumption per capita is projected to decrease by 0.5% per year, from 32.5 kg in 2021 to 31 kg in 2031, in line with the trend in the past decade (EC, 2021).

In Spain, a tendency of pork consumption decrease (17.5%) has occurred for the last 15 years. In contrast, pork products consumption in Spain was of 489 thousand tonnes in 2007 and 489 thousand tonnes in 2022, reaching a maximum of 576 thousand tonnes in 2013 (MAPA, 2022).

Registering a slight decrease in pork consumption in Spain but not in pork products, that include cured meat products and DFS.

Aiming attention at the consumption of dry fermented sausages in Spain, the consumption per capita (kg/habitant/year) of *chorizo*, *salchichon/salami* and *fuet/llonganissa* was of 0.89, 0.39 and 0.59, respectively, in 2022, and of 0.95, 0.41 and 0.66, respectively in 2021 (MAPA, 2023). Historically (2004-2020), the consumption of *chorizo* and *salchichon/salami* decreased a 14% and 27%, respectively, and *fuet/llonganissa* consumption increased a 30% (Figure 3).

2. Hurdle technology and preservation strategies of DFS

Dry fermented sausages are classified as both shelf-stable and ready-to-eat (RTE) meat products. The terms are defined as:

- Shelf Stable (SS) product is free of microorganisms (pathogens and spoilage) capable of growing in the product at non-refrigerated conditions at which the product is intended to be held during distribution and storage (FSIS, 2021).

- Ready-to-eat food means food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level the microorganisms of concern (EC, 2005).

It has to be considered, however, that shelf-stability does not involve food safety, one aspect of DFS that has to be guaranteed through the application of the hurdle technology along the production process.

2.1. Hurdle technology

The combination of several factors (hurdles) that prevent the survival and/or growth of microorganisms present in foods is the base of microbial stability and food safety. Usual hurdles in foods include pH, water activity, preservatives, temperature, redox potential and accompanying microbiota. The intelligent combination of these hurdles, that is called "Hurdle technology", secures microbial stability and safety, and sensory and nutritive food properties. Between the different hurdles applied, microorganisms present at start of the process or in raw materials (i.e., spoilage and pathogenic microorganisms) should not be able to overcome the set barrier, otherwise the food would spoil or allow the growth or survival of foodborne pathogens.

In Mediterranean style dry fermented sausages different hurdles succeed during the fermentation and ripening process, from meat batter to the final product. Figure 4 illustrates a combination of hurdles that affect microorganisms' survival along DFS elaboration process (Leistner, 1995).

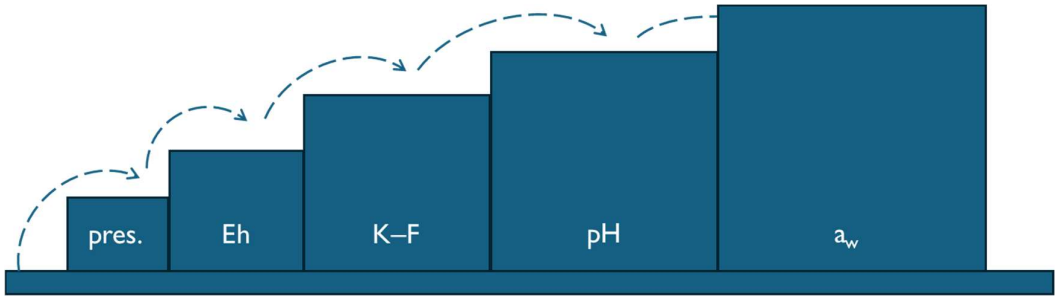

Figure 4. Example illustration of the effect of the combination of antimicrobial hurdles, typical from Mediterranean style dry fermented sausages. Pres.: preservatives, E_h: redox potential, K–F: competitive microbiota, pH means acidification, a_w: water activity. Figure adapted from Leistner (1995).

The mixture of pork, pork back fat and other ingredients (i.e., spices and herbs) harbours the initial microbiota of the meat batter, which is stuffed into casings that can also shelter intrinsic microorganisms (i.e., natural casings) or be colonized by moulds during ripening. Pork has been reported to have a high bacterial diversity, with up to 51 bacterial orders, where *Pseudomonadales* (45%), *Enterobacterales* (13%) and *Lactobacillales* (11%) are the main bacterial orders, and initial total viable counts ranging between 2.0 to 5.8 Log CFU/g (Dorn-In et al., 2024). Species and herbs are mostly dry products that do not support the microbial growth due to the low water activity on the seed/leaf surface, but up to 7 Log CFU/g of total viable counts have been reported, mainly from spore-forming bacteria (de Boer et al., 1985). The species can be contaminated during harvesting and post processing with human pathogens that are spore-forming (e.g., *Bacillus cereus* and *Clostridium perfringens*) or non-spore-forming bacteria able to survive a certain degree of dryness (e.g., *Salmonella* spp., *L. monocytogenes*) (Hertwig et al., 2015). Salt preserved natural casings can contain gut bacteria in a latent phase, including pathogens and well-adapted LAB, and interfere with the fermentative process and, therefore, in the resulting ripening properties (Houben, 2005; Pisacane et al., 2015).

Preservatives, such as salt and nitrifying salts (i.e., nitrates and nitrites), and sugars (e.g., dextrose and maltodextrin) are also applied as additives in the meat batter (further developed in section 1.3.2). Salts are the first hurdle for the inhibition of many bacteria in the initial product. Salt (NaCl) reduces the water availability (i.e., decreases a_w) in a matrix and nitrifying salts can be bacteriostatic or bactericidal, causing oxidative stress, thus reducing the oxygen availability and creating a hostile environment for *Pseudomonadales* and other Gram-negative and positive microorganisms, which rapidly multiply and spoil fresh meats in the presence of oxygen (Dorn-In et al., 2024; Leistner & Gould, 2002). Usually in industrial DFS formulation, starter cultures (further developed in section 2.3) are mixed with the DFS meat batter to promote a rapid and controlled acidification and characteristic flavour, thus enhancing DFS food safety and quality.

After manufacturing, DFS are hanged in drying chambers to ferment and dry. The process conditions are product and company specific but a in the case of *fuet*-type DFS process conditions could include a fermentation at 18–20 °C of temperature with a 90–95% of relative humidity until pH decreases to 5.0 and then, to dry, at 12–14 °C with a 70–80% of relative humidity for 15 days until the end of the whole process (Gou et al., 1996). During a long ripening

of sausages, the nitrifying salts are depleted, pH and redox potentials increase again, and its hurdle effects become weak. Reducing the water activity (i.e., drying) is the last hurdle effect to achieve a long shelf life and product stability.

In moulded DFS (e.g. Mediterranean style DFS), the outer part of the casing is covered by desirable (non-mycotoxigenic) moulds e.g., *Penicillium nalgiovense*, that are inoculated through the application of a spore's solution after stuffing (Figure 5). The growth of desirable moulds contributes to the sensory properties of fermented sausages through lactate oxidation, proteolysis, conversion of amino acids, and lipolysis (J. Flores, 1997; Sunesen et al., 2004; Sunesen & Stahnke, 2003). In contrast, Northen-Europe style DFS are smoked to avoid the growth of undesirable moulds on the surface of the sausages (Leistner & Gould, 2002).

The sequence of hurdles is a good combination of barriers for spoilage and pathogenic microorganisms' outgrowth, as shown in Figure 4, that can be present at raw materials or at the first's steps of sausage manufacturing. These sequential processes ensure food safety and self-stability of dry fermented sausages.

In the framework of the hurdle technology, formulation (addition of antimicrobials, starter cultures, etc.) and process parameters can be added or modified during the manufacturing and ripening process to better control certain spoilage and pathogenic microorganisms.

2.2. Antimicrobial additives

In the past, saltpetre (salt containing nitrate) was used as a meat preservative. Nowadays, chemical preservatives commonly applied in cured meat products, including DFS, are salt (NaCl) and nitrifying salts (i.e., nitrate and nitrite salts) (Scientific Panel on Biological Hazards, 2003).

Curing effects of nitrate and nitrite on meat and meat products thereof are not only antimicrobial but sensorial. The main effects of nitrate and nitrite on cured meat products are:

- Antimicrobial. Nitrite can function as bacteriostatic or bactericidal agent, depending on the NO_2^-, NO, O_2^- and H_2O_2 concentrations and directly related to the oxidative stress caused by $ONOO^-$/ONOOH. The toxic effect of peroxynitrite may stem from its oxidation of zinc fingers, protein thiols, membrane lipids, cysteine and arginine biosynthesis and iron-sulphur proteins, which mainly affects Gram-positive bacteria, whereas Gram-negative bacteria are hardly affected. The metabolic pathways due to the limitation of oxygen uptake supress the growth of certain bacteria such as *Clostridium botulinum* and the survival of its spores, and *S. aureus*, *L. monocytogenes*, *Bacillus cereus* and enterobacteria in acidic pH ($pH \leq 6.0$) conditions when nitrate is oxidised to nitrite (Alahakoon et al., 2015; Scientific Panel on Biological Hazards, 2003). The concentration levels of nitrite reactive oxygen species and their production and reaction kinetics also depend on pH, NaCl and ascorbate rates (Majou & Christieans, 2018).

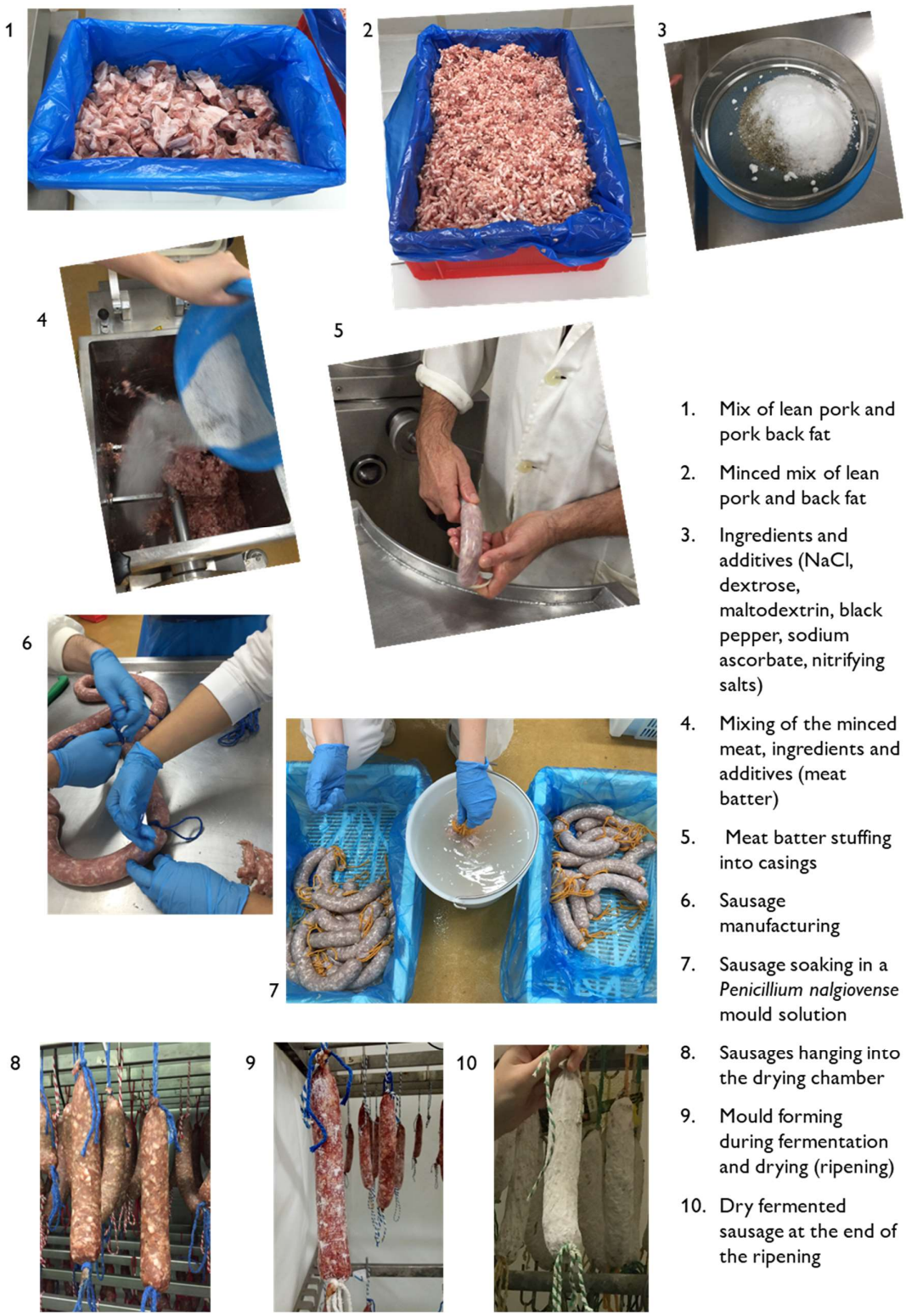

Figure 5. Dry fermented sausage manufacturing process. Photos taken during the manufacturing and ripening process of the DFS to conduct the study entitled "Dynamics of microbial communities in nitrite-free and nutritionally improved dry fermented sausages" (Article I, in the RESULTS section).

- Colour formation. Oxidation of nitrite salts form nitric oxide which able to react with myoglobin Fe^{2+} and metmyoglobin Fe^{3+}, leading to the development of the red Bordeaux cured meat colour (Honikel, 2004).
- Antioxidant. Retards oxidative reactions and rancidity development due to their rapid reaction with oxygen (Velasco et al., 2010).
- Cured flavour. The suppression of rancidity enhances the formation of nitrite-related flavours and aromas (M. Flores et al., 2021; Terns et al., 2011).

Despite the benefits of nitrifying salts in cured meat products, there are some concerns on the impact of nitrites on health and its consumption related to the formation of nitrosamines (i.e., carcinogenic N-nitroso compounds). These compounds are eventually generated when nitrifying agents react with nitrosatable amines or amides (e.g., putrescin and cadaverine) (Bernardo et al., 2021). Nitrosamines are of high concern because they have genotoxic and carcinogenic potential (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2018) and that fact motivated a study on the risks associated with the consumption of nitrites and nitrates, which was conducted by Anses. On the Anses report, it specifies that the use of nitrates and nitrites should undergo a specific assessment (for each pathogenic microbial hazard (i.e., *L. monocytogenes*, *Salmonella* sp. and *C. Botulinum*)/food pair) based on impact data concerning their effectiveness and safety for human health (ANSES, 2022). The relationship between consumption of cured meat products and cancer, together with misinformation propagated by social media, has raised concern among consumers, who has reinforced the consumers claim for "clean-label" products (Franco-Arellano et al., 2020; Martínez et al., 2019). Industry seeks for natural compounds (e.g., vegetal extracts) as nitrifying salts substitutes that can replace the beneficial effects of colouring, aroma and antimicrobial properties, and produce clean-label meat products (Bernardo et al., 2021). The opinion of experts recommends reducing the population's exposure to nitrates and nitrites by taking proactive measures to limit dietary exposure (i.e., 150 g of processed meat products) (ANSES, 2022).

A diversity of natural alternatives to nitrate/nitrite in DFS have been evaluated to test the replacement of nitrites on quality characteristics, physicochemical, aromatic, sensory, antioxidant, and antimicrobial characteristics (Bernardo et al., 2021). Some plant-based alternatives are *Kitaibelia vitifolia* extract (Kurcubic et al., 2014), lyophilized plant powder (containing celery, celery juice, parsnip, and leek) (Eisinaiti et al., 2016), grape seed, chestnut extract (Aquilani et al., 2018), rosemary (Martínez et al., 2019), radish and beetroot powders (Ozaki et al., 2021). Nitrate/nitrite alternatives form animal origin such as porcine heart and liver extracts as pork industry by-products have also been proposed as colouring agents in nitrite-free meat products regarding the Zinc-protoporphyrin pigment[2] formation (Kauser-Ul-Alam et al., 2020). The addition of single bacterial strains (e.g., *Staphylococcus xylosus* or *Lactococcus lactis* subsp. *cremoris*) or bacterial cocktails (e.g., *S. xylosus* and *Pediococcus pentosaceus*) alone or together with plant and animal nitrite-alternatives have also been proved to enhance the sensory

[2] Zinc-protoporphyrin complex is naturally present in meat products and contributes to the bright red colour, for instance, of Parma hams (Wakamatsu et al., 2004).

characteristics of DFS in the absence of nitrifying salts (Bernardo et al., 2021, Kauser-Ul-Alam et al., 2020).

2.3. Starter cultures

The most important technological microbiota found in DFS are *Lactobacillale*s as the lactic acid bacteria (LAB) and the Gram-positive catalase-positive cocci (GCC+), including lactobacilli, and/or pediococci, giving a characteristic flavour of fermented sausages and promoting the acidification of the meat matrix (Leroy et al., 2006; B. Martín et al., 2006). Both bacterial groups are used as starter cultures in DFS mainly in industrial productions to achieve homogeneous sensorial traits between production batches though its use is not mandatory. Starter cultures are carefully selected strains or a cocktail of strains or bacterial species that are well adapted to the food environment, hence will rapidly grow and colonize the matrix. The starter culture will also compete for nutrients with the intrinsic microorganisms of the food matrix, thus controlling the outgrowth of non-desired bacteria that will lead them to the stationary phase (i.e., Jameson effect (Jameson, 1962, Cornu et al., 2011)). In general, the use of starter culture will contribute to food safety, organoleptic properties, technological or nutritional advantages, helping to achieve one or some desired properties for final products.

Typical starter cultures used in meat fermentations and dry fermented sausages are *Lactiplantibacillus plantarum*, *Latilactobacillus curvatus*, *Latilactobacillus sakei*, *Lacticaseibacillus casei*, *Lactiplantibacillus pentosus*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *Enterococcus faecium*, as LAB, and some staphylococcal species, such as *Staphylococcus xylosus* and *Staphylococcus carnosus* (Leroy & De Vuyst, 2004; Stegmayer et al., 2023). Commercialized DFS starter cultures are various and different regarding the final sensorial characteristics and style of fermented sausage. For instance, SM-194 is a marketed mixed culture combination of *Pediococcus pentosaceus* and two different *Staphylococci* spp. give a mild lactic taste and accelerated pH-drop at higher temperatures and a more intense colour and mild aroma development, respectively, employed to produce stable salamis with a pronounced Mediterranean flavour (WESTCOMBE, 2024).

An example of GCC+ species are *Staphylococcus xylosus*, *S. carnosus* and *S. epidermis*, that at mild temperatures are able to use the oxygen to multiply and perform lipolysis, provoking a change in the redox potential of the matrix that inhibits aerobic bacteria. The suppression of indigenous bacteria improves the meat product quality and prevents it from spoilage events. During processing, proteolysis of skeletal muscle meat proteins (e.g., sarcoplasmic and myofibrillar proteins) occurs through endogenous proteolytic enzymes of meat and microorganisms (Hughes et al., 2002). The resulting small peptides and amino acids serve as substrate for GCC+ to convert them in flavour compounds (Berardo et al., 2017). LAB are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals, and these products makes them good functional starter cultures. Regarding the beneficial potential for LAB use in DFS, several LAB species have received the Qualified presumption of safety (QPS) in EU (and Generally Recognised As Safe or GRAS in USA),

however the application of their metabolites in foods is not currently allowed in Europe and requires specific EFSA authorisation. The QPS status is the result of a pre-assessment that covers safety concerns of microorganisms for humans, animals and the environment. During this process, experts assess the taxonomic identity of the microorganism, the related body of knowledge and potential safety concerns (EFSA, 2024). Some QPS LAB are *Latilactobacillus sakei*, *Latilactobacillus curvatus* and *Lactiplantibacillus plantarum,* formerly *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum*, respectively, which are homofermentative and the optimal conditions of growth and metabolic pathways activation are anaerobiosis and mild temperatures of *ca.* 20 °C, common in the Mediterranean countries (Aymerich et al., 2003). During fermentation and ripening, bacterial diversity decreases and *L. sakei*, which has shown a species' excellent adaptation, competitiveness and assertiveness, colonises the meat matrix (Barbieri et al., 2021; Chaillou et al., 2005; Janssens et al., 2013; Talon & Leroy, 2011). Homofermentative bacteria (*e.g.*, *L. sakei*) catabolises fermentable sugars producing lactic acid as a fermentation subproduct; hence a pH drop is observed in the meat matrix inhibiting the acid sensitive bacteria.

2.4. Biopreservation

Biopreservation is a technique of food preservation in which antimicrobial potential of naturally occurring organisms and their metabolites are exploited, providing increased food safety and extension of shelf-life (Aymerich et al., 2011). It can provide extra protection during temperature abuse conditions, decrease the risk of transmission of foodborne pathogens, reduce the economic losses due to food spoilage, reduce the application of chemical preservatives and permits the application of less severe heat treatments, with preservation of organoleptic properties of foods (Delboni & Yang, 2017).

Strategies of biopreservation pass through lactic acid bacteria which naturally produce antimicrobials such as bacteriocins, organic acids, carbon dioxide, hydrogen peroxide, diacetyl and ethanol (Messens & De Vuyst, 2002). Antimicrobials in combination of one or more can enhance its potential against food-borne pathogens and increase the food safety, as Castellano et al. (2017) showed in research with organic acids and bacteriocin on antilisterial activity (Castellano et al., 2018).

2.4.1. Bacteriocinogenic starter cultures and bacteriocins

Bioprotective or bacteriocinogenic starter cultures are microbial species that produce specific molecules characterised by bacteriostatic or bactericidal effects, such as bacteriocins, and other antimicrobial peptides (García-López et al., 2023).

Bacteriocins are small or low-molecular peptides or proteins (~3 to 10 KDa) that exhibit antibacterial activity against specific Gram-positive bacteria, including strains of the same species and different pathogens, mainly those closely related such as *Listeria monocytogenes* and *Staphylococcus aureus* (Martín et al., 2022). These peptides are synthesized naturally in the

ribosomes by several LAB, are non-toxic, can be degraded by digestive proteolytic enzymes and they are usually thermostable and active over wide pH range (Zendo, 2013). The chemical characteristics of bacteriocins are its hydrophobicity and positive charged peptides that make them stable proteins in many organic solvents and polymers. The genes coding for bacteriocin production are mostly organized in operon clusters comprising four genes, which may be located on the chromosome, on plasmids or transposons (Klaenhammer, 1993; Knoll et al., 2008).

Bacteriocins from LAB have been classified to three different classes according to its affinity or not to lantibiotics (i.e., class I and class II, respectively) and thermal instability (i.e., class III). Class I bacteriocins are nano peptides (< 5 kDa) which have been incorporated lanthionine (i.e., lanthipeptides with antimicrobial activity) during the post-translational modifications after its synthesis and have affinity to cell wall precursor lipids, where they can anchor forming pores to the wall (Ongpipattanakul et al., 2022). Class II bacteriocins are heat stable proteins (< 10 kDa) with no post-translational modifications and can be divided into four categories: a) pediocin-like Listeria-active peptides (this class IIa includes sakacin K, the bacteriocin studied in the PhD thesis), b) bacteriocins that need two bacteriocins to be active, c) bacteriocin with covalent linkage of the N- and C- terminal residues, d) miscellaneous, non-pediocin like. Class III bacteriocins are heat-labile large bacteriocins (> 10 kDa) (Yi et al., 2022).

The general mode of action of bacteriocins is pore forming to bacterial cell walls, membrane permeabilization, dissipation of the transmembrane electrical potential and change in the pH gradient. The specificity of bacteriocins to the target (e.g., lipid I or II of the bacterial cell wall), diversity in terms of structure and modes of action, and additionally for not affecting gut microbiota and host health, makes them of special interest for food industry and pharmaceutics (Yadav & Tiwari, 2023).

In food industry, there are conventional strategies for using bacteriocins (i.e., bacteriocin-production live cells, bacteriocin-containing fermentates[3] and applying pure bacteriocins) and novel and emerging applications for bacteriocins (e.g., incorporation of bacteriocins into food packaging films/coatings) (Yi et al., 2022). Several examples of bioprotective bacteriocin producing LAB use and bacteriocin application in DFS and other meat products are described in Table 2.

Forty-nine species of the genera *Carnobacterium, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus* and the former *Lactobacillus* genus, have the QPS status granted by the European Food Safety Authority (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards) et al., 2022) and the FDA (USA Food and Drug Administration) has granted *L. lactis, L. fermentum* and *L. bulgaricus* in the GRAS status (FDA, 2018).

Nisin was the first and only approved bacteriocin in the EU and the most widely used bacteriocin in food industry, since it has been used for more than 50 years to combat food-borne pathogens

[3] Fermentates are powdered preparation derived from fermented raw materials or food ingredients including milk, sugar, and corn or wheat starch. They can contain fermenting microorganisms, culture supernatants, fermented substrates, and bioactive components including bacteriocins (Yi et al., 2022).

(Barcenilla et al., 2022; Lubelski et al., 2008). In the European Union, nisin is the only regulated bacteriocin to be considered as a food additive (E-234), and its application is only authorised in some dairy products such as cheese (not all types) according to the Commission Regulation (EC) No 1333/2008 of 16 December 2008 (and subsequent modifications) on food additives (EC (European Commission), 2008).

Nowadays, research has been conducted to discover new bacteriocins and potential target bacteria. Emerging bacteriocin application technologies are in consideration to increase the food safety of many products as a replacement strategy of chemical antimicrobial additives. Food industry is enthusiastic about the further development of bacteriocins as preservatives as the need for biopreservation is continuously growing (Yi et al., 2022).

Table 2. Summary of bacteriocin types, mode of action, targeting bacteria and industrial applications, based on and modified from Barcenilla et al. (2022).

Bacteriocin (subcategory, producer)	**Structure, mode of action and target bacteria**	**Industrial application (status)**	**Reference**
Nisin (Class I/Type A, *Lactococcus lactis*)	 Killing gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *Enterococci*, *Staphylococci* and *Streptococci*	Food-preservative (commercial)	(Lubelski et al., 2008)
Enterocin (Class IIa, *Enterococcus avium* DSMZ17511)	 Mannose phosphotransferase system is the membrane-specific receptor for class IIa bacteriocins, which attach and form pores to *Listeria monocytogenes*	Incorporated into agar edible films for active packaging (research)	(Guitián et al., 2019)
Pediocin (Class IIa, *Pediococcus acidilactici*)		Fermentate (Commercial (ALTA 2341®, Quest International, USA)	(Mills et al., 2011)
Enterocin A and B (Class IIa, *Enterococcus faecium* CTC492)		Additive to formulation (research)	(Jofré et al., 2009)
Sakacin K (Class IIa, *Latilactobacillus sakei* CTC494)		Starter culture (commercial and research)	(Garriga et al., 2002; Hugas et al., 1995)
Plantaricin EF and KJ (Class IIb, *Lactiplantibacillus plantarum* C11)	Parallel or anti-parallel transmembrane dimer complex that permeabilises cell membrane of *Enterococcus faecalis*	Starter culture for malolactic fermentation (research)	(Knoll et al., 2008; Moll et al., 1999)

Latilactobacillus sakei CTC494, producer of sakacin K

An object of study in this thesis was the *Latilactobacillus sakei* CTC494 (formerly *Lactobacillus sake* or *sakei*) isolated from a non-inoculated DFS in 1995 by Hugas et al. (1995) showing great antilisterial potential against *Listeria* genus and specifically bacteriostatic properties against *Listeria innocua* and bacteriolytic properties against the species *L. monocytogenes*.

Firstly, the antimicrobial compound produced by *L. sakei* CTC494 was tested for heat stability, showing heat-resistance, proteolytic enzymes sensitivity, which was sensible, and displayed a bacteriolytic mode of action, like other bacteriocins produced by LAB. These criteria outlined by Tagg et al. (1976) was used for the bacteriocin identification (Hugas et al., 1995).

Aymerich et al. (2000) further characterized the sakacin K bacteriocin. Sakacin K is a class IIa bacteriocin composed by 40 amino acid residues and a molecular weight of 3,802 Da. Its structural gene is located on a 60 Kbp plasmid harboured by the *L. sakei* CTC494 strain. The sakacin K mode of action is similar to other bacteriocins such as sakacin A and curvacin A and belongs to the pediocin-like Listeria-active peptides category (Table 2). These bacteriocins specifically attach and form pores in the membrane of *Listeria* disrupting the cell homeostasis and thereby reducing cell viability.

L. sakei CTC494 has been used in research as a bioprotective starter culture for controlling the outgrowth of *L. monocytogenes* in a variety of animal-derived products such as seabream (Bolívar, Correia Peres Costa, et al., 2021; Costa et al., 2019), ready-to-eat fish products (Bolívar et al., 2021), vacuum-packed cold-smoked salmon (Aymerich et al., 2019), cooked ham (Serra-Castelló et al., 2022), and dry fermented sausages (Austrich-Comas et al., 2022).

2.4.2. Organic acids

Lactic acid bacteria produce organic acids (i.e., lactic acid, acetic acid, formic acid, phenylacetic acid and caproic acid) as a subproduct of sugar fermentation (Leroy & De Vuyst, 2004). LAB can be homo- or heterofermentative and use monosaccharides and disaccharides (e.g., saccharose, lactose, glucose and xylose) or fermentable sugars (i.e., hexoses) to produce energy. For instance, homofermentative bacteria perform the homolactic fermentation, from 1 mol of glucose can obtain 2 mols of lactic acid and 2 ATP (Castillo et al., 2013). Organic acids can be either naturally present in as constituents of foods or latter added directly or indirectly. Moreover, the levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions.

Matrix or media acidification by organic acids lead to a pH decrease. Acidic environments give to organic acids the liposoluble property, which allows cell membrane breaking and bacterial inhibition, interference of the cell membrane potential maintenance, active transport inhibition, intracellular pH reduction and a variety of metabolic functions inhibition (Özcelik et al., 2016). On the other hand, organic acids can affect the quality of the food due to the production of flavour compounds such as diacetyl and acetaldehyde, as well as compounds which may have

positive health implications such as vitamins, antioxidants and bioactive peptides (Ross et al., 2002).

Organic acids and their salts greatly potentiate the activity of bacteriocins, while acidification enhances the antibacterial activity of both organic acids and bacteriocins; the increase in the net charge of bacteriocins at low pH would facilitate translocation of bacteriocin molecules through the cell wall (Gálvez et al., 2007). In contrast to bacteriocins, organic acids have a very broad mode of action and inhibit both Gram-positive and Gram-negative bacteria as well as yeast and moulds (Blom & Mørtvedt, 1991; Caplice & Fitzgerald, 1999). Moreover, organic acids such as lactic and acetic acid are non-toxic preservatives that have been recognised as GRAS by the FDA and QPS by the EU for use as additive in food industry (Ameen & Caruso, 2017; FDA, 2023). Specifically, the EC 1333/2008 European regulation establishes that the maximum level of organic acids application is *quantum satis* which shall mean that no maximum numerical level is specified, and substances shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled (EC, 2008).

2.4.3. Other antimicrobials produced by LAB

Other natural antimicrobials produced by LAB are carbon dioxide, hydrogen peroxide, diacetyl and ethanol (De Vuyst & Vandamme, 1994; Leroy & De Vuyst, 2004). These are subproducts of heterolactic fermentation, which is an alternative pathway to obtain energy (i.e., ATP) from sugar or pyruvate catabolism.

For instance, the heterolactic fermentation follows the pentose phosphate pathway (e.g., *L. reuteri*), which from 1 mol of glucose results (a) 1 mol of lactic acid, 1 mol of CO_2, 1 mol of ethanol and 1 ATP, or (b) 1 mol of lactic acid, 1 mol of CO_2, 1 mol of acetic acid, 2 ATP and 2 NADH. Under stress conditions of for example in glucose scarcity, LAB can perform mixed acid fermentation and produce formic acid (Hofvendahl & Hahn–Hägerdal, 2000). Moreover, some heterofermentative LAB, such as *L. plantarum*, and can catabolise both hexose and pentose sugars. When using pentoses (i.e., fructose) for LAB fermentation the yield of 1 mol of fructose rises 1 mol of lactic acid, 1 mol of acetate and 2 ATP (Castillo et al., 2013).

3. Microbial ecology of DFS

3.1. Meat microbiota

As detailed in previous sections, the basis for DFS manufacturing is pork. Pork is a food matrix rich in nutrients (e.g., fatty acids, amino acids, and water-soluble proteins) that can support microbial colonization and growth in adequate temperature and physicochemical conditions (i.e., neutral pH and high-water content) (Papadochristopoulos et al., 2021). Meat microbiota is a complex ecosystem formed by endogenous and exogenous sources, i.e., microorganisms that

live in the animal intestinal tract and are transferred to animal carcass after slaughtering and environmental microorganisms of the meat processing facility (Xu et al., 2023).

During the last decades, fresh pork microbiota has been identified by culture-dependent analysis, evaluating the Total Viable Counts (TVC) to check the hygienic quality of the processed meat. Nowadays, culture-independent techniques (i.e., metataxonomic studies, further developed in section 1.7.2) permit a broad meat microbiota characterization, including non-culturable microorganisms. Several studies identified *Pseudomonas* spp., *Acinetobacter* spp., *Pantoea* spp., *Raoultella* spp., *Serratia* spp., *Psychrobacter* spp., *Enterobacter* spp. *and Citrobacter* spp., belonging to *Pseudomonadota* (formerly *Proteobacteria*) phylum, *Brochothrix* spp. *and Lactobacillus* spp., belonging to *Bacillota* (formerly *Firmicutes*) phylum, as the 10 top genera found in fresh pork (Nychas et al., 2008; Wang et al., 2022). Environmental conditions in a meat processing and DFS manufacturing plants typically include low temperatures (i.e., < 10 °C) and high relative humidity (i.e., > 70%) (Carpentier et al., 2014) which promote psychrotrophic bacteria growth. *Pseudomonas* spp., *Acinetobacter* spp. and *Psychrobacter* spp., are bacterial genus frequently found in environmental surface samples from meat facilities. During the fermentation and ripening, microbial communities change regarding the oxygen, temperature and nutrients availability. Bacterial families such as *Lactobacillaceae* and *Streptococcaceae* are also part of the fresh pork niche and reduced oxygen and mild temperatures (i.e., between 15 – 25 °C) conditions favour its growth and meat colonization. A dynamic transition from *Pseudomonas* spp., *Acinetobacter* spp. and *Psychrobacter* spp. as the most abundant genera in fresh pork to *Lactobacillus* spp. and *Streptococcus* spp. as the most abundant genera in DFS (Franciosa et al., 2018).

The safety of DFS can be compromised by microbiological hazards surviving the hurdle steps of fermentation (i.e., reducing the pH through product acidification), drying (i.e., reducing the water content of the product) and smoking (hurdle technology information is extended in section 2.1) and remain in the product during its shelf-life. The main pathogenic microorganisms found in DFS are *Salmonella* spp. and *L. monocytogenes*. The origin of these microbial hazards is usually the raw materials (in general meat) although can also be cross contaminated during manipulation of the final product (e.g., slicing and packaging). Contaminated DFS have been related with withdrawals and recalls, and in some cases involved in outbreaks.

3.2. Microbial hazards

3.2.1. *Salmonella* spp.

Salmonella spp. is a Gram-negative, facultative anaerobic, rod-shaped, and non-spore-forming genus form the Enterobacteriaceae family (ICMSF, 1996). The genus *Salmonella* was proposed to be a single species, i.e., *S. enterica*, and divided in six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *bongori* (V) and *indica* (VI) (Le Minor et al., 1986). The first four subspecies correspond to Kauffmann's classification, while each subspecies is divided into serovars based on O (somatic) and H (flagellar) antigens (Le Minor, 1988). The Kauffmann-White scheme establishes the existence possibility of 20,000 different *Salmonella*

serovars regarding the antigen combination. Among them, more than 2,600 *Salmonella* serovars have been identified word-wide and some are well adapted or restricted to a single host species (Stevens & Kingsley, 2021); for example S. Dublin is well adapted to cattle (Holschbach & Peek, 2018), S. Derby to porcine and *Gallus gallus* (Sévellec et al., 2018) and S. Gallinarium restricted to fowl (Eswarappa et al., 2009).

The aetiology of *S. enterica* subsp. *enterica* is the intestinal track of warm-blooded animals, which is a good reservoir for the pathogen. The optimal conditions of temperature, pH and a_w are 35–43 °C, 7–7.5 and 0.99 while the limits of growth of *Salmonella* are at temperatures between 5.2–46.2 °C, pH range between 3.8–9.5 and a_w range between 0.94–>0.99. However, salmonellae can survive for long periods in foods and other substrates at freezing and room temperatures (e.g., at −23 °C for more than 10 weeks in butter or at 2–4 °C for more than 28 days in vegetables) (ICMSF, 1996).

Salmonella is the major causative agent of outbreaks in the EU and salmonellosis is the second most reported foodborne zoonosis in humans in the EU. The EU health care, social and economic costs due to Non Typhoidal *Salmonella* cases (i.e., zoonotic *Salmonella* serovars that can infect warm-blood animals, including humans) are estimated as high as €3 billion per year and "Meat and meat products" is one of the major sources of human infection (EFSA and ECDC, 2022). Salmonellosis causes gastroenteritis with symptoms of diarrhoea, fever, vomiting and abdominal pain, and can be treated with antibiotics, but it can be severe if the infected person is immunocompromised (Arya et al., 2017). Data reported in the EU from the last three years (2020-2022) indicates that, within the identified *Salmonella* serovars that caused salmonellosis (i.e., 80.3% in 2021 and 72.3% in 2022) of the total number of confirmed cases (i.e., 47,122 cases out of 65,208 in 2022 (Table 3)), the three most reported serovars were Enteritidis, Typhimurium and the monophasic S. Typhimurium (1,4,[5],12:i:-). The latest data published from 2022 set on 54.6% for Enteritidis, 12.1% for Typhimurium and 10.4% for 1,4,[5],12:i:-, the confirmed cases per serovar, englobing the 77.1% of the confirmed cases (EFSA and ECDC, 2023). According to the last One Health 2022 Zoonoses report, Spain is one of the European countries reporting more cases of salmonellosis after France and Germany, confirming a total of 8,832 salmonellosis cases in 2022, similar to the pre-pandemic COVID-19 value of 8,730 reported in 2018 (Table 3).

In pigs, the primary agents of *Salmonella* infection, i.e., salmonellosis, are S. Typhimurium and S. 1,4,[5],12:i:- serovars and can be asymptomatic or symptomatic with different clinical characteristics (diarrhoea, dehydration, fever and wasting) that are indistinguishable from the serovar origin. In a study of piglets inoculation with S. Derby, S. Typhimurium and S. 1,4,[5],12:i:, piglets inoculated with the monophasic variant shed *Salmonella* continuously and in higher proportions in comparison to S. Typhimurium and S. Derby ($p<0.05$) (Cevallos-Almeida et al., 2019), exhibiting a high transmission ability and becoming a global public and animal health hazard (Sun et al., 2020).

Table 3. Salmonellosis and listeriosis confirmed cases in the European Union, highlighting the countries with the highest figures, during the 2018 to 2022 period.

Country	Year and total foodborne disease (S; salmonellosis, L; listeriosis) confirmed cases									
	2018		2019		2020		2021		2022	
	S	L	S	L	S	L	S	L	S	L
Germany	13,293	678	13,494	571	8,664	546	8,144	562	9,064	548
France	8,936	338	8,935	373	7,071	334	9,315	435	11,162	451
Spain	**8,730**	**370**	**5,087**	**504**	**3,526**	**191**	**6,168**	**355**	**8,832**	**437**
Italy	3,635	178	3,256	202	2,713	155	1,776	230	3,302	345
Other EU MS[1]	56,528	812	47,417	817	30,716	661	34,766	783	32,848	957
Total EU MS	**82,392**	**2,376**	**78,189**	**2,467**	**52,690**	**1,887**	**60,169**	**2,365**	**65,208**	**2,738**

[1] MS: EU Member States, excluding United Kingdom, Iceland, Norway, Liechtenstein, and Switzerland.

The transmission ability could also be related to the bacteria mobility. Most of the *Salmonella* serovars are motile, including *S.* Typhimurium, and produce two types of flagella that are regulated by the expression of *fliC*, *fljBA* and *hin* genes. Non-motile *S.* Typhimurium was first described in the late 1987 in chicken carcasses in Portugal (Machado, 1990), in the 1990s it was spread in Spain pointing pork as the original source (Echeita et al., 1999). The atypical *S.* Typhimurium serovar was named monophasic variant of *S.* Typhimurium and was identified by the antigenic formula: 1,4,[5],12:i:-. The monophasic variant is characterized by the lack of expression of the phase 2 flagellar antigen (H2), due to partial or complete deletion of the *fljBA* locus, or due to different mutations in *fljA*, *hin* and the promoter controlling the expression of *fljB* and *fliC* (Cadel-Six et al., 2021; Sun et al., 2020).

In the pig sector, *Salmonella* is present, persists and spreads in farms and lairage environments, and an appropriate cleaning and disinfection regimes must be implemented. The preferred class of disinfectants appears to be peroxygen-based products (Gradel et al., 2004) although other disinfectants such as glutaraldehyde, quaternary ammonium compounds (QACs), iodine-based compounds, and chlorocresols have been reported to have good performance (De Lucia & Ostanello, 2020). Despite the intensive disinfection, *Salmonella* can be introduced in farms through faecal contamination of feed, drinkers, or farm equipment by rodents, wild birds, insects, and pets (dogs and cats) which represent other possible ways for the introduction and transmission of *Salmonella* to pigs (Zamora-Sanabria & Alvarado, 2017). Pigs can be *Salmonella* reservoirs since there are latent and undetectable infections (asymptomatic), that for example under the stress of transport of the pig to the slaughter, start shedding *Salmonella* and contaminate healthy animals and transport trucks (Hurd et al., 2004). Contamination and cross-contamination of *Salmonella* in slaughterhouses and processing pork facilities can succeed regardless periodical intensive cleaning and disinfection procedures (Soliani et al., 2023).

3.2.2. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, facultative anaerobic, rod-shaped and non-spore-forming bacterial species from *Listeriaceae* family, which is divided in two genera *Listeria* and *Brochothrix*. *Listeria* genus has been classically divided into seven species: *L. innocua* and *L. murray*, classified as non-pathogenic, *L. seeligeri*, *L. ivanovii* and *L. welshimeri*, which have rarely caused

human infection, and *L. monocytogenes*, related to human infection. Since 2010, the genus *Listeria* has had the addition of 22 new species, sixteen of these are distantly related to *L. monocytogenes* species, and several of these present phenotypes that distinguish them from classical *Listeria* species (Orsi et al., 2024).

L. monocytogenes is divided in four lineages (I, II, III, and IV) and 13 serotypes that are present in different and overlapping niches. Lineage III and IV strains are predominantly isolated from animal sources whereas lineage II strains are common in foods and lineage I strains are common in human clinical cases, thus including 1/2a, 1/2b and 4b serotypes that represent the 90–95% of the human disease cases (Radoshevich & Cossart, 2018; Schiavano et al., 2021). Lineage II strains have a high rate of horizontal gene transference, in comparison to lineage I strains, which facilitates the adaptation to the environment for instance in the acquisition of plasmids that carry resistant genes to toxic metals or to be resistant to bacteriocins or environments where bacteriocinogenic compounds are present (Orsi et al., 2011).

The genetic diversity of *Listeria* is high as the conditions for the genus survival and growth in different environments are multiple. *Listeria* is encountered in a wide range of habitats (e.g., soil, water and animal digestive tracts) and can grow in aerobic and anaerobic conditions (Kurpas et al., 2018; Lagarde et al., 2024; WHO, 2018), which makes food industry to be specific to the detection of the foodborne *L. monocytogenes* instead of *Listeria* spp. The optimal conditions of temperature and pH are 37 °C and 7 while the limits of growth of *L. monocytogenes* are at temperatures between −0.4–45 °C, pH range between 4.4–9.4 and a_w > 0.92. However, this pathogenic microorganism can survive for long periods in foods and other substrates at freezing, refrigeration and room temperatures (e.g., at −18 °C for more than 6 months in minced meat or at 4.4 °C for more than 6 weeks days in a Summer sausage vacuum packed with a pretreatment of 18h at 37 °C and final pH of 4.8–5.1) (ICMSF, 1996). This ubiquitous microorganism can survive at extreme environmental circumstances of temperature (from −18 to 50 °C), pH (from 4.6 to 9.2) and water activity (i.e., a_w) (from 0.90 to 0.99) (ICMSF, 1996; Välimaa et al., 2015).

This microorganism is the causative agent for listeriosis, a foodborne zoonosis that affects both animal and humans, producing clinical symptoms of diarrhoea, fever, headache, and myalgia after a few days of pathogen ingestion if it is non-invasive, or septicaemia, abortions, meningitis and rhombencephalitis if it is invasive and reaches high-risk people (e.g., pregnant woman, elderly people or infants) (WHO, 2018). Listeriosis was the fifth most commonly reported zoonosis in humans in the European Union in 2022 and one of the most serious foodborne diseases, which accounts for the highest mortality rate within the foodborne pathogens under EU surveillance. In the EU there were 2,738 cases of listeriosis, 1,330 hospitalizations and 286 deaths related to *L. monocytogenes* in 2022, with 35 strong evidence outbreaks where pig meat and products thereof (5) and fish and fish products (4) were the implicated food vehicles (EFSA and ECDC, 2023). According to the last One Health 2022 Zoonoses report, Spain is one of the European countries reporting more cases of listeriosis after Germany and France, confirming a total of 437 listeriosis cases in 2022 and oscillating values from 2018, 2019, 2020 and 2021 were 370, 504, 191 and 355 confirmed listeriosis cases were confirmed, respectively (Table 3).

The main transmission route of the pathogen to humans is by contaminated food, pointing to ready-to-eat products from animal origin (Henriques et al., 2017; Kurpas et al., 2018), since pasteurization or thoroughly cook processes would kill the pathogen. *L. monocytogenes* may occur in raw and processed foods that are contaminated during and/or after processing (Ricci et al., 2018). Afterwards, the pathogen can survive and grow in foodstuff stored at refrigeration temperatures and in vacuum or atmosphere modified packages as a result of its facultative anaerobiosis. The ability to form biofilms in industrial food processing surfaces where food is in contact is notable, adhering to stainless steel or polystyrene (Mahoney et al., 2022; S. Silva et al., 2008) and persisting for several months or years (E. P. da Silva & De Martinis, 2013; Orsi et al., 2008). Once the biofilm is formed, bacterial cells are protected from harsh cleaning and disinfection procedures and may result in cross-contamination when food contacts the biofilm (Carpentier & Cerf, 2011).

The ubiquitous nature of *L. monocytogenes* makes it to be found all along the pig and pork production chain. The pathogen has been found in pens of pig farms, rarely found in live pigs from rectal tonsil scrapings (0.3%) but sampling the carcasses of the same pigs after slaughtering increased prevalence (4.1%) was observed, suggesting *L. monocytogenes* contamination during the slaughtering (Hellström et al., 2010; Kanuganti et al., 2002). The bacteria can also remain in the intestinal track of the pig, becoming a *L. monocytogenes* reservoir, without manifesting symptoms of disease (Stein et al., 2018). After pig slaughtering and evisceration, the intestinal microbiota increases the pathogen prevalence in surfaces (24%) and is reduced (*ca.* 9%) after cleaning and disinfection procedures (Cherifi et al., 2020; Meloni et al., 2013). Several studies have reported high prevalence (from 1 to 33%) of *L. monocytogenes* in pig carcasses after slaughtering in Europe (Hellström et al., 2010; Meloni et al., 2013). Raw meat is an important source of contamination, mainly associated with the contamination of working environments and equipment, where the most contaminated zones are the reception of raw materials and refrigeration and the processing rooms. The prevalence of *L. monocytogenes* in minced meat intended to be processed ranges between 16% and 50% and can also be contaminated through the cross-contamination with work surfaces and equipment (Chasseignaux et al., 2002; Thevenot et al., 2006). For instance, the prevalence *L. monocytogenes* was studied at small-scale Spanish factories producing traditional fermented sausages and 16.9% of the samples tested positive for the pathogen: 11.8% samples of the equipment (i.e., mincing, mixing, and stuffing machines, cutting tables, knives, and cold rooms), 28.9% of the raw material (i.e., pork meat batters and casings) and 15.8% of the DFS. In this case, the *L. monocytogenes* positive samples were in compliance with the EC 2073/2005 regulation (i.e., *L. monocytogenes* < 100 CFU/*g*, n=5, c=0) (EC, 2005; Martin et al., 2011). Good practices of hygiene, cleaning and disinfection products (i.e., chlorine substances and quaternary ammonium compounds) can reduce the persistence in surfaces and the cross-contamination, impacting directly to the food safety risk (Meloni, 2015). The implementation of Good Hygienic Practices (GHP) and Good Manufacturing Practices (GMP) as well as implement a food safety management system based on the principles of Hazard Analysis Critical Control Points (HACCP) is crucial to reduce the hazard and should be implemented by food manufacturers following the "Guidelines on the Application of General Principles of Food Hygiene to the Control of *Listeria monocytogenes* in Foods" set by Codex Alimentarius (FAO,

2007). Food manufacturers should also test, validate, and verify the correct functioning of their hygiene control measures and HACCP procedures.

4. Product notification and outbreaks linked to DFS consumption

In the EU, there were 48 alert notifications in the Rapid Alert System for Food and Feed (RASFF, (European Commission, 2023)) from 2020 to 2023 linked to pathogen detection in DFS, where 43 notifications were related to only dried sausages. According to the product, 7 were related to *fuet* (the country origin of 6 out of 7 alerts was Spain), 6 to *chorizo* (the country origin of 5 out of 6 alerts was Spain) and 12 to *salami* (the country origin of 8 out of 12 alerts was Italy, and only 1 was from Spain), so only these three products grouped more than the half of the DFS notifications (58%).

The most notifying country of alerts related to DFS in the EU during the 2020-2023 period was France (17), followed by Italy (6) and Germany (5) (Figure 6). On the other hand, the data described that the country of origin of the DFS products that registered the most alerts was Spain (13), followed by Italy (12) and France (9) (Figure 7). In that sense, *fuet* and *chorizo* from Spain and *salami* from Italy were the products that produced the most alerts, thus accounting for the 19 alerts out of 43 (44%).

From the 43 alerts, the pathogenic bacteria encountered were *Salmonella* spp. (26), *L. monocytogenes* (13), Shiga toxin *Escherichia coli* (STEC) (3) and *Campylobacter* spp. (1) (Figure 8). *Salmonella* was the major foodborne pathogen encountered (60%) in DFS, and when focusing to the products, *Salmonella* was the hazard found in 6 out of 7 notifications for *fuet*, in 4 out of 6 for *chorizo* and 5 out of 12 for *salami*. *L. monocytogenes* was found in 2 out of 6 for *chorizo* and 5 out of 12 for *salami*. The STEC was encountered in DFS made or containing beef meat, while *Campylobacter* spp., typically sampled from poultry meat, was found in pork meat DFS.

Out of the 43 notification alerts, four were classified as food outbreak (Refs. 2020.3378, 2021.0203, 2021.3787 and 2023.6367), two of them were from *Salmonella* spp. detected in *fuet* from Spain, one from *Salmonella* spp. detected in salami from Italy and one from *L. monocytogenes* detected in dry sausages from Italy. In that sense, *Salmonella* is the pathogen the most related to outbreaks while the other pathogens (i.e., *L. monocytogenes*, STEC and *Campylobacter*) are most related to product recalls and withdrawals of the product from the market.

In the USA, some European and Mediterranean style DFS have been linked to product recalls and outbreaks. According to the FSIS database, in 2011 there was a recall from Pennsylvania firm for Lebanon Bologna products due to possible *E. coli* O157:H7 contamination (FSIS & USDA, 2011). In 2021, two recalls of salami sticks (FSIS & USDA, 2021) and Italian style meats that were fermented and dried due to possible *Salmonella* contamination and suspicious to be under-processed.

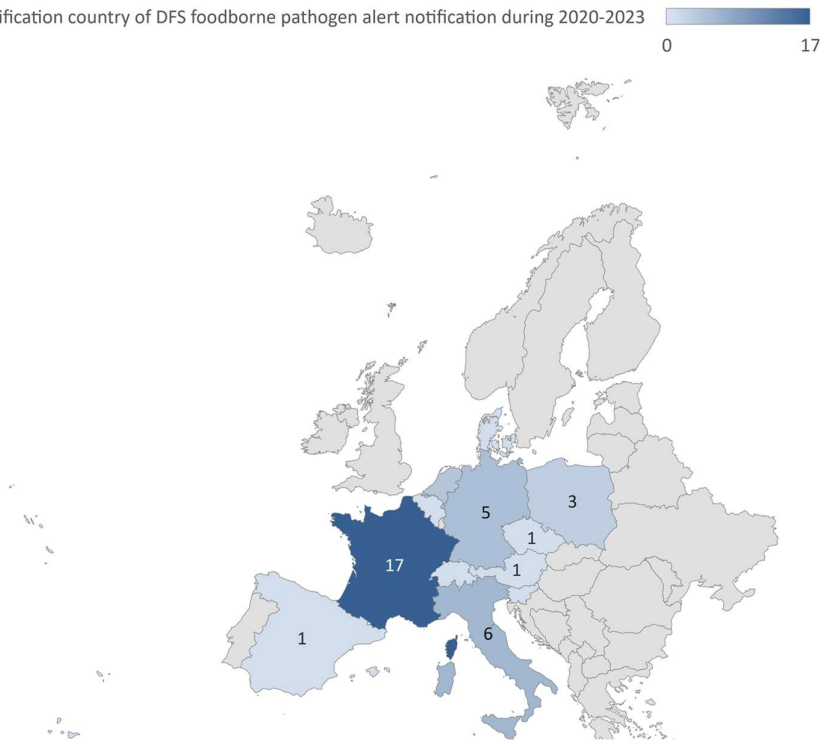

Figure 6. Country notifying alerts associated with DFS in the EU during the 2020-2023 period, data retrieved form RASFF (European Commission, 2023).

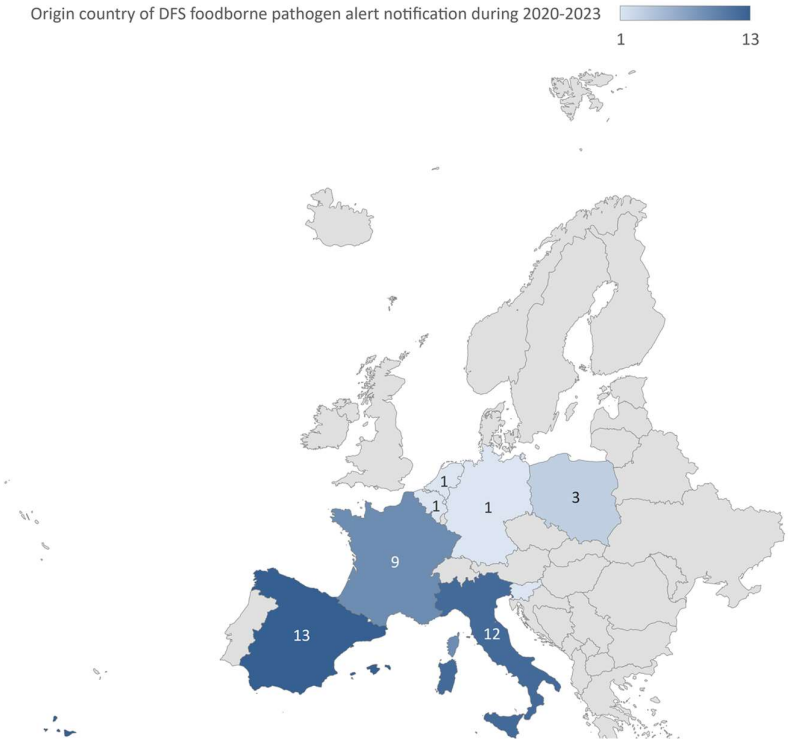

Figure 7. Country of origin of DFS causing alert notifications in the EU within the 2020-2023 period, data retrieved form RASFF (European Commission, 2023).

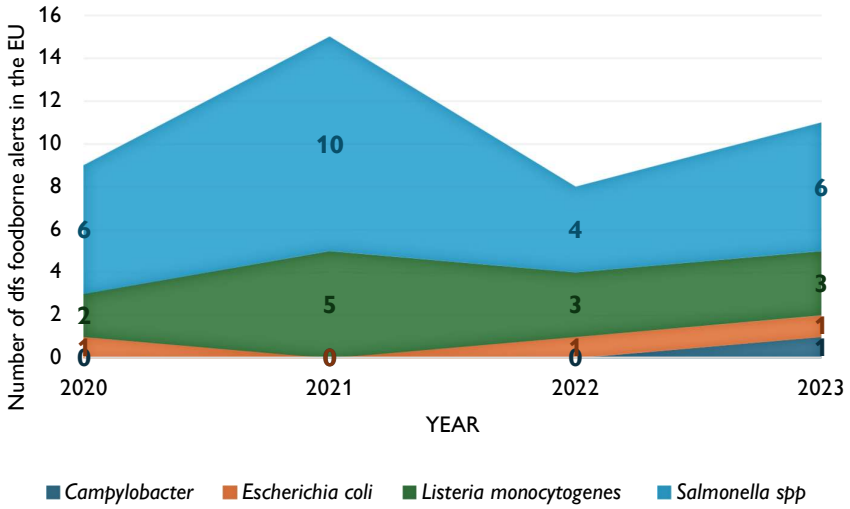

Figure 8. Distribution of the foodborne pathogen species involved in DFS alert notifications in EU during the 2020-2023 period, data retrieved form RASFF (European Commission, 2023).

5. Legislation regarding DFS food safety

The application of preventive measures in the framework of the Hazard Analysis and Critical Control Points (HACCP) is crucial to guarantee the food safety and consumers' health. Despite the efforts made by Food Business Operators (FBO) during quality controls checking and competent authorities during official samplings, two main pathogens (i.e., *Salmonella* spp. and *Listeria monocytogenes*) are periodically involved in recalls, withdrawals, and outbreaks. Regulations on the detection and levels of such pathogens have been established to control and limit its presence on meat and meat products. Regulation on microbiological criteria based on detection and/or enumeration of pathogens for products placed on the market (e.g., DFS) is particular for each country, making FBO to consider product exportation regarding the restriction of every specific regulation.

5.1. European Union

In the European Union, the Commission Regulation (EC) No 2073/2005 of 15 November 2005 (and subsequent modifications) on microbiological criteria for foodstuffs establishes the Food safety criteria and Process hygiene criteria requirements for foods placed on the market during their shelf life (EC, 2005).

Process hygiene criteria require a sampling of pig carcasses after slaughtering, dressing and before chilling. If aerobic colony counts between 4.0 to 5.0 Log CFU/cm^2 or *Enterobacteriaceae* between 2.0 to 3.0 Log CFU/cm^2 are detected in the samples, improvements in slaughter hygiene and review of process controls should be performed. The sampling plan for *Salmonella* (here as a hygiene indicator) establishes that 50 carcasses shall be derived and randomly selected from

10 consecutive sampling sessions, where *Salmonella* should not be detected in the area tested per carcass, allowing only 3 samples to pass the criteria limitation. In the case of unsatisfactory results, improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin, should be made.

Food safety criteria for DFS includes criteria for both *Salmonella* and *L. monocytogenes*. For *Salmonella*, DFS are classified in the "1.8 Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the *Salmonella* risk" food category. In products placed on the market during their shelf-life (i.e., DFS) *Salmonella* shall not be detected in 5 sampling units, 25 g each, following the analytical method established in the ISO 6579-1:2017 (ISO, 2017).

For *L. monocytogenes*, DFS are generally classified in the "1.3 Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" food category, due to DFS a_w is below 0.92. The criteria establishes that for products placed in the marked during their shelf-life the maximum level of *L. monocytogenes* is 100 CFU/g in each of the 5 samples analysed. The analytical method applicable is established in ISO 11290-2:2018 (ISO, 2018).

5.2. United States of America

In the United States of America (USA), the Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) established the zero-tolerance policy against *Salmonella,* Shiga-Toxin *Escherichia coli* (STEC), *L. monocytogenes* and *Staphylococcus aureus* (FDA, 2017) in ready-to-eat foodstuffs.

The last "Ready-to-Eat Fermented, Salt-Cured, and Dried Products" guideline (FSIS-GD-2023-0002) established that a 5-log reduction should be demonstrated for *Salmonella* and STEC during the production process of ready-to-eat fermented, salt-cured, and dried products (e.g., dry fermented sausage), while a 3-Log reduction for *L. monocytogenes* (a 5-Log reduction is more desired) is recommended. *S. aureus* has to be controlled through the degree/hour concept (FSIS, 2023).

It should be considered that USA standards are quite stringent and can be fulfilled by DFS elaborated in USA (considerably acid and usually submitted to a thermal treatment, see Table *1*) but can be difficult to accomplish by the low acid Mediterranean style DFS.

5.3. Canada

In Canada, regulation of dry fermented sausages depends on the meat type. For pork DFS, industry should validate through microbiological testing program that "their process will not result in the presence of *E. coli* O157:H7 or *Salmonella* in the finished product". In contrast, for beef dry fermented sausages industry should follow one of five options (i.e., heat process (1),

manufacturing process that achieves a > 5 Log reduction of the pathogens (2), test 30 samples (25 g/sample) for the absence of pathogens (3), manufacturing process that reduces 2 Log of the pathogen scientifically validated and test 15 samples of raw meat batter (25 g/sample) for the absence of the pathogen (4), manufacturing process validation through a challenge test the reduction of > 5 Log of the pathogen (5)) to control verotoxigenic *E. coli* O157:H7 or *Salmonella*. *L. monocytogenes* and other pathogens (i.e., *S. aureus*, *E. coli* O157:H7, *Salmonella* and *Clostridium botulinum*) have to be checked in the products only if degree-hours limit has been exceeded and the product intends to be distributed (Canadian Food Inspection Agency & Government of Canada, 2024).

5.4. Other standards

CODEX Alimentarius establishes the international principles for food safety. Regarding DFS, *L. monocytogenes* is controlled if pH < 4.4 or aw < 0.92, but in the case of unfit these criteria, microbiological criterion for ready-to-eat foods in which growth of *L. monocytogenes* will not occur describes a sample plan of 5 samples that should not overcome 100 CFU/g of the pathogen (FAO, 2007).

The control measures for *Salmonella* in pork is based on the good hygiene practices performed by manufacturers, early on the pig carcasses and pork, through the control of temperature that do not permit the pathogen growth (FAO & WHO, 2016).

International Organization for Standardization (ISO) is a global network of experts that develops and publishes standards for quality, safety and sustainability. The standard ISO23854:2021 (ISO, 2021) establishes microbial criteria for fermented meat products which is similar to those established by the Commission Regulation (EC) No 2073/2005 in relation to *Salmonella* spp. and *L. monocytogenes* (EC, 2005).

5.5. Methodologies used to validate DFS food safety

Challenge tests, mathematical modelling for predictive microbiology and information from the scientific literature are methodologies approved by the competent authority to validate that the pathogen lethality of the DFS production process ensures food safety of the commercialised product. In other cases, these methodologies can be used to demonstrate that the DFS will not support the growth of *L. monocytogenes* during shelf life (Buchanan et al., 2017).

Challenge tests are laboratory test studies where the pathogen of concern (e.g., *L. monocytogenes* cocktail) is artificially contaminated (100 CFU/g) to the food.

The methodology of challenge test is recognised and widely used to validate the safety of food products during the shelf life and the expected storage, usage, and distribution conditions.

The EC Regulation 2073/2005 establishes that FBO should perform challenge test studies that investigate the accomplishment of the regulation in the food products (especially those RTE)

when necessary. For instance, in the EU, the European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm) established the "GUIDANCE DOCUMENT to evaluate the competence of laboratories implementing challenge tests and durability studies related to *Listeria monocytogenes* in ready-to-eat foods" for FBO (Bergis et al., 2023). There is also an ISO standard (i.e., ISO 20976-1:2019) for performing growth (i.e., Part 1, Challenge tests to study growth potential, lag time and maximum growth rate (ISO, 2019)) and inactivation (i.e., Part 2, Challenge tests to study inactivation potential and kinetics parameters (ISO, 2022)) challenge tests.

Predictive microbiology is the science that estimates the microbial ecology to environmental responses (e.g., temperature, pH, water activity and time) through mathematical equations and models. Food predictive microbiology gives quantitative information on microbial behaviour (i.e., growth/no growth, if so growth potential, growth rate, time to environmental adaptation (lag)) in foods evaluating the perishability and food safety.

In DFS, mathematical modelling of the response of pathogenic bacteria to the hurdles applied (e.g., bacteriocinogenic LAB strains, storage time, temperature and pH) has been studied providing decision support tools to validate manufacturing processes and enhance food safety (Austrich-Comas et al., 2023; Hashemi et al., 2023; Serra-Castelló et al., 2021).

6. Sequencing technologies and genomic applications in food microbiology

The use of sequencing technologies and bioinformatic analyses allows the genomically characterization of the bacterial community of a sample (e.g., a food matrix) and of a specific bacterial isolate, by metagenomics and whole genome sequencing, respectively (Figure 9). Metagenomic studies can be divided in two categories, metataxonomic (also known as metabarcoding) and shotgun metagenomics, due to its objective and methodologies used. The metataxonomics approach and whole genome sequencing have been applied in the research conducted in this PhD thesis.

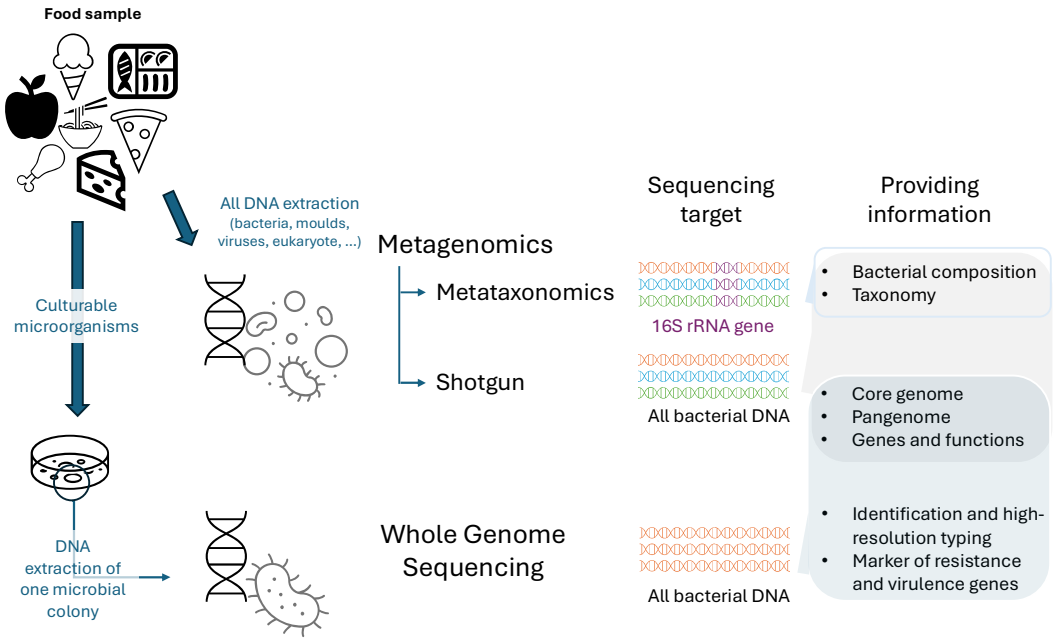

Figure 9. Genomic approaches commonly used in food microbiology.

6.1. Sequencing technologies

The technology based on nucleic acids (i.e., desoxyribonucleic and ribonucleic acids, DNA and RNA, respectively) is broad, thus comprising genomics and transcriptomics. For the last 40 years, DNA-based sequencing technologies have developed in three generation stages: first generation sequencing, starting in 1977 with Sanger method, second generation sequencing with pyrosequencing and sequencing by synthesis (SBS) technologies in 2005 and third generation sequencing by single molecule sequencing (SMS) in the 2010s. Second and third generation sequencing technologies are also called Next Generation Sequencing (NGS) technologies (Figure *10*) and the main high throughput sequencing (HTS) platforms are Roche 454, Ion Torrent, Illumina, MGI, Nanopore and PacBio (Table 4) (Satam et al., 2023; Quail et al., 2012).

First generation sequencing technology, usually called 'Sanger sequencing', was developed by Frederick Sanger and colleagues in 1977 and was based on the chain termination method by the addition of the modified di-deoxynucleotide triphosphates (ddNTPs)[4] instead of the deoxynucleotide triphosphates (dNTPs) during the polymerase chain reaction (PCR).

Second generation sequencing technologies can be divided in two major categories regarding the methodology used: sequencing by hybridization and SBS. The main second generation sequencing platforms are based on SBS methodology and can analyse billions of sequences at

[4] ddNTPs terminate the DNA strand elongation during PCR because these nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labelled for detection in automated sequencing machines.

the same time by massive parallel amplification. Sequencing by synthesis relies on the DNA immobilization on a solid substrate, where massive parallel sequencing produces high amounts of DNA sequences and nucleotide composition is reported by high-resolution detectors (Cao et al., 2017; G. Cardinali et al., 2017). There are four types of SBS second generation sequencing technologies: (a) pyrosequencing technology, (b) semiconductor technology, (c) bridge amplification approach and, (d) DNA nanoball approach (Figure 10). Pyrosequencing technology was developed by Marcel Margulies, Michael Egholm and colleagues in 2005 (Margulies et al., 2005), and then Roche 454 sequencing platform was developed using this approach (Petrosino et al., 2009). Pyrosequencing technology is based in two enzymatic reactions where the incorporation of dNTPs during the DNA elongation step can be quantitated through conversion of the released pyrophosphate into a light signal in real time. The consecutive reactions start when the dNTP is incorporated in the nucleotide sequence and the released pyrophosphate is used in a sulfurylase reaction releasing ATP that is subsequently used by luciferase to produce light. Semiconductor technology, from which Ion Torrent technology is based, was developed by Nader Pourmand, Miloslav Karhanekin and colleagues in 2006 (Pourmand et al., 2006), where the incorporation of a dNTP during the DNA elongation releases a hydrogen ion (H^+) in the media and a chemical potential or pH change is recorded by a chip. In 1997, Cambridge University scientists Shankar Balasubramanian and David Klenerman developed a methodology to observe the motion of a polymerase at the single molecule level as it synthesized DNA immobilized to a surface through fluorescently labelled nucleotides and the sequence of this fluorescence would decipher the nucleotide sequence. These scientists thought that the fluorescent labelling sequencing using the solid phase could be performed in parallel and massively, and funded Solexa start up to commercialize the technology which was further acquired by Illumina company in 2007. Bridge amplification approach was developed by Illumina platform and popped out in 2011 (Akintunde et al., 2023). In bridge amplification, DNA molecules are ligated to adapters whose complementary sequences are attached to the solid platform, then adapters are used as primers for the elongation step where the different dNTPs are fluorescent labelled and its fluorescence measured for nucleotide detection (Slatko et al., 2018). DNA nanoballs sequencing (DNBSEQ) approach was developed by Radoje Drmanac, Dennis G. Ballinger and colleagues in 2009 in Complete Genomics USA company (Drmanac et al., 2010), which was purchased by Beijing Genomics Institute and then MGI Chinese company developed MGI platform for this technology. The DNBSEQ technology consists of the fragmentation of the double stranded DNA (dsDNA), then adaptors are ligated, and DNA fragments are separated to single stranded DNA (ssDNA) molecules, which are then circularized and ligated. A prove hybridizes in the adaptors as a substrate for DNA polymerase and rolling circle amplification, generating a DNA nanoball (DNB) which is negatively charged and loaded to a solid glass chip with positively charged spots. Each DNB will be loaded to one positively charged spot and then sequenced through SBS methodology using labelled dNTPs. Apart from the latest developed DNBSEQ technology, second generation sequencing is based in short-read (i.e., 100-300 base pairs in length) sequencing approaches that, for example Illumina platform, provide lower-cost, higher-accuracy data that are useful for population-level research and clinical variant discovery and have been the mainly used approach (Table 4). Illumina sequencing is the

platform that has been used in this PhD thesis, for metataxonomics and whole genome sequencing.

Third generation sequencing aims to sequence long DNA molecules (i.e., 10-50 Kilo bases in length) and is based on single molecule sequencing at real-time (SMRT) set in the early 2010s by Pacific Biosciences (i.e., PacBio) (A. Yang et al., 2020). PacBio sequencing technology has a modified DNA polymerase attached to the bottom of a well, where the fluorescent labelled dNTPs are added to the new DNA strain and the fluorescence is registered. In that sense, PacBio is classified as a SBS sequencing technology. In contrast, Oxford Nanopore technology which some scientists classify as fourth generation sequencing, is not a SBS technology since the ssDNA passes through a protein channel and the sequence detection is performed by measuring electrical impedance (Slatko et al., 2018). Third generation sequencing provides long-read sequence data that are well suited for *de novo* genome assembly applications and full-length isoform sequencing (Goodwin et al., 2016).

All these sequencing technologies have been developed since 1977 leading to the two first published bacteria genome sequences in 1995 by Fleischmann et al. (1995) and Fraser et al. (1995), and in 1996 the first sequenced complete eukaryotic cell (A. Yang et al., 2020). Sequencing technologies costs have also been reduced as new technological advances were succeeding, leading to wide and international projects such as the "Human microbiome project" set in 2008 and still going on.

Nowadays, sequencing technologies are becoming cost-affordable for many research and public health centres, leading them to implement sequencing technologies for genomic or transcriptomic studies at, for instance, research projects and microbial outbreak investigations. Sequencing technologies together with bioinformatic analysis of the nucleotide sequences permit gene identification and whole genome reconstruction of bacterial cells, that are further developed in the next sections.

Table 4. Summary of the Next generation sequencing platforms. Table modified from Akaçin et al. (2022).

Sequencing platform	Methodology	Sequencing technology	Initial PCR amplification	Length of the reads (bp)	Speed	Pros	Cons
Roche 454	Pyrosequencing	Sequencing by synthesis	Emulsion PCR	Medium (400–1000)	Medium speed, up to 8h	Fast run time. Longer read length. Small data files.	Reagent cost is high. InDels in homopolymers.
Ion Torrent	Electrochemical change in the media	Sequencing by synthesis	Emulsion PCR	Short (200–400)	Fast, up to 4h	Equipment cost is low compared to TGS platforms. Fast run time.	Loose of signal in homopolymers. Short read length.
Illumina	Bridge amplification	Sequencing by synthesis	Bridge PCR	Short (36–300)	Fast, up to 4h	High sequencing depth and yield	Overlapping signals in overcrowded samples. Equipment cost can be expensive.
MGI	DNA nanoballs	Sequencing by ligation	Amplification by Nanoball PCR	Short (50–150)			Multiple PCR cycles.

Table 4 (continuation)

Nanopore	ssDNA passing through a protein channel	Sequence detection through electrical impedance	No PCR	Long (10,000–50,000)	Fast (1 bp/ns) 1 min-72 h	Fast run time. Long reads. Inexpensive. Selective sequencing. Portable. Rapid and accurate identification with PCR-free metagenomics, can reach greater sequencing depths, and was specifically reported to be more effective with species located in well-referenced databases.	High error rates. Sensitivity of nanopores changes depending on the sample and the environment.
PacBio	DNA elongation through a DNA polymerase fixed in a solid platform	Sequencing by synthesis	PCR amplification while sequencing	Long (10,000–16,000)	Not very fast (up to 4h)	Long-read technology. High accuracy and sensitivity. Run time is fast. Efficient for metabarcoding of complex samples.	Low throughput and low flow cell success Low yield at high accuracy. Equipment cost is high. Complex sample preparation protocols and lower throughputs.

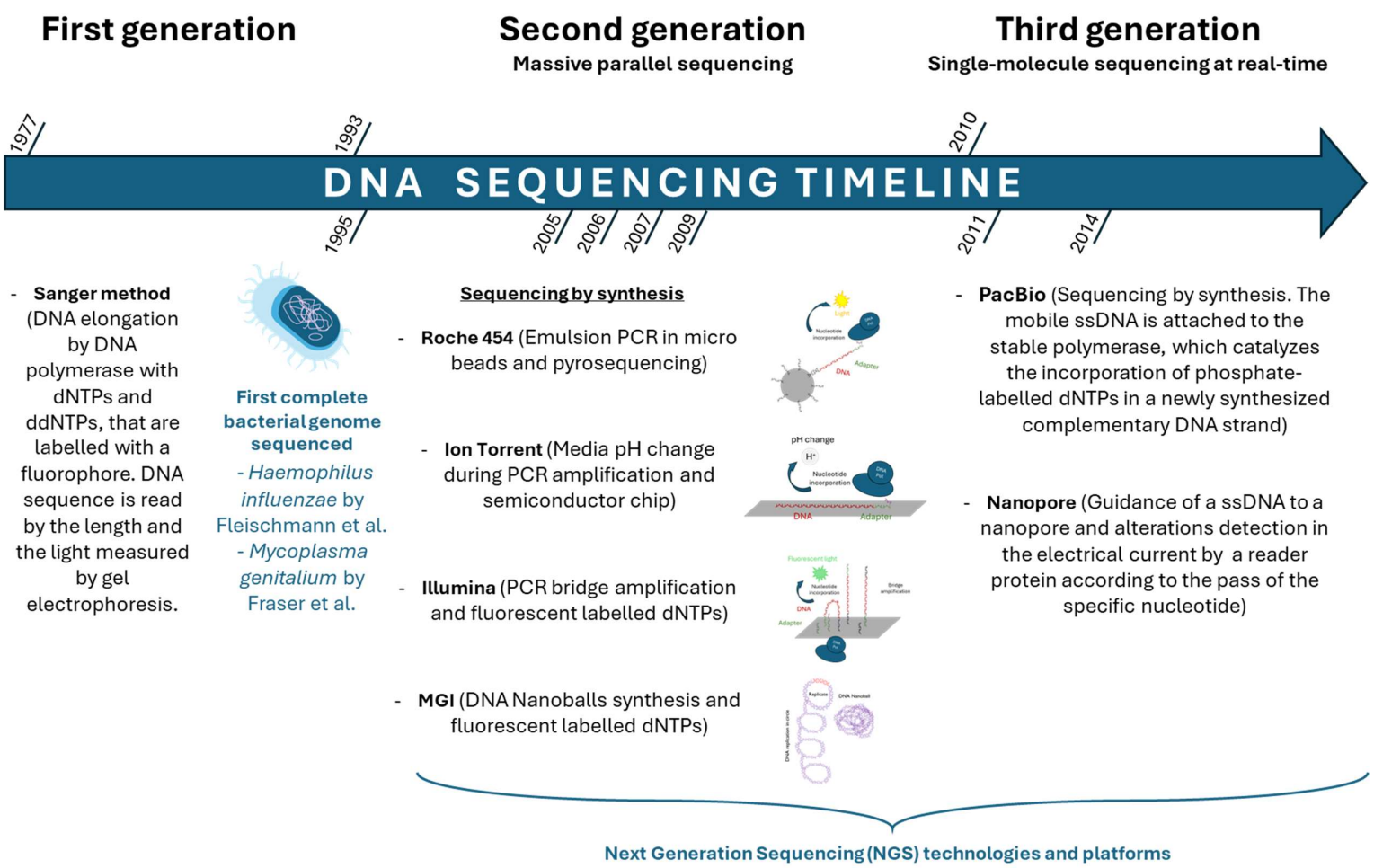

Figure 10. Timeline of the DNA sequencing technologies. Data retrieved from Athanasopoulou et al. (2021); Fleischmann et al. (1995); Fraser et al. (1995); Heather & Chain (2016); Land et al. (2015); Yang et al. (2020).

6.2. Metataxonomics

Metataxonomics (also known as metabarcoding) approach consists in the targeted sequencing of marker genes, usually the highly conserved ribosomal genes in microorganisms (e.g., 16S rRNA gene in bacteria and nuclear ribosomal internal transcribed spacer (ITS) in fungi) (Kõljalg et al., 2013). The 16S rRNA gene is constitutive in any bacteria and its sequence consists of nine hypervariable regions (named from "V1" to "V9"), that harbour the phylogenetic information of the gene, separated by highly conserved regions (Yang et al., 2016). Culture-independent studies of microbial populations were pioneered in the Pace lab in 1985 (Pace et al., 1985, 1986) and continued through improvement sequence alignments and developing new tools for 16S rRNA gene analysis. For instance, UniFrac tool was developed for comparing microbial communities by Lozupone & Knight in 2005 (Lozupone & Knight, 2005) and the Ribosomal Database Project was set and leaded by Cole et al. in 2008 to create a curate database of rRNA sequences (Cole et al., 2009). Specifically, the 16S rRNA gene (i.e., sixteen Swedberg unit or small subunit of the ribosomal RNA gene) is found in all bacteria and accumulate mutations at a slow and constant rate over time. Highly variable portions of the 16S rRNA sequence provide unique signatures to any bacterium species and useful information about relationships between them (M. Kim et al., 2011; Woese, 1987; Xu, 2004).

Metataxonomics is the most used approach in food microbial ecology and is useful to provide an overall taxonomic picture of the microbial community (Sequino et al., 2022). Metataxonomics allows the culture-independent identification of microbial communities, from food and environmental samples and clinical specimens, of non-culturable and difficult to detect foodborne disease-causing agents (Miller et al., 2013). The 16S rRNA gene sequencing and sequence identification is performed through, usually, second generation sequencers that produce short reads with high accuracy and coverage[5]. Additionally, the variable regions V3-V4 are highly recommended for bacteria identification and discrimination (M. Kim et al., 2011) and recommended by Illumina sequencing technologies (Jeong et al., 2021).

The usefulness of metataxonomics implementation within the food industry is for microbiome mapping in the processing plant environment, microbial source tracking investigation or for monitoring the product shelf-life, identifying the presence of microbial spoilers and how processing/storage conditions may affect microbial dynamics (Sequino et al., 2022).

The most studied fermented foods for metataxonomic studies have been conducted on dairy and fermented milks, beverages and vegetables (De Filippis et al., 2017), while they have also been conducted in meat matrixes and dry fermented sausages. For instance, Garofalo et al. (2024) studied the bacterial composition during the fermentation of Italian salamis with saturated fatty acids reduced by substituting pork fat by avocado pulp, Pini et al. (2020) studied the bacterial composition of Cinta Senese salamis formulated with grape seed and chestnut

[5] The coverage or depth of a sequencing read states for the number of times that a known nucleotide will be "covered" of amplified.

extracts and Yang et al. (2023) studied the bacterial composition of sheep meat based DFS formulated with cranberry powder.

Bioinformatic analysis

Bioinformatic analysis need to be performed on the results obtained from the sequencers, after base-calling[6] to evaluate and characterize the microorganisms present in the sample. Large and heavy files (i.e., ~1 GB) are generated after sequencing with the nucleotide sequences and its related information. These files are called raw reads and its format is FASTQ or BAM.

Commonly the results retrieved from sequencing platforms or after sequencing are raw reads that contain the nucleotide sequences results with the sequencing information in FASTQ or BAM format files. First, raw reads should be checked for sequencing quality with the FastQC software (Linux environment), then should be pre-processed by trimming and quality checked again to control that the trimming step have improved the quality of the sequences. Pre-processing consists in discarding the low-quality sequences and cut the sequencing adapters and other extra sequences that have been added during library preparation of the DNA sequences, ligated to the template DNA before sequencing. There are many tools for trimming reads and removing adapters, such as Trimmomatic, cutadapt or fastp. After quality checking and pre-processing the raw reads, specific analyses are conducted depending on the samples and genomic studies to be conducted.

For metataxonomic studies, various bioinformatic software have been developed such as QIIME2 or DADA2, which throughout the pipeline several steps are taken for filter/trimming, learn error rates, merge pair-end reads, taxonomy assignation with GreenGenes or SILVA data base. Taxonomy assignation databases link the sequencing data with the taxonomic identifiers, called operational taxonomic unit (OTU) or amplicon sequence variant (ASV), obtaining PHYLOSEQ files that are used for biostatistics and graphic representation.

Statistical analysis of microbial diversity

Phylogenetic relationships and diversity indexes (i.e., alpha and beta diversity indexes, Table 5) can be calculated to exploit the degree of divergence between different 16S rRNA bacterial sequences and to explore the composition of the bacterial community, respectively. Concretely, phylogenetic relationships and diversity indexes can be calculated for metataxonomic and shotgun metagenomic studies. Bacterial taxonomic classification (i.e., phylum, class, order, family, genus, and species) can be performed due to the divergence of the 16S rRNA gene.

Within diversity indexes, alpha diversity permits the determination of the bacterial richness and proportion within a sample (i.e., how many different bacterial species are in whole bacterial community of a sample and the weight of each in the total sample). Alpha diversity metrics are

[6] Base calling is the process of assigning nucleobases to chromatogram peaks, light intensity signals, or electrical current changes resulting from nucleotides added in the elongation step of the DNA polymerase or passing through a nanopore.

obtained from different indexes such as Chao1, Shannon and Simpson. Chao1 index calculates the richness of a sample, which is the number of taxa (i.e., number of OTUs or ASVs) observed (Callahan et al., 2017). Shannon and Simpson indexes are estimators of taxa diversity, combining richness and evenness. Shannon index places a greater weight on taxa richness than Simpson index, which considers taxa evenness more than taxa richness in its measurement and varies from 0 to 1, increasing the index while diversity decreases (Kim et al., 2017).

The beta diversity permits the comparison of the bacterial community composition between different samples and this information is very useful to compare the resemblance of the bacterial composition in different environments. Beta diversity metrics include Bray-Curtis Dissimilarity (BD), Jaccard Distance, Aitchison and UniFrac Distance indexes. BD index measures the compositional dissimilarity between the microbial communities of two samples based on counts on each sample, ranges between 0 (the two samples share all taxa) and 1 (the two samples do not share any taxa) and is computed pairwise between all samples (Legendre & Legendre, 2012). The Jaccard index is the ratio between the number of members that are common between the two samples and the number of members that are distinct; it is a measure of similarity for the two communities and ranges between 0 (the communities are different) and 1 (the two communities are identical). UniFrac index considers the phylogenetic tree and thus phylogenetic distances between community members (Kers & Saccenti, 2022; Lozupone & Knight, 2005). Aitchison index metric corresponds to Euclidean distances between centred log ratio (i.e., CLR) transformed sample abundance and proportional vectors (Egozcue & Pawlowsky-Glahn, 2019).

6.3. Shotgun metagenomics

Shotgun metagenomics sequences the whole genomic content of the sample, providing the taxonomic composition of the whole microbial community (i.e., bacteria, fungi, archaea and protozoa), plus the description of its functional capability, identifying the presence and abundance of specific genes of interest (e.g., genes involved in spoilage activities, antimicrobial resistance or virulence) (De Filippis et al., 2018; Jagadeesan et al., 2019). Regarding the sequencing platform and sequences length, shotgun metagenomics technique involves small size fragmentation (*ca.* 300 to 500 bp) of genomic DNA and frequently preamplification, while long-read sequencing utilizes much larger genomic fragments (*ca.* 30 Kbp) and does not require preamplification (Billington et al., 2022).

As an example, the methodology followed for conducting shotgun metagenomics starts by the fragmentation of the whole DNA or RNA (after the synthesis of complementary DNA) by enzymatic or mechanical methods, addition of adaptors in the DNA fragments, and then sequencing of these DNA fragments without targeting specific regions. Bioinformatic analysis then reconstructs the genomes found, by trimming and annotation of the sequences found in the sample.

Table 5. Diversity indexes for metagenomic studies.

Diversity index	Definition	Metric index	Definition	Reference
Alpha	Microbial diversity assessment within an individual sample	Chao1	Calculates the richness of a sample regarding the number of taxa observed	Chao, 1984
		Shannon	Estimates the taxa diversity, combining richness and evenness, considering more taxa richness than evenness. The higher the value, the highest the microbial diversity.	Kim et al., 2017
		Simpson	Considers taxa evenness more than taxa richness in its measurement and varies from 0 to 1, increasing the index while diversity decreases.	Kim et al., 2017
Beta	Microbial diversity assessment across all samples	Bray-Curtis Dissimilarity (BD)	Compositional dissimilarity between two sample microbial communities based on counts on each sample. Range between 0–1 (i.e., 0: the two samples share all taxa and 1: the two samples do not share any taxa).	Legendre & Legendre, 2012
		Jaccard Distance	Measures the similarity for two sample bacterial communities and ranges between 0 (the communities are different) and 1 (the two communities are identical).	Kers & Saccenti, 2022
		Aitchison	Takes into account the Euclidean distances of CLR transformed samples regarding the proportional abundances of the bacterial communities between samples.	Egozcue & Pawlowsky-Glahn, 2019
		UniFrac Distance	Takes into account the phylogenetic distances between community members.	Kers & Saccenti, 2022

A high sequencing coverage[7] is highly recommended in shotgun metagenomics to overcome errors in the base-calling and assembly bioinformatic steps (Bergholz et al., 2014). The assembly bioinformatic steps in shotgun metagenomics can be referenced to an already sequenced bacterial species and use it for genome comparison, while *de novo* assembly has allowed the identification of new bacterial species. The reconstruction of microbial genomes directly from metagenomics reads (Metagenome-Assembled Genomes, MAGs) is a promising approach to track specific microbial strains across the food chain, as well as for epidemiological purposes (Sequino et al., 2022).

In comparison to metataxonomics, shotgun metagenomics is more expensive than metataxonomics as is giving much more information and higher sequencing coverage (i.e., deep sequencing) is required to avoid sequence errors and to reconstruct whole or partial metagenomes. The benefits of shotgun metagenomics include no amplification bias, greater specificity of identification and representation of diversity, and ability to detect organisms from different kingdoms. According to the objectives of the study, the assessment of the taxonomic diversity of the community, gene prediction and functional annotation, or associating community data with a particular phenotype information may be included (Kovac et al., 2017). One of the benefits for pathogenic or non-pathogenic bacteria identification through shotgun metagenomics is the identification of bacteria at the species and subspecies level, and the presence of toxin genes (e.g., in STEC or *Bacillus cereus*). On the other hand, the shotgun approach has also been applied in food matrix samples for the detection of antibiotic resistance genes, mobile genetic elements, genes encoding secondary metabolites and antimicrobial compounds such as bacteriocins.

Food matrixes contain a high microbial diversity, which may reduce the benefits of using shotgun metagenomics in food samples. Some of the disadvantages of shotgun approach are that only dominant genomes are well represented, is less sensitive for pathogen detection than metabarcoding sequencing, a high proportion of taxonomically uninformative sequences are discarded, and large volumes of data can be generated that require high-performance computational tools for analysis and storage. When high concentration of host DNA is expected or the target is a low-abundance or rare taxa, then a higher throughput sequencer may be required, and moderate-to-high technical bioinformatic and scientific expertise required (Billington et al., 2022).

Bioinformatic analysis

For metagenomic studies, high quality reads are assembled obtaining contiguous reads (i.e., contigs) and after binning, metagenome-assembled genomes (MAGs) which are typically thought to represent the average, composite genome of a population present in a sample (C. Yang et al., 2021). These high-quality reads, contigs or MAGs are mapped to reference libraries or genomes

[7] Sequence coverage (or depth) is the number of reads that include a specific nucleotide position in the reconstructed sequence. Deep sequencing refers to the general concept of aiming for high number of unique reads of each region of a sequence.

of external references to obtain the OTUs or ASVs. Taxonomy assignation is performed as metataxonomic studies and further detailed in section 6.2 Bioinformatic analysis, using packages such as Kraken2 or MetaphiAN (Ye et al., 2019).

6.4. Whole Genome Sequencing

Whole genome sequencing (WGS) technology characterizes the whole genome content of a single cultured bacterial isolate, including the chromosomal and extrachromosomal DNA (e.g., mobile genetic elements such as plasmids) (Jagadeesan et al., 2019). In comparison to the shotgun metagenomics, WGS also retrieves the genetic information regarding gene functionality and species and subspecies identification from only one single bacterial isolate and not the genetic information from the whole DNA found in the food matrix. WGS technology is increasingly replacing traditional microbial typing and characterisation techniques (i.e., Pulse Field Gel Electrophoresis (PFGE)), providing faster and more precise answers (Miller et al., 2013). From 2011 onwards, WGS has been adopted by public health surveillance in at least four countries, these are the United Kingdom, Denmark, France and USA (Allard et al., 2016; Ashton et al., 2016; Jackson et al., 2016; Kvistholm Jensen et al., 2016; Moura et al., 2016).

The information retrieved from WGS is diverse and includes information about resistance/virulence/stress genes and genomic information from highly and low conserved regions. This information allows the complete genomic characterization of a single strain, that can be compared with other bacterial isolates and provide information of genetic variants between them. WGS allows the inference of phylogenomic relationships between bacterial isolates that can be used for the identification of clonally related strains. To study the phylogenomic relationships between isolates or comparative genomic studies, the access to the core and accessory genome nucleotide content of the bacteria is needed. The core genome (cg-) comprises hundreds or thousands of highly conserved genes within the species of study and the accessory genome comprises the variable regions of the bacterial genome. Each information provides data to describe the evolution of a strain (at allele and single point mutations (SNPs) level) and the adopted strategies for survival. This information is analysed in whole genome (wg-) sequencing studies.

The benefits of application of WGS in the food industry to get safer products are magnified in a pathogen or spoilage contamination event. WGS allows to distinguish one bacterial species from another at a very resolutive level (strain or isolate) and thus makes it a perfect tool for tracking and tracing microorganisms along the food chain, helping to identify sources of contamination and the transmission route (Rantsiou et al., 2018). Moreover, one of the main goals of surveillance programs are to identify, control and prevent foodborne outbreaks, determine the causes of foodborne disease, and monitor trends in occurrence of foodborne disease, hence WGS suitable fits to this aim (WHO, 2008). The coronavirus disease 2019 (COVID-19) pandemic marked a breakpoint for genomic surveillance where the speed of data sharing and pathogen characterization was unprecedented and crucial for the development of

countermeasures (e.g., the first diagnostic assay was made available on 13 January 2020 (Corman et al., 2020; WHO, 2024)) (Carter et al., 2022). After that, the world health organization established guiding principles for pathogen genome data sharing to protect global public health (i.e., preventing, detecting, and responding to epidemics and pandemics at national and international levels) (WHO, 2022). One example of the WGS use and benefits in food safety management occurred recently, from February to April 2022, in the EU/EEA (i.e., European Economic Area) and United Kingdom, where one international outbreak of multidrug-resistant monophasic *Salmonella* Typhimurium associated with chocolate products took place. In this outbreak, the food contamination source could be identified when comparing *S.* Typhimurium strains from clinical and industrial isolates, which resulted in the same genomic sequences. Traceback investigations could identify the industrial surface where the pathogen resided and corrective measures could be taken by the FBO (Larkin et al., 2022).

Bioinformatic analysis

Bioinformatic workflow and main software used in WGS are summarized in Table 6. In WGS, sequences can be used to perform *de novo* genomic assembly, for instance Velvet bioinformatic tool is indicated for short read sequencing technologies (e.g., Illumina) and SPAdes is indicated for both short and long sequencing reads resulting from Illumina, PacBio or Nanopore. If *de novo* assembly is not required, sequences can be mapped by an available reference genome using, for example, Bowtie2 tool which is an ultrafast and memory-efficient tool for aligning sequencing read to long reference sequences. Then, to perform genome annotation, Prokka tool is indicated, this tool annotates bacterial, archaeal, and viral genomes quickly and produce standard-compliant output files. Finally, for variant or SNP calling, SRST2 and BFCtools or SAMtools, and Snippy are indicated. These bioinformatic tools are designed to take sequence data and a known database to report the differences of the annotated genome to a reference genome. From WGS sequences, there are web-based tools and publicly available databases that identify the genes of interest by aligning draft genomes to a gene database. For instance, the genome data can query to predict virulence profiles, stress response, biofilm formation, antimicrobials and biocide resistance traits. Some tools to perform bioinformatic analysis of phylogenetics and phylogenomics (e.g., FastTree and RAxML), mobile element detection (e.g., PlasmidFinder and SPIFinder), virulence and resistome analysis (e.g., PlasmidFinder and VirulenceFinder) and visualization (e.g., PHYLOViZ). Other bioinformatic programs, such as BioNumerics and Ridom SeqSphere+, have integrated diverse functions to quality control, genome assembly, reference mapping, SNP calling, wg-Multi Locus Sequence Type (MLST) and cgMLST phylogenetic analysis (Jagadeesan et al., 2019). It is important to mention that genomic information does not necessarily translate into gene expression and the phenotypic characteristics should be studied in parallel (Jagadeesan et al., 2019; Rantsiou et al., 2018). In that sense, WGS data may be used for phenotypic prediction regarding the genetic results therefore many disciplines including predictive food microbiology are likely to benefit from WGS and metagenomics and finally improve food safety and consequently public health (Miller et al., 2013).

Table 6. General bioinformatic pipeline and software for whole-genome sequencing data analysis.

Content (file type)	General step	Tool	Reference
Raw sequencing reads (.fastq or .bam)	**Quality control and trimming**	**FastQC**: Sequence quality control **Trimmomatic**, **cutadapt**, **fastp**...: Remove the adapters and other sequences added during library preparation	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ Bolger et al., 2014 Martin, 2011 Chen et al., 2018
Clean sequences (.fastq or .bam)	***De novo* assembly**	**Velvet**: De novo genomic assembler specially designed for short read sequencing technologies **SPAdes**: De novo assembly toolkit containing various assembly pipelines and taxonomic identification of bacterial reads	Zerbino & Birney, 2008 Bankevich et al., 2012
	Reference mapping	**Refseq**: Search for a reference genome on databases. **Burrows-Wheeler Aligner (BWA_MEM)**: Align the contigs of the unknown strain with the reference genome. **Bowtie2**: Ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences. **Bedtools**: Overview of the mapping	O'Leary et al., 2016 Li & Durbin, 2009 Langmed, 2010 Quinlan & Hall, 2010
	Genome annotation	**Kraken2**: Generate the contigs of the trimmed raw sequences. **PROKKA**: Genome annotation producing standards-compliant output files.	Wood & Salzberg, 2014 Seemann, 2014
	Base/Variant calling	**SRST2**: Designed to take Illumina sequence data, a MLST database and/or a database of gene sequences. **Snippy**: SNP calling and phylogenetic tree maker. **BFCtools/SAMtools**: Set of utilities that manipulate variant calls in the Variant Call Format (VCF) and its binary counterpart BCF.	Inouye, et al., 2014 Seemaann, 2015 Li, 2011

OBJECTIVES

The thesis project focused on low-acid DFS (mainly *fuet*-type) by addressing both the microbial communities, including lactic acid bacteria as the main technological microbiota with recognized bioprotective capacity, and the most relevant microbial hazards: *Salmonella* and *Listeria monocytogenes*. The field of action was multidisciplinary, mainly including food microbiology, molecular microbiology, and bioinformatics, but also considering aspects of food technology and mathematical modelling. Genomics and bioinformatic/biostatistics were applied as powerful approaches to in-depth characterize both microorganisms and microbial communities.

The **general objective** was to enhance the quality and safety of DFS through the study of their microbial communities and by developing strategies to control *Salmonella* and *L. monocytogenes*. The studied strategies were focused on the use of genomics and microbial-based technological interventions.

To achieve the general objective, three **specific objectives** were set, each one linked to the scientific articles that compile this PhD thesis, i.e.:

1. To develop *fuet*-type DFS elaborated with different innovative and clean-label formulations and characterize their culture-independent microbial communities (Article I).

2. To characterize *Salmonella* spp. isolates from the Catalan and French DFSs production chains to establish phylogenomic relationships between the isolates and to describe their antimicrobial resistance profiles and virulence potential (Article II).

3. To explore the potential of the bacteriocinogenic *Latilactobacillus sakei* CTC494 as bioprotective starter culture and identify conditions promoting the inactivation of *L. monocytogenes* (Article III).

METHODOLOGY

This doctoral thesis complies three articles that have used different methodologies to assess and characterize the microbiota and main pathogenic strains related to dry fermented sausages. Table 7 summarizes the methodologies used in the different studies and main research topics, that are fully described in each article of the following Results section.

Table 7. Summary of the main methodologies used in each article.

Article	**I**	**II**	**III**
General methodology			
Classical microbiology	✓	-	✓
Genomics	Metataxonomics	Whole genome sequencing	-
Mathematical approach	-	-	Response Surface Methodology
Scale			
Research - Laboratory	✓	✓	✓
Pilot plant	✓	-	-
Industry	-	✓	-
Specific methodology			
Product/Source	DFS	Pig carcass, pork, DFS	Meat Simulation Media (broth)
Microorganisms			
Starter culture	*L. sakei* CTC494	-	*L. sakei* CTC494 *L. sakei* 23K
Pathogen	-	*Salmonella* spp.	*L. monocytogenes* CTC1034
Formulation modifications	NaCl substitution by KCl Liver ingredient Nitrifying agents replacement	-	NaCl Mn Glucose
Process modification	Temperature Relative humidity	-	Temperature
Statistics	ANOVA Tukey–Kramer Honest Significant Difference (HSD)	-	F-test ANOVA t-test
Software	JMP v16.0.0	SeqSphere+ v7.0.4 Bionumerics v7.6.3	JMP v16.0.0

Table 7 (continuation)

Genomics methodology			
➢ **Wet laboratory**			
DNA extraction	DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) QIAcube sample preparation system (QIAGEN)	QIAamp DNA Mini QIAcube Kit (QIAGEN, Hilden, Germany) QIAcube sample preparation system (QIAGEN)	-
Sequencing			
- Platform	Illumina NovaSeq6000	Illumina NovaSeq6000	
- Library preparation	Nextera DNA XT	Nextera DNA XT	
- Single/Pair-end reads	Pair-end reads (2 × 150 bp)	Pair-end reads (2 × 150 bp)	
- Target	16S rRNA gene (V3-V4)	All DNA	-
➢ **Dry laboratory**			
Repositories/databases	-	ENTEROBASE Center for Genomic Epidemiology – ResFinder, Plasmid Finder, SPIFinder Vfdb database Bacmet IToL (phylogenetic tree visualization)	-
Bioinformatic pipelines	QIIME2 DADA2 SILVAdatabase	Trimmomatic v0.40 FastQC v0.11.5 ConFindr v0.8.1 Snippy v4.6.0 Gubbins v2.4.1 snp-dists v0.8.2 RaxML v8.2.10	-
RStudio packages	Phyloseq (Alpha diversity) CodaSeq zComposition PERMANOVA RVAideMemoire (beta diversity) ALDEx2	-	-

RESULTS

Article I

Dynamics of microbial communities in nitrite-free and nutritionally improved dry fermented sausages

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Article

Dynamics of Microbial Communities in Nitrite-Free and Nutritionally Improved Dry Fermented Sausages

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Abstract: Dry fermented sausage innovation trends are linked to consumer preferences for clean label and sodium-reduced foods. This study aims to evaluate the effect of the formulation and production process temperature on the dynamics of bacterial communities in fuet-type dry fermented sausages using metataxonomics. Six fuet batches were manufactured, including formulations without and with the addition of nitrifying salts (replaced or not by pork liver auto-hydrolysate as a colouring agent), processed at 3 to 12 °C, and a partial replacement of NaCl by KCl, processed at 12 °C. Fermentation was performed spontaneously or by a starter culture. Physicochemical characterisation and culture-dependent and independent bacterial analyses were performed at day 0, 4 and 12, at the end of ripening (a_w < 0.90) and after storage. Temperature was the most important factor determining the change in pH, a_w and lactic acid bacteria levels while the presence of a starter culture promoted a pH decrease. Metataxonomic analysis showed that low temperature processes and the absence of nitrifying salts allowed the growth of spoilage-related species, while sausages submitted to a mild temperature containing a starter culture and nitrifying salts showed less bacterial diversity. Liver auto-hydrolysate added putative probiotic species to the product. This study provides valuable information to manufacturers who want to innovate safely.

Keywords: metataxonomics; sodium reduction; meat fermentation; clean label; 16S rRNA; Zn(II)PPIX

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1. Introduction

Dry fermented sausages (DFS) are a traditional meat product appreciated for their organoleptic properties and sensory traits and are industrially produced through standardised but traditional and empirical processes and formulations [1]. Fuet is a Catalan low-acid and small-calibre DFS whose basic formulation consists of a mixture of lean pork and backfat added with salt (NaCl), pepper, carbohydrates (e.g., dextrose), ascorbate and nitrates/nitrites. Fuet is elaborated by stuffing meat batter in ca. 35–40 mm diameter natural casings, surface inoculation with mould spores (usually *Penicillium nalgiovense*) and ripening at 10–18 °C [2,3]. In some cases, the production process starts with a fermentation phase at 20–25 °C, promoting the rapid development of fermentative microbiota (endogenous LAB or inoculated starter cultures) and ensuring early acidification. Industrially, this typical Mediterranean DFS can also be formulated with starter cultures comprising lactic acid bacteria (LAB) and/or Gram-positive catalase-positive cocci (GCC+) whose main roles are to ensure controlled fermentation and to improve the sensory properties through its lipolytic and proteolytic activities, respectively [4,5].

The initial microbiota of DFS mainly depends on the microorganisms present in the meat and any other ingredient added. In the slaughterhouse, meat is contaminated with microorganisms from the gastrointestinal tract and external sources, such as hides, skin or slaughterhouse surfaces [6]. Subsequently, the microbiota of DFS is modulated by the combination of antimicrobial factors (hurdles) coming from additives and processing conditions that promote the growth of LAB/GCC+ and inhibit pathogenic and spoilage

microorganisms throughout the so-called hurdle technology [7]. The drying that occurs during the fuet ripening process results in a shelf-stable end product.

Nowadays, the meat industry is experimenting with new challenges since consumers are becoming more demanding regarding the quality, safety, and nutritional aspects of processed foods, wanting improved nutritional profiles (e.g., NaCl reduction), and the removal of additives, such as nitrifying agents (i.e., clean label products) [8]. Nitrifying agents have been described to play different roles in cured meat products, including antimicrobial effects, the outgrowth inhibition of pathogens, especially *Clostridium botulinum*, and aroma and colour formation [9,10]. However, the use of nitrates and nitrites in dry-cured meat products is controversial due to the potential formation of carcinogenic nitrosamines [11]. As a consequence, the use of alternative natural ingredients enhancing reddish colour formation, such as liver, with high capacity to form zinc protoporphyrin (Zn(II)PPIX), have been reported [12,13]. A reduction in dietary sodium intake is encouraged by health authorities to prevent cardiovascular diseases [14]. However, NaCl is an ingredient with a multifunctional role in both the technological aspect and food safety. Accordingly, sodium reduction in DFS requires its replacement by other salts, with KCl being the most frequently used [15,16]. Considering that nitrifying agents and NaCl concentration have an impact on the microbial growth [17] and the role microorganisms play on the quality and safety of DFS, the characterisation of microbial population dynamics is of upmost importance.

The study of complex microbial communities through classical microbiology techniques is time-consuming and has a limited taxonomic resolution. In contrast, a metataxonomic approach provides deep information of the microbiota present in foods by high-throughput sequencing (HTS) of the 16S rRNA gene [18]. In recent years, metataxonomics has been used to characterise the microbiota of different types of DFS and to study the dynamics of the bacterial communities of fermented meats as a function of the processing factors [19,20]. However, little is known about the influence of improved formulations and production processes on the bacterial composition of Mediterranean DFS by HTS.

In this work, metataxonomics was applied to evaluate the dynamics of microbiota during the production of fuet with innovative formulations, including a liver auto-hydrolysate ingredient rich in Zn(II)PPIX as a colouring substitute of nitrites and nitrates and a salt-reduced product.

2. Materials and Methods

2.1. Dry Fermented Sausage Manufacturing

Six batches of fuet-type DFS were manufactured. The basic formulation of the different batches consisted of meat batter containing pork mince (70:30, shoulder:belly proportion) and the following common additives (g per kg of meat): dextrose (2), maltodextrin (20), white pepper (3) and sodium ascorbate (0.5). The components of the six different formulations are detailed in Table 1. Specifically, batch 1 to 3 were formulated without nitrifying salts. Batch 1 and 2 included an innovative ingredient, based on auto-hydrolysed pork liver described as a rich source of Zn(II)PPIX [13,21] to improve the colour of DFS without nitrifying salts. In these batches, glucono-delta-lactone (GDL) was added to compensate for the pH increase produced by the liver auto-hydrolysate. In batch 4 to 6, nitrifying salts were added at concentrations usually applied to fuet. Additionally, batch 6 was formulated with an equimolar substitution of 50% of NaCl by KCl to obtain a sodium reduced product. Fermentation was led either by spontaneous LAB in batch 1 or by the bioprotective starter culture *Latilactobacillus sakei* CTC494 [22] added at an initial concentration of 6 log CFU/g in the rest of the batches. Meat batter was thoroughly mixed for 3 min at 4 °C (Mixer AVT-150, Castellvall, Girona, Spain) and stuffed (Junior continuous vacuum stuffer, SIA, Barcelona, Spain) into 36–38 mm diameter natural pig casings (Collelldevall, Girona, Spain). Sausages of 20 ± 5 cm in length were soaked in a *P. nalgiovense* solution (Danisco, France) and underwent different drying processes, as described in Table 1.

Table 1. Assessed dry fermented sausage formulations and process conditions.

Specific Additives/Ingredients (g/kg)					Process Conditions [3]		
Batch	**KNO_3/ $NaNO_2$**	**Liver Auto-Hydrolysate/GDL**	**NaCl/ KCl**	**Starter Culture [2]**	**Days**	**Temperature (°C)**	**Relative Humidity (%)**
1	na	300/3	20/0	na	20 2 18	3.4 ± 0.3 7.8 ± 2.1 12.5 ± 0.4	87.7 ± 8.6 90.0 ± 8.3 76.0 ± 8.6
2	na	300/3	20/0	+			
3	na	na [1]	20/0	+			
4	0.10/ 0.15	na [1]	20/0	+			
5	0.10/ 0.15	na [1]	20/0	+	27	12.4 ± 0.6	86.2 ± 7.6
6	0.10/ 0.15	na [1]	10/ 12.76	+			

[1] Instead of liver auto-hydrolysate, 300 g of water were added. [2] *Latilactobacillus sakei* CTC494 bioprotective starter culture. [3] Temperature and relative humidity values correspond to the mean ± standard deviation of the recorded profiles. Process conditions were the same for batches 1–4 (dynamic) and batches 5–6 (constant). na: not added; +: added.

Fuets without nitrifying agents added (batches 1 to 3) underwent cold ripening aiming to control the growth of *Clostridium botulinum*, both psychrotrophic (i.e., initially at temperature ca. 3 °C until a_w was <0.97) and mesophilic (at <10 °C until a_w was <0.94) [23,24] for 32–40 days. Batch 4 underwent the same ripening conditions in an independent drying chamber (separated from the batches 1, 2 and 3) to avoid nitric oxide cross-contamination through air. For batches 5 and 6, sausages were ripened at 12–13 °C for 27 days. Table 1 describes the temperatures and relative humidity (RH) recorded for each process. At the end of the process, DFS were vacuum packed in PA/PE bags (oxygen permeability of 50 $cm^3/m^2/24$ h and a low water vapor permeability of 2.8 $g/m^2/24$ h; Sistemvac, Estudi Graf S.A., Girona, Spain) and stored for 15 days at 4 °C [25].

For each batch, two independent manufacturing processes of DFS were performed on different days.

2.2. Microbial Counts, pH, a_w and Weight Loss

For the microbiological analysis of the DFS, first, the casing was removed aseptically and then ca. 25 g of chopped sausage was 10-fold diluted in a saline solution (0.85% NaCl and 0.1% Bacto Peptone; Beckton Dickinson, Franklin Lakes, NJ, USA), homogenised in a bag blender (Smasher®, bioMérieux, Marcy-l'Etoile, France) for 1 min and, if necessary, 10-fold serially diluted in a saline solution. LAB were enumerated in MRS (de Man, Rogosa and Sharpe; Merck, Darmstadt, Germany) agar plates incubated at 30 °C for 72 h under anaerobiosis using sealed jars with an AnaeroGen sachet (Oxoid Ltd., Altrincham, UK). GCC+ were enumerated in MSA (Mannitol Salt Agar; Oxoid™, Thermo Scientific™) plates incubated at 30 °C for 24 h.

The pH was measured with a puncture electrode 5232 and a portable pH meter PH25 (both from Crison Instruments S.A., Alella, Spain) and a_w with an Aqualab 3TE device (Decagon Devices, Inc., Pullman, WA, USA) at 25 °C.

All the analyses were performed in triplicate (three sausages randomly selected from the drying chamber) throughout the production process, which comprised day 0 (t0), 4 (t4), 7 (t7), 12 (t12) and 20 (t20), the end of ripening (tRip; day 32–40 for batches 1 to 4 and day 27 for batches 5 and 6) and at the end of the 15-day refrigerated storage (tStor), which was day 52–53 for batches 1 to 4 and day 40–41 for batches 5 and 6.

Five DFS from every batch located at different positions in the drying chamber were selected and labelled to follow the weight loss along the process. The weight loss (%) was calculated with respect to the initial weight of the sausage.

2.3. Monitoring of Starter Culture Implantation

To verify the implantation of the starter culture *L. sakei* CTC494, eight colonies per batch were isolated from MRS plates at t0, t4 and tRip, and were submitted to Enterobacteria Repetitive Intergenic Consensus (ERIC)-PCR with primers FW-ERIC R1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and RV-ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) [26] for typing under the conditions described in Rubio et al., 2014 [3].

2.4. DNA Purification, qPCR and High Throughput Sequencing

Samples processed for sequencing were those representing the microbial community of the meat batter used for the sausages production, the liver auto-hydrolysate and the DFS at the different times of the process: t0, t4, t12, tRip and tStor.

A volume of 200 mL of 10-fold diluted homogenates was filtered in sterile conditions with a nonwoven filter with a 22–25 µm diameter pore (475855-1R, Millipore Corp, Burlington, MA, USA). The filtered samples were centrifuged at 30.000× *g* for 15 min at 4 °C (Avanti® JXN-30, Beckman Coulter, Pasadena, CA, USA) in 35-mL capacity sterile centrifuge tubes (Nalgene, Rochester, NY, USA). The supernatant was discarded and up to 100 mg of pellet was recovered and stored at −20 °C until further processing.

DNA was extracted with the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany). The protocol followed was that described by the manufacturer. Mechanical lysis was performed through bead beating for 10 min at 30 Hz in a ball mill (MM200, Retsch, Haan, Germany). DNA purification and isolation steps were automated using the QIAcube sample preparation system (QIAGEN).

DNA was quantified spectrophotometrically (µDrop plate, Thermo Fisher Scientific, Waltham, MA, USA) and fluorometrically (Quant-iT^{TM} 1X dsDNA HS Assay Kit, Invitrogen, Merelbeke, Belgium) in a Varioskan^{TM} multiplate reader (Thermo Fisher Scientific, USA), and the concentration was adjusted to 5 ng/µL.

16S rRNA gene amplicons were obtained following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The gene-specific sequences used in this protocol target the 16S rRNA gene V3 and V4 regions (459 bp) with the primers designed by Klindworth et al. (2013) [27]. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. After 16S rRNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit (FC-131-1096). After normalisation and pooling, libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, CA, USA).

2.5. Bioinformatic Analysis

Sequencing reads were imported into the QIIME2 platform [28] and quality filtering, denoising, paired-ends joining and chimera depletion were performed using the DADA2 pipeline [29]. Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in the QIIME2 and SILVA database.

2.6. Statistical Analysis

Statistical differences regarding physicochemical parameters, weight loss and microbial counts between batches at each sampling time were assessed by one-way analysis of variance (ANOVA) and the Tukey–Kramer Honest Significant Difference (HSD) test (level of significance 0.05) using JMP v16.0.0 software (SAS Institute Inc., Cary, NC, USA).

Amplicon Sequence Variants (ASVs) table with the assigned taxonomy constructed in QIIME2 was exported to RStudio (v 1.4.1103) [30]. Alpha diversity metrics (including Shannon, Simpson evenness, Simpson dominance and Chao1 indexes) were calculated using "phyloseq" R package [31] after rarefaction of the samples (subsample without replacement) to a depth of 15,000 sequences per sample.

For the statistical analysis of the bacterial composition across samples, compositional data analysis methods [32] were used. OTU counts were normalised using the centred

log ratio (CLR) transformation after removing low-abundance OTUs (minimal proportion abundance: 0.5%) and including a pseudo-count using CodaSeq [32,33] and zComposition [34,35] R packages. Beta diversity was evaluated using principal component analysis (PCA) performed by plotting a singular value decomposition of the CLR-transformed data. Permutational Multivariate Analysis of Variance (perMANOVA) [36] was used to evaluate differences in beta diversity using the "RVAideMemoire" package. To identify treatment/time specific OTUs, the ANOVA-like different expression (ALDEx) was performed in the ALDex2 package [37,38].

3. Results and Discussion

3.1. Physicochemical Characteristics of DFS

Acidification and drying profiles of DFS depended on the sausage formulation and production process parameters (Table 1). The results of the physicochemical analysis, including pH, water activity (a_w) and weight loss, are reported in Table 2.

The initial meat batter pH ranged from 5.83 to 5.96. Subsequently, the pH profile was determined by the product formulation and process temperature. For batch 1, formulated without the *L. sakei* CTC494 starter culture and submitted to a low temperature fermentation and ripening process, the same pH was maintained throughout the production process. For batches 2, 3 and 4, formulated with the starter culture and following a low temperature process, a pH decrease was observed from t7, registering the minimum pH values at t20 (5.11 ± 0.04, 5.09 ± 0.02 and 5.13 ± 0.01, respectively). The pH drop did not show significant differences among these batches at t12, t20 and tRip. In contrast, batches 5 and 6, formulated with the starter culture and submitted to a mild temperature process, registered a fast and strong product acidification, with the lowest pH values at t7 (4.81 ± 0.03 and 4.83 ± 0.02, for batches 5 and 6, respectively, $p > 0.05$). *L. sakei* CTC494 was previously reported to show a higher capacity to reduce the pH and produce lactic acid in production processes at 21–23 °C than at 10–14 °C [2]. Often, low-acid DFS acidification is followed by a gradual increase in the pH due to proteolysis, a process generating small peptides and free amino acids and amines [4,39]. In this regard, pH increases up to 0.5–0.7 pH units (end product pH values of 5.3–5.5) were shown in the present study for batches 5 and 6, corresponding to batches with a higher temperature process (12 °C).

The a_w value of the initial meat batter was above 0.982 in all batches. At t12, the a_w of batches ripened at a low (3 °C) temperature (1, 2, 3 and 4) was 0.98, and was slightly lower (0.97, $p < 0.05$) in batches ripened at a mild (12 °C) temperature (5 and 6). Afterwards, batches 1, 2, 3 and 4 continued ripening at 3 °C until day 20 of the process, when a_w decreased below 0.97. Then, the ripening temperature was raised to 8 °C and after 2 days, when the a_w decreased below 0.94, a final ripening phase of 18 days at 12 °C was applied until the a_w was below 0.9. The overall process time for batches ripened at low temperature was 32–40 days, reaching final a_w values of 0.866, 0.846, 0.840 and 0.857 for batches 1, 2, 3 and 4, respectively ($p > 0.05$). On the other hand, the process for batches ripened at 12 °C (batches 5 and 6) lasted 27 days, and the final a_w ranged from 0.865 to 0.858 ($p > 0.05$). Weight loss decreased to values of 61–65% in the final products.

A_w is a key factor for the food safety of DFS, especially in the Mediterranean-type, which usually show an a_w below 0.92 [3,40]. A particular reference should be made to the DFS formulated without the addition of nitrifying salts as they require additional control measures (e.g., temperature, a_w/NaCl and pH) to guarantee inhibition of *Clostridium botulinum* growth and toxin production [24,41]. In the present study, process temperature was linked to product a_w (see details in Section 2.1), and no remarkable differences in a_w were observed between fuets formulated with and without nitrifying salts nor with the batch formulated with liver auto-hydrolysate.

Table 2. Weight loss (%), physicochemical parameters, lactic acid bacteria (LAB) and Gram-positive catalase positive cocci (GCC+) counts (in log CFU/g). Values are the mean ± standard deviation for the replicates. Specifically, for each sampling time, significant differences between batches are marked with different capital letters ($p < 0.05$). NA: Not applicable; ND: Non-determined.

Time	Batch [1]	Weight Loss	a_w	pH	LAB	GCC+
t0	1	NA	0.983 ± 0.001^{AB}	5.96 ± 0.04^{A}	5.01 ± 0.19^{B}	4.13 ± 0.19^{AB}
	2	NA	0.982 ± 0.000^{B}	5.93 ± 0.03^{A}	6.19 ± 0.00^{A}	4.38 ± 0.03^{A}
	3	NA	0.985 ± 0.001^{AB}	5.87 ± 0.11^{A}	6.26 ± 0.13^{A}	4.03 ± 0.01^{AB}
	4	NA	0.985 ± 0.001^{AB}	5.83 ± 0.12^{A}	6.08 ± 0.04^{A}	3.76 ± 0.09^{B}
	5	NA	0.985 ± 0.001^{AB}	5.84 ± 0.08^{A}	6.11 ± 0.04^{A}	4.04 ± 0.33^{AB}
	6	NA	0.985 ± 0.000^{A}	5.90 ± 0.05^{A}	6.06 ± 0.02^{A}	4.03 ± 0.15^{AB}
t4	1	2.49 ± 0.85^{C}	0.982 ± 0.000^{B}	5.94 ± 0.11^{A}	5.17 ± 0.03^{C}	3.84 ± 0.03^{A}
	2	2.70 ± 0.92^{C}	0.982 ± 0.000^{B}	5.95 ± 0.13^{A}	8.11 ± 0.16^{B}	4.13 ± 0.15^{A}
	3	3.68 ± 0.58^{C}	0.985 ± 0.001^{A}	5.90 ± 0.12^{A}	8.24 ± 0.17^{B}	3.97 ± 0.16^{A}
	4	12.45 ± 1.23^{A}	0.983 ± 0.001^{B}	5.90 ± 0.13^{A}	8.09 ± 0.28^{B}	3.70 ± 0.05^{A}
	5	6.37 ± 0.87^{B}	0.984 ± 0.000^{A}	5.11 ± 0.08^{B}	8.90 ± 0.11^{A}	3.40 ± 0.00^{A}
	6	6.00 ± 0.71^{B}	0.984 ± 0.000^{A}	5.09 ± 0.09^{B}	8.85 ± 0.03^{A}	3.40 ± 0.00^{A}
t7	1	6.87 ± 1.03^{C}	0.980 ± 0.000^{B}	5.95 ± 0.08^{A}	5.27 ± 0.07^{C}	4.14 ± 0.27^{A}
	2	8.05 ± 2.15^{C}	0.980 ± 0.001^{B}	5.74 ± 0.04^{B}	8.43 ± 0.16^{B}	4.38 ± 0.09^{A}
	3	9.74 ± 0.65^{BC}	0.983 ± 0.001^{A}	5.58 ± 0.02^{C}	8.47 ± 0.06^{B}	4.07 ± 0.26^{A}
	4	12.16 ± 1.27^{AB}	0.983 ± 0.000^{AB}	5.66 ± 0.14^{BC}	8.40 ± 0.02^{B}	3.95 ± 0.30^{A}
	5	13.21 ± 2.59^{AB}	0.983 ± 0.002^{AB}	4.81 ± 0.03^{D}	8.92 ± 0.04^{A}	3.52 ± 0.11^{B}
	6	13.46 ± 0.73^{A}	0.982 ± 0.001^{AB}	4.83 ± 0.02^{D}	8.97 ± 0.04^{A}	3.51 ± 0.12^{B}
t12	1	23.24 ± 2.06^{B}	0.976 ± 0.000^{A}	5.94 ± 0.09^{A}	5.48 ± 0.25^{D}	3.77 ± 0.29^{A}
	2	26.85 ± 3.47^{B}	0.976 ± 0.001^{A}	5.30 ± 0.01^{B}	8.77 ± 0.04^{ABC}	3.55 ± 0.01^{A}
	3	30.85 ± 1.86^{B}	0.979 ± 0.000^{A}	5.27 ± 0.02^{B}	8.54 ± 0.04^{C}	3.58 ± 0.21^{A}
	4	24.17 ± 1.70^{B}	0.979 ± 0.000^{A}	5.31 ± 0.06^{B}	8.63 ± 0.09^{BC}	3.40 ± 0.05^{A}
	5	44.45 ± 3.82^{A}	0.971 ± 0.003^{B}	4.85 ± 0.04^{C}	9.00 ± 0.16^{A}	2.76 ± 0.02^{B}
	6	46.36 ± 3.72^{A}	0.970 ± 0.003^{B}	4.90 ± 0.06^{C}	8.88 ± 0.05^{AB}	2.75 ± 0.37^{B}
t20	1	37.61 ± 2.36^{CD}	0.968 ± 0.004^{A}	5.85 ± 0.02^{A}	ND	ND
	2	41.38 ± 3.44^{CD}	0.967 ± 0.001^{A}	5.11 ± 0.04^{A}	ND	ND
	3	45.20 ± 2.10^{B}	0.967 ± 0.000^{A}	5.09 ± 0.02^{A}	ND	ND
	4	37.01 ± 2.28^{D}	0.970 ± 0.004^{A}	5.13 ± 0.01^{A}	ND	ND
	5	59.19 ± 2.22^{A}	0.939 ± 0.017^{AB}	5.30 ± 0.32^{A}	8.62 ± 0.10	2.73 ± 0.45
	6	59.86 ± 2.13^{A}	0.928 ± 0.007^{B}	5.59 ± 0.37^{A}	8.61 ± 0.07	2.53 ± 0.24
tRip	1	61.41 ± 1.81^{B}	0.866 ± 0.016^{A}	5.82 ± 0.00^{A}	8.25 ± 0.11^{B}	1.98 ± 0.73^{A}
	2	61.52 ± 1.37^{B}	0.846 ± 0.009^{A}	5.20 ± 0.05^{C}	8.34 ± 0.00^{AB}	1.27 ± 0.01^{A}
	3	64.75 ± 0.90^{A}	0.840 ± 0.031^{A}	5.20 ± 0.02^{C}	8.18 ± 0.00^{AB}	1.40 ± 0.12^{A}
	4	63.54 ± 1.25^{A}	0.857 ± 0.027^{A}	5.19 ± 0.01^{C}	8.17 ± 0.00^{B}	1.90 ± 0.08^{A}
	5	63.95 ± 1.47^{A}	0.865 ± 0.012^{A}	5.49 ± 0.17^{B}	8.32 ± 0.03^{AB}	1.90 ± 0.44^{A}
	6	64.24 ± 1.32^{A}	0.858 ± 0.013^{A}	5.54 ± 0.08^{B}	8.53 ± 0.00^{A}	2.88 ± 0.92^{A}
tStor	1	ND	ND	5.83 ± 0.00^{A}	8.02 ± 0.06^{B}	2.83 ± 0.16^{A}
	2	ND	ND	5.35 ± 0.01^{C}	8.21 ± 0.00^{AB}	2.31 ± 0.23^{AB}
	3	ND	ND	5.23 ± 0.01^{C}	8.22 ± 0.12^{AB}	2.24 ± 0.11^{AB}
	4	ND	ND	5.23 ± 0.08^{C}	8.20 ± 0.08^{AB}	1.97 ± 0.01^{B}
	5	ND	ND	5.32 ± 0.04^{C}	8.29 ± 0.00^{A}	2.25 ± 0.05^{AB}
	6	ND	ND	5.52 ± 0.12^{B}	8.30 ± 0.03^{A}	2.68 ± 0.24^{AB}

[1] Batch 1: With liver auto-hydrolysate at a low temperature; Batch 2: With liver auto-hydrolysate and the starter culture at a low temperature; Batch 3: With the starter culture at a low temperature; Batch 4: With the starter culture and nitrifying salts at a low temperature; Batch 5: With the starter culture and nitrifying salts at a mild temperature; Batch 6: With the starter culture, nitrifying salts, and sodium reduction at a mild temperature.

3.2. Culture-Dependent Microbial Dynamics

The levels of LAB and GCC+ of the fuets are reported in Table 2. For batches containing the *L. sakei* CTC494 starter culture, the LAB concentration at t0 was ca. 6 log CFU/g, whereas the initial LAB concentration for batch 1, without the starter culture, was 1 log CFU/g lower (5.01 ± 0.19 log CFU/g) ($p < 0.05$). The low temperature processed batches (2, 3 and 4) registered LAB values ca. 8 log CFU/g at t4 and reached maximum levels at t12, which slightly decreased until tStor. For batch 1, endogenous LAB growth was slow (0.5 log after 12 days at a low temperature), compared to other batches formulated with the starter culture, and stationary phase levels (8.25 ± 0.11 log CFU/g) were not registered until tRip. Consequently, the pH of batch 1 did not decrease and was maintained above 5.80 during the whole process. In contrast, for batches 5 and 6, ripened at 12 °C, the LAB grew the fastest and reached values ca. 9 log CFU/g at t4, maintained the population density until t20, and thereafter slightly decreased until tStor. Monitoring of the *L. sakei* CTC494 by ERIC-PCR showed 100% implantation in batches inoculated with the starter culture at t0, t4 and tRip.

The behaviour of GCC+ was similar in all batches of fuet, although the process was shorter for 12 °C-processed fuets. Initial GCC+ values were ca. 4 log CFU/g in all the batches and a progressive decrease was observed along the process time, reaching levels of 1.3–2.0 log CFU/g at tRip, without significant differences between batches ($p > 0.05$). During subsequent refrigerated storage, GCC+ slightly increased in batches 1 to 3 (a maximum of 1 log in batch 2) and was maintained in batches 4 to 6.

The initial increase in the LAB population in batches formulated with the starter culture highly depended on the process temperature and was not affected by the addition of the liver auto-hydrolysate, the removal of nitrifying salts or the NaCl reduction. *L. sakei* CTC494 is a psychrotrophic strain well adapted to the meat fermentation environment; it is able to rapidly grow and acidify DFS processed at both mild (12 °C) and low (3 °C) temperatures [2,22,42]. Given the importance of a rapid pH drop for food safety, the selection of starter cultures suitable for the conditions of the production process (e.g., low temperature) is a key aspect. GCC+ development is strongly modulated by the levels of LAB, which are able to grow at a lower pH and a_w and, thus, can impact the sensory characteristics of the final product given that flavour development is highly influenced by GCC+ proteolytic and lipolytic activities [43].

3.3. Diversity and Taxonomic Composition of the Bacterial Communities

A total of 17,894,929 high-quality reads were obtained after filtering and denoising, with an average of 101,101 sequences per sample. The rarefaction curve showed that the sequencing depth was sufficient to infer microbial composition.

Taxonomic assignment of ASV resulted in the identification of 800 taxa belonging to 14 different phyla, which was mainly represented by *Bacillota* (formerly *Firmicutes*), *Pseudomonadota* (formerly *Proteobacteria*) and *Actinomycetota* (formerly *Actinobacteriota*). A total of 171 different genera and 158 species (Figure 1, Supplementary Table S1) were identified.

Alpha diversity, which describes the intra-sample diversity, was evaluated through the Shannon index (Supplementary Table S2). Extended alpha diversity indexes (Simpson and Chao1) are also represented in Supplementary Table S2. Results show that batches formulated with liver auto-hydrolysate and/or without curing agents (batches 1, 2 and 3) had the highest microbial diversity during the first four days of ripening (i.e., Shannon index values of 2.84, 1.71 and 1.79, respectively), which decreased along the ripening process, especially in batches with the starter culture. Batch 1, formulated without the starter culture and with liver auto-hydrolysate, was the only one that maintained the index above 1.5 at tStor. Batch 2 registered the highest alpha diversity value at t0, which decreased during the process, reaching 0.66 at tStor. Liver auto-hydrolysate contributed to the presence of microorganisms that are not typically present in meat batters and, thus, increased the microbial richness of the samples. Batch 3 registered the highest Shannon

diversity index at t4 (1.79), which progressively decreased along the process, with a value of 0.80 at the end of storage. Alpha diversity decrease was related to the application of the starter culture. Starter cultures promote fermentation, ensure food safety, standardise product properties and shorten ripening times. However, they also decrease the microbial biodiversity of the fermented product [18,44]. Nitrified batches formulated with the starter culture (4, 5 and 6) had less diverse microbial communities, showing values $\leq$0.82 at t0 and $\leq$0.66 throughout the ripening process and storage, without differences between batches ($p > 0.05$). The combined application of nitrifying salts and the starter culture contributed to the reduction in the alpha diversity. The use of nitrifying agents has been described to cause acidic, oxidative and nitrative stresses to sensitive microorganisms [17], which could explain the microbial diversity reduction.

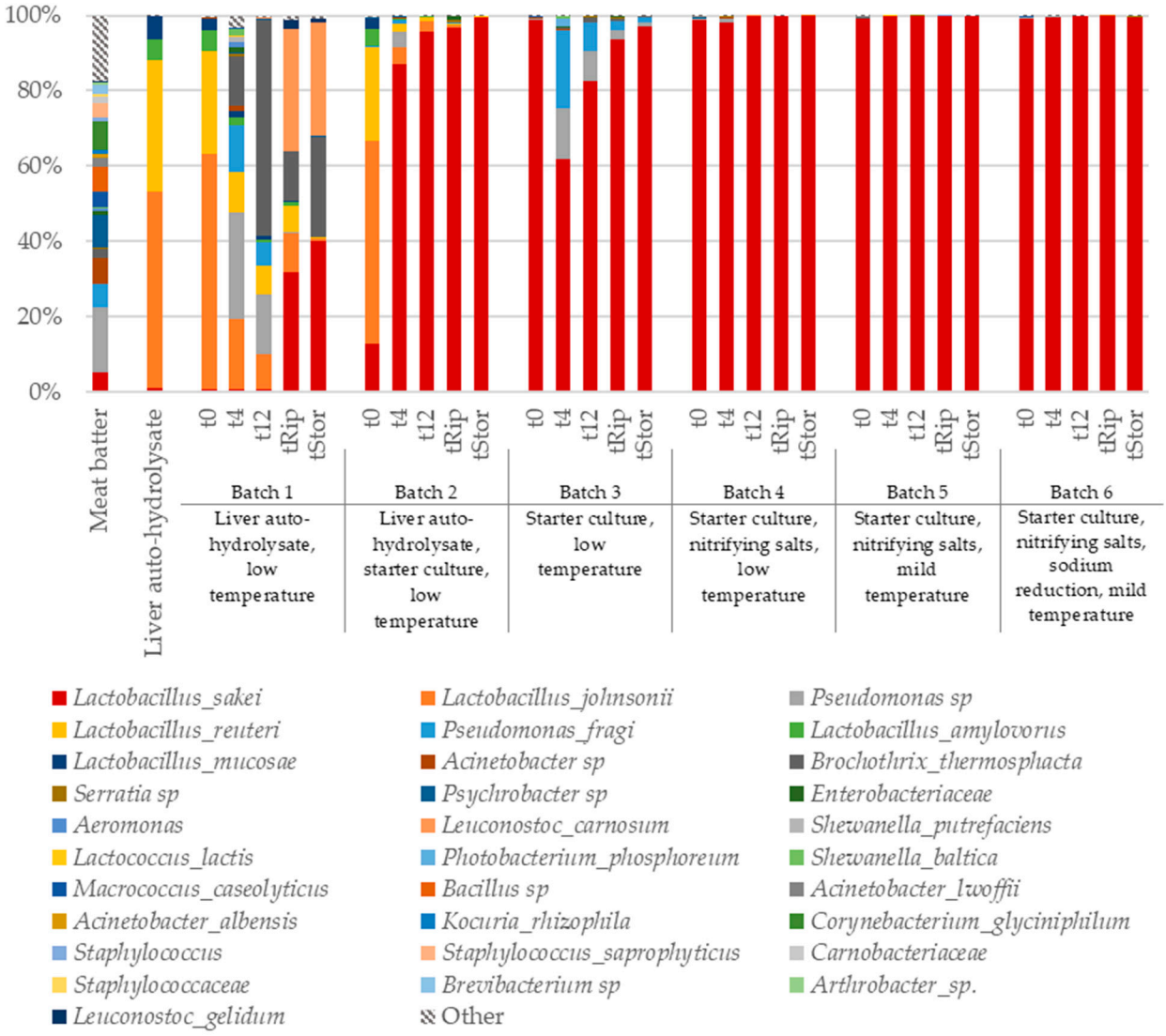

Figure 1. Taxonomic composition represented in the relative abundance (%) of microbial communities at the species level identified in the meat batter, liver hydrolysed ingredient and DFS batches (1, 2, 3, 4, 5 and 6) by time (days) 0, 4, 12, end of ripening and end of storage. Only species with an incidence above 1% in at least one sample are represented; the remaining are classified as "Other".

The bacterial relative abundances of DFS at the genus/species level were different depending on the type of fuet (i.e., formulation and production process) and sampling time (Figure 1, Supplementary Table S1). The initial meat batter was mostly characterised by the presence of *Pseudomonas* sp. (17.37%), *Psychrobacter* sp. (8.43%), *Corynebacterium glyciniphilum* (7.32%), *Acinetobacter* sp. (6.84%), *Bacillus* sp. (6.53%), *Pseudomonas fragi* (6.27%) and *L. sakei* (5.12%); all have been previously described in raw meat stored under refrigeration [6,45,46]. The psychrotrophic genus *Pseudomonas* sp. is the main spoilage bacteria of aerobically stored fresh meat kept at refrigeration temperatures since it can grow from 2 to 35 °C [6]. Specifically, the *P. fragi* species stands out for meat spoilage among the *Pseudomonas* spp. as meat can be considered its ecological niche [47]. *B. thermosphacta* can easily colonise the meat matrix since it can grow under aerobic and anaerobic conditions and has been classified as a fresh meat and cooked meat spoilage microorganism due to its off-odour metabolite production, mainly upon depletion of glucose [48]. Similar results were obtained in the meat batter of Fabriano-like fermented sausages, detecting *Pseudomonas* sp. and *B. thermospacta* [49]. In contrast, the pork liver auto-hydrolysate contained mainly LAB species, with the most abundant being *Lactobacillus johnsonii* (62.64%), *Limosilactobacillus reuteri* (34.89%), *Limosilactobacillus mucosae* (6.34%) and *Lactobacillus amylovorus* (5.43%). These species have been classified as putative probiotic [50], and are included in the EFSA QPS (Qualified Presumption of Safety) list [51].

At t0 (before fermentation), in batches 1 and 2 of DFS, the most abundant species were *L. johnsonii*, representing more than 50% of the total species, and *L. reuteri* (*ca*. 25%), both coming from the added liver auto-hydrolysate. The most abundant microorganisms found in the meat batter (*Pseudomonas* sp., *Psychrobacter* sp. and *C. glyciniphilum*) were detected at very low percentages (<0.14%). Batch 2 also showed 12.9% *L. sakei*, corresponding to the applied starter culture, as confirmed by ERIC-PCR. At t0, *L. sakei* was the most abundant species (>98%) in batches 3 to 6.

After 4 days at 3 °C, the batch 1 bacterial community changed as the relative abundance of *L. johnsonii* and *L. reuteri* decreased to 18.7% and 10.9%, respectively, and *Pseudomonas* sp., *B. thermosphacta* and *P. fragi* increased to 28.3%, 13.1% and 12.6%, respectively. At t12, there was still a high abundance of microorganisms coming from both the meat batter and the liver auto-hydrolysate (i.e., *B. thermosphacta* (57.6%), *Pseudomonas* sp. (15.9%), *L. johnsonii* (9.2%), *L. reuteri* (7.6%) and *P. fragi* (6.1%)). *Pseudomonas* sp. and *B. thermosphacta* have been classified as spoilage microorganisms mainly found in chilled fresh meat products [46,52] and were also present along the ripening process of sausages submitted to low temperatures. From t20 to tRip, when the process temperature was raised to 12 °C, an increase in the abundance of *L. sakei* (to 31.9%) and *Leuconostoc carnosum* (32.3%) was observed. In contrast, *B. thermosphacta* (13.1%), *Pseudomonas* sp. (0.2%) and *P. fragi* (0.1%) were decreased, and *L. johnsonii* and *L. reuteri* were maintained (10.3% and 7.0%, respectively). Those putative probiotic strains, *L. johnsonii* and *L. reuteri*, have been described to play a role in the intestines, increasing lipid absorption and stimulating host immunity against infectious agents, respectively [53]. By tStor, the most relevant species were *L. sakei* (40.1%), *L. carnosum* (30.3%) and *B. thermosphacta* (26.5%), while *L. johnsonii* and *L. reuteri,* decreased (<1%). *L. carnosum* is frequent in meat-based products and plays a controversial role as it can participate in the spoilage, affecting sensorial properties, or act as a bioprotective culture, through organic acid release or bacteriocin production [54]. Interestingly, specific strains of the putative probiotic species provided by the liver auto-hydrolysate (*L. reuteri* and *L. amylovorus*) have been proposed for incorporation, through microencapsulation, in fermented foods to ensure a desired level of probiotic microorganisms in the final product [55]. Moreover, other authors have concluded that the addition of LAB probiotic bacteria, such as *Lacticaseibacillus casei* (formerly *Lactobacillus casei)* and *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), improve the quality of DFS [56].

Considering batches formulated with the starter culture, *L. sakei* CTC494 led the fermentation process and was maintained at high levels until the end of storage of fuets. *L. sakei* has been reported to be very competitive in meat fermentations [57] and, specifically for *L. sakei* CTC494, it has been described to grow in a wide range of temperatures and formulations [2,57,58], explaining the ability to dominate among other bacteria, even in DFS submitted to a low temperature ripening process [59]. At t4 in batch 2, *L. sakei* represented 86.9% of the total bacterial population; the abundance of *L. johnsonii* (4.7%) and *Pseudomonas* sp. (4.2%) was also remarkable. In batch 3, the main species was *L. sakei* (61.8%) followed by *P. fragi* (21.0%) and *Pseudomonas* sp. (13.4%). *L. sakei* was also the dominant species (>98%) in batches 4 to 6 from t4 until the end of storage. From t12, *L. sakei* dominance continued, representing 95% of the bacterial community in all batches except for number 3, which was formulated without nitrifying salts, whose relative abundance was 82.4%. In this batch, *Pseudomonas* sp. and *P. fragi* had a relative abundance of 8.2% and 7.6%, respectively, although they progressively decreased to 1.1% and 1.2%, respectively, at tStor. In parallel, *L. sakei* progressively increased from 82.4% (t12) to 97.2% (tStor). Strain typing by ERIC-PCR showed the competitiveness of the applied starter culture, *L. sakei* CTC494, being the dominant species until the end of ripening.

Beta diversity was studied through a compositional approach to analyse microbial abundance differences between batches. The PCA (Figure 2) based on ASVs table (Supplementary Table S1), showed samples from batches 1 and 2 clustered together and separated from the other batches at t0. The perMANOVA with Aitchison distances indicated a significant effect on the bacterial community between these two groups ($p < 0.05$). ALDEx results showed that the abundance of the main species of the liver auto-hydrolysate (i.e., *L. reuteri*, *L. mucosae*, *L. johnsonii*, *L. amylovorus*, *Lactobacillus salivarius* and *Lactobacillus delbrueckii*) were enriched in batches 1 and 2, while the abundance of *Bacillus* sp., *M. caseolyticus* and *L. sakei* was higher in batches 3 to 6.

During the fermentation and drying processes, microbial communities progressively changed (Figure 2), and from t12 until tStor, batches without curing salts (1, 2 and 3) showed significant beta diversity differences between them and with the rest of the batches ($p < 0.05$). No differences were found between batches containing nitrites and nitrates ($p > 0.05$); therefore, neither salt concentration nor temperature process exerted a significant effect on the bacterial community composition. Charmpi et al. [60] also reported no taxonomic differences among fermented meat with different salt concentrations. The ALDEx analysis showed some taxa being differentially abundant between batches 1 and 2. At t12, the abundance of *L. johnsonii*, *L. amylovorus* and *L. sakei* was increased in batch 2 while *Leuconostoc* spp., *C. divergens*, *B. thermosphacta* and *Pseudomonas* spp. had a significantly higher abundance in batch 1. After ripening and storage, only the abundance of *L. sakei* was increased in batch 2 when compared to batch 1, which maintained a significantly higher abundance of *B. thermosphacta*, *L. gelidum*, *L. carnosum* and *C. divergens*. During the whole process, the most important differences between batches with/without the liver auto-hydrolysate (i.e., batch 1 and 2 vs. batches 3–6) were the liver auto-hydrolysate associated species being significantly more abundant in batches 1 and 2, whereas batches 3 to 6 were enriched in *L. sakei*. When comparing batches containing nitrifying salts against batch 3 (no nitrite or nitrate added), the ALDEx analysis showed that *L. sakei* was enriched in batches 4 to 6 throughout the process and *P. fragi*, *Pseudomonas* sp. and *B. thermosphacta* were enriched in batch 3. It was shown in minced turkey meat that the application of nitrite lowered the relative abundance of both *Pseudomonas* spp. and *Brochothrix* spp. during storage at 4 °C [61].

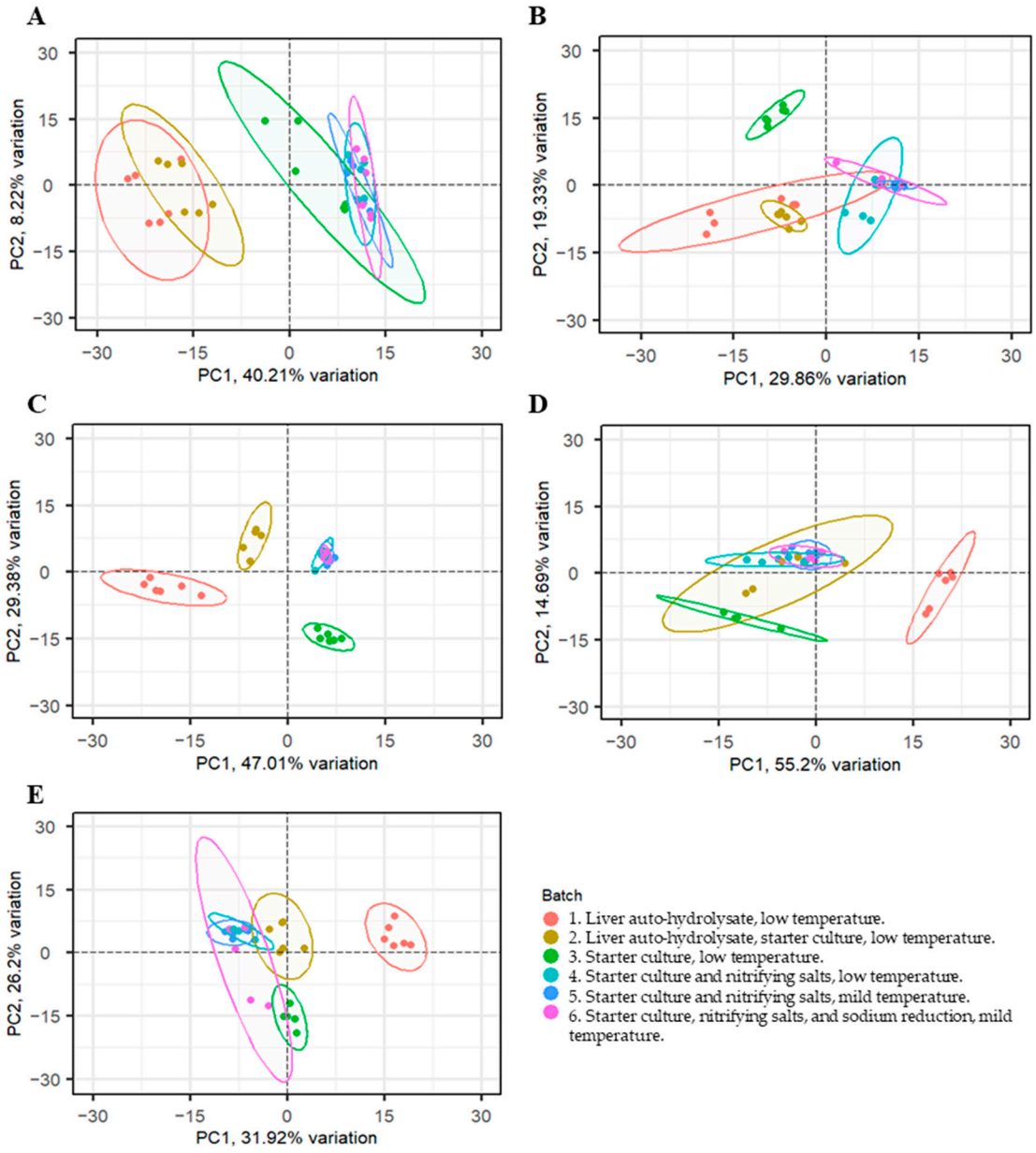

Figure 2. Principal component analysis plots representing the beta diversity analysis comparing every batch by time t0 (**A**), t4 (**B**), t12 (**C**), tRip (**D**) and tStor (**E**).

4. Conclusions

This research provides valuable microbiological information to DFS manufacturers who want to innovate safely. Nutritionally improved (low in sodium) and clean label (without nitrifying salts) formulations and low-temperature processes only cause minor shifts in the physicochemical characteristics of DFS when using a competitive starter culture to ensure product acidification. Accordingly, production processes requiring a low temperature to guarantee food safety can be used to produce DFS with similar characteristics to those fermented and ripened at mild temperatures. Irrespective of the formulation and process parameters, the microbial communities gradually change over time, decreasing their diversity due to the progressively harsher conditions that occur throughout fermentation and ripening. However, the factors that most impact the bacterial community composition are the addition of a starter culture and/or liver auto-hydrolysate, that provides putative probiotic species, and the presence of nitrate/nitrite salts that reduce the meat spoilage-

related microorganisms. Further studies are in progress to evaluate the sensory aspects of the newly developed fuet-type dry fermented sausages.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040403/s1, Table S1: Taxonomic composition represented as the relative abundance (%) of all samples at the species level detected above 1% in at least one sample; Table S2: Alpha diversity indexes Shannon, Simpson and Chao1.

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Article II

Genomic insights of *Salmonella* isolated from dry fermented sausage production chains in Spain and France

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Genomic insights of *Salmonella* isolated from dry fermented sausage production chains in Spain and France

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The presence of *Salmonella* in dry fermented sausages is source of recalls and outbreaks. The genomic diversity of 173 *Salmonella* isolates from the dry fermented sausage production chains (pig carcasses, pork, and sausages) from France and Spain were investigated through their core phylogenomic relationships and accessory genome profiles. Ten different serovars and thirteen sequence type profiles were identified. The most frequent serovar from sausages was the monophasic variant of *S*. Typhimurium (1,4,[5],12:i:-, 72%) while *S*. Derby was in pig carcasses (51%). Phylogenomic clusters found in *S*. 1,4,[5],12:i:-, *S*. Derby, *S*. Rissen and *S*. Typhimurium serovars identified closely related isolates, with less than 10 alleles and 20 SNPs of difference, displaying *Salmonella* persistence along the pork production chain. Most of the *S*. 1,4,[5],12:i:- contained the *Salmonella* genomic island-4 (SGI-4), Tn21 and IncFIB plasmid. More than half of *S*. Derby strains contained the SGI-1 and Tn7. *S*. 1,4,[5],12:i:- genomes carried the most multidrug resistance genes (91% of the strains), whereas extended-spectrum β-lactamase genes were found in Typhimurium and Derby serovars. *Salmonella* monitoring and characterization in the pork production chains, specially *S*. 1,4,[5],12:i:- serovar, is of special importance due to its multidrug resistance capacity and persistence in dry fermented sausages.

Keywords cgMLST, SNPs, Accessory genome analyses, Dry fermented sausages (DFS), *Salmonella*, Whole genome sequencing (WGS)

Europe is the world's second largest producer of pork (22 million tonnes in 2022)[1] after China and the biggest exporter of pork and products thereof[2]. *Salmonella* contamination is a persistent problem in the pork production chain of many European countries. In the European Union (EU) on 2021, food business operators (FBO) samplings of pig carcasses at slaughterhouse detected 1.4% of *Salmonella*-positive samples, with values above the EU mean for France (4.6%) and Spain (3.9%). Subsequently, data gathered by the EU Member States for "Meat and meat products from pigs" category showed proportions of *Salmonella* positive samples up to 0.82% for ready-to-eat (RTE) (with an average of 0.23%) and 1.5% for non-RTE (average of 2.1%)[3]. Recently, during 2020–2023, dry fermented sausages (DFS), classified as RTE meat products, were responsible of 22 alerts related with *Salmonella* contamination as reported in the Rapid Alert System for Food and Feed (RASFF), being France the most notifying country and Spain the most identified country within the notification alerts (9 out of 22)[4]. From those, five were specifically linked to "fuet" (Refs. 2020.2344, 2020.3378, 2021.2535, 2021.3787, 2023.2633), a low-acid DFS traditional from the northeast region of Spain, Catalonia, made from lean pork, fat, salt, and pepper[5], and two out of these five were linked to salmonellosis outbreaks (i.e., 2020.3378 and 2021.3787).

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The most frequently reported serovars in pigs, as a food-animal source, and associated with human salmonellosis due to consumption of pork and its thereof products in the EU in 2021[3] were the monophasic variant of *S.* Typhimurium (1,4,[5],12:i:-, 28.2%), *S.* Derby (22.3%), *S.* Typhimurium (15.3%) and *S.* Rissen (6.6%). Although these serovars are closely related genetically at subspecies level (they belong all to *Salmonella enterica* subsp. *enterica*), they can differ significantly in their pathogenic potentials[6–8]. Furthermore, within the same serovar, clones with a higher virulence and resistance potential may exist. Indeed, pathogenicity is directly associated with resistance to antimicrobials, biocide or heavy metal and virulence profile, traits usually acquired through mobile genetic elements (MGE) (i.e., transposons, integrons and plasmids)[9]. Subsequently, the dissemination of these specific and emerging clones can be favoured by international goods trade and human travelling[10].

Whole genome sequencing (WGS) is currently the most robust method used in surveillance, microbial trace-back investigation, source attribution and risk assessment of food-borne microorganisms[11–14], including *Salmonella* strains and circulating clones. The two main WGS-typing techniques are single nucleotide polymorphism (SNP) or allelic based methods. In particular, core-genome single nucleotide polymorphisms (cgSNP) and core-genome multilocus sequence typing (cgMLST, with 3002 loci in the case of *Salmonella* spp.) are largely used for bacterial typing and phylogenomic analysis[12,15]. Elseways, the accessory genome analyses allow exploring the most variable part of the microbial pan-genome, comprising the vertically or horizontally transferred DNA incorporated in the bacterial chromosome or contained in plasmids[16].

By analysing 173 *Salmonella* isolates from the pork production chain, and more particularly from the DFS production chain, from France and Spain collected in the 1997–2021 period, the present study aimed to characterize the circulating clones with a high potential for resistance and virulence. The dissemination of the prevalent clones was also considered within the pork sector (from farm to fork) and possible trade between France and Spain, two countries among the largest producers of DFS and pork in Europe.

Results

Description of *Salmonella* serovars isolated in the French pork production chain

A total of 74 different serovars were identified among the 4717 *Salmonella* references of the French *Salmonella* Network collection from the pork production chain between 2002 and 2022 within the context of alerts, official control, surveys, surveillance, and control plans. Most of the references (97.9%) were isolated from 2008 to 2020 and, within this period, the main *Salmonella* serovars were *S.* 1,4,[5],12:i:-, *S.* Derby, *S.* Rissen and *S.* Typhimurium. Remarkably, *S.* 1,4,[5],12:i:- progressively increased from 2009 (6.4%) to become the most predominant serovar in the pork production chain in 2014 (41.3%) and then stabilized (Fig. 1). On the contrary, *S.* Typhimurium remarkably decreased its proportion from 2008 (45.5%) to 2020 (5.3%). From 2008 to 2020, there was a slight decrease in the proportion of *S.* Derby (from 36.4 to 26.0%) and *S.* Rissen (from 9.1 to 7.9%).

Genome panel characteristics

Whole genome sequence data of 173 *Salmonella enterica* originating from pork and different stages of the DFS production chain in the northeast area of Spain and France were analysed. The sources of the strains were pig carcass (49), pork (38), fresh sausage (16) and pork DFS (70) (Table 1). A total of 125 isolates were collected during *Salmonella* surveillance, 31 in the context of outbreaks (27 specifically isolated from DFS), 14 and 3 were from IRTA and ANSES culture collections, respectively (Supplementary Table S1).

Among the 173 *Salmonella* genomes analysed there were 10 different serovars: *S.* 1,4,[5],12:i:-, *S.* Derby, *S.* Rissen, *S.* Typhimurium, *S.* Worthington, *S.* Infantis, *S.* Kedougou, *S.* London, *S.* Wien and *S.* Goettingen

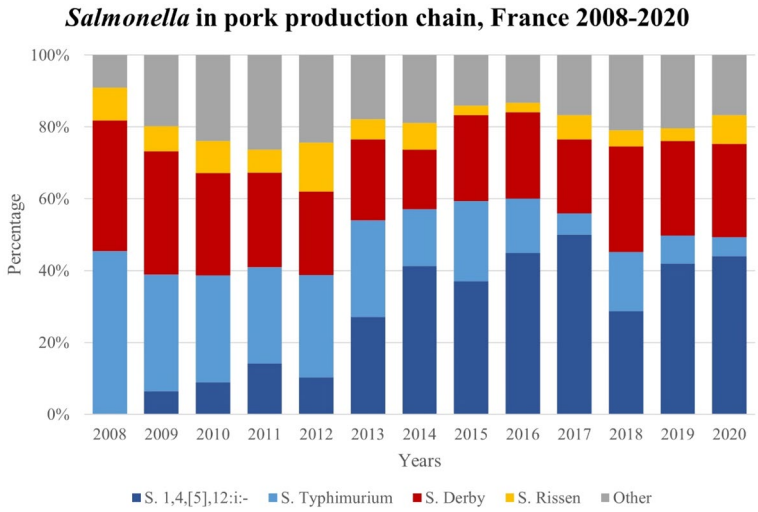

Figure 1. Serovar distribution of the main *Salmonella* serovars in the pork production chain in France from 2008 to 2020.

Serovar	Pig carcass	Pork Meat	Fresh sausage	DFS	Total isolates by serovar
S. 1,4,[5],12:i:-	29%	56%	100%	72%	102 (59%)
S. Derby	51%	42%	–	7%	45 (26%)
S. Rissen	14%	–	–	6%	11 (6%)
S. Typhimurium	4%	3%	–	7%	8 (5%)
S. Worthington	–	–	–	3%	2 (1%)
S. Goettingen	2%	–	–	-	1 (1%)
S. Infantis	–	–	–	1%	1 (1%)
S. Kedougou	–	–	–	1%	1 (1%)
S. London	–	–	–	1%	1 (1%)
S. Wien	–	–	–	1%	1 (1%)
Total isolates by matrix	49 (28%)	38 (22%)	16 (9%)	70 (40%)	173

Table 1. Summary of the *Salmonella* serovars distribution (%) in the different matrixes studied (pig carcass, pork meat, fresh sausage, dry fermented sausages (DFS)) determined in silico using SeqSero+ and monophasic variant of *S.* Typhimurium confirmed by in silico PCR. Dash (–): Serovar not present in the panel.

(Table 1). *S.* 1,4,[5],12:i:- was the most prevalent serovar in DFS (72%), fresh sausages (100%) and pork (56%) and *S.* Derby was the most frequent in pig carcasses (51%).

S. Worthington, *S.* Infantis, *S.* Kedougou, *S.* London, *S.* Wien have only been detected in DFS (with only 1 or 2 isolates each). *S.* Goettingen has only been detected in pig carcasses from Spain.

Multilocus sequence type (MLST) and cgMLST analysis

SeqSphere+ results revealed thirteen different MLST profiles in the 173 *Salmonella* genome panel including ST34 (58.4%) and ST5239 (0.6%) for *S.* 1,4,[5],12:i:-, ST40 (20.8%), ST39 (3.5%) and ST71 (1.7%) for *S.* Derby, ST469 (6.4%) for *S.* Rissen, ST19 (4.6%) for *S.* Typhimurium, ST9253 (1.2%) for *S.* Worthington, ST32 (0.6%) for *S.* Infantis, ST1543 (0.6%) for *S.* Kedougou, ST155 (0.6%) for *S.* London, ST9248 (0.6%) for *S.* Wien and ST20 (0.6%) for *S.* Goettingen (Supplementary Table S2, "ST_Summary" tab).

Considering the cgMLST results, a maximum likelihood phylogenomic tree (Supplementary Fig. S3) and a minimum spanning tree (Fig. 2) were built, and both clustered the isolates per each serovar except for *S.* Derby which had two different lineages due to its polyphyletic nature (ST39 and ST40 belonging to the lineage 1 and ST71 to the lineage 2)[17].

Clustering association analysis revealed 22 clusters (named with the letter of the alphabet from A to V) using as cut-off a maximum of 10 alleles of difference between genomes and grouped 61 out of the 173 genomes (Supplementary Table S2, "cgMLST" tab). Within these 22 clusters, 2 belonged to *S.* Typhimurium (A-B), 14 to *S.* 1,4,[5],12:i:- (C-P), 4 to *S.* Derby (Q-T), 1 to *S.* Rissen (U) and 1 to *S.* Worthington (V) serovars (Fig. 2). Nine of the clusters included *Salmonella* isolated specifically from DFS (B, F, H, I, J, L, M, O, V), 4 from pig carcass (K, R, S, U), 2 from pork (P, Q),1 from fresh sausages (E), and the remaining 6 clusters included isolates from different matrixes. Two (D, G) out of 22 included DFS and pig carcass matrixes, 2 (A, S) pork and pig carcass, 1 (C) fresh sausage and pig carcass, and 1 (N, including six genomes) DFS, pork and pig carcass matrixes (Supplementary Table S2).

SNP-based phylogenomic analysis

The most abundant serovars (i.e., *S.* 1,4,[5],12:i:-, *S.* Typhimurium, *S.* Derby and *S.* Rissen accounted for 95.9% of the genomes analysed in this study) were further explored with the cgSNP phylogenomic analysis for epidemiological investigation and source tracking (see maximum likelihood phylogenomic trees in Figs. 3, 4, 5).

Using a cutoff of 20 cgSNPs, 26 clusters (labelled numerically and with letters according to the serovar, *e.g.*, 1T stands for Cluster **1** of *S.* **T**yphimurium) were identified according to the cgSNP phylogenetic results that grouped 68/173 genomes (Supplementary Table S2, "cgSNP" tabs). This clustering analysis was in accordance with the cgMLST results (Table 2) for *S.* Typhimurium (2 clusters), *S.* 1,4,[5],12:i:- (14 clusters), and *S.* Rissen (1 cluster) serovars. In contrast, in *S.* Derby, 9 and 4 clusters were identified in the cgSNP and cgMLST analysis, respectively (Supplementary Table S2).

Clusters formed by isolates sharing the same metadata and with a difference of ≤ 2 SNPs between isolates were formed by clonal isolates coming from the same sampling day, batch or belonging to the same outbreak. For *S.* 1,4,[5],12,:i:- serovar, six clusters (4M, 6M, 7M, 10M, 11M, 14M) shared the same metadata, for each *S.* Derby, *S.* Rissen and *S.* Typhimurium serovars, only one cluster grouped isolates sharing the same metadata and with ≤ 2 SNPs of difference between core genomes (1D, 1R, and 2T, respectively).

The largest cluster (5M) contained a total of nine closely related *S.* 1,4,[5],12,:i:- isolates, six of them isolated in 2018 from a DFS outbreak occurred in Occitanie. Only one cgSNP differed between the outbreak related isolates and the strain isolated in 2019 also from DFS in Occitanie, and there were six cgSNPs of difference with the strain isolated in 2019 from a pig carcass in Nouvelle-Aquitaine. In cluster 1T (identical cgSNP profiles), *S.* Typhimurium strains were isolated in 2013 and 2014 from a pig carcass and pork in Nouvelle-Aquitaine and Bretagne, respectively. Clusters 5M and 1T are examples of genotype persistence and survival along time in the pork sector in France.

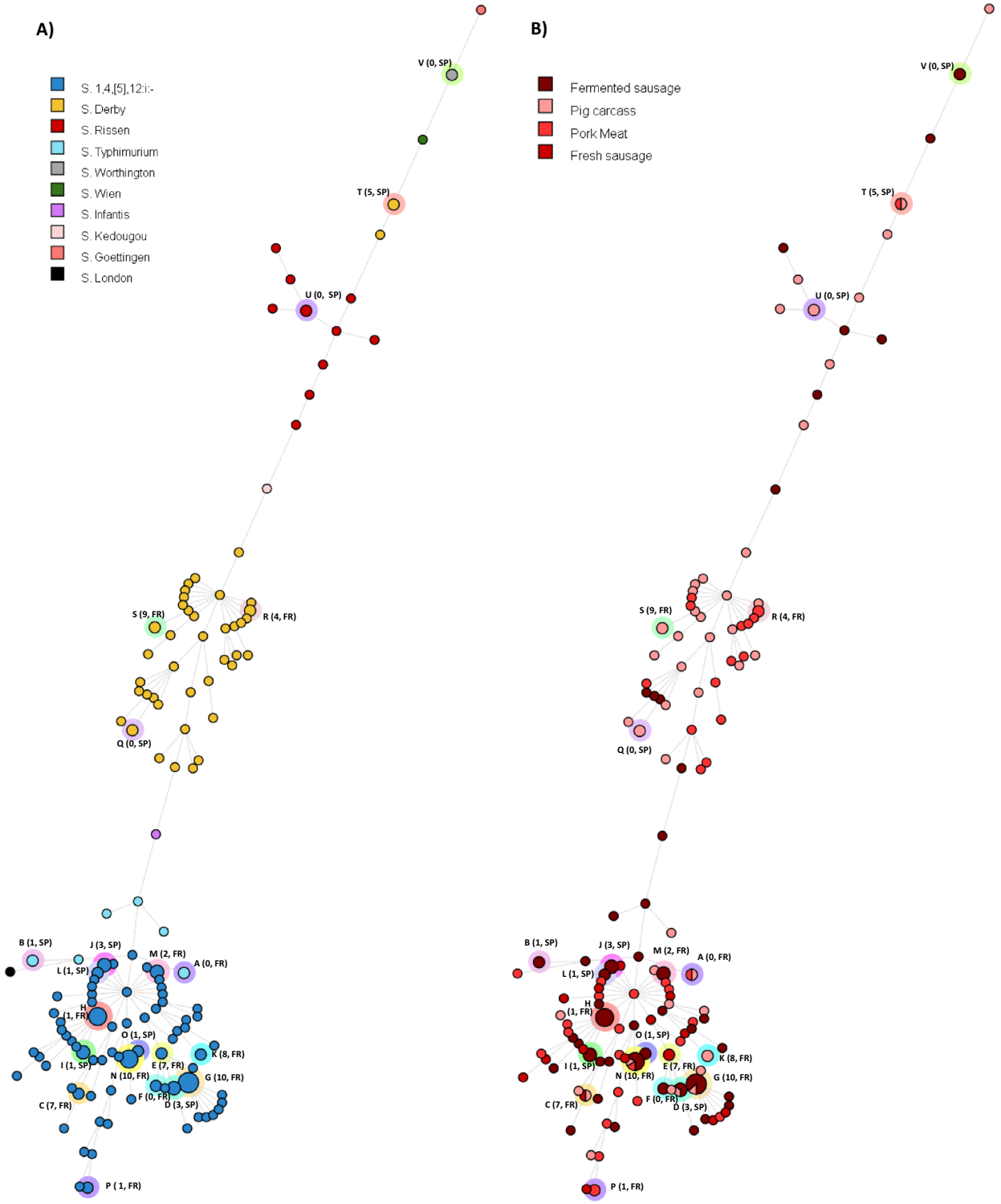

Figure 2. Minimum-spanning tree based on cgMLST analysis of the 173 *Salmonella* isolates. Each node represents a cgST. The node size is proportional to the number of isolates sharing the same genotype. The branch lengths correspond to allelic differences in log-scale. Clusters formed by nodes with a maximum of 10 allelic differences were labelled with coloured halos and, in parenthesis, the number of different alleles between the most distanced isolates in the cluster and the country of origin (SP: Spain; FR: France). Node colouring corresponds to the serovar in A and to the matrix type in B.

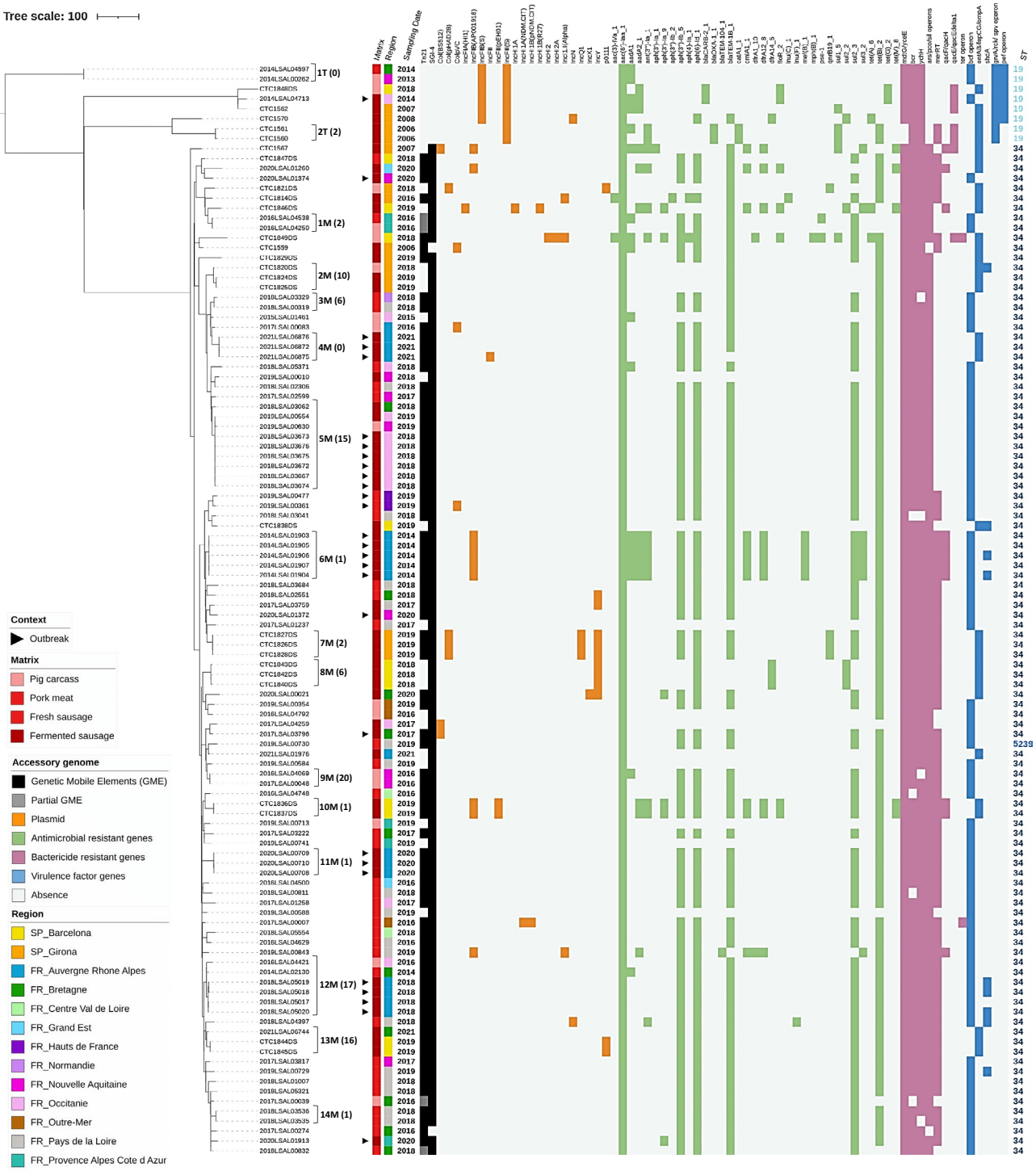

Figure 3. SNP core phylogenomic tree of 110 *S.* Typhimurium and *S.* 1,4,[5],12:i:- isolates including metadata, ST and accessory genome. Tree was constructed using LT2 reference genome. A cutoff of ≤ 20 SNPs highlighted 16 clusters indicated with numbers and letters (*e.g.*, 1T stands for Cluster 1 of *S.* Typhimurium and 1M stands for Cluster 1 of the monophasic variant, *S.* 1,4,[5],12:i:-) and, in parenthesis, the number of different cgSNPs between the most distanced isolates in the cluster. Outbreak related isolates are indicated with a black triangle. Matrix origin and geographic location are indicated with a coloured strip. Sampling year and sequence type (ST) are indicated as labels. Mobile genetic elements (black), plasmids (orange), antimicrobial resistance genes (green), biocide resistance genes (pink) and virulence factor genes (blue) are indicated as a heat map. The accessory genome genes that were found in all the isolates are not represented.

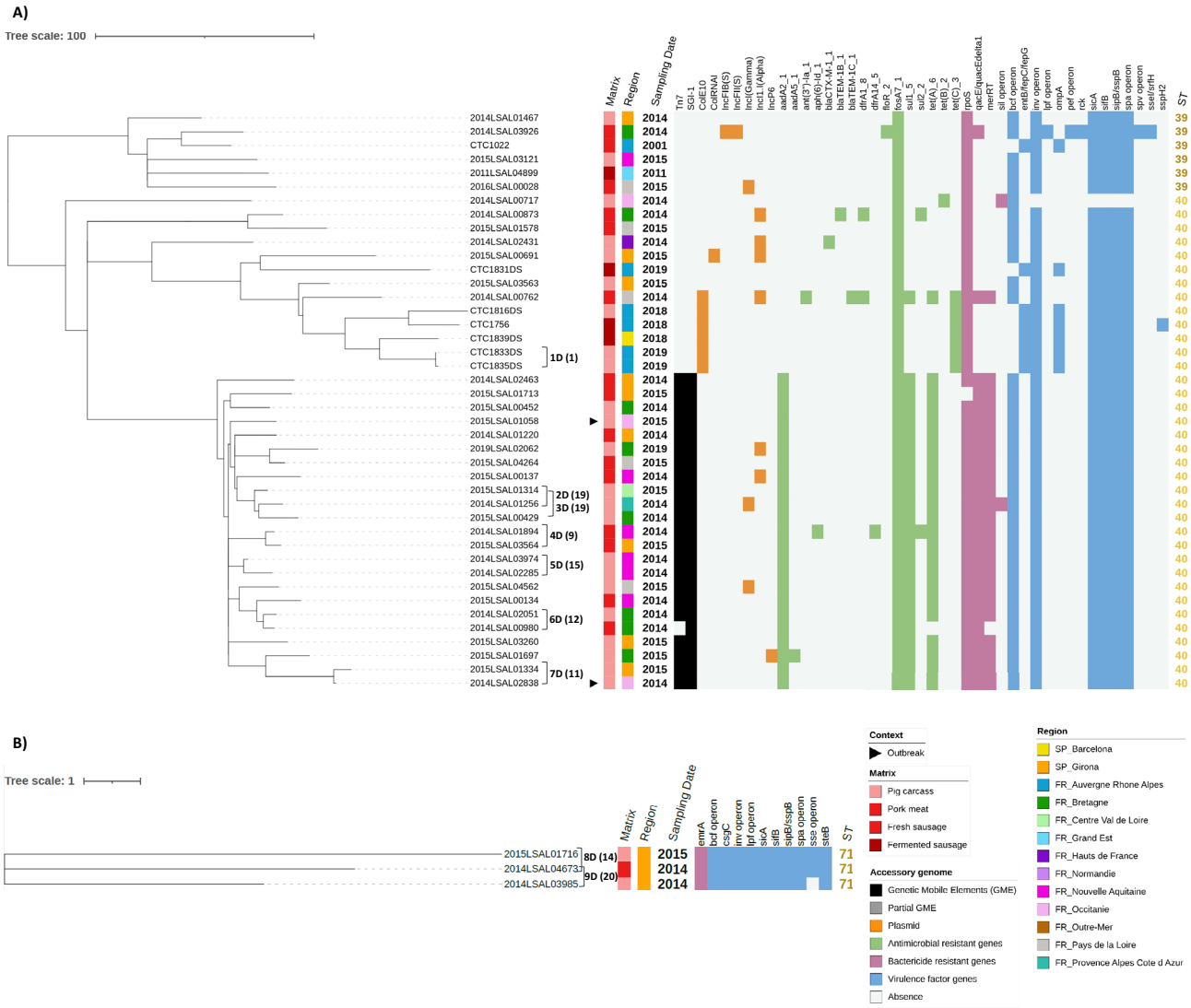

Figure 4. SNP core phylogenomic tree of 45 *S.* Derby isolates, including metadata, ST (ST39 and ST40 (**A**) and ST71 (**B**)) and accessory genome. Tree was constructed RM006 as reference genome A cutoff of ≤ 20 SNPs highlighted 9 clusters indicated with numbers and letters, *e.g.*, 1D stands for Cluster 1 of S. Derby, next to the strain label with the cgSNP value in parenthesis. Outbreak related isolates are indicated with a black triangle. Matrix origin and geographic location or region are indicated with a coloured strip. Sampling year and sequence type (ST) are indicated as labels. Mobile genetic elements (black), plasmids (orange), antimicrobial resistance genes (green), biocide resistance genes (pink) and virulence factor genes (blue) are indicated as a heat map. The accessory genome genes that were found in all the isolates of the *S.* Derby serovar are not represented in the figure.

Cluster 12M provided evidence of *S.* 1,4,[5],12:i:- genotype persistence in the French DFS production chain. The oldest strain of cluster 12M was sampled in 2014 in Centre-Val-de-Loire from pork, then another isolate was collected in 2016 in Occitanie from pig carcass, and four isolates in 2018 in Auvergne-Rhône-Alpes from outbreak related DFS. According to these results, this clone was circulating in the DFS production chain (slaughterhouse > cutting plant > retail) and different France regions for at least four years (2014–2018).

Other examples of clones circulating from pig carcasses to final sausage products are shown by the clusters 1M and 2M including region-specific isolates. Cluster 1M isolates were collected in November 2016 in the same region in France (Provence–Alpes-Côte-d'Azur), from both a pig carcass and a fresh sausage. Cluster 2M isolates were collected in the same region in Spain (Girona) with one strain isolated in 2018 from a pig carcass and two isolated in 2019 from DFS (same batch).

Clusters 4D, 7D (*S.* Derby ST40), and 8D (*S.* Derby ST71) isolates were collected in different years in pig carcasses and pork from the same or different geographic locations, and had few SNPs of difference, suggesting a common *S.* Derby ancestor circulating in the pork sector and within French regions.

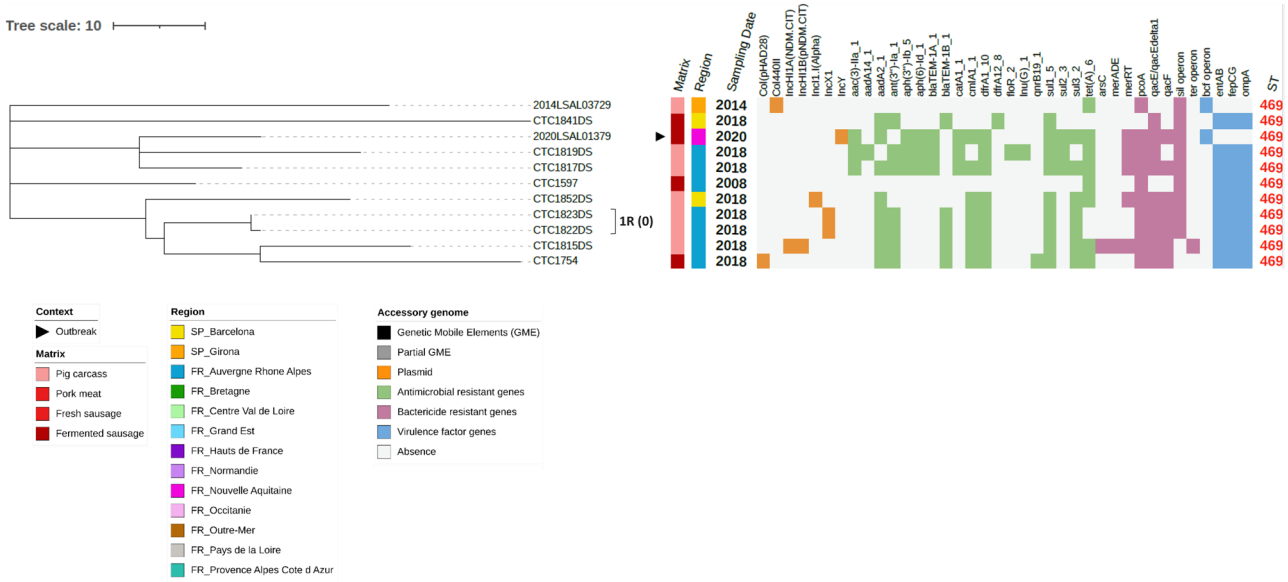

Figure 5. SNP core phylogenomic tree of the 11 S. Rissen including metadata, ST and accessory genome. Tree was constructed using GJ0703-2 as reference genome. A cutoff of ≤ 20 SNPs highlighted 1 cluster indicated with a number and letter, *e.g.*, 1R stands for Cluster 1 of *S.* Rissen, next to the strain label with the cgSNP value in parenthesis. Outbreak related isolates are indicated with a black triangle. Matrix origin and geographic location or region are indicated with a coloured strip. Sampling date, in years, and sequence type (ST) are indicated as labels. No mobile genetic elements were observed. Plasmids (orange), Antimicrobial resistance genes (green), biocide resistance genes (pink) and virulence factor genes (blue) are indicated as a heat map. The accessory genome genes that were found in all the isolates of the *S.* Rissen serovar are not represented in the figure.

Interestingly, the cluster 13M is an example of a multi-country occurrence with *S.* 1,4,[5],12:i:- isolates collected in Spain (Barcelona) in 2019 and in France (Bretagne) in 2021. The genomic distance within all the isolates is of 16 SNPs.

Characterization of *Salmonella* isolates through accessory genome analysis

Resistome, virulome and MGE of all the genomes were examined to characterize the antimicrobial resistances, virulence potential, heavy metal, and biocide tolerances (Figs. 3, 4 and 5) (extended data in Supplementary Tables S4, S5 and S6).

Antimicrobial resistance genes

The antimicrobial resistance (AMR) gene analysis showed that all genomes possessed the cryptic aminoglycoside resistance gene *aac(6')-Iaa*. AMR prediction indicates that *S.* 1,4,[5],12:i:- serovar had a high variability in AMR genetic profile between isolates and more than the 91% of the isolates had multidrug resistance (MDR) genes. The *blaTEM-1B_1*, *aph(3'')-Ib_5* and *aph(6)-Id_1*, *sul2_3* and *tet(B)* genetic profile, described as conferring resistance to ampicillin (beta-lactamase), streptomycin (aminoglycoside), sulphonamide and tetracycline (ASSuT profile) typical of the epidemic clone was observed in 65.7% of the *S.* 1,4,[5],12:i:- genomes. A total of 10.8% isolates had the ACSSuTTm genetic profile which presents simultaneously the ASSuT profile and genes *cmlA1_1* and *dfrA12_8*, coding for chloramphenicol and trimethoprim resistances, respectively. Finally, 3.9% *S.* 1,4,[5],12:i:- isolates had a genetic profile *blaTEM-1B_1*, *aph(3'')-Ib_5* and *aph(6)-Id_1* and *sul2_3* coding for resistances to ampicillin, streptomycin and sulphonamide, respectively.

Fosfomycin resistance, *fosA7_1* gene, was only detected in *S.* Derby. The 55% of the *S.* Derby analysed genomes had the Tn7 and SGI-1 profiles, including *aadA2_1*, *sul1_5* and *tet(A)_6* genes, which code for resistance to aminoglycoside, sulphonamide, and tetracycline, respectively. These isolates closely clustered (ST40) and were recovered in both France and Spain from pig carcass and pork during 2014 and 2015. In contrast, *S.* Derby ST71 showed no AMR genes (except for *aac(6')-Iaa*) (Fig. 3).

Third generation extended-spectrum β-lactamase (ESBL) resistance genes were found in *S.* Typhimurium and *S.* Derby serovars. Specifically in *S.* Typhimurium, two isolates contained *blaCARB-2_1* and two other strains contained *blaOXA-1_1* ESBL genes, coding for resistance to carbenicillinase and carbapenemase, respectively. *blaCTX-M-1_1* ESBL resistance gene, coding for cefotaxime resistance[18], was only found in one isolate of *S.* Derby ST40.

S. Rissen serovar showed a MDR genetic profile (81.8%) where the most frequent resistance genes were *sul1_5*, *aadA2_1*, *tet(A)_6* and *dfrA1_10*, coding for resistance to sulfamethoxazole, aminoglycoside, tetracycline, and trimethoprim, respectively.

Serovar	Clusters		Total isolates	Metadata		
	cgMLST	cgSNP		Geolocation	Matrix origin	Sampling year
S. Typhimurium	A	1T	2	Nouvelle-Aquitaine, Bretagne	Pig carcass, pork	2013, 2014
	B	2T	2	Girona	Dry fermented sausage (DFS)	2006
S. 1,4,[5],12:i:-	C	1M	2	Provence–Alpes-Côte-d'Azur	Pig carcass, fresh sausage	2016
	D	2M	3	Girona	Pig carcass, DFS	2018, 2019
	E	3M	2	Pays de la Loire, Normandie	Fresh sausage	2018
	F	4M	3	Auvergne-Rhône-Alpes	DFS	2021
	G	5M	7/9[a]	Occitanie, Nouvelle-Aquitaine/ Occitanie, Nouvelle Aquitaine, Bretagne[a]	DFS, pig carcass	2018, 2019
	H	6M	5	Auvergne-Rhône-Alpes	DFS	2014
	I	7M	3	Girona	DFS	2019
	J	8M	3	Barcelona	DFS	2018
	K	9M	2	Nouvelle-Aquitaine	Pig carcass	2016
	L	10M	2	Barcelona	DFS	2019
	M	11M	3	Auvergne-Rhône-Alpes	DFS	2020
	N	12M	6	Bretagne, Occitaine, Auvergne-Rhône-Alpes	Pig carcass, pork, DFS	2014, 2016, 2018
	O	13M	2/3[b]	Barcelona/Barcelona, Bretagne[b]	DFS	2019
	P	14M	2	Pays de la Loire	Pork	2018
S. Derby	R	1D	2	Nouvelle-Aquitaine, Auvergne-Rhône-Alpes	Pork	2014, 2015
	-	2D	2	Provence–Alpes-Côte-d'Azur, Centre Val de Loire	Pig carcass	2014, 2015
	-	3D	2	Centre Val de Loire, Bretagne	Pig carcass	2014, 2015
	Q	4D	2	Girona	Pig carcass	2019
	-	5D	2	Nouvelle-Aquitaine	Pig carcass	2014
	-	6D	2	Bretagne	Pig carcass, pork	2014
	S	7D	2	Occitanie, Auvergne-Rhône-Alpes	Pig carcass	2014, 2015
	T	8D	2	Auvergne-Rhône-Alpes	Pig carcass, pork	2014, 2015
	-	9D	2	Auvergne-Rhône-Alpes	Pig carcass, pork	2014
S. Rissen	U	1R	2	Girona	DFS	2018
S. Worthington	V	-	2	Girona	DFS	1997, 1999

Table 2. Description of the phylogenomic clusters resulting from cgMLST (22 clusters, cutoff ≤ 10 alleles) and cgSNP (26 clusters, ≤ 20 SNPs) results encountered in *S.* Typhimurium *S.* 1,4,[5],12:i:-, *S.* Derby, *S.* Rissen and *S.* Worthington serovars. Differences in the isolate metadata and accessory genome profile are indicated. Underlined clusters share the same profile in the accessory genome (MGE, plasmids, AMR, virulence genes and biocide resistance genes). [a]Seven isolates from Occitanie, Nouvelle Aquitaine clustered by cgMLST and 9 from Occitanie, Nouvelle Aquitaine, Bretagne by cgSNP. [b]Two isolates from Barcelona clustered by cgMSLT and 3 from Barcelona and Bretagne by cgSNP.

Virulence genes

The virulence potential of the *Salmonella* isolates was evaluated through the presence of 141 virulence genes. Remarkably, a co-exclusion pattern was found in all the genomes: the presence of *bcf* locus (encoding for fimbrial related proteins) excluded the enterobactin related dehydrogenase and synthase genes (*entA* and *entB*, respectively), adhesin fimbriae genes (*fepC* and *fepG*) and outer membrane protein A (*ompA*) (Figs. 2, 3 and 4). The autotransporter *shdA* gene was found in *S.* Infantis (Supplementary Table S5) and unevenly found in *S.* 1,4,[5],12:i:- isolates from the same cluster (i.e., clusters D, H and N) (Fig. 2). The superoxide dismutase gene, *sodCI*, and the anti-inflammatory effector, *gogB,* were exclusively found in *S.* Typhimurium and *S.* 1,4,[5],12:i:- serovars. The type III secretion system genes, *sseI* and *srfH*, were found in *S.* Typhimurium (100%) and *S.* 1,4,[5],12:i:- (99%) and in one strain of *S.* Derby ST39 (Supplementary Table S5). *Salmonella* pathogenicity island (SPI) 3 (SPI-3) and 9 (SPI-9) were found in all the *Salmonella* isolates analysed. CG54 island was found in all *S.* Typhimurium, *S.* 1,4,[5],12:i:- and *S.* Infantis isolates, SPI-2 in all *S.* Typhimurium and *S.* 1,4,[5],12:i:- isolates and SPI-13 was exclusively found in *S.* 1,4,[5],12:i:- serovar. SPI-8 was found in all *S.* Rissen, *S.* Derby (ST71), *S.* Worthington, *S.* Kedougou, and *S.* Wien isolates (Supplementary Fig. S3, Supplementary Table S8).

Genes implied in biofilm formation, biocide and stresses tolerances

A total of 121 genes related to biofilm formation, stress adaptation and biocide and chemical/metal compounds resistance, among others, were evaluated (Supplementary Table S6). The profile of bactericide resistance genes highly depended on the serovar (Figs. 2, 3 and 4). All serovars carried 11 biocide resistance genes, five stress adaptation protein genes, and, apart from *S.* Goettingen, 10 biofilm formation genes. The *ars*/*pco*/*sil* operons, described to confer resistance to arsenic, cooper and silver, were simultaneously observed in 98% of *S.* 1,4,[5],12:i:- genomes, whereas *pco*/*sil* were simultaneously observed in 81.8% of *S.* Rissen isolates. Quaternary

ammonium compounds resistance genes *qacE/qacEdelta1* (*qacEdelta1* is the truncated *qacE* and does not express itself) and *qacF/qacH* were present in *S.* Typhimurium (62.5%) and *S.* Derby (55.6%), and in *S.* Rissen (63.6%) and *S.* 1,4,[5],12:i:- (10.8%), respectively. *Ter* operon, linked to tellurite resistance, was detected in *S.* Rissen (9.1%) and *S.* 1,4,[5],12:i:- (2.0%). The three *S.* Derby ST71 isolates had the *emrA* gene, coding for an efflux pump associated to chromate resistance[19]. The truncated *mer* operon (*merRTPC-ΔmerA*), involved in mercury resistance, was present in 1,4,[5],12:i:- (66.7%), Derby (51.1%) and Rissen (45.5%) serovars. The MGE Tn21 and SGI-4 were highly prevalent in *S.* 1,4,[5],12:i:- (80.4 and 98.0%, respectively) and Tn7 and SGI-1 were present in half of the *S.* Derby ST40 isolates (48.9% and 51.1%, respectively). In contrast, they were not detected among other serovar isolates.

Plasmids

The presence of plasmid replicons in *Salmonella* genomes was different among serovars (Supplementary Table S7). At least one plasmid was identified in *S.* 1,4,[5],12:i:- (33 genomes out of 102) (Fig. 2), *S.* Derby (16/45) (Fig. 3), *S.* Typhimurium (8/8) (Fig. 2), *S.* Rissen (7/11) (Fig. 4), *S.* Infantis (1/1), *S.* London (1/1) and, *S.* Wien (1/1) genomes, though no plasmids were identified in *S.* Goettingen, *S.* Kedougou and *S.* Worthington. IncFII(S) was detected in all *S.* Typhimurium and one *S.* Derby. Other Inc plasmid replicons were sparsely detected, being the most frequent IncFIB(S) in *S.* Typhimurium and, IncFIB(AP001918) and IncY in some *S.* 1,4,[5],12:i:-. However, the most abundant plasmid replicons in *S.* Derby were colE10 and IncI1.I(Alpha) (Figs. 2, 3 and 4). The presence of plasmid replicons was linked to phylogenomically related clusters H (IncFIB(AP001918), concurrently with the ACSSuTTm resistance genes profile) and I (col(pHAD28), IncQ1 and IncY) in the *S.* 1,4,[5],12:i:- serovar.

Discussion

The relevance of *S.* 1,4,[5],12:i:- was raised in the recent decades and the serovar increase observed in France agrees with the information reported from Spain[20] and worldwide[21]. *S.* 1,4,[5],12:i:- was first described as an atypical monophasic *S.* Typhimurium in 1987[22] and it was spread in Spain during the 1990s[23]. From then on, within the context of pork industry globalization[24], its dominance among the existing 2,600 *Salmonella* serovars has occurred in the pig herds specially[25–27]. *S.* 1,4,[5],12:i:- (ST34) is also the most abundant serovar from the evaluated panel of *Salmonella* genomes, corresponding to isolates from the pig production chain (i.e., pig carcasses, pork, pork sausages and dry fermented sausages) from both France (65%) and Spain (44%) during the 1997–2021 period.

Pig carcasses, before being cut, are cooled down to refrigeration temperatures (0−4.4 °C), which has been reported to cause a *Salmonella* decrease in meat though not eliminating it completely[28]. Interestingly, the number of *S.* 1,4,[5],12:i:- isolates is higher in fresh sausages and DFS than in pig carcasses, its main source of contamination. These two facts could indicate that there is a selection towards the 1,4,[5],12:i:- serovar along pork and DFS production chain, a process which ends when food matrix is fermented (i.e., acidified) and dried[5]. DFS are a harsh environment for *Salmonella* and a progressive decrease of the pathogen has been described[29]. Under these circumstances, the high stress tolerance described for *S.* 1,4,[5],12:i:- among *Salmonella* serovars[30] and the efficient colonization and survival abilities displayed above its parent *S.* Typhimurium strain[31], could account for the higher prevalence of this serovar at the end of the pig production chain (i.e., fresh sausages and, particularly DFS). *S.* Derby and *S.* Rissen serovars seem less well adapted to the environment of DFS processing plants and to the production processes of DFS. Indeed, despite its prevalence during the last 20 years in pig herds has been stable[32], as shown by French *Salmonella* Network data, our genomic panel showed a decrease of *S.* Derby and *S.* Rissen serovars along the DFS production chain, from pig carcass (51% and 14%, respectively) to the final product (7% and 6%, respectively).

The phylogenomic relationship between the 173 isolates shows *Salmonella* clusters of two or more isolates with equal or less than 10 allelic and 20 SNP differences in the core genome from *S.*1,4,[5],12:i:-, *S.* Derby, *S.* Rissen, *S.* Typhimurium and *S.* Worthington. Among the clusters, most of them indicated genotype persistence and survival along time in the pork sector and DFS production chain while others are related with region-specificity. Within our panel only three isolates clustered together in the cgSNP analyses although having different origin country, suggesting a witness of international trade exchange.

Salmonella Typhimurium and *S.* 1,4,[5],12:i:- isolates ancestry and phylogeny has been studied in several pig-related environments (i.e., pig farms and slaughterhouses)[20,33,34] and only a few studies[35] have focused in the production chain of pork products from official control sampling. The phylogenomic results have unveiled that 7 out of 9 *Salmonella* clusters identified within DFS matrix were due to the monophasic *S.* 1,4,[5],12:i:-, thus increasing its concern for official authorities and industry. *S.* Typhimurium short-term substitution rate has been reported to be of 1–2 SNPs per genome per year, thus providing information of strain clonality or common ancestor[36]. WGS-derived SNPs provided great cluster resolution in our panel that showed *S.* Typhimurium clonal isolates dissemination and transmission between regions (cluster 1 T, 0 cgSNPs) and *S.* 1,4,[5],12:i:- isolates with a common ancestor in the DFS production chain (cluster 12 M, 17 cgSNPs). Cross-country spread of *Salmonella* due to exportation of DFS was found in our phylogenetic results. Out of 173 samples analysed, three noticed cross-border contamination with a prevalence of 1.8% in our study. Other studies also reported *Salmonella* dissemination due to pig trade in Europe[20].

In agreement with previous studies[37], *S.* Derby STs mainly found in the French pork sector were ST40 and ST39 and the same was observed for Spanish genomes. Regardless of its polyphyletic nature, cgSNP analysis of *S.* Derby isolates was highly resolutive and closely related clusters were identified within ST40 and ST71 and genotypes with matrix or geographic persistence were shown. WGS approach has also been used for trace-back microbial investigations, which have indicated DFS as the main source of *Salmonella* outbreaks[38,39]. In our study, cluster 5M (15 cgSNPs) revealed seven 1,4,[5],12:i:- isolates from DFS related with an outbreak in 2018 and from

pig carcass in 2019, which emphasizes the importance of following good manufacturing practices and validating the DFS production process[40] together with adequate sampling plans and monitoring. Successful implementation of continuous monitoring of *Salmonella* has shown an effective control of the pathogen dissemination[41]. On the other hand, further studies should be carried out on the ability of some *S.* 1,4,[5],12:i:- clones to resist cleaning and disinfection practices applied in DFS manufacturing processes.

MGE determine the potential for genomic plasticity and pathogenicity of a bacteria[42,43]. Among the identified MGE, plasmid replicon types IncF and Col are the two most abundant replicon families in the dataset. IncFIB and IncFII virulence plasmids are among the best characterized and abundant plasmids within the genus[44] and have been described to be part of the ancestral virulence plasmids together with *rck*, *spv* and *pef* virulence operons[45]. The inheritance of these plasmids is primarily vertical and serovar divergence theory may explain why only one *S.* Derby strain and all genomes of *S.* Typhimurium contain these plasmids[46]. Colicinogenic (Col) plasmids, which encode colicin bacteriocins, are typical from *Enterobacteriaceae* and are abundant in animal guts[47]. ColE10_1 is usually found in *Salmonella* and its relationship with quinolone resistance spread through *qnrS1* and *qnrB19* genes has been described[48]. In our panel, Col plasmids were found in genomes from *S.* 1,4,[5],12:i:- (9.80%), *S.* Derby (15.56%) and *S.* Rissen (18.18%), isolated from all studied matrixes. ColE10_1 plasmid was the most detected between the Col plasmids, specifically, it was found in 6 *S.* Derby genomes (13.33%), but *qnrB19_1* gene, that confers resistance to quinolone, was not detected concurrently.

Furthermore, transposons and *Salmonella* Genomic Islands are MGE usually integrated in the chromosome and carry specific antimicrobial resistance genes (ARG), virulence factors and biocide resistance genes. In 1980s, the acquisition of Tn21 and SGI-4 favoured the expansion of 1,4,[5],12:i:- European epidemic clone[49]. The majority of 1,4,[5],12:i:- genomes in the panel showed the Tn21 genetic element, which encodes mercury resistance (*merRT*) and antibiotic (ASSuT profile) genes, and SGI-4, encoding genes involved in arsenic (*ars* operon) and copper (*pco* operon) resistances[31,49,50]. Isolates of 1,4,[5],12:i:- mainly from DFS (90.9%) had the ACSSuTTm profile, which is usually related to the acquisition of the class 1 integron[51]. Stress conditions (*e.g.*, cleaning and disinfection procedures) promote the gain of MGE[52] that can include genes conferring resistance to heavy metals, biocides and biofilm formation, providing the ability to overcome stress conditions and favouring *S.* 1,4,[5],12:i:- serovar survival and its selection[51,53]. In *S.* Derby ST40 there is a big cluster of isolates that carry resistance genes to quaternary ammonium and mercury compounds, co-occurring with *aadA2*, *sul1* and *tet(A)* AMR genes. This fact was already described by Sévellec et al.[17] for the presence of the SGI-1, which also included *tetA* gene and extra mercury resistance genes (*merA* and *merC*) located in a Tn7 transposon.

Salmonella Typhimurium and its monophasic variant shared some genomic particularities (i.e., presence of SPI-2 and SPI-13) in comparison to the other studied serovars. SPI-2, which was found exclusively in *S.* Typhimurium and *S.* 1,4,[5],12:i:- isolates, is a 5-kb locus of horizontally acquired virulence genes that encodes a type III secretion system responsible for delivering effector proteins to the host cell after infection[54]. SPI-13, which was found in some genomes of *S.* 1,4,[5],12:i:-, harbours genes that encode proteins putatively involved in bacterial metabolism, however, their functions remain largely uncharacterized[55].

Virulence factors related to *Salmonella* adherence, *bcf* operon, and infection, *entAB*/*fepCG*/*ompA*, were excluding each other in the *Salmonella* genomes. The *bcf* gene, standing for bovine colonization factor, is an operon encoding for cryptic fimbriae and plays a role in the regulation of biofilm formation when *Salmonella* colonizes the intestines[56,57], though has not been described to promote the biofilm formation in industrial surfaces. In contrast, *ent* operon encodes for the ferric iron binding siderophore enterobactin and *fep* operon encodes for the siderophore ABC transporter[58]. Both *ent* and *fep* operons, together with the ferric iron binding siderophore salmochelin constitute the primary ferric iron import system of *Salmonella* and are required for its persistent infection in macrophages[58]. Functions of outer membrane proteins (OMPs) are multiple and iron regulation function has also been attributed, specifically for the take up of ferri-siderophore complexes[59,60]. Nonetheless, *ompA*, encoding for the outer membrane protein A, plays an important role in the intracellular virulence of *Salmonella* due to the self-protection from the macrophages nitrosative stress[61] and the activation of the immune system response[62]. The *shdA* gene was exclusively found in *S.* 1,4,[5],12:i:- and *S.* Infantis, unequally found in *S.* 1,4,[5],12:i:- isolates from the same sampling and in different proportions in the studied matrixes (7.1% in pig carcass, 5.0% in pork, 6.3% in fresh sausages and 11.5% in DFS). Gene *shdA* encodes for an OMP that is expressed while the pathogen inhabits the animal intestine and allows its specific binding through fibronectin[63], an extracellular adhesion molecule involved in muscular tissue repair. The presence of *shdA* could be an advantage for *S.* 1,4,[5],12:i:- isolates attachment to pig carcasses and fresh pork, enhancing its selection along the production chain and together with the abovementioned stress tolerance result in the serovar persistence and survival.

Multidrug resistancet (MDR) *Salmonella* strains represent a serious challenge worldwide in the treatment and control of *Salmonella* infections, since these strains exhibit resistance to three or more antimicrobial classes[64]. MDR *Salmonella* isolates from pigs was of 39.1% in the EU in 2021[65]. Our results show that the most prevalent serovar in DFS, *S.* 1,4,[5],12:i:-, is also the serovar described to harbour more ARG in its genomes (i.e., 91% of *S.* 1,4,[5],12:i:- genomes had three or more ARG), thus proving the warning for its worldwide spread. Notwithstanding, extended-spectrum β-lactamase (ESBL) genes, *blaCARB-2_1* and *blaOXA-1_1*, were found in *S.* Typhimurium, and *blaCTX-M-1_1* in *S.* Derby ST40, in pig carcasses and DFS from both countries, France and Spain, since 2006. WGS approach allowed the detection of ESBL in a large genome dataset without in vitro susceptibility testing and the monitoring of MDR *Salmonella* profiles which is of interest for tracking resistome evolution and transfer in different ecosystems and to identify emerging resistance hazards more quickly[66].

Several genetic markers of resistance to antibiotics and biocides, virulence factors and MGE have been found in the analysed *Salmonella* genomes, especially in *S.* 1,4,[5],12:i:-. Considering the high figures of the pig and pork derivatives industry and the fact that DFS are RTE products (i.e. eaten without the need for cooking), the transmission of *Salmonella* isolates and the corresponding resistance genes along the pork production chain is of concern. The ability of the enteric pathogen to survive along the DFS production process, overcoming

disinfection cycles and DFS harsh conditions and the remarkable presence of strains with MDR genetic profile emphasize the need for *Salmonella* monitoring globally, paying special attention to *S.* 1,4,[5],12:i:- serovar. Further research on phenotype verification would confirm the survival advantage provided by the genetic markers encountered in the genomic *Salmonella* panel. In this context, WGS technology is a powerful tool to establish precise phylogenetic relationships between genomic clusters of persistent and transmissible strains in the pork sector, confirming the spread of the *S.* 1,4,[5]12:i:- European epidemic clone and characterizing the differences in the resistome and virulence profile between *Salmonella* serovars and food matrixes. Sharing genome sequences of isolates together with the corresponding metadata is essential to perform international pathogen surveillance, quickly identify outbreaks, and move forward towards the One Health approach.

Materials and methods

Salmonella isolates origin and selection

Fifty Spanish *Salmonella* isolates were analysed for this study, 36 isolates were provided by the official control food services of the Department of Health (Catalan Public Health Agency, Government of Catalonia) and 14 were from the IRTA culture collection. Isolates originated from different matrixes (pig carcass, n = 14, pork, n = 1, and DFS, n = 21) sampled in the frame of the "Biological Hazards Surveillance Program" (BHSP) and "*Salmonella* control program" (SCP), from 2016–2019 and 2018–2019, respectively. IRTA culture collection provided 12 *Salmonella* spp. genomes isolated from dry fermented sausages and 2 from pork from 1997–2018.

For French data, ANSES database of *Salmonella* Network was inquired for *Salmonella* spp. isolates collected from 2002 to 2022 from pig carcass, pork, and sausages (including DFS and "fresh sausages" made from pork and species). A total of 4717 references were obtained and evaluated to determine the proportion of the main serovars over time in the pork production chain in France. From those, 143 isolates had the genome available, and 123 genomes were selected for this study. For pig carcasses and pork origin genome isolates, sample duplicates were removed and the French regional pig carcass and pork production data[67] was considered to balance the number of genome isolates for each French region (i.e., n = 35 genomes from pig carcasses and n = 35 from pork)[67]. All *Salmonella* genomes available from dry fermented sausages (n = 37) and fresh sausages (n = 16) were selected.

Specific information for Spanish and French *Salmonella* genomes, including matrix, is summarised in Supplementary Table S1.

Genome sequencing and bioinformatics

Genomic DNA of the 50 IRTA *Salmonella* spp. isolates was extracted and isolated with the QIAamp DNA Mini QIAcube Kit (QIAGEN) with the automatic QIAcube sample preparation system (QIAGEN). DNA was quantified spectrophotometrically (µDrop plate, Thermo Fisher Scientific, Waltham, MA, USA) and fluorometrically (Quant-iT™ 1X dsDNA HS Assay Kit, Invitrogen, Merelbeke, Belgium) in a Varioskan™ multiplate reader (Thermo Fisher Scientific, USA). DNA samples were sent to Macrogen, Inc (South Korea) for library preparation and sequencing. Nextera DNA XT technology (Illumina) was used for library preparation and indexing according to the manufacturer recommendations. Paired-end sequencing (2 × 150 bases) was performed with an Illumina NovaSeq6000 sequencer.

The 123 French isolates were previously sequenced using Illumina chemistry producing paired-end reads as described by Radomski et al.[68] and Sévellec et al.[17].

Spanish and French raw reads were quality checked and filtered as described by De Sousa Violante et al.[69] and with an in-house pipeline. In brief, Trimmomatic v0.40[70] was used for the trimming step, FastQC v0.11.5 to check the read quality and ConFindr v0.8.1 to identify intra-and cross-species contamination[71]. An in silico PCR was performed to confirm the monophasic *S.* Typhimurium variant according to the primers described in the ISO/CD TS 6579-4[72].

The metadata of the final panel of 173 *Salmonella* spp. genomes set for bioinformatic analysis are reported in the Supplementary Table S1.

Phylogenomic analyses

All the genomes were uploaded and deposited in EnteroBase database (https://enterobase.warwick.ac.uk/). Whole genome sequences of 173 *Salmonella* spp. isolates were analysed trough in silico MLST, using the seven housekeeping loci (including *aroC, dnaN, hemD, hisD, purE, sucA* and *thrA*)[73], and through cgMLST, using 3002[74] loci based on SeqSphere+ v7.0.4 (Ridom R GmbH, Münster, Germany) scheme. A maximum likelihood phylogenetic tree was built considering the cgMLST SeqSphere+ results (Supplementary Fig. S3) and a minimum spanning tree was built with Bionumerics v7.6.3 (bioMérieux/Belgium) (Fig. 2).

For high-resolution genotyping, the cgSNP analysis was carried out by aligning the sequences of the most prevalent serovars (i.e., *S.* Typhimurium and its monophasic variant, *S.* Derby and *S.* Rissen), using snippy-core command, within Snippy v4.6.0 (https://github.com/tseemann/snippy). Reference strains used for cgSNP analysis were strains LT2 (NCBI NC_003197.1), RM006 (NCBI GCF_028892955.1) and GJ0703-2 (NCBI GCF_011057955.1), for the *S.* Typhimurium and its monophasic variant, *S.* Derby and *S.* Rissen serovars, respectively. The full-length whole-genome alignment was cleaned with the snippy-clean function and then used as an input to Gubbins v2.4.1 to filter and remove recombination artifacts[75]. The pairwise SNP differences were calculated using snp-dists v0.8.2 (https://github.com/tseemann/snp-dists). The alignment length for LT2 was 4,857,450 nucleotide and pairwise SNP differences ranged between 0 and 999. For RM006, the alignment length was 4,825,435 nucleotide sites and pairwise SNP differences ranged between 0 and 40,793. The alignment length for GJ0703-2 of 4,930,938 nucleotide sites and pairwise SNP differences ranged between 0 and 183.

The maximum likelihood phylogenomic trees were constructed from cgSNP results using RaxML v8.2.10[76], with the evolutionary model GTRCAT and 100 bootstraps. Trees were visualized and annotated using interactive

Tree Of Life (iTOL)[77]. A cutoff of 20 cgSNPs was set to define clusters of closely related isolates, based on the short-term substitution rate of 1–2 SNPs per genome per year for *Salmonella*[34,36,78] and the range of strain isolation dates (1999–2021), as recommended by the European Food Safety Authority (EFSA) for *Salmonella* epidemiologically related strains[11].

In silico detection of resistance and virulence genes, from ResFinder v4.4.2, Bacmet v2.0 and VFDB v4.0 databases was performed using an in-house pipeline whereas the detection of SPI and plasmid track down was performed through SPIFinder v2.0 and PlasmidFinder v2.0.1 databases, respectively, available online at the Center for Genomic Epidemiology (CGE) (Denmark). The minimum threshold of genetic identity was set at 90% for the in-house pipeline and 95% for the online databases and, the coverage at 80% in both cases.

Data availability

All genome sequences were deposited in EnteroBase database (https://enterobase.warwick.ac.uk/). Data is provided within the manuscript tables and figures and supplementary tables and figures.

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The authors declare no competing interests.

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Article III

The antilisterial effect of *Latilactobacillus sakei* CTC494 in relation to dry fermented sausage ingredients and temperature in meat simulation media

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Article

The Antilisterial Effect of *Latilactobacillus sakei* CTC494 in Relation to Dry Fermented Sausage Ingredients and Temperature in Meat Simulation Media

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Abstract: *Listeria monocytogenes*, the causative agent of listeriosis, is a relevant pathogen in dry fermented sausages (DFSs), and the application of antilisterial starter cultures is an effective intervention strategy to control the pathogen during DFS production. The effect of factors in relation to DFS formulation and production, NaCl (0–40 g/L), Mn (0.08–0.32 g/L), glucose (0–40 g/L) and temperature (3–37 °C), on the behaviour of *L. monocytogenes* when cocultured with *Latilactobacillus sakei* 23K (non-bacteriocinogenic) and CTC494 (bacteriocinogenic) strains was studied through a central composite design in meat simulation media. *L. sakei* and *L. monocytogenes* counts, pH, lactic acid production and bacteriocin activity were determined in mono and coculture. The pH decrease and lactic acid production were highly influenced by glucose, while production of sakacin K by *L. sakei* CTC494 was observed at moderate (10 and 20 °C), but not at the lowest (3 °C) and highest (37 °C), temperatures. Coculture growth had no effect on the acidification and bacteriocin production but inhibited and inactivated *L. monocytogenes* when *L. sakei* 23K entered the early stationary phase and when *L. sakei* CTC494 produced sakacin K. Optimal conditions for achieving a 5-log units reduction of *L. monocytogenes* were at 20 °C, 20 g/L of NaCl, 0.20 g/L of Mn and 40 g/L of glucose, those highlighting the importance of considering product formulation and fermentation conditions for bioprotective starter cultures application.

Keywords: *Listeria monocytogenes*; starter culture; bioprotection; bacteriocin; meat fermentation

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1. Introduction

Listeria monocytogenes is a facultative anaerobe foodborne pathogen found in a wide range of environments, including water, soil, faeces, food processing environments, and food [1]. This pathogen, when ingested, is the causative agent for listeriosis, a very severe foodborne illness with high mortality rate between 20 and 30%, making *L. monocytogenes* one of the most significant pathogens encountered in food [1,2]. Ready-to-eat (RTE) products (e.g., deli meats, soft cheeses, and seafood) are the main source of listeriosis outbreaks. Dry fermented sausages (DFSs) are not generally linked to food poisoning [1,3], due to the low probability of growth of the pathogen in these foods thanks to the effect of hurdles combination, with pH and water activity (a_w) reductions achieved during fermentation and drying processes [4]. However, DFSs (especially low-acid type) are occasionally involved in withdrawals and recalls if the raw meat is contaminated with *L. monocytogenes* and the lethality of the fermentation process is not able to decrease to compliance levels (i.e., <2 log CFU/g) [5]. In the last 4 years, the European Rapid Alert System for Food and Feed portal (RASFF) [6] has recorded 13 notifications of *L. monocytogenes* in DFSs. The ubiquitous nature

of *L. monocytogenes* allows it to be found at all stages of the pork processing chain, including raw pork. Specifically, pork can be contaminated in industrial environments when in contact with equipment and surfaces, where the pathogen can adapt and persist for extended periods, for instance in biofilm formations (i.e., aggregation ability of microorganisms on wet surfaces and grow in microcolonies, persisting in the environment) [7–9]. This increases the risk of cross-contamination during post-processing stages, which can pose a listeriosis risk to vulnerable consumer groups such as pregnant woman and individuals with weakened immune systems. Chilling temperatures during pork storage before DFS manufacturing can support *L. monocytogenes* growth [10], since it can multiply under relatively broad range of conditions of temperature (−1.5–45 °C), pH (4.5–9.0), a_w (0.92–0.99) and salt concentrations (<16%) [10–12]. Despite European Regulation (EC) 2073/2005 establishing a maximum of 100 CFU/g in ready-to-eat (RTE) foods like DFSs, which are unable to support the growth of *L. monocytogenes*, 1.24% of fermented sausage units tested positive, and 0.11% exceeded the maximum limit according to enumeration tests for the pathogen in EU official samplings conducted during the 2018–2022 period [3,13,14]. As shown by several authors [8,15], *L. monocytogenes* is highly present in the equipment, industrial surfaces, the meat batter used for DFS, and final products, which is of concern.

Mediterranean-style DFSs are produced with a mixture of lean pork and fat with ingredients and additives including salt, dextrose, black pepper or paprika, sodium ascorbate and nitrifying agents. The production process of DFSs consists of fermentation and drying steps that reduce the meat batter pH and a_w, turning DFSs into self-stable meat products not supporting bacterial growth [16]. Acidification and decrease in water activity transform nutritious fresh meat environments to harsh conditions for microorganism growth and development [10]. In the framework of the hurdle technology, both the formulation and production processes (i.e., decrease in pH and a_w) provide antimicrobial barriers that contribute to the food safety of the final product. In industrial production, lactic acid bacteria (LAB) are frequently used as starter cultures to promote the product acidification through fermentation. Some LAB strains (the so-called bioprotective LAB) produce small antimicrobial peptides (i.e., bacteriocins such as sakacin and enterocin) with strong antimicrobial activity against *L. monocytogenes* [17] that can be used as a strategy to control the pathogen [18,19]. In fermented meat products, the bioprotective culture *Latilactobacillus sakei* CTC494 (formerly *Lactobacillus sakei* CTC494), a producer of sakacin K, has been used as starter culture in different types of DFSs, enhancing food safety due to its antilisterial effect [20,21]. The sakacin K antilisterial effect of *L. sakei* CTC494 has been previously tested with *Listeria innocua* coculture in meat simulation media [22], but it is the first time that it is experimented in coculture with the pathogenic *L. monocytogenes*. Sakacin K is a class IIa bacteriocin composed of 40 amino acid residues and a molecular weight of 3802 Da. Its structural gene is located on a 60 Kbp plasmid harboured by the *L. sakei* CTC494 strain. The sakacin K mode of action is similar to other bacteriocins such as sakacin A and curvacin A. These bacteriocins specifically attach and form pores in the membrane of *Listeria*, disrupting the cell homeostasis and thereby reducing cell viability [21,23]. Among the DFS ingredients influencing the behaviour of LAB, glucose and black pepper have shown to promote the growth, while NaCl can reduce their growth. For instance, increasing the amount of salt, from 2 to 3.5% or up to 6%, was reported to interfere with the LAB growth, bacteriocin production or the binding of the bacteriocins to the target microbiota cell membrane receptors [24,25]. In contrast, glucose, as carbon source, assists bacteriocin biosynthesis and bacterial growth [26]. Similarly, black pepper, with a high manganese content (76 ppm), promotes the LAB growth and fermentation of the meat batter [27,28]. Environmental conditions during fermentation, including temperature and sausage ingredients and additives (e.g., salt, nitrite, magnesium, manganese, oxygen, and fat), were reported to influence the growth of *L. sakei* CTC494 and sakacin K production [29,30]. Accordingly, in the DFS production process, ingredients, additives, temperature, and initial microbial communities have cross-related effects on the fermentation; hence [31], that can have an impact on the bacterial growth and metabolism.

Response Surface Methodology (RSM) is a robust and cost-effective approach [32] that allows to study the influence of several factors on bacterial growth response and metabolite production behaviour through a multifactorial experiment [33]. Previous RSM studies have shown reliable inferences when testing the influence of ingredients and additives used in RTE food on the growth and inhibition of *Listeria* spp. [2,33,34]. The optimal application of factors promoting the production of antimicrobials (e.g., bacteriocins and organic acids) and inhibition of pathogens can be used as a tool to improve food safety.

The objective of the present study was to evaluate the effect of factors related with the DFS formulation and production (glucose, manganese, salt, and temperature) on the behaviour of a non-bacteriocinogenic and bacteriocinogenic *L. sakei* strains growing in mono and coculture with *L. monocytogenes*. An RSM approach was applied to identify those combinations enhancing the antilisterial potential of *L. sakei* used as starter culture in DFSs.

2. Materials and Methods

2.1. Experimental Design

The effect of NaCl, manganese (Mn) and glucose (Gluc) concentration and temperature (T) on the behaviour of *L. sakei* and *L. monocytogenes* in monoculture and coculture was evaluated through two complete factorial designs ($\alpha = 2^{3/4}$), using a circumscribed Central Composite Design (CCD) [35], with three independent factors each (Table 1). The central levels of the independent factors were selected regarding the typical concentrations and fermentation temperatures employed on industrial DFS production [20,31]. A total of 40 experimental runs were designed, of which 30 were conducted. Overlapping combinations between CCD1 and CCD2 (10 experiments) were performed only once (Table 2, in italics). For three factors, a second-degree polynomial equation was modelled for each CCD.

Table 1. Independent factors of the circumscribed Central Composite Designs (CCDs) 1 and 2, operating at five levels for each factor, used to evaluate the effect of ingredients and temperature of DFS on *L. sakei* and *L. monocytogenes* behaviour.

Levels [a]	CCD1 Factors			CCD2 Factors
	x1 [b]	x2 [c]	x3 [d]	x2 [c]
−1.6818	0.00	0.08	0.00	3.01
−1.0000	8.18	0.13	8.18	9.90
0.0000	20.18	0.20	20.18	20.00
+1.0000	32.18	0.27	32.18	30.11
+1.6818	40.36	0.32	40.36	37.00

[a] To maintain rotatability and orthogonality, the scaled value for α relative to the coded values ± 1 was 1.68 ($2^{3/4}$). [b] Factor 1 represents the concentration of NaCl (g/L) for CCD1. The corresponding a_w theoretical values regarding NaCl concentration were 1.00 (0.00 g/L), 1.00 (8.18 g/L), 0.99 (20.18 g/L), 0.98 (32.18 g/L) and 0.98 (40.36 g/L) [36]. [c] Factor 2 represents the concentration of Mn (g/L) and temperature (°C) for CCD1 and CCD2, respectively. [d] Factor 3 represents the concentration of glucose (g/L) for CCD1.

Table 2. Concentrations of the evaluated factors according to the CCD1 and CCD2 and observed and predicted values of *L. monocytogenes* reduction (log) and inactivation rate constant (k_d; h^{-1}) when in coculture with the bacteriocinogenic *L. sakei* CTC494.

Experiment	x1	x2	x3	NaCl (g/L)	Mn (g/L)	T (°C)	Gluc (g/L)	Observed Reduction (log) [1]	Predicted Reduction (log) [1]	Observed k_d (h^{-1}) [1]	Predicted k_d (h^{-1}) [1]
1	−1.00	−1.00	−1.00	8.18	0.13	NE [2]	8.18	−3.24	−2.81	−0.12	−0.06
2	−1.00	−1.00	1.00	8.18	0.13	NE	32.18	−4.14	−4.29	−0.17	−0.09
3	−1.00	1.00	−1.00	8.18	0.27	NE	8.18	−4.56	−2.81	−0.20	−0.06
4	−1.00	1.00	1.00	8.18	0.27	NE	32.18	−4.59	−4.29	−0.15	−0.09
5	1.00	−1.00	−1.00	32.18	0.13	NE	8.18	−2.59	−2.39	−0.07	−0.06
6	1.00	−1.00	1.00	32.18	0.13	NE	32.18	−2.37	−3.87	−0.06	−0.09
7	1.00	1.00	−1.00	32.18	0.27	NE	8.18	−3.34	−2.39	−0.08	−0.06
8	1.00	1.00	1.00	32.18	0.27	NE	32.18	−2.64	−3.87	−0.07	−0.09

Table 2. *Cont.*

Experiment	x1	x2	x3	NaCl (g/L)	Mn (g/L)	T (°C)	Gluc (g/L)	Observed Reduction (log) [1]	Predicted Reduction (log) [1]	Observed k_d (h^{-1}) [1]	Predicted k_d (h^{-1}) [1]
9	*0.00*	0.00	*1.68*	*20.18*	0.20	*NE*	*40.36*	*−5.59*	*−5.46*	*−0.13*	*−0.09*
10	*0.00*	0.00	*−1.68*	*20.18*	0.20	*NE*	*0.00*	*0.35*	*−2.96*	*−0.01*	*−0.03*
11	0.00	1.68	0.00	20.18	0.32	NE	20.18	−5.62	−4.21	−0.12	−0.13
12	0.00	−1.68	0.00	20.18	0.08	NE	20.18	−5.16	−4.21	−0.19	−0.13
13	*1.68*	0.00	*0.00*	*40.36*	0.20	*NE*	*20.18*	*−1.75*	*−1.40*	*−0.06*	*−0.04*
14	*−1.68*	0.00	*0.00*	*0.00*	0.20	*NE*	*20.18*	*−2.07*	*−2.11*	*−0.04*	*−0.04*
15	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.69*	*−4.21*	*−0.14*	*−0.13*
16	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.56*	*−4.21*	*−0.10*	*−0.13*
17	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−5.61*	*−4.21*	*−0.13*	*−0.13*
18	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−3.72*	*−4.21*	*−0.16*	*−0.13*
19	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−3.10*	*−4.21*	*−0.14*	*−0.13*
20	*0.00*	0.00	*0.00*	*20.18*	0.20	*NE*	*20.18*	*−4.15*	*−4.21*	*−0.10*	*−0.13*
21	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−3.26	−2.48	−0.02	−0.01
22	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−5.29	−3.97	−0.04	−0.04
23	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.52	−0.89	−0.03	−0.02
24	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.53	−2.37	−0.03	−0.05
25	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−3.26	−2.06	−0.02	−0.01
26	0.00	−1.00	0.00	*20.18*	NE	9.90	*20.18*	−2.81	−3.55	−0.02	−0.04
27	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.60	−0.47	−0.02	−0.02
28	0.00	1.00	0.00	*20.18*	NE	30.11	*20.18*	−0.60	−1.95	−0.02	−0.05
29	0.00	1.68	0.00	*20.18*	NE	37.00	*20.18*	−1.62	0.31	−0.05	−0.02
30	0.00	−1.68	0.00	*20.18*	NE	3.01	*20.18*	−0.02	−2.37	0.00	0.00

[1] *L. monocytogenes* in coculture with the bacteriocinogenic *L. sakei* CTC494. [2] NE: Factor Not Evaluated in this experiment. In italics: Overlapped combinations between CCD1 and CCD2.

2.2. Bacterial Strains and Inoculum Preparation

The bacteriocinogenic strain *L. sakei* CTC494, known for antilisterial activity [21] and sourced from the Institute of Agrifood Research and Technology (IRTA) Food Safety and Functionality Programme culture collection, and the non-bacteriocinogenic strain *L. sakei* 23K [37] were selected for this study. *L. monocytogenes* CTC1034 (serotype 4b), sourced from IRTA's collection, was used as the target pathogen. *L. sakei* and *L. monocytogenes* strains were isolated from DFS and stored at −80 °C in De Man Rogosa and Sharpe broth (MRS, Oxoid, UK) and Brain Heart Infusion broth (BHI, Beckon Dickinson, Sparks, MD, USA), respectively, both supplemented with 20% glycerol as a cryoprotectant.

Before the experiments, *L. sakei* and *L. monocytogenes* strains were cultured in MRS at 30 °C and BHI at 37 °C, respectively, for 8 h. Subsequently, the strains were pre-adapted in Meat Simulation Media (see Section 2.3), supplemented with NaCl (20.18 g/L), Mn (0.20 g/L), and glucose (20.18 g/L) (i.e., to concentrations at the central conditions of the CCD), and then incubated for 16 h at 20 °C.

2.3. Experiments with L. sakei Strains and L. monocytogens in Mono and Coculture in Meat Simulation Media

The experiments with *L. sakei* strains and *L. monocytogenes*, both in mono and coculture, were carried out in Meat Simulation Media (MSM) based on the formulation by Sánchez Mainar et al. [38]. The basic composition per litter included 11.0 g of Bacto proteose peptone No. 3 (Gibco™, Thermo Fisher Scientific, San Diego, CA, USA), 8.8 g of Beef extract (Difco™, Thermo Fisher Scientific, San Diego, CA, USA), 2.2 g of Yeast Extract (Liofilchem, Roseto degli Abruzzi, Italy), 0.038 g of $MnSO_4$:$4H_2O$ (Merck KGaA, Darmstadt, Germany), and 1 mL of Tween 80 (Merck KGaA, Darmstadt, Germany). The pH was adjusted to 5.80, simulating the pH of a DFS meat batter.

Following pre-adaptation, the strains were inoculated into tubes containing 10 mL of MSM with different initial concentration of *ca.* 5 log CFU/mL for *L. sakei* (Ls) strains, within the typical range of concentrations of *L. sakei* starter cultures [20,31], and *ca.* 3 log CFU/mL for *L. monocytogenes* (Lm), a level expected in pork [5], under mono and coculture conditions. For the coculture experiments, the inoculation of *L. sakei* (CTC494 or 23K):

L. monocytogenes resulted in an inoculation ratio of 5:3, corresponding to a logarithmic scale. The set of experiments in both mono and coculture were stored at 20 °C for CCD1 and at 3, 10, 20, 30 and 37 °C for CCD2 (Table 2) during a period from 2 to 28 days.

Samples for microbiological, pH, lactic acid and bacteriocin activity analysis were taken periodically (one tube per sampling time) with a temperature-dependent frequency: every hour for 30–37 °C cultures, every 3 h for 10–20 °C and every 24 h for 3 °C.

2.4. Microbiological Analyses

Microbiological determinations were performed by 10-fold serially diluting the cultures in physiological saline solution (1 g/L of peptone and 8.5 g/L of NaCl). *L. sakei* strains were enumerated in MRS agar plates (de Man, Rogosa and Sharpe; Merck, Darmstadt, Germany) and incubated at 30 °C for 72 h under anaerobic conditions using sealed jars with AnaeroGen sachets (Oxoid Ltd., Altrincham, UK). *L. monocytogenes* was enumerated in CHROMagar™ *Listeria* plates (CHROMagar, Paris, France) incubated at 37 °C for 48 h.

2.5. Physicochemical and Metabolite Determinations

pH values were measured using a puncture electrode model 5232 connected to a portable pHmeter PH25 (Crison Instruments S.A., Alella, Spain). The minimum pH (pH_{min}) was recorded, while the pH reduction was calculated as the difference between the initial pH (5.80) and the pH_{min}.

The determination of lactic acid concentration (LA; [g/L]) was indirectly measured by spectrophotometry directly from the culture supernatant, as by Borshchevskaya (2016) [39]. The coloured product resulting from the reaction of lactate ions with iron (III) chloride was measured at a wavelength of 390 nm.

Sakacin K activity of *L. sakei* CTC494 cultures was analysed through the spot-on lawn test [34,35]. In brief, culture supernatant was pasteurized (10 min at 80 °C), and proteins were precipitated with ammonium sulphate (0.3 g/mL) and stored overnight at 4 °C. The pelleted proteins were then resuspended in phosphate buffer and 2-fold serially diluted. Subsequently, 10 µL drops were placed onto the surface of a semisolid TSAYE overlay (Tryptone Soya agar with 0.6% yeast extract and 7.5 g/L of agar) inoculated with an overnight culture of *L. monocytogenes* CTC1034 in TSBYE (Tryptone Soya broth with 0.6% yeast extract). Plates were incubated overnight at 30 °C for 24 h. The results of sakacin K activity were expressed in arbitrary units (AU/mL), defined as the highest dilution causing a clear inhibition zone on the lawn of the target strain.

The maximum values of lactic acid concentration (LA_{max}) and bacteriocin activity (BAC_{max}) were determined. Yield of lactic acid (Y_{LA}; mg/log CFU·h) and bacteriocin (Y_{BAC}; AU/log CFU·h), expressing the metabolite production as a function of bacterial biomass, were estimated by fitting the modified Luedeking and Piret model [40].

2.6. Assessment of Growth and Inactivation Parameters

Plate counts for *L. sakei* CTC494 and 23 K, as well as *L. monocytogenes* in mono and coculture, were transformed into decimal logarithmic values. The Logistic growth model without delay ($\lambda = 0$) [41] was fitted to the growth curves obtained from the experimental datasets generated by CCD1 and 2, using the MS Excel 2016 Solver add-in (Microsoft, Redmond, WA, USA). This model was used to obtain the kinetic parameters of growth, including the maximum specific growth rate (μ_{max}; h^{-1}) and maximum population density (N_{max}; log CFU/mL). The goodness of fit of the Logistic model was assessed by root-mean-square error (RMSE) and coefficient of determination (R^2), respectively.

The growth potential (i.e., log increase) of *L. sakei* (CTC494 and 23K) and *L. monocytogenes* strains was calculated as the difference between the maximum and the initial bacterial concentrations (log CFU/mL) using concentrations estimated by the Logistic model. For coculture experiments, pathogen inactivation (i.e., log reduction) was calculated as the difference between the maximum and the minimum concentrations (log CFU/mL).

The inactivation rate constant (k_d; h^{-1}) of *L. monocytogenes* in coculture with bacteriocinogenic *L. sakei* CTC494 was estimated as the slope of the first-order equation fitted to the log counts of the pathogen between 12 and 36 h, the interval of time in which *L. monocytogenes* showed a linear decrease.

2.7. Response Surface Methodology and Data Analysis

The effects of the independent factors (i.e., NaCl, Mn, glucose, and temperature) on the dependent physicochemical (i.e., pH_{min}, LA_{max}, Y_{LA}, BAC_{max} and Y_{BAC}) and microbiological factors (i.e., μ_{max}, N_{max}, growth potential, log reduction and k_d) were assessed by the Response Surface Methodology. The JMP v16.0.0 software (SAS Institute Inc., Cary, NC, USA) was used to fit a polynomial model for each response applying a stepwise regression approach, with forward direction and combined rules of the Bayesian Information Criteria (BIC) to achieve a parsimonious model with only statistically significant factors (p-value < 0.05). This methodology allowed the evaluation of linear, quadratic, and interactive effects of independent factors on microbial behaviour (dependent factors). Statistic tests of lack of fit, summary of fit, and Analysis of Variance (ANOVA) were used to evaluate the goodness of fit of polynomial equations and test significant differences (p-value < 0.05). The significance of the regression model and estimated parameters were evaluated by the lack-of-fit test. Two- and three-dimensional surface plots were drawn to illustrate the effect of the independent factors on significant models with insignificant lack of fit. Differences between the mono and cocultures responses were calculated using the t-test within the replicates of the central level of CCD.

3. Results and Discussion

The present study evaluated the effect of NaCl (0, 8.18, 20.18, 32.18 and 46.36 g/L), Mn (0.08, 0.13, 0.20, 0.27 and 0.32 g/L), glucose (0, 8.18, 20.18, 32.18 and 40.36 g/L) and temperature (3, 9.9, 20, 30 and 37 °C) (Table 1) on the physicochemical parameters (pH_{min}, LA_{max}, BAC_{max}, Y_{LA} and Y_{BAC}) and microbiological parameters (μ_{max}, N_{max}, growth potential, log reduction and k_d) associated with the behaviour in mono and coculture of *L. sakei* (CTC494 and 23K) and/or *L. monocytogenes*. The factor's impact main results on mono and coculture are described in the following sections, while detailed results are reported in Supplementary Table S1. All polynomial equations and the results of statistic tests (i.e., lack of fit, summary of fit and ANOVA) for *L. sakei* and *L. monocytogenes* strains are described in Supplementary Table S2.

3.1. Assessment of the Effect of NaCl, Mn, Glucose, Temperature and Coculture in the Acidification and Bacteriocin Production in Meat Simulation Media

3.1.1. pH Decrease and Lactic Acid Formation

The decrease in pH values and the production of lactic acid depended on the bacterial species and were clearly influenced by the NaCl and glucose concentrations in the medium and the incubation temperature. Sodium chloride has been described to interfere with bacterial growth due to its role as an a_w-lowering agent [25], and glucose is the main ATP-energy source for LAB fermentation and is fully converted to lactate by homofermentative *L. sakei*. Temperature modulates cell growth and, consequently, the use of the nutrient resources available in the media [42].

The strongest acidification, representing a decrease of 2.0 pH units from an initial pH of 5.80, was observed for both *L. sakei* strains (CTC494 and 23K) in mono and coculture with *L. monocytogenes*, reducing the pH of the medium to pH_{min} values of *ca.* 3.80 at glucose concentrations $\geq$ 8 g/L (i.e., all experiments except for experiment 10) (Figure 1A). Specifically, the lowest pH_{min} was achieved when the glucose concentration was the highest (40 g/L) by the strains *L. sakei* 23K and CTC494 both in mono and coculture, with pH_{min} ranging from 3.6 to 4.0 (reduction of *ca.* 2.0 pH units). When glucose was not added (i.e., experiment 10), the highest pH_{min} was achieved by *L. sakei* 23K (pH of 5.1) and *L. sakei* CTC494 (pH of 5.4), showing significant differences with the lowest values (p < 0.05). The

same results were observed in coculture experiments (pH_{min} *ca.* 5.1), resulting in a reduction of only *ca.* 0.7 pH units and the lowest LA_{max} concentrations (*ca.* 0.55 g/L), as detailed in Supplementary Table S1. Conversely, in *L. sakei* monoculture experiments with higher NaCl concentration (32.18 g/L) at 20 °C (experiments 5, 6, 7 and 8), the pH_{min} values were slightly higher, *ca.* 4.00, representing a reduction of 1.8 pH units (Figure 1A). Barbieri et al. (2022) [43] showed that in fermentations of *L. sakei* in defined liquid medium (initial pH of 6.50), when glucose was not a limiting factor, the pH was reduced by 2.6 units, whereas when glucose was limiting, the reduction of pH was only 0.5 units.

In contrast, *L. monocytogenes* reached pH_{min} values in MSM of *ca.* 4.4, with the exception of experiment 10, where glucose was not added and the pH_{min}, with a value of 5.1, was the highest, corresponding to a reduction of 0.7 pH units (Figure 1A). Different pH reductions were due to the different availability of glucose, and when it was limiting (i.e., not added to the MSM), there was a lower production of lactic acid and, therefore, a less pronounced reduction in pH. On the contrary, when glucose was not a limiting factor (i.e., present in sufficient quantity, such as 40 g/L), the metabolic activity of LAB and the pathogen strains was accelerated, and a more significant reduction in the pH of the MSM was observed. Glucose is a fermentable sugar used by bacteria to generate lactic acid as a fermentation metabolite that promotes a pH drop. Specifically, for homofermentative bacteria (e.g., *L. sakei*), lactic acid is obtained as the sole product of glucose metabolization (i.e., 1 mole of glucose yields 2 moles of lactic acid and 2 moles of ATP, or 1 g of lactic acid per 1 g of substrate) [44].

As expected, higher LA_{max} values were registered when higher pH reductions occurred and lower pH_{min} were observed (Supplementary Table S1), as previously shown for *L. sakei* CTC494 [42]. On the contrary, and similar to the pH_{min} results, the lowest values of LA_{max} were achieved when glucose was not added (0.5, 0.7 and 0.6 g/L of LA_{max} for *L. sakei* 23K, CTC494 and *L. monocytogenes*, respectively). *L. sakei* strains produced more LA (>3 g/L) than *L. monocytogenes* (*ca.* 1.5 g/L) ($p < 0.001$) (Figure 1A). This fact could be related to metabolic differences between *L. sakei* and *L. monocytogenes*. *L. sakei* strains are homofermentative, performing glycolysis as the most efficient way to produce energy (i.e., ATP), and LA is the main fermentation product. In contrast, *L. monocytogenes* also produces ethanol and formate as products of its metabolism [45]. In the coculture experiments, the same observation (i.e., higher reductions in LA_{max} and pH in *L. sakei* monocultures) was made due to the acid produced by *L. sakei* strains with no acidification differences regarding the *L. sakei* strain in coculture ($p > 0.05$). However, coculture of the 23K strain and *L. monocytogenes* often showed higher values (e.g., 5.86 g/L of LA_{max} concentration in experiment 4) than the CTC494 coculture (e.g., 5.09 g/L of LA_{max} concentration in experiment 4) [45].

Overall, the highest LA_{max} and pH reductions registered by both coculture experiments were similar to those observed by monoculture (>3 g/L and a decrease of 2 pH units) and slightly higher than those of *L. monocytogenes* monoculture (*ca.* 1.5 g/L and 1.5 pH units) ($p < 0.01$) (Figure 1A). In the central point experiment runs, no coculture effect was observed, and similar pH_{min} (i.e., 3.72 ± 0.01 and 3.75 ± 0.03, for the 23Kco and CTC494co, respectively) and LA_{max} (i.e., 4.47 ± 0.12 g/L and 4.12 ± 0.32 g/L) were similar to those obtained in monoculture experiments for *L. sakei* strains ($p > 0.05$) (Figure 1A). These results indicated that simultaneous growth of *L. sakei* with *L. monocytogenes* had no relevant effect on acidification.

The RSM showed in experiments with *L. sakei* strains that glucose and NaCl had quadratic and linear effects, respectively, on LA_{max} values, whereas in coculture with *L. monocytogenes*, only glucose had a quadratic effect ($p < 0.05$) on the pH_{min} and LA_{max} concentrations (see polynomial equations coefficients in Supplementary Table S2). LA yield (Y_{LA}) was influenced by glucose with linear or quadratic effects for all strains. However, the polynomial models obtained showed poor goodness of fit to observed Y_{LA} data (Supplementary Table S2). In food fermentations, high LA production is desirable from a food safety perspective, and, in DFS, the antilisterial effect of LAB has been linked to the antimicrobial effect of LA (i.e., pH drop) [20].

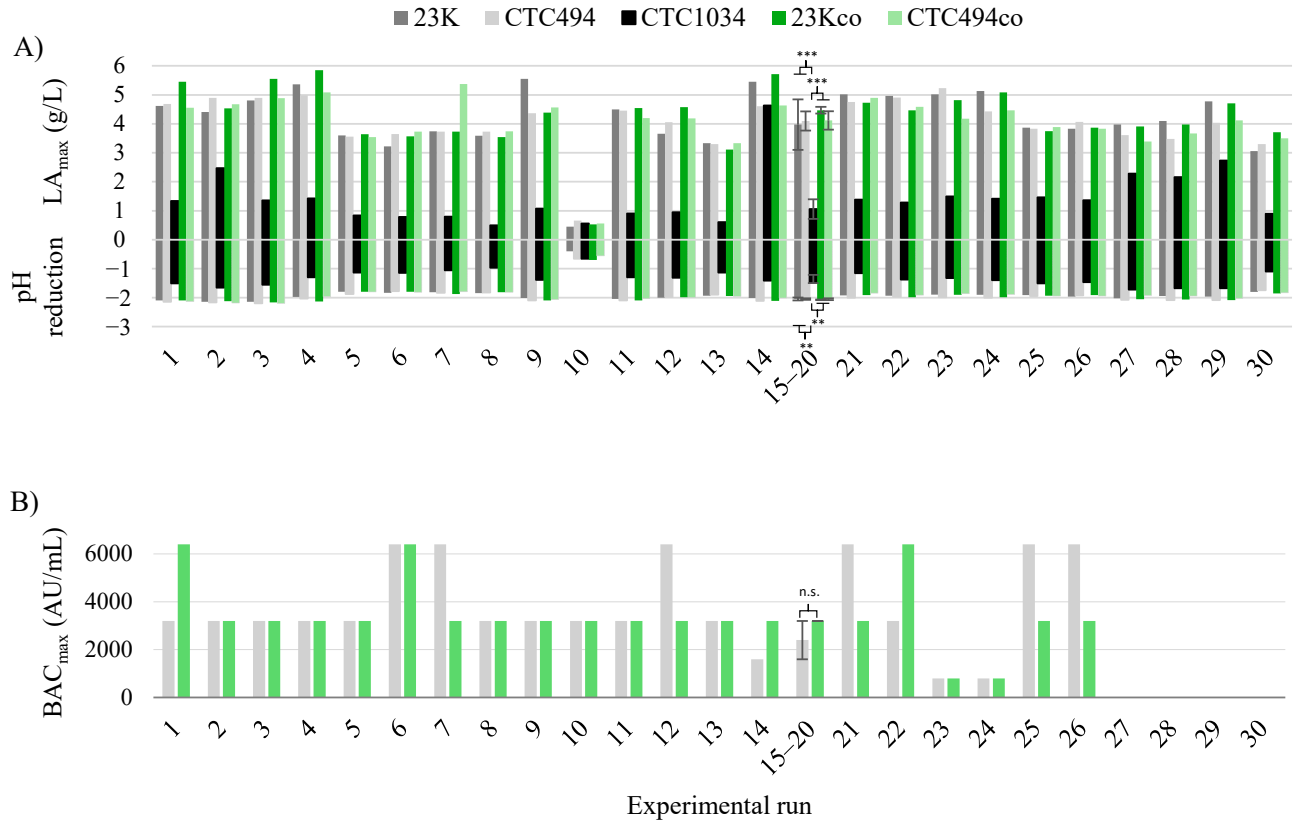

Figure 1. Maximum concentration of lactic acid (LA_{max}) and pH reduction of *L. sakei* 23K, CTC494 and *L. monocytogenes* CTC1034 in monoculture and in coculture with *L. sakei* 23K (23Kco) and *L. sakei* CTC494 (CTC494co) (**A**), and maximum bacteriocin activity (BAC_{max}) registered by *L. sakei* CTC494 (**B**). Central points (experiments 15–20) are expressed as mean and standard deviation of 6 replicates, and statistical significance is indicated: *p*-value $p < 0.01$ (**), $p < 0.001$ (***), $p > 0.05$ (non-significant, n.s.).

3.1.2. Sakacin K Activity

The main factors affecting bacteriocin production by *L. sakei* CTC494, measured as maximum activity recorded during the experiment (i.e., BAC_{max}), were temperature and Mn concentration (g/L). Leroy and de Vuyst [30] studied the effect of Mn limitation, which resulted in a considerable decrease in cell growth and lactic acid and bacteriocin production. The most favourable temperatures for bacteriocin activity in monoculture experiments were moderate (i.e., 10 and 20 °C), reaching a maximum activity of 6400 AU/mL in experiments 6, 7, 12, 25 and 26, whereas in coculture the higher BAC_{max} values were observed in experiments 1, 6 and 22 (Figure 1B), indicating that there were no consistently higher values in coculture. Consequently, sakacin K production by *L. sakei* CTC494 was not stimulated by the presence of the pathogen, although induction of bacteriocin production has been reported in *Lactiplantibacillus plantarum* strains. This trait may be associated with a quorum sensing-related mechanism involving cell-to-cell contact and is therefore specific to the strain and determined by the bacteriocin production regulatory operon [46].

BAC activity was detected at the late exponential phase (t = 15 h) and in general increased to reach its maximum (BAC_{max} of 3200 AU/mL) at the early stationary phase (see Figure 2 as an example). However, when experiments were conducted at higher temperatures (i.e., 30 and 37 °C), sakacin K activity was ≤800 AU/mL for experiments 23, 24, 27, 28 and 29, while at low temperature (i.e., 3 °C; experiment 30), no *BAC* activity was detected (Figure 1B). Previous studies with the CTC494 strain showed that at pH 6.5, the optimal temperature for *BAC* production was between 20 and 25 °C and was undetectable at 34 °C [29]. Furthermore, it was observed that at NaCl concentrations of 2%, *L. sakei* bacteriocin activity was decreased [25], and above 8% of NaCl, the sakacin

K activity was not detected [29]. For another *L. sakei* strain, CCUG 42687, the maximum sakacin P production was also reported at 20 °C, with a concentration seven times higher than at 30 °C [47]. Temperatures for some fast-fermented European-style DFS fermentation coincide with the highest values of bacteriocin activity, maximising the antilisterial effect of the bioprotective starter culture. At temperatures higher than 25 °C, although cells grow faster, bacteriocin activity drops significantly due to the combined effect of a lower specific productivity and a higher bacteriocin degradation rate, and cell yield decreases as the energy needed for maintenance becomes more important [42].

The concentrations of Mn and NaCl (CCD1) showed a significant effect ($p < 0.05$) on BAC_{max} and Y_{BAC}, which was also influenced by temperature (CCD2) (Supplementary Table S2). Y_{BAC} in coculture also showed similar yields to those obtained in monoculture, with values <300 AU/log CFU·h at extreme temperatures (3 °C ≤ and ≥ 30 °C), while at tested star concentrations of Mn (i.e., experiments 11 and 12), Y_{BAC} was >2000 AU/log CFU·h (Supplementary Table S1). Polynomial models for Y_{BAC} highlight temperature and Mn as the main factors influencing sakacin K production with quadratic effects (Supplementary Table S2). Leroy and De Vuyst [42] observed that the production of sakacin K by *L. sakei* CTC494 was highly influenced by pH and that the optimal temperature and pH for growth were different from those maximising bacteriocin production. Specifically, the optimum temperatures and pH values for *L. sakei* CTC494 growth were reported to be 33.5 °C and 6.15, while a pH of 5.0 and 23 °C were the most favourable conditions for bacteriocin production [30,42]. Enhancing the production of lactic acid and sakacin K can be used not only to maximise the antilisterial properties of *L. sakei* CTC494 when applied as a bioprotective culture in foods but also to increase the antimicrobial activity of the strain prepared as a postbiotic (i.e., microbial cells inactivated with/without metabolites) [48].

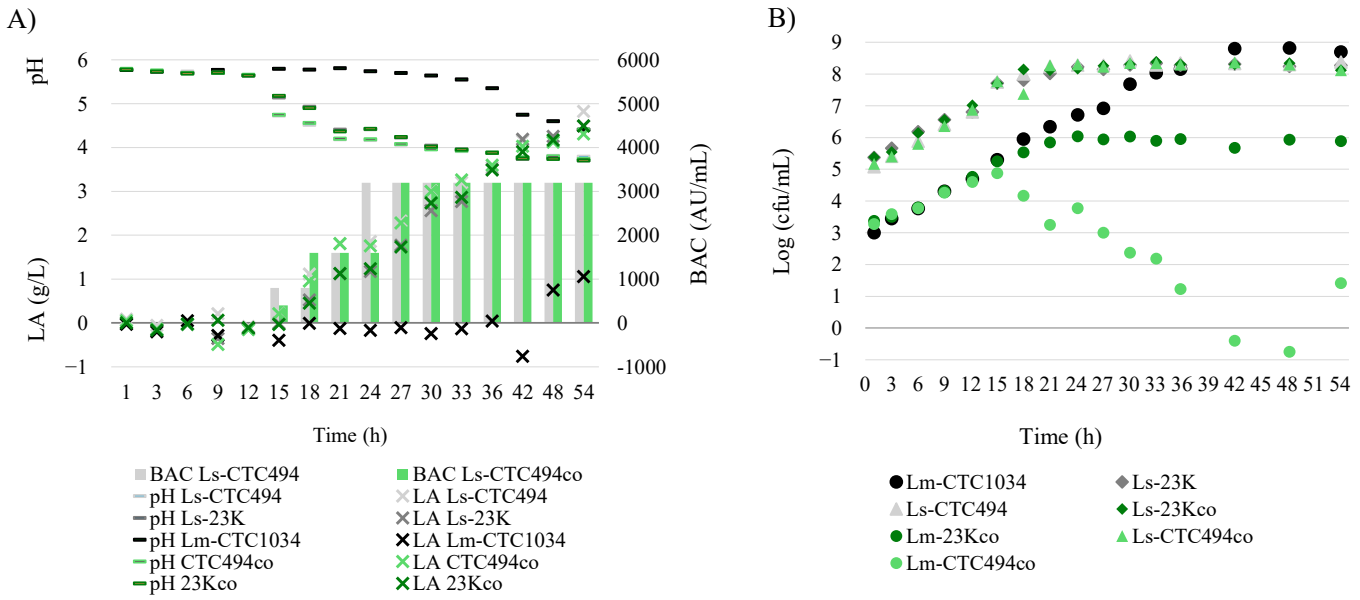

Figure 2. Physicochemical parameters (pH, lactic acid (*LA*) and bacteriocin activity (*BAC*)) (**A**) and microbial counts (**B**) of strains in mono- and coculture of experiment run 9 (star point of glucose, 20 g/L of NaCl, 0.20 g/L of Mn, 40 g/L of glucose at 20 °C). Ls: *L. sakei*, Lm: *L. monocytogenes*, co: coculture.

3.2. Impact of NaCl, Mn, Glucose, Temperature and Coculture in the Behaviour of L. sakei and L. monocytogenes

3.2.1. Bacterial Growth under DFS Fermentation Conditions in MSM

Microbiological parameters (μ_{max}, N_{max} and growth potential) of the strains were highly influenced by temperature. In both mono- and coculture, the highest and the lowest μ_{max} values were observed at 37 °C and at 3 °C for the two *L. sakei* and *L. monocytogenes* (around 1.0 h^{-1} at 37 °C and 0.025 h^{-1} at 3 °C) (Figure 3A in monoculture; Figure 3D,G,H in

coculture; Supplementary Table S1), which is in agreement with previous studies evaluating the effect of temperature on the growth of *L. sakei* CTC494 [42].

In monoculture experiments, the N_{max} ranged between 7.5 and 8.7 log CFU/mL for *L. sakei* strains and were slightly higher (8.4–9.1 log CFU/mL) for *L. monocytogenes* (Supplementary Table S1). Previous studies in cooked ham also showed higher N_{max} values for *L. monocytogenes* CTC1034 than *L. sakei* CTC494 [19]. NaCl, Mn, glucose and temperature had a microbiologically and statistically relevant effect on N_{max}, although they were only significant for the CTC494 CCD1 model (Figure 3B).

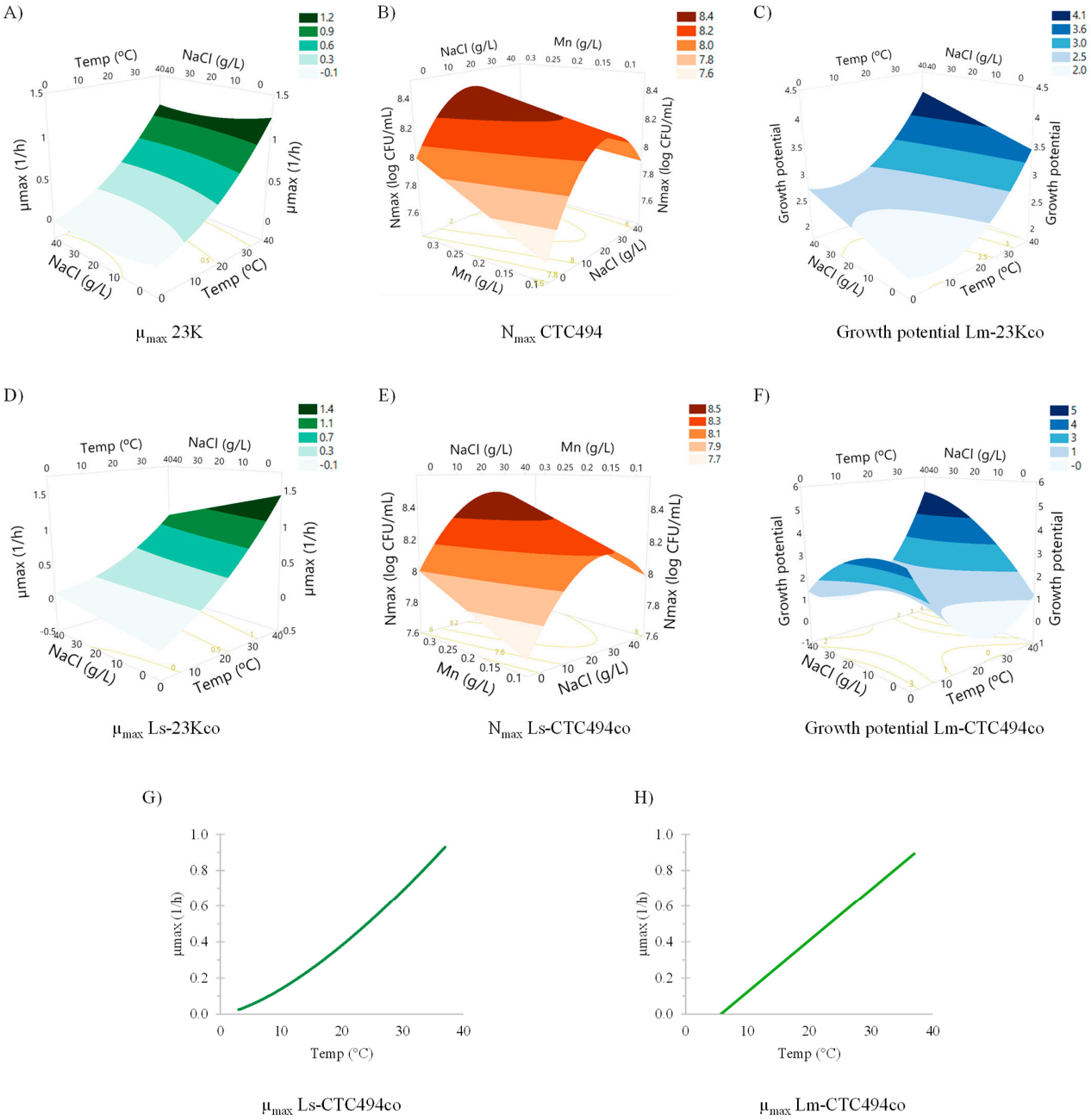

Figure 3. Microbiological parameters with significant effects response to independent factors. In monoculture, μ_{max} of *L. sakei* 23K (**A**) and N_{max} of *L. sakei* CTC494 (**B**). In coculture, growth potential of *L. monocytogenes* CTC1034 in coculture with *L. sakei* 23K (Lm-23Kco) (**C**), μ_{max} of *L. sakei* 23K in coculture (Ls-23Kco) (**D**), N_{max} of *L. sakei* CTC494 in coculture (Ls-CTC494co) (**E**), growth potential of *L. monocytogenes* CTC1034 in coculture with *L. sakei* CTC494 (Lm-CTC494co) (**F**), μ_{max} of Ls-CTC494co (**G**) and of Lm-CTC494co (**H**).

Overall, the growth potential of the strains was affected by the independent factors NaCl and glucose, with temperature having the most remarkable effect. In monoculture experiments with *L. sakei* strains, growth potential ranged from 2.4 to 5.1 for 23K and 2.3 to 3.8 for CTC494, with the highest values observed in experiments with low salt concentrations and intermediate temperatures (5.1 log for 23K in experiment 3 and 3.8 log for CTC494 strains in experiment 23). In contrast, in monoculture, *L. monocytogenes* exhibited higher growth potential, ranging from 4.8 to 6.0 log, attributed to its lower initial concentration and higher N_{max} compared to *L. sakei* (Figure 4). Coculture did not affect *L. sakei* N_{max}, as shown for CTC494 strain in Figure 3B,E. This is in agreement with other experiments with this strain in cooked ham [19] and the strain *L. sakei* 706 (sakacin A producer) in MRS broth [49].

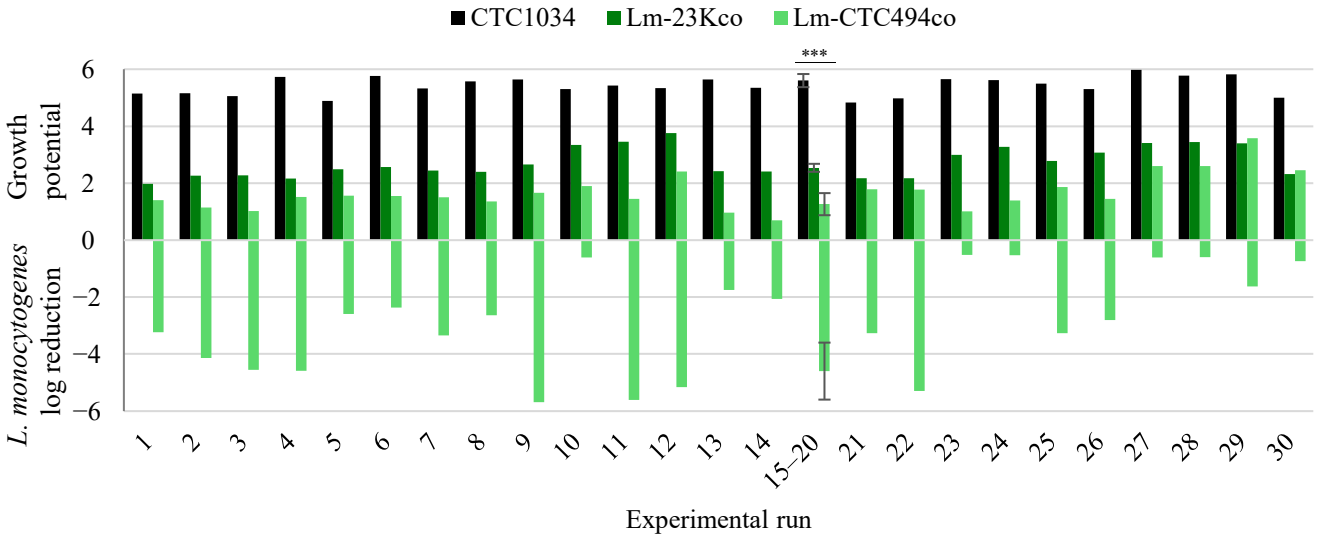

Figure 4. Growth potential of *L. monocytogenes* CTC1034 in monoculture and coculture with *L. sakei* 23K (Lm-23Kco) and CTC494 (Lm-CTC494co) and log reduction of *L. monocytogenes* in coculture with *L. sakei* CTC494 (Lm-CTC494co). Growth potential statistical significance ($p < 0.001$; ***) in central points (experiments 15–20) is indicated.

However, it is remarkable that coculture with *L. sakei* strains considerably reduced N_{max} of *L. monocytogenes* ($p < 0.001$), especially the bacteriocinogenic CTC494 strain (Figure 4). Specifically, *L. monocytogenes* reached N_{max} values ranging from 4 to 7 log CFU/mL in coculture with *L. sakei* strains, while in monoculture experiments, the pathogen always exhibited $N_{max} > 8.4$ log CFU/mL (Supplementary Table S1).

Multiple factors may be involved in interactions between bacterial populations in the same ecosystem, including competition for nutrients, production of metabolites (e.g., organic acids and bacteriocins), signalling molecules and cell-to-cell contact mechanisms [50]. The results in coculture experiments showed that inhibition of *L. monocytogenes* growth occurred when the dominant population, i.e., *L. sakei* 23 K, reached its N_{max}, leading to an early entry of the pathogen into the stationary phase. This phenomenon is known as the Jameson effect [51], which has been observed by several authors studying non-bacteriocinogenic competition between *L. sakei* and *L. monocytogenes* in broth media simulating DFS fermentation conditions [52–54]. On the other hand, the bacteriocinogenic strain CTC494 not only inhibited the growth of *L. monocytogenes* but also promoted its inactivation by more than 5 log units. Similar results were observed for the non-pathogenic *Listeria innocua* by Leroy et al. [47] in broth. The different behaviour of *L. monocytogenes* in coculture with 23K and CTC494 strains is shown in Figure 2B, which provides a representative example of the microbial kinetics together with the corresponding pH, *LA* and *BAC* production profiles (Figure 2A).

Considering the impact of the evaluated factors on the coculture experiments between *L. sakei* CTC494 and *L. monocytogenes*, the highest N_{max} (5.5 log CFU/mL) for the pathogen was observed at extreme temperatures of 3 and 37 °C, and at 30 °C with high NaCl concentration of 32.18 g/L (Supplementary Table S1). These conditions correspond to those combinations of factors that did not favour bacteriocin production (i.e., experiments 23, 24, 27, 28, 29 and 30) (Figure 1B). Accordingly, the growth potential of *L. sakei* and *L. monocytogenes* in coculture was also influenced by temperature and NaCl ($p < 0.05$), and in the case of *L. monocytogenes*, it was significantly reduced ($p < 0.001$) by the presence of the dominant population, i.e., *L. sakei*, especially in experiments where the CTC494 strain produced bacteriocin (Figure 3C,F). More specifically, *L. monocytogenes* growth potential in monoculture was above 5 log and decreased, depending on the experimental conditions, to 1.4–3.5 log in coculture with *L. sakei* 23K and to 0.9–3.9 log in coculture with *L. sakei* CTC494 (Figure 4). In contrast, for both *L. sakei* strains, experiments at the lowest temperature (3 °C), at the highest temperature (37 °C), and at high temperature and high NaCl (30 °C and 32 g/L), the lowest reductions in the growth potential of *L. monocytogenes* (<1 log) were observed (Figure 4).

3.2.2. *L. monocytogenes* Inactivation by the Sakacin K Producer *L. sakei* CTC494

L. monocytogenes inactivation was only observed in the presence of *L. sakei* CTC494, with reductions ranging from −0.6 to −5.7 log. The lowest reductions correlated with experiments with lower BAC (i.e., experiments 23, 24, 27 and 28) (Figures 4 and 1B, respectively). The promotion of *L. sakei* CTC494 by glucose or Mn at 20 or 10 °C (e.g., experiments 9 and 11) enhanced inactivation of the pathogen and reduced N_{max} the most (i.e., >5.2 log). The mechanism explaining this phenomenon would be related with the model of action of sakacin-type bacteriocins, which specifically targets *Listeria* spp. by interacting with bacterial cell surface and cell membrane and forming pores, leading to cell permeabilization and ultimately to cell death [17,55]. Specifically for experiment 9, the highest reduction was observed with the maximum glucose concentration, which increased *LA* and sakacin K production. Similarly, high inactivation of *L. monocytogenes* was also observed by Pleasants et al. [49] in coculture experiments with *L. sakei* 706, a sakacin A producer, under conditions of 20 °C and pH 7 in MRS broth.

The highest pathogen inactivation rate (k_d; h^{-1}) in coculture with CTC494 was $-0.20\ h^{-1}$, at 20 °C, with 8 g/L of NaCl and glucose and 0.27 g/L of Mn (experiment 3). Under these conditions, the k_d coincided with the highest reductions in pH, high LA production (*ca.* 5 g/L) (Figure 1A) and a reduction in the growth potential of *L. monocytogenes* by approximately 1 log compared to the pathogen in monoculture (e.g., 5 log) (Figure 4).

Experiments 9 and 3 reinforce that the combination of mild temperature (20 °C) and high glucose (≥32 g/L) determines the best strategy to inactivate the pathogen. As shown in Figure 5, the combined concentrations of NaCl (i.e., 10 to 20 g/L), glucose (i.e., 20 to 30 g/L) and temperature (i.e., 10 to 20 °C) had a statistically significant effect on pathogen inactivation in terms of inactivation rate and log reduction. Usual formulation of DFSs (g per kg of meat) consists of NaCl (20), maltodextrin (20), pepper (3) (i.e., Mn (0.228)) and ripening temperature of 13 °C [31], coinciding with central values of the studied factors. The obtained results show that low NaCl (i.e., 0.8% [*w*/*v*]) and abundant glucose and Mn availability in the media at mild temperature (i.e., 20 °C) enhance bacteriocin activity, therefore *L. monocytogenes* inactivation.

The polynomial equation indicated that the highest predicted pathogen reduction (−5.5 log) and k_d ($-0.13\ h^{-1}$) would be achieved at the optimal combinations of 40.36 and 20.18 g/L of glucose, respectively, together with 20.18 g/L of NaCl at 20 °C (Table 2). The analysis also showed two and three quadratic two-way interactions for NaCl ($p < 0.05$) and temperature ($p < 0.01$) for *L. monocytogenes* reduction and for all independent factors ($p < 0.01$) for k_d (Supplementary Table S2).

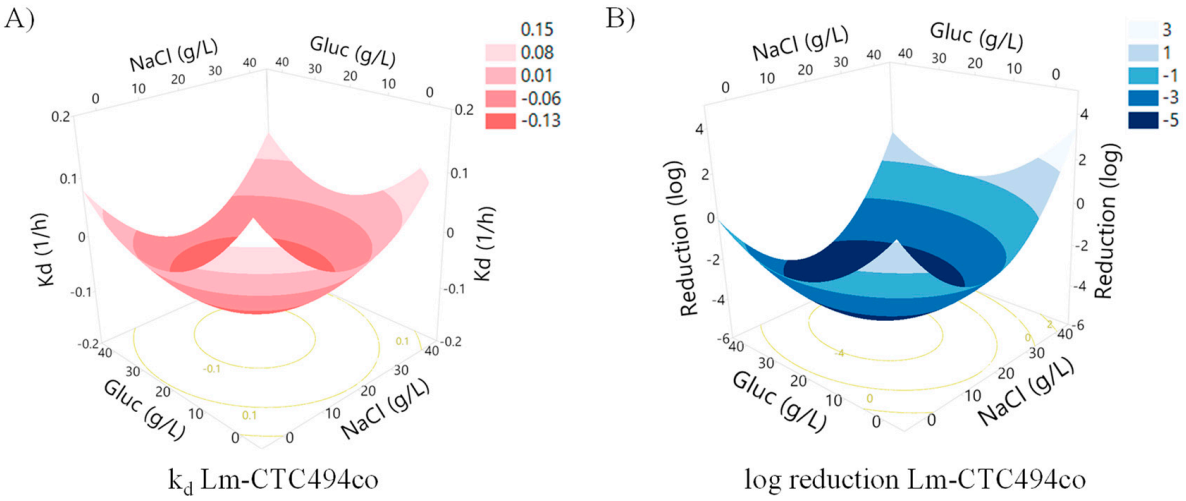

Figure 5. Inactivation parameters of *L. monocytogenes* in coculture with the bacteriocinogenic *L. sakei* CTC494 showing significant effects in response to independent factors. (**A**) Inactivation rate (k_d; h^{-1}). (**B**) log reduction.

The optimal conditions to increase pathogen inactivation mainly depended on glucose concentration, as shown by the growth potential. In this regard, Figure 4 shows that the lowest pathogen growth also resulted in higher inactivation rates, such as in experiment 9 (with a maximum glucose concentration of 40 g/L). However, the combination of NaCl and glucose have also shown an effect on sakacin K production and, consequently, on the pathogen inactivation. In agreement, Leroy and De Vuyst [30] showed that when high glucose was combined with 10–20 g/L of NaCl, increased *L. innocua* inactivation rates were observed. Additionally, as temperature also determined the inactivation rate, sakacin K amounts have been reported to be enhanced at 20 °C in comparison to higher temperatures of 30 °C. Temperatures $\leq$ 20 °C are recommended to promote the antilisterial potential of bacteriocinogenic strains in DFSs [30].

4. Conclusions

The inhibition of *L. monocytogenes* through the application of *L. sakei* represents a control measure to enhance the food safety of dry fermented sausages as this species reduces the growth potential of the pathogen through the reduction of N_{max}. However, only the application of the bacteriocinogenic *L. sakei* CTC494 has an antilisterial effect, providing an additional level of control by the reduction in the pathogen population. At mild temperatures around 20 °C and formulations promoting *L. sakei* growth (such as high glucose concentrations and Mn), together with lactic acid and sakacin K production, the inactivation of *L. monocytogenes* was enhanced, resulting in a reduction of more than 5-log units.

The safety of DFSs must be achieved through reduction of the levels of *L. monocytogenes* during the production process. The findings of this study underscore the critical role of starter cultures within the framework of the hurdle technology. Furthermore, their efficacy can be enhanced by application under optimal conditions for *L. sakei* growth and/or bacteriocin production.

The response surface methodology was a useful approach to identify the most appropriate temperature, NaCl and glucose conditions, taking into account the complex interactions between multiple variables to increase the inactivation of *L. monocytogenes* in meat simulation media in coculture with *L. sakei* CTC494. Further research needs to be performed on a real DFS food matrix to confirm the optimal conditions of application as a useful strategy for DFS manufacturers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060326/s1, Table S1: Experimental and predicted data for each experiment run of CCDs 1 and 2; Table S2: Polynomial equations and Response Surface statistical parameters.

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DISCUSSION

Dry fermented sausages are very appreciated by the consumers who expect safe products of high organoleptic quality. Although DFS are shelf-stable meat products, they have a risk of being contaminated with pathogens, mainly *L. monocytogenes* and/or *Salmonella,* at levels above the regulated limits. In fact, DFS have been involved in multiple recalls and outbreaks. Meat used as raw material is usually the main source of contamination. DFS producers must ensure their production processes are effective and rigorous enough to inhibit the growth of spoilage microorganisms and inactivate (reduce) the relevant microbial hazards to obtain end-products of the desired quality and safe. In this framework, the objectives of this thesis were focused on the study of the quality and safety of DFS from three different perspectives applying three different methodologies, based on genomic and technological (formulation and process) approaches:

1. Evaluation of the impact of the formulation and production process on the microbial communities in standard and nitrite-free DFS through metataxonomics (Article I).

2. Characterization of the dissemination, resistance and virulence potential of *Salmonella* clones circulating within the pork chain, from carcasses to DFS, using whole genome sequencing of isolates from contaminated samples (Article II).

3. Optimization of the application of the sakacin K producer *L. sakei* CTC494 bioprotective starter culture with antilisterial activity using central composite design and response surface methodology (Article III).

1. Microbiota evaluation of DFS with innovative formulation and production process

Microbiota of DFS has traditionally been characterized by culture-dependent techniques, evaluating the main microbial groups playing a technological role in the meat product that are lactic acid bacteria (LAB) and gram-positive catalase-positive cocci (GCC+). Both bacterial groups are well adapted to the initial meat batter and subsequent conditions and are indicative of the fermentation process.

In the study "Dynamics of Microbial Communities in Nitrite-Free and Nutritionally Improved Dry Fermented Sausages" (Article I) six batches (numbered from 1 to 6) of *fuet*-type DFS were elaborated with different product formulation and processing conditions. Batches 1 and 2 were formulated without nitrifying salts and with an innovative pork liver auto-hydrolysate ingredient, to substitute the colour enhancing effect of nitrifying salts. Batch 3 was formulated without nitrifying salts and batches 4, 5 and 6 with nitrate and nitrite salts. Batch 6 was NaCl-reduced and KCl-replaced. All batches except for batch 1 were formulated with *L. sakei* CTC494 starter culture. Batches 1, 2, 3 and 4 were processed at low temperatures to control the outgrowth of non-desired microorganisms in the nitrite/nitrate free formulated batches (i.e., batches 1, 2 and 3), though batch 4 was the control nitrified batch. Batch 5 was formulated and processed as an

industrial product, with nitrites and nitrates and dry-cured at 12-14 °C. Nitrifying salts contribute to control the outgrowth of spoilage and pathogenic microorganisms, for instance *Brochothrix thermosphacta* and *Clostridium botulinum,* which can grow in minimally processed meat products (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2016). In the nitrite-free DFS assessed in this thesis, the growth of both non-proteolytic and proteolytic *C. botulinum* was controlled by managing the process temperature. A low temperature process profile was set at 3 °C for the first 20 days until the a_w of the products was < 0.97, then slowly raised for 2 days at 8 °C and maintained at <10ºC until product a_w was <0.94, and finally ripened at 12 °C for 18 days until the product a_w was < 0.87. Batches 5 and 6 were processed at mild temperatures, mimicking a DSF artisanal production process. The mild temperature process profile was set at 12 °C during all the production process.

The initial LAB concentrations registered in the batches formulated with starter culture were of *ca.* 6 log CFU/g as desired by controlled inoculation. At the end of the ripening process, the LAB concentrations achieved in all batches were *ca*. 8 log CFU/g. Nonetheless, LAB concentrations were affected by the ripening temperature and the addition of starter culture, but not by the formulation. For GCC+, endogenous levels (no GCC+ starter culture was applied) were *ca.* 4 log CFU/g and progressively decreased in all batches until the end of the ripening, reaching values of 1.3–2.0 log CFU/g. During the final refrigerated storage period of 15 days under vacuum packaging, LAB and GCC+ levels maintained or slightly increased. In accordance, Serra-Castelló et al. (2021) showed that the application of a corrective storage at room temperature (aiming to enhance pathogen reduction) did not affect the viability of LAB and GCC+ of DFS.

Regarding the physicochemical parameters, the addition of the *L. sakei* CTC494 starter culture promoted a moderately rapid acidification, especially in batches submitted to mild temperatures (i.e., pH values < 5.0 in less than 7 days), that allowed faster growth of LAB. In contrast, autochthonous LAB of batch 1 (not containing starter culture and ripened at low temperatures) were not able to acidify the DFS and the pH was maintained at *ca.* 5.9, thus eliminating one of the typical hurdles of the DFS production. In this sense, the use of LAB starter cultures is recommended to ensure the pH decrease. Otherwise, the a_w decrease became the main hurdle. Progressive a_w decrease during drying is an important factor for Mediterranean style DFS, which are usually classified as slightly fermented because they have final pH values above 5.0. In the present study, a_w of the final products were below 0.88, which guarantees the shelf stability of the final product by preventing microbial outgrowth.

Culture-independent techniques allowed a deeper characterization of the microorganisms present in the DFS than the enumeration of LAB and GCC+. Results showed important differences between batches, mainly due to the addition of the pork liver auto-hydrolysate ingredient, starter culture and nitrifying agents.

Initially, the most abundant bacterial genus found in the meat batter of the total bacterial composition was *Pseudomonas* sp. (17%) which is a spoilage microorganism frequently found in raw meat stored under refrigeration (Nychas et al., 2008) and in pork that is going to be used

for DFS elaboration (Cardinali et al., 2018). The putative probiotic bacterial species *Lactobacillus johnsonii* and *Limosilactobacillus reuteri* composed the main bacterial species identified in the pork liver auto-hydrolysate ingredient and were dominant in the meat batter of batches 1 and 2 before fermentation while *L. sakei* CTC494 was the most abundant species in batches 3 to 6. Temperature (3 °C) and formulation conditions (no starter culture nor nitrifying salts and with the liver auto-hydrolysate ingredient) of batch 1 led to a predominance of *Pseudomonas* spp. during the first days of the process although from day 12 it was succeeded by the facultative anaerobic *B. thermosphacta* as the most abundant species. Both species have been found in chilled fresh meat products, related to meat spoilage processes (Doulgeraki et al., 2012), and were found in all DFS processed at low temperatures. After the temperature rose to 12 °C, *L. sakei* and *Leuconostoc carnosum* abundance increased, composing up to the 60% of the DFS bacterial community. In batches formulated with starter culture, *L. sakei* CTC494 led the fermentation process, rapidly colonizing the meat matrix and promoting the pH drop regardless of the process temperature. The culture-independent metataxonomic approach provided evidence of *L. sakei* DFS colonization at species level. Afterwards, more than 20 LAB strains per sample were characterized by the ERIC-PCR molecular typing approach confirming that DFS matrix colonization was performed by *L. sakei* CTC494 strain.

Alpha diversity index (Shannon) for intrasample composition was the highest in batches formulated without nitrifying agents and/or with the liver auto-hydrolysate ingredient (i.e., batches 1, 2 and 3). The incorporation of the innovative pork ingredient in DFS provided bacterial species that are not commonly present in the DFS meat batter, and thus increased the bacterial richness of the samples. Curing agents are technologically employed to control the outgrowth of mesophilic and psychrophilic microorganisms and this effect was verified in batch 3 (without nitrifying agents) with high richness index during the first days of DFS production process. The growth of the starter culture to maximum levels led to a bacterial alpha diversity (i.e., bacterial richness and evenness) decrease. Beta diversity index (Atchison) for intersample composition grouped batches 1 and 2 due to the high bacterial diversity provided by the liver auto-hydrolysate ingredient and batches 3 to 6 because of the predominance of the *L. sakei* starter culture. During the DFS production process, bacterial communities changed and those batches without nitrifying agents were significantly different between them and from the rest of the batches (PERMANOVA p-value < 0.05). For instance, ALDEx results showed that throughout the process, *Pseudomonas* spp. and *B. thermosphacta* were enriched in batch 3 (no nitrifying salts), while *L. sakei* was enriched in batches 4 to 6. Bacterial communities of NaCl-reduced DFS (salt content substitution with KCl) were similar to DFS containing NaCl. By the end of ripening, batch 1 had the most different bacterial community ($p < 0.05$) in comparison to the batches containing starter culture.

The metataxonomic approach based on 16S rRNA gene sequencing allowed the characterization of the initial bacterial communities and their change during the DFS production process due to formulation, starter culture and temperature processing, providing an exhaustive and accurate characterization of the bacterial composition and taxonomy at genus or species level. The genomic emerging technologies such as metagenomics allow an in-deep characterization of the

meat matrix microorganisms, but pathogenic microorganisms of interest are hard to find within a food matrix through metagenomics due to their usually low prevalence and concentration. Notwithstanding, other powerful genomic approaches such as shotgun metagenomics and whole genome sequencing can go beyond the bacterial taxonomy, providing extra genomic information about bacterial species, plus information related to the genetic content for specific functions (e.g., antimicrobial resistance genes). In this PhD thesis, the powerful whole genome sequencing genomic approach has been used to explore the phylogenomic relationships and genomic composition of *Salmonella* isolates sampled from DFS and DFS raw materials (i.e., pig carcasses and pork) of industrial contexts as discussed in the next section.

2. Genomic characterization of *Salmonella* spp. isolated from the DFS production chain

The foodborne pathogen *Salmonella* spp. is responsible for salmonellosis which is the second most reported foodborne gastrointestinal disease in humans in the EU and has been a major cause of foodborne outbreaks in the EU for years. The enteric pathogen can survive in pigs' intestinal tracts, developing or not infection disease symptoms, and those animals become a pathogen reservoir. According to the "The European Union One Health Zoonoses Report", in 2022 there were 18 outbreaks linked to the "Pig meat and products thereof" and 14 were registered in 2021 (EFSA and ECDC, 2022, 2023). Additionally, during 2020-2023 period, the Rapid Alert System for Food and Feed (RASFF) reported 22 notifications related with DFS contaminated with *Salmonella*, some of them associated with salmonellosis outbreaks (European Commission, 2023).

The study "Genomic insights of *Salmonella* isolated from dry fermented sausage production chains in Spain and France" (Article II) showed that within the context of the pork industry globalization, the monophasic variant of the *Salmonella* Typhimurium serovar (1,4,[5],12:i:-) has raised and spread for the last 20 years worldwide and in the pig herds especially. The increase of *S.* 1,4,[5],12:i:- in the pork sector in France was shown in Article II from data gathered by the French *Salmonella* Network, while its increase and circulation in pigs and pig-related environments in Spain was previously characterized by Andres-Barranco et al. (2016). The evaluated *Salmonella* spp. genomic panel (Article II) included 173 genomes isolated from pig carcasses, pork, fresh sausages and DFS in France and Spain during the 1997-2021 period. Within the DFS production chain, it was observed a strong selection towards *S.* 1,4,[5],12:i:-, which could be related to a higher capacity of this serovar to survive the hurdles happening during pig carcass refrigeration, cleaning and disinfection processes, and acidification and drying during DFS production. The main *Salmonella* serovars identified in the panel were 1,4,5,12:i:-, Typhimurium, Derby and Rissen. Focusing on the serovars encountered within the genomic panel, an increase of 1,4,[5],12:i:- and Typhimurium serovars and a decrease of Derby and Rissen serovars were observed along the DFS production chain, from pig carcass to DFS.

Several antimicrobial resistance genes (ARG) were identified in all *Salmonella* genomes of the panel. The serovar that had more ARG co-occurrence was *S.* 1,4,[5],12:i:-, which exhibited resistance genes to three or more different antimicrobial classes, proving its worldwide spread warning. Extended-spectrum β-lactamase (ESBL) resistance genes were identified in *S.* Typhimurium and *S.* Derby isolated from 2006 on, but not in the monophasic variant of *S.* Typhimurium. Pathogens carrying ARG are of special concern for human health since foodborne infections, are the main cause morbidity and mortality worldwide and would be difficult to treat. In that sense the controlled use of antibiotics throughout food production and the antimicrobial is recommended to prevent the gain and transmission of ARG between bacteria and therefore the spread and transmission from food to consumers (Samtiya et al., 2022).

Through the whole genome characterization of the *Salmonella* isolates, mobile genetic elements (MGE) such as transposons (Tn) and *Salmonella* Genomic Islands (SGI) that are usually integrated in the *Salmonella* chromosome were identified. MGE carry specific ARG, virulence factors and biocide resistance genes that bring survival advantages by genetic strategies. For example, it has been described that the acquisition of Tn21 and SGI-4 favoured the expansion of the 1,4,[5],12:i:- European epidemic clone in 1980s (Cadel-Six et al., 2021) and both elements have been identified in the majority of 1,4,[5],12:i:- genomes within the panel. The Tn21 encodes mercury and antibiotic resistance genes, and SGI-4 encodes genes involved in arsenic and copper resistances. Genes encoding for quaternary ammonium and mercury compounds resistances co-ocurred with *aadA2*, *sul1* and *tet(A)* ARG in *S.* Derby sequence type 40 (ST40), which are linked to the presence of SGI-1 and Tn7.

Bacterial cells under stress conditions (e.g., in cleaning and disinfection processes) promote the gain of MGE (e.g., plasmids) to overcome the harsh conditions and improve cell survival (Ortega et al., 2013). IncF and Col were the most abundant plasmid replicon families in the genomic panel. *S.* Typhimurium presented IncFIB and IncFII, which are *Salmonella* ancestral virulence plasmids containing *rck*, *spv* and *pef* virulence operons. Colicinogenic plasmids are related to quinolone resistance (contain *qnrS1* and *qnrB19* genes) and were identified in *S.* 1,4,[5],12:i:-, *S.* Derby and *S.* Rissen genomes although at low prevalences.

Salmonella pathogenicity islands (SPI) are unstable large regions not belonging to the core genome that can be acquired by horizontal gene transfer and encode virulence factors of pathogenic bacteria (Hacker et al., 2003). SPI-2 and SPI-13 were shared by Typhimurium and 1,4,[5],12:i:- serovars but not by the other serovars characterized in the panel. Specifically, SPI-2 encodes for a type III secretion system responsible for delivering effector proteins to the host cell after infection. In contrast, SPI-13 functions remain largely uncharacterised and unclear. SPI-13 was identified only in some *S.* 1,4,[5],12:i:- that, interestingly, were isolated from DFS and some were outbreak related. This fact opens further research studies for SPI-13 complete genetic and phenotypic characterization and relatedness to virulence.

Within the most relevant virulence factors identified, it was noticed that the occurrence of *bcf* operon (related to *Salmonella* adherence) was associated with the absence of *ent*/*fep*/*ompA* genes

(related to adherence, biofilm formation and macrophage infection, respectively) as both sets of genes were not found in the same genome. Outer membrane protein (OMP) functions are numerous, for instance related to *Salmonella* iron regulation for the siderophore complexes up take or *Salmonella* intercellular virulence of macrophage for the activation of the immune system response during infection episodes. The *shdA* gene encodes for an OMP expressed during pig intestine survival and allows its specific binding to fibronectin, which can be further explored for the relation with *Salmonella* survival along the DFS production chain.

Phylogenomic relationships could be established between *Salmonella* genomes, forming clusters of two or more isolates with equal or different matrix, year, and country origin. *Salmonella* genomic clusters were set by allelic (i.e., ≤ 10 alleles) and SNP (i.e., ≤ 20 SNPs) differences identified between the isolates core genome of 1,4,[5],12:i:-, Typhimurium, Derby, Rissen and Worthington serovars. A total of 22 cgMLST and 27 cgSNP clusters were identified, mainly within the *S.* 1,4,[5],12:i:- genomes. Phylogenomic relationships indicate genotype persistence in the pork sector and DFS production chain, region-specificity, clonal isolates dissemination and interregional transmission and, cross-country *Salmonella* spread. The largest cluster identified (5M) was formed by nine *S.* 1,4,[5],12:i:- genomes closely related with a maximum of 15 cgSNPs between the most distant genomes. Cluster 5M is an example of strain persistence and interregional transmission in the pork sector in France since six out of nine strains were sampled in the French region of Occitanie in 2018 with ≤ 1 cgSNP of difference between them, one year later the same strain was isolated in the same region (1 cgSNP of difference between the strains isolated in 2018) and, also in 2019, one strain was isolated from pig carcass in the French region of Nouvelle-Aquitaine with only 6 cgSNPs of difference between the strains isolated in 2018. An example of *Salmonella* strain surviving the DFS production chain is cluster 2M (1,4,[5],12:i:- serovar) grouping three strains isolated from pig carcass and DFS in Girona (Spain) in 2018 and 2019, respectively, showing the same accessory genome pattern except for *shdA* gene and 10 cgSNPs of difference.

Bioinformatic repositories of genomic sequences of pathogen isolates shared online with the corresponding metadata is of special interest for pathogen international surveillance and advance in the One Health approach to promote public health. Countries that have implemented the WGS technology for pathogen surveillance and monitoring have reduced the dissemination incidence of pathogenic strains in food production chains and final products (Viltrop et al., 2023). The ability of *Salmonella* to survive along the DFS production process, paying special attention to *S.* 1,4,[5],12:i:-, and the presence of MDR genetic profiles emphasize the need for a global monitoring of pathogens.

Trends in genetic markers of antimicrobial and biocide resistances provide theoretical information in both *Salmonella* surveillance and monitoring. Withal, further phenotypic testing can be required to verify the linkage of those genetic markers with biocide and antimicrobial susceptibility and providing further insights for the identification of future targets for *Salmonella* inactivation strategies.

Nowadays, *Salmonella* control measures of DFS industrial production are based on raw material sampling and product acidification and drying. An important strategy to inhibit *Salmonella* during the production process is the application of a LAB starter culture to control the pathogen mainly through the production of lactic acid and the corresponding pH reduction. Some bioprotective cultures and few Gram-negative bacteria with antisalmonella activity have been identified (Chalón et al., 2012). For example, Ananou et al. (2010) identified that *Enterococcus faecalis* A-48-32 strain produced enterocin AS-48, active against *S. enterica* and Gong et al. (2010) identified that *Lactiplantibacillus plantarum* KLDS1.0391 (formerly *Lactobacillus plantarum*) strain produced plantaricin MG active against *S.* Typhimurium. As a consequence of the lack of bioprotective control measures, the best strategy for controlling *Salmonella* spp. in the DFS industry is related to the use of high microbiological quality raw materials and validated production processes ensuring enough lethality of the pathogen. As shown in Article II, in addition to the HACCP own-check procedures that DFS producers have to apply, exhaustive monitoring and control plans for *Salmonella* spp. are conducted by official and competent authorities to detect and identify the pathogen in the early stages of the DFS process before finishing the ripening process and ending in final products and consumers. Specific *Salmonella* serovars are predominant to specific animal hosts (e.g., *Salmonella* Derby is associated with the pork sector) (Sévellec et al., 2019)) and the genetic differences may explain the predominance to specific sectors. The genomic characterization provides information about genetic resistances to antimicrobials used in veterinary medicine and specific biocides employed in food industry, and thus helps in the design for strategies to control the pathogen.

In addition to *Salmonella*, *Listeria monocytogenes* is a significant pathogen of concern in DFS. In contrast to *Salmonella*, many bioprotective starter cultures with antilisteria activity have been identified, one of them is the sakacin K producer *L. sakei* CTC494, and those can be applied as additional control measures against *L. monocytogenes* (Barcenilla et al., 2022). Genomic characterization of *L. monocytogenes* isolates is also of interest for agricultural sectors due to its differences in serotypes and hypo- and hypervirulent profiles and its severity when reaches RTE products and consumed (Lagarde et al., 2024). Further research on the use of *L. monocytogenes* genomic information for serotype severity identification should be performed. Currently, stakeholders can apply bioprotective strategies, the effectiveness of which can be further can be enhanced by optimization methodologies, as discussed in next section.

3. *L. sakei* CTC494 antilisteria activity optimization

LAB starter cultures are frequently used to control fermentation during DFS production, ensuring a pH drop and specific organoleptic characteristics. A rapid acidification of the meat batter through the application of a competitive LAB constitutes an important hurdle to prevent acid-sensitive spoilage and pathogenic bacteria to grow and even promote its inactivation. Additionally, if LAB that are applied in DFS formulation are bacteriocinogenic an extra hurdle will be present, hence enhancing the food safety of the final product. Additionally, the rapid LAB

colonization in meat matrixes fermented at low and mild temperatures, confers a competitive advantage to other bacteria present in the meat environment, preventing from their growth.

In the study "The antilisteria effect of *Latilactobacillus sakei* CTC494 in relation to dry fermented sausage ingredients and temperature in meat simulation media" (Article III), the effect of the formulation and temperature on the behaviour of *L. sakei* CTC494 and 23K (non-bacteriocinogenic) strains in monoculture and coculture with *L. monocytogenes* CTC1034 strain was investigated. A meat simulation media facilitating the control of the concentrations of the evaluated factors was used. The *L. monocytogenes* CTC1034 strain was selected for this study because it was originally isolated from a DFS and belongs to serotype 4b, Clonal Complex (CC) I, and ST1 (Martín et al., 2014), frequently associated with hypervirulent traits (Lagarde et al., 2024), thus representing a worst-case scenario.

The studied independent factors and its range were: NaCl, 0.00–40.36 g/L, manganese (Mn), 0.08–0.32 g/L, glucose, 0.00–40.36 g/L and temperature, 3–37 °C. The physicochemical dependent factors were the pH of the media and the lactic acid (LA) and bacteriocin (BAC) production. These dependent factors were periodically evaluated and the minimum pH (pH_{min}) and maximum LA concentration (LA_{max}) and sakacin K activity (BAC_{max}) determined. Additionally, the yields of LA (Y_{LA}) and BAC (Y_{BAC}) were calculated. The growth-related dependent factors evaluated were the maximum growth rate (μ_{max}), the maximum population density (N_{max}), the growth potential, the log reduction, and the inactivation rate constant (k_d).

The effect of the independent factors was assessed in monoculture and coculture experiments of *L. sakei* and *L. monocytogenes*. The initial concentrations of NaCl and glucose and incubation temperature influenced the most on physicochemical parameters. NaCl high concentrations limited acidification and the highest glucose concentration in the media (i.e., 40 g/L of glucose), the lowest pH values were registered. According to these results, when glucose was abundant and NaCl concentration was low in the media, the metabolic activity of the studied bacteria was stimulated, and a notable pH drop was observed due to the lactic acid production. From the microbiological safety point of view, a high pH drop (i.e., a high LA production) in food fermentations is desirable to inhibit pathogens.

A response surface methodology (RSM) was employed to find the effect of independent to dependent factors and their relationships, which have been characterised through polynomial equations. It is remarkable the quadratic and linear effects drawn by glucose and NaCl, respectively, on the lactic acid maximum values (LA_{max}) of *L. sakei* strains and cocultures.

Sakacin K producer, *L. sakei* CTC494, showed the highest bacteriocin activity (i.e., 3200 UA/mL in most of the experiments but 6400 UA/mL in a few) at the early stationary phase of cultures grown at 10 and 20 °C, irrespective of the coculture. At higher and lower temperatures, BAC was almost undetectable (i.e., < 800 AU/mL). NaCl negatively influenced the production of sakacin K and in high NaCl concentrations, low BAC activity was detected. Leroy & De Vuyst (1999b, 1999a) studied the temperature, pH and NaCl effect on sakacin K activity by the CTC494 strain and observed an optimum at 23 °C and pH of 5.0 while no sakacin K activity was

detected at NaCl concentrations ≥ 8%. For sakacin K production RSM results reported that temperature and Mn concentration were the factors affecting the most, resulting in BAC_{max} and Y_{BAC} values similar to those in *L. sakei* CTC494 monoculture with quadratic polynomial effects.

Microbiological parameters of strains were mainly influenced by temperature and in coculture experiments, formulation-related factors were not relevant but the dominant strain (*L. sakei:L. monocytogenes* ratio; 5:3). The highest growth rates (μ_{max}) and maximum population densities (N_{max}) were registered at high temperatures (i.e., ≥ 30 °C) and the lowest at 3 °C. In monoculture experiments, all the evaluated independent factors influenced the maximum population densities and NaCl, glucose and, mainly, temperature significantly affected the growth potential. The highest growth potential values (difference between the maximum and the initial bacterial concentrations) were achieved at low NaCl concentrations and at intermediate temperatures for *L. sakei* strains (5.1 log for 23K and 3.8 log for CTC494), while it was achieved at high NaCl concentrations (i.e., 32.18 g/L) and temperature (i.e., 30 °C) for *L. monocytogenes* (6.0 log). It is especially remarkable the fact that the coculture reduced the N_{max} of *L. monocytogenes*, particularly with the *L. sakei* CTC494 bacteriocinogenic strain. The early entry of the *L. sakei* 23K (non-bacteriocinogenic strain) to the stationary phase inhibited the *L. monocytogenes* growth, leading it to the stationary phase (i.e., Jameson effect). In contrast, *L. sakei* CTC494 (bacteriocinogenic strain) population not only inhibited the *L. monocytogenes* growth but inactivated (decreased the levels) the pathogen in experiments where BAC activity was detected. In the experiments where temperature and NaCl combinations impeded the production of bacteriocin (i.e., extreme temperatures and high NaCl concentrations at 30 °C), the highest N_{max} and lowest reductions of *L. monocytogenes* were registered. Furthermore, up to 5-log reduction of *L. monocytogenes* were achieved in experiments where *L. sakei* CTC494 growth was promoted by high glucose or Mn concentrations (i.e., increasing LA and BAC production) at 20 or 10 °C. Additionally, the combination of mild temperature, high glucose and low NaCl concentrations is the best strategy to obtain the highest inactivation rates of the pathogen (> 0.1 h^{-1}). RSM predictions indicated that the best factor combination for *L. monocytogenes* inactivation was glucose concentrations up to 20 g/L, 20 g/L of NaCl and 0.27 g/L of Mn at 20 °C. The RSM approach allowed the identification of the most appropriate conditions for sakacin K production and *L. monocytogenes* inactivation. Further studies will be necessary to confirm the results on real DFS.

CONCLUSIONS

1. The metataxonomic approach allows an in-deep taxonomic characterization of the bacterial communities' succession of different *fuet*-type DFS, including those elaborated with the innovative pork liver auto-hydrolysate ingredient. Results provide valuable microbiological information to DFS manufacturers who want to have a better knowledge of their production process and innovate safely.

2. The most impacting factors on the bacterial community composition of DFS are (i) the addition of a starter culture (i.e., *Latilactobacillus sakei* CTC494) and/or a liver auto-hydrolysate, that provides putative probiotic species (e.g., *Lactobacillus johnsoni* and *Limosilactobacillus reuteri*), and (ii) the elimination of nitrifying agents which increases the occurrence of meat spoilage-related microorganisms (e.g., *Pseudomonas* genus).

3. Irrespective of the formulation and process parameters, the microbial communities of DFS gradually change over time, decreasing their diversity due to the progressively harsher conditions that occur throughout fermentation and drying and the dominance of the competitive and well adapted *L. sakei* CTC494 starter culture.

4. Changes in formulation, towards nutritionally improved (low in sodium) and clean-label (without nitrifying salts) options, and low-temperature processes only cause minor shifts in the physicochemical characteristics of DFS when using a competitive starter culture to ensure product acidification. Low temperature production process (< 10 °C) to guarantee food safety can be used to produce DFS with similar characteristics to those fermented and ripened at mild temperatures (13 °C).

5. *Salmonella* serovars along the French pork production chain (carcasses, pork and DFS) show a progressive increase in the *S.* Typhimurium monophasic variant (1,4,[5],12:i:-) serovar during the last 15 years and a reduction in the Typhimurium serovar.

6. Core genome phylogenomic cluster analysis is a powerful tool that grouped *Salmonella* genomes isolated at different stages of the pork production chain (i.e., different matrixes) and at different years, which reflects strain persistence and contamination due to international trade exchange.

7. Accessory genome characterization discovered relevant antimicrobial and biocide resistance genes, virulence genes, *Salmonella* genomic islands and plasmids in *Salmonella* isolates from the pork production chain and DFS from Spain and France. The ability of *Salmonella* to survive along the harsh conditions occurring during DFS production process, and the remarkable presence of MDR genetic profile strains emphasize the need for pathogen monitoring globally, paying special attention to 1,4,[5],12:i:- serovar.

8. Matrix formulation and process temperature modulate the lactic acid production (i.e., acidification capacity) and growth of *L. monocytogenes* CTC1034, *L. sakei* 23K and CTC494. High glucose concentrations (i.e., 40 g/L) promote acidification, while low concentrations limit the lactic acid production and pH decrease. Temperature is the most influencing factor on growth parameters reaching the highest μ_{max} at the highest tested temperature.

9. Mild temperatures (10–20 °C) favour the production of sakacin K by *L. sakei* CTC494 achieving maximum bacteriocin activity values at the early stationary phase of the culture. The bacteriocin production by *L. sakei* CTC494 was not stimulated by the coculture with *L. monocytogenes.*

10. Bacteriocinogenic *L. sakei* CTC494 contribute to the hurdle technology not only inhibiting the growth of *L. monocytogenes* but promoting its inactivation to up to 5-log. Optimal conditions for the highest *L. monocytogenes* reduction were at 20 °C with 20 g/L NaCl, 0.20 g/L Mn and 40 g/L glucose.

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