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"Characterisation of *Listeria monocytogenes* and *Listeria innocua* to investigate contamination scenarios in dairy processing facilities"

verfasst von / submitted by

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“blessed are the cheesemakers”

Monty Python, Life of Brian

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I. ABSTRACT

Adaptive characteristics enable *Listeria (L.)* spp. to survive and grow under the stressful conditions in dairy processing environments. Knowledge of the entry routes, contamination patterns and growth niches of *L. monocytogenes* and *L. innocua* is essential to implement control strategies and prevent food contamination with *L. monocytogenes*, the causative agent of listeriosis.

The aim of study phase I was to investigate *L. monocytogenes* contamination in an Austrian dairy processing environment over time by environmental sampling and isolate genotyping. During three years of monitoring, *L. monocytogenes* was detected in 19.5% of the samples, with initial identification of multiple contamination routes and high strain heterogeneity. Interventions successfully reduced the overall *L. monocytogenes* occurrence and confined the contamination to floors and drains with one ineradicable, dominant sequence type 5 (ST5). In study phase II, previously recovered *L. monocytogenes* and *L. innocua* isolates from five Austrian dairy processors were characterised by geno- and phenotyping. PFGE and MLST subtyping of *L. innocua* and *L. monocytogenes* isolates yielded 33 distinguishable PFGE profiles (*Asc*I) and 27 STs, including seven novel *L. innocua* STs (ST1595 to ST1601). Persistent contamination by *L. monocytogenes* as well as *L. innocua* for up to eleven and seven years respectively was detected, with the most prevalent STs being ST121, ST14 and ST1597 and ST603. *L. monocytogenes* as well as *L. innocua* strains present for a long period of time in the environment and products of the same facility harboured either stress survival islet (SSI)-1 or SSI-2 and were rhamnose fermenters.

Subtyping proved to be an invaluable tool for the identification of *L. monocytogenes* and *L. innocua* contamination patterns over time and to reveal contamination hot-spots in dairy processing facilities. Our results demonstrate that it is possible to reduce *L. monocytogenes* diversity and presence in the food processing environment (FPE) by adequate sampling, subtyping, management and personnel awareness and by applying adequate control measures. Due to the similarities in terms of habitat and adaptive properties, incorporation of *L. innocua* into *Listeria* monitoring programmes as an index organism for possibly undetected *L. monocytogenes* presence is recommended.

II. ZUSAMMENFASSUNG

Die Fähigkeit zur Anpassung an vorherrschende Umweltbedingungen ermöglicht es *Listeria (L.)* spp. über einen langen Zeitraum in Milchverarbeitungsbetrieben zu überleben. Die Eintrittswege, Kontaminationsmuster und Wachstumsnischen von *L. monocytogenes* und *L. innocua* zu kennen ist daher unerlässlich, um Bekämpfungsstrategien umzusetzen und die Kontamination von Lebensmitteln mit *L. monocytogenes*, dem Erreger der Listeriose, zu verhindern. Das Ziel der ersten Studienphase war die Untersuchung des *L. monocytogenes* Kontaminationsgeschehens im Zeitverlauf in einem österreichischen Milchverarbeitungsbetrieb durch Probenahme und Genotypisierung. Während der dreijährigen Monitoringphase war *L. monocytogenes* in insgesamt 19,5% der Proben nachweisbar, wobei anfänglich mehrere Kontaminationsrouten und eine hohe Stammheterogenität festgestellt wurden. Den Monitoringergebnissen angepasste Interventionen führten zur Reduktion des *L. monocytogenes* Vorkommens und zu einer eingegrenzten Kontamination der Böden und Gullies mit einem persistenten, dominanten Sequenztyp 5 (ST5). In der zweiten Studienphase wurden zuvor isolierte *L. monocytogenes* und *L. innocua* von fünf österreichischen Milchverarbeitern mittels Geno- und Phänotypisierung charakterisiert. Die PFGE- und MLST-Subtypisierung von *L. innocua*- und *L. monocytogenes*-Isolaten ergab 33 unterscheidbare PFGE-Profile (*Asc*I) und 27 STs. Darunter fanden sich sieben bisher nicht dokumentierte *L. innocua*-STs (ST1595 bis ST1601). Wir konnten eine anhaltende Kontamination mit *L. monocytogenes* sowie *L. innocua* von bis zu elf bzw. sieben Jahren nachweisen, wobei die STs ST121, ST14 und ST1597 sowie ST603 am häufigsten detektiert wurden. Persistente *L. monocytogenes* sowie *L. innocua*-Stämme enthielten entweder SSI-1 oder SSI-2 und waren Rhamnose-Fermenter.

Die Subtypisierung erwies sich als wertvolles Werkzeug zur Identifizierung von *Listeria* spp. Kontaminationsmustern und um Kontaminations-Hot-Spots in Milchverarbeitungsbetrieben zu ermitteln. Die Ergebnisse zeigen, dass es möglich ist, die Diversität und das Vorkommen von *L. monocytogenes* insbesondere durch systematische Probenahme, Subtypisierung, Steigerung des Bewusstseins bei QM-Verantwortlichen und dem Personal, sowie die Anwendung geeigneter

Kontrollmaßnahmen zu reduzieren. Aufgrund der Ähnlichkeiten in Bezug auf Habitat und adaptive Eigenschaften, sowie den Studienergebnissen, wird empfohlen, *L. innocua* als Indexorganismus für ein möglicherweise unentdecktes *L. monocytogenes* Vorkommen in Listerien-Monitoring-Programme aufzunehmen.

III. PUBLICATIONS

This thesis is based on the following publications:

Original research article 1:

Rückerl, I., Muhterem-Uyar, M., Muri-Klinger, S., Wagner, K. H., Wagner, M., Stessl, B. & (2014). ***L. monocytogenes*** **in a cheese processing facility: learning form contamination scenarios over three years of sampling.** *International Journal of Food Microbiology, 189*, 98-105.

Original research article 2:

Kaszoni-Rückerl, I., Mustedanagic, A., Muri-Klinger, S., Brugger, K., Wagner, K. H., Wagner, M., & Stessl, B. (2020). **Predominance of Distinct *Listeria Innocua* and *Listeria Monocytogenes* in Recurrent Contamination Events at Dairy Processing Facilities.** *Microorganisms, 8*(2), 234.

Further publications and scientific presentations are listed in chapter 9.

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VII. ABBREVIATIONS

ADI	arginine deiminase
ANI	average nucleotide identity
BC	benzalkonium chloride
bp	base pair
CC	clonal complex
CDC	(US) Centers for Disease Control and Prevention
CFR	case fatality rate
cfu	colony-forming unit
cg	core genome
DNA	deoxyribonucleic acid
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EDTA	ethylene diamine tetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
FBO	food business operator
FCS	food contact surface
FF	full-strength Fraser broth
FPE	food processing environment
g	relative centrifugal force
GABA	glutamate-γ-aminobutyrate
GAD	glutamate decarboxylase
GC	guanine-cytosine
GHP	good hygiene practices
GMP	good manufacturing practices
h	hour(s)
HF	half-strength Fraser broth
HGT	horizontal gene transfer
ISO	International Organization for Standardization
kb	kilobase
L.	*Listeria*
Mbp	mega base pairs

MGE	mobile genetic element
MIC	minimal inhibitory concentration
MLST	multi-locus sequence typing
mRNA	messenger RNA
n	number (of samples)
NFCS	non-food contact surface
ORF	open reading frame
PAA	peracetic acid
PCR	polymerase chain reaction
PDO	Protected Designation of Origin
PI-PLC	phosphatidylinositol-specific phospholipase C
PFGE	pulsed-field gel electrophoresis
QAC	quaternary ammonium compound
QM	quality management
(r)RNA	(ribosomal) ribonucleic acid
rpm	revolutions per minute
RTE	ready-to-eat
sRNA	small non-coding RNA
SSI	stress survival islet
spp.	species pluralis
ST	sequence type
T(B)E	tris-(borate-)ethylene diamine tetraacetic acid
U	unit(s)
UK	United Kingdom
US(A)	United States (of America)
V	volt
wg	whole-genome
WGS	whole-genome sequencing
WHO	World Health Organization
w/v	weight per volume

1. INTRODUCTION

Listeria are ubiquitously found in the natural environment, with most species being non-pathogenic saprophytes (Sauders & Wiedmann, 2007). *Listeria (L.). monocytogenes* however, is able to switch from saprophytic to intracellular lifestyle and is an important human and animal pathogen (Freitag et al., 2009). The history of *L. monocytogenes* as the causative agent of listeriosis in humans and animals is a relatively recent compared to other pathogenic agents. The first report about the bacterium by Murray et al., the observation of rabbits suddenly dying of leucocytosis, was published in 1926. The name „*Bacterium monocytogenes*“ was suggested (Murray et al., 1926). The pathogenicity of *L. monocytogenes* in humans was described a few years later (Nyfeldt, 1929). The first substantial evidence for foodborne transmission of *L. monocytogenes* to humans was reported in the 1950s. Around that time a significant increase in stillbirths was noted in a clinic in Halle, Germany. The same *L. monocytogenes* serovar was successfully isolated from the milk of a cow with mastitis and from stillborn twins of a mother reportedly having consumed the cow's raw milk (Potel, 1953).

Following the development of selective media, various *Listeria* spp. strains were isolated from diverse environments. Thereafter, *L. monocytogenes* and *L. grayi* had been described, as well as *L. denitrificans,* which was later excluded from the genus and *L. murrayi*, which was later found to be a member of *the L. grayi* species (Rocourt & Buchrieser, 2007). Given the non-haemolytic, non-pathogenic “innocuous“ nature of the bacterium, *L. innocua* was first described in 1977 as the (then) fifth species of the genus *Listeria* (Seeliger, 1981). The definite evidence of *L. monocytogenes* transmission via food was successfully provided in the 1980s, when coleslaw was identified as source of a listeriosis outbreak in Nova Scotia, Canada. Manure from shedding sheep, which was used to fertilise the cabbage, was determined as the source of contamination (Schlech et al., 1983).

Transmission of *L. monocytogenes* to humans occurs almost exclusively via consumption of contaminated food, especially ready-to-eat (RTE) food (Swaminathan & Gerner-Smidt, 2007). Previous listeriosis outbreaks underline the fact that *L. monocytogenes* is hard to eradicate from foodstuffs and from the food processing environment (FPE) despite hygiene measures, being obviously well

adapted to the often inhospitable conditions during food production and processing (V. Ferreira et al., 2014; Norton & Braden, 2007).

According to the European Union (EU) One Health 2020 Zoonoses Report, the notification rate of listeriosis was 0.46, which corresponds to 1,876 reported invasive listeriosis cases in the EU in 2020. This is relatively low, compared to the most prevalent foodborne diseases in the EU, campylobacteriosis (40.3 per 100,000 population in 2020) and salmonellosis (13.7 cases per 100 000 population in 2020). Listeriosis is however one of the most serious foodborne diseases, with a high hospitalisation rate of 97.1% in 2020 (and 92.1% in 2019) and a case fatality rate (CFR) of 13.0% in 2020 (and 17.6% in 2019) in the EU and poses a major concern for public health (European Food Safety Authority [EFSA] & European Centre for Disease Prevention and Control [ECDC], 2021). Surveillance of food products at retail and production level in the EU in 2020 revealed a low level of samples non-compliant to Regulation (EC) No. 2073/2005. At retail level 0.0-1.4% of samples were positive for *L. monocytogenes* and at processing level 0.0-3.8% resulted in positive results. The food categories most often non-compliant were fish and fishery products, meat products and soft and semi-soft cheeses (EFSA & ECDC, 2021).

Molecular subtyping methods are essential for surveillance purposes and to control the prevalence of *Listeria* spp. in FPEs and foodstuffs. Genotypic methods such as pulsed-field gel electrophoresis (PFGE) are helpful tools to characterise strains and facilitate the identification of contamination routes. Sequence-based techniques, e.g. multi-locus sequence typing (MLST) and whole-genome sequencing (WGS) are now widely used for outbreak investigation as well as to gain insight into physiology and epidemiology of *Listeria* spp (Lüth et al., 2018).
Recently, *L. innocua* received increased attention as atypical strains with a haemolytic phenotype and virulence genes were discovered (Moreno et al., 2014, 2012; Moura et al., 2019). The species and its atypical strains are also of interest for gaining insight into the evolution of the genus *Listeria* and the development of pathogenicity within the genus (Buchrieser et al., 2003; J. Chen et al., 2010).

1.1. Characteristics of the genus *Listeria*

The genus *Listeria* belongs to the phylum *Firmicutes,* class *Bacilli,* order *Bacillales* and together with the genus *Brochotrix* to the family *Listeriaceae.* The prokaryotes exhibit a Gram positive cell wall structure and a low guanine-cytosine (GC)-content (McLauchlin & Rees 2009).

With numerous new descriptions during the last years, the genus *Listeria* currently comprises 27 recognised species (as of 11 February 2022): *L. aquatica, L. booriae, L. cornellensis, L. cossartiae, L. costaricensis, L. farberi, L. fleischmannii, L. floridensis, L. goaensis, L. grandensis, L. grayi, L. immobilis, L. innocua, L. ivanovii, L. kieliensis, L. marthii, L. monocytogenes, L. newyorkensis, L. portnoyi, L. riparia, L. rocourtiae, L. rustica, L. seeligeri, L. thailandensis, L. valentina, L. weihenstephanensis, L. welshimeri* (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=1637&lvl=3&lin=f&keep=1&srchmode=1&unlock; accessed on: 11 February 2022). An overview of the currently described *Listeria* species is provided in Table 1 (*Listeria* spp. characteristics) and in Supplemental Table 1 (*Listeria* spp. source of isolation).

According to Orsi and Wiedmann the genus *Listeria* can be classified into *Listeria sensu stricto* and *Listeria sensu lato* by genomic and phenotypic analysis (Orsi & Wiedmann, 2016). The *sensu lato* clade solely comprises environmental bacteria without the ability to invade mammalian cells. The *sensu stricto* clade includes the pathogenic species *L. ivanovii*, which causes abortion, enteritis and mastitis in animals, especially small ruminants and *L. monocytogenes*, which is the causative agent of human and animal listeriosis (Chiara et al., 2015; Low & Donachie, 1997; Orsi & Wiedmann, 2016; Vázquez-Boland et al., 2001b; Orsi & Wiedmann, 2016). Furthermore, *L. cossartiae, L. farberi, L. immobilis, L. marthii*, *L. innocua, L. seeligeri* and *L. welshimeri* are grouped in the *senu stricto* clade (Orsi & Wiedmann, 2016). These species are widely recognised as non-pathogenic, however rare clinical cases of infections with the latter two species have been reported (Favaro et al., 2014; Perrin et al., 2003; Rocourt et al., 1986).

1.1.1. **Morphology and growth conditions**

Listeria spp. are rod-shaped, non-spore forming, aerobic, facultative anaerobic, facultative intracellular bacteria. *Listeria* hydrolyse aesculin and are catalase positive (except for *L. costaricensis*), oxidase negative, measuring 1-2 µm in length and 0.4-0.5 µm in diameter on average (Low & Donachie, 1997). Flagellatic motility in *L. monocytogenes* occurs below 30 °C at room temperature, but not or very weakly at mammalian physiological temperature (37 °C) (see Table 1).
Under laboratory conditions, colonies are 0.5-1.5 mm in diameter after 24-48 h of incubation and appear round, translucent, low convex with a smooth surface an entire margin and non-pigmented. Older colonies are larger and sometimes have a sunken centre (McLauchlin & Rees 2009).

Listeria are able to survive and grow under harsh environmental conditions.
Listeria grow within the following ranges of different environmental factors (D. Liu et al., 2005; Renier et al., 2011):

- temperature: growth between 0 and 45 °C
- pH: growth between 4.3 and 9.6
- NaCl concentration: growth up to 10% (w/v) NaCl, survival possible at concentrations as high as 40% (w/v) NaCl
- water activity (a_w): growth at or above 0.9

Generally, *Listeria* spp. are of ubiquitous occurrence in the natural environment (Sauders & Wiedmann, 2007). Diverse isolation sources have been described, including soil, water, sewage, plant material, animal feed, food, farm- and FPEs (Rocourt & Buchrieser, 2007; Sauders & Wiedmann, 2007; Supplemental Table 1).

Table 1: *Listeria* species and their biochemical characteristics

Listeria species	biochemical characteristics												acid production from:																						reference
	Motility	Growth at 4 °C	Voges-Proskauer test	Nitrate reduction	Nitrite reduction	PI-PLC	Catalase	Oxidase	β-haemolysis	D-Arylamidase (DIM)	Hydrolysis of Aesculin	α-Mannosidase	D-Arabitol	D-Xylose	L-Rhamnose	Methyl-α-D-Glucopyranoside	D-Ribose	D-Glucose-1-Phosphate	D-Tagatose	Glycerol	L-Arabinose	D-Galactose	D-Glucose	L-Sorbose	Inositol	D-Mannitol	Methyl-α-D-Mannopyranoside	Maltose	Lactose	Melibiose	Sucrose	Inulin	Melezitose	Turanose	D-Lyxose	
L. aquatica	-	-	V	+	-	-	+	-	-	-	+	+	-	+	+	-	+	-	+	V	+	-	+	-	V	-	+	-	-	-	-	-	-	V	den Bakker et al., 2014
L. booriae	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+	+	V	-	-	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	Weller et al., 2015
L. cornellensis	-	+	-	+	-	-	+	-	-	-	+	-	-	+	-	+	+	-	-	V	V	-	+	-	-	-	-	+	(+)	-	-	-	-	-	den Bakker et al., 2014
L. cossartiae	+	+	+	-	-	-	+	-	-	-	+	+	+	-	-	+	-/V	-	-	+	-	-	+	-	-	-	+	+	+	-	-	-	-	-	Carlin et al., 2021
L. costaricensis	+	-	+	+	-	-	-	-	-	-	+	-	+	+	+	+	+	-	-	+	-	+	+	-	-	-	+	+	+	-	+	-	-	-	Núñez-Montero et al., 2018
L. farberi	+	+	+	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	Carlin et al., 2021
L. fleischmannii	-	-	-	+	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	+	-	+	+	V	V	V	V	+	+	V	V	-	V	V	Bertsch et al., 2013
L. floridensis	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	-	-	+	den Bakker et al., 2014
L. goaensis	-	-	-	-	-	-	+	-	(α)	-	+	-	+	+	+	-	-	-	-	(+)	-	+	-	-	-	-	-	+	+	-	-	-	-	-	Doijad et al., 2018
L. grandensis	-	+	-	+	-	-	+	-	-	-	+	-	V	+	-	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	den Bakker et al., 2014
L. grayi	+	+	+	V	-	-	+	-	-	+	+	V	+	-	-	+	+	-	-	V	-	+	+	V	-	+	+	+	+	-	-	-	-	V	Larsen & Seeliger, 1966
L. immobilis	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	-	V	-	-	+	-	-	-	-	V	V	-	V	-	V	-	Carlin et al., 2021
L. innocua	+	+	+	-	-	-	+	-	-	+	+	+	+	-	V	+	-	-	-	+	-	-	V	V	-	-	+	+	+	V	+	V	V	V	Seeliger, 1981
L. ivanovii	+	+	+	-	-	+	+	-	+	V	+	-	+	+	-	+	+	V	-	+	-	V	V	V	-	-	-	+	+	-	+	-	V	-	Seeliger et al., 1984
L. marthii	+	+	+	-	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	V	V	-	-	+	+	+	V	-	-	-	+	Graves et al., 2010
L. monocytogenes	+	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	-	-	-	V	-	V	V	V	-	-	+	+	+	V	+	V	V	-	Pirie, 1940
L. newyorkensis	-	+	-	+	-	-	+	-	-	-	+	-	-	+	V	+	+	-	-	+	+	+	+	-	-	+	-	+	+	-	-	-	-	-	Weller et al., 2015
L. portnoyi	-	+	+	+	-	-	+	-	-	-	+	-	(+)	+	+	+	-	-	-	-	-	+	+	-	-	+	-	-	(+)	-	-	-	-	-	Carlin et al., 2021
L. riparia	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	+	V	-	-	V	+	+	+	-	V	V	+	+	+	V	-	-	-	-	den Bakker et al., 2014
L. rocourtiae	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	+	+	-	-	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	Leclercq et al., 2010
L. rustica	-	+	+	+	-	-	+	-	-	-	+	-	(+)	+	+	+	-	-	-	-	+	+	+	-	-	+	-	+	+	-	-	-	-	-	Carlin et al., 2021
L. seeligeri	+	+	+	-	-	-	+	-	+	+	+	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	V	+	+	-	+	-	V	-	Rocourt & Grimont, 1983
L. thailandensis	-	-	+	+	-	-	+	-	-	-	+	-	+	+	+	+	+	-	+	(+)	-	-	+	-	+	-	-	-	-	-	-	-	-	-	Leclercq et al., 2019b
L. valentina	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	V	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	Quereda et al., 2020
L. weihenstephanensis	(+)	+	-	+	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	+	V	-	-	-	-	-	Halter et al., 2013
L. welshimeri	+	+	+	-	-	-	+	-	-	V	+	+	+	+	V	+	-	-	+	+	-	-	+	-	-	-	+	+	+	-	+	-	V	-	Rocourt & Grimont, 1983

Abbreviations: + positive; (+) weak positive; - negative; V: variable; *L. kieliensis* is not shown here, because biochemical characteristics have not been determined (Kabisch, 2018); further references: Orsi & Wiedmann, 2016; https://bacdive.dsmz.de/; accessed on: 17 February 2022.

1.1.2. **Stress tolerance**

The ability to survive and multiply under a broad range of environmental stress conditions encountered in the natural environment, the food processing chain and in the susceptible host, contributes to the relevance of *L. monocytogenes* as a foodborne pathogen (Gandhi & Chikindas, 2007). Stress survival mechanisms of *L. monocytogenes* include changes in membrane composition and gene expression, induction of proteins as well as uptake and accumulation of compatible solutes such as osmo- and cryoprotectants (Bucur et al., 2018). Biofilm formation and sublethal adaptation to sanitisers and disinfectants are further factors contributing to stress survival (Bucur et al., 2018; Wiktorczyk-Kapischke et al., 2021). It has been demonstrated that adaptation to stress factors can be co-selected and can be increased when pre-exposure takes place (Davis et al., 1996; Faleiro et al., 2003; Gandhi & Chikindas, 2007; Schmid et al., 2009; Lou & Yousef, 1997). Previous studies confirmed that environmental conditions determine growth and survival limitations and that stress tolerance can vary widely between strains and lineages (Aalto-Araneda et al., 2020; Bergholz et al., 2010; Redfern & Verran, 2017).

On a molecular level, survival under harsh environmental conditions e.g. acid stress, cold temperatures or osmotic stress is enabled by various protein and RNA transcriptional regulators (Guerreiro et al., 2020).

The major transcriptional regulators in *L. monocytogenes* are the sigma factors, which are specialised protein subunits needed for the initiation of transcription (Kazmierczak et al., 2005; Feklístov et al., 2014). A sigma factor promotes binding of the RNA polymerase to specific initiation sites and thereby defines which genes are transcribed (Wösten, 1998). The majority of *L. monocytogenes* strains harbour five sigma factors, *L. monocytogenes* housekeeping sigma factor σ^A, as well as σ^B, σ^C, σ^H and σ^L, which determine the transcription of genes in response to the current environmental conditions (Glaser et al., 2001; O'Byrne & Karatzas, 2008).

The σ^B factor regulates the transcription of about 300 genes (which equates to about 10% of the genome), mostly including general stress response genes and is essential for the survival of *L. monocytogenes* in challenging environments (Chaturongakul et al., 2011; O'Byrne & Karatzas, 2008). A cytoplasmic complex,

termed stressosome, is responsible for the activation of the σ^B regulon (Pané-Farré et al., 2017). This regulon has been well studied in *Bacillus (B.) subtilis*. The latter having a high level of sequence homology of the stressosome components and the signalling pathway with *L. monocytogenes* (A. Ferreira et al., 2004). The existence of a stressosome had recently been demonstrated in *L. monocytogenes* as well and was reviewed by Impens et al. Structure wise, similar to *B. subtilis*, it is composed of protein subunits, RsbR, RsbL, RsbS and RsbT. RsbR is involved in sensing stress. A stress signal results in a conformation change in the stressosome and the release of RsbT, which activates the phosphatase RsbU. RsbU then dephosphorylates RsbV, an anti-anti-sigma factor and antagonist of the anti-sigma factor RsbW. This interaction between RsbV and RsbW triggers an interaction of σ^B with RNA polymerase and subsequently the transcription of the general stress response regulon (Impens et al., 2017). With the exception of a previously discovered miniprotein, Prli42, involved in stress sensing and the characterisation of the blue light sensing protein RsbL (Lmo0799), details about the mechanism of stress sensing in *L. monocytogenes* still need to be elucidated (Dorey et al., 2019; Impens et al., 2017).

Genes regulated by σ^B contribute to a wide range of stress coping mechanisms (NicAogáin & O'Byrne, 2016). Key osmotic stress resistance systems include OpuCABCD (encoded by *opuCABCD*), GbuABC (encoded by *gbuABC*) and BetL (encoded by *betL*). These are transporters of carnitine (OpuC) and glycine betaine (Gbu, BetL), which transport the solutes from outside the cell into the cytoplasm to cope with stress from high salt concentrations (Angelidis & Smith, 2003; Fraser et al., 2003; Sleator et al., 1999). Among the mechanisms for acid stress protection, the glutamate decarboxylase (GAD) and the arginine deiminase (ADI) systems have previously been described (Cotter et al., 2001; Ryan et al., 2009): the GAD system, encoded by the five genes *gadA-E,* is composed of glutamate decarboxylase enzymes (GadD1, GadD2 and GadD3) and glutamate-γ-aminobutyrate (GABA) antiporters (GadT1 and GadT2). Acid stress is counteracted by a reduction of acidity of the cell cytoplasm in a twofold manner, via elimination of protons from the cytoplasm by a decarboxylation reaction and furthermore via the build-up of GABA in the cell cytoplasm, which is less acidic than glutamate (Cotter et al., 2001; Gahan & Hill, 2014; Karatzas et al., 2010). Through the ADI system, the acidity in the cell cytoplasm is reduced via

conversion of arginine to ornithine, carbon dioxide and ammonia. The regulator involved is ArgR, which regulates the arginine biosynthesis genes *arcA*, *arcB, arcD* (Ryan et al., 2009).

σ^B not only regulates stress tolerance, there is also evidence for *L. monocytogenes* σ^B regulation of various metabolic pathways, especially carbon metabolism functions (Y. Liu et al., 2019).

Previous transcriptomic and phenotypic studies imply a vital role for *L. monocytogenes* σ^B in the modulation of transcriptional networks during the saprophytic as well as the intracellular phase of the life cycle (Toledo-Arana et al., 2009; Wemekamp-Kamphuis et al., 2004). The greatest regulon overlaps were found between σ^B and other transcriptional regulators such as AgrA, CodY, CtsR, HrcA, MogR, PrfA, and other sigma factors (Chaturongakul et al., 2011; Garmyn et al., 2012; Guariglia-Oropeza et al., 2014; Hu et al., 2007a; Hu et al., 2007b; Lobel & Herskovits, 2016). Small non-coding RNAs (sRNAs) regulate gene expression in bacteria by controlling target genes post-transcriptionally by base pairing with their messenger RNAs (mRNAs). This mechanism enables pathogenic bacteria to react to changes in environmental conditions by modulating metabolism, stress response and virulence accordingly (Waters & Storz, 2009). The transcription of numerous sRNAs is σ^B dependent (Wurtzel et al., 2012). Control over sRNAs can also be exercised through the σ^B-dependent RNA-binding protein Hfq (Christiansen et al., 2004). Details about regulatory sRNAs of *L. monocytogenes* dependent on σ^B and their respective functions can be found in a recent review by Dorey et al. (Dorey et al., 2019).

Moreover, σ^B plays a role in virulence of *L. monocytogenes.* Several virulence genes are co-regulated by σ^B and PrfA (pleiotropic regulatory factor) (Gaballa et al., 2019). Examples are the internalin genes required for adhesion and invasion, *inlA* and *inlB* (Kim et al., 2005). Previous studies also found that a regulatory network exists between σ^B and PrfA. σ^B regulates PrfA activity directly through transcriptional activation from the prfAP2 promoter and indirectly through posttranscriptional repression under certain environmental circumstances (Nadon et al., 2002; reviewed by Gaballa et al., 2019). An overview of the main *L. monocytogenes* stress response, virulence and metabolic genes regulated by σ^B and their respective role is provided in Supplemental Table 2.

The genus *Listeria* has a highly conserved genome (den Bakker et al., 2010b). A comparison of the genomes of *L. monocytogenes* (EGD-e, lineage II, serovar 1/2a) and *L. innocua* (CLIP 11262, serovar 6a) showed a high degree of similarity between these species, with *L. innocua* CLIP 11262 sharing around 88% of orthologous, protein-coding genes with *L. monocytogenes* EGD-e (den Bakker et al., 2010b; Glaser et al., 2001). Actually, the clonality of *Listeria* strains can be assessed by analysing the average nucleotide identity (ANI). A genome-wide 95% ANI cut-off point has been suggested, with genomes sharing ≥95% ANI belonging to the same species (Goris et al., 2007; Richter & Rosselló-Móra, 2009; Jain et al., 2018; Olm et al., 2020).

It was demonstrated that *L. monocytogenes* and *L. innocua* have species specific σ^B-dependent genes and display variations in stress resistance phenotypes, particularly in acid stress resistance (Raengpradub et al., 2008). Strain-specificities can be assumed for both species, as variations in σ^B-regulated stress response were found among *L. monocytogenes* strains of different lineages (Oliver et al., 2010).

In *L. monocytogenes,* the five-gene islet *lmo0444-0448* accommodates *gadD1* and *gadT1*, which are part of the GAD system contributing to acid stress survival of the bacterium, as well as a penicillin V acylase (*pva*), involved in bile tolerance (Begley et al., 2005; Ryan et al., 2010). These genes are regulated by the σ^B-dependent *lmo0445* gene, which might contribute to adaptation to stress encountered by *L. monocytogenes* in food-associated environments or the host. This 9.7 kb genetic islet is present in certain *L. monocytogenes* strains and was designated stress-survival islet 1 (SSI-1) (Ryan et al., 2010). SSI-1 was also associated with serovar-specific differences in biofilm formation ability of *L. monocytogenes*, with predominantly SSI-1^+ serovar 1/2b strains forming the strongest biofilms and mostly SSI-1^- serovar 4b strains forming the weakest biofilms (Keeney et al., 2018). Instead of the SSI-1, a 2.2 kb fragment was found at the corresponding location in *L. innocua* and in some *L. monocytogenes* strains, especially in sequence type 121 (ST121) strains (Hein et al., 2011). The 2.2 kb insert *lin0464-0465* was previously identified as SSI-2 and it was suggested that its presence contributes to alkaline and oxidative stress survival (Harter et al., 2017).

1.1.3. **Virulence**

In the natural environment, the lifestyle of *Listeria* spp. is saprophytic (Sauders & Wiedmann, 2007). In the susceptible mammalian host *L. monocytogenes* is able to switch to intracellular pathogen (Freitag et al., 2009; Tiensuu et al., 2019). Two main regulators are responsible for the adaptability of *L. monocytogenes* to different environmental conditions: adaptation to environmental stresses in and outside the host is enabled by σ^B (see chapter 1.1.2.), (A. Ferreira et al., 2001; Kazmierczak et al., 2003). The other, master virulence regulator PrfA, is activated by environmental conditions during host cell invasion and controls a multitude of virulence factors in *L. monocytogenes* (de las Heras et al., 2011).

Metabolism is an essential factor in stress tolerance as well as in intracellular survival of *L. monocytogenes* (Sauer et al., 2019). Metabolites like amino acids and carbon sources encountered by *Listeria* fulfil the metabolic growth needs, but also their availability dictates the regulation of *L.monocytogenes* virulence (Tapia et al., 2020; Sauer et al., 2019). Intracellular, the regulation of PrfA is to the majority moderated by the existence of carbon sources in the host cytosol (G. Y. Chen et al., 2017). Hexose-phosphates and glycerol are the primary carbon sources in the cytosolic metabolism. These stimulate full activity of PrfA, while PrfA activity is inhibited by glucose or other phosphotransferase system (PTS) substrates, which are associated with survival in non-host environments (Joseph et al., 2008; Stoll & Goebel, 2010).

In *L. monocytogenes,* the major virulence genes are located on the *Listeria* pathogenicity island-1 (LIPI-1) (Vázquez-Boland et al., 2001a; Johansson & Freitag, 2019),

- encoding a pore-forming toxin listeriolyin O (LLO),
- an actin-polymerising surface protein ActA, enabling intracellular motility and cell-to-cell spread,
- two phospholipases C, phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by *plcA*) and phosphatidylcholine phospholipase C (PC-PLC, encoded by *plcB*), involved in lysis of the phagocytic vacuole membrane,

- a metalloprotease (Mpl) maturing the PC-PLC precursor to its mature configuration,
- and a thermo-regulated transcriptional activator PrfA in control of LIPI-1 gene expression and further chromosomal virulence determinants.

Further chromosomal virulence determinants include (Johansson & Freitag, 2019)

- the *inlAB* operon, encoding the surface proteins InlA and InlB responsible for host cell invasion,
- a small secreted internalin (InlC) aiding spread to neighbouring cells,
- a bile salt hydrolase (Bsh) conferring bile resistance,
- a secretion chaperone (PrsA2) required for virulence factor secretion and bacterial viability and
- *hpt*, encoding a hexose phosphate transporter (Hpt) needed for replication in the cytosol.

L. monocytogenes is able to invade and multiply in macrophages as well as in non-phagocytic (e.g. epithelial) cells (Vázquez-Boland et al., 2001a). This process takes place as follows: the surface proteins InlA and InlB promote attachment and entry into the cell. Depending on the species and type of cell invaded, an interaction of InlA with cell surface receptors E-cadherin or InlB with the hepatocyte growth factor receptor tyrosine kinase c-Met takes place (Mengaud et al., 1996; Shen et al., 2000). InlA interacts with human and guinea pig E-cadherin, but not mouse and rat E-cadherin. InlB on the other hand interacts with human and mouse Met, but not guinea-pig Met (Khelef et al., 2006; Lecuit et al., 1999). Further proteins play a role in crossing the epithelial and placental barrier. Among them the *Listeria* adhesion protein Lap and the virulence factor InlP respectively (Drolia & Bhunia, 2019; Faralla et al., 2016). As a reaction to the interaction of bacterial surface proteins with the cell receptors, the bacterium is internalised into a vacuole (also called phagosome). Subsequently, lysis of the vacuole influenced by phospholipases C and pore-forming toxin LLO occurs and *L. monocytogenes* is released into the cytosol, where multiplication takes place (Pizarro-Cerdá et al., 2016; Phelps et al., 2018). Hpt permease is responsible for the uptake of hexose phosphates from the host cell (Chico-Calero et al., 2002). The surface protein ActA mediates polymerisation of actin, enabling the bacterium to move through

the cell and with the help of an internalin (InlC) to spread directly from cell-to-cell by protruding the membrane (Costa et al., 2020). *L. monocytogenes* enters neighbouring cells; a double-membrane vacuole is formed, subsequently lysed and the bacterium begins a new infection cycle (a visual description of the above delineated mechanism is available from: Hamon et al., 2006; Radoshevich & Cossart 2018).

While the key virulence factors located on LIPI-1 are present in all typical *L. monocytogenes* strains, LIPI-2 was found to be species-specific to *L. ivanovii* (Domínguez-Bernal et al., 2006). Recently, however, a truncated LIPI-2 was identified in hypervirulent *L. monocytogenes* strains, encoding sphingomyelinase (SmcL), a virulence factor needed for intracellular proliferation (Yin et al., 2019).
The LIPI-3, encoding listerioloysin S (LLS), a cytolytic peptide, was originally found to be exclusive to *L. monocytogenes* lineage I strains (Cotter et al., 2008). LLS is a bacteriocin and contributes to haemolytic properties of LIPI-3^{+} strains (Quereda et al., 2017). Some *L. innocua* strains harbour an intact LIPI-3, while in other *L. innocua* strains fragments of the island were detected. *L. innocua* strains with an intact LIPI-3 displayed a haemolytic phenotype when under constitutive expression (Clayton et al., 2014). Recently, Maury and co-workers identified a fourth *Listeria* pathogenicity island, LIPI-4, in clonal complex 4 (CC4) *L. monocytogenes* strains. The gene cluster expresses the cellobiose-family PTS, which confers CC4 strains with a fetal-placental as well as a central nervous system tropism (Maury et al., 2016). An intact LIPI-4 was also found to be present in typical and atypical *L. innocua* strains and it was suggested that the presence of this sugar transport system might be essential for carbon metabolism of the environmental saprophyte (Moura et al., 2019).

While the typical *L. innocua* strain is non-pathogenic, few reports of meningitis and septicaemia in humans and animals exist (Favaro et al., 2014; Perrin et al., 2003; Rocha et al., 2013; Walker et al., 1994).
Atypical *L. innocua* have been described, with haemolysis as the most prominent phenotypic feature of the studied strains (Table 2).

Table 2: Atypical *L. innocua* and their pheno- and genotypic characteristics

phenotype	genotype	source	reference
non-haemolytic	*gtcA*	unknown	Lan et al., 2000
haemolytic, rhamnose-	LIPI-1	seafood	Johnson et al., 2004
haemolytic	LIPI-1*, inlA*	FPE, seafood	Volokhov et al., 2007
haemolytic	*hly* , *inlAB*	FPE, pork, market	Moreno et al., 2012
haemolytic	nd	farm environment, poultry	Milillo et al., 2012
haemolytic	LIPI-3, *llsA*	cheese, human, sheep, silage	Clayton et al., 2014
haemolytic	*hly, plcA, inlC*	FPE, pork, market	Moreno et al., 2014
haemolytic, PI-PLC+, virulent	*inlA,* LIPI-1, LIPI-3, LIPI-4	bird feces	Moura et al., 2019
non-haemolytic	*inlA, hly, maz* EF	FPE, food	Rossi et al., 2020
survival of *Danio rerio* larvae between 4.6% and 80.9%	*sigB, rrn, luxS, actA, plcB, hlyA*	fish	Zakrzewski et al., 2020

Abbreviations: + positive; - negative; nd: not determined.

Some strains were also found to be virulent, although less than *L. monocytogenes* (Moura et al., 2019). Genetically, LIPI-1 (or parts of the island), LIPI-3, and/or LIPI-4 were found to be present in atypical *L. innocua* strains, as well as *inlA* in some of the studied strains (Table 2).

1.1.4. **Genetic and phylogenetic information**

According to den Bakker and colleagues, the *Listeria* core genome consists of about 2,000 genes and is highly conserved. In evolutionary terms, the *Listeria* genome is characterised by limited gene acquisition and gene loss (den Bakker et al., 2010a).

The size of *Listeria* species genomes is around 3 Mbp with ca. 3,000 protein coding genes (https://www.ncbi.nlm.nih.gov/datasets/genomes/?taxon=1637; accessed on: 18 February 2022). In comparison to other bacterial genomes, *Listeria* harbour a high number of surface proteins, including the major virulence genes, transport proteins, particularly for carbohydrate transport and regulatory proteins (Buchrieser et al., 2003). Variations in genome size mainly result from hypervariable genomic hotspots, where foreign chromosomal DNA, i.e. mobile genetic elements (MGE), is integrated in the form of bacteriophages, transposons, plasmids and genomic islands (Kuenne et al., 2013; Nelson et al., 2004). Genomes of *Listeria* spp. typically contain at least one prophage or partial bacteriophage genomes (Buchrieser & Glaser, 2011). In *L. monocytogenes* prophages and plasmids have been observed more frequently in serovar 1/2a strains than 4b strains (Hain et al., 2012). Plasmids, although comparably rare in *L. monocytogenes*, have been implicated in resistance to heavy metals, antibiotics and benzalkonium chloride (BC) (Elhanafi et al., 2010; Kuenne et al., 2010; Lebrun et al., 1992; Lemaître et al., 1998; Parsons et al., 2019b). Previous research has demonstrated that antibiotic resistance is significantly more prevalent in *L. innocua* than in *L. monocytogenes* strains, implying a potential reservoir function of *L. innocua* regarding antibiotic resistance gene transfer to *L. monocytogenes* (Bertsch et al., 2014; Walsh et al., 2001).

The genus *Listeria* can be divided into distinct clades by phylogenetic analysis, revealing the genetic relationship between *Listeria* species and providing an indication for *Listeria* evolution (Carlin et al., 2021; den Bakker et al., 2014; Leclercq et al., 2019b):

- a clade comprising *Listeria sensu stricto*: *L. monocytogenes, L. marthii, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. farberi, L. immobilis* and *L. cossartiae*
- a clade containing *L. grayi*
- a clade consisting of *L. booriae, L. riparia, L. newyorkensis, L. cornellensis, L. rocourtiae, L. grandensis, L. weihenstephanensis, L. portnoyi, L. rustica*
- a clade representing *L. fleischmannii, L: goaensis, L. thailandensis, L. aquatica, L. floridensis, L. costaricensis, L. valentina*

The classical serotyping scheme, developed by Seeliger and Höhne, is based on somatic (O) and flagellar (H) surface antigens and allows differentiation of *Listeria* strains according to variations in the structure of the antigens, which are conferred by flagellum proteins and the cell wall teichoic acids, respectively (Seeliger & Höhne, 1979). Combining O and H antigens yields at least 15 *Listeria* serovars. *L. monocytogenes* serovars are: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7, *L. seeligeri* associated serovars are: 1/2a, 1/2b, 3b, 4a, 4b, 4c 4d and 6b, *L. innocua, L. welshimeri* and *L. grayi* serovars include: 1/2b, 6a and 6b, and serovar 5 in *L. invanovii* (Seeliger & Höhne, 1979; D. Liu, 2006a; Seeliger & Jones, 1986). Recently, novel serovar 4h was detected in *L. monocytogenes* isolates with hypervirulent features (Yin et al., 2019; Feng et al., 2020).

The latter method requires extensive technical expertise, is time-consuming and expensive and includes the use of antisera, which are to a certain extent prone to variations in quality and cross-reactivity (Kérouanton et al., 2010). To overcome these disadvantages, several conventional and real-time multiplex polymerase chain reaction (PCR) assays were developed, which allow a less costly and rapid differentiation between *Listeria* species and the grouping of commonly isolated *L. monocytogenes* serovars prior to further subtyping (Bubert et al., 1999; Doumith et al., 2004a; D. Liu et al., 2007; Oravcova et al., 2006; Rossmanith et al., 2006; Ryu et al., 2013; Salcedo et al., 2003; Leclercq et al., 2011; Feng et al., 2020; F. Li et al., 2021). Applied multiplex PCRs include an assay targeting the invasion-associated gene (*iap*), encoding protein p60, which is shared among the species and enables discrimination of *L. monocytogenes*, *L. innocua*, *L. grayi*, and the three grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* (Bubert et al.,

1999). The PCR-essays developed by Doumith et al. and Leclercq et al., targeting virulence-associated genes, classifies *L. monocytogenes* into four serogroups i) 1/2a and 3a, ii) 1/2c and 3c, iii) 1/2b, 3b, and 7, iv) 4b, 4d, and 4e) and one serogroup variant referred to as “IVb-v1” (Doumith et al., 2004a; Leclercq et al., 2011).

Various subtyping methods were employed to identify four distinct genetic lineages in *L. monocytogenes* (lineages I-IV) (Nightingale et al., 2005; Orsi et al., 2011; Wiedmann et al., 1997). Each lineage groups specific serovars: lineage I includes serovars 1/2b, 3b, 4b, 4e, lineage II groups serovars 1/2a, 1/2c, 3a and 3c, lineage III and IV include serovars 4a, 4c and some 4b strains. Lineage IV was initially described as a lineage III subgroup, however, phylogenetic differences warranted a reclassification into a distinct fourth lineage (D. Liu et al., 2006b; Orsi et al., 2011; Roberts et al., 2006; Ward et al., 2008; Wiedmann et al., 1997).

The major *L. innocua* serovars according to somatic and flagellar antigen serotyping include 6a and 6b (D. Liu, 2006a). Chen et al. performed internalin gene profiling and MLST, which revealed four *L. innocua* subgroups (A-D). While the majority of the tested isolates grouped into the A and B subgroup, which both exhibited the same phylogenetic distance to *L. monocytogenes*, designated subgroup D strain contained *inlJ* and formed the subgroup genetically nearest to *L. monocytogenes* (J. Chen et al., 2010).

The need to analyse genetically similar isolates for outbreak detection and contamination source tracing using molecular subtyping methods led to the definition of epidemic clones (ECs), CCs, STs and core genome MLST (cgMLST) cluster types (CTs) (Bergholz et al., 2016; Y. Chen et al., 2007; Kathariou, 2002; Lomonaco et al., 2013). An EC is a clonal group of isolates, associated with distinct, geographically and temporally unrelated outbreaks (Cheng et al., 2008). The first ECs were defined based on typing methods like ribotyping and multilocus enzyme electrophoresis (MEE). Later, multi-virulence-locus sequence typing (MVLST) and WGS have been used to detect ECs (Bergholz et al., 2016; Y. Chen et al., 2007; Kathariou, 2002; Lomonaco et al., 2013). CCs are defined according to a MLST scheme targeting seven genes, as groups of closely related STs, a

distinct combination of MLST allele designations used in MLST, i.e. differing by only one allele from other STs in the group (Chenal-Francisque et al., 2011; Ragon et al., 2008). Cantinelli et al. demonstrated that ECs (determined by MVLST) correspond well to CCs (determined by MLST) while conversely, the EC system does not fully reflect the heterogeneity of the CC terminology. Another advantage of the MLST-based typing scheme is the public, online availability of the platform (Cantinelli et al., 2013). A CT was initially proposed as a cgMLST profile that has ≤10 allelic mismatches from a pair of neighbouring *L. monocytogenes* isolates (Ruppitsch et al., 2015).

Whole-genome-approaches as well as analysis of variations in specific genes, such as *Listeria* housekeeping genes, internalins or the virulence gene cluster have been used to identify distinct molecular attributes responsible for niche-adaptation and *L. monocytogenes* lineage, serovar- and strain-specific adaptation to FPEs, host adaptation and virulence properties (Disson et al., 2021; Petit & Lebreton, 2022).

While *Listeria* species are ubiquitously found in nature, species-specificities in colonisation of different habitats have been documented. In samples collected across New York State, USA, *L. innocua* and *L. monocytogenes* were significantly more prevalent in urban environmental specimens, while *L. welshimeri* and *L. seeligeri* were associated with natural environments (Sauders et al., 2012). *L. seeligeri* has been the most frequently isolated species in natural environments, while *L. ivanovii* has been found to have a low prevalence in the environment and is most commonly isolated from farm environments (Linke et al., 2014a; Sauders et al., 2012; Stea et al., 2015a). *Listeria* spp. have been detected in various foods and food processing or preparation environments with differences in the overall *Listeria* spp. prevalence among facilities and foods sampled as well as variation in the prevalence of the isolated species (Kornacki & Gurtler, 2007). *L. innocua,* however, has often been the most frequently isolated species, frequently co-existing with *L. monocytogenes* (Bouayad et al., 2015; Chambel et al., 2007; John et al., 2020a; Rudolf & Scherer, 2001; Sergelidis et al., 1997; Vilar et al., 2007).

The majority of human listeriosis outbreaks are caused by *L. monocytogenes* serovars 1/2a, 1/2b and 4b (belonging to lineages I and II), with varying

prevalence of either serovar according to geographical location (Lopez-Valladares et al., 2018; Orsi et al., 2011). The latter serovar, especially serovar 1/2a, are also frequently detected in foods and FPEs (Lomonaco et al., 2015; Cheng et al., 2008).

Lineage I, which is most frequently implicated in human listeriosis outbreaks, seems to be the most clonal one, whereas a substantial horizontal gene transfer (HGT) and recombination has been observed in the other lineages, especially in lineage II strains, which are commonly isolated from FPEs, foods, natural habitats and implicated in sporadic human listeriosis cases (Meinersmann et al., 2004; Orsi et al., 2011). Lineage III and IV strains are rare in comparison to lineage I and II, seldom associated with listeriosis outbreaks and have been isolated mainly from animals (den Bakker et al., 2008; D. Liu et al., 2006b; Orsi et al., 2011). While the nucleotide diversity of *L. innocua* is comparable to *L. monocytogenes* lineage I, it is lower than lineage II-IV (J. Chen et al., 2010).

For distinct ECs, CCs, STs and CTs niche-specificity was observed and the terms hyper- and hypovirulent *L. monocytogenes* CCs were coined (Maury et al., 2016). While hypervirulent clones (CC1, CC2, CC4, CC6) are linked to human infections, potential hypovirulent clones (CC8, CC9 and CC121) are food-related (S. Lee et al., 2018; Maury et al., 2019, 2016; Muchaamba et al., 2021). When comparing hyper- and hypovirulent CCs, Maury et al. concluded that hypovirulent, food-associated clones display better biofilm formation ability and genes involved in stress and BC tolerance are more prevalent. CC1 is strongly associated with infection in ruminants and with contamination of dairy products. It has been demonstrated that CC121 strains harbour a truncated *inlA* and persist in FPEs (Maury et al., 2016).

Different models for the evolution of the genus *Listeria* and specifically *L. monocytogenes* have been proposed (J. Chen et al., 2013; den Bakker et al., 2008; Orsi et al., 2011). One model proposes a common *Listeria* ancestor with a full set of virulence factors and virulence genes (LIPI-1, *inlABC*), which were lost over time, resulting in distinct *L. monocytogenes* lineages and avirulent species like *L. innocua* (den Bakker et al., 2010a; den Bakker et al., 2010b; Lomonaco et

al., 2015; Orsi et al., 2007; Y. H. L. Tsai et al., 2011). The time to the most recent common ancestor was calculated to be 30 to 67 million years (den Bakker et al., 2010b; Meinersmann et al., 2004).

Another model suggests stepwise evolution, where a common ancestor formed *L. monocytogenes* lineages I and II. In subsequent events, some strains of the latter lineages evolved into lineages III and IV through gene deletion, with some lineage III strains serving as intermediates on the way to *L. innocua* evolution through additional gene deletion. These evolutionary intermediates harbour several genes common to *L. monocytogenes*, e.g. LIPI-1, *inlAB, bsh, hpt*, but also share gene deletions common to *L. innocua*, e.g. *inlC, inlI, inlJ, ascB-dapE* cluster, ADI system (J. Chen et al., 2013; J. Chen et al., 2009a; Doumith et al., 2004b; H. Zhao et al., 2011).

Eugster and co-workers suggested that *L. monocytogene*s serovar 3 and 7 strains evolved from serovar 1/2 from a common ancestor, as a result of point mutations in glycosylation genes and exposure to phages (Eugster et al., 2015).

Bacterial evolution is driven by recombination events, positive selection, adaptive loss, point mutations and HGT. The recombination rate of *L. monocytogenes* is low compared to other bacterial species (Ragon et al., 2008). Den Bakker et al. found recombination in almost 50% of the core genome of *L. monocytogenes* and *L. innocua* and a higher genetic diversity, recombination and HGT in *L. monocytogenes* lineage II, compared to the other lineages (den Bakker et al., 2008). This might explain why lineage II strains are associated with adaptation to a wide range of environmental conditions, prevailing in nature and especially in the FPE (Cheng et al., 2008). This can equally be hypothesised for *L. innocua*, where an adaptive loss of virulence genes might have rendered the species fit for survival in environmental niches (J. Chen et al., 2009a).

The evolution of *inlA* is characterised by recombination in lineage II strains and positive selection resulting in heterogeneity of *inlA* across *Listeria* spp. (Orsi et al., 2007). Point mutations in *inlA* with consequent premature stop codons and truncation in secreted proteins result in reduced virulence. The complete loss of *inlA* was also observed in some lineage II strains and resulted in the inability to spread from cell to cell (Nightingale et al., 2008). Den Bakker et al. proposed that a complete *prfA* gene cluster was present in the common *Listeria* ancestor and

loss of the cluster occurred several times during *Listeria* evolution. Furthermore, they suggested, that this loss of virulence genes might confer superior environmental adaptation and survival, which could explain the high prevalence of *L. innocua* in non-host environments. Atypical *L. innocua* with an intact *prfA* cluster and *inlA* have been described previously and might constitute evolutionary intermediates (den Bakker et al., 2010b).

1.2. *Listeria* spp. relevance in dairy production

Milk and dairy products are characterised by a high nutrient-density, supplying energy and containing high-quality protein (Muehlhoff et al., 2013). In human nutrition, milk can contribute to many of the required micronutrients, especially for calcium, riboflavin, vitamin B12, phosphorus, zinc, magnesium, potassium and vitamin A, in an easily absorbable form (Park & Haenlein, 2013). Especially fermented dairy foods like cheese contain bioactive peptides, which have been demonstrated to have antioxidant, anti-hypertensive, anti-microbial and immuno-modulatory properties (López-Expósito et al., 2012; Tagliazucchi et al., 2019).

Milk contributes to 9% of the dietary energy supply in Europe and Oceania, to 19% of the dietary protein supply in Europe and to 12 to 14% of the dietary fat supply in Europe, Oceania and the Americas, compared to 3 to 4% energy supply, to 6 to 8% protein supply and to 7% fat supply in Africa and Asia (http://www.fao.org/dairy-production-products/products/en/; accessed on: 21 February 2022). Dairy products are consumed by an estimated six billion people worldwide, primarily in developing countries, where a twofold increase in per capita milk consumption was recorded since the 1960s (http://www.fao.org/dairy-production-products/products/en/; accessed on: 21 February 2022). A growth of 1.7% annually to 980 million tons by 2028 is projected for worldwide milk production, with India and Pakistan anticipated accounting for over half of this growth (Organisation for Economic Cooperation and Development [OECD] and Food and Agriculture Organization [FAO], 2019). In international comparison, milk consumption is the highest with over 150 kg/capita/year in Europe, as well as in Argentina, Armenia, Australia, Costa Rica, Israel, Kyrgyzstan, North America and Pakistan (http://www.fao.org/dairy-production-products/products/en/; accessed on: 21 February 2022). Cheese is consumed mainly in Europe, North America and

Oceania, with a foreseen increase in per capita consumption (at present around 20 kg/capita/year for the latter regions) according to OECD's and FAO's projections. With the EU as the world's main cheese exporter, the share of the EU's global cheese production was projected to reach almost 50% in 2028 (OECD-FAO, 2019).

Cheese is the dairy product most frequently contaminated with *L. monocytogenes* (Martinez-Rios & Dalgaard, 2018). Due to the intrinsic characteristics and the method of manufacture, smeared and brined soft and semi-soft cheeses are especially prone to contamination, supporting the survival and growth of the pathogen (Martinez-Rios & Dalgaard, 2018; Mazaheri et al., 2021).

EFSA and ECDC reported the detection of *L. monocytogenes* in 0.54% of cheese samples tested (n=11,934; all milk origin and all types of cheeses) in 2020 (EFSA and ECDC, 2021). However, Martinez-Rios and Dalgaard conducted a meta-analysis using literature from 2005-2015 and reported a mean prevalence of *L. monocytogenes* in cheeses of 2.3%, over three times higher than the EFSA/ECDC results in the corresponding period. *L. monocytogenes* prevalence varied according to cheese type, with 0.8% in fresh cheeses, 2.0% in ripened cheeses, 2.4% in veined cheeses, 5.1% in smeared cheeses, 11.8% in brined cheeses. No significant differences were observed in mean *L. monocytogenes* prevalence in pasteurised or un-pasteurised soft and semi-soft cheeses (Martinez-Rios & Dalgaard, 2018).

Despite these low levels of RTE product contamination, dairy products, especially cheeses, have been implicated repeatedly in listeriosis outbreaks and caused large-scale product recalls globally during the last years (Desai et al., 2019; Feng et al., 2013; Fretz et al., 2010; Heiman et al., 2016; K. A. Jackson et al., 2018; Lundén et al., 2004; Magalhães et al., 2015; Makino et al., 2005; McIntyre et al., 2015; Qiu et al., 2021).

Listeriosis is one of the most serious foodborne diseases and characterised by a high hospitalisation rate and a high CFR in susceptible people (Charlier et al., 2017). The proportion of elderly citizens, a particularly susceptible group, is going to increase over the next few years in the EU and in many other developed regions of the world (https://ec.europa.eu/eurostat/cache/digpub/ageing/; accessed on: 26 February 2022). The economic consequences caused by

listeriosis outbreaks for both public health and the food industry are serious (Thomas et al., 2015).

Listeria can enter the dairy production chain at any point from farm to the finished product (Kallipolitis et al., 2020). The sources of dairy product contamination with *L. monocytogenes* are raw milk, unhygienic processing practices along the dairy chain and for the most part post-processing cross- or recontamination from the production or food-handling environment (Melo et al., 2015). Conditions in dairy processing facilities (e.g. low temperature in many processing steps, high relative humidity, protein residues on hard-to-clean equipment) combined with the ability of *Listeria* spp. to adapt to a wide range of environmental stresses such as low pH, high salt concentration or low temperature, may further contribute to *Listeria* spp. growth or survival (Anast et al., 2020; Magalhães et al., 2016).
Persistence is recognised as a major contributing factor to post-processing contamination of dairy foods (Fox et al., 2018). A variety of intrinsic, geno- and phenotypic features of *L. monocytogenes* contribute to FPE adaptation and persistence of the pathogen, as well as external factors prevailing in the FPE (Carpentier & Cerf, 2011; V. Ferreira et al., 2014). One of the likely contributors to continuous *L. monocytogenes* presence in FPEs is exposure to subinhibitory concentrations of disinfectants, especially quaternary ammonium compounds (QACs) (Martínez-Suárez et al., 2016).

Thorough knowledge of *Listeria* spp. contamination pathways, mechanisms behind adaptation ability and interaction with the environment or the host is essential to develop efficient strategies to control dairy processing environmental contamination and to ensure consumer safety.

1.2.1. **Listeriosis**

According to the EU case definition, laid down in the Commission Implementing Decision (EU) 2018/945, a confirmed case of listeriosis is any person meeting the laboratory criteria of *L. monocytogenes* isolation or detection of *L. monocytogenes* nucleic acid from a normally sterile site (EC, 2018). Listeriosis can manifest itself as self-limiting febrile gastroenteritis in healthy individuals (Allerberger &

Wagner,2010). Typically, the incubation period for gastrointestinal forms ranges from six hours to ten days and symptoms may last for up to one week (Goulet et al., 2013). In the severe, invasive form of listeriosis, *L. monocytogenes* is able to pass the blood-brain and the blood-placenta barrier, which can result in septicaemia, meningoencephalitis, stillbirth or infection in neonates. The severe form affects immunocompromised individuals, elderly, pregnant women or new-borns (Allerberger & Wagner, 2010). The incubation period for invasive listeriosis depends on the clinical manifestation and varies between 14 days up to six weeks (Goulet et al., 2013). The long incubation period often complicates outbreak investigations due to incomplete food consumption histories (Yde et al., 2012). Asymptomatic faecal carriage of *Listeria* has been documented and may occur in 1-15% population wide, with a higher prevalence among food plant workers (Grif et al., 2003; El-Shenawy, 1998; Hafner et al., 2021).

Listeriosis is a rare disease, with a notification rate of 0.42/100,000 population in the EU in 2020. The CFR in the EU in 2020 was 13%, while globally ranges between 20% and 30% have been reported (EFSA and ECDC, 2021; Desai et al, 2019; W. Li et al., 2018). About 73% of listeriosis cases and ca. 80% of fatal cases reported in the EU in 2020 affected the age group over 64 years (EFSA and ECDC, 2021).

Transmission occurs almost exclusively through the consumption of contaminated food (Allerberger & Wagner, 2010). *L. monocytogenes* contamination of a food processing plant can occur for extended periods and finished products are often distributed within a wide geographical area (Lüth et al., 2019). These facts together with the long incubation period of listeriosis, exacerbate the identification of an outbreak source, despite continuous improvements in subtyping techniques and databases for comparing strains from clinical, animal and food sources (Lüth et al., 2018; Pietzka et al., 2019). Transmissions from animals to humans and between humans rarely occur and have been described in the context of occupational exposure to infected animals, e.g. in veterinarians or farmers (McLauchlin & Low, 1994; Allerberger & Wagner, 2010).

The infective dose for oral transmission is unknown and depends on host susceptibility, the bacterial strain and the food matrix. In immunocompromised persons however, already low levels of contamination (10^2-10^4 cfu/g) may lead to

infection (McLauchlin et al., 2004; Swaminathan & Gerner-Smidt, 2007; Vázquez-Boland et al., 2001b).

A recent study describes an increase of international events during the period from 1996-2018 due to products contaminated with *L. monocytogenes,* reflecting a trend towards more complex global distribution networks (Desai et al., 2019). Recent multicountry listeriosis outbreaks in the EU were linked to RTE meat products, fish products and frozen vegetables (https://www.ecdc.europa.eu/en/listeriosis/threats-and-outbreaks; accessed on: 27 February 2022). In the USA, previous major multistate listeriosis outbreaks were linked to salads, cooked chicken, queso fresco, deli meats, mushrooms and hard-boiled eggs (https://www.cdc.gov/*Listeria*/outbreaks/index.html; accessed on: 27 February 2022). Dairy products, especially cheeses, are among the high-risk RTE foods, causing sporadic and epidemic listeriosis outbreaks (Table 3).

Table 3: Major listeriosis outbreaks linked to dairy products

country	year(s)	implicated product	serotype	cases (n=)	CFR (%)	reference
Germany	1949-1957	raw milk	nr	ca 100	nr	Potel, 1953
Switzerland	1983-1987	soft cheese	4b	57	32	Büla et al., 1995
USA	1985	mexican-style soft cheeses	4b	142	34	Linnan et al., 1988
Austria	1986	raw milk	1/2a	28	18	Allerberger & Guggenbichler, 1989
Denmark	1989-1990	blue cheese	4b	26	23	Jensen et al., 1994
France	1995	raw milk cheese	4b	37	30	Goulet et al., 1995
France	1997	soft cheese	4b	14	nr	Lundén et al., 2004
Finland	1998-1999	butter	3a	25	24	Lyytikäinen et al., 2000
Sweden	2001	soft cheese	1/2a	33	nr	Carrique-Mas et al., 2003
Japan	2001	cheese	1/2b	86	nr	Makino et al., 2005
Czech Republic	2006	cheese	1/2b	78	17	Denny & McLauchlin, 2008
Germany	2006-2007	acid curd cheese	1/2a, 1/2b, 4b	189	14	Koch et al., 2010
Canada	2008	pasteurized cheese	1/2a	38	5	Gaulin et al., 2012
Austria, Germany, Czech Republic	2009-2010	acid curd cheese	1/2a	34	24	Fretz et al., 2010
Portugal	2009-2012	cheese	4b	30	37	Magalhães et al., 2015
Belgium	2011	hard cheese	1/2a	12	33	Yde et al., 2012
USA	2012	ricotta salata cheese	1/2a	22	18	Heiman et al., 2016; CDC, 2012
Spain	2012	fresh cheese	1/2a	2	0	de Castro et al., 2012
USA	2013	farmstead cheeses	nr	6	17	CDC, 2013
USA	2013-2014	hispanic-style cheese	1/2b	8	13	Y. Chen et al., 2017a; CDC, 2014
USA	2015	ice cream	nr	10	30	CDC, 2015a
USA	2015	soft cheese	nr	30	10	CDC, 2015b
USA	2017	raw milk soft cheese	nr	8	25	CDC, 2017
USA	2019	deli sliced meats and cheeses	nr	10	10	CDC, 2019
Switzerland	2018/2020	brie	4b	34	29	Nüesch-Inderbinen et al., 2021
USA	2021	queso fresco	nr	13	8	CDC, 2021

Abbreviations: nr: not reported; CDC, 2012 https://www.cdc.gov/*Listeria*/outbreaks/cheese-09-12/index.html; CDC, 2013 https://www.cdc.gov/*Listeria*/outbreaks/cheese-07-13/index.html; CDC, 2014 https://www.cdc.gov/*Listeria*/outbreaks/cheese-02-14/index.html; CDC, 2015a https://www.cdc.gov/*Listeria*/outbreaks/ice-cream-03-15/index.html; CDC 2015b https://www.cdc.gov/*Listeria*/outbreaks/soft-heeses-09-15/index.html; CDC 2017 https://www.cdc.gov/*Listeria*/outbreaks/soft-cheese-03-17/index.html; CDC 2019 https://www.cdc.gov/*Listeria*/outbreaks/deliproducts-04-19/index.html; CDC 2021 https://www.cdc.gov/*Listeria*/outbreaks/hispanic-soft-cheese-02-21/index.html; accessed on: 27 February 2022.

The economic consequences of listeriosis are serious, both on an individual and on a population level, for the health system as well as for the food industry. In recent years, different studies have estimated the costs associated with listeriosis outbreaks. Hussein and Dawson mentioned financial losses of $7 billion US dollars (about €6 billion Euros) per year for the US economy caused by food safety incidents including consumer notifications, product recalls and lawsuit-associated costs (Hussain & Dawson, 2013). Costs of different food-borne pathogens for the health care sector were estimated as cost-of-illness and disease burden represented by Disability-Adjusted Life Years (DALY) in the Netherlands in 2011. Perinatal listeriosis infections were associated with the highest DALY per case and also with the highest cost-of-illness of €275,000 Euros per case (in comparison the cost-of-illness per case of *Clostridium perfringens* toxi-infection was €150 Euros) (Mangen et al., 2015). A similar estimation for annual cost-of-illness and Quality-Adjusted Life Year (QALY) was employed for the USA. Among 14 pathogens considered, about 90% of the costs and QALY loss were attributed to five pathogens, among them *L. monocytogenes* with $2.6 billion US dollars (ca. €2.2 billion Euros) and 9,000 QALY loss (Hoffmann et al., 2012).

Thomas et al. estimated the costs associated with a listeriosis outbreak in Canada in 2008 caused by delicatessen meat. In total 57 cases and 24 deaths could be linked to this outbreak. Case costs, which included loss of life, medical costs, non-medical costs, and productivity losses, were estimated at $2.8 million Canadian dollars (€1.8 million Euros). Total costs comprising case costs, costs for the implicated food plant and outbreak response costs were calculated to be almost $242 million Canadian dollars (€143 million Euros) (Thomas et al., 2015). From 2017 to 2018 a large listeriosis outbreak, caused by deli meat, occured in South Africa. Over 1,000 cases and over 200 deaths were reportedly linked to the outbreak (Smith et al., 2019). Costs attributable to productivity loss and ceasing of food production by the affected company amounted to $15 million US dollars (€13 million Euros). Hospitalisation costs were calculated to be $10.4 million US dollars (€9 million Euros) and total economic loss due to the listeriosis outbreak were estimated at $260 US dollars (€220 million Euros) (Olanya et al., 2019).

1.2.2. **Regulation**

While some countries, such as the USA, have a zero tolerance policy for *L. monocytogenes*, the incidence of listeriosis cases is not necessarily higher in countries that tolerate minimal levels of the pathogen in foods that cannot support *L. monocytogenes* growth, such as the EU, Canada, Australia, and New Zealand (EC, 2005; Health Canada, 2011; Food Standards Australia New Zealand [FSANZ], 2014;
US Department of Agriculture [USDA] - Food Safety and Inspection Service [FSIS], 2014). Since complete elimination of *L. monocytogenes* in foods is deemed impossible, minimising the risk to consumers is the primary aim of such regulatory policies. In the EU, Regulation (EC) No. 2073/2005 lays down microbiological criteria for *L. monocytogenes* in RTE foods.

Table 4: Microbiological criteria for *L. monocytogenes* in RTE foods according to Regulation (EC) No. 2073/2005

sampling stage	**RTE-food category**		
	RTE foods for infants and special medical purposes	**RTE foods able to support *L. monocytogenes* growth**	**RTE foods unable to support *L. monocytogenes* growth***
processing	na	ISO 11290-1: *L. monocytogenes* not detected in 25 g (n=5)**	na
retail[+]	ISO 11290-1: *L. monocytogenes* not detected in 25 g (n=10)	ISO 11290-2: <100 cfu/g (n=5)***	ISO 11290-2: <100 cfu/g (n=5)

Abbreviations: na: not applicable; * Products with pH ≤ 4.4 or a_w ≤ 0.92, products with pH ≤ 5.0 and a_w ≤ 0.94, products with a shelf-life of less than five days are automatically classified into this category. Other products can also belong to this category, when scientifically justified. ** This criterion is applicable, when the FBO is not able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life. *** This criterion is applicable, when the FBO is able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life. [+] until the end of shelf-life.

In the context of this regulation, RTE foods are defined as “Food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to cut out or reduce to acceptable level microorganisms of concern”. It is the obligation of the food business operator (FBO) to organise food batch controls and ensure compliance to the regulation. According to the regulation, *L. monocytogenes* must be absent from foods intended for infants and for special medical purposes. For all other RTE foods, different criteria apply, according to the intrinsic food properties (a_w, pH, NaCl

concentration) and stricter criteria apply for RTE food products able to support the growth of *L. monocytogenes* (EC, 2005). Requirements for the testing method, batch based number of samples and the legal limits of *L. monocytogenes* per RTE food category according to Regulation (EC) No. 2073/2005 are summarised in Table 4.

Challenge tests to estimate the growth or non-growth potential of *L. monocytogenes* in certain RTE products, storage tests during shelf- life, and mathematical modelling are recommended (EC, 2005; ISO, 2019). Several studies have been published describing challenge testing to assess the growth potential of *L. monocytogenes* in different RTE foods. The methodologies used vary widely among the studies and make it difficult for FBOs and ultimately for the competent authorities to use these studies for categorising a specific product into the *L. monocytogenes* growth or no-growth category according to Regulation (EC) No. 2073/2005 (Álvarez-Ordóñez et al., 2015; Gérard et al., 2018). To overcome this issue, the EU reference laboratory for *L. monocytogenes* (EURL *Lm*) developed a guideline for laboratories carrying out challenge tests on behalf of FBOs. The guideline describes two types of tests, which should be applied according to the desired needs: i) a challenge test to determine the growth potential and ii) a challenge test to determine the maximum growth rate. A key principle when designing a challenge test is that the most favourable growth conditions for *L. monocytogenes*, adding parameter ranges, should be chosen (French Agency for Food, Environmental and Occupational Health & Safety [ANSES] & EURL *Lm*, 2021).

The difference between the $\log_{10}$ cfu/g at the end of the shelf-life and the $\log_{10}$ cfu/g at the beginning of the challenge test gives an indication of the growth potential of *L. monocytogenes* in the RTE food under consideration. A difference of more than 0.5 $\log_{10}$ cfu/g classifies the product into the category of RTE foods able to support the growth of *L. monocytogenes*. A difference of less than 0.5 $\log_{10}$ cfu/g classifies the product into the category of RTE foods not able to support the growth of *L. monocytogenes* (ANSES & EURL *Lm*, 2021).

Sampling the FPE for *L. monocytogenes* is required, without detailing the frequency or number of samples, according to EU law (EC, 2005). Guidelines on sampling the food processing area and equipment for the detection of

L. monocytogenes are available from ANSES/EURL *Lm* (ANSES & EURL *Lm*, 2012).

In Austria, food producers, public and private laboratories detecting *L. monocytogenes* from human, food – as well as food-associated environmental-samples are required to submit the isolates to the Austrian National Reference Laboratory for typing according to § 38 Abs 1 Z 6 Lebensmittelsicherheits- und Verbraucherschutzgesetz (LMSVG) and § 74 LMSVG.

1.2.3. *Listeria* spp. contamination and persistence in the dairy processing environment

Several studies have suggested that *L. innocua* and *L. monocytogenes* are the *Listeria* species most abundant in FPEs and foods, including dairy processing and milk products with *L. innocua* being more frequently detected than *L. monocytogenes* in many cases. Tirloni et al. reported an overall recovery of *L. innocua* in 77.6% and *L. monocytogenes* in 1.6% of samples from four different dairy plants producing PDO Taleggio (Tirloni et al., 2020). J. Chen et al. studied Chinese food products and samples were positive at a rate of 28.9% for *L. innocua* and 25.3% for *L. monocytogenes* (J. Chen et al., 2009b). *L. innocua* (8.2%) and *L. monocytogenes* (1.2%) were isolated from frozen vegetables and the respective processing environment (Aguado et al., 2004). Samples from a small scale mushroom processing facility revealed the prevalence of *L. innocua* and *L. monocytogenes* in 10.3% and 1.6% respectively (Viswanath et al., 2013). In a range of RTE meat products in Jordan, 23% of the samples were positive for *L. innocua* and 17.1% positive for *L. monocytogenes* (Awaisheh, 2010). Regarding these observations, it has to be considered that *L. innocua* has been found to outcompete *L. monocytogenes* during enrichment (Cornu et al., 2002; Zitz et al., 2011). Also, it has been suggested that *L. innocua* can negatively influence the attachment and growth of *L. monocytogenes* (Koo et al., 2014).

The prevalence of *L. monocytogenes* and other *Listeria* spp. in dairy processing environments varies widely, due to multifactorial influences (see Table 5). The hygienic practices applied, the size of the plant, the complexity of the processing

line, the type of food processed, the equipment and technology used, the time of processing, sampling sites, time of sampling and the methods used for detection are some of the factors influencing the observed *Listeria* spp. prevalence in the FPE (Brito et al., 2008; Leong et al., 2014).

A dairy processing plant may be considered free of *Listeria* spp. at the time of sampling. However, a prevalence of *L. monocytogenes* of over 30% has been documented previously (Kells & Gilmour, 2004). An overview of *L. monocytogenes* prevalence in dairy processing environments reported in recent literature is given in Table 5.

Table 5: *L. monocytogenes* prevalence and persistence in dairy processing environments

FPE type; total (n=)	milk	products manufactured	study period (years)	prevalence of *L. monocytogenes* (%)	prevalence of *Listeria* spp. (%)	persistence	reference
farmhouse (16)	cow, ewe, goat	soft, semi-hard, hard cheese, blue mould, smear-ripened, fresh cheese	2	0-20.9	nd	yes	Fox et al., 2011a
producer unspec (34)	cow, ewe, goat	fresh cheese, ricotta, pasta filata, ripened cheese	3	6.4*	*Listeria* spp.:11.0*; *L. innocua* : 7.1*	yes	Parisi et al., 2013
producer unspec (2)	ewe	Pecorino Romano PDO, ricotta salata	1	13.1-23.2	nd	yes	Ibba et al., 2013
artisanal; small-scale industrial; industrial (3)	cow, ewe, goat	raw ewe's milk cheese, pasteurised cow, ewe and goat milk cheese varieties, semi hard pasteurised cow milk cheese	4	0-15.4	nd	yes	Almeida et al., 2013
farmhouse; industrial (19)	unspec	acid curd, ripened soft, blue-veined, semihard/hard cheese	1	13.5*	nd	yes	Stessl et al., 2014
small-scale (3)	cow	minas frescal cheese, Prato cheese/pasteurised cow's milk cheese	1	0-12.5	*L. innocua:* 0-24.0; *L. seeligeri:* 0-2.9; *L. welshimeri:* 0-0.7	yes	Barancelli et al., 2014
small-scale; medium-scale (3)	ewe	Bryndza/raw ewe's milk cheese	3	3.1*	*Listeria* spp: 48.4*	yes	Véghová et al., 2015
industrial (13)	ewe	Pecorino Romano PDO, Ricotta salata cheese	2	0-25.9	*Listeria* spp: 14.8-48.5;	nd	Spanu et al., 2015
farmhouse; industrial (9)	cow, ewe, goat	fresh cheese, ripened soft/semi-soft-, brined cured-, and hard cheeses, raw and pasteurised milk cheeses	1	0.5-26.0	nd	nd	Muhterem-Uyar et al., 2015

Abbreviations: * overall prevalence; nd: not determined; unspec: unspecified.

Table 5 continued: *L. monocytogenes* prevalence and persistence in dairy processing environments

FPE type; total (n=)	milk	products manufactured	study period (years)	prevalence of *L. monocytogenes* (%)	prevalence of *Listeria* spp. (%)	persistence	reference
artisanal; small-scale; medium-scale (5)	cow, buffalo	pasteurised and raw milk cheeses	1	0-1.3	nd	yes	Oxaran et al., 2017
small-scale artisanal (16)	unspec	cheeses div	3	4.2*	nd	yes	Leong et al., 2017
small-scale (5)	unspec	ice cream, yoghurt, cheese from pasteurised milk	1.5	0*	nd	nd	Madden et al., 2018
producer unspec (1)	unspec	grated cheese from pasteurised milk	1	8.4*	nd	yes	Melero et al., 2019b
producer unspec; ripening plant (4)	cow	PDO Taleggio,other PDO cheeses, brined, ripened, blue-cheese, ricotta, whey butter	1	0-2.4	*Listeria* spp.: 0-73.1	nd	Tirloni et al., 2020
artisanal; small-scale; medium scale (5)	cow	Chanco/semi-hard cheese	1	0-22.5	nd	yes	Barría et al., 2020

Abbreviations:* overall prevalence; nd: not determined; unspec: unspecified; div: diverse.

Dairy farms constitute a natural environment for saprophytic *Listeria* spp. (Fox et al., 2018). *Listeria* spp. can be transferred from the farm environment, silage and faecal excretions to milking equipment and raw milk (D'Amico, 2014).

Data on the occurrence of *L. monocytogenes* in raw milk varies widely between studies, with reported ranges from <1% up to 16% (D'Amico, 2014; Melo et al., 2015). Raw milk is thus a potential source for the introduction of *Listeria* spp. into the dairy processing environment and into the finished product. This poses the risk of transferring the pathogen to consumers, especially if no or inadequate pasteurisation is performed (Verraes et al., 2015).

While a thermal inactivation step such as cooking or pasteurisation is effective in destroying *Listeria* spp., post-processing contamination is a common phenomenon (Jordan et al., 2018). The sources for several listeriosis outbreaks have been traced back to cross-contamination events within the FPE (Mazaheri et al., 2021). In dairy processing and cheese production, *L. monocytogenes* can be disseminated through various manufacturing steps. Specifically cheese brining, smearing and cutting, slicing or grating of cheeses have been named as high-risk operations regarding *L. monocytogenes* transfer to the finished product (Melo et al., 2015). During the manufacturing process, niches may be present, which favour biofilm formation and are difficult to access through hygiene and disinfection measures. Existing food residues provide a food source for bacteria and have the potential to further facilitate *Listeria* spp. contamination (Tompkin, 2002). Biofilms can be defined as microbial communities, which adhere to biotic or abiotic surfaces with cell- membrane bound structures like polysaccharides and proteins (da Silva & De Martinis, 2013; Pan et al., 2006). Equipment parts, which have been described to provide niches for *Listeria* spp. and can contribute to post-processing contamination events, include fillers, slicers, valves, gaskets and conveyor belts (Kornacki & Gurtler, 2007).

In addition to growth niches, transfer sites allow the carryover of bacteria from one location to another (Simmons & Wiedmann, 2018). Areas especially prone to *Listeria* spp. contamination and promotion of re-contamination are wet, humid and often cold production areas, such as drains, stagnant water on floors and food contact surfaces, condensate from pipes and air conditioning (Berrang & Frank, 2012; Redfern & Verran, 2017; Tompkin, 2002; T. Zhao et al., 2006). Deficiencies

in the design, maintenance, and repair of facilities and equipment, as well as disregard of good manufacturing practices (GMP), are major contributors to microbial and therewith *L. monocytogenes* and *Listeria* spp. contamination of FPEs (Aalto-Araneda et al., 2019; Angelo et al., 2017; Cramer, 2013; Holah, 2014a).

Once introduced into the dairy processing environment, *Listeria* are able to survive for long periods of time and tolerate various stresses, e.g. acid, alkaline, temperature or salt stress, sanitisers and they are able to form biofilms. The latter factors are contributing to *L. monocytogenes* and *Listeria* spp. persistence (Bucur et al., 2018). Persistence of *L. monocytogenes* over months or years has been reported in dairy processing facilities (Almeida et al., 2013; Latorre et al., 2011; Lomonaco et al., 2009; further studies mentioned in Table 5), meat processing facilities (Pasquali et al., 2018; Simmons et al., 2014), in poultry processing facilities (Fox et al., 2015; Melero et al., 2019a), in fish processing facilities (B. Y. Chen et al., 2016; Malley et al., 2013) and in various other RTE food processing facilities (Keto-Timonen et al., 2007; Leong et al., 2014; Murugesan et al., 2015).
A basic definition of *L. monocytogenes* persistence in FPEs was given by Ferreira et al. as “repeated isolation of *L. monocytogenes* strains at different time points that are subsequently identified as identical subtypes” (V. Ferreira et al., 2014). A uniform definition of *L. monocytogenes* persistence is nevertheless missing to date (Belias et al., 2021). In their recent review, Unrath et al. compiled an overview of criteria used in literature to define persistence of *L. monocytogenes,* showing the inconsistent use of the term “persistence” in different studies (Unrath et al., 2021).
There is currently a lack of knowledge about persistence of *Listeria* species other than *L. monocytogenes* in FPEs, with only a limited number of studies published to date (Belias et al., 2021). Alvarez-Ordonez et al. investigated the occurrence, persistence and virulence potential of *L. ivanovii* in various foods and FPEs. The authors were able to detect one persistent pulsotype for a period of ten months in the environment of a dairy food business out of 48 FBOs sampled (Alvarez-Ordóñez et al., 2015). Aguado and colleagues repeatedly sampled a vegetable processing plant and characterised *L. monocytogenes* and *L. innocua* isolates. They detected two major subtypes of *L. innocua* on several sampling occasions.

The authors are inconclusive as to whether these subtypes were either common in vegetables and repeatedly introduced into the plant, or whether the strains were able to survive cleaning operations during processing (Aguado et al., 2004). Furthermore, Keeratipibul and Techaruwichit investigated the transmission routes of *Listeria* in a chicken meat processing plant. The authors recovered *L. innocua* (82.3%), *L. welshimeri* (11.2%), *L. seeligeri* (5.5%) and *L. monocytogenes* (1%) from processing environment samples and identified the persistence of one specific *L. innocua* strain from surface samples of the processing line (Keeratipibul & Techaruwichit, 2012).

When studying persistence of *Listeria* spp. in the FPE, isolates should be assigned to the categories of persistent and transient or sporadic with caution. The discriminatory power of the subtyping method used and the sampling plan applied, among other factors, affect categorisation, and a presumably sporadic strain could persist in future sampling occasions or persist in a different environment (Kastbjerg & Gram, 2009; Carpentier & Cerf, 2011; V. Ferreira et al., 2014). On the other hand, a designated persistent strain might not be “truly persistent”, but rather the result of repeated re-introduction into the FPE (Carpentier & Cerf, 2011; V. Ferreira et al., 2014). Belias and Wiedmann have recently suggested to designate the latter category of strains as “persistent transient” (Belias & Wiedmann, 2021).

Current knowledge offers two main concepts to explain the persistence of *L. monocyotgenes* and *Listeria* spp. in FPEs, one pointing to external, environmental circumstances and the other to intrinsic strain characteristics as the underlying cause. In support of external sources as the primary cause of *Listeria* persistence, niches and harbourage sites within the FPE were frequently cited as enabling the survival and subsequent emergence of persistence, which can be further facilitated by insufficient cleaning and disinfection (Abee et al., 2016; Carpentier & Cerf, 2011; V. Ferreira et al., 2014; M. H. Larsen et al., 2014). With regard to external factors that promote the persistence of *Listeria* spp. in FPEs, Belias and co-workers in a late breaking review identified the lack of hygienic zoning and inadequate separation of different areas or employees in FPEs, as well as equipment cleanability as the most common risk factors for the occurrence

of persistent *Listeria*. Other identified risk factors included cleaning routines, raw materials, product type and dripping condensate (Belias et al., 2021).

A variety of intrinsic, phenotypic or genotypic strain characteristics have been studied to date with the aim of uncovering the mechanisms underlying the persistence of *L. monocytogenes*. Investigated parameters, strain features and specific genes of *L. monocytogenes* as well as any observed association with persistence in FPEs are listed in Supplemental Table 3. As shown in Supplemental Table 3, results of these studies have frequently been inconclusive, contradictory and for the most part hard to compare due to different study set-ups. In summary, it is now widely accepted, that persistence of *L. monocytogenes* in the FPE is caused by an interplay of multiple factors, including influences from the surroundings of the bacteria and adaptation processes triggering the evolvement of distinct genetic features (Kallipolitis et al., 2020; Palaiodimou et al., 2021).

The presence of persistent *L. monocytogenes* strains in an FPE can increase the risk of food product contamination (Nüesch-Inderbinen et al., 2021). Therefore, subtyping is a prerequisite for the identification of persistent strains and detection of the latter should optimally prompt efficient *L. monocytogenes* control measures (Jooste et al., 2016; Mäesaar et al., 2021; Stasiewicz et al., 2015; Stessl et al., 2014).

1.2.1. **Disinfectant susceptibility**

According to the Codex Alimentarius, disinfection is “the reduction, by means of chemical agents and/or physical methods, of the number of micro-organisms in the environment, to a level that does not compromise food safety or suitability” (Joint FAO/WHO Codex Alimentarius Commission, 1969/2020).

In the food industry disinfectants are used as part of a sanitising protocol, optimally specifically tailored to the respective FPE, where prior cleaning is required to ensure efficient disinfection (Holah, 2014b). The following groups of disinfectants are commonly used in the food industry: alkylamines, quaternary ammonium agents, peroxygens, alcohols, aldehydes, halogen-releasing agents, bisphenols and biguanides (McDonnell & Russell, 1999; https://www.desinfektion-dvg.de/index.php?id=1801; accessed on: 1 March 2022). The concern about the use of disinfectants in the food processing industry is that inadequate use may

impose selective pressure on bacteria, which may develop tolerance mechanisms by mutation or by acquiring genetic material (Ferreira et al., 2014; McDonnell & Russell, 1999).
Bacteria are not capable of surviving lethal doses of disinfectants, i.e. the manufacturer's recommended concentrations; however, sublethal concentrations may allow bacterial survival (Aarnisalo et al., 2000). Excessive bacterial survival after disinfection may be the result of a disinfection failure caused by i) the presence of organic material not removed by prior cleaning, ii) the formation of biofilms that protect bacteria from accessing the disinfectant, iii) dilution of the disinfectant, e.g. by stagnant water, iv) the presence of niches in the environment that are difficult for disinfectants to reach, v) acquired bacterial tolerance or resistance mechanisms (Cerf et al., 2010; Martínez-Suárez et al., 2016).

There is no universal definition of bacterial survival or elimination in the context of disinfectant use, but there is common ground when using the term "resistance" when analysing bacterial removal by biocides and the term "tolerance" when examining adaptation to inhibitory concentrations of biocides.
(Cerf et al., 2010). In our work, we prefer to use the word “susceptibility” to describe the outcome of minimal inhibitory concentration (MIC) determination studies (Gilbert & McBain, 2003). In contrast to antibiotics, which are clinically used at levels close to their MICs, in-use concentrations of disinfectants for the FPE are higher than the bacterial MICs determined and when used as instructed, are sufficiently lethal (Meyer, 2006).

Repeated exposure of *L. monocytogenes* to sublethal concentrations and prolonged persistence in the FPE may lead to adaptation and increased tolerance to the applied agent (Ortiz et al., 2014). Several studies were carried out to investigate possible underlying genetic changes of disinfectant - especially QAC-tolerant *L. monocytogenes* strains. Some authors demonstrated that, on a genetic level, this adaptation may be explained by mutations resulting in decreased permeability of the cell for QACs due to modifications in the cell membrane (C. Ferreira et al., 2011a; To et al., 2002).
Activation of efflux pumps belonging to the small multidrug resistance (SMR) protein family or MdrL (multidrug resistance *Listeria*) and Lde (*Listeria* drug efflux),

belonging to the major facilitator superfamily (MFS), has been associated with adaptation of *L. monocytogenes* to BC (Blair et al., 2015; Rakic-Martinez et al., 2011; Romanova et al., 2006; Wessels & Ingmer, 2013). Several *L. monocytogenes* genes encoding for those efflux pumps have been discovered and have been studied in relation to their ability to confer tolerance to BC: the plasmid-associated BC resistance cassette *bcrABC* was detected in *L. monocytogenes* H7550, a strain from the 1998-1999 outbreak caused by contaminated hot dogs in the USA (Elhanafi et al., 2010). The SMR transporter genes *emrE, located* on a chromosomal genetic islet and *emrC,* located on a plasmid, were found to confer reduced susceptibility towards QAC in *L. monocytogenes* (Kovacevic et al., 2016; Kremer et al., 2017). The *L. monocytogenes* transposon Tn*6188*, harbouring the *qacH* gene has recently been described. The transposon has been found to confer tolerance to QACs via a QAC resistance protein QacH, a small multidrug resistance protein family transporter (Müller et al., 2013, 2014).

Adaptation processes may not only lead to increased tolerance to the stress immediately acting on *L. monocytogenes*, but may result in cross-protection against other stresses. For example, acid tolerant strains were found to tolerate thermal and osmotic stresses and adaptation to osmotic stress led to increased resistance to peroxide stress (Bergholz et al., 2012; Hill et al., 2002). Previous studies have suggested a connection between *L. monocytogenes* susceptibility to biocides and antibiotic resistance for different substances (Christensen et al., 2011; Rakic-Martinez et al., 2011). Also authors argued that antibiotic resistance triggered by the use of food-industry disinfectants may favour *L. monocytogenes* persistence in FPEs (Alonso-Hernando et al., 2009; Møretrø et al., 2017a). However a recent study failed to identify any relationship between biocide tolerance and antibiotic resistance (Roedel et al., 2019). While data on the susceptibility of *L. innocua* to commonly used food industry disinfectants are scarce, it has been shown that antibiotic resistance was more prevalent in *L. innocua* than in *L. monocytogenes* strains (B. Y. Chen et al., 2010; Gómez et al., 2014; Baquero et al., 2020).

1.3. *Listeria* spp. detection in the FPE and differentiation

Internationally, besides national food legislation, the Codex Alimentarius standards are applied and several food safety management standards, systems and practices (such as Hazard Analysis and Critical Control Points [HACCP], GMP, Good Hygiene Practices [GHP], Sanitation Standard Operating Procedure [SSOP], ISO standards) are in place throughout the dairy processing industry (Papademas & Bintsis, 2010; https://www.fao.org/fao-who-codexalimentarius/en/; accessed on: 2 March 2022). However, the presence of *L. monocytogenes* is still a common occurrence in dairy and cheese processing environments (see Table 5) and necessitates the implementation of efficient control measures (J. C. Lee et al., 2021). Therefore, an environmental *Listeria* monitoring scheme tailored to the respective FPE is required (Zoellner et al., 2018). When developing a *Listeria* control strategy, a well-designed sampling plan and thorough documentation of detection and differentiation, along with appropriate *Listeria* spp. detection and subtyping methods should be selected and implemented (Jooste et al., 2016).

1.3.1. Sampling and *Listeria* spp monitoring

If present in finished products, *L. monocytogenes* is most likely present in low numbers, making contamination difficult to detect. Therefore, a combination of FPE monitoring, and testing of raw material and finished products is required (Jordan et al., 2018).

Listeria FPE monitoring is the routine (microbiological) sampling of the facility environment, equipment and work clothing with the aim of detecting *Listeria* contamination and monitor the applied control measures (Zoellner et al., 2018). The responsibility for *Listeria* FPE monitoring lies with the FBO and is mandatory according to Regulation (EC) No. 2073/2005 (EC, 2005). Comparable to the regulation in the EU, in the USA, Canada, Australia and New Zealand, RTE food processing facilities are required to perform environmental monitoring for *L. monocytogenes.* Regulatory agencies classify food products according to the ability to support the growth *of L. monocytogenes* and the risk of being associated with listeriosis. While no further distinction is made in the EU, specific RTE processing environments in the USA, Canada, Australia, and New Zealand must be sampled more frequently depending on the associated risk profile (US Food

and Drug Administration [FDA], 2017; Health Canada, 2011; Australian Government, 2018). It is important that the monitoring program for each FPE is established in accordance with the legal food safety requirements (Spanu & Jordan, 2020).

The main principles for designing an environmental monitoring program have been described in recent literature, e.g. by Simmons and Wiedmann, Spanu and Jordan, Wagner and Stessl or Zoellner et al (Simmons & Wiedmann, 2018; Spanu & Jordan, 2020; Wagner & Stessl, 2021; Zoellner et al., 2018).

In summary, as reviewed by Zoellner et al., when designing an environmental monitoring programme for *Listeria*, areas should be defined according to the contamination risk implied (so called “zoning”). Sampling locations, sample size, frequency and time of sampling should then be established accordingly. Areas where contamination is likely to occur include for example, floors, drains, and wet areas, hard-to-clean niches in the equipment, doors, or air handling systems. Sampling procedures might vary according to the relevant regulations and taking into account the risk-profile of the manufactured products and the manufacturing process as well as the available resources (Zoellner et al., 2018). In addition, the methods for detection and typing should be defined. Data recording and mapping, as well as data interpretation and trend analysis methods need to be determined. The design should provide for corrective measures such as test sampling in the event of positive findings and the implementation of cleaning and disinfection measures (Simmons & Wiedmann, 2018; Spanu & Jordan, 2020).

The Austrian *Listeria* Environmental Monitoring Program for Dairies and Cheesemakers, established in 1988 at the Unit of Food Microbiology (University of Veterinary Medicine Vienna) is an example of a multistage environmental monitoring program (Asperger et al., 2001; Wagner & Stessl, 2021):

Level 1 is routine monitoring in which 1000 ml of product-associated liquid samples (smear, brine, wash water) and/or drain water are microbiologically analysed semiquantitatively for *Listeria* spp. Product-associated liquids may indicate *L. monocytogenes* cross-contamination, while drain water harbouring *Listeria* spp. can indicate processing facility contamination with potentially persistent *Listeria* spp. exposed to sublethal stress due to disinfection residues.

Level 2 is an interventional level. Once *L. monocytogenes* is detected in environmental samples, sampling is increased from previously contaminated sampling points and includes additional areas. To determine the extent of contamination in the facility, food-lots are also analysed for *L. monocytogenes*.
Level 3 is the sanitation of the FPE, when investigations at level 2 confirm further *L. monocytogenes* contamination. The sanitation should be systematic and guided by external experts. This phase should include subtyping of recovered isolates to identify contamination hot-spots within the FPE and detect in-house persistent strains.
Level 4 is the confirmation of successfully established control measures through intensive FPE monitoring and attempts to eliminate *L. monocytogenes* contamination (Asperger et al., 2001; Wagner & Stessl, 2021).

Generally, samples from the FPE should include food-contact (e.g. cheese smear or brine) and non-food contact samples (various equipment surfaces), liquid samples (e.g. drain water) and swab samples of various surfaces (door handles, floors, areas of water condensation, equipment). Swabbing is performed, using either a stick swab for hard-to-reach small areas, or sponges, clothes or gauze pads for larger areas. The sampled surface should ideally incorporate at least 10 x 10 cm^2, to increase the likelihood of *L. monocytogenes* detection. Sample transportation needs to take place at a cool temperature (ideally 4 °C) and sample processing should happen as soon as possible. Solid samples, such as food samples are homogenised prior to further analysis. Guidelines for sampling a FPE are provided by ANSES/EURL *Lm* (ANSES & EURL *Lm*, 2012). Furthermore, in 2018, ISO standard 18593 was published, providing a reference method on horizontal methods for surface sampling (ISO, 2018).

1.3.2. *L. monocytogenes* and *Listeria* spp. isolation and identification

Listeria spp. detection traditionally involves a two-stage enrichment culture followed by plating on a selective differential agar (Beumer & Curtis, 2012).
Internationally, the major culture-based reference methods for the isolation and detection of *L. monocytogenes* from food are the FDA - Bacteriological and Analytical Manual (BAM), the USDA-FSIS and the ISO 11290 methods (ISO,

2017a; ISO 2017b; https://www.fda.gov/food/laboratory-methods-food/bam-chapter-10-detection-*Listeria*-monocytogenes-foods-and-environmental-samples-and-enumeration; https://www.fsis.usda.gov/news-events/publications/microbiology-laboratory-guidebook; accessed on: 3 March 2022). These methods vary slightly, in the enrichment media, plating media as well as procedural parameters (incubation times, temperatures) employed. Regulatory bodies propose different selective enrichment media for standard *L. monocytogenes* detection: Buffered *Listeria* enrichment broth (BLEB) is recommended by the FDA-BAM for the isolation and identification of *L. monocytogenes*. BLEB contains added disodium phosphate for increased buffering capacity, which improves enrichment properties. Selective agents (acriflavine, cycloheximide and nalidixic acid) are added to the medium following an initial four hour non-selective enrichment (Law et al., 2015; Magalhães et al., 2014). The USDA-FSIS isolation protocol foresees the use of the University of Vermont (UVM) broth. UVM is suggested as primary enrichment specifically for the recovery of heat-injured *L. monocytogenes.* In the second enrichment step, nalidixic acid and acriflavine are added as selective agents.

The ISO horizontal method for the detection and enumeration of *L. monocytogenes* and of *Listeria* spp. is recommended in EC regulation 2073/2005 (EC, 2005). The ISO 11290 method is a prolonged culture-based method, which includes a qualitative as well as a quantitative protocol (ISO 11290-1 and 2). Fraser broth is used as selective enrichment of *L. monocytogenes* and *Listeria* spp. from food and environmental samples. Fraser broth, based on the Fraser and Sperber formulation, is a modified UVM-broth, whereby ferric ammonium citrate and lithium chloride are added. In the first, pre-enrichment step, 25 g of the sample are used with 225 ml of half-strength Fraser broth (HF) to revive injured *Listeria* spp. and dilute inhibitory compounds present in the sample. For swab samples 50-100 ml HF are recommended. Incubation is at 30 °C for 24 h. In the second enrichment step, the selective enrichment (full Fraser, [FF]) nalidixic acid and acriflavine are added as selective agents. Selective enrichment suppresses competing bacterial growth, while providing a nutritional basis for the target pathogen, which enables isolation and detection. Then 0.1 ml is transferred to 9 ml FF broth and incubated for 48 h at 37 °C (ISO, 2017a, 2017b). Incubation in the second enrichment step can be reduced to 24 h, which

makes detection of presumptive *L. monocytogenes* or *Listeria* spp. possible within 48 h using Fraser broth (Fraser & Sperber, 1988; Gnanou Besse et al., 2016).

Differential plating media recommended by FDA-BAM, USDA or ISO for the isolation of *Listeria* spp. include Oxford, modified Oxford (MOX), Polymyxin-Acriflavine-Lithium-Chlorid-Ceftazidime-Aesculin Mannitol (PALCAM) and chromogenic media, such as *Listeria*-Agar according to Ottaviani and Agosti (ALOA) (Curtis et al., 1989; McClain & Lee, 1989; Ottaviani et al., 1997; van Netten et al., 1989). In addition to selective agents, selective agar media also contain indicator substrates that allow visual identification of *Listeria* spp. and other bacteria (Manafi, 1996). While all *Listeria* spp. exhibit the same morphology on selective agar plates containing ferric ammonium citrate and aesculin, such as PALCAM or Oxford, *Listeria* spp. are distinguishable from each other on chromogenic or blood agars (T. Johansson, 1998). Chromogenic agars contain chromogenic substrates, e.g. in ALOA agar substrate for the detection of PI-PLC and ß-D-Glucosidase produced by *L. monocytogenes* and *Listeria* spp. respectively or Rapid'L. mono Agar (a selective, chromogenic medium) PI-PLC and Xylose (Ottaviani et al., 1997; Reissbrodt, 2004; https://www.bio-rad.com/de-at/product/rapidl-mono-medium?ID=35bad7d6-36ac-4044-b859-d5febbda101a; accessed on: 2 March 2022).
The ISO 11290-1 protocol requires the primary and secondary enrichment to be streaked on differential and selective agar plates. ALOA agar and a second, selective agar such as PALCAM are used and subsequently incubated at 37 °C for 24-48 h. Typical *L. monocytogenes* or *Listeria* spp. colonies are then isolated for further testing (ISO, 2017a). ISO 11290-2 describes the enumeration of *L. monocytogenes,* whereby a 1:10 dilution of contaminated foods and samples in HF broth or buffered peptone water is prepared, homogenised and incubated for one hour at room temperature. 0.1 ml of the suspension is then plated on ALOA agar and a second selective agar medium such as PALCAM. Incubation follows for 24-48 h at 37 °C (ISO, 2017b).

Traditionally, identification of *Listeria* spp. has been performed by culture-based reference methods using biochemical tests or phenotypic markers. These include catalase testing, Gram-staining, tests for haemolysis (e.g. Christie–Atkins–Munch-

Peterson - CAMP) or carbohydrate utilisation tests (Gasanov et al., 2005). Commercially available identification kits such as Analytical Profile Index (API®) *Listeria* have widely replaced these traditional identification methods due to simple handling and rapid results (Bille et al., 1992). Following culture-based isolation and DNA extraction, PCR-based identification of *L. monocytogenes* and *Listeria* spp. is employed routinely. Conventional and real-time PCR protocols, reverse transcription (RT)-PCR, microarray and isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) or nucleic acid sequence-based amplification (NASBA) PCR protocols for *L. monocytogenes* and *Listeria* spp. identification have been established (J. Q. Chen et al., 2017; Gasanov et al., 2005; Law et al., 2015; A. Liu et al., 2018).

Examples for PCR targets in *L. monocytogenes* and *Listeria* spp. are the 16S rRNA and 23S rRNA genes (Paillard et al., 2003; Sallen et al., 1996; R. F. Wang et al., 1992), 16S/23S rRNA intergenic regions (Graham et al., 1997; Rantsiou et al., 2008), *hly* (Furrer et al., 1991; Rodríguez-Lázaro et al., 2004), *plcA, plcB* (Jaradat et al., 2002; Volokhov et al., 2002), *actA* (Longhi et al., 2003; Oravcova et al., 2006), *prfA* (Rossmanith et al., 2006; Ward et al., 2004; Wernars et al., 1991), *inlA and inlB* (Jung et al., 2003), *iap* (Bubert et al., 1999) and further *L. monocytogenes* or *Listeria spp.* specific genes (J. Q. Chen et al., 2017; D. Liu, 2006a).

Rapid test kits enable in-house testing in food production facilities and can be a valuable addition to routine *Listeria* monitoring in external, fully equipped laboratories. The tests most commonly used in these areas are based on amplification and hybridisation techniques, immunological and cultural methods (Välimaa et al., 2015; Wiedmann et al., 2014). The FSIS published a list of foodborne pathogen test kits, which are validated by independent organisations (Association of Official Agricultural Chemists [AOAC], French National Organization for Standardization [AFNOR], MicroVal or NordVal), US regulatory bodies (USDA-FSIS or FDA) or the ISO (USDA-FSIS, 2020).

1.3.3. **Subtyping**

Subtyping is the method of discriminating bacterial isolates beyond the species or sub-species level (Sandora et al., 2014). Subtyping is used in disease surveillance, to detect outbreaks and to trace the outbreak source within the food production chain. *L. monocytogenes* and other *Listeria* spp. are furthermore subtyped to study the ecology, epidemiology, evolutionary genetics and strain or subtype characteristics, e.g. virulence or stress resistance (Wiedmann, 2002; Sabat et al., 2013; Datta & Burall, 2018). Differentiation by subtyping can be based on the bacterial phenotype, employing conventional or phenotypical methods or the genotype, using genetic or DNA-based methods. The latter has seen substantial advances by the development of rapid and increasingly sensitive methods throughout previous years (Datta & Burall, 2018). Depending on the intended purpose and aim of *Listeria* spp. subtyping, certain criteria for subtyping methods can help decide which subtyping method is best suited (Zunabovic et al., 2011). These include the discriminatory ability (characterised for example by the Simpson's Index of Discrimination), reproducibility, cost, ease of application, the available options for data management and standardisation to ensure international comparability and the possibility of automation (Datta & Burall, 2018; Jadhav et al., 2012; Wiedmann, 2002; Zunabovic et al., 2011).

1.3.3.1. Phenotypic methods

Conventional phenotypic subtyping methods include serotyping, phage typing and multilocus enzyme electrophoresis (MEE) (Gasanov et al., 2005). The latter typing methods have nowadays been widely replaced by genotypic methods, which generally offer a higher degree of discriminatory power, epidemiological concordance, reproducibility and typeability (D. Liu, 2006a; Wei & Zhao, 2021). Biotyping and antimicrobial resistance profiling also fall into the category of phenotypic subtyping techniques (Gebreyes & Thakur, 2010).

Serotyping was the first method employed for *L. monocytogenes* subtyping (Seeliger & Höhne 1979). Serotyping is based on the flagellar (H) and somatic cell wall (O) antigens and involves antisera to distinguish between at least 15 serovars (see chapter 1.1.4.). Serotyping is nowadays used to a limited extent, e.g. in reference laboratories, and has been predominantly replaced by genotypic

subtyping methods (Seeliger & Höhne 1979; Liu 2006; Seeliger & Jones, 1986; Wei & Zhao, 2021).

Phage typing was developed to discriminate between strains of the same serovar (Rocourt et al., 1985). Phage typing uses the principle of bacteriophages being able to invade and lyse specific bacterial cells. The susceptibility of bacteria to the lytic action of a standard set of bacteriophages is tested and the phage type determined according to the reaction (lytic plaque formation) of the bacterial culture on the agar plate (Rocourt et al., 1985; Gasanov et al., 2005). Low typeability of some *L. monocytogenes* strains and variability in results is a drawback of the method, however the use of bacteriophages has increasingly been researched as a method for *L. monocytogenes* biocontrol in FPE (Capita et al., 2002; Gasanov et al., 2005; Kawacka et al., 2020; Rocourt et al., 1985).

MEE has been frequently used as a typing technique in population genetics studies (Selander et al., 1986). The method is based on the principle of amino acid sequence variations of enzymes resulting in different charges of the proteins, which can be detected by different electrophoretic migration patterns of the enzymes. Electrophoretic types can be deducted based on the velocity of electrophoretic migration. Differences in the mobility directly translate to the gene sequence encoding for the enzyme (D. Liu, 2006a). The method is relatively simple, with lysis of bacterial cells, subsequent electrophoresis of the enzyme extracts and staining of the enzymes (Selander et al., 1986; Tibayrenc, 2009; Gasanov et al., 2005).

In biotyping, enzyme and carbohydrate testing is used for sub-species discrimination. In both cases colour changes are an indicator for carbohydrate fermentation or enzyme reaction. As one of the early subtyping methods developed, it is nowadays used only infrequently, due to the low discriminatory power in comparison to other genotypic and phenotypic methods (Gebreyes & Thakur, 2010).

Antimicrobial susceptibility testing is performed to determine resistance patterns to different classes of antimicrobials. Various methods have been established, e.g. the disc diffusion, broth macro- and microdilution methods or automated systems with microtiter plates containing lyophilised antimicrobial agents in different concentrations, which are read in a photometer and analysed with specific software (Khan et al., 2019). International agencies have published guidelines for

MIC cut-offs, e.g. the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/ast_of_bacteria/; accessed on: 4 March 2022). Although antibiotic resistance is usually not a concern in clinical *L. monocytogenes* isolates, individual cases of resistant, including multi-resistant, strains have been described. Hence testing the susceptibility of *L. monocytogenes* strains to antimicrobials is used in clinical routine as well as in research settings (Kuch et al., 2018).

1.3.3.2. Genotypic methods

Genotypic subtyping methods have gained importance over the last decades and are today for the most part methods of choice for subtyping *L. monocytogens* and *Listeria* spp. Especially the application of WGS-based methods for foodborne bacteria subtyping has rapidly been gaining momentum in different settings (Baert et al., 2021; Pietzka et al., 2019; Wei & Zhao, 2021).

Restriction digestion based subtyping methods include ribotyping and PFGE (Wiedmann, 2002). Ribotyping is a molecular typing method, which makes use of polymorphisms in the bacterial rRNA (Matloob & Griffiths, 2014). The technique involves restriction digestion of *Listeria* chromosomal DNA with frequently cutting enzymes such as *Eco*RI, followed by separation of DNA fragments using gel electrophoresis and subsequent Southern blot transfer and DNA hybridisation with an *E. coli* derived rRNA gene probe (Matloob & Griffiths, 2014; Nadon et al., 2001).

Due to its high discriminatory power, PFGE has been the gold standard for subtyping *L. monocytogenes* for epidemiological studies in foodborne outbreaks (Dalmasso & Jordan, 2015; Luque-Sastre et al., 2015). A standardised protocol by the US CDC facilitates trans-national outbreak strain matching through PulseNet, a network of public health and food regulatory laboratories using PFGE for foodborne pathogen subtyping (CDC, 2017). In summary, the PFGE process includes the lysis of *L. monocytogenes* in agarose plugs, followed by restriction digest, which is performed with restriction enzymes such as *Asc*I and *Apa*I yielding between eight and 25 large DNA bands of 40–600 kb in size. The DNA fragments are then electrophoresed with alternating currents for at least 24 h

resulting in DNA banding patterns, which allow subtype (pulsotype) classification of strains. The discriminatory power of PFGE exceeds that of other molecular subtyping techniques such as MLST and multilocus variable number of tandem repeat analysis (MLVA), but one has to consider that PFGE is a multi-step, laborious and time-consuming technique, which requires technical experience (Ranjbar et al., 2014).

Methods such as amplified fragment length polymorphism (AFLP) and PCR-restriction fragment length polymorphism (RFLP) combine restriction digestion and PCR-based techniques (D. Liu, 2006a). AFLP is a technique for DNA polymorphism detection. It comprises DNA digestion with two restriction enzymes and ligation of the restriction fragments with double stranded adaptors. The fragments are amplified by PCR. PCR products are then electrophoresed and resulting patterns visualised (Keto-Timonen et al., 2003; Vos et al., 1995). In PCR-RFLP, amplification of specific *L. monocytogenes* housekeeping genes or genes associated with virulence (e.g. *actA, inlA, hly*) is followed by restriction enzyme digest and agarose gel electrophoresis to achieve band patterns allowing *L. monocytogenes* subtype identification (Jeffers et al., 2001; Rousseaux et al., 2004; Wiedmann et al., 1997). PCR-RFLP has the advantage over AFLP that no adaptor linkage is needed. When combined with other subtyping methods, PCR-RFLP can provide discriminatory and reproducible results (D. Liu, 2006a).

PCR-based typing methods include random amplified polymorphic DNA (RAPD) analysis, whereby short arbitrary primers (ca. 10 bp) are used in a PCR at low annealing temperature (Farber, 1996). The resulting amplified products show strain specific profiles of unknown DNA fragments after gel electrophoresis (Mazurier & Wernars, 1992). The method is less discriminatory than other subtyping techniques, however has been used due to its simplicity, low cost and being rapid to perform (Jadhav et al., 2012; D. Liu, 2006a).

In REP- and ERIC-PCR either repetitive extragenic palindromes (REP) or enterobacterial repetitive extragenic palindromes (ERIC) sequences are used as primer binding sites (D. Liu, 2006a). *L. monocytogenes* genomes harbour multiple repetitive sequences in different genomic regions. Following PCR amplification, strain specific patterns are obtained, allowing subtype discrimination (Jeršek et al., 1999). Repetitive elements-based PCRs have been found to be a rapid and simple addition to other subtyping techniques (Nyarko & Donnelly, 2015).

L. monocytogenes serogrouping by multiplex PCR-based methods has been widely replacing the conventional phenotypic serotyping (Chen & Knabel, 2007; Doumith et al., 2004a). Also, these type of assays have been further developed to accomplish *L. monocytogenes* grouping to one of the major clonal groups (Chenal-Francisque et al., 2015).
L. monocytogenes strains can also be further discriminated by using PCR assays targeting different *L. monocytogenes* genes. Various PCR assays to detect the presence or absence of genes associated with virulence, e.g. listeriolysin S or genes associated with persistence in food processing e.g. SSI-1 and SSI-2 or Tn*6188* have been developed (Cotter et al., 2008; Harter et al., 2017; Müller et al., 2013; Ryan et al., 2010).

DNA sequence-based molecular methods include MLVA, MLST, microarray techniques and WGS (D. Liu, 2006a).
Bacterial genomes harbour repeated short nucleotide sequences, so called variable number tandem repeats (VNTRs), which differ in their location on the genome, their size and structure (Van Belkum, 2007). MLVA is a technique to detect differences in the number of tandem repeats at specific loci in the genome of microorganisms (Murphy et al., 2007). In practice, a software-assisted selection for the loci with highest allelic variations is conducted, then the repeat sequences are amplified by PCR and fragments are subsequently separated on agarose gels. Sequencing or electrophoresis are used to size the fragments (S. Chen et al., 2011; Chitlapilly Dass et al., 2010). MLVA is a robust method with generally good discriminatory power and option for high throughput. However, the method has been lacking international harmonisation, a prerequisite to allow inter-laboratory surveillance (Nadon et al., 2013).
MLST is useful to determine the ancestral and evolutionary relationship between *L. monocytogenes* isolates based on genetic variations (Ragon et al., 2008). MLST involves sequencing of seven housekeeping genes to detect allelic polymorphisms. In *L. monocytogenes* these housekeeping genes are *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase) and *lhkA* (histidine kinase) (Salcedo et al., 2003; Ragon et al., 2008). For each of these loci every unique sequence is allocated a unique

allele number. This denomination results in a seven digit allelic profile or a specific ST number (Sabat et al., 2013). The allelic profile or ST number is compared to an online database, e.g. the Institut Pasteur databases (https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022). MLST has been established for inter-laboratory comparison and has been widely used to study the genetic relatedness of pathogenic bacteria (Maiden et al., 2013; Stessl et al., 2021a).

In order to improve the discriminatory ability and to enable the detection of outbreak clusters, *L. monocytogenes* MLST has been further developed in recent years by including the analysis of additional genes, MVLST and WGS techniques such as core or whole-genome MLST (cgMLST, wgMLST) (Y. Chen et al., 2007; Gerner-Smidt et al., 2019; B. R. Jackson et al., 2016; Jadhav et al., 2012; Moura et al., 2016; Ruppitsch et al., 2015).

DNA microarrays arrange thousands of DNA molecules of known sequence (probes) on a matrix. They function based on the binding of complementary sequences to each other (Gresham et al., 2008). In summary, DNA microarray analysis consists of array fabrication, probe preparation, hybridisation on the array, and data analysis. Fluorescently labelled target sequences interact with specific immobilised probes. This interaction results in a signal whose strength depends on the amount of target sample that binds to each of the probes (Gerner-Smidt et al., 2019; Miller, 2011).

DNA microarrays can be applied to detect either DNA (e.g. in comparative genomic hybridisation [CGH], or for single-nucleotide polymorphism [SNP] analysis) or RNA, for measuring gene expression. The latter has been the most prevalent application of DNA microarrays (Bumgarner, 2013). Different approaches, including *Listeria* species identification (Bang et al., 2013; Call et al., 2003; Hmaïed et al., 2014; Volokhov et al., 2002), *Listeria* subtyping (Borucki et al., 2004; Borucki et al., 2003; Call et al., 2003; C.Zhang et al., 2003), and studies on virulence (Doumith et al., 2004b; Kazmierczak et al., 2003) and epidemiology (Borucki et al., 2004; Laksanalamai et al., 2012a; Laksanalamai et al., 2012b) using DNA microarray have been applied. DNA microarrays have been rapidly replaced by WGS during recent years (Kwong et al., 2015). This is for the most part due to optimisation and cost reduction in WGS technology, but also due to limitations of the DNA microarray technique. The latter include the reliance upon

annotations of a reference genome, which means that many genes in a test strain not present in the reference genome might not be detected (Sabat et al., 2013). When analysing highly repetitive regions, the efficiency of hybridisation can be variable, leading to false positives. Also cross-hybridisation, i.e. hybridisation between non-complementary sequences can occur, negatively affecting the specificity (Bumgarner, 2013; Gresham et al., 2008).

Before WGS has been introduced for outbreak investigation and source tracing, PFGE has been regarded as the gold standard for *L. monocytogenes* subtyping (Swaminathan et al., 2001; Salipante et al., 2015). Although PFGE is still used today in routine food safety and public health laboratories, as well as for tracing *L. monocytogenes* contamination routes and persistence in FPEs, its resolution is limited in comparison to WGS, because it exploits only a fraction of an isolates' genetic information (CDC, 2017). The emergence of WGS in recent years has enabled determination of nucleotide sequences of DNA molecules and of the whole genome (Gerner-Smidt et al., 2019). Following the development of Sanger-sequencing in the 1970s, the introduction of next generation sequencing (or second-generation sequencing) in the early to mid-2000s has led to an increased automation by massive parallel sequencing and a reduction in cost (Heather & Chain, 2016). With further development of the technology and the introduction of third generation sequencing, international efforts to standardise protocols have increased, which has furthered the widespread use of WGS in food safety, clinical and public health laboratory practice (Deng et al., 2016; Heather & Chain, 2016; W. Li et al., 2021; Moura et al., 2017; Ronholm et al., 2016).

Giani et al. and Segerman recently reviewed currently used sequencing and assembly methods. In short, the process of WGS involves DNA extraction from a bacterial culture followed by purification of the DNA. The DNA is sheared into short fragments, either by enzymes or mechanically. Subsequent amplification of the fragments generates the DNA library, which is then loaded onto a sequencer. The sequencer reads the nucleotide combination of each DNA fragment and generates millions of DNA reads. After quality analysis of the reads, bioinformatic tools are employed to assemble the reads to contigs (consecutive sequences). A genome can either be recreated with no prior knowledge, using de novo sequence assembly, or by mapping against a known and closely related reference genome (Giani et al., 2020; Segerman, 2020).

The selection of a sequencing pathway should be carefully considered, based on the bacterial species at hand, the intended analysis and study goals as well as on the available laboratory resources (Seth-Smith et al., 2019).

For short-read WGS (50-400 bp), as used for sequencing bacterial genomes, the sequencing-by-synthesis approach, offered by Illumina (e.g. MiSeq, HiSeq or NovaSeq) or Ion Torrent semi-conductor sequencing (PGM, S5, Proton), are examples for currently available platforms (https://www.illumina.com; https://www.thermofisher.com/at/en/home/life-science/sequencing/next-generation-sequencing.html; accessed on: 4 March 2022). Illumina systems offer short read lengths of high accuracy and good throughput, however the initial investment costs are higher compared to other sequencing platforms. The Ion Torrent product line offer rapid sequencing at relatively low initial and operating costs. A weakness however is the inaccurate length detection of homopolymers, which can hinder detection of genomic variations (Besser et al., 2018).

In recent years, third generation sequencing technologies, such as Oxford Nanopore long read sequencing or single-molecule real-time (SMRT) sequencing by Pacific Bioscience have become available (Cao et al., 2017; https://nanoporetech.com/applications/dna-nanopore-sequencing; https://www.pacb.com/smrt-science/smrt-sequencing/; accessed on: 4 March 2022). Genome assemblies generated from next-generation sequencing are usually fragmented due to the short reads. To overcome this issue, platforms generating longer reads have been introduced. These technologies allow rapid sequencing of single DNA-molecules and determination of the base sequence of very long DNA molecules (commonly 10-30 kb) in one piece (Giani et al., 2020). Thus, the platforms are well suited for de novo sequencing and for generating finished (reference) genomes of high quality. The long read length results in higher error rates, which make error correction at the assembly step necessary. A high number of subreads can also help to lower the error rate (Besser et al., 2018).

There are currently a number of different approaches to compare genome sequences for foodborne outbreak investigation. The most common approaches are i) cgMLST, taking into account thousands of genes present in isolates of a species or genus, ii) wgMLST, taking into account all genes including the variable additional genes of a species and iii) reference mapping of SNPs (Kovac et al.,

2017; Pérez-Losada et al., 2018; Schürch et al., 2018). With SNP-based methods, all differences that occur in comparison to the reference genome are recorded, while with cg/wgMLST only allele differences are taken into account without differentiating the number and type of mutations between the isolates under comparison. Therefore, a higher resolution can be achieved with the SNP analysis than with cg/wgMLST (Halbedel et al., 2018). An advantage of cg/wgMLST is the availability of nomenclature schemes that allow the creation of a unique identification of the sequence under analysis, which can be easily communicated between laboratories, without the need to exchange raw sequence data. However, this requires the use of a centrally managed and continually updated nomenclature (Nadon et al., 2017). WGS data can also be used to investigate isolates e.g. for virulence genes, resistance genes of genes that play a role in biofilm formation and other markers of functional properties (Ronholm et al., 2016). With gene-by-gene approaches as well as SNP mapping, the results are only comparable if generated with the same or equivalent software programs, algorithms and identical parameter settings. Specifically, evaluation programs for cluster detection using cgMLST or SNP analysis offer multiple options, which makes direct comparison of WGS results between laboratories difficult. Also, at present, comparative analysis of isolate sequences cannot be carried out decentralised (Lüth et al., 2018). There is now a working group established at ISO (ISO TC34/SC9/WG25), tasked with developing an international standard for the application of WGS for typing and genomic characterisation of foodborne pathogens (https://www.iso.org/standard/75509.html; accessed on: 4 March 2022). For *L. monocytogenes* cgMLST there is still a need for coordination to make data directly comparable between laboratories using different schemes. However, it has been shown that the two most commonly used cgMLST schemes acc. to Moura et al. and Ruppitsch et al. lead to highly consistent results (Moura et al., 2016; Ruppitsch et al., 2015; Walle et al., 2018).

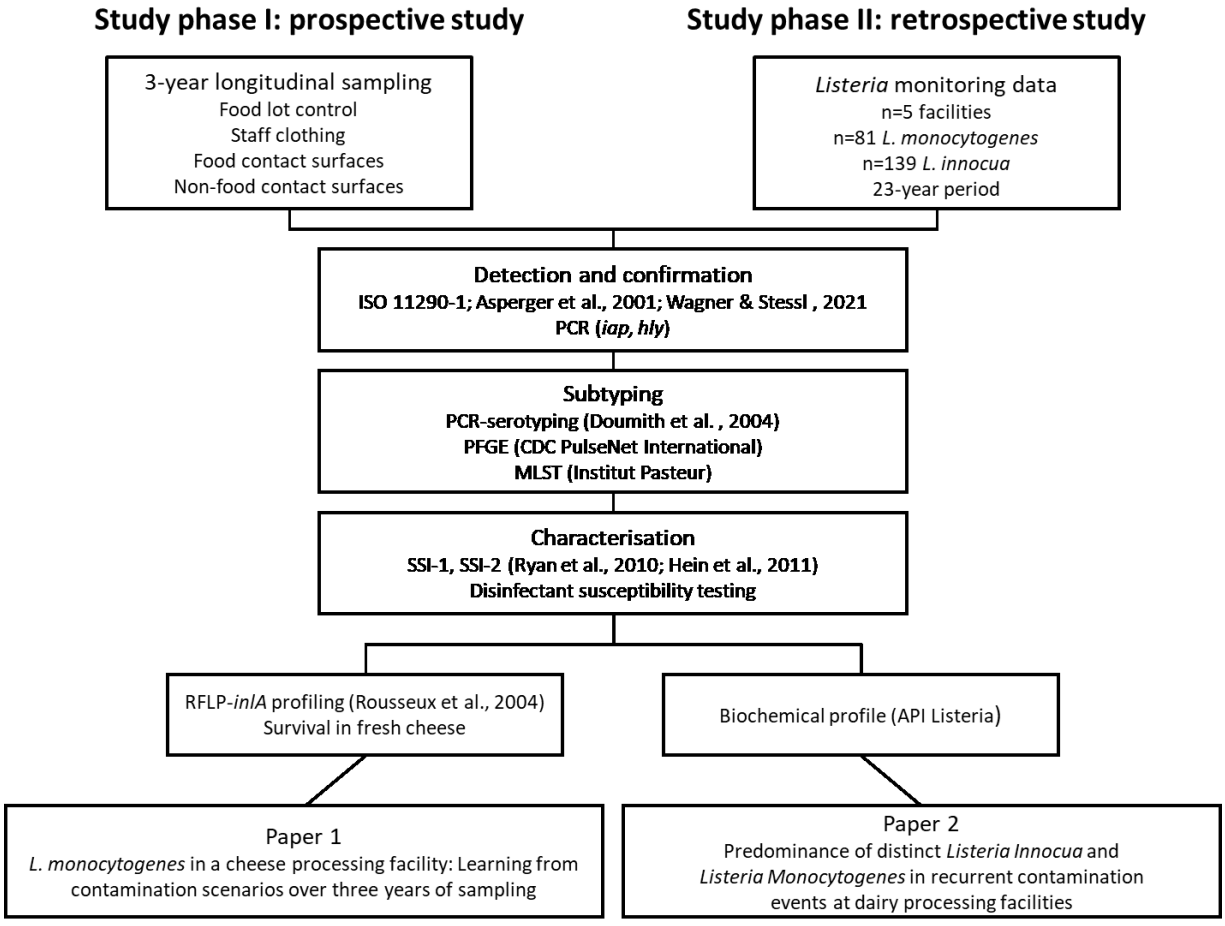
2. AIM AND HYPOTHESES

Recurrent *L. monocytogenes* contamination in dairy processing environments and the identification of *L. monocytogenes* contamination routes as well as the rarely

studied fate of *L. innocua* in dairy processing environments were the cornerstones of the studies conducted within this thesis.

The aim of the first study phase was to (i) analyse *L. monocytogenes* contamination patterns over three years, (ii) trace potential sources of contamination in a dairy processing facility and to iii) further characterise *L. monocytogenes* strains by genotypic subtyping (PFGE and MLST), disinfectant susceptibility testing and assessing the survival of recovered *L. monocytogenes* strains in food matrix. Furthermore results from this analysis were aimed to iv) advice the FBO on intervention strategies to help reducing *L. monocytogenes* contamination in the production environment.

Figure 1: Overview of the studies conducted within this thesis



The aim of the second study phase was to i) characterise *L. innocua* and *L. monocytogenes* isolated from FPEs and cheese products from five dairy

processing facilities by geno- and phenotypic subtyping to ii) gain insight into *L. innocua* ability to adapt and potentially persist in *L. monocytogenes*-colonised dairy FPEs. The outline of the study is shown in Figure 1.

Based on current scientific evidence, the studies were designed to test the following hypotheses:

Study phase I (Paper 1):

- Initial entry of *L. monocytogenes* into a processing facility is caused by raw material.
- *L. monocytogenes* contamination of product occurs due to the sudden detachment of biofilm from equipment during production (i.e. sloughing effect).
- Transmission of *L. monocytogenes* within the processing facility is due to the violation of manufacturing workflow from raw product processing to areas of higher hygiene requirements.
- *L. monocytogenes* recurrently recovered from FPE have specific geno- or phenotypic traits, which allow certain strains to better adapt to FPEs.
- Dilution of applied disinfectants by stagnant water in the FPE triggers biocide tolerance in *L. monocytogenes*.
- FPE monitoring for *L. monocytogenes* is necessary for controlling the contamination.
- Fresh cheese is a RTE food product able to support the growth of *L. monocytogenes.*

Study phase II (Paper 2):

- *L. innocua* is more prevalent in dairy processing environments than *L. monocytogenes.*
- *L. innocua* contamination in a production facility can serve as an index organism for *L. monocytogenes* contamination.
- *L. innocua* and *L. monocytogenes* recurrently recovered from FPE have specific geno- or phenotypic traits, which allow certain strains to better adapt to FPEs.

- Dairy processing environments provide harbourage sites, where *Listeria* spp. can successfully establish, which subsequently leads to recurrent isolation.
- *L. innocua* as well as *L. monocytogenes* strains recurrently isolated from FPEs exhibit decreased disinfectant susceptibility.

3. MATERIALS AND METHODS

The present thesis is based on a two-tiered methodical approach. In phase I, a prospective sampling approach was chosen to determine changing patterns of *L. monocytogenes* contamination within a cheese production facility. Each sampling was followed by culture based detection, isolation, determination and subtyping of *Listeria* spp. For phase II of the study, *L. innocua* and *L. monocytogenes* isolates were retrospectively selected, and subtyped to screen for genetic and phenotypic characteristics that might contribute to the adaptation of the strains to cheese processing environments.

The selected isolates were collected in a strain set. An overview of the two study phases and the following methodical steps is provided in Figure 1.

3.1. Sampling (Paper 1)

For this study, samples were collected over a period of almost three years (from 1 June 2010 to 20 February 2013) at an Austrian cheese production facility manufacturing a wide range of fresh cheese and semi-hard cheese products, made from pasteurised cow's, ewe's and goat's milk. When the company was founded about two decades ago, it was an artisanal dairy. The main production premise of the company consisted of an old building, which had previously been used as an abattoir. When the product range was enlarged and the dairy grew to a medium sized enterprise, with steadily increasing sales and export activity, an additional building was constructed in the years 2010-2011. The sampling of the cheese production facility in 2010 was initiated to investigate the source of a sporadically occurring *L. monocytogenes* contamination in cheese samples. Therefore, an extensive sampling of the FPE according to a sampling plan was carried out. During the sampling period, the sampling plan and the scope of

analyses performed were adapted according to the changing circumstances in the production plant. When we noticed that the one-way principle from raw material processing to finished product areas was frequently violated, and there was heavy (re)contamination with *L. monocytogenes* during construction work, employee training and process improvements were implemented. The sampling plan was modified also to monitor the adapted cleaning and disinfection routines.

After the construction works, the cheese production plant consisted of the following departments:

- department I included rooms for the preparation of raw material, a cooling chamber and an office,
- department II consisted of several rooms for the preparation of different types of cheese (e.g. fresh cheese production, ripening room, yogurt production) and
- department III was also used for the preparation of raw material.

An outline of the cheese processing facility can be found in the original paper (Paper 1), enclosed in Annex II of this thesis.

Overall, 1284 samples from the FPE were collected for further analysis, which included swab samples from fillers, conveyor belts, tables, slicers, mixers and molds (FCS, n=393), swabs from floors, drains, transport boxes and trolleys (NFCS, n=615) and drain water as well as swab samples associated with work clothing, e.g. shoes and aprons (FPE, n=276). In addition, 71 *L. monocytogenes* isolates from raw materials and final products were available from the district laboratory.

A surface of up to 10 x 10 cm^2 of floors and walls, NFCS and FCS was swabbed by using sterile sponge sticks, hydrated in 10 ml Buffered Peptone Water (3M, St. Paul, Minnesota, USA). Drain water samples and other liquid samples were collected with a sterile syringe (Omnifix®, Braun Melsungen AG, Melsungen, Germany) and transferred into sterile polypropylene (PP) bottles (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

The samples were kept in cooling boxes at 4 °C for the transport to the laboratory of investigation (Unit of Food Microbiology, University of Veterinary Medicine Vienna, Austria). The final cheese products were routinely subjected to food lot

control at the local district laboratory according to ISO 11290-1. Raw materials were analysed on a sample basis by the local district laboratory.

3.2. Selection of isolates (Paper 2)

L. innocua and *L. monocytogenes* isolates for this study were selected from the collection of *Listeria* spp. isolates at the Unit of Food Microbiology (University of Veterinary Medicine Vienna, Austria). The isolates deposited in this collection have been obtained by analysing samples from Austrian cheese production facilities within the Austrian *Listeria* monitoring program according to the ISO 11290-1 protocol and as described by Asperger et al. and Wagner and Stessl (Asperger et al., 2001; ISO, 2017a; Wagner & Stessl, 2021).

Isolates were selected from the collection if they met the following inclusion criteria:

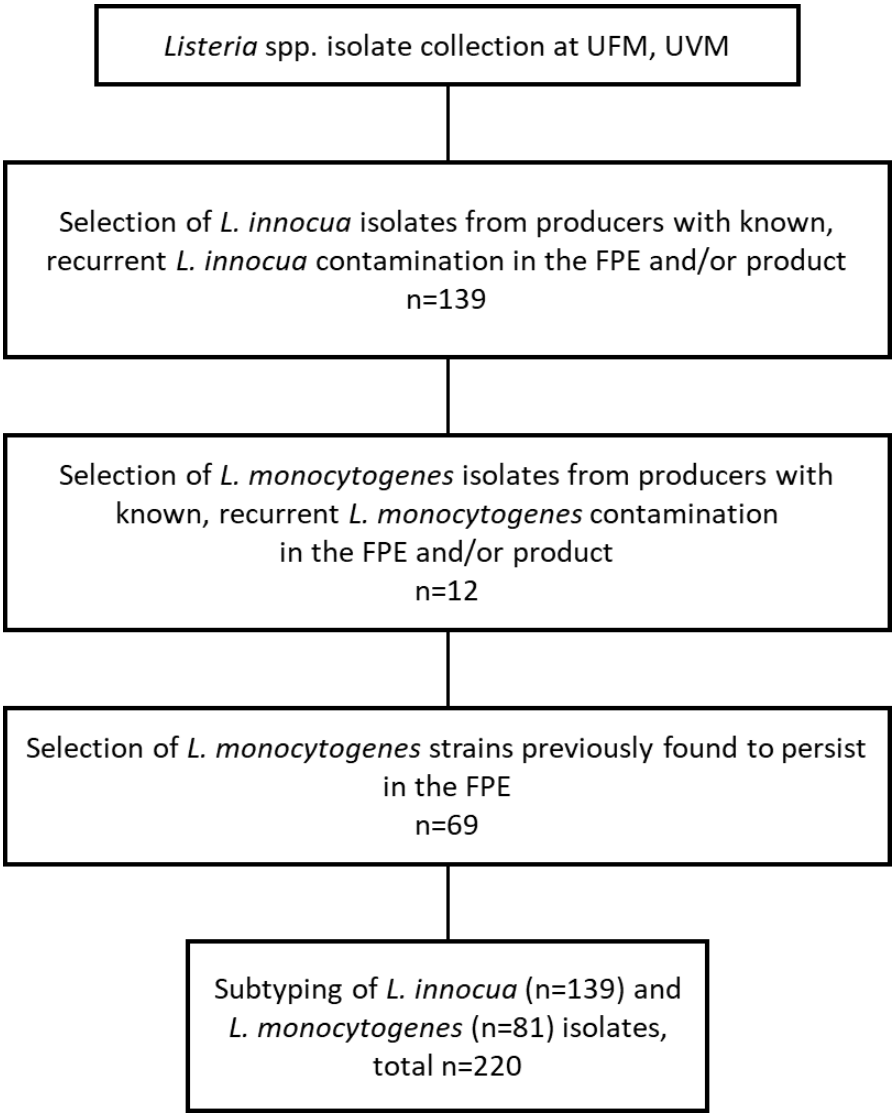
- In a first step, isolates from cheese producers with a history of *L. innocua* and *L. monocytogenes* contamination in the processing environment were chosen.
- In a further step, *L. monocytogenes* strains previously found to persist in the processing environment were added to the set of isolates (Stessl et al., 2014).

In total 139 *L. innocua* and 81 *L. monocytogenes* isolates from five Austrian cheese-producing facilities were chosen for further analysis. A detailed schematic representation of the selection process of *L. innocua* and *L. monocytogenes* isolates for this study is shown in Figure 2.

The selected isolates originated from samples analysed between the years 1987 and 2010 from five Austrian cheese production companies (Producers A-E). Producer A produced primarily grated, sliced semi-hard cheese and cheese ripening was another line of production, producers B and C each produced a range of white milk and fresh products as well as a semi-hard and hard cheese product line, producer D produced a range of semi-hard and red smear cheeses, and producer E produced sour milk cheese.

The isolates were obtained from cheese samples, raw material, product associated samples (i.e. surface ripening culture, enrichment), product associated liquids (i.e. smear, brine), from FCS as well as from NFCS, and environmental liquids samples (i.e. floor water, drain water).

Figure 2: Selection process of *L. innocua* and *L. monocytogenes* isolates obtained from five Austrian dairy and cheese producers



Abbreviations: UFM: Unit of Food Microbiology; UVM: University of Veterinary Medicine Vienna, Austria.

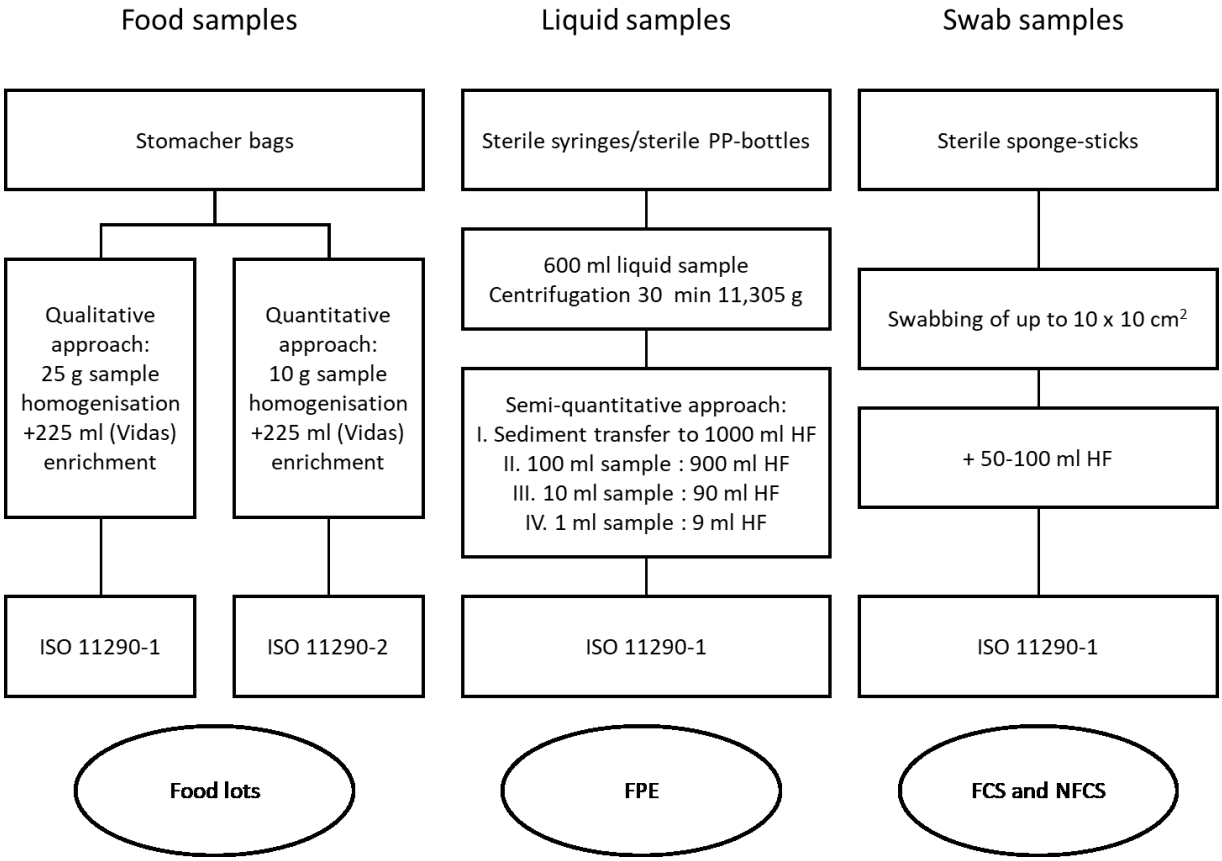
3.3. Culture based detection (Paper 1 and 2)

Swab samples were enriched in 50-100 ml of HF (Biokar Diagnostics, Beauvais Cedex, France) and analysed according to the ISO 11290-1 protocol (ISO, 2017a). Liquid samples were centrifuged at 11,305 g for 30 minutes (Beckman Coulter GmbH, Krefeld, Germany). The sediment was transferred to 1000 ml of

HF. The liquid samples were also investigated semi-quantitatively following the protocol for *Listeria* spp. detection of the Austrian *Listeria* Monitoring at the Unit of Food Microbiology (University of Veterinary Medicine Vienna, Austria) (Asperger et al., 2001; Wagner & Stessl, 2021).

Therefore, 100 ml, 10 ml and 1 ml of each liquid sample were enriched 1:10 in HF. Following incubation for 24 h at 30 °C, 0.1 ml HF was transferred into 10 ml FF (Biokar Diagnostics) and incubated for 48 h at 37 °C. HF and FF enrichments were each streaked onto ALOA (Merck KgA, Darmstadt, Germany) and PALCAM agar (Biokar Diagnostics). The applied process for food, liquid and swab sample processing are shown in Figure 3.

Figure 3: Sample processing and *Listeria* spp. detection approach



In case of study phase I (Paper 1) raw materials and products were tested for *Listeria* spp. by the local district laboratory using a VIDAS® *Listeria* DUO test kit (bioMerieux, Marcy l'Etoile, France). Food lots found positive for

L. monocytogenes were further quantified applying the ISO 11290-2 enumeration on ALOA agar (ISO, 2017b).

In case of study phase II (Paper 2) *Listeria* spp. detection using the above described method was accomplished immediately after sampling and isolation between the years 1987 and 2010.

3.4. Isolation and confirmation of *Listeria* spp. (Paper 1 and 2)

Listeria spp. suspect colonies were collected from ALOA (turquoise with and without surrounding halos) or PALCAM agar (concave grey-green colonies surrounded by black halos) for further confirmation by PCR.

One *Listeria* spp. colony was dispersed in 100 µl 0.1 M Tris-HCl buffer (Sigma Aldrich, St. Louis, Missouri, USA). Additionally, the whole agar surface was swabbed and dispersed in 1 ml 0.1 M Tris-HCl buffer (Sigma Aldrich). A DNA isolation protocol using Chelex® 100-Resin (BioRad, Hercules, California, USA) described by Walsh et al. was followed on the bacterial suspensions prior to PCR (Walsh et al., 1991). *Listeria* species were confirmed by a multiplex PCR according to Bubert et al., targeting the invasion-associated protein p60 (*iap*) gene. The *iap* genes within the species *Listeria* have conserved gene portions at the 5' and 3' ends. However, the internal gene portions are species-specific and therefore allow for the differentiation of *L. monocytogenes*, *L. innocua*, *L. grayi*, and the three grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* (Bubert et al., 1999). The species-specific PCR-amplicons were subsequently distinguished by size comparison in agarose gel electrophoresis. For this purpose the gel was prepared by dissolving 1.5 g of agarose (Sigma Aldrich) in 100 ml 1 x Tris-Borat EDTA buffer (TBE; Sigma Aldrich) over heat and 3.5 µl of SYBR Safe (Invitrogen, Lofer, Austria) were mixed in for staining. The amplicons (10 µl), together with 3 µl of loading buffer (33% glycerine, 0.07% bromophenol blue; MBI Fermentas, St. Leon-Rot, Germany) were applied to the gel slots. The agarose gel was subjected to electrophoresis for 30 minutes at 120 V. Bands were visualised by ultraviolet light using Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, California, USA).

In study phase II (Paper 2) the biochemical profile of the *L. innocua* and *L. monocytogenes* isolates was determined using the API®-*Listeria* system (Biomérieux, Marcy l'Etoile, France).

3.5. Subtyping and characterisation of *L. monocytogens* and *L. innocua*

The isolates were serogrouped by PCR and subjected to PFGE and MLST. The following subsections detail the subtyping techniques used and describe the further analyses performed to characterise the geno- and phenotype of the recovered *L. monocytogenes* and *L. innocua* isolates.

3.5.1. *L. monocytogenes* serogroup determination (Paper 1 and 2)

L. monocytogenes serogroups were determined by a multiplex PCR according to Doumith et al. This PCR allows for differentiation between the four major *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c and 4b by separating them into four distinct groups. Serovars 1/2a and 3a form group 1 with only the *lmo0737* DNA fragment being amplified. Group 2 comprises serovars 1/2c and 3c with the DNA fragments *lmo0737* and *lmo1118* being amplified. Group 3 comprises serovars 1/2b, 3b, 7 (only open reading frame *[ORF]2819* DNA fragment amplified) and group 4 is made up of serovars 4b, 4d and 4e (with *ORF2819* and *ORF2110* amplified). As an internal amplification control, *prs*, which is specific to all *Listeria* species was used (Doumith et al., 2004a).

3.5.2. PFGE (Paper 1 and 2)

PFGE subtyping of *L. innocua* and *L. monocytogenes* strains was performed according to the latest PulseNet protocol (CDC, 2017). An overnight culture of *L. innocua* and *L. monocytogenes* strains was grown on Tryptic Soy Agar with 6% yeast (TSA-Y), (Merck KgA) at 37 °C. Culture material was picked from the agar plates and adjusted to an optical density (OD) of 1.00 in 2 ml Tris-EDTA (TE; Sigma-Aldrich). To fabricate plugs, 40 µl of lysozyme (Sigma-Aldrich) were mixed with 400 µl of cell suspension (10 mg/ml) and incubated at 56 °C for 30 minutes. Then 20 µl of Proteinase K (20 mg/ml; Lonza Group Ltd. , Basel, Switzerland) were added and mixed with 400 µl melted 1% SeaKem Gold agarose (Lonza Group Ltd.). The mixture was pippeted into plug mould wells and solidified at room temperature for 20 minutes. To make the bacterial DNA inside the plugs available for enzyme digest, the bacterial cells were disrupted in a cell lysis step. The agarose plugs were lysed overnight in 5 ml of cell lysis buffer (50 mM Tris : 50 mM

EDTA, pH 8.0 + 1% Sarcosyl; Sigma-Aldrich) containing 25 µl Proteinase K (stock solution 20 mg/ml) in a shaking (175 rpm) water bath at 56 °C. To remove proteinase K, cell debris, lytic enzymes or proteases which may interfere with DNA digestion, the plugs were washed at 50 °C twice with 10 ml sterile grade water (Mayrhofer Pharmazeutika, Leonding, Austria) and three times with TE buffer (10mM Tris : 1 mM EDTA, pH 8.0; Sigma-Aldrich). The plugs were stored in the refrigerator in 5 ml TE buffer.

DNA macrorestriction digest was accomplished with 50 U *Asc*I (Thermo Fisher Scientific Inc.) at 37 °C and with 50 U *Apa*I (Thermo Fisher Scientific Inc.) at 30 °C for 4 h per plug. *Salmonella* Braenderup isolate H9812 was used as a size standard to enable the establishment of reference positions within the pattern and to allow normalisation of different images of the PFGE patterns in the analysis software. DNA macrorestriction digest of the size standard strain was accomplished using 50 U *Xba*I (Thermo Fisher Scientific Inc.) and incubation at 37 °C for 4 h.

Restricted DNA was separated in a 1% SeaKem Gold agarose gel (Lonza Group Ltd, Basel, Switzerland) in 0.5 × TBE at 6 V/cm in a CHEF-DR® III Pulsed Field Electrophoresis System (Bio-Rad Laboratories). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and an included angle of 120° was applied for 22-22.5 h. The gel was stained with ethidium bromide (Sigma Aldrich) and digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.). The Tag Image File Format (TIFF) images were compared using BioNumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium), and normalised using the PFGE global standard *Salmonella* Braenderup isolate H9812. Pattern clustering was performed using algorithms within Fingerprinting II Cluster Analysis. Specifically, the unweighted pair group method using arithmetic averages (UPGMA) and the Dice correlation coefficient was applied with a position tolerance of 1.0%. In order to identify indistinguishable PFGE types, a Dice coefficient similarity of 100% was used. PFGE patterns with no fragment difference were considered indistinguishable and patterns with two to three fragment differences were interpreted as closely related PFGE types (Tenover et al., 1995).

3.5.3. **MLST (Paper 1 and 2)**

Nucleotide differences of the seven housekeeping loci *acbZ, lmo2752* (ABC transporter), *bglA, lmo0319* (betaglucosidase), *cat, lmo2785* (catalase), *dapE, lmo0265* (succinyl diaminopimelate desuccinylase), *dat, lmo1617* (D-amino acid aminotransferase), *ldh, lmo0210* (L-lactate dehydrogenase), and *lhkA, lmo1508* (histidine kinase) were analysed by MLST according to Ragon et al. (Ragon et al., 2008). Primers used and PCR amplification conditions can also be found at the Institut Pasteur MLST dedicated website (https://bigsdb.pasteur.fr/listeria/primers-used/; accessed on: 4 March 2022). Amplified products were sequenced using primers with universal sequencing tails (*oF*: GTT TTC CCA GTC ACG ACG TTG TA; *oR*: TTG TGA GCG GAT AAC AAT TTC; LGC Genomics, Berlin, Germany). Allele-specific sequences were submitted to the Institute Pasteur sequence and profile database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef; accessed on: 4 March 2022).

Different sequences for each of the seven housekeeping genes were assigned to allelic numbers, which were combined to form an allele profile and subsequently used to determine STs. The STs of *L. monocytogenes* and *L. innocua* determined in this study were compared with MLST profiles stored in the Institute Pasteur MLST database to assess their global relevance and previous isolation sources (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates; accessed on: 4 March 2022).

In study phase I (Paper I) allelic profile data was subjected to a split decomposition analysis based on the seven housekeeping genes analysed, using a web version of SplitsTree (Huson & Bryant, 2006).

3.5.4. **Screening for SSIs (Paper 1 and 2)**

Sporadically and recurrently isolated *L. monocytogenes* strains (n=15) were screened for the presence of the SSI-1 in study phase I. In study phase II *L. innocua* and *L. monocytogenes* strains were screened for SSI-1 and SSI-2 according to Ryan et al. and Hein et al. SSI-1 is a 9.7 kb fragment and comprises five *L. monocytogenes* EGDe genes: *lmo0444, lmo0445, lmo0446* (*pva*), *lmo0447* (*gadD1*) and *lmo0448* (*gadT1*). The corresponding genomic region in

L. monocytogenes strains lacking the SSI (SSI-1$^-$; e.g. in *L. monocytogenes* F2365) is an ORF transcribed in the opposite direction of 1.1 kb length. SSI-2 is a 2.2 kb fragment and consists of the SSI-1 homologous *L. innocua lin0464* and *lin0465* genes (Harter et al., 2017; Hein et al., 2011; Ryan et al., 2010).

Primers *lmo0443* Fwd and *lmo0449* Rev were used to amplify the target region (Ryan et al., 2010). PCR for the detection of SSI-1$^+$ and SSI-1$^-$ was performed using 0.2 µM of each primer, 2 mM MgCl2, 1 mM deoxynucleoside triphosphates (dNTPs), 2.5 U long range DNA polymerase, 10x PCR buffer, diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific Inc.) with 2 µL DNA template in a final volume of 25 µL. PCR for the detection of SSI-2 slightly differed from the SSI-1 PCR reaction: 1 U Platinum Taq DNA Polymerase and 1 µL DNA template in a final reaction volume of 25 µL was used. PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). PCR products were subjected to gel electrophoresis on a 1% agarose gel containing 1x Tris-Acetate-EDTA (TAE) buffer and SYBR Safe (Paper 1) and 1.5% agarose gel containing 0.5 × TBE buffer and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, Pennsylvania, USA) (Paper 2). The DNA ladders Thermo Scientific™ GeneRuler™ 100 bp and 1 kb plus (Thermo Fisher Scientific Inc.) were applied for sizing.

3.5.5. **Disinfectant and cleaner susceptibility testing (Paper 1 and 2)**

The MIC of various disinfecting agents and industrial cleaners against six recurrently isolated *L. monocytogenes* in study phase I (Paper 1) and ten recurrently isolated *L. innocua* and *L. monocytogenes* in study phase II (Paper 2) was determined by an agar spotting test according to Mereghetti et al. (Mereghetti et al., 2000). *L. monocytogenes* and *L. innocua* were grown on Mueller–Hinton agar at 37 °C overnight (Thermofisher, Oxoid Ltd., Hampshire, UK). Bacterial suspension was adjusted to a turbidity of 0.5 McFarland U and 5 µL of the suspension were spotted onto Mueller– Hinton agar (7.5 × 10^5 cfu/spot), containing the disinfectant or cleaner to be tested.

Disinfectant compounds tested in study phase I included peracetic acid (PAA), BC, hydrogen peroxide (H_2O_2) (all Sigma-Aldrich, St. Louis, Missouri, USA),

multiple composite disinfectants Prodesan PE 15 (composition: H_2O_2, acetic and peracetic acid) and Weiquat (QAC) and industrial cleaners Citrosan SR (acidic), Rimalkan SR mild (alkaline) (the latter four Tensid Chemie, Muggensturm, Germany). Concentrations tested ranged from 31.3 to 2500 mg/l for PAA, H_2O_2, Prodesan PE 15, and Citrosan SR, from 3.9 to 2500 mg/l for BC and Weiquat I, and from 1250 to 25,000 mg/l for Rimalkan SR mild. This included working concentrations of disinfectants and cleaners applied in the cheese processing facility.

In phase II of the study the following disinfectants were used for MIC determination: PAA, BC, H_2O_2, sodium hypochlorite (NaOCl) and isopropanol (all Sigma-Aldrich). Concentration ranges of the disinfectants tested were 31.3–1000 mg/L for PAA and H_2O_2, 0.5–1000 mg/L for BC and 125–10,000 mg/L for NaOCl.

Agar plates were incubated at 37 °C for 24–48 h. The MIC was evaluated following incubation and recorded as the lowest concentration of the tested disinfectant or cleaner preventing growth of the spotted bacteria. All experiments were carried out in duplicates. Mean MIC values were calculated in Excel (Microsoft Corporation, Redmond, Washington, USA).

3.5.6. **PCR-RFLP (Paper 1)**

A PCR- RFLP method for the detection of *L. monocytogenes* point mutations in the 733 bp fragment *inlA* gene was performed on a selection of 15 *L. monocytogenes* isolates according to Rousseaux et al. (Rousseaux et al., 2004). This PCR-RFLP method allows the identification of *L. monocytogenes* strains that express a truncated *inlA*, which hinders adhesion to epithelial cells. Therefore, 1 µl of amplified DNA was digested for 1 h with 10 U of the restriction enzyme *Alu*I (Thermo Fisher Scientific Inc.). An inactivation step was performed after 20 minutes at 65 °C on a Thermomixer (Eppendorf Austria GmbH, Vienna, Austria). The restricted fragments were electrophoresed on a 2% agarose gel (Sigma Aldrich) for 1.5 h in 1 x TBE buffer (Rotiphorese®, Carl Roth GmbH + Co. KG., Karlsruhe, Germany) with 3.5 µl of SYBR Safe (Invitrogen) for staining. Bands were visualised by ultraviolet light using Gel Doc 2000 (Bio-Rad Laboratories, Inc.).

3.5.7. **Artificial *L. monocytogenes* contamination experiments in fresh cheese (Paper 1)**

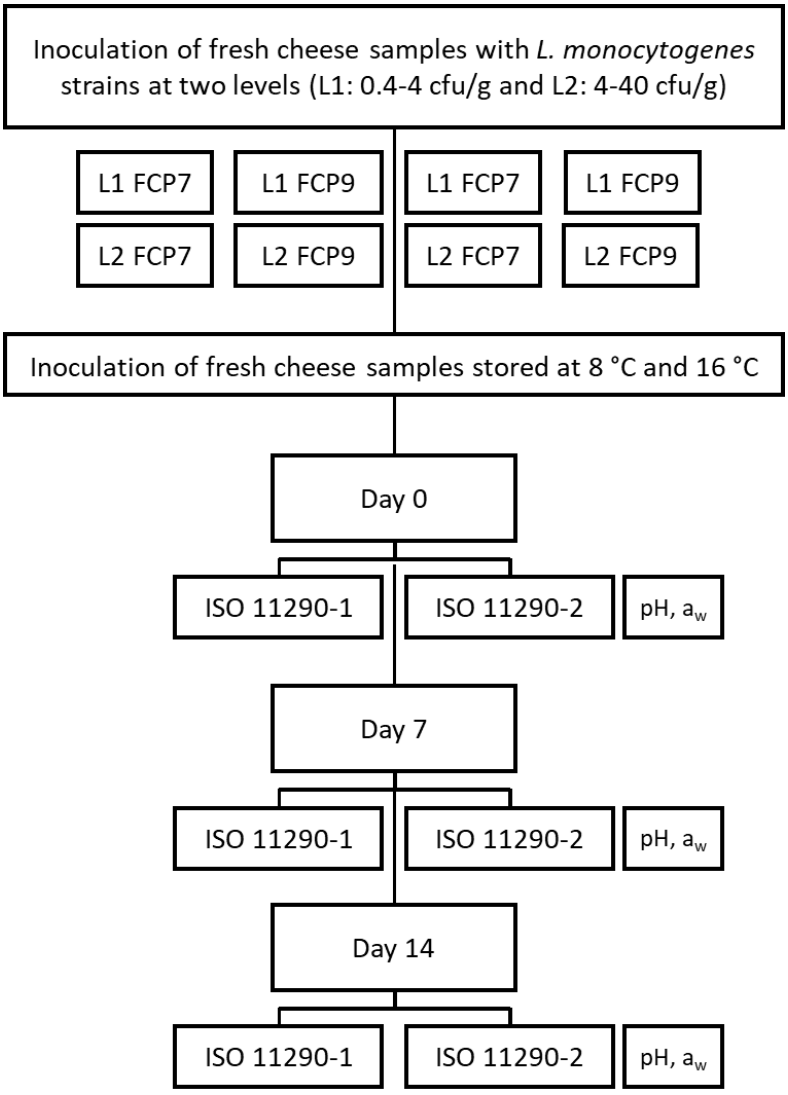
When in doubt, determining whether a RTE product is unable or able to support the growth of *L. monocytogenes* is important, because different regulatory limits apply to either category according to EC regulation No. 2073/2005 (see Table 4). Fresh cheese may support the growth of *L. monocytogenes* because of pH-values ≥ 5.0 and a_w ≥ 0.94. To further test this hypothesis, fresh cheese was artificially inoculated to investigate the ability of *L. monocytogenes* to survive and grow in this matrix.

The cheese samples were provided by the manufacturer and were tested negative for the presence of *Listeria* spp. before inoculation according to ISO 11290-1 and 2 (ISO, 2017a, 2017b). A graphical overview of the experiment is given in Figure 4. For inoculation of the fresh cheese *L. monocytogenes* strains were grown in Brain Heart Infusion (BHI; Merck KgA) and a serial dilution in sterile Ringer's solution was prepared up to dilution 10^{-7}. Fresh cheese samples were inoculated with two different spike levels (level 1: 0.4-4 cfu/g and level 2: 4-40 cfu/g) of two different *L. monocytogenes* strains (FCP7, PCR serogroup 1/2b, 3b, ST5 and FCP9, PCR serogroup 1/2c, 3c, ST9) and longitudinally (at days 0, 7, 14) surveyed for the presence/absence of *L. monocytogenes*. The inoculated fresh cheese batches were incubated at 8 °C and 16 °C respectively throughout the experiment.

At days 0, 7 and 14 of the experiment the pH (pH 526, WTW, Weilheim, Germany) and the a_w (LabMaster-aw, Novasina AG, Lachen, Switzerland) of the cheese samples was recorded. The samples were investigated following ISO 11290-2 protocol (ISO, 2017b) by plating each 1 ml of initial suspension (1:10) onto three plates of ALOA agar at days 0, 7 and 14. In addition; the ISO 11290-1 protocol was followed for qualitative investigation of the samples (ISO, 2017a): 25 g were diluted with 225 ml HF and homogenised for 180 seconds in a Stomacher 400 (Seward Inc., West Sussex, UK).

The samples were incubated for 24 h at 30 °C and subsequently 0.1 ml of the primary enrichments were transferred to 10 ml FF and incubated for 48 h at 37 °C.

Figure 4: Experimental procedure for the determination of *L. monocytogenes* growth potential in fresh cheese



Loop inoculation of ALOA and PALCAM agar was carried out after 24 h of HF and 48 h of FF enrichment. Presumptive *L. monocytogenes* colonies were subjected to DNA extraction and PCR (targeting the *iap* gene) confirmation according to Bubert et al. (see chapter 3.4.) (Bubert et al., 1999). The experiments were performed in duplicates.

3.5.8. **Calculations (Paper 2)**

To quantitatively assess the discriminatory ability of the subtyping methods used, the Simpson's index of diversity was calculated for PFGE and MLST with the Comparing Partitions online tool (http://www.comparingpartitions.info/; accessed

on: 4 March 2022). The strength of relationship between the variables i) *Listeria* species (*L. innocua, L. monocytogenes*), ii) rhamnose utilisation (yes/no) and iii) the isolation frequency (sporadic/persistent) was assessed by calculating the association coefficient, Cramer's V (Microsoft Excel 2010, Microsoft Corp., Redmond, Washington, USA).

4. RESULTS AND DISCUSSION

In summary, the studies conducted within this thesis demonstrate i) the relevance and benefit of FPE monitoring in dairy processing to identify growth niches and contamination patterns and therefore enable targeted *L. monocytogenes* control, ii) various sources of *L. monocytogenes* contamination in cheese production (raw material, construction works, uncontrolled personnel movement, contaminated equipment), iii) that it is possible to reduce *L. monocytogenes* diversity and presence in the FPE by systematic sampling, subtyping and by applying adequate control measures and staff awareness, iv) the relevance of *L. innocua* presence in cheese processing, v) the value of using different subtyping methods to gain insight into *L. monocytogenes* - and the comparably seldom studied non-pathogenic *L. innocua* – contamination patterns and strain properties, which might affect the adaptability of the bacteria to the dairy processing environment.

4.1. *L. monocytogenes* contamination routes in the dairy processing environment (Paper 1)

Previous studies have documented *L. monocytogenes* contamination for a limited period of time in dairy farm settings (Castro et al., 2018; Dalzini et al., 2016; Fox et al., 2011a; Papić et al., 2019; Rodriguez et al., 2021; Rossmanith et al., 2013; Schoder et al., 2011; Tahoun et al., 2017) or in dairy processing environments (Barancelli et al., 2014; Ibba et al., 2013; S. H. I. Lee et al., 2017; Rodríguez-López et al., 2019). Some studies have focused on investigating the processing of one specific product e.g. the production of kaymak, PDO Gorgonzola or Taleggio (Tirloni et al. 2020; Filipello et al. 2017; Kara & Aslan 2021). In a number of studies the aim was to document *L. monocytogenes* occurrence in dairy FPEs, contamination patterns and to characterise strains, without providing recommendations to sanitise the affected FPE or implement further intervention or control measures (Leong et al., 2017; Oxaran et al., 2017; Spanu et al., 2015; Véghová et al., 2015).

To our knowledge, our study is the first to demonstrate the variation of *L. monocytogenes* contamination patterns in an Austrian dairy FPE over an extended period of time, i.e. over a three-year period. Based on the contamination

scenarios identified by subtyping methods, advice was given to the FBO, which evidently helped to reduce *L. monocytogenes* contamination.
The main objective of this study was to analyse the fluctuation of *L. monocytogenes* contamination in an Austrian cheese processing facility during 2010-2013 by sampling the FPE and subsequently employing subtyping and strain characterisation techniques.

Listeria spp. were detected in 21.9% (n=281) of the processing environment samples. *L. monocytogenes* was predominant and was recovered from 19.5% (n=251) of the samples. These results classify the investigated dairy processing facility as heavily contaminated compared to other dairy and cheese processing environments, where *L. monocytogenes* prevalence ranged between 0 and 26% (see Table 5).

Strains of serogroups 1/2b, 3b, 7 and 1/2a, 3a as well as 4b, 4d, 4e are often related to human listeriosis cases and outbreaks (Jensen et al. 2016; Amato et al. 2017; Leclercq et al., 2019a; Bundesministerium für Soziales, Gesundheit, Pflege und Konsumentenschutz, 2021). The latter serogroups were dominant in our dataset, with 67.4% of the isolates belonging to serogroup 1/2b, 3b, 7, 15.8% to serogroup 4b, 4d, 4e and 14.8% belonging to 1/2a, 3a.
Sporadic listeriosis cases as well as large-scale listeriosis outbreaks have been linked to *L. monocytogenes* 1/2b (Bergholz et al., 2018; Fan et al., 2019; McCollum et al., 2013; Pérez-Trallero et al., 2014).
In contrast to our findings, *L. monocytogenes* 1/2a was found to be the dominant serovar in several studies analysing isolates from foods and FPEs in different geographical locations (Ciolacu et al., 2015; Leong et al., 2017; Shimojima et al., 2016; S. Wu et al., 2015). As several studies from South America found a high prevalence of serovar 1/2b in FPEs, some authors have suggested a strain prevalence varying according to geographic location (Barría et al., 2020; Braga et al., 2017; Haubert et al., 2016). However, we suggest that FPE-specificities play a significant role in strain variation as well. This includes the type of raw material used and specific niches neglected during cleaning and disinfection, which are hotspots for product contamination and recontamination after pasteurisation.

PFGE analysis of the *L. monocytogenes* isolates resulted in 17 indistinguishable profiles (FCP1-12 including subtypes; typing with *Asc*I and *Apa*I), which correlated to nine STs (ST1, ST5, ST7, ST9, ST21, ST37, ST87, ST204 and ST570) determined by MLST.

Longitudinal monitoring of the dairy processing facility and cheese products for a period of three years revealed fluctuating patterns of *L. monocytogenes* contamination, which allowed classification into three phases:

i) At the beginning of the study in 2010, sporadic, low level (below 10 cfu/g) contamination of food lots was detected and samples from FCS, including fillers, yielded 10.8% *L. monocytogenes* positive results. PFGE analysis revealed up to eight predominant genotypes in the FPE, raw materials and cheese products. Of these, ST1 (pulsotype FCP1, serogroup 4b, 4d, 4e) was most prevalent in FCS, drains and floors and was also detected in shoe swab samples and cheese products.
The detection of ST1 throughout the FPE and especially in cheese lots was particularly concerning, as ST1 is strongly associated with human and animal infection and proved to be hypervirulent and hyperinvasive under experimental conditions (Gözel et al., 2019; Maury et al., 2016). For certain hypervirulent serovar 4b strains, among them CC1, a higher likelihood to cause invasive forms of listeriosis was shown with prolonged survival in vivo in liver and spleen (Vázquez-Boland et al., 2020). Maury et al. found a strong association of hypervirulent clones, especially CC1, with dairy products (Maury et al., 2019). ST1/CC1 is the most prevalent genotype worldwide and throughout recent years has been frequently involved in outbreaks and sporadic listeriosis cases (Ariza-Miguel et al., 2015; den Bakker et al., 2010c; Cabal et al., 2019; Moura et al., 2020; Institut Pasteur database: https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022).

ii) In 2011, a reduction of *L. monocytogenes* positive FCS samples was noticeable (from 10,8% to 4.0%), as well as a reduction of genotype diversity (from seven STs to five, namely ST1, ST5, ST9, ST87 and ST570). As demonstrated before, increasing the efforts in FPE monitoring may lead to heightened awareness of

L. monocytogenes presence and could therefore contribute to a reduction of *L. monocytogenes* prevalence in FPE (Leong et al., 2017; Zwietering et al., 2016). However, several studies have documented that this observational approach alone is not enough to ensure an effective *L. monocytogenes* control (D'Arrigo et al., 2020; Manios et al., 2015; Simonetti et al., 2021; Véghová et al., 2015; Vongkamjan et al., 2017). Previous studies have shown that intervention is warranted and proved successful in different FPEs by reducing the overall *L. monocytogenes* prevalence and strain diversity. For example, a reduction of *L. monocytogenes* strain diversity was achieved in a meat processing plant by modifying the disinfection routine (Ortiz et al., 2014), or in another case, by setting up strict hygiene barriers between raw meat handling and cooked meat processing areas (Fagerlund et al., 2020). *L. monocytogenes* contamination levels were successfully reduced in ham processing by lowering the a_w levels through drying of ham surfaces (Prencipe et al., 2012). *L. monocytogenes* prevalence decreased in the FPE as well as in finished products of a smoked fish plant through plant specific intervention control strategies, such as improved sanitation and employee training (Lappi et al., 2004). A decrease of *L. monocytogenes* occurrence in a mushroom processing facility was demonstrated by improving cleaning and sanitation procedures (Murugesan et al., 2015). *L. monocytogenes* was almost eliminated from an avocado processing plant by altering the ambient temperature in the plant and by restricting workers movements (Strydom et al., 2016). *L. monocytogenes* presence was effectively reduced by increasing the monitoring of footbath sanitiser concentration in a farmstead dairy (Ho et al., 2007). During the second monitoring phase a building extension was constructed and reorganisation of the production lines and premises took place. During that time novel genotypes were introduced into the processing environment. The role of building reconstruction on FPE contamination with *L. monocytogenes* was investigated in a recent study, where the FPE of a meat processing facility was monitored for *L. monocytogenes* while reconstruction works were ongoing. The reconstruction works caused an increase in *L. monocytogenes* prevalence in the FPE, as well as a shift towards genotypes linked to listeriosis cases (ST1, ST6, ST8, ST11, ST77, ST398) (Stessl et al., 2020). Post-outbreak investigations of a major listeriosis episode in 2009/2010 in Austria and neighbouring countries, involving acid curd cheese, suggested construction works as a potential entry-

point of *L. monocytogenes* into the FPE (Schoder et al., 2010; Pichler et al., 2009). Our findings offer further evidence that renovation or construction works facilitate *L. monocytogenes* FPE contamination, thus posing a serious food safety risk. Subtyping revealed the introduction of three novel genotypes into the FPE: ST9 (FCP9, 1/2c, 3c), ST570 (FCP11, 1/2a, 3a) and ST5 (FCP7, 1/2b, 3b, 7). ST9 and ST570 were most probably introduced via raw materials. A number of studies have demonstrated the association of CC9 – which includes ST9 - with meat products (Alvarez-Molina et al., 2021; Fagerlund et al., 2020; Félix et al., 2018; Maury et al., 2019). Our findings are congruent with these reports, as we detected ST9 in bacon and final product samples containing bacon. Globally, CC9 is the fourth most prevalent clone (Institut Pasteur database: https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022). Along with CC121/ST121, CC9/ST9 strains are often detected in FPEs and were found to frequently harbour a truncation in the virulence gene *inlA*, resulting in lower virulence but higher adaptability to environments outside the host, thus deemed hypovirulent (Guidi et al., 2021; Pérez-Baltar et al., 2021; van Stelten et al., 2010; Maury et al., 2016). Beside a truncated *inlA*, we could detect SSI-1 in the ST9 strain, a 9.7 kb genetic region, reportedly responsible for adaptation of *L. monocytogenes* to various FPE stresses, such as a low pH and high salt concentrations (Ryan et al., 2010).

At the time of publication in 2014, ST570 was a novel sequence type and has since been detected only rarely (Caruso et al., 2020; Painset et al., 2019). As ST570 was isolated from plant-based raw material, associated raw material processing areas and final food lots, introduction into the FPE via raw material is highly likely. However, this strain was successfully eradicated following the implementation of more rigorous hygiene measures. Our results suggest that the manufacture of a wide range of products implying a broad palette of incoming raw materials and complex processing lines are factors influencing the risk of *L. monocytogenes* contamination in a dairy processing environment and subsequently the final product. Another genotype, ST5 (FCP7, 1/2b, 3b, 7, SSI-1^{+}), was first isolated in the second study phase (2011) during ongoing construction works, in drain water samples. CC5/ST5 is among the ten most frequently isolated allelic profiles worldwide (Painset et al., 2019; Institut Pasteur database: https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022), and was

associated with several US outbreaks, e.g. caused by ice cream (Y. Chen et al., 2017b), cantaloupe (Lomonaco et al., 2013) and stone fruit (B. R. Jackson et al., 2015). A previous study in China found a large proportion of ST5-caused infections under investigation to be pregnancy-associated, all of which resulted in fetal loss or stillbirth (X. Zhang et al., 2019). With onset in the early 2000s, ST5 is found with increased frequency in human clinical samples and is also commonly isolated from the FPE and food-derived samples (Bergholz et al., 2018; Kurpas et al., 2020; Maury et al., 2016; G. Wang et al., 2015). During 2011, ST5 (FCP7) became the dominant genotype (detected in 74.6% of *L. monocytogenes* positive samples). ST5 (FCP7) spread throughout the processing environment, most probably facilitated by the ongoing construction works, where additional traffic by workers and a disregard of hygiene barriers occurred and established persistence in the dairy processing facility, with repeated detection in cheese products as well.

iii) In the third and last study phase, in 2012, following further improvements in hygiene management and staff training, the strain heterogeneity was significantly decreased, from initially seven genotypes, to the detection of the predominant genotype ST5 (FCP7, 1/2b, 3b, 7, SSI-1^{+}, detected in 95.2% of *L. monocytogenes* positive samples), and sporadic detection of ST204 (FCP12, 1/2a, 3a, SSI-1^{+}, truncated *inlA*) in drains and on floors. ST204 strains have been isolated from clinical, food and environmental sources and therefore were suggested to adapt to a wide range of niches (Fox et al., 2016). Persistence of ST204 strains in the dairy FPE was demonstrated recently (Stessl et al., 2014). Interestingly, in another study, the first isolate detected in a newly established dairy plant was characterised as ST204 (Melero et al., 2019b).

The strains detected in *L. monocytogenes* positive samples during this last study phase were able to survive despite enhanced hygiene and sanitation measures. Muhterem-Uyar et al. further investigated predominant ST5 strains and sporadically occurring strains from the present study and found that *L. monocytogenes* strains detected in the last phase of the study, ST5 and ST204 shared the same plasmids, most likely beneficial for the survival in the FPE and also harbouring the SSI-1, conferring acid and salt adaptation properties. In addition, a *bcrABC* resistance cassette against QACs was detected in the persistent ST5 strains, which yielded higher MICs to QACs in our investigation

compared to other analysed strains (Muhterem-Uyar et al., 2018; Schmitz-Esser et al., 2021).

L. monocytogenes contamination is a highly dynamic process with patterns of introduction, elimination, recontamination and persistence. Our study suggests multiple factors contributing to the contamination of the dairy FPE with *L. monocytogenes*: biofilm formation and detachment, raw material originating from a wide geographic area, a wide product range, construction works on the premise, inadequate compartmentalisation of the FPE, lack of staff training and globally widely distributed STs. While complete elimination of *L. monocytogenes* from the FPE is not realistic, our study demonstrated that a reduction of *L. monocytogenes* prevalence and strain diversity is achievable through adequate FPE monitoring and plant-specific interventions.

4.2. Controlling *L. monocytogenes* in the dairy processing environment (Paper 1)

In addition to knowledge about *L. monocytogenes* contamination routes and dynamics in the FPE, an insight into the influence of different processing steps and prevailing environmental conditions along the processing chain is necessary to control *L. monocytogenes* in the FPE.

Besides the longitudinal monitoring and the documentation of the changing *L. monocytogenes* contamination patterns, this study aimed to provide guidance to the FBO on the control of *L. monocytogenes* contamination and to apply intervention strategies tailored to the production environment and the actual contamination scenario.

Sampling of the cheese production facility in 2010 was initiated to investigate where the sporadic, low level *L. monocytogenes* contamination in cheese samples originated from. Visiting the production facility is essential to gain insight into processing peculiarities and evaluate adherence to GMP (John et al., 2020; Lappi et al., 2004; Malley et al., 2015; Rotariu et al., 2014). We visited the cheese processing facility on several occasions during the sampling process and were able to observe and discuss the manufacturing steps and the applied hygiene and sanitation measures with the quality management team of the FBO.

When sampling was initiated in 2010, the cheese producing facility comprised two building compartments. In sampling phase two, at the end of 2010 and at beginning of 2011 an extension building was added to the manufacturing premise. Across the whole FPE, representative sampling points were chosen based on initial inspection of the FPE and critical control points identification, which were adapted as needed, based on the contamination scenario detected at the previous sampling occasion. We conducted extensive sampling and included samples from commonly recognised good indicator sites, such as FCS after heat-treatment of the product, which are hard-to-reach places where biofilm could build up (e.g. in the present facility: fillers), product-associated liquids (e.g. cheese smear, brine) as well as floors and drains (Asperger et al., 2001; Magdovitz et al., 2020; Simmons & Wiedmann, 2018; Wagner & Stessl, 2021; Zoellner et al., 2018).

At the initial stage of monitoring, overall samples from the production environment, as well as FCS samples were found to be positive for *L. monocytogenes* at a high rate (19.5% and 10.8% respectively) and 4.7% of filler samples were positive for *L. monocytogenes.* The sparse and infrequent food lot contamination together with the relatively high occurrence of *L. monocytogenes* in fillers was most likely contributable to “sloughing”, where biofilm, containing *L. monocytogenes,* amassed in the fillers and the release of biofilm during filler operation contaminated the final product. Sloughing occurs in the late growth stages of biofilm and is a form of passive cell dispersion initiated by forces, e.g. fluid shear or abrasion. It is characterised by a sudden detachment of large pieces of the biofilm or the entire biofilm, which can lead to contamination of food during processing (Alonso & Kabuki, 2019; Bremer et al., 2015).
The above described scenario underlines that food lot control is not sufficient as a *L. monocytogenes* control strategy. Furthermore, previous listeriosis outbreaks point towards the importance of continuous FPE monitoring as an opportunity to detect and control *L. monocytogenes* contamination within the FPE, before contaminated products reach retail or further processing (Elson et al., 2019; McLauchlin et al., 2021; EFSA: https://www.efsa.europa.eu/en/press/news/180703; accessed on: 4 March 2022).
The FBO raised another question, whether the fresh cheese produced was a RTE food that supports the growth of *L. monocytogenes* according to EU 2073/2005,

i.e. products that do not fall into the no-growth support category with a pH below 4.4 and an a_w below 0.92 OR a pH below 5.0 and an a_w below 0.94 OR when scientifically justified. In case of products which support the growth of *L. monocytogenes*, the FBO needs to ensure *L. monocytogenes* absence in 25 g before the food leaves the immediate control of the producer in addition to complying to the limit of 100 cfu/g during the shelf life of the product (EC, 2005).

The pH of the fresh cheese samples ranged from 4.7 to 5.6 and the water activity (a_W) ranged from 0.97 to 0.98. Judging from these chemico-physical properties, due to the recorded pH-values ≥ 5.0 and a_w ≥ 0.94, the fresh cheese studied may support the growth of *L. monocytogenes*. Although *L. monocytogenes* is psychrotrophic and multiplication at temperatures around 0 °C was observed in some strains, it was demonstrated that only a slight elevation of the refrigeration temperature can increase the growth rate of *L. monocytogenes* in various food matrices (Carpentier & Cerf, 2011; Castro et al., 2017; Leclair et al., 2019; Ziegler et al., 2019). Studies have shown, that RTE foods are often stored at temperatures well above the recommended 5 °C at retail level, during transport or in consumer's homes (Dumitrașcu et al., 2020; González et al., 2013; James et al., 2017; Morelli et al., 2012; Ndraha et al., 2018; Ricci et al., 2018). We therefore performed challenge experiments in fresh cheese with storage at inadequate cooling temperatures of 8 °C and 16 °C.

Our results show that *L. monocytogenes* was not only able to survive, but also to multiply at 8 °C as well as at 16 °C. Besides the parameters discussed above, the growth/no-growth ability of *L. monocytogenes* is also influenced by other factors such as the food's composition, microstructure, storage conditions and naturally occurring or added antimicrobial compounds (Ziegler et al., 2019; Hunt et al., 2018). Thus it is necessary to assess the ability of *L. monocytogenes* growth for each specific food product (Hunt et al., 2018). In the case of fresh cheese, a certain extent of growth inhibition might be achieved by the addition of bacteriocin producing starter cultures, e.g. *Enterococcus* spp. (Coelho et al., 2014) or *Lactococcus lactis* (Ribeiro et al., 2016), which could be added as an additional element in a multi-hurdle technology to control *L. monocytogenes* (Falardeau et al., 2021). As our case demonstrates, FBOs might not always be aware of their products' *L. monocytogenes* growth potential. Without further studies, this might

lead to an underestimation of the risk for *L. monocytogenes* growth and/or survival in the product, thus posing a potential risk to consumer safety.
At these initial stages of the study, compartmentalisation of the production areas was observably deficient and traffic of staff and equipment lacked clear rules. Non-adherence to GMP, such as uncontrolled movement between zones of raw material processing and post-processing areas, is a major contributor to post-processing contamination, persistence, highly contaminated FPEs in general and may cause contamination of the final product (Fox et al., 2015; John et al., 2020; Muhterem-Uyar et al., 2015).
Therefore, the high level of *L. monocytogenes* contamination in the FPE, in combination with the heterogeneity of genotypes detected at this stage of monitoring (see chapter 4.1.) were a clear reflection of prevailing production flaws. At the end of the first sampling phase, the awareness of *L. monocytogenes* contamination throughout the production areas was raised and enhanced hygiene measures were adopted. As a result, the occurrence of *L. monocytogenes* on FCS and NFCS (conveyor belts, transport trolleys, pallets, and tables) was significantly reduced (from 10.8% to 4.0% positive FCS samples and 8.7% to 1.6% positive NFCS samples). Furthermore, the number of genotypes detected by PFGE subtyping decreased from initially seven to five genotypes.

In the second phase of the study, at the end of 2010 and beginning of 2011 construction works were undertaken at the production plant and an additional building was erected, which subsequently served as a production area for yoghurt, fresh- and semi-hard cheese. Subtyping of *L. monocytogenes* isolates revealed the spread of contamination from the major production area (building I) to the newly erected building II. Construction works at and around a food processing plant pose a major challenge to maintain GMP and adequate hygiene measures. To limit the potential for cross-contamination, monitoring activities and sanitation should be increased throughout the phase of building work and additional hygiene barriers should be set up (FDA, 2017; New Zealand Government, 2017). The location of RTE production facilities often close to agriculturally used areas might play a role in the introduction of *L. monocytogenes* into the FPE as well, as soil, vegetation, water and manure naturally harbour *L. monocytogenes* (Gorski et al., 2016; Linke et al., 2014; Raschle et al., 2021; Rodriguez et al., 2021; Stea et al.,

2015; Strawn et al., 2013). This has to be considered especially during construction works, as increased movements from the outside to the inside of a plant and within the plant, pose a high risk of introducing *L. monocytogenes* from outside environmental residues and for further spread within the FPE (Jooste et al., 2016).

At the same time, while the number of different *L. monocytogenes* genotypes could be reduced, new genotypes were introduced to the processing environment, most likely through raw material and due to the ongoing construction works. While randomised microbiological control of received raw material is an integral part of GHP, it is essential to implement a one-way traffic system for produce, workers and equipment throughout the processing line that prevents cross-contamination from a pre-processing side to post-processing areas. Controlling the traffic within the FPE and clearly separating zones with different hygiene requirements has been recognised as an efficient measure to control *L. monocytogenes* contamination and persistence in the FPE (Aalto-Araneda et al., 2019; Alvarez-Ordóñez et al., 2018).

L. monocytogenes could also be detected in the packaging areas of building III. This suggests that critical control points in the packaging areas and other parts of the processing areas were still not sufficiently attended to. As the potential for *L. monocytogenes* survival on packaging materials and therewith cross-contamination in retail and at consumers' households has been demonstrated (Di Ciccio et al., 2020), our finding underline the importance of including packaging areas in a *Listeria* monitoring scheme of the FPE.

After further improvements to the existing hygiene measures had been implemented, i.e. strict compartmentalisation of the processing areas and traffic routes through the facility, the occurrence of *L. monocytogenes* on FCS was reduced further (to 1.7% positive samples). Moreover, a significant reduction of *L. monocytogenes* genotypes detected by PFGE subtyping was achieved, from initially seven to one predominant genotype and two sporadically detected genotypes in the last monitoring phase. Other authors have also recently noted the successful reduction of strain diversity in FPEs, highlighting the role of FPE monitoring and introduction of corrective measures according to the results derived from sampling (Jordan et al., 2013; Larivière-Gauthier et al., 2014).

At the site visits during the monitoring phases, it was noticed that advice was not entirely followed, due to the fact that a lot of water and steam were applied - in great part with pressure hoses - during cleaning and sanitation procedures, which accumulated on floors and in drains and the production environment was not allowed to dry off before recommencing production. These circumstances very likely were one of the main causes for the high positivity rate of samples from the FPE (drains, walls, doors and floors) throughout the three-year monitoring period (15.8% *L. monocytogenes* positive) and the frequent occurrence of *L. monocytogenes* on shoe swab samples (48.4%), which increased over the period under observation. Other authors have also recognised perpetually wet surfaces, especially floors, in the food production plants as the cause for increased occurrence of *Listeria* spp. in the FPE (El Hag et al., 2021; Estrada et al., 2020; Murugesan et al., 2015). Applying water or steam via high-pressure hoses can also contribute to cross-contamination by producing aerosols, which can travel through the air and reach FCS or food products (Berrang et al., 2013; Conficoni et al., 2016).

A further concern is that cleaners and disinfectants might become diluted by stagnant water, leading to an in-use-concentration lower than recommended and potentially compromising their efficacy. Several studies have found that exposure to sublethal concentrations of disinfectants was associated with *L. monocytogenes* tolerance and persistence in the FPE (Conficoni et al., 2016; Møretrø et al., 2017; Olszewska et al., 2016; Rodríguez-Melcón et al., 2019) as well as stress adaptation (Bansal et al., 2018; Yu et al., 2018). Results of the disinfectant susceptibility test showed, that all disinfectants used in the facility were effective against the tested *L. monocytogenes* strains. However, the persistent strains ST5 (FCP7) and ST204 (FCP12), which were isolated in the last monitoring phase, yielded higher MICs for QACs (BC and Weiquat) than the other tested strains.

Our results are in accordance with other authors, who noted that disinfectants and cleaners commonly applied in the food industry, when used as instructed by the manufacturer, are highly efficient against *L. monocytogenes* (Gerba, 2015; Hoelzer et al., 2012). However, bacterial biofilm and food residue removal through adequate cleaning prior to disinfectant application as well as thorough rinsing and optimally drying after the disinfection procedure is prerequisite to achieve the

desired bactericidal effect (Boucher et al., 2021; Cruz & Fletcher, 2012; Duze et al., 2021; Luque-Sastre et al., 2018; Overney et al., 2017). In a follow-up study, the genetic features of the dominant *L. monocytogenes* ST5 strains from the present study were investigated and compared to sporadically isolated genotypes from the same habitat. The authors found that ST5 and ST204, STs recovered in the last study phase, both harbour plasmids with the *bcrABC* cassette, which presumably allowed ST5 to establish at the processing environment and ST204 to emerge despite enhanced hygiene measures. Strains isolated in the first monitoring phase (ST1 and ST21 and the sporadically isolated ST37 strains) did not contain plasmids, a *bcrABC* cassette, or other genes or transposon conferring tolerance to disinfectants (Tn*6188*, *qacH*, *emrB*, *emrE*) (Muhterem-Uyar et al., 2018).

We therefore suggest that the strains tolerating higher disinfectant concentrations in comparison to other tested strains in our study might have undergone a niche-adaptation following high selective pressure in the form of disinfectant stress in their habitat (i.e. in floor drains).

External expert assessment of the GMP and hygiene measures in place on site is essential to help introducing improved hygiene and corrective sanitation management practices. FBO risk perception and staff behaviour play a vital role in *L. monocytogenes* contamination processes and a hygiene management program will only be successful if staff is well trained in sanitation measures relevant within the scope of their duties, understands the hygiene management program, and the vital role they play in hygiene management (Evans et al., 2021; Lakicevic & Nastasijevic, 2016). Besides observational assessment of the production processes, questionnaires can also help in establishing the level of awareness of FBO and staff on *L. monocytogenes* related hygiene measures (Aalto-Araneda et al., 2019; Magdovitz et al., 2020; Nayak & Waterson, 2017; Stadlmüller et al., 2017). Knowledge of the nature of contamination in an FPE and detected inadequacies in manufacturing and hygiene procedures, as described in this study, facilitate improvements in hygiene management to direct sanitation activities, facilitate communication with staff and implement corrective action.

Our study demonstrates that it is possible to reduce and control *L. monocytogenes* contamination in a heavily contaminated dairy processing facility and restrict contamination to areas less sensitive for product contamination (i.e. non-FCS). This can be achieved by a suitable monitoring scheme and adequate subtyping methods to detect routes of transmission, contamination sources and persistence and thereby raise awareness with the FBOs quality management team and allow the FBO to take corrective actions. In our study, corrective actions, namely restricting and channelling the movements of staff within the FPE, adapting the cleaning and disinfection routine and generally raising awareness for *L. monocytogenes* contamination with the staff, were effective in reducing the overall *L. monocytogenes* prevalence in the dairy processing plant. FBOs need to be aware how to translate current food regulation into practice, as negligent FPE and food lot monitoring or the selection of inadequate control points could lead to an underestimation of *L. monocytogenes* prevalence (Cabal et al., 2019; Centorotola et al., 2021). FBOs knowledge on the susceptibility of the produced foodstuffs for *L. monocytogenes* growth or survival is also indispensable to ensure consumer safety and to abide by regulations (Dufour, 2011).

We were able to identify a particular high abundance and persistence of *L. monocytogenes* ST5 (pulsotype FCP7) in the surveyed dairy processing facility. A variety of studies have identified geno- and phenotypic characteristics common to persistent *L. monocytogenes* strains (listed in Supplemental Table 3), which increased adaptation to FPE and renders persistence a major contributor to FPE and food contamination (Castro et al., 2021; Cherifi et al., 2020; Knudsen et al., 2017; Manso et al., 2019; Martínez-Suárez et al., 2016; Rodríguez-Campos et al., 2019; Stessl et al., 2020). However, it has to be considered, that persistence is a complex, multifactorial phenomenon, driven also by environmental influences. Moreover, limitations frequently apply, as only a small number of strains have been investigated in many of the aforementioned studies. A deeper insight into the underlying mechanisms of persistence and the options for controlling persistent *L. monocytogenes* contamination is therefore needed.

4.3. *L. innocua* and *L. monocytogenes* persistence in the dairy processing environment (Paper 2)

L. innocua is many times the most frequently detected species within the genus *Listeria* in food processing and preparation environments (Bouayad et al., 2015; Gwida et al., 2020; Simmons et al., 2014; Tirloni et al., 2020), foods (Akrami-Mohajeri et al., 2018; Arslan & Baytur, 2019) as well as on animal farms (Jamali et al., 2013; Q. Zhao et al., 2021). Although influencing factors and the underlying mechanism have not yet been revealed and are controversially discussed, dairy FPE contamination by persistent *L. monocytogenes* strains has been widely recognised and investigated, while strain characteristics and potential persistence of the non-pathogenic species *L. innocua* remain relatively uncharted (Almeida et al., 2013; Barría et al., 2020; Carpentier & Cerf, 2011; V. Ferreira et al., 2014; Guidi et al., 2021; Ibba et al., 2013; M. H. Larsen et al., 2014; Leong et al., 2017; Oxaran et al., 2017). Systematic monitoring of the FPE and adequate subtyping techniques to identify persistent *L. monocytogenes* strains has proven to be critical, due to the high risk for food lot contamination posed by persistent *L. monocytogenes* strains (Alvarez-Ordóñez et al., 2018; Stasiewicz et al., 2015; Véghová et al., 2017).

In our study we used molecular genetics and phenotypic subtyping methods with the aim to characterise recurrently isolated *L. innocua* and *L. monocytogenes* from five different Austrian cheese producers. Longitudinal observations of contamination dynamics in dairy processing environments over an extended period of time are seldom found in the present literature, and those that are existing have been focusing almost exclusively on *L. monocytogenes* (Alessandria et al., 2010; Almeida et al., 2013; Fox et al., 2011a; Ho et al., 2007; Ibba et al., 2013; Melero et al., 2019b; Muhterem-Uyar et al., 2015). Another objective of our research therefore was to determine and describe *L. innocua* contamination scenarios in the co-habitat of *L. monocytogenes* over an extended period of time.

The isolates included in this study (n=139 *L. innocua* and n=81 *L. monocytogenes*) were collected within the scope of the Austrian *Listeria* monitoring program from 1987 to 2010 and comprised samples from cheese products (24 *L. innocua* and 3 *L. monocytogenes*), product associated samples

and product associated liquids (100 *L. innocua* and 64 *L. monocytogenes*), the production environment (including swabs from FCS and NFCS and environmental liquid samples; 14 *L. innocua* and 14 *L. monocytogenes*), and one *L. innocua* isolate from raw milk.

Currently, there is no universal definition for the term persistence, meaning the recurrent isolation of clonal bacterial strains from the same location (e.g. within the FPE), identified by a suitable subtyping method (Belias et al., 2021; Forauer et al., 2021; Leong et al., 2017). In our study, we defined the following categories of *L. innocua* and *L. monocytogenes* isolates: i) recurrent: isolates with identical PFGE-types (*Asc*I digest) from dairy producers A-E detected on at least two sampling occasions, ii) persistent: isolates with identical PFGE types detected recurrently at producers A-E over a period of at least six months and iii) sporadic: isolates with unique PFGE-types (*Asc*I) detected once at producers A-E.

PFGE typing with *Asc*I yielded 33 individual profiles, 11 distinct *L. monocytogenes* and 22 distinct *L. innocua* profiles. In total, 27 STs were identified by MLST, with nine clonal complexes (CCs) and one singleton among *L. monocytogenes* and three CCs and 14 singletons among *L. innocua* isolates.

Comparison with sequences deposited in the Institut Paseur MLST database revealed seven novel *L. innocua* STs: ST1595 to ST1601 (https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022).

Most of the genotypes were detected only on one sampling occasion (i.e. sporadic; n=7 *L. monocytogenes* and n=17 *L. innocua* PFGE types), while several PFGE types were associated with either recurrent isolation over a short (n=3 *L. monocytogenes* and n=2 *L. innocua* PFGE types) or over a long period of time (i.e. persistent; n=4 *L. monocytogenes* and n=5 *L. innocua* PFGE types).

Persistent *L. monocytogenes* strains have been detected in dairy FPEs across the world and have been described extensively (Acciari et al., 2016; Barría et al., 2020; V. Ferreira et al., 2014; Leong et al., 2014; Oxaran et al., 2017). Melero et al. delineate the spread of *L. monocytogenes* and subsequent detection of persistent strains within one year in a newly built dairy processing facility (Melero et al., 2019b). In the latter study, *L. monocytogenes* 1/2a strains represented the most common serovar, which is in accordance with our findings of 95% serogroup 1/2a, 3a among the *L. monocytogenes* isolates investigated (the other isolates

were confirmed as serogroup 1/2b, 3b and 4b, 4d, 4e [n=3, 3.75% and n=2, 1.25% respectively]).
This is especially relevant since an increase in human listeriosis cases caused by *L. monocytogenes* 1/2a strains had been reported in the 2000s (Jensen et al., 2016; Mammina et al., 2013). Our results support the findings of various other studies, which found 1/2a strains to be highly abundant in various FPEs and food isolates (D'Arrigo et al., 2020; Jennison et al., 2017; Sonnier et al., 2018; Stessl et al., 2020). Several authors have suggested genetic and phenotypic factors responsible for the high prevalence of *L. monocytogenes* 1/2a strains in the FPE, especially an advantage in biofilm formation compared to serovar 4b strains (Huang et al., 2018; Keeney et al., 2018).

L. monocytogenes PFGE types M11/M7 (ST14) and M5 (ST121) were attributable to more than one producer, with M11/M7 (ST14) detected at producers A and D and M5 (ST121) isolated at producers B, D and E. Of the 22 *L. innocua* profiles, three profiles were attributable to more than one producer - in one case even to four different producers. IN4 (ST603) was detected once at producer C and recurrently in producer E, IN5 (ST1597) was found recurrently at producers A, C, D and E and IN6 (ST1599) was detected once at producer C and recurrently at producers A and D.
The detection of identical *L. monocytogenes* subtypes across different processing facilities and retail delis has been reported previously (Leong et al., 2014; Morganti et al., 2016; Stasiewicz et al., 2015). Chambel et al. investigated the processing environments of ewe and cow's milk cheese dairies in Portugal for the presence of *Listeria* spp. While they did not identify persistence of *Listeria* spp., they detected clonal types common to different dairies, predominantly among *L. innocua* types (Chambel et al., 2007).
Different scenarios have been suggested as a cause, e.g. habitat-specific colonisation or widespread global distribution favouring repeated re-introduction via raw materials or via other external sources (D'Arrigo et al., 2020; Martín et al., 2014).

We found co-colonisation of *L. innocua* and *L. monocytogenes* with varying degrees of heterogeneity in genotype occurrence and contamination patterns in

four of the five producers included in the study. Producers A, C and E harboured the greatest genotype heterogeneity (n=11, n=12 and n=9 individual genotypes). In contrast, in producer B, a persistent *L. monocytogenes* genotype M5 (ST121) was recurrently detected for a period of seven years, but no other *L. monocytogenes* or *Listeria* spp. was introduced during the monitoring period. In producer D, *L. innocua* and *L. monocytogenes* genotypes IN5 (ST1597) and M5 (ST121) were the significantly dominating genotypes (n=68/74 *L. innocua* and n=7/9 *L. monocytogenes* isolates respectively) detected over a period of six years and recurrently for four months. Studies have shown that *Listeria* spp. colonisation patterns in dairy processing environments vary from one processing facility to another and over time. Either *L. monocytogenes* or *L. innocua* might be predominately recovered in a FPE and previous studies demonstrated the dominance of one or more *L. monocytogenes* or *L. innocua* strains for several months and years as well as a variety of contamination scenarios (Leong et al., 2017; Muhterem-Uyar et al., 2015; Parisi et al., 2013; Tirloni et al., 2020). One particularly concerning contamination scenario is persistence of *L. monocytogenes* for several months or years, a widespread and well documented phenomenon in the dairy processing chain and frequent cause for food contamination (see Table 5).

The recurrent detection of a clonal *L. monocytogenes* population has been associated previously with a provenance from the same geographic area or adaptation to a certain type of food product (Bechtel & Gibbons, 2021). Furthermore, specific FPEs or niches within FPEs were mentioned in association with the recovery of the same genotypes (Antoci et al., 2019; Filipello et al., 2017; Finazzi et al., 2020; Guidi et al., 2021). Similarly, we found persistent *L. monocytogenes* as well as *L. innocua* strains, present in the dairy FPE for a period of up to eleven years. We found that the majority of those strains had adapted to specific niches within the FPE, namely brine, smear and in floor drains. *L. monocytogenes* genotypes most often detected were M5[B]=M5[D]=M5[E] (ST121, n=55/81) and M11[A]=M7[D] (ST14, n=12/81). These types were found to persist for seven years in cheese smear at producer B and for eleven years in drain water at producer A respectively. The same genotypes were also detected recurrently for a short period of time at producer D and once at producer E (M5) and sporadically in producer D in smear (M7). The *L. innocua* genotypes most

often detected were IN5[A]=IN5[C]=IN5[D]=IN5[E] (novel ST1597, n=94/139) and IN4[C]=IN4[E] (ST603, n=13/139). Genotype IN5 (ST1597) was found to persist in four different producers (A, C, D and E) for one – up to almost seven – years, where it was recurrently isolated from drain water, brine, cheese smear and cheese products. PFGE-type IN4 (ST603) was recurrently isolated from cheese smear in producer E and from non-food contact surfaces as well as the final cheese product and detected once at producer C, where it was isolated from hard cheese. Following the occurrence in cheese products, these genotypes could not be detected anymore at the affected producer hence had been successfully eliminated from the FPE. Similarly, *L. innocua* and *L. monocytogenes* genotypes found recurrently in the same producer for six months and above (ST14, ST637, ST1595, ST1597), were detected in product associated liquids (i.e. brine and smear). Recurrent isolation of the same genotype of up to eleven years strongly suggests niche-adaptation. Cross-contamination from these niches to the cheese surface (e.g. in the case of hard cheese) or from environmental contamination most probably via equipment (e.g. in the case of grated cheese) was observed. Our results support previous findings of cheese brine and smear acting as growth niches for *L. monocytogenes* and constituting a source for post-processing re- or cross-contamination events (Fox et al., 2014; Stessl et al., 2020). Including cheese brine and smear in the *Listeria* monitoring scheme is therefore recommended as a means of early indication of cheese contamination (Zangerl et al., 2021).

While we detected a number of *L. monocyotgenes* genotypes, which have been previously associated with human infections (namely ST1, ST3, ST7, ST59, ST398, ST403) (Institut Pasteur database: https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022), *L. monocytogenes* ST14 and ST121 were found recurrently over a timespan of seven and elven years suggesting adaptation to niches within the FPE. Both sequence types have frequently been reported to persist in different FPEs. ST14 was one of three genotypes found to persist in a meat processing facility in Slovakia and among the most commonly detected STs recovered from Finnish dairy farms (Véghová et al., 2017; Castro et al., 2021). ST121 was among the more frequently recovered genotypes from seafood facilities producing RTE foods in France and a newly established meat processing

facility (Alvarez-Molina et al., 2021; Palma et al., 2020). ST14 and ST121 were found to persist in a rabbit meat processing plant in Italy (Cesare et al., 2017). Further analysis of *L. monocytogenes* ST14 and ST121 strains isolated at the aforementioned plant revealed strain specificities, namely a pre-mature stop codon in *inlA* in ST121 strains, associated with reduced virulence and the presence of *qacH* and *cadA1C* genes, associated with adaptation to QAC-disinfectants and cadmium stress. Furthermore, a truncation in *actA* was detected in ST121, a gene involved in motility of *L. monocytogenes* and onset of biofilm formation. Congruently, ST14 was phenotypically confirmed to be a stronger former of biofilm than ST121 (Palma et al., 2017; Pasquali et al., 2018).
The most abundant *L. innocua* genotypes were ST603 and ST1597, which were recurrently detected for a period of almost six and almost seven years respectively. ST1597 was one of the *L. innocua* genotypes newly identified in our study and ST603 was previously described in connection with a human case of bacteraemia and an isolation from a natural wild animal feeding trough (Moura et al., 2019; Palacios-Gorba et al., 2021; Perrin et al., 2003).

To further elucidate strain characteristics of *L. monocytogenes* and *L. innocua* strains isolated from different dairy processing facilities and cheese products, we applied biochemical profiling, screening for presence/absence of SSIs (SSI-1 and SSI-2) and assessed the adaptation towards commonly used disinfectant compounds. Biochemical profiling was performed for *L. monocytogenes* (n=81) and *L. innocua* isolates (n = 124). While all *L. monocytogenes* isolates yielded a arylamidase (DIM) negative and rhamnose positive profile (API® profile 6510), three different profiles were obtained for the *L. innocua* isolates: the most common profile included a DIM and rhamnose positive reaction (n=124; API profile 7501), further isolates were DIM positive and rhamnose negative (n=13; API profile 7110) and DIM, D-ribose and D-tagatose positive (n=2; API® profile 7531). The power of association was determined between *L. innocua*, *L. monocytogenes*, rhamnose fermentation (yes/no), sporadic or persistent occurrence by calculating the Cramer's V. The association for "persistence and rhamnose positive" was highly significant ($p<0.01$), while only a weak association between the other variables was found. Typically, *L. monocytogenes* is able to acidify rhamnose, a deoxy sugar occurring in bacteria and plants, while *L. innocua* show variable rhamnose

utilisation (Allerberger, 2003; Giraud & Naismith, 2000). Atypical rhamnose-negative *L. monocytogenes* have been described previously and a growth disadvantage, potentially leading to a decreased ability to environmentally adapt and to an impaired host colonisation has been suggested for *L. monocytogenes* and *L. innocua* lacking *pdu* genes needed for 1,2-propanediol utilisation (Xue et al., 2008; Zeng et al., 2021a; Zeng et al., 2021b; Schardt et al., 2017). Investigations into the role of rhamnose utilisation by *L. monocytogenes* and the effect on virulence have been conducted; e.g. a subgroup of atypically avirulent *L. monocytogenes* lineage III strains deficient in rhamnose acidification was described by Liu et al., while Yin et al. investigated atypic *L. monocytogenes* strains lacking rhamnose utilisation genes and exhibiting a rhamnose negative phenotype (D. Liu et al., 2006b; Yin et al., 2019). We therefore advocate for further investigation of rhamnose fermentation ability of *L. monocytogenes* and *L. innocua* strains and the potential influence on strain behaviour in the FPE.

Screening of *L. innocua* and *L. monocytogenes* isolates for the SSI-1^{+} (9.7 kb fragment), SSI-1^{-} (F2365_0481 homologous gene; 1.1 kb fragment) and SSI-2^{+} (2.2 kb fragment) revealed presence of SSI-1 (SSI-1^{+}) in *L. monocytogenes* genotypes M6[D] (ST3), M8[A] (ST7), M10[C] (ST155), M3[E] (ST403), M11[A]=M7[D] (ST14) and presence of the 1.1 kb F2365_0481 homologous gene (SSI-1^{-}) in *L. monocytogenes* genotypes M9[C] (ST1), M4[E] (ST398), M12[A] (ST529) and M1[E] (ST59) and presence of SSI-2 (2.2 kb fragment) in *L. monocytogenes* ST121 (M2[E] and M5[B]=M5[D]=M5[E]). In accordance with our results, all tested ST155 as well as ST3, ST7 in a study characterising strains from various food sources from the Swiss National Reference Centre for Enteropathogenic Bacteria and *Listeria* were found to harbour SSI-1 (Ebner et al., 2015). Wagner and co-authors found that all ST155 (a sequence type frequently detected from clinical as well as food-associated samples) strains analysed, harboured SSI-1. The genetic islet has been suggested to confer a survival advantage in food matrices, FPE as well as in the human host due to tolerance towards a range of different stresses (Ryan et al., 2010; E. Wagner et al., 2020). We could also detect SSI-1 in the long-term persistent ST14 strain.

The presence of the SSI-2 had been associated with heightened alkaline and oxidative stress tolerance (Harter et al., 2017). SSI-2 was detected in all ST121

and *L. innocua* strains, which supports previously published study results (Centorotola et al., 2021; Toledo et al., 2018). Besides the occurrence of SSI-1 and SSI-2 in presumably FPE adapted strains, various geno- and phenotypic factors (e.g. plasmids, transposons, genetic islands, biofilm formation) have been suggested to facilitate *L. monocytogenes* adaptation and persistence in FPEs (see Supplemental Table 3). However, persistence is multi-faceted and not merely the result of geno- or phenotypic traits of a contaminant strain. Several major contributing factors to *L. monocytogenes* persistence in the FPE have been mentioned in literature: the survival in hard-to-access niches and harbourage sites is a key factor. For example, the survival in an FPE-niche was suggested as main contributor to persistence of a *L. monocytogenes* strain, implicated in a listeriosis outbreak caused by RTE meat products in Germany from 2013 to 2018 (Lüth et al., 2020). Niches can evolve in hard-to-clean equipment, with certain surfaces and materials favouring *L. monocytogenes* growth (Nyarko et al., 2018). Also, shortcomings in GMP and hygienic measures, including hygiene barrier-breach are important contributors to *L. monocytogenes* persistence previously identified (Belias et al., 2021; Castro et al., 2018; Leong et al., 2017; Nüesch-Inderbinen et al., 2021). Another reason for *Listeria* spp. persistence in the FPE can be inappropriate cleaning and disinfection (i.e. dilution of disinfectants when applied on wet or not sufficiently cleaned surfaces and inadequate cleaning and disinfectant routine), which may result in the adaptation to sublethal concentration of biocides (Martínez-Suárez et al., 2016; Møretrø et al., 2017). Detecting *L. innocua* and *L. monocytogenes*, which have lost the sensitivity towards biocides with techniques beyond genotypic subtyping methods is therefore important. We assessed the MIC towards disinfectant compounds commonly used in FPEs (PAA, BC, hydrogen peroxide and sodium hypochlorite) for four *L. monocytogenes* and six *L. innocua* recurrently isolated genotypes. MIC comparison between the genotypes investigated revealed adaptation to higher concentrations of BC in M5[B]=M5[D]=M5[E] (ST121), IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597), and IN2[E] (ST637) (1.3 to 2-fold higher; mean MIC 15.6 mg/L in comparison to 11.7 and 7.8 mg/L). *L. innocua* genotypes IN1[E] (ST1595) and IN2[E] (ST637) were adapted to higher concentrations of sodium hypochlorite (2.7-5.7-fold higher; mean MIC 10,000 mg/L in comparison to 1750-3750 mg/L). A slightly better adaptation to hydrogen peroxide was

determined for M5[B]=M5[D]=M5[E] (ST121) and M1[E] (ST59) (1.5-fold higher; 188 mg/L in comparison to 125 mg/L) while all tested *L. innocua* strains except IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597) and IN3[E] (ST1601) were adapted to higher concentrations of PAA (1.7-fold higher; 250 mg/L in comparison to 141 mg/L). Our results suggest that some strains have adapted to certain disinfectant compounds, frequently applied in the food industry. As a cause, exposure to sublethal concentrations of disinfectants due to inadequate application or the acquisition of resistance genes e.g. BC resistance genes such as the *bcrABC* cassette, *qac* genes, Tn*6188*, *EmrE* have previously been named (Gelbicova et al., 2021; Kovacevic et al., 2016; Minarovičová et al., 2018; Müller et al., 2013).
We strongly advocate to characterise *L. monocytogenes* as well as *L. innocua* isolates on a genetic level as well as phenotypically, to uncover the mechanisms behind the adaptive nature of *Listeria* spp.

4.4. The role of *L. innocua* in the dairy processing environment (Paper 2)

In various habitats including food, FPE and the natural environment, *L. innocua* is more frequently detected than the pathogenic *L.* monocytogenes (Rossi et al., 2020; Stea et al., 2015; H. C. Tsai et al., 2018; Q. Zhao et al., 2021). However, as a non-pathogenic member of the genus *Listeria*, *L. innocua* is rarely the focus of research. A few selected topics of relevance to food safety have been investigated previously: i) case descriptions of human and animal infections caused by *L. innocua* (Favaro et al., 2014; Perrin et al., 2003; Rocha et al., 2013; Walker et al., 1994), ii) atypical *L. innocua* isolated from foods and FPEs (Johnson et al., 2004; Milillo et al., 2012; Moreno et al., 2014, 2012; Rosimin et al., 2016; Volokhov et al., 2007), iii) the use of *L. innocua* as a surrogate for the pathogenic *L. monocytogenes* in strain behaviour studies e.g. challenge tests (Bonilauri et al., 2021; Giacometti et al., 2020; Hamilton et al., 2021; Michelon et al., 2016) or iv) growth advantage of *L. innocua* over *L. monocytogenes* in selective enrichment and agar media (Heir et al., 2018; Keys et al., 2013; Locatelli et al., 2017). The frequent presence of *L. innocua* in FPE, sometimes alongside the pathogenic *L. monocytogenes,* the knowledge gap on adaptation mechanisms and persistence in FPEs of this non-pathogenic *Listeria* species and the description of atypical *L. innocua* with intact virulence genes or haemolytic properties detected in FPE warrant further research on this species. With our study, we aimed to

contribute to a deeper knowledge of the role of *L. innocua* in dairy FPEs cohabited by *L. monocytogenes.*

Of the *L. innocua* isolates subtyped (n=139) PFGE profile IN5[A]=IN5[C]=IN5[D]=IN5[E], newly identified ST1597, was most often detected (68%, n=94/139), followed by IN4[C]=IN4[E], ST603 (9%, n=13/139).
Genotype IN5 was isolated from product associated liquids (brine and smear), drain water and cheese products in four different producers (A, C, D and E). In producers C and D, this genotype was recovered on multiple sampling occasions for 6.8 years and 6.2 years respectively. Genotype IN4 (ST603) was recovered in producer E, predominantly from cheese smear samples and on singular occasions from NFCS and the final cheese product over a period of 5.6 years. Other genotypes were recurrently isolated over periods ranging from 1.3 years (IN7[C], ST1085), six months (IN1[E], ST1595 and IN3[E], ST1601), five months (IN6[A]=IN6[C]=IN6[D], ST1599) and four months (IN2[E], ST637).
L. innocua ST603 (CC600), identified as persistent in our study, was previously recovered from different types of foods, silage, forest environment and an atypical case of human listeriosis (Palacios-Gorba et al., 2021; Perrin et al., 2003; Institut Pasteur database: https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates; accessed on: 4 March 2022). *L. innocua* ST1085 was isolated recently from a meat processing environment (Moura et al., 2019). *L. innocua* ST637 (CC140), recurrently isolated in a dairy processing facility, was isolated from foods and FPEs in the past (Institut Pasteur database: https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates; accessed on: 4 March 2022; L. Wu et al., 2021). The group of *L. innocua* CC140 includes the reference strain CLIP 11262, the first fully sequenced *L. innocua* genome which diverges from ST637 (*ldh* 192) by the *ldh* housekeeping gene (CLIP 11262: *ldh* 74; ST637: *ldh* 192) (Glaser et al., 2001; https://www.ncbi.nlm.nih.gov/genome/genomes/1024?; https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates; accessed on: 4 March 2022). Together with six *L. innocua* STs (ST1595 to ST1601), abundantly recovered persistent *L. innocua* ST1597 was newly deposited in the Institut Pasteur MLST database (https://bigsdb.pasteur.fr/listeria/; accessed on: 4 March 2022).

We found housekeeping genes common to *L. innocua* STs 605 and ST1085, the newly identified *L. innocua* ST1595, ST1597, ST1599 and ST1601 and *L. monocytogenes* genetic lineage III (*abcZ* 25, *bglA* 73, *dapE* 96, *dat* 45). Close relation to *L. monocytogenes* genetic lineage III, ST267 was established for the housekeeping gene *abcZ* 40 in *L. innocua* ST1600. Compared to genetic lineage I and II, *L. monocytogenes* lineage III strains are relatively rare and show a high number of recombination events (Y. H. L. Tsai et al., 2011; Zamudio et al., 2020). Furthermore, lineage III strains have been associated predominantly with animal clinical cases (Orsi et al., 2011). It has been suggested that *L. monocytogenes* serovar 4a, grouped into lineage III, might be an evolutionary intermediate between *L. monocytogenes* 1/2a, 4b and *L. innocua* (J. Chen et al., 2009a).
Despite the close genetic relatedness of certain *L. innocua* strains determined on the basis of *Listeria* housekeeping genes, we did not find atypical haemolytic *L. innocua* strains. The latter have been reportedly recovered from different types of food, FPEs, animal and environmental sources and harbour *L. monocytogenes* specific genes such as LIPI-1, *inlA,* LIPI-3 and exhibit phenotypic features, such as the ability to lyse red blood cells (see Table 2). These atypical *L. innocua* strains with *L. monocytogenes* virulence traits are likely evolutionary artefacts, descending from a *L. monocytogenes* progenitor (Moura et al., 2019).

We performed biochemical testing using API® *Listeria* rapid kit, to further characterise *Listeria* spp. isolates. Three different profiles for the *L. innocua* isolates were obtained: 7510 including a DIM and rhamnose positive reaction (n=124/139), API® profile 7110 with a DIM positive and rhamnose negative reaction (n=13/139), and API profile 7531 with a DIM, rhamnose, D-ribose, and D-tagatose positive reaction (n=2/139). The vast majority of the persistent *L. innocua* isolates exhibited a rhamnose positive profile (API® profiles 7510), except for genotype (IN7[C], ST1085), which yielded a rhamnose negative profile (API® profile 7110). A highly significant association for persistence and the ability to utilise rhamnose was found by computing the Cramer's V coefficient of association ($p = 0.0065$; Cramer's V rV = WERT, $p < 0.01$). Considering our results and taking into account previous findings of atypical *L. monocytogenes* lineage III strains and *L. innocua* unable to utilise rhamnose, those strains have been associated with a survival disadvantage in FPEs, reduced stress tolerance

and reduced virulence compared to rhamnose-fermenters (Roberts et al., 2006; Salazar et al., 2013; Xue et al., 2008).

We observed that the recurrently recovered *L. innocua* isolates of genotypes IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597) and IN2[E] (ST637) alongside the abundantly detected *L. monocytogenes* M5[B]=M5[D]=M5[E] (ST121), were better adapted to BC, when compared to the other isolates investigated (1.3- and 2-fold higher; mean MIC 15.6 mg/L in contrast to 11.7 and 7.8 mg/L). Resistance to BC in *L. innocua* has been documented in earlier studies, with Xu et al. reporting significantly higher MICs of BC in *L. innocua* than that in *L. monocytogenes* isolates from swine and observing a correlation between BC and heavy metal resistance (Xu et al., 2019). Genes conferring resistance to different agents, including biocides, metals, toxic dyes or antibiotics are frequently localised together on bacterial mobile genetic elements such as plasmids, transposons or genetic islands (Luque-Sastre et al., 2018). Several authors have provided evidence for the transfer of MGEs from other bacterial species to *L. monocytogenes* and between *L. innocua*, *L. welshimeri* and *L. monocytogenes* by sequence analysis and in vitro (Katharios-Lanwermeyer et al., 2012; Parsons et al., 2019b). BC and cadmium resistance genes have been detected not only in *L. monocytogenes*, but also in non-pathogenic *Listeria* spp., including *L. innocua*. Resistance genes *cadA1, cadA2* and *bcrABC,* extensively described in *L. monocytogenes,* were also detected in *L. innocua* (Katharios-Lanwermeyer et al., 2012; Korsak et al., 2019). The role of *L. innocua* as a resistance-gene donor with the potential to confer enhanced biocide tolerance, as well as the potential effect on the environmental fitness in connection with the virulence potential of the human pathogen *L. monocytogenes* should be considered when recovering *L. innocua* from the FPE.

We found that smear and brine provided growth niches and enabled the *L. innocua* genotypes ST603 and ST1597 to persist alongside *L. monocytogenes* in the dairy FPEs under observation. To our knowledge, our study is the first to report identification of *L. innocua* persistence in dairy FPEs co-colonised by *L. monocytogenes*. The fact that *L. monocytogenes* and *L. innocua* were frequently detected in samples of the same category, underlines the benefit of

using the non-pathogenic *L. innocua* as a hygiene indicator and index organism. As such, several authors have recently pointed out that recovery of *L. innocua* in the FPE could indicate the presence of undetected pathogenic *L. monocytogenes* (Gwida et al., 2020; Jemmi & Stephan, 2006; Lakićević et al., 2010; Townsend et al., 2021). Although this was most likely not the case in our study, as various *L. monocytogenes* strains were recovered from the dairy FPEs alongside *L. innocua*, microbiological detection and isolation of *L. monocytogenes* alongside accompanying bacteria can be challenging, with *L. innocua* masking the presence of *L. monocytogenes* in enrichment or on selective agar plates (Engelhardt et al., 2016; Fgaier et al., 2014; Zittermann et al., 2016).

Besides the significance of *L. innocua* as an index for *L. monocytogenes* presence, the possibility of HGT from *L. innocua* to other *Listeria* species including *L. monocytogenes* should also be considered. Besides the HGT of BC and heavy metal resistance genes described above, the emergence of atypical haemolytic *L. innocua* strains is also very likely a result from HGT transfer events with the potential of introducing the virulence genes, e.g. LIPI-4 genes, to other bacteria (M. Li et al., 2021; Moreno et al., 2012). Furthermore, although the rate of antimicrobial resistance in *Listeria* spp. is low compared to other bacteria, resistance to antibiotics, including multi-drug resistance, is more common in *L. innocua* than in any other *Listeria* species and provides the potential for resistance transfer to the human pathogen *L. monocytogenes* (Baquero et al., 2020; Bertsch et al., 2014; Chin et al., 2018; Escolar et al., 2017; Gray et al., 2021; M. Li et al., 2021; Q. Li et al., 2007; H. C. Tsai et al., 2018). The incidental aggregation of an elevated number of *Listeria* spp. provides the potential for intra- and inter-species transfer of resistance to antibiotics, biocides, heavy metals or a tolerance towards environmental stresses. Due to the high genetic homology between *L. innocua* and *L. monocytogenes* and the frequent co-occurrence along the food processing chain, the potential for the abovementioned genes being transferred from *L. innocua* to *L. monocyogenes* or to various other commensal bacteria should be considered.

Detection of *L. innocua* may facilitate the assessment of hygienic and sanitising measures in place at the FPE, as some *Listeria* spp. are considered a good index organism for *L. monocytogenes* contamination (Nastasijevic et al., 2017; Joint FAO/WHO Codex Alimentarius Commission, 2007).

We therefore strongly support the incorporation of *L. innocua* into FPE monitoring programmes and food surveillance.

5. CONCLUSIONS AND OUTLOOK

The ability of *Listeria* spp. to adapt to a broad range of environmental stresses, including high salt concentrations, low temperature or biocide residues, makes the dairy processing environment and RTE cheese products especially susceptible to colonisation by these bacterial saprophytes (Martinez-Rios & Dalgaard, 2018; Melo et al., 2015; Spanu et al., 2015). Due to the intrinsic characteristics of the foodborne pathogen *L. monocytogenes*, complete elimination from the FPE is considered impossible. The overarching aim therefore must be the reduction of the overall occurrence and the limitation of the presence of *Listeria* spp. and particularly of the pathogenic *L. monocytogenes* within the FPE to areas precluding direct food contact. Accordingly, systematic monitoring of the FPE is not only legally required, but also a necessity to identify entry routes, contamination patterns and persistent strains and the key leverage point to control contamination within the dairy processing environment (Jooste et al., 2016; Wagner & Stessl, 2021). Choosing adequate sampling, detection and identification methods is essential for efficient *L. monocytogenes* control (Spanu & Jordan, 2020; Zangerl et al., 2021). Additionally, subtyping is warranted to identify contamination routes and *L. monocytogenes* persistence on FPE level and is indispensable for outbreak investigations, cluster detection and to analyse isolate characteristics. At the time the principal research for this thesis was conducted (2010-2013), PFGE was unanimously established as the gold standard molecular subtyping method for *L. monocytogenes,* used internationally in research settings as well as in routine surveillance (Dalmasso & Jordan, 2015; Michelon et al., 2015). PFGE together with MLST are nowadays still valuable tools for further strain differentiation and correlation and for tracing contamination routes and are used in diverse settings (Caruso et al., 2020; Neoh et al., 2019; Zwirzitz et al., 2021). However, as WGS methods have advanced during the recent years, costs have significantly dropped and techniques have become more user friendly, WGS, including plasmid and bacteriophage analysis, as well as cgMLST, which provide superior typing resolution, are today extensively used in standard surveillance, outbreak investigations, contamination tracing as well as for research purposes (Halbedel et al., 2018; Mohan et al., 2021; Moura et al., 2017; Smith et al., 2019; Stasiewicz et al., 2015; Stessl et al., 2021). Advanced typing and characterisation

methods are needed, specifically to further investigate *Listeria* spp. persistence and environmental adaptation phenomena, frequently described but with the underlying mechanism still not fully understood (Torresi et al., 2020; Unrath et al., 2021). WGS methods offer the opportunity to distinguish “truly” persistent isolates from reintroduced ones and further investigate the genomic basis of adaptive, resistance, persistence and virulence mechanisms in *Listeria* spp. (Demaître et al., 2021; Hurley et al., 2019; Unrath et al., 2021). WGS-derived data can furthermore be used for in-silico typing, e.g. for MLST or plasmid typing (M. V. Larsen et al., 2012; Uelze et al., 2020). Although bacterial subtyping in the clinical setting as well as in food surveillance will be dominated by WGS technology in the future, there is a continuous need for the further development of microbiological and molecular biological methods for isolation and differentiation, rapid and cheap identification methods and in-vitro and in-vivo phenotypic assays, e.g. to analyse the nutrient metabolism, biofilm formation or the susceptibility to biocides and antibiotics and establish the virulence potential of specific *Listeria* spp. strains (Lianou & Koutsoumanis, 2013; Sintchenko et al., 2007). However, further improvement is necessary regarding the data exchangeability and the comparability of results from geno- as well as phenotypic studies. Molecular typing methods, especially WGS, offer a multitude of possibilities in terms of data analysis, interpretation and storage. A harmonised protocol is therefore urgently needed, especially for international epidemiological surveillance purposes to allow inter-laboratory data exchange and comparison (Jagadeesan et al., 2019; Lüth et al., 2021). In spite of the advances in subtyping methods, prevention of *L. monocytogenes* in foods and in FPEs remains the prime public health protection measure. An optimal *Listeria* monitoring scheme is tailored to the respective facility and should create awareness of *L. monocytogenes* FPE contamination as the first step for successful control (Beno et al., 2016). Besides the public health and food safety relevance of the human pathogen *L. monocytogenes*, our results also suggest that the typically non-pathogenic *L. innocua* should be integrated in *Listeria* monitoring and control concepts. We were able to document year-long persistence of *L. innocua* in dairy processing environments, alongside *L. monocytogenes* and given the occurrence of strains with increased tolerance to biocides, an atypically haemolytic phenotype and virulence features and reports of multidrug-resistant strais from various sources,

this species should be investigated further and its role as innocuous member of the *Listeria* genus may need reconsideration (M. Li et al., 2021; Moura et al., 2019; Parsons et al., 2019a). Consistent record keeping including longitudinal mapping of contamination, adapting the cleaning and disinfection routine, eliminating possible harbourage sites and niches through sustainable structural modifications, such as hygienically designed equipment, hygienic zoning and regulating personnel and equipment traffic are some of the main components of an effective *Listeria* spp. control strategy (Kallipolitis et al., 2020; Zoellner et al., 2018).

Despite highly automated production processes, especially in large scale milk processing and cheese making facilities, the human factor should not be forgotten when it comes to food hygiene (Evans et al., 2021; Manning, 2017). Awareness and engagement of the FBO and the management level in *Listeria* control measures is an essential step to ensure adequate implementation of basic hygiene measures by the personnel. Personnel, who are aware of their responsibility to contribute to safe food and who are trained in the implementation of essential and easily integrable hygiene measures, are another main factor to prevent re- and cross-contamination from the FPE to food and thereby decrease the public health burden of *L. monocytogenes*.

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8. FURTHER PUBLICATIONS AND SCIENTIFIC PRESENTATIONS

Publication:

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., Tichy, A., Wagner, M., & Stessl, B. (2014). Reservoirs of *Listeria* species in three environmental ecosystems. *Applied and Environmental Microbiology, 80*(18), 5583-5592.

Book chapter:

Stessl, B., Rückerl, I., & Wagner, M. (2014). Multilocus Sequence Typing (MLST) of *Listeria monocytogenes*. In J. Kieran (Ed.), *Listeria monocytogenes*: Methods and Protocols. *Methods in Molecular Biology, VoL. 1157 (pp.73-85)*. New York, NY: Humana Press.

Oral and poster presentations:

Rückerl, I., Stabler, R., Klinger, S., Wagner, K. H., Wagner, M., & Stessl, B. (2011, May 18-20). *Is there a genetic relationship between potentially persistent L. innocua and L. monocytogenes strains?* [Conference presentation]. IAFP's European Symposium on Food Safety, Ede, Netherlands.

Rückerl, I., Stabler, R., Klinger, S., Wagner, M., & Stessl, B. (2011, August 31-September 2). *Molekulare Epidemiologie von persistierenden Listerien isoliert aus Molkereibetrieben* [Conference presentation]. 44. DACH Epidemiologietagung, Wien, Austria.

Rückerl, I., Pin, C., Stabler, R., Klinger, S., Wagner, K. H., Wagner, M., Stessl, B. (2012, May 21-24). *Surveying a common marker region in persistent L. innocua and L. monocytogenes strains* [Conference poster]. Thirty-third Meeting of the Austrian Society for Hygiene, Microbiology and Preventive Medicine, Salzburg, Austria. (Poster Award 2012, best poster).

9. ANNEX I – Supplemental tables

Supplemental Table 1: *Listeria* species and their first-time isolation sources

	first reported					
***Listeria* species**	**sample category**	**source**	**location**	**GC-content (mol%)**	**type strain**	**reference**
L. aquatica	ANE	running water	Florida, USA	40.9	DSM 26686	den Bakker et al., 2014
L. booriae	FPE	NFCS, dairy processing	north-eastern USA	45.2	DSM 28860	Weller et al., 2015
L. cornellensis	ANE	water	Colorado, USA	42.5	DSM 26689	den Bakker et al., 2014
L. cossartiae	ANE	soil	North Carolina, Georgia, Alabama, USA	38.6/38.7	FSL L7-1447, FSL L7-0993	Carlin et al., 2021
L. costaricensis	FPE	drainage system	Alajuela, Costa Rica	43.7	DSM 105474	Núñez-Montero et al., 2018
L. farberi	ANE	soil	Texas, Florida, USA	36.8	FSL L7-0091	Carlin et al., 2021
L. fleischmannii	FPE, food	cheeses ripening cellars	Switzerland	39.0	DSM 24998	Bertsch et al., 2013
L. floridensis	ANE	running water	Florida, USA	41.8	DSM 26687	den Bakker et al., 2014
L. goaensis	ANE	mangrove swamps	Goa, India	40.3	DSM 29886	Doijad et al., 2018
L. grandensis	ANE	water	Colorado, USA	43.0	DSM 26688	den Bakker et al., 2014
L. grayi	animal	chinchilla faeces	Denmark	38.0	DSM 20601	Larsen & Seeliger, 1966
L. immobilis	ANE	soil	Montana, South Dakota, Wyoming, USA	35.9	FSL L7-1519	Carlin et al., 2021
L. innocua	animal	bovine brain	unk	37.4	DSM 20649, FSL S4-378*	Seeliger, 1981
L. ivanovii	animal	sheep	Bulgaria	38.0	DSM 20750	Seeliger et al., 1984
L. kieliensis	ANE	wastewater plant	Germany	45.7	nd	Kabisch, 2018
L. marthii	ANE	environment	Finger Lakes region, New York, USA	37.7	DSM 23813	Graves et al., 2010
L. monocytogenes	animal	rabbit	UK	38.0	DSM 20600, 10403s**	Pirie, 1940
L. newyorkensis	FPE	NFCS, seafood processing	north-eastern USA	45.2	DSM 28861	Weller et al., 2015
L. portnoyi	ANE	soil	South Dakota, USA	41.9	FSL L7-1582	Carlin et al., 2021
L. riparia	ANE	running water	Florida, USA	41.9	DSM 26685	den Bakker et al., 2014
L. rocourtiae	food	pre-cut lettuce	Salzburg, Austria	40.3	DSM 22097	Leclercq et al., 2010
L. rustica	ANE	agricultural water source	New York, USA	42.3	FSL W9-0585	Carlin et al., 2021
L. seeligeri	ANE	soil	Germany	36.0	DSM 20751	Rocourt & Grimont, 1983
L. thailandensis	food	fried chicken	Thailand	40.3	DSM 107638	Leclercq et al., 2019b
L. valentina	animal	sheep faeces	Valencia, Spain	40.2	DSM 110544	Quereda et al., 2020
L. weihenstephanensis	ANE	water plant *Lemna trisulca,* freshwater pond	Germany	41.8	DSM 24698	Halter et al., 2013
L. welshimeri	ANE	decaying vegetation	USA	36.0	DSM 20650	Rocourt & Grimont, 1983

Abbreviations: ANE: agricultural and natural environment; unk: unknown; nd: not determined; * isolated from puddle of water, New York, USA, 2002; ** streptomycin resistant human skin lesion isolate, Montana, USA; further references: Orsi & Wiedmann, 2016; https://bacdive.dsmz.de/; accessed on: 4 March 2022.

Supplemental Table 2: Major σ^B regulated genes and their function in *L. monocytogenes*

function	gene	protein	role	reference
osmotic stress response	*opuCA*	carnitine transport ATP-binding protein OpuCA	carnitine transport from outside the cell membrane into cytoplasm to combat high salt stress	Fraser et al., 2003
	opuCB	carnitine transport permease protein OpuCB		
	opuCC	carnitine transport binding protein OpuCC		
	opuCD	carnitine transport permease protein OpuCD		
	gbuA	lycine betaine/carnitine transport ATP-binding protein Gbu	glycine betaine transport from outside the cell membrane into cytoplasm to combat high salt stress	Angelidis & Smith, 2003
	gbuB	glycine betaine/carnitine transport permease protein GbuB		
	gbuC	glycine betaine/carnitine transport binding protein GbuC		
	betL	glycine betaine transporter BetL		Sleator et al., 1999
	hfq	RNA-binding protein Hfq	regulator binding to sRNAs during intracellular growth	Christiansen et al., 2004
	dtpT	di- and tripeptide transporter DtpT	peptide transmembrane transporter activity	Wouters et al., 2005
	ctc	general stress protein, 50S ribosomal protein L25	high osmolarity resistance in the absence of other osmoprotectants	Gardan et al., 2003
oxidative stress response	*qoxA*	quinol oxidase subunit II QoxA	oxidase required for growth under low oxygen conditions, murine infection	Corbett et al., 2017
	qoxB	quinol oxidase subunit I QoxB		
	qoxC	quinol oxidase subunit III QoxC		
	qoxD	quinol oxidase subunit IV QoxD		
	spxA	transcriptional regulator SpxA	vacuolar escape and peroxide and disulfide stress protection	Reniere et al., 2016
	sod	superoxid dismutase Sod	protection from superoxides and reactive oxygen species	Archambaud et al., 2006
	uspA	universal stress protein A UspA	promotion of extracellular survival under acid and oxidative stress	Gomes et al., 2011
acid stress response	*gadB*	glutamate decarboxylase beta GadB	GAD system: increase of cytoplasmic pH via extracellular glutamate transport into the cell and conversion to GABA	Cotter et al., 2001; Feehily et al., 2013
	gadC	glutamate/gamma-aminobutyrate antiporter GadC		
	gadD2	glutamate decarboxylase GadD		
	argR	arginine repressor ArgR	regulation of arginine biosynthesis genes	
	arcA	arginine deiminase ArcA	ADI system: increase of cytoplasmic pH via conversion of arginine to ornithine, carbon dioxide and ammonium	Ryan et al., 2009
	arcB	ornithine carbamoyltransferase ArcB		
	arcD	arginine/ornithine antiporter ArcD		

further reference: Y. Liu et al., 2019.

Supplemental Table 2 continued: Major σ^B regulated genes and their function in *L. monocytogenes*

function	gene	protein	role	reference
anitbiotic resistance	*mpoA*	PTS system mannose-specific EIIAB component	carbon metabolism and resistance to class IIa bacteriocins	Arous et al., 2004
	mpoB	PTS mannose transporter subunit IIAB		
	mpoC	PTS mannose/fructose/sorbose transporter subunit IIC		
	mpoD	PTS mannose transporter subunit IID		
	fosX	fosfomycin resistance protein FosX	response to antibiotic stress by catalysing the hydration of fosfomycin	Kerry et al., 2007
bile response	*bsh*	bile-salt hydrolase Bsh	bile resistance, virulence	Begley et al., 2005
	bilEA	ABC transporter ATP-binding protein BilEA	*bilE* operon (bile-exclusion system), osmotic stress response, bile stress response and intestinal	Sleator et al., 2005
	bilEB	ile exclusion ABC transporter, substrate binding protein BilE		
cold stress response	*ltrA*	low temperature requirement protein A LtrA	growth at low temperature	Zheng & Kathariou, 1995
	ltrB	low temperature requirement protein B LtrB		
	ltrC	low temperature requirement C protein LtrC		
response to other stresses	*htrA*	serine protease DegP/HtrA do-like	growth under salt, heat and oxidant stress	Wonderling et al., 2004
	fri	non-heme iron-binding protein Fri	growth under iron limitation and oxidative stress, intracellular growth	Olsen et al., 2005
	clpC	endopeptidase Clp ATP-binding chain C clpC	growth under iron limitation, heat and osmotic stress, virulence	Rouquette et al., 1996
	clpP	ATP-dependent Clp protease proteolytic subunit		
	mcsA	protein-arginine kinase activator protein McsA		
	mcsB	protein-arginine kinase McsB		
	ctsR	transcriptional regulator CtsR		
	hrcA	heat-inducible transcription repressor HrcA	control of heat-shock response	Raengpradub et al., 2008

Abbreviations: PTS: phosphotransferase system; ATP: adenosine triphosphate; further reference: Y. Liu et al., 2019.

Supplemental Table 2 continued: Major σ^B regulated genes and their function in *L. monocytogenes*

function	gene	protein	role	reference
virulence	*prfA*	positive regulatory factor A PrfA	key transcription activator of virulence gene expression in *L. monocytogenes,* growth in and outside the host	de las Heras et al., 2011; Vasanthakrishnan et al., 2015
	inlA	internalin A InlA	adhesion and invasion	Gaillard et al., 1991
	inlB	internalin B InlB		
	srtA	sortase A SrtA	infection, proteolysis, processing of internalin proteins	Garandeau et al., 2002
	chiA	chitinase A1 ChiA	infection, chitin catabolic process	M. H. Larsen et al., 2010; Chaudhuri et al., 2010
	mogR	motility gene repressor MogR	regulation of flagellar motility genes	Gründling et al., 2004
	flaA	flagellin FlaA	polymerization to form filaments of bacterial flagella	
	gmaR	glycosyl transferases flagella synthesis antirepressor GmaR	control of temperature-dependent transcription of flagellar motility genes	Kamp & Higgins, 2011
	lapB	LPXTG cell wall anchor domain-containig protein LapB	entry into eukaryotic cells	Reis et al., 2010
carbon metabolism	*gap*	glyceraldehyde 3-phosphate dehydrogenase Gap	glycolysis, binds to human plasminogen (virulence)	Schaumburg et al., 2004
	eno	enolase Eno	glycolysis, binds to human palsminogen (virulence)	
	nagA	N-aceltylglucosamine-6-phosphate deacetylase NagA	cell wall peptidoglycan and teichoic acid biosysnthesis	Popowska et al., 2011
	nagB	glucosamine-6-phosphate deaminase NagB	GlcNAc degradation	
	pduD	propanediol dehydratase medium subunit PduD	1,2-propanediol utilization during infection	Xue et al., 2008
nucleotide metabolism	*pyrE*	orotate phosphoribosyltransferase PyrE	intracellular proliferation	Klarsfeld et al., 1994
cell wall metabolism	*murC*	UDP-N-acetylmuramate-L-alanine ligase MurC	peptidoglycan biosynthesis	Mahapatra et al., 2000

Abbreviations: ATP: adenosine triphosphate; GlcNAc: N-acetylglucosamine; further reference: Y. Liu et al., 2019.

Supplemental Table 3: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
physical	heat stress	no	meat processing, meat	no significant differences in heat tolerance betw persistent and non-persistent strains; large variation in heat tolerance betw strains (3 log units)	Lundén et al., 2007
		no	mussel processing, mussels	no consistent difference betw sporadic and persistent isolates after heat treatment	Nowak et al., 2017
	cold stress	no	fish processing, human clinical, EGD	persistent strain no less sensitive to cold-smoked salmon processing steps than clinical strain or EGD	Porsby et al., 2008
		assumed	na (review)	cold stress appears to be signal received by *L. monocytogenes* from its environment causing metabolic changes that allow persistence in diverse habitats, including FPEs	Tasara & Stephan, 2006
	desiccation	no	fish processing	persistent *L. monocytogenes* not more tolerant to desiccation than presumed non-persistent; long-term survival at low relative humidity may enable *L. monocytogenes* strains to persist in FPEs	Vogel et al., 2010
		no	FPE div, food div	persistent strains were not more resistant to desiccation than presumed non-persistent ones; persistent strains were present in all three groups (sensitive, intermediate, or resistant)	Zoz et al., 2017
	pressure	no	meat processing	persistent and non-persistent *L. monocytogenes,* artificially inoculated in dry-cured ham, exhibited a similar resistance against high pressure processing	Pérez-Baltar et al., 2021
	light	no	FPE div, food div, lab	exposed to blue light, *L. monocytogenes* food strains showed variation regardless of whether they were persistent or not; showed significant differences compared to lab isolates	NicAogáin & O'Byrne, 2016

Abbreviations: betw: between; div: diverse; na: not analysed.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
chemical	disinfectant tolerance	yes	meat processing	persistent strain biofilms resuscitated faster after chitosan treatment; recovery rates of persistent group doubled those of non-persistent group; persistent strains biofilms slightly more tolerant to PAA treatment	Orgaz et al., 2013
		no	chilled foods processing, chilled foods, lab	resistance to commercial disinfectants of *L. monocytogenes* strains not significantly different from the laboratory control strain	Holah et al., 2002
		yes	dairy processing, milk, cheese	resistance to QACs was shown to be higher among persistent strains	Fox et al., 2011b
		no	dairy processing, cheese	no relation betw persistence and sanitizer resistance observed	Magalhães et al., 2016
		assumed	na (review)	low-level resistance to QACs in *L. monocytogenes* may contribute to environmental adaptation and persistence in FPEs; tolerant strains have survival advantages, e.g. the ability to form biofilms in presence of increased biocide concentrations	Martínez-Suárez et al., 2016
		no	retail delis	no significant difference in sanitizer tolerance betw persistent and transient strains	J. Wang et al., 2015
		no	dairy processing	persistence of *L. monocytogenes* isolates in Gorgonzola cheese processing plants was not linked to their susceptibility to hydrogen peroxide-based disinfectants	Costa et al., 2016
		no	fish processing, fish, human clinical	persistent *L. monocytogenes* strains as susceptible to disinfectants as presumed non-persistent strains and attachment did not make the strains more tolerant to disinfectants	Kastbjerg & Gram, 2009
		yes	fish processing	persistent strain produced greater amounts of biofilm and extracellular polymeric substances than the transient strain; this resulted in greater resistance of the persistent strain to disinfectants	Nakamura et al., 2013

Abbreviations: betw: between; div: diverse; na: not analysed

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
chemical	disinfectant tolerance	no	retail delis	no significant difference in growth rate or ability to grow for retail deli *L. monocytogenes* isolates of persistent strains compared to sporadic strains for sanitizer treatment (BC, 0, 2, and 5 µg/mL) at 37 °C	Taylor & Stasiewicz, 2019
		yes/no	poultry processing	greater susceptibility to disinfectants for biofilms of persistent strains than for sporadic strains; resistance to disinfectants in *L. monocytogenes* sessile cells is strain-dependent, not associated with persistence	Rodríguez-Campos et al., 2019
		assumed	FPE div, food div	BC resistance may be factor among others influencing the ability of a strain to persist in the FPE	Aase et al., 2000
		no	meat processing	association betw resistance to sublethal concentrations of BC and long term survival for specific strains; long-term persistence probably not due to observed low-level disinfectant resistance but related to cleaning/disinfection	Ortiz et al., 2014
		yes	food div.	persistent strains more likely resistant to cadmium than sporadic strains	Harvey & Gilmour, 2001
		assumed	na (review)	no direct evidence for role of cadmium resistance plasmids in environmental persistence; high prevalence of cadmium resistance in strains from food, FPEs reported; suggestive evidence for foods or FPEs driving acquisition/retention of cadmium resistance determinants, or that cadmium resistance promoting *L. monocytogenes* persistence and fitness in foods or FPEs	Parsons et al., 2020
		no	meat processing	no associations betw cadmium, arsenic resistance and persistent strains; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b

Abbreviations: betw: between; div: diverse; na: not analysed.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
chemical	pH	yes	meat processing	persistent strains showed higher tolerance to acidic conditions than non-persistent strains; large variation in acid tolerance betw strains (> 6 log units)	Lundén et al., 2007
		yes	dairy processing, cheese	persistent cells showed faster growth rate and lower lag phase when grown in the presence of NaCl and at pH 5	Magalhães et al., 2016
		yes	cheese, cheese smear water, smoked salmon, EGDe	persistent strains showed increased survival under alkaline stress	Rychli et al., 2016
		no	retail delis	no significant difference in growth rate or ability to grow for retail deli isolates of persistent strains compared to sporadic strains at pH 5.2, 7.2, 9.2 at 37 °C	Taylor & Stasiewicz, 2019
	NaCl	yes	dairy processing, cheese	persistent cells showed faster growth rate and lower lag phase when grown in the presence of NaCl and at pH 5	Magalhães et al., 2016
		no	retail delis	no significant difference in growth rate or ability to grow for retail deli isolates of persistent strains compared to sporadic strains at concentrations of 0, 5, and 10% NaCl at 37 °C	Taylor & Stasiewicz, 2019
	energy sources	no	retail delis	no significant difference in growth rate/ability for persistent retail deli isolates compared to sporadic strains when exposed to different energy sources: 25 mM glucose, cellobiose, glycogen, fructose, lactose, sucrose at 37 °C	Taylor & Stasiewicz, 2019

Abbreviations: betw: between.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
biological	biofilm formation	yes	mussel processing, mussels	persistent isolates formed more biofilm than sporadic isolates	Nowak et al., 2017
		no	meat processing	persistent strains did not perform better at adherence or biofilm formation; persistent strains biofilms no more tolerant to chitosan treatment	Orgaz et al., 2013
		yes/no	poultry	no significant difference in biofilm formation at 30 °C; persistent strains produced significantly more biofilm at 37 °C; persistent strains alter biofilm formation in response to changing environmental factors	Ochiai et al., 2014
		no	FPE div, food div	no clear association betw biofilm formation efficiency and persistent or prevalent genotypes	B. H. Lee et al., 2019
		no	meat processing, dairy processing	significant differences in biofilm formation among strains belonging to same genetic clusters; indicating long-term persistence not determined by level of biofilm production	Guidi et al., 2021
		no	meat processing	no significant differences betw persistent and non-persistent strains in biofilm formation ability; all isolates were able to form a 48 h mature biofilm	Cherifi et al., 2018
		no	dairy processing	persistence of isolates in Gorgonzola cheese processing plants not linked to biofilm-forming ability	Costa et al., 2016
		yes	fish processing	persistent strain produced greater amounts of biofilm and extracellular polymeric substances; greater resistance of persistent strain to disinfectants	Nakamura et al., 2013
		no	FPE div, food div, human clinical, ovine	persistent FPE isolates produced less biofilm than sporadic ones; none of the persistent strains produced significantly greater levels of biofilm compared to all other strains, while those recovered as sporadic contaminants did; greater biofilm production among serotype 1/2a strains observed	Nilsson et al., 2011
		yes	poultry processing	persistent strains showed larger biofilm formation than sporadic strains	Rodríguez-Campos et al., 2019

Abbreviations: betw: between; div: diverse.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
biological	surface attachment	yes	poultry processing, dairy processing	persistent *L. monocytogenes* strains showed enhanced adherence at short contact times, promoting survival in FPEs; possibly allowing initiation of persistent plant contamination	Lundén et al., 2000
		yes/no	fish processing	persistent isolates attached better than transient ones at 21 °C but not at 10 °C	Harrand et al., 2020
		yes	retail delis	persistent strains showed enhanced adhesion on day 1 of 5-day adhesion-biofilm formation assay	J. Wang et al., 2015
		no	meat processing, meat	*L. monocytogenes* clone persisted despite weak adhesion ability; atypical persister strain: serogroup (IVb); virulence potential and antibiotic resistance (ampicillin) demonstrated	Sereno et al., 2019
	motility	no	dairy processing	bacterial motility itself should not be considered a key feature for persistence of *L. monocytogenes* in the FPE	Cabrita et al., 2015
		no	mussel processing, mussels	isolates analysed were motile at 20 °C and 30 °C; motility was fractionally higher for sporadic isolates, but no significant difference observed	Nowak et al., 2017
	protozoa interactions	assumed	sausage	viable and nonviable amoebal cysts can protect internalized bacteria against stressful conditions; cyst passage can induce cross-tolerance in bacteria, increasing survival when exposed to certain stressors; potential importance of free-living amoebae in FPEs and impact on persistence of food-borne bacteria	Lambrecht et al., 2017
		assumed	sausage	*L. monocytogenes* survive inside cysts of *Acanthamoeba castellanii* up to 2 weeks, even when exposed to antibiotic treatment or highly acidic conditions; important role of free-living protozoa in persistence and epidemiology of foodborne bacterial pathogens in FPEs suggested	Lambrecht et al., 2015
		no	lab strains, mutant strains	clear evidence that *L. monocytogenes* is unable to persist in *Acanthamoeba castellanii* and *A. polyphaga*	Doyscher et al., 2013

Abbreviations: betw: between.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
biological	(low) virulence	yes	fish processing, ScottA, human clinical	persistent group showed lower virulence potential in all four virulence models than Scott A and clinical listeriosis strain	Jensen et al., 2008
		no	meat processing	no significant association betw invasion efficiency in Caco-2 cells and persistence; significant effects on invasion efficiency of either molecular serogroup or lineage; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b
	bacteriophage resistance	no	fish processing	persistent *L. monocytogenes* strains include phage susceptible and resistant isolates; persistent strains with wide range of phage susceptibility (4.6%-95.4%)	Vongkamjan et al., 2013
		assumed	poultry processing	exposure to phage in FPEs may select for loss of teichoic acid decorations in *L. monocytogenes;* resulting resistance to phage may contribute to FPE persistence of the ST321 and ST391 strains	Brown et al., 2021
		no	meat processing	no significant association betw persistence and phage susceptibility; no significant association betw lysogeny and persistence; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b
	antibiotic resistance	no	food div.	only 2/45 strains showed resistance to any of the nine antibiotics tested; two sporadic strains were resistant to tetracycline	Harvey & Gilmour, 2001
		no	meat processing	no association betw tetracycline resistance and persistent strains; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b

Abbreviations: betw: between; div: diverse.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

	parameter investigated	association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	gene expression	yes	FPE div	*L. monocytogenes* exposure to lethal nisin concentrations alters transcriptional regulation; functional genes associated with persister population identified: heat-shock-related stress response, cell wall synthesis, ATP-binding cassette (ABC) transport system, PTS and SOS/DNA repair	S. Wu et al., 2018
		yes	dairy processing	persistent strain showed significantly higher transcript levels of *dtpT* and *sigB* genes and significantly lower levels of *flaA, oppA* genes and helicase-encoding genes *lmo1722* and *lmo0866*	Cabrita et al., 2015
		yes	cheese, dairy processing, fish, EGDe	gene expression of the proteins linked to stress response (Lmo2637, NamA, Fhs and QoxA) was higher in the persistent strain not only at 37 °C but also at 10 °C	Rychli et al., 2016
		no	meat processing	persistent *L. monocytogenes* hardly affected by pressure treatments, whereas non-persistent exhibited significant increase of *prfA, hly, sigB* gene expression	Pérez-Baltar et al., 2021
		no	fish processing, meat processing	salt stress induced higher transcript levels for all stress and virulence genes analysed in wildtype strains; transcript levels for *clpB* induced at significantly higher levels in non-persistent strains; significantly higher transcript levels of *gadD* 3 and *clpB* after salt shock in lineage I compared to lineage II strains; no clear association betw stress gene transcript levels and persistence	Ringus et al., 2012
		yes	dairy processing, milk, cheese	comparing persistent and non-persistent strains, transcription of many genes upregulated among persistent strains including *pdu, cob-cbi,eut* ; these genes may play a role in persistence of *L. monocytogenes* outside the human host	Fox et al., 2011b
		no	dairy processing, cheese	no markers correlating with ability of strains to persist in FPEs; upregulation of *pocR;* potential response of persistent *L. monocytogenes* strains to BC	Mazza et al., 2015

Abbreviations: betw: between; div: diverse.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	specific genes	yes	meat processing	disinfectant resistance genes *bcrABC, emrE* contribute to persistence; either *bcrABC* resistance cassette or *emrE* gene, or both, present in persistent *L. monocytogenes* strains	Cherifi et al., 2020
		no	mussel processing, mussels	wg analysis revealed no genetic markers for persistent *L. monocytogenes* isolates	Nowak et al., 2017
		yes	fish processing	compared to non-persistent strains, all three persistent strains distinguished by two genome deletions: one contains the gene for *inlF*, the other three genes potentially related to bacteriocin production and transport (*lmo2774, lmo2775,* and the 3'-terminal part of *lmo2776*)	Holch et al., 2013
		no	retail delis	no individual genes enriched among persistent compared to sporadic isolates	Stasiewicz et al., 2015
		no	meat processing, dairy processing	all studied strains carried multidrug efflux-pumps genetic determinants (*sugE, mdrl, lde, norM, mepA*); all CC2 carried a full-length *inlA* while CC9 and CC121 presented a PMSC correlated with lower virulence	Guidi et al., 2021
		assumed	meat processing	presence of the *bcrABC* cassette may account for persistence of isolates in the slaughterhouse environment	Cherifi et al., 2018
		no	retail delis	only 10/442 isolates had *inlA* PMSCs; strains with PMSCs were not persistent, even in delis with other persistent strains	J. Wang et al., 2015
		no	retail delis	no genetic content found that explained persistence; presence of sanitizer tolerance elements not significantly correlated with phenotypic sanitizer tolerance	Assisi et al., 2021
		yes	dairy farms	resistance genes against bacitracin, arsenic and cadmium significantly more prevalent among persistent than sporadic strains; *L. monocytogenes* is potential reservoir of mobile elements harbouring resistance genes against antimicrobials, biocides, heavy metals	Castro et al., 2021

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	specific genes	yes	meat processing, vegetable processing	occurrence of resistance genes in 62% strains, increasing to 73% among presumed persistent strains; most prevalent genes were *emrC* followed by bcrABC, *qacH*- Tn*6188* , and *qacC*	Hurley et al., 2019
		assumed	meat, dairy, fish, seafood, fruit and vegetable and processing environments	QAC resistance genes may be indicators for persistence; not specified, if strains analysed did persist; *bcrABC* in 59.2% of FPE and 38.6% of food isolates; *bcrABC* in strains from varying lineage and origin; *bcrABC* most common BC resistance genetic element; *L. monocytogenes* with *bcrABC* resistant to BC	Cooper et al., 2021
		no	meat processing	no single genetic marker responsible for a strain's ability to persist; persistent and presumed non-persistent cohorts encode a range of stress resistance markers; trends associated with each cohort (e.g., *qacH, cadA1C* resistance more frequent in persistent isolates); persistent isolates more likely contain mutations associated with hypovirulence, including truncated *InlA*	Palaiodimou et al., 2021
		no	FPE div	no particular gene linked to persistence phenotype; all isolates within a ST have the potential to be 'truly persisting' under optimal conditions	Knudsen et al., 2017
		yes	food processing div., food div.	SSI-1 and *inlA* may play a role in evolution of Canadian *L. monocytogenes* strains into either virulent (4b clinical isolates) or environmentally persistent (1/2a food isolates) phenotype	Upham et al., 2019
		no	meat processing	no associations betw *inlA* PMSC and persistent strains; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b

Abbreviations: betw: between; div: diverse.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	MGEs	no	mussel processing, mussels	differences in prophages and plasmids not associated with persistence	Nowak et al., 2017
		no	meat processing, dairy processing	CC121 harbored the Tn*6188* specific for tolerance to BC; only CC9 and CC121 carried a SSI and presented high-level cadmium resistance genes (*cadA1C1*) carried by different plasmids	Guidi et al., 2021
		yes	FPE div	presence of SSI-1, SSI-2, Tn*6188*, and ability to form biofilm support persistent strains	Manso et al., 2019
		yes	dairy farms	prophages and other mobile elements significantly more numerous in persistent than sporadic strains; mobile elements may support persistence of *L. monocytogenes; L. monocytogenes* potential reservoir of mobile elements harbouring resistance genes against antimicrobials, biocides, and heavy metals	Castro et al., 2021
		no	meat processing	no single genetic marker universally responsible for a strain's ability to persist; plasmids and transposons widespread betw cohorts	Palaiodimou et al., 2021
		assumed	seafood processing, seafood	MGEs possibly responsible for successful adaptation to FPEs; prophage- and transposon-related clusters of genes major loci of genetic diversity across different CCs; presence of closely related plasmids in *L. monocytogenes* CCs supports HGT hyptothesis leading to enhanced survival under FPE-associated stressors, especially in hard-to-clean areas	Palma et al., 2020
		assumed	dairy processing	ST5 plasmids harbored an efflux pump system (*bcrABC* cassette) and heavy metal resistance genes possibly providing a higher tolerance to disinfectants; pLM80 prototype plasmids are likely genetic determinants for FPE survival of *L. monocytogenes*; plasmids are important for persistence of ST5 strains in FPE	Muhterem-Uyar et al., 2018
			meat processing, poultry processing	model for high-frequency recombination driving rapid niche-specific adaptation and persistence of *L. monocytogenes* in FPEs	Verghese et al., 2011

Abbreviations: betw: between; div: diverse.

Supplemental Table 3 continued: Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	genetic islands	assumed	FPE div, food div, human clinical	SSI-2 predominantly in *L. monocytogenes* ST121 strains; benefits alkaline and oxidative stress survival; potentially supporting adaptation and persistence of *L. monocytogenes* in FPEs	Harter et al., 2017
		assumed	meat processing, dairy processing	presence of SSI-1 or SSI-2 may be associated with persistence	Guidi et al., 2021
		yes	FPE div	presence of SSI-1, SSI-2, Tn*6188*, and ability to form biofilm support persistent strains	Manso et al., 2019
		no	retail delis	presence of SSI-1 correlated to increased attachment capacity but not to persistence	Assisi et al., 2021

Abbreviations: betw: between; div: diverse

.**Supplemental Table 3 continued:** Parameters investigated in association with *L. monocytogenes* persistence in FPEs

parameter investigated		association betw persistence and parameter	*L. monocytogenes* isolate origin	effect observed	reference
genetic	lineage/ST/CC	no	meat processing	*L. monocytogenes* subtyped: 1/2a (36.8%), 1/2c (34%), 1/2b (17.9%) and 4b (11.3%); ST9 most common allelic profile (33% of isolates) followed by ST121 (16%); ST9 and ST121 are frequently isolated from food and seem to be better adapted to FPEs, including meat processing	Martín et al., 2014
		no	meat processing, dairy processing	hypo-virulent clones CC9 and CC121 most adapted to FPEs with a higher prevalence of stress resistance, presence of BC-specific tolerance genes and higher biofilm production capability	Guidi et al., 2021
		assumed	FPE div, food div	common adaptations in ST121 genomes potentially related to persistence in FPEs: presence of Tn*6188* , an insertion harboring recombination hotspot proteins, likely involved in competition against other bacteria, and homologs of the *L. innocua* genes *lin0464* and *lin0465*	Schmitz-Esser et al., 2015
		yes	FPE div	ST6, ST7, ST101 and ST121 categorised as 'true persisting' STs; 'true persistence' = clonal: limit of ≤4SNPs for clonal groups AND isolation over extended period of time (>2 months)	Knudsen et al., 2017
		yes	food div, human clinical	(i) hypervirulent, host-associated clones (i.e., CC1 and CC4), prevalent in dairy products, low adaptation to FPEs, BC tolerance genes rare; (ii) hypovirulent, efficient FPE persister clones (i.e., CC9 and CC121), efficient biofilm formation, tolerance to disinfectants, BC tolerance genes; and (iii) intermediary clones (i.e., CC2 and CC6) may transition from host-associated to saprophytic lifestyles by virulence loss or acquisition of disinfectant tolerance	Maury et al., 2019
		assumed	meat processing	enrichment observed in ST121 genomes concerning SSI-2, *qacH* gene, *cadA1C* gene cassette and a truncated *actA* gene (biofilm formation); ST14 genomes enriched with full-length version of *actA* gene along with the *Listeria* Genomic Island 2 (LGI 2) including the *ars* operon and *adA4C* gene cassette; greater ability to form biofilms	Pasquali et al., 2018
		no	meat processing	no significant association betw persistence and molecular serogroup or lineage; persistent strains showed considerable phenotypic and genetic diversity	V. Ferreira et al., 2011b

Abbreviations: betw: between; div: diverse.

10. ANNEX II – Publications

10.1. Original article 1

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L. monocytogenes in a cheese processing facility: Learning from contamination scenarios over three years of sampling

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ABSTRACT

The aim of this study was to analyze the changing patterns of *Listeria monocytogenes* contamination in a cheese processing facility manufacturing a wide range of ready-to-eat products. Characterization of *L. monocytogenes* isolates included genotyping by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Disinfectant-susceptibility tests and the assessment of *L. monocytogenes* survival in fresh cheese were also conducted. During the sampling period between 2010 and 2013, a total of 1284 environmental samples were investigated. Overall occurrence rates of *Listeria* spp. and *L. monocytogenes* were 21.9% and 19.5%, respectively. Identical *L. monocytogenes* genotypes were found in the food processing environment (FPE), raw materials and in products. Interventions after the sampling events changed contamination scenarios substantially. The high diversity of globally, widely distributed *L. monocytogenes* genotypes was reduced by identifying the major sources of contamination. Although susceptible to a broad range of disinfectants and cleaners, one dominant *L. monocytogenes* sequence type (ST) 5 could not be eradicated from drains and floors. Significantly, intense humidity and steam could be observed in all rooms and water residues were visible on floors due to increased cleaning strategies. This could explain the high *L. monocytogenes* contamination of the FPE (drains, shoes and floors) throughout the study (15.8%). The outcome of a challenge experiment in fresh cheese showed that *L. monocytogenes* could survive after 14 days of storage at insufficient cooling temperatures (8 and 16 °C). All efforts to reduce *L. monocytogenes* environmental contamination eventually led to a transition from dynamic to stable contamination scenarios. Consequently, implementation of systematic environmental monitoring via in-house systems should either aim for total avoidance of FPE colonization, or emphasize a first reduction of *L. monocytogenes* to sites where contamination of the processed product is unlikely. Drying of surfaces after cleaning is highly recommended to facilitate the *L. monocytogenes* eradication.

1. Introduction

Human listeriosis is a severe food-borne disease caused by *Listeria monocytogenes*, a Gram-positive environmental saprophyte, that is capable of switching into a facultative intracellular pathogen (Freitag et al., 2009). The main route of transmission to susceptible individuals, such as pregnant women, the elderly and immunocompromised patients, is contaminated food (McLaughlin et al., 2004). Over the last eight years, incidence rates ranging from 0.3 to 1.3 per 100,000 capita have been reported in European countries, the U.S., Canada and Australia (Todd and Notermans, 2011). Cheese-related foodborne outbreaks were often linked to major deficiencies in good hygiene practices, inadequate process monitoring and fluctuation in *L. monocytogenes* contamination dynamics (Fretz et al., 2010; Koch et al., 2010; Jackson et al., 2011; Schoder et al., 2012; Yde et al., 2012). *L. monocytogenes* is difficult to eradicate due to surface and niche colonization, resistance conditions of low pH (<4.4), water activity (<0.94) and growth at refrigeration temperatures (Ortiz et al., 2010; Tasara and Stephan, 2006). Recently an increase in the prevalence of *L. monocytogenes* serotype 1/2a has been observed among food and human sources (Gilmour et al., 2010; Lambertz et al., 2013; Lomonaco et al., 2013; Mammina et al., 2013). Although *Listeria* spp. are generally sourced to soil environments, carriage by wild and domesticated animals, and humans, specific routes of entry to food processing facilities are still not fully understood.

Even low grade contamination (<10 cfu/g) of raw materials with *L. monocytogenes* poses a major challenge to the food industry, with

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respect to the ease of potential equipment and plant colonization (Alessandria et al., 2010; Carpentier and Cerf, 2011; Reij and den Aantrekker, 2004; Williams et al., 2011). Consequently, subtyping of *L. monocytogenes* isolated from food and local environments is necessary to trace contamination events back to potential sources, to detect potential persistent strains and to optimize sanitation and sampling strategies (Almeida et al., 2013; Schoder et al., 2013b). Pulsed-field gel electrophoresis (PFGE), the 'gold standard' among molecular typing methods, is highly applicable for the detection of large-scale genomic re-arrangements in genomic islands and mobile elements resulting in gain or loss of restriction sites (Cooper and Feil, 2004; Tenover et al., 1995). To reconstruct the ancestral and evolutionary linkage between *L. monocytogenes* isolates based on genetic variations, PFGE can be complemented by multi-locus sequence typing (MLST).

To add more insight to the *L. monocytogenes* contamination dynamics in food processing environments (FPEs) this study set out to: (i) analyze contamination scenarios of *L. monocytogenes* in an Austrian cheese producing facility by applying PFGE typing and (ii) characterize *L. monocytogenes* isolates by PCR-restriction fragment length polymorphism (RFLP) of *inlA*, stress survival islet 1 (SSI-1) PCR, multi locus sequence typing (MLST) and disinfectant susceptibility testing. Fresh cheese may favor the growth of *L. monocytogenes* due to pH-values ≥ 4.4 and aw ≥ 0.94 (European Commission (EC) regulation, 2073/2005). Hence, a challenge experiment was performed including two storage temperatures (8 and 16 °C) representing temperature fluctuations at retail level and in consumers households (Dufour, 2011; Garrido et al., 2010; Marklinder et al., 2004).

2. Materials and methods

2.1. Cheese processing facility characteristics and sampling

Producing a wide range of artisan cheese products requires the highest microbiological standards in processing facilities. For this reason, an Austrian dairy company producing fresh cheese and fresh cheese products, ripened brine curd cheese and semi-hard cheeses, all made from pasteurized cow, ewe and goat milk, was selected. Occurrence and contamination patterns of *L. monocytogenes* in the FPE were determined in a sampling approach between 01.06.2010 and 20.02.2013. The production facility comprised three compartments: one for the production of fresh cheese products, including rooms for preparation of raw materials and a cooling chamber (BI), an extension building, mainly for dairy processing (yogurt, semi-hard cheese processing, fresh cheese processing) (BII), and a third for the storage and preparation of raw materials (BIII) (Fig. 1). Sampling included non-food contact surfaces (NFCS; drain water, floors, walls, tables, trays, and trolleys), food-contact surfaces (FCS; e.g. fillers, molds, milk vats, slicers; tables, trays, and trolleys), and gloves, work aprons and shoes from dairy staff. Sterile sponge-sticks (3M, St. Paul, MN, USA) were used for FCS and NFCS sampling by swabbing an area up to 900 cm^2. Liquid samples as drain water (500–1000 ml) were collected in sterile polypropylene bottles (Thermo Fisher Scientific, Nalgene®, Waltham, MA, USA). All samples were transported to the laboratory of the Institute of Milk Hygiene, Milk Technology and Food Science (IMMF) in standardized cooling boxes (cooling temperature 4 °C), and investigated immediately. Examination of routine food lot controls was undertaken by the local district laboratory.

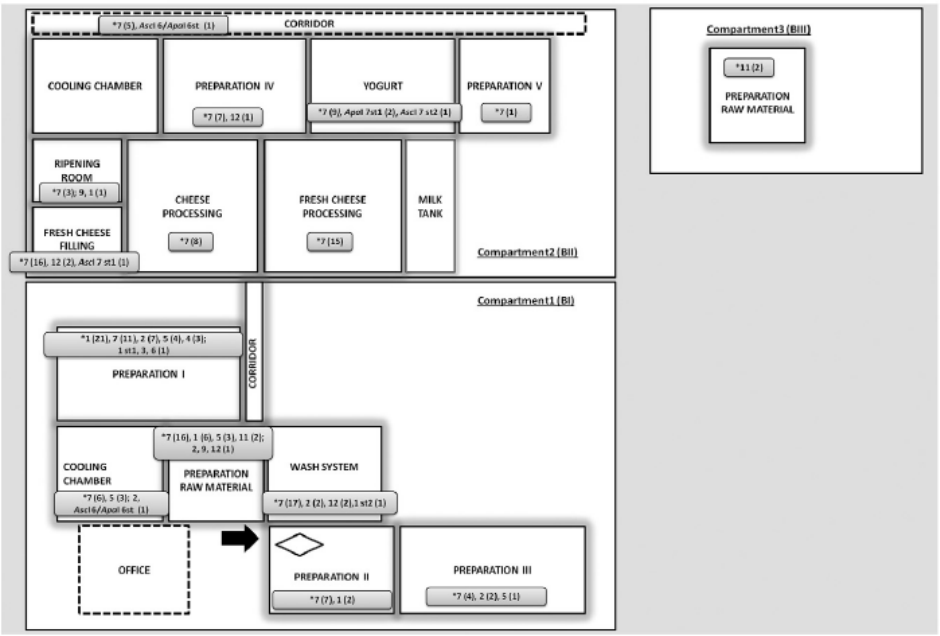


Fig. 1. Distribution of *Listeria monocytogenes* PFGE types in the building compartments (BI, BII, and BIII) of the cheese processing facility investigated during the study phase (2010–2013). PFGE types marked with an asterisk (*) are listed in gray boxes and frequencies of isolation are shown in brackets. The restriction enzyme is supplemented for PFGE-types differing in one enzyme. The rectangle presents the hygiene barrier.

2.2. Microbiological investigation of samples

Environmental swabs were enriched in 50–100 ml half-strength Fraser (HF; Biokar Diagnostics, Beauvais Cedex, France) and investigated according to the ISO 11290-1 protocol (1996; Amd. 2004).

Liquid samples (600 ml) were centrifuged for 30 min at 11,305 g. The sediment was transferred to 1000 ml HF. Additionally, 100 ml, 10 ml and 1 ml of each liquid sample were enriched 1:10 in HF following a semi-quantitative approach applied in the Austrian Listeria Monitoring by IMMF (Asperger et al., 2001; Stessl et al., 213). After incubation at 30 °C for 24 h, 0.1 ml HF each was transferred to 10 ml full-strength Fraser (FF) (Biokar Diagnostics) and incubated for 48 h at 37 °C. HF and FF enrichments were both separately streaked onto Agar Listeria according to Ottaviani and Agosti (ALOA; Merck KgA, Darmstadt, Germany) and Palcam agar (Biokar Diagnostics).

The local district laboratory applied the VIDAS Listeria DUO test kit (bioMerieux, Marcy l'Etoile. France) for the simultaneous detection of *L. monocytogenes* and *Listeria* spp. in food products. *L. monocytogenes* positive food lots were enumerated on ALOA agar according to ISO 11290-2 (1998).

2.3. Isolate confirmation

Colonies suspected for *Listeria* spp. on ALOA (turquoise with and without surrounding halo) and on Palcam agar (concave gray-green colonies surrounded by black halos in the medium) were confirmed by PCR method. Therefore, one *Listeria* spp. colony was dispersed in 100 µl of 0.1 M Tris–HCl buffer (Sigma Aldrich, St. Louis, MO, USA). Additionally, the whole agar surface was swabbed and dispersed in 1 ml of 0.1 M Tris–HCl buffer (Sigma Aldrich). DNA isolation followed a simple protocol based on Chelex® 100-Resin (BioRad, Hercules, CA, USA; Walsh et al., 1991). Subsequently, *Listeria* species were confirmed and differentiated by multiplex-PCR targeting the invasion-associated protein (*iap*) gene (Bubert et al., 1999).

2.4. Subtyping and epidemiological analysis

L. monocytogenes serogroups were defined using a multiplex-PCR targeting genes *lmo0737*, *lmo1118*, ORF2819, ORF2110 and *Listeria* spp. specific *prs*, as published by Doumith et al. (2004).

L. monocytogenes confirmed isolates were incubated overnight in Brain Heart Infusion (BHI; Merck KgA) at 37 °C and cryo-conserved in 15% glycerol (Merck KgA) at −80 °C. PFGE typing applying the restriction enzymes *Asc*I and *Apa*I was performed according to the most up-to-date PulseNet International protocol (http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL04_ListeriaPFGEProtocol.pdf; accessed on: 05.04.2014).

Restricted DNA was electrophoresed on 1% (w/v) SeaKem gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and an included angle of 120° was applied for 22.5 h. Gels were stained with ethidium bromide (Sigma Aldrich), digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.) and normalized as TIFF images (BioNumerics 6.6 software Applied Math NV, Sint-Martens-Latem, Belgium) using the PFGE global standard *Salmonella* ser. Braenderup H9812. Pattern clustering was performed using the unweighted pair group method, including arithmetic averages (UPGMA) and the Dice correlation coefficient with a position tolerance of 1.0%. PFGE types with less than three band difference were considered as closely related, according to Tenover et al. (1995).

2.5. Characterization of potentially persistent strains

MLST typing of seven housekeeping loci *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase) and *lhkA* (histidine kinase) was performed according to Ragon et al. (2008). Sequence types (ST) were determined using the Institute Pasteur Database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html; accessed on: 05.04.2014). A split decomposition analysis was performed on allelic profile data (based on the seven housekeeping genes analyzed), using a web version of SplitsTree (http://pubmlst.org/analysis/; Huson, 1998). *L. monocytogenes* STs detected in this study were compared with *L. monocytogenes* MLST profiles stored in the Institute Pasteur MLST database.

A subset of 15 sporadically and recurrently isolated *L. monocytogenes* PFGE types was characterized by PCR-restriction fragment length polymorphism (RFLP) for detection of point mutations in the 733-bp fragment of the *inlA* gene, according to Rousseaux et al. (2004). Therefore, 1 µl of amplified DNA was digested with 10 U *Alu*I (1 h at 37 °C) and separated on a 2% (w/v) agarose gel containing 3.5 µl SYBR Safe DNA gel stain (Invitrogen, Eugene, Oregon, USA).

Furthermore, the 15 sporadically and recurrently isolated *L. monocytogenes* PFGE types were screened for the presence or absence of the stress survival islet 1 (SSI-1) by PCR, targeting the intergenic region between lmo0443 and lmo0449, according to Ryan et al. (2010). PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and amplicons were resolved on 1% (w/v) agarose gels containing 1× Tris-Acetate-EDTA (TAE) buffer and SYBR Safe at 70 V for 1 h.

2.6. Disinfectant and cleaner susceptibility testing

An agar spotting test was performed in duplicate to determine the minimal inhibitory concentration (MIC) of disinfectants and industrial cleaners against six recurrently isolated *L. monocytogenes* (Mereghetti et al., 2000). *L. monocytogenes* isolates were grown on Mueller–Hinton agar at 37 °C for 18 h (Thermofisher, Oxoid Ltd., Hampshire, UK). Aliquots of 5 µl of bacterial inoculum adjusted to a turbidity of 0.5 McFarland unit (7.5×10^5 cfu per spot) were spotted onto Mueller–Hinton agar, containing the respective cleaner or disinfectant to be tested. Following basic disinfectants [peracetic acid, benzalkonium chloride and hydrogen peroxide, (H_2O_2); Sigma-Aldrich], commercially available multiple composited disinfectants [Prodesan PE 15 (H_2O_2, acetic and peracetic acid), Weiquat (quaternary ammonium compounds); Tensid Chemie], and cleaners [Citrosan SR (acidic), Rimalkan SR mild (alkaline); Tensid Chemie, Muggensturm, Germany] were tested. The test concentrations included the working concentrations applied in the company (representing each highest test concentration) and ranged for peracetic acid, H_2O_2, Prodesan PE 15, and Citrosan SR from 31.3 to 2500 mg/l, for benzalkonium chloride and Weiquat from 3.9 to 2500 mg/l, and for Rimalkan SR mild from 1250 to 25,000 mg/l. Agar plates were incubated at 37 °C for 24–48 h.

2.7. Artificial contamination of fresh cheese samples with L. monocytogenes

The ability of *L. monocytogenes* to survive in fresh cheese was investigated by artificial inoculation. Initially, fresh cheese samples were negative before inoculation, in accordance with ISO 11290-1, 2 (1996; 1998; Amd. 2004). Subsequently, subsamples of fresh cheese were spiked on the surface with spike level 1 (0.4–4 cfu/g) and spike level 2 (4–40 cfu/g) of *L. monocytogenes* (FCP7, PCR-serogroup 1/2b, 3b and FCP9, PCR-serogroup 1/2c, 3c). The *L. monocytogenes* strains were grown in BHI and serially diluted in sterile Ringer's solution up to dilution 10^{-7}. The cheese samples were longitudinally (days 0, 7, 14) surveyed at two incubation temperatures (8 °C and 16 °C). Samples were enumerated after days 0, 7 and 14, according to ISO 11290-2, by plating 1 ml of the initial suspension (1:10) onto three ALOA agar plates. Additional subsamples of 25 g each were diluted with 225 ml HF (Biokar Diagnostics), homogenized for 180 s in a Stomacher 400 (Seward Inc., West Sussex, UK). After incubation at 30 °C for 24 h, 0.1 ml of the primary enrichment was transferred to 10 ml FF (Biokar Diagnostics) and

incubated for 48 h at 37 °C. Loop inoculation of ALOA and Palcam agar was performed according to ISO 11290-1. Colony confirmation was performed as described in Section 2.3. All experiments were performed in duplicate.

3. Results

3.1. Longitudinal environmental monitoring of L. monocytgenes

During the sampling period between 2010 and 2013, a total of 1284 environmental samples were tested for the presence or absence of *Listeria* spp. including *L. monocytogenes*. *L. monocytogenes* isolated (n = 71) from raw materials and products were provided by the district laboratory and compared to local environmental isolates to trace potential routes of contamination (Supplemental Table 1).

Specifically, 183 drain water samples and 1101 swab samples were taken from three compartments of the facility. Surface sampling comprised the following: 393 swabs from FCS (conveyor belts, fillers, tables, slicers, mixers and molds), 615 swabs from NFCS (including transport boxes and trolleys, floors and drains), and 93 swabs from shoes, work aprons, and personnel gloves.

The overall *Listeria* spp. occurrence rate was 21.9%, which was dominated by *L. monocytogenes* (19.5%). In detail, 15.3% of the investigated samples were contaminated with *L. monocytogenes*, 3.4% contained *Listeria* species mixtures (*L. monocytogenes*, *L. innocua*, and *L. welshimeri*), and 0.9%, mainly floor and drain samples, contained *L. monocytogenes* mixtures.

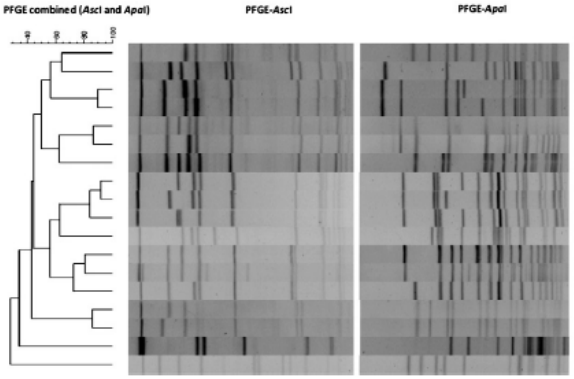
Sampling began in 2010 to trace the source of a *L. monocytogenes* contamination in fresh cheese during processing. FCS swabs from fillers were mainly positive for *L. monocytogenes* (4.7%) in the first sampling phase. After improving hygiene management, e.g. strict compartmentalization of the hygiene areas, and staff training, e.g. following to one-way traffic routes and correct usage of hygiene locks, FCS were *Listeria* spp. negative. In the subsequent monitoring period, several extensions and reconstructions of the facility took place. Further testing of NFCS (conveyor belts, transport trolleys, pallets, and tables) resulted in 1.4% of samples positive for *L. monocytogenes*. The processing environment (drains, walls, doors and floors) was found to be *L. monocytogenes* positive during the whole investigation (15.8%). *L. monocytogenes* contamination spread from the processing environment in building compartment BI to BII. As floor contamination increased, shoes were frequently found to be positive for *L. monocytogenes* (48.4%). In 2013 NFCS and FCS sampling was performed by the quality management team (QMT) of the company and not available for the actual publication. Further details on *L. monocytogenes* occurrence during the study phase are depicted in Supplemental Table 2.

3.2. Subtyping and epidemiological analysis

In total, 226 and 71 *L. monocytogenes* were isolated from FCS/NCFS and from raw materials/cheese products, respectively. The majority of isolates were classified by PCR as serogroup 1/2b, 3b, 7 (67.4%) followed by serogroups 4b, 4d, 4e (15.8%), 1/2a, 3a (14.8%) and 1/2c, 3c (2%). Therefore, 247 isolates were assigned to genetic Lineage I or III (1/2b, 3b, 4b) and 50 isolates assigned to Lineage II (1/2a, 1/2c). PFGE analysis of the 297 *L. monocytogenes* resulted in 17 *Asc*I and 17 *Apa*I profiles. The restriction digest applying *Asc*I and *Apa*I resulted in at least one subtype (st) for FCP1, FCP2, FCP6, FCP7, and FCP9. A combined PFGE-cluster analysis revealed a high genetic relatedness (≥ 90%) among FCP7 and FCP7 st1 and FCP6 and FCP6 st when digested with both *Asc*I and *Apa*I. Furthermore, ≥ 85% relatedness was found among FCP9 and FCP9 st, FCP2 and FCP2 st, and FCP7 st2 and FCP7/FCP7 st1 (Fig. 2).

At the beginning of monitoring in 2010, seven different genotypes dominated the food processing environment (FCP1, FCP1 st2, FCP2, FCP3, FCP4, FCP5 and FCP6). FCP1 st2, FCP2 and FCP6 were also isolated from raw materials and cheese products during lot control (Supplemental Table 1). Food lots tested positive for *L. monocytogenes* were found to be contaminated below 10 cfu/g. PFGE type FCP1 (PCR-serogroup 4b, 4d, 4e) represented the majority of strains isolated from the food associated equipment, floors and drains in 2010. Subtype FCP1 st2 was most often found on workers' shoes and was also isolated from the end product. During 2011, two novel PFGE types were introduced into the food processing area probably via raw materials: FCP9 (PCR-serogroup 1/2c, 3c) and FCP11 (PCR-serogroup 1/2a, 3a). FCP11 was solely detected in the storage rooms at BIII. In 2011, a decrease in *L. monocytogenes* strain variability was observed. Nevertheless, a new genotype appeared: FCP7 was initially detected in drain water samples. The latter type could not be eradicated from the environment and was also found in the cheese products. During the final period of sampling (2012 to 2013) another genotype FCP12 (serotype 1/2a, 3a) was colonizing the equipment and drains.

MLST typing resulted in nine *L. monocytogenes* MLST types. The SplitsTree analysis of *L. monocytogenes* ST types yielded a fit value of 83.2 (Fig. 3). The *inl*A analysis applying the restriction enzyme *Alu*I



Year	Source	AscI profile	n	ApaI profile	n
2010-2011	FCS[a]/cheese products	FCP11	9	FCP11	9
2010	FCS	FCP4	3	FCP4	3
2010	NFCS[b]/raw material	FCP6	3	FCP6	2
2010	NFCS	FCP6	1	FCP6st[c]	2
2011	NFCS/raw material	FCP9	5	FCP9	4
2010	raw material	FCP9 st	1	FCP9 st	2
2011-2012	FCS/NFCS	FCP12	6	FCP12	6
2011-2013	FCS/NFCS/cheese products	FCP7	187	FCP7	186
2012	NFCS	FCP7 st1	1	FCP7 st1	2
2012	NFCS	FCP7 st2	1	FCP7	1
2010-2011	FCS/NFCS	FCP5	11	FCP5	11
2010-2011	FCS/NFCS	FCP1	30	FCP1	30
2010	FCS	FCP1 st1	1	FCP1 st1	1
2010-2011, 2013	NFCS/cheese products	FCP1 st2	15	FCP1 st2	15
2010	FCS/NFCS/cheese products	FCP 2	20	FCP 2	20
2010	cheese products	FCP2 st	1	FCP2 st	1
2010	cheese products	FCP10	1	FCP10	1
2010	NFCS	FCP 3	1	FCP3	1

Fig. 2. Combined PFGE cluster analysis of *Listeria monocytogenes* isolates from this study (restriction enzymes *Asc*I & *Apa*I). The TIFF images were compared using BioNumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium), and normalized using the PFGE global standard *Salmonella* ser. Braenderup H9812. Pattern clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) and the Dice correlation coefficient was applied with a position tolerance of 1.5%. The table indicates the year of isolation, the source, and the number of isolates (n). [a] FCS, food contact surfaces; [b]NFCS, non-food contact surfaces; st[c], subtype.

revealed five major PCR-RLFP profiles: ST37 strains corresponded to PCR-RLFP profile 1; ST1, ST5, ST7 and ST87 shared the PCR-RLFP profile 2; ST21 and ST570 had PCR-RLFP profile 3; ST9 and ST204 PCR-RLFP had profile 4 and ST7 was typed as PCR-RLFP profile 5. According to Rousseaux et al. (2004), PCR-RLFP profiles 1 and 4 include *L. monocytogenes* strains with truncation in *inlA*. Additional screening for the presence of genetic SSI-1 revealed that *L. monocytogenes* ST5, ST7, ST9 and ST204 had a typical 9.7 kbp fragment.

3.3. Disinfectant and cleaner susceptibility testing

In general, all *L. monocytogenes* test strains (FCP1 st2, FCP2, FCP7, FCP7 st2, FCP6, FCP12) were susceptible to peracetic acid (MIC 250 mg/l), H_2O_2 (MIC 125 mg/l), Prodesan PE 15 and Citrosan (MIC 500 mg/l), and Rimalkan (MIC 15714 mg/l). No variability among *L. monocytogenes* test strains could be detected with these disinfectants and cleaners. Additionally, all strains were sensitive to benzalkonium chloride (at concentrations in the range of 7.8–31.3 mg/l) and Weiquat (at concentrations in the range of 31.3–187.5 mg/l). FCP7 showed the highest MIC at 31.3 mg/l for benzalkonium chloride and FCP12 for Weiquat (MIC 187.5 mg/l). These strains were recurrently isolated in the last phase of the project (2012–2013) when hygiene measures and changes in sanitation routines were implemented.

3.4. Artificial contamination of fresh cheese samples with L. monocytogenes

Fresh cheese samples were spiked with 1.4 (spike level 1) and 15.0 cfu (spike level 2) *L. monocytogenes* per g. The pH ranged from 4.7 to 5.6 and the water activity (a_W) ranged from 0.97 to 0.98. After seven days of storage at 8 °C, the enumeration of *L. monocytogenes* (ISO 11290-2) yielded <10 and 10 cfu/g for the spike levels of 1.4 and 15 cfu/g. At 16 °C, <10 and 20 cfu/g *L. monocytogenes* were detected. After further seven days of storage at 8 °C *L. monocytogenes* counts for both spike levels were <10 cfu/g. At 16 °C and 14 days of storage a *L. monocytogenes* and pH (5.6) increase could be observed, as 120 and 8.0×10^4 cfu/g were detected at spike levels of 1.4 and 15 cfu/g, respectively. All test samples were positive in the qualitative test, according to ISO 11290-1.

4. Discussion

L. monocytogenes is widely distributed in the environment, capable of entering the food chain via raw materials, persisting in niches and recontaminating final products by cross-contamination (Almeida et al., 2013; Ferreira et al., 2011; Fox et al., 2011; Holch et al., 2013; Parisi et al., 2013). Following the most recent outbreaks of human listeriosis, it has been recognized that food lot analyses are not sufficient to control *L. monocytogenes* transmission during food production (Fretz et al., 2010; Gilmour et al., 2010; Schoder et al., 2013a).

Therefore, this study is the first to focus on analysis of changing *L. monocytogenes* environmental contamination patterns in respect of an Austrian cheese processing facility within a certain timeframe (2010 to 2013). The study was complemented by application of epidemiological tools for strain characterization, recording changes in hygiene management strategies, susceptibility tests of recurrently isolated *L. monocytogenes* strains to disinfectants and cleaners, and *L. monocytogenes* survival experiments during storage on the food matrix.

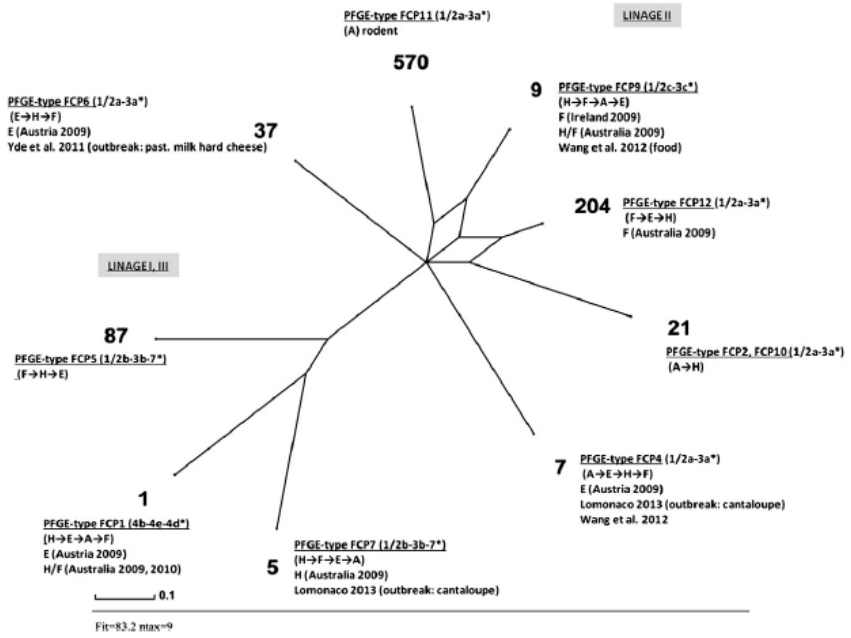


Fig. 3. Genetic relationships of *Listeria monocytogenes* isolates from this study based on MLST compared with isolates from the Institute Pasteur database and literature searches. MLST profiles of the Institute Pasteur database were selected within a timeframe between 2009 and 2013. Figure shows a split decomposition analysis based on allelic profiles using a PubMLST software tool by Huson (1998). Each number represents a multilocus sequence typing genotype (ST). Source abbreviations for food, factory (F), human (H), animal (A), and environment (E) are listed, according to the serotype. The arrow marks decreasing isolation frequencies in the respective sources. PFGE-types and PCR-serogroups (marked with an asterisk) are depicted.

In the course of monitoring cheese production, three major contamination scenarios were observed: initially (2010) filler contamination was predominant; a complex *L. monocytogenes* biofilm might have led to a "sloughing effect", where food lots were probably minimally contaminated by particles of the biofilm (Van Houdt and Michiels, 2010). During food lot testing by the district laboratory most of these lots were negative. In the second phase of sampling, awareness of the ubiquity and persistence of *L. monocytogenes* during food processing was heightened and sanitation measures were improved. Hence, the initial strain variability (up to eight different genotypes) decreased. A second contamination scenario was observed: the remaining and newly introduced *L. monocytogenes* strains (probably via raw materials and building extensions) were further transmitted to the processing environment due to a lack of one-way personnel and equipment traffic routes. Insufficient attention was paid to critical control points in the processing and packaging areas. Further, weak points in hygiene management were eliminated by extended training and greater compartmentalization of the processing and packaging areas. The third contamination scenario in the final phase (2012–2013) was total reduction to one predominant genotype FCP7 (ST5), with occasional detection of FCP12 (ST204) in the drains and on the floors. A reduction of certain *L. monocytogenes* strains in food processing plant was also observed by other authors (Ferreira et al., 2011; Ortiz et al., 2010). Malley et al. (2013) stated that *L. monocytogenes* was successfully eliminated from FCS in a fish plant. Nonetheless, *L. monocytogenes* could be still isolated from niches on NFCS. Therefore, the authors suggested implementation of statistical tools, including typing data, to support identification of persistent *L. monocytogenes*.

In this study, intensive humidity and steam could be observed in all rooms, and water residues were visible on the floors due to enhanced cleaning vigor. This could even explain the high *L. monocytogenes* contamination of the FPE (shoes, drains and floors) throughout the study (15.8%). Non-stop processing of foodstuffs and permanently wet processing rooms may lead to two negative consequences of improved sanitation: *Listeria* spp. transmission to FCS and food by aerosols, and reduced effectiveness of disinfectants (Berrang and Frank, 2012).

However, the impact of a dilution effect and sublethal concentrations of disinfectants triggering resistance and virulence is still controversial (Carpentier and Cerf, 2011; Pricope et al., 2012; Rodrigues et al., 2011). All sanitizers included in this study were effective against recurrently isolated *L. monocytogenes*. In the last monitoring phase, the persistent strains FCP7 and FCP12 resulted in higher MICs for quaternary ammonium compounds (QAC) (benzalkonium chloride and Weiquat). Industrial disinfectants and cleaners applied in accordance with the manufacturer's instructions are still efficient on pre-dried surfaces (Kastbjerg and Gram, 2012). These authors stated that the QAC Triquart SUPER could still actively kill *L. monocytogenes* at a concentration of 0.0125%.

The molecular typing of recurrently isolated *L. monocytogenes* genotypes revealed that the facility harbored the worldwide second most prevalent ST1 in the first period of monitoring. ST1 was also found to be persistent in two Austrian dairies during 1996–2003 (Stessl et al., 2013). ST87 and ST5 (both PCR serogroup 1/2b, 3b, 7), could be isolated from the local environment for more than six months. Recently, Wang et al. (2012) reported that ST87 was the third most prevalent genotype isolated from meat and vegetable samples in China. ST5, SS-1$^+$ and persistent in the cheese processing environment of the current study, was also isolated during the epidemic outbreak related to cantaloupes (Lomonaco et al., 2013). Ryan et al. (2010) claimed that SSI-1$^+$ *L. monocytogenes* strains are better adapted to low pH and high salt concentrations. Evidence that certain strains possessing a SSI-1$^+$ seem to withstand adverse environmental conditions and sanitation is rising and warrants further research. ST7 (SSI-1$^+$), isolated once in 2010, was comparable to a second US cantaloupe outbreak strain (Lomonaco et al., 2013). ST7 is distributed worldwide, and was frequently isolated from animals, particularly from farm outbreaks (silage feeding) and sporadic cases in humans (Institute Pasteur MLST database:http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html; accessed: 05.04.2014). ST37 (truncated *inl*A), occasionally isolated from the cheese processing environment, was comparable to the genotype responsible for an outbreak in Belgium linked to consumption of hard cheese (Yde et al., 2012). ST570 represents a new allelic profile and was probably introduced into the cheese processing environment via raw materials. The latter was probably eradicated due to implementation of hygiene measures as the application of correct concentrations of disinfectants and one-way traffic routes of staff and equipment. ST21 was prevalent during the first contamination phase and was not re-isolated during further *L. monocytogenes* monitoring. This occasionally isolated genotype is strongly linked to rodents (Institute Pasteur MLST database). ST9 (truncated *inl*A, SSI-1$^+$) represents the genotype most often isolated from meat and fish in China (Wang et al., 2012), and from Irish and Australian food samples (MLST database Institute Pasteur). A premature stop codon causing a truncation in the virulence gene *inl*A is often present in *L. monocytogenes* strains CC9 and CC121. *L. monocytogenes* ST assigned to the latter CC is suggested to be better adapted to environmental conditions (Holch et al., 2013; Ragon et al., 2008). In the current study, ST9 was introduced most likely via raw materials. *L. monocytogenes* strain ST204 (truncated *inl*A, SSI-1$^+$), identified during the final phase of monitoring, was also found to be persistent in two food processing facilities in the Czech Republic (Stessl et al., 2013; Fig. 3).

To summarize, a high number of *L. monocytogenes* strains enter a dairy plant and cycles of elimination, recontamination and persistence frequently occur (Chambel et al., 2007). It appears that *L. monocytogenes* contamination could establish as a multifactorial random event, influenced by the global distribution of STs, cross-border trade of raw materials, building renovations of the facility, potential biofilm formation, missing hygiene compartmentalization, inadequately trained dairy staff and a large product range (Nilsson et al., 2011).

The outcome of challenge experiments in fresh cheese showed that *L. monocytogenes* was able to survive, even after 14 days of storage, and to multiply at insufficient cooling temperatures (8 and 16 °C). This has been proposed to be influenced by heterogeneity of immobilized bulk water present in the cheese matrix (Møller et al., 2013). Successful control of *L. monocytogenes* growth could be achieved by adding bacteriocinogenic *Lactococcus lactis* starter cultures (Dal Bello et al., 2012).

In conclusion, a better incorporation of systematic environmental monitoring (FCS, NFCS, and product associated samples) into food surveillance systems is needed (Anonymous, 2012; Luber et al., 2011). Infrequent or inadequate sampling could easily overlook *L. monocytogenes* contamination and could pose a risk of transmission to food under disguise of false security. Contamination may also be the result of persistent strains. Consequently, further research is required to understand this persistence and how it may be controlled. In this study, identification of *L. monocytogenes* FPE contamination scenarios was important to increase FBOs awareness of possible transmission routes to food lots. Contamination scenarios identified were subject to change and thereby must be viewed as dynamic processes.

Improving awareness of FBOs and dairy staff of good hygiene practices should be estimated by questionnaires as published by Lehto et al. (2011) and Rotariu et al. (2014). Knowledge of the type of contamination in an FPE, as described in this study, could facilitate improved hygiene management to direct sanitation activities, facilitate communication with staff and implement corrective action.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.001.

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10.2. Original article 2

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Predominance of Distinct *Listeria Innocua* and *Listeria Monocytogenes* in Recurrent Contamination Events at Dairy Processing Facilities

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Abstract: The genus *Listeria* now comprises up to now 21 recognized species and six subspecies, with *L. monocytogenes* and *L. innocua* as the most prevalent sensu stricto associated species. Reports focusing on the challenges in *Listeria* detection and confirmation are available, especially from food-associated environmental samples. *L. innocua* is more prevalent in the food processing environment (FPE) than *L. monocytogenes* and has been shown to have a growth advantage in selective enrichment and agar media. Until now, the adaptive nature of *L. innocua* in FPEs has not been fully elucidated and potential persistence in the FPE has not been observed. Therefore, the aim of this study is to characterize *L. innocua* ($n = 139$) and *L. monocytogenes* ($n = 81$) isolated from FPEs and cheese products collected at five dairy processing facilities (A–E) at geno- and phenotypic levels. Biochemical profiling was conducted for all *L. monocytogenes* and the majority of *L. innocua* ($n = 124$) isolates and included a rhamnose positive reaction. *L. monocytogenes* isolates were most frequently confirmed as PCR-serogroups 1/2a, 3a (95%). Pulsed-field gel electrophoresis (PFGE)-typing, applying the restriction enzymes *AscI*, revealed 33 distinct *Listeria* PFGE profiles with a Simpson's Index of Diversity of 0.75. Multi-locus sequence typing (MLST) resulted in 27 STs with seven new *L. innocua* local STs (ST1595 to ST1601). *L. innocua* ST1597 and ST603 and *L. monocytogenes* ST121 and ST14 were the most abundant genotypes in dairy processing facilities A–E over time. Either SSI-1 (ST14) or SSI-2 (ST121, all *L. innocua*) were present in successfully FPE-adapted strains. We identified housekeeping genes common in *Listeria* isolates and *L. monocytogenes* genetic lineage III. Wherever there are long-term contamination events of *L. monocytogenes* and other *Listeria* species, subtyping methods are helpful tools to identify niches of high risk.

Keywords: *Listeria monocytogenes*; *Listeria innocua*; dairy processing; subtyping; persistence

1. Introduction

The genus *Listeria*, assigned to the phylum Firmicutes, comprises Gram-positive facultative anaerobe bacteria that are found ubiquitously in environments such as soil, water, or plant material [1,2]. The genus comprises up to 21 recognized species and six subspecies, with *L. monocytogenes* and *L. innocua* as the most prevalent sensu stricto associated species [3] (http://www.bacterio.net/listeria.html). *L. monocytogenes* has been implicated in human listeriosis outbreaks, most often associated with ready-to-eat (RTE) food products (https://www.cdc.gov/listeria/outbreaks/index.html) [4,5]. Food is most commonly contaminated by *L. monocytogenes* from niches in the food processing environment (FPE) [6]. As an environmental saprophyte, *L. monocytogenes* is highly adapted to harsh conditions, such as those associated with osmotic and cold stress, low pH, desiccation, and competitive microflora [7]. The adaptive strategies of *L. monocytogenes* to ecological niches are clearly divergent. Genetic lineage I (serovar 1/2b and 4b) has a tropism to human and animal host tissues and cell types, whereas genetic lineage II (serovar 1/2a and 1/2c) is more adapted to environmental conditions [8,9].

The ecological co-habitation, genomic synteny, and phenotypic similarities of *L. monocytogenes* and *L. innocua* qualify the latter as surrogate for *L. monocytogenes* behavior prediction in FPEs [10]. Nevertheless, the *prfA*-virulence gene cluster that was present in the common ancestor of *Listeria* species was lost in two separate recombination events with *L. innocua* and *L. welshimeri* [11]. In fact, *L. innocua* comprises two major subgroups A and B, with one atypical subgroup D (e.g., hemolytic, LIPI regions and *inlJ* positive) serving as a link between *L. monocytogenes* and *L. innocua* in the evolutionary chain [12]. Internalin genes (*lin0354, lin0661, lin1204, and lin2539*) have been suggested as potential genetic markers for *L. innocua* subgroups A-D [13]. Hemolytic *L. innocua* strains have now been characterized by whole genome sequencing (WGS) and virulence testing. The strains harbor a pathogenic island, LIPI-1, and internalin *inlA*, required for mammalian cell invasion. These findings will challenge risk management in the food chain, as *L. innocua* was often assessed in the past as non-hazardious [12]. Currently, *L. innocua* genome sequences are still limited [14,15]. Eleven *L. innocua* genome assemblies are currently available from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/genomes/1024?).

The loss of full virulence in *L. monocytogenes* lineage II strains (e.g., sequence type (ST) 9, ST121), due to point mutations in *prfA*, *inlA*, *inlB*, and *plcA*, indicates the formation of monophyletic groups present in FPEs [16]. The latter strains were mentioned, amongst others (e.g., ST5, ST7, ST8), in past long-term FPE contamination scenarios, which were then referred to as in-house clones or persisters [17–20]. Until now, the adaptive nature of *L. innocua* in FPEs has not been fully characterized and potential persistence has not been observed. Accordingly, the aim of this study is to characterize potential *L. innocua* persistent isolates originating from FPEs and cheese products on geno- and phenotypic levels and to compare them to *L. monocytogenes* strains from the same habitat.

2. Materials and Methods

2.1. Isolate Selection and Listeria Species Confirmation

In total, 220 *Listeria* isolates (139 *L. innocua* and 81 *L. monocytogenes*) originating from different cheese types, product-associated liquids (PAL; smear, brine), product-associated samples (culture, enrichment), raw material (RM), food contact surfaces (FCS), non-food contact surfaces (NFCS), and environmental liquid samples (EL; floor and drain water) were included in this study. The isolates were collected during a *Listeria* monitoring program between 1987 and 2010 from five dairy producers (A–E) [21,22]. The selection criteria for this study were as following: (1) producer with a history of frequent *L. innocua* or *L. monocytogenes* isolation, and (2) *L. monocytogenes* previously identified as potentially persistent in the FPE. Producer A was mainly involved with ripening cheese and packaging grated and sliced semi-hard cheeses. Producers B and C manufactured a broad range of white dairy and fresh products and a product line of semi-hard and hard cheeses. Producer D manufactured a range of semi-hard and red smear cheeses and producer E manufactured acid curd cheeses (Table 1).

The *L. innocua* and *L. monocytogenes* isolates were stored at −80 °C at the *Listeria* collection located at the Unit of Food Microbiology, University of Veterinary Medicine Vienna, Austria. *L. innocua* and *L. monocytogenes* isolates were re-cultivated on tryptic soy agar (TSA; Merck KgA, Darmstadt, Germany) at 37 °C overnight. DNA isolation followed a rapid protocol using Chelex 100-Resin (Bio-Rad Laboratories Inc., Hercules, CA, USA), as published by Walsh et al. [23]. For *Listeria* species differentiation, a multiplex PCR targeting the invasion-associated protein (*iap*) gene was performed [24]. *L. monocytogenes* isolates were subtyped by serogroup PCR, targeting the *Listeria* spp. specific *prs*, and *lmo0737*, *lmo1118*, *ORF2819*, and *ORF2110* [25]. Biochemical profiling was performed for each *Listeria* isolate by applying the API-*Listeria* identification system (Biomérieux, Marcy l'Etoile, France).

To evaluate a possible association between the categorical variables, i.e., *Listeria* species, rhamnose (yes/no), and sporadic/persistence, the association coefficient (Cramer's V) was calculated (Microsoft Excel 2010, Microsoft Corp., Redmond, WA, USA).

2.2. Molecular Epidemiological Analysis

DNA macrorestriction digest, applying *Asc*I and *Apa*I of 220 *Listeria* isolates (139 *L. innocua* and 81 *L. monocytogenes*), was performed according to the latest CDC PulseNet International PFGE protocol (https://www.cdc.gov/pulsenet/pdf/listeria-pfge-protocol-508c.pdf). In brief, the cell suspension was standardized to an optical density of 1.0 in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8; Sigma-Aldrich Corp, St. Louis, MO, USA) and lysed by adding 20 mg/mL lysozyme (Sigma-Aldrich Corp) to an incubation step at 55 °C for 30 min. After incubation, 20 mg/mL proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) was added to the cell suspension followed by 1% SeaKem Gold agarose (1:1; each 400 µL; Lonza Group, Basel, Switzerland). The suspension was poured into plug molds and the solidified plugs were lysed overnight in 5 mL of cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8, 1% Sarcosyl + 0.1 mg/mL proteinase K; Sigma-Aldrich Corp). Subsequently, the plugs were washed with sterile water (twice; Mayrhofer Pharmazeutika, Leonding, Austria) and TE buffer (three times). The *Listeria* DNA macrorestriction digest was performed by applying 50 U each of *AscI* and *ApaI* at 37 °C and 30 °C for 4 h (Thermo Fisher Scientific Inc., Waltham, MA, USA). The universal standard *Salmonella* ser. Braenderup H9812 was digested with 50 U *Xba*I (Thermo Fisher Scientific Inc.) at 37 °C for 4 h (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). The restriction digested plugs were loaded into 1% SeaKem Gold Agarose gel in 0.5 × Tris borate EDTA buffer (45 mM Tris, 45 mM borate, 1 mM EDTA; Sigma-Aldrich Corp) and electrophorezed for 22.5 h at 6 V/cm with a linear ramping factor and pulse times from 4.0 to 40.0 s at 14 °C and an included angle of 120° (CHEF DR III system; Bio-Rad Laboratories Inc., Hercules, CA, USA).

The gel was stained with ethidium bromide (Sigma-Aldrich Corp) and digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.). The TIFF images were normalized with BioNumerics 6.6 software package (Applied Math NV, Sint-Martens-Latem, Belgium) to the universal standard *Salmonella* ser. Braenderup H9812. Pattern clustering utilized the unweighted pair group method with arithmetic mean (UPGMA) and the dice correlation coefficient with a position tolerance of 1.5%. PFGE types were considered identical when the patterns were indistinguishable. The Simpson's Index of diversity was calculated with the online tool of Comparing Partitions (http://www.comparingpartitions.info/).

The *L. monocytogenes* multi-locus sequence typing (MLST) scheme included the following seven housekeeping genes: ABC transporter (*acbZ*, *lmo*2752), beta glucosidase (*bglA*, *lmo*0319), catalase (*cat*, *lmo*2785), succinyl diaminopimelate desuccinylase (*dapE*, *lmo*0265), D-amino acid aminotransferase (*dat*, *lmo*1617), L-lactate dehydrogenase (*ldh*, *lmo*0210), and histidine kinase (*lhkA*, *lmo*1508). Protocols for target-specific primers and PCR conditions are provided at https://bigsdb.web.pasteur.fr/listeria/primers_used.html. Target-specific PCR products were sequenced with universal sequencing primers (*oF*: GTT TTC CCA GTC ACG ACG TTG TA; *oR*: TTG TGA GCG GAT AAC AAT TTC; LGC Genomics, Berlin, Germany) and allele-specific sequences were submitted to the Institute Pasteur sequence and profile database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef). The sequence types (ST) were determined by the combination of the seven housekeeping loci.

The STs were compared to the Institute Pasteur isolate database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates_public) to estimate their global presence and potential niche attribution.

2.3. Screening for Stress Survival Islets (SSI-1 and SSI-2)

L. innocua and *L. monocytogenes* isolates were screened for the presence of SSI-1^{+} (9.7 kbp fragment), SSI-1^{-}(F2365_0481 homologous gene; 1.1 kbp fragment) and SSI-2 (2.2 kbp fragment). PCR primers targeting the *L. monocytogenes* flanking genes *lmo0443* and *lmo0449* were used according to Ryan et al. [26]. The homologous genes related to *L. innocua lin0464* and *lin0465* (2.2 kbp fragment) were investigated according to Hein et al. [27]. PCR reactions contained 0.2 µM each primer, 2 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates (dNTPs; Thermo Fisher Scientific), 1 U Platinum *Taq* DNA polymerase (Thermo Fisher Scientific), 10× PCR buffer, diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific), and 1 µL DNA template in a final volume of 25 µL.

The PCR reaction for the detection of SSI-1^{+} (9.7 kbp) and SSI-1^{+} (1.1 kbp) differed from the latter mix by the following components: 2.5 U long range DNA polymerase and 2 µL DNA template in a final volume of 25 µL. The gel electrophoresis of PCR-reactions was determined in a 1.5% agarose gel containing 0.5 × Tris–borate–EDTA (TBE) buffer and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, PA, USA). The DNA standard Thermo Scientific™ GeneRuler™ 100 bp and 1 kb plus (Thermo Fisher Scientific Inc.) were applied for fragment length comparison.

2.4. L. Monocytogenes and L. Innocua Minimum Inhibitory Concentration (MIC) towards Biocides

The minimum inhibitory concentrations (MIC) of five disinfectant compounds (peracetic acid, benzalkonium chloride, sodium hypochlorite, hydrogen peroxide, and isopropanol; all supplied by Sigma AldrichCorp) were determined for 10 recurrent *L. monocytogenes* and *L. innocua* genotypes: M1[E] (ST59, 1/2b); M5[B]=M5[D]=M5[E] (ST121, 1/2a); M10[C] (ST155, 1/2a); M11[A]=M7[D] (ST14, 1/2a); IN1[E] (ST1595); IN2[E] (ST637); IN3[E] (ST1601); IN4[C]=IN4 [E] (ST603); IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597); IN7[C] (ST1085).

The disinfectant components were tested at concentration ranges of 31.3–1000 mg/L for peracetic acid and hydrogen peroxide, 0.5–1000 mg/L for benzalkonium chloride and 125–10,000 mg/L for sodium hypochlorite. An agar dilution method was performed in duplicates to determine the minimal inhibitory concentration (MIC) of the disinfectants against *L. innocua* and *L. monocytogenes* strains. As previously described, 5 µL of bacterial culture was spotted onto Mueller–Hinton agar (Oxoid, Basingstoke, UK) containing the disinfectants to be tested [28,29]. Plates were incubated at 37 °C for 24 to 48 h. Following incubation, the lowest disinfectant concentration that showed no bacterial growth was recorded as MIC. Mean MIC values were calculated using Excel (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Isolate Characteristics

The isolate set comprised 139 *L. innocua* and 81 *L. monocytogenes* PCR-confirmed isolates targeting the *iap* (invasion associated protein p60) gene [24]. *Listeria* spp. originated from the following Austrian cheese processing facilities: A (*L. innocua*/*L. monocytogenes*: (n = 9/13), B (n = 0/47), C (n = 34/3), D (n = 72/9), and E (n = 24/9). The *Listeria* isolate collection was established over a monitoring period of 23 years (1987–2010). *Listeria* spp. were isolated from cheese samples (24 *L. innocua* and 3 *L. monocytogenes*), product associated samples (PA, PAL; 100 *L. innocua* and 64 *L. monocytogenes*), production environment (FCS, NFCS, environmental liquids (EL); 14 *L. innocua* and 14 *L. monocytogenes*), and one *L. innocua* isolate from raw milk (RM) (Table 1).

Table 1. *L. innocua* (n = 139) and *L. monocytogenes* (n = 81) isolate characteristics included in this study.

PRODUCER	SOURCE	OCCURRENCE	TIME-FRAME	ISOLATES (n)	PFGE PROFILE (*ASCI/APAI*)	CC [f]	ST (Serogroup)	*abcZ*	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*	SSI-1 + [g]	SSI-1- [h]	SSI-2 [i]
***L. monocytogenes* (API profile 6-5-1-0)**																	
C	milk filter (PA)	spor[c]	once	1	M9[C]	1 (I)	1 (4b, 4d, 4e)	3	1	1	1	3	1	3	0	1	0
D	smear (PAL)	spor	once	1	M6[D]	3 (I)	3 (1/2b, 3b)	4	4	4	3	2	1	5	1	0	0
A	soft cheese (P)	spor	once	1	M8[A]	7 (II)	7 (1/2a, 3a)	5	8	5	7	6	404	1	1	0	0
E	smear (PAL)	spor	once	1	M4[E]	398 (II)	398 (1/2a, 3a)	7	13	19	6	1	7	1	0	1	0
A	smear (PAL)	spor	once	1	M12[A]	ST529 (III)	529 (4b, 4d, 4e)	25	73	82	96	45	211	67	0	1	0
E	acid curd cheese (P)/smear (PAL)	rec [b]	2 mo [d]	2	M1[E]	59 (I)	59 (1/2b, 3b)	11	1	12	16	3	1	7	0	1	0
C	swab (FCS)	rec[c]	1 yr [d]	2	M10[C]	155 (II)	155 (1/2a, 3a)	7	10	16	7	5	2	1	1	0	0
E	smear (PAL)	rec[c]	7 mo	2	M3[E]	403 (II)	403 (1/2a, 3a)	7	7	10	4	5	24	1	1	0	0
A/D	drain water (EL)/smear (PAL)	rec[c]/spor	11 yr/once	11/1	M11[A]=M7[D]	14 (II)	14 (1/2a, 3a)	8	6	13	6	5	2	1	1	0	0
E	acid curd cheese (P)/culture (PA)/environment (NFCS)	rec [b]	1 mo	3	M2[E]	121 (II)	121 (1/2a, 3a)	7	6	8	8	6	37	1	0	0	1
B/D/E	smear (PAL)	rec[c]/rec[b]/spor	7 yr/4mo/once	47/7/1	M5[B]=M5[D]=M5[E]21 (II)		121 (1/2a, 3a)	7	6	8	8	6	37	1	0	0	1
***L. innocua* (API profile 7-5-1-0)**																	
C	smear (PAL)	spor	once	1	IN10[C]	ST1596	1596	26	21	33	33	48	213	216	0	0	1
C	cheese (P)	spor	once	1	IN18[C]	ST530	530	28	62	40	97	45	214	53	0	0	1
A	smear (PAL)	spor	once	1	IN12[A]	140	637	28	23	33	35	23	192	16	0	0	1
C	floor water (NFCS)	spor	once	1	IN15[C]	140	637	28	23	33	35	23	192	16	0	0	1
E	smear (PAL)	rec [b]	4 mo	2	IN2[E]	140	637	28	23	33	35	23	192	16	0	0	1
E	smear (PAL)	spor	once	1	IN4ST[E]	600	603	36	21	40	108	65	243	81	0	0	1

Table 1. *Cont.*

PRODUCER	SOURCE	OCCURRENCE	TIME-FRAME	ISOLATES (n)	PFGE PROFILE (*ASCI*/*APAI*)	CC [f]	ST (Serogroup)	*abcZ*	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*	SSI-1 + [g]	SSI-1- [h]	SSI-2 [i]
C/E	hard cheese (P)/acid curd cheese (P)/smear (PAL) environment (NFCS	spor/rec[c]	once/5.6 yr	1/12	IN4[C]=IN4[E] /n. t.[e]	600	603[a]	36	21	40	108	65	243	81	0	0	1
A/C/D/E	grating cheese (P)/smear, brine(PAL)/floor water (EL)	rec [c]/rec [c]/rec [c]/rec [c]	1 yr/6.8yr/6.2yr/1yr	2/22/68/2	IN5[A]=IN5[C]=IN5[D]=IN5[E]/n. t.	ST1597	1597 [a]	36	23	30	96	195	19	16	0	0	1
A	smear (PAL)	spor	once	1	IN9[A]	448	448	65	21	40	33	45	170	53	0	0	1
C	raw milk (RM)	spor	once	1	IN14[C]	ST1598	1598	79	21	33	97	20	356	58	0	0	1
A	smear (PAL)	spor	once	1	IN16[A]	ST43	43	143	21	40	167	55	307	16	0	0	1
C	hard cheese (P)	spor	once	1	IN21[C]	ST43	43	143	21	40	167	55	307	16	0	0	1
A/C/D	soft cheese (P)/smear (PAL)	rec [b]/spor/rec [b]	5 mo/once/4mo	02.01.2003	IN6[A]=IN6[C]=IN6[D]	ST1599	1599[a]	143	95	30	96	55	180	16	0	0	1
***L. innocua* (API profile 7-1-1-0)**																	
E	acid curd cheese (P)/enrichment (PAL)/drain water (EL)	rec [c]	6 mo	3	IN1[E]	ST1595	1595 [a]	25	73	237	130	55	19	16	0	0	1
D	semi-hard cheese (P)	spor	once	1	IN8[D]	ST1482	1482	26	21	40	33	45	19	53	0	0	1
C	cheese (P)	spor	once	1	IN17[C]	ST605	605	36	21	30	35	45	69	17	0	0	1
C	cheese (P)	spor	once	1	IN20[C]	ST605	605	36	21	30	35	45	69	17	0	0	1
C	cheese (P)	spor	once	1	IN19[C]	ST1087	1087	191	21	184	110	45	356	16	0	0	1
C	smear (PAL)	rec [c]	1.3 yr	2	IN7[C]	ST1085	1085 [a]	188	157	182	223	136	353	148	0	0	1
E	acid curd cheese (P) smear (PAL)	rec [c]	6 mo	4	IN3[E]	ST1601	1601 [a]	250	140	73	223	136	341	214	0	0	1

Table 1. *Cont.*

PRODUCER	SOURCE	OCCURRENCE	TIME-FRAME	ISOLATES (n)	PFGE PROFILE (*ASCI*/*APAI*)	CC [f]	ST (Serogroup)	*abcZ*	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*	SSI-1 + [g]	SSI-1- [h]	SSI-2 [i]
***L. innocua* (API profile 7-5-3-0)**																	
A	smear (PAL)	spor	once	1	IN13[A]	ST1600	1600	40	62	30	33	55	356	17	0	0	1
A	smear (PAL)	spor	once	1	IN11[A]	ST1008	1008	173	140	173	208	136	341	138	0	0	1

[a] sporadically isolated *Listeria* spp. genotypes; [b] recurrently isolated *Listeria* spp. genotypes; [c] recurrently isolated *Listeria* spp. genotypes over a period of ≥6 months defined as persistent; [d] mo, month; yr, year; [e] non-typable; [f] CC, clonal complexes; genetic lineages are provided in brackets; [g] SSI-1+, stress survival islet positive strains; [h] SSI-1-, stress survival islet negative strains, F2365_0481 homologous gene; [i] SSI-2, stress survival islet 2 positive genotypes. Abbreviations: Product associated liquids (PAL; smear, brine), product associated samples (PA, culture, and enrichment), raw material (RM), food contact surfaces (FCS), non-food contact (NFCS) surfaces, and environmental (E) liquid samples (floor and drain water). Red marked housekeeping genes are present in *L. monocytogenes* genetic lineage III and *L. innocua*.

The biochemical profiling resulted for all *L. monocytogenes* isolates in the typical naphthylamidase (DIM; Differentiation/*Innocua*/*Monocytogenes*) negative and rhamnose positive profile (API profile 6510). *L. innocua* isolates exhibited three different biochemical profiles: API profile 7510 with a DIM and rhamnose positive reaction (n = 124), API profile 7110 with a DIM positive and rhamnose negative reaction (n = 13), and API profile 7531 with a DIM, D-ribose, and D-tagatose positive reaction (n = 2) (Table 1).

L. monocytogenes isolates (n = 81) were most frequently confirmed as PCR-serogroups 1/2a, 3a (n = 76; 95%), followed by 1/2b, 3b (n = 3; 3.75%), and 4b, 4d, 4e (n = 2; 1.25%).

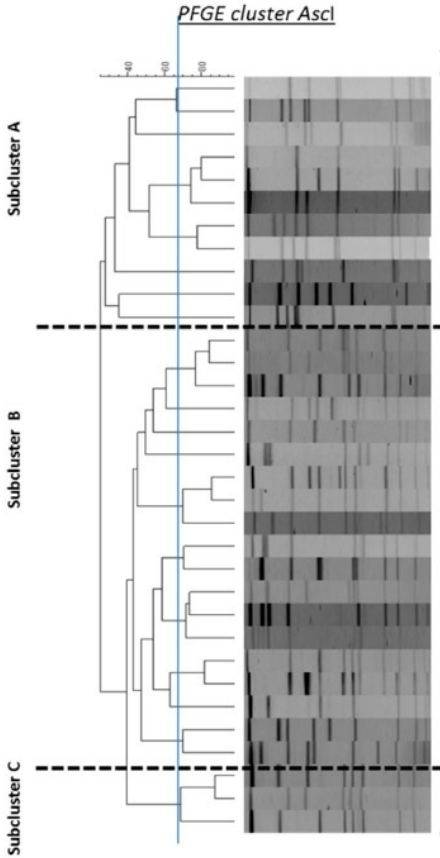
The PFGE-typing, applying the restriction enzyme *AscI*, revealed 33 distinct *Listeria* PFGE profiles with a Simpson's Index of Diversity of 0.75. Thereof, 11 and 22 *Asc*I were specific for *L. monocytogenes* and *L. innocua*, respectively (Simpson´s Index 0.519 and 0.533). The *Apa*I macrorestriction digest resulted in fewer PFGE profiles for *L. innocua* (n = 20). *L. innocua* PFGE profiles IN5[A]=IN5[C]=IN5[D]=IN5[E] and IN4[C]=IN4[E] were non-typable by *Apa*I.

PFGE-types with identical *Asc*I profiles assigned to dairy producers A-E and isolated during two or more sampling events were classified as recurrent, whereas *Listeria* isolates with unique *Asc*I profiles and isolated once were defined as sporadic genotypes. *Listeria* spp. genotypes recurrently isolated over a period of six or more months were defined as persistent. Generally, *L. monocytogenes* clustered together in a distinct subcluster A and could be clearly distinguished at a similarity level of 25% from *L. innocua* subcluster B and C (similarity level 40%) (Table 1, Figure 1).

The MLST typing resulted in 27 STs (Simpson´s Index of Diversity of 0.742). In total, 9 and 3 clonal complexes (CCs) and 1 and 14 singletons were identified among *L. monocytogenes* and *L. inncoua* isolates, respectively. The discriminatory power of MLST analysis was comparable to PFGE-typing for *L. monocytogenes* (10 STs; Simpson´s Index 0.469). ST121 could be differentiated by applying PFGE typing into two *L. monocytogenes* distinct fingerprints. MLST analysis for *L. innocua* isolates was less discriminative in comparison to PFGE (*Asc*I), resulting in 17 STs (Simpson´s Index 0.531).

Seven new *L. innocua* STs (ST1595 to ST1601) were defined by submitting the sequences to the Institute Pasteur MLST database (https://bigsdb.web.pasteur.fr/listeria/listeria.html; Table 1). The ST attribution to dairy processing facilities A–E is depicted in Figure 2.

The stress survival islet (SSI-1) inserted into intergenic region *lmo0443* to *lmo0449* in *L. monocytogenes* was present in ST3 (genetic lineage I; PCR serogroups 1/2b, 3b), ST7, ST14, ST155, and ST403 (genetic lineage II; PCR serogroup 1/2a, 3a). The *L. monocytogenes* homologous gene to *F2365_0481* (1.1 kbp) was present in genetic lineage I isolates assigned to ST1 (serogroup 4b, 4d, 4e) and ST59 (1/2b, 3b), ST398 (genetic lineage II (PCR-serogroup 1/2a, 3a), and ST529 (genetic lineage III, PCR-serogroup 4b, 4d, 4e). *L. monocytogenes* ST121 and all *L. innocua* isolates harbored the SSI-2 (2.2 kbp fragment; Table 1).



SPECIES	PRODUCER	ISOLATES (n)	SOURCE	OCCURRENCE	PFGE PROFILE (ASCI)
M	E	2	P/PAL	rec	M1[E]
M	E	2	PAL	rec	M3[E]
M	D	1	PAL	spor	M6[D]
M	E	3	P/PA/EL	rec	M2[E]
M	A/D	11/1	PAL/EL	rec/spor	M11[A]=M7[D]
M	B/D/E	47/7/1	PAL	rec/rec/spor	M5[B]=M5[D]=M5[E]
M	C	2	FCS	rec	M10[C]
M	A	1	P	spor	M8[A]
M	C	1	PA	spor	M9[C]
IN	C	1	P	spor	IN17[C]
M	E	1	PAL	spor	M4[E]
IN	A	1	PAL	spor	IN16[A]
IN	C	1	P	spor	IN21[C]
M	A	1	PAL	spor	M12[A]
IN	E	3	P/PA/EL	rec	IN1[E]
IN	A/C/D	2/1/2	P/PAL	rec/spor/rec	IN6[A]=IN6[C]=IN6[D]
IN	C	2	PAL	rec	IN7[C]
IN	C/E	1/12	P/PAL/NFCS	spor/rec	IN4[C]=IN4[E]
IN	E	1	PAL	spor	IN4ST[E]
IN	C	1	P	spor	IN18[C]
IN	C	1	RM	spor	IN14[C]
IN	C	1	PAL	spor	IN9[]
IN	A	1	PAL	spor	IN10[A]
IN	C	1	P	spor	IN19[C]
IN	C	1	P	spor	IN20[C]
IN	A/C/D/E	2/22/68/2	P/PAL/EL	rec	IN5[A]=IN5[C]=IN5[D]=IN5[E]
IN	D	1	P	spor	IN8[D]
IN	E	4	P/PAL	rec	IN3[E]
IN	A	1	PAL	spor	IN11[A]
IN	A	1	PAL	spor	IN13[A]
IN	A	1	PAL	spor	IN12[A]
IN	C	1	EL	spor	IN15[C]
IN	E	2	PAL	rec	IN2[E]

Dotted lines indicate PFGE subcluster A-C.

Figure 1. PFGE cluster analysis (*Asc*I) of *L. innocua* (n = 139) and *L. monocytogenes* (n = 81) isolates included in this study.

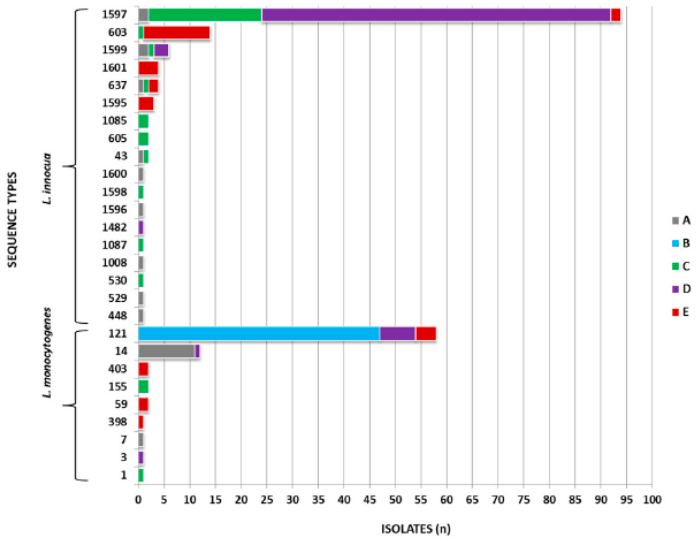


Figure 2. Comparison of the *L. innocua* and *L. monocytogenes* STs identified in this study to the Institute Pasteur MLST isolate database (https://bigsdb.pasteur.fr/listeria/listeria.html) was performed to identify highly abundant housekeeping genes, CC and STs (Table S1). Interestingly, some housekeeping genes were not specific for *L. monocytogenes* genetic lineage III or *L. innocua*.

3.2. Molecular Epidemiological Interpretation

The majority of *L. innocua* and *L. monocytogenes* PFGE types were isolated once (n = 20/33; 60.61%), but certain genotypes were recurrently isolated from process associated samples and cheese for a short period of time (n = 6/33; 18.18%). These genotypes were present in PAL (smear) and environmental samples and after contamination events in cheese for between one and six months and were successfully eliminated. Other *Listeria* spp. PFGE types were persistent in the dairy processing environment for a long period and somehow adapted to niches (smear, brine, drain water). The latter *L. monocytogenes* and *L. innocua* PFGE types cross-contaminated the surface of cheeses (e.g., hard cheese). Almost all of the persistent *L. innocua* and *L. monocytogenes* isolates were rhamnose positive (API profiles 7510 and 6510), except for one genotype with a rhamnose negative profile (IN7[C], ST1085). Almost all of the persistent *L. innocua* and *L. monocytogenes* isolates were rhamnose positive (API profiles 7510 and 6510) except for one genotype with a rhamnose negative profile (IN7[C], ST1085). The association coefficient Cramer's V showed a weak association between *L. innocua*, *L. monocytogenes*, rhamnose positive, rhamnose negative sporadic and persistent occurrences, although the result "for persistence and rhamnose positive" was highly significant (p = 0.0065; Cramer's V rV = WERT, $p < 0.01$).

The most abundant *L. monocytogenes* genotypes related to persistence in cheese processing associated samples and environments were PFGE profiles M5[B]=M5[D]=M5[E] (ST121, 1/2a, 3a) and M11[A]=M7[D] (ST14, 1/2a, 3a), which were present for 7 and 11 years at dairy processing facility B and A in smear and drain water, respectively. The latter profiles were also isolated once and after four months at producers D and E. PFGE profiles M10[C] (ST155, 1/2a, 3a), M3[E] (ST403, 1/2a, 3a) were present at producers C and E for one year and seven months. M1 (ST59, 1/2b, 3b) and M2 (ST121, 1/2a, 3a) were recurrently isolated during a shorter timeframe (one and two months), both at producer E. Other sporadically isolated *L. monocytogenes* genotypes (ST1, ST3, ST7, and ST398) were isolated once during the monitoring period.

The most common *L. innocua* genotypes were IN5[A]=IN5[C]=IN5[D]=IN5[E] (newly identified ST1597), IN4[C]=IN4[E] (ST603), and IN7[C] (ST1085), which were repeatedly isolated for a year up to 6.8 years in the same dairy processing environment (floor water) and product-associated liquids (smear, brine). IN1[E] (newly identified ST1595) and IN 3[E] (newly identified ST1601) were recurrently isolated during a six-month period at processing facility E.

IN6[A]=IN6[C]=IN6[D] was present during a short contamination event (four and five months) at processing facilities A and D. The *L. monocytogenes* and *L. innocua* PFGE clusters were heterogeneous (25% similarity). Interestingly, the persistence-related genotypes *L. monocytogenes* ST14 (M11[A]=M7[D]), ST155 (M10[C]), and ST121 (M5[B]=M5[D]=M5[E]) clustered together in subcluster A at a 75% and 80% similarity level. *L. innocua* ST43 (IN16[A], IN21[C]), ST603 (IN4ST[E], IN4[C]=IN4[E]), and ST637 (IN12[A], IN15[C]) related PFGE profiles clustered in subcluster B and C at a similarity level ≥80%.

L. innocua ST1597, ST603 and *L. monocytogenes* ST121 and ST14 were the most abundant genotypes in dairy producing facilities A–E over time (n = 178/220 isolates). The highest genotype diversity was identified in dairy producing facilities A, C, and E (n = 11, 12, and 9 different genotypes; Figure 2).

The following housekeeping genes were common in *Listeria* isolates included in this study and *L. monocytogenes* genetic lineage III: ST529, ST1595 (*abcZ* 25, *bglA* 73), ST529, ST1597 and ST1599 (*dapE* 96), ST529, ST530, ST448, ST1482, ST605, and ST1087 (*dat* 45) (Table 1). Furthermore, the housekeeping gene *abcz* 40 in *L. innocua* ST1600 was identified in the Institute Pasteur MLST database to be more related to *L. monocytogenes* genetic lineage III (ST267). Further details concerning the prevalence of housekeeping genes present in the MLST database are provided in Table S1.

3.3. Susceptibility to Biocides

The MIC towards biocides was determined for four recurrent *L. monocytogenes* and six *L. innocua* genotypes. All test strains except *L. innocua* genotype IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597) and IN3[E] (ST1601), both 141 mg/L, were adapted to higher concentrations of peracetic acid (250 mg/L; 1.7 fold higher). M5[B]=M5[D]=M5[E] (ST121, 1/2a), IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597), and IN2[E] (ST637) were better adapted to benzalkonium chloride (1.3- and 2-fold higher; mean MIC 15.6 mg/L in contrast to 11.7 and 7.8 mg/L). M5[B]=M5[D]=M5[E] (ST121, 1/2a) and M1[E] (ST59, 1/2b) were slightly better adapted to hydrogen peroxide (1.5-fold higher; 188 mg/L in contrast to 125 mg/L). IN1[E] (ST1595) and IN2[E] (ST637) were better adapted to sodium hypochlorite (2.7–5.7-fold higher; 10,000 mg in contrast to 1750–3750 mg/L).

4. Discussion

Dairy and cheese processing environments are frequently colonized by *Listeria* spp., including pathogenic *L. monocytogenes*. Even newly established dairy processing facilities become colonized after a short period of time [29,30].

Generally, prevalence and concentrations of *L. monocytogenes* in cheeses and cheese processing environments are low. Its growth is supported by the presence of fresh, ripened, veined, and smear cheeses (0.8%–5.1% prevalence in cheese lots). Brined cheeses are most often contaminated by *L. monocytogenes* (11.8%), according to a meta-analysis-based literature review [31]. This suggests that product-associated liquids (smear, brine) contribute to *L. monocytogenes* contamination of cheese lots [21,32]. In our study, smear and brine samples were indeed most often associated with *L. monocytogenes* and *L. innocua* surface contamination and supported the persistence of certain genotypes (ST121, ST14, ST603, and ST1597) in the dairy environment.

Floor drains are further niches for efficient *Listeria* spp. colonization of the FPE and hot-spots for cross and recontamination events [33,34]. These niches were also identified in our study. To a certain extent, drain waters harbored recurrent and persistent *Listeria* spp. (ST14, ST637, ST1595, ST1597; Table 1). Despite having a cleaning potential, the introduction of high-pressure water from hoses into a

contaminated drain can cause the airborne spread of *Listeria* and further contribute to the successful establishment of persistent *Listeria* spp. strains in a facility [35].

As reports about dairy processing environment contamination scenarios in the literature are sparse, our goal was to identify any potential long-term contamination with certain *L. innocua* and *L. monocytogenes* genotypes.

Some studies indeed allude to wider contamination of dairy facilities. Parisi et al. [36] isolated *Listeria* spp. at 19/34 cheese factories (55.8%). Occasionally, *L. innocua* and *L. monocytogenes* were detected at the same sampling site (2/19 plants) and persisted in floor drains, which were identified as ideal sampling sites to be included in a monitoring system.

Relevantly, we clearly identified a higher fluctuation of *L. innocua* and *L. monocytogenes* genotypes in parallel in 4/5 dairy processing facilities (Figure 1, Table 1). One dairy plant (B) harbored a persistent *L. monocytogenes* genotype (ST121) for seven years without further introduction of other *Listeria* spp. genotypes.

Lomonaco et al. [37] reported two persistent *L. monocytogenes* genotypes in the Gorgonzola processing chain. About 88% of the *L. monocytogenes* strains were serotype 1/2a, which is consistent with our findings (95% of the isolates were serotype 1/2a). However, genotypes were not comparable due to the lack of common nomenclature in *Listeria* spp. subtyping, which is urgently in need of rectification [38]. In this respect, Jagadeesan et al. [39] highlighted the need to include metadata for genotypic approaches, which should be sufficiently cleaned with the removal of replicates and unintended information. Actual studies indicate that whole-genome sequencing (WGS) and core genome (cg) MLST approaches already contribute to the real-time exchange of information on the emergence and geographic dispersal of clones [40–42]. Maury et al. [43] reported that *L. monocytogenes* CC1 are strongly associated with dairy products, whereas hypovirulent clones, CC9 and CC121, are related to meat products.

In the presented study, several disease-related genotypes that are globally distributed were introduced into the dairy processing environment (ST1, ST3, ST7, ST59, ST155, ST398, and ST403). However, ST14 and ST121 established themselves for a longer time (7 and 11 years, respectively) in the FPE and tended to be persistent. Almost all of the persistent *L. innocua* and *L. monocytogenes* isolates that we identified were rhamnose-positive (API profiles 7510 and 6510; Table 1). Rhamnose is a naturally occurring monosaccharide present in plant material and important for saprophytes such as *Listeria* spp.

Atypical *L. innocua* and *L. monocytogenes* lacking the ability to ferment rhamnose (where the *pdu* operon for propanediol utilization is missing) are potentially less capable of exploiting nutritional sources important for adaptation to the FPE [44].

Further, atypical strains with deficient rhamnose fermentation have been reported to be attenuated in virulence and have reduced resistance to temperature changes [45]. In contrast, *L. monocytogenes* serotype 1/2a mutants confer phage-resistance due to a loss of rhamnose. This is important when there is a field application of lytic phage cocktails as biocontrol measures [46]. Therefore, detailed characterizations of *L. monocytogenes* and *L. innocua* rhamnose-positive and -negative field strains in respect of persistence in the FPE and resistance to biocontrol measures are important.

Pasquali et al. [47] also identified ST14 and ST121 as persisters in a rabbit meat processing plant. Several strain-specific features, such as a stronger biofilm-forming potential (ST14) and the presence of the *qacH* gene associated with adaptation to BAC in ST121, contributed to successful establishment in the FPE.

The stress survival islet (SSI-1) inserted into the intergenic region *lmo0443* to *lmo0449* in *L. monocytogenes* is present in long-term persister ST14 and is related to acid tolerance [26]. *L. monocytogenes* ST121 and all *L. innocua* isolates that we identified harbored the SSI-2 (2.2 kbp fragment), which is related to elevated tolerance to oxidative and alkaline stress (Table 1) [48].

The entrance and establishment of certain successful genotypes in the FPE are influenced by several factors. These include strain properties (e.g., prophage diversification, transposons, plasmids),

the possibility of reintroduced genotypes by raw material, over-diluted biocides on wet surfaces, and a permanent change in cleaning regimes by external cleaning companies and consultants [49]. Consequentially, Muhterem-Uyar et al. (2018) reported that in a heavily contaminated cheese processing environment, the strain variability (ST1, ST7, ST21, and ST37) was reduced to a persistent genotype (ST5) harboring a plasmid type (*plM*80 related), which is present in successful clones worldwide [20].

In corroboration, we observed higher MICs towards biocides in M5[B]=M5[D]=M5[E] (ST121, 1/2a), IN5[A]=IN5[C]=IN5[D]=IN5[E] (ST1597), and IN2[E] (ST637), which were better adapted to benzalkonium chloride (up to two-fold higher). IN1[E] (ST1595) and IN2[E] (ST637) were better adapted to sodium hypochlorite (2.7–5.7-fold higher).

In FPEs, resident *Listeria* are frequently exposed to sublethal concentrations of biocides due to the dilution effect of wet surfaces and the presence of food soil [50]. Therefore, testing the sensitivity of *Listeria* spp. to sublethal concentrations of biocides should be performed routinely to identify potential strain adaptations. Particularly, *L. monocytogenes* and *L. innocua* isolated from the pork processing chain have been shown to harbor efflux pumps and resistance genes *(cadA1-cadA4, arsA1, arsA2)* that confer resistance to benzalkonium chloride and heavy metals [51].

The exchange of genetic material between *L. innocua* and *L. monocytogenes* has been observed in a few studies. The possibility of horizontal gene transfer (HGT) of plasmids, including heavy metal resistance, enhanced tolerance to QACs and DNA intercalating dyes between *L. welshimeri*, *L. innocua* and *L. monocytogenes*, has been described by sequence analysis in experimental settings and by comparison of FPE isolates [52–54].

The presence of atypical hemolytic *L. innocua* strains in the food chain might also have been introduced by HGT, and this constitutes a reservoir of virulence genes transferable to other species [12,55]. This could be the same for genetic exchange between *L. innocua* and *L. monocytogenes* related to environmental adaptation. More research focusing on the uptake of genetic material by *Listeria* spp. in the FPE is warranted.

We identified housekeeping genes common in novel identified *L. innocua* ST1595, ST1597, ST1599, and ST1601 in STs 605 and ST1085 that are recorded in the MLST database, and *L. monocytogenes* genetic lineage III (*abcZ* 25, *bglA* 73, *dapE* 96, *dat* 45) (Table 1, Table S1).

Comparing *L. innocua* STs from this study to the MLST Institute Pasteur database, we identified ST603 (CC600) and ST637 (CC140) (Figure 2), commonly isolated from different niches (e.g., silage, food, human blood samples). Comparing the NCBI available strains (n = 11) to *L. innocua* from this study, no complete match was found based on STs. The nearest match was *L. innocua* reference strain CLIP 11,262 assigned to CC140, which differed by the *ldh* housekeeping gene *(ldh* 74) in comparison to ST637 (*ldh* 192) (https://www.ncbi.nlm.nih.gov/genome/genomes/1024?; https://bigsdb.web.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates).

In fact, the draft genome of *Listeria innocua* UAM003-1A, available from NCBI, is also related to highly abundant CC140 [14].

We did not identify any atypical hemolytic ST188 or ST437 *L. innocua* strains (LIP-1 positive, *hly* positive), which have recently been isolated from wild bird feces in Finland [12] and previously described by Volokhov [56]. The *L. innocua* strains included in our study showed no relationship to the newly announced MEZLIS26 genome, due to their different housekeeping genes. The latter *L. innocua* was assigned to the highly abundant CC537 [15].

L. innocua has been reported to be more commonly found in the FPE than *L. monocytogenes* [57], which is supported by our findings. We fully agree with Jemmi and Stephan [58] who suggest that *L. innocua* is a good hygiene indicator and also a marker for unrecognized *L. monocytogenes* contamination events in the FPE. What should now be considered in retrospect is the demanding nature of the microbiological reference method, for example, ISO 11290-1, concerning the differentiation of *L. innocua* and *L. monocytogenes* [59]. Reports focusing on the challenges associated with *Listeria* detection and confirmation are available, including atypical strains or *L. monocytogenes* present in lower concentrations due to competitive *L. innocua* strains during enrichment or that mask detection of *L. monocytogenes* on

selective agar plates such as ALOA medium [60,61] (https://www.fda.gov/food/laboratory-methods-food/guidelines-bam-users-identification-atypical-hemolytic-listeria-isolates). This might also have contributed to a higher isolation rate of *L. innocua* compared to *L. monocytogenes* in our study.

In respect of long-term contamination events with *L. monocytogenes* and other *Listeria* species, subtyping methods are helpful tools to identify the true nature of persister candidates.

5. Conclusions

L. monocytogenes is a foodborne pathogen of significance to human health, and it is able to co-survive in the dairy FPE in microbial communities with other *Listeria* species and with other bacteria (e.g., *Proteobacteria*, lactic acid bacteria) [33,62]. Our study identified for the first time the recurrent isolation and persistence of *L. innocua* in *L. monocytogenes*-colonized habitats. Novel local *L. innocua* sequence types (ST1595 to ST1601) were identified, which shared, to a certain extent, the housekeeping genes that are also common in *L. monocytogenes* genetic lineage III. Either SSI-1 (ST14) or SSI-2 (ST121, all *L. innocua*) were present in strains successfully adapted to the FPE. There is a great need for further insight into the processes of FPE adaptation and exchange of genetic information between *Listeria* species so that appropriate food safety control measures can be designed.

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