Aus dem Institut für Lebensmittelsicherheit und –hygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

und

der Abteilung Planung und Lenkung Lebensmittelsicherheit des Bayerischen
Landesamtes für Gesundheit und Lebensmittelsicherheit

Listeria monocytogenes in food of non-animal origin

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Simone Erika Wartha
Tierärztin aus Schwandorf

Berlin 2023
Journal-Nr.: 4412

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List of Abbreviations

ActA	protein ActA (gene)
AMR	antimicrobial resistance
ANT(6)-Ia	aminoglycoside nucleotidyltransferase (gene)
bcrABC	efflux pump (gene)
CC	clonal complex
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
cgMLST	core genome Multilocus Sequence Typing
CT	complex type
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECDC	European Centre for Disease Prevention and Control
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
emrC	efflux pump (gene)
emrE	efflux pump (gene)
e.g.	exempli gratia, for example
esp.	especially
EU	European Union
FAO	Food and Agriculture Organization of United Nations
FAO	food of animal origin
FNAO	food of non-animal origin
g	gramm
GSH	glutathion
GSR	general stress response
HAV	Hepatitis A Virus
HIV	Human Immunodeficiency Virus

HP	hexose phosphates
hly	Listeriolysin O (gene)
hpt	hexose phosphates (gene)
InlA	Internalin A
InlB	Internalin B
InlP	Internalin-like protein
ISO	International Standards Organization
lde	Listeria drug efflux (gene)
LIPI	*Listeria* pathogenicity island
LLO	Listeriolysin O
L. monocytogenes	*Listeria monocytogenes*
LPSN	List of Prokaryotic names with Standing in Nomenclature
$\log_{10}$	logarithm
mdrl	efflux pump (gene)
MLST	multilocus sequence typing
mpl	zinc metalloproteinase (gene)
mprF	cationic peptides (gene)
MST	minimum spanning tree
N.A.	not applicable
NGS	next-generation sequencing
OHHLEP	One Health High Level Expert Panel
OIE	Office International des Epizooties, World Organization for Animal Health
P. aeruginosa	*Pseudomonas aeruginosa*
pH	potential of hydrogen
plcA	phosphatidylinositol-specific phospholipase C (gene)
plcB	nonspecific phosphatidylcholine phospholipase C (gene)

PI-PLC/PLC-A	phosphatidylinositol-specific phospholipase C
PC-PLC/PLC-B	nonspecific phosphatidylcholine phospholipase C
PrfA	Positive regulatory factor A
qacH	efflux pump (gene)
QACs	Quaternary ammonium compounds
RTE	ready-to-eat
spp.	species
ST	sequence type
SW	sample ID Simone Wartha
TESSy	The European Surveillance System
UNEP	United Nations Environment Programme
USA	United States of America
VBNC	viable but non-cultivable
WHO	World Health Organization
WGS	whole-genome sequencing
YOPI	young, old, pregnant, immunosuppressed
° C	degree Celsius
σ	Sigma factor

Introduction

“One health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and inter-dependent. […]” (FAO, OIE, WHO, UNEP 2021).

The idea of One Health is ubiquitous. The One Health High Level Expert Panel (OHHLEP), an advisory panel of the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), the World Organization for Animal Health (OIE, Office International des Epizooties) and the United Nations Environment Programme (UNEP), publicized the definition of One Health on 1st December 2021 (FAO, OIE, WHO, UNEP 2021). As Figure 1 demonstrates, the environment, animals, humans and their society are interacting and forming one unit. It has to be considered as a whole. Communication, cooperation and coordination are essential pillars in creating a healthy ecosystem and healthy animals and humans.

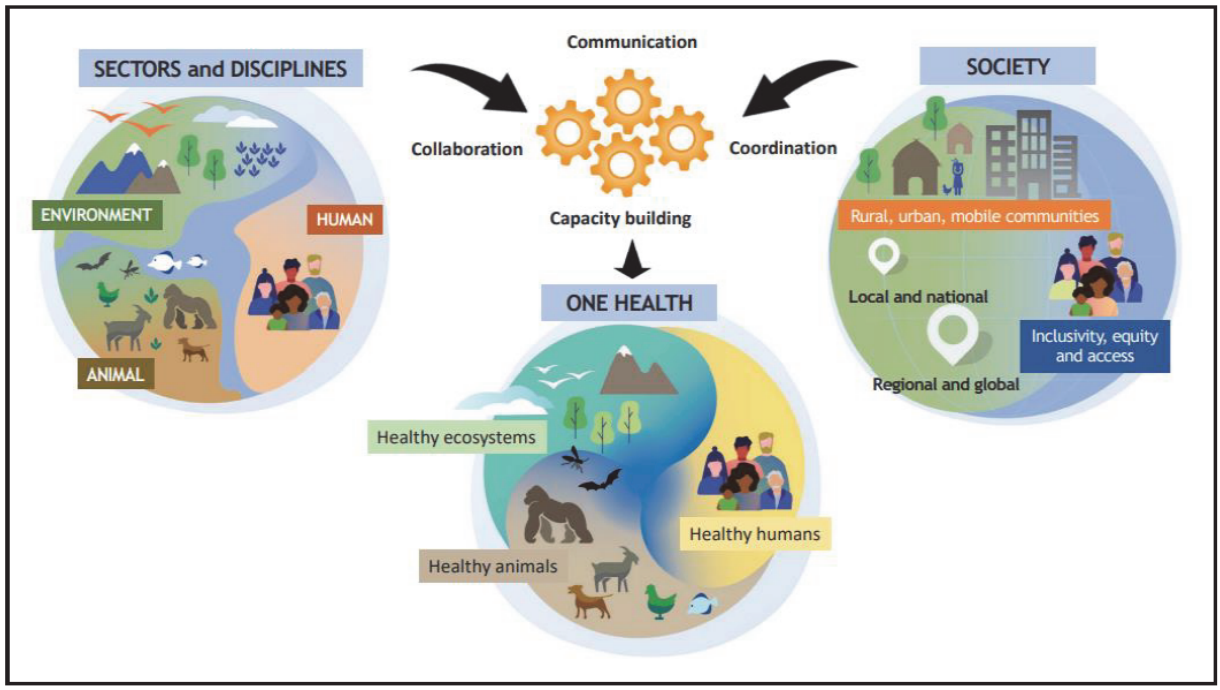

Figure 1. Definition of One Health by OHHLEP (FAO, OIE, WHO, UNEP 2021).

The challenges of infectious diseases have been exposed by the Covid-19 pandemic. Over 60% of contagious diseases are zoonotic diseases and there are approximately 1.7 million unknown viruses in wild animals which can spread different kinds of diseases (Federal Ministry for Economic Cooperation and Development 2020). The term zoonosis originates from the Greek words “*zoon*”

(living beings) and "*nosos*" (disease). It defines an infectious disease caused by bacteria, viruses, parasites, fungi or prions. The transmission to human beings happens via direct contact, vectors (mosquitoes, ticks) or food (meat, milk, plant-based food, eggs) (Federal Institute for Risk Assessment 2022). For example, the Human Immunodeficiency Virus (HIV) or the Ebola Virus are direct contact diseases, which are transmitted from animals to humans. The West Nile Virus or Zika Virus are transmitted by vectors, means spread by mosquitoes (Federal Ministry for Economic Cooperation and Development 2020). Foodborne zoonotic diseases originate from contaminated food with potential pathogenic microorganisms and/or their toxins (EFSA). In the United States of America (USA), there is an estimate of 48 million cases of foodborne diseases each year (U.S. Food and Drug Administration 2020). Most of the common foodborne pathogens are *Salmonella* spp., *Listeria* spp., *Escherichia (E.) coli*, *Campylobacter*, Hepatitis A Virus (HAV), norovirus or *Cyclospora* (EFSA; U.S. Food and Drug Administration 2020). Especially, the pathogenic species of *Listeria* spp., namely *Listeria (L.) monocytogenes* is an important zoonosis microorganism because of its high mortality rate (20–30%) in vulnerable population groups (Centers for Disease Control and Prevention 2022a; Newell et al. 2010). Because of a multi-country outbreak linked to blanched frozen vegetables caused by *L. monocytogenes* from 2015 until 2018 in the European Union (EU), food of non-animal origin (FNAO) has come into focus (Koutsoumanis et al. 2020). The question is, how widespread is the prevalence of *Listeria* spp., especially *L. monocytogenes* in FNAO-producing and processing companies.

The prevention of foodborne illnesses is complex and requires global cooperation. The One Health concept will help to broaden the science and combine the environmental sector with human and animal health (FAO, OIE, WHO, UNEP 2021). One of the primary goals in preventing foodborne illnesses should be fulfilling the One Health requirements, which means collaborative approach between human, animal and environmental health sectors (Centers for Disease Control and Prevention 2022c).

Chapter 1: Literature Overview

1.1 Taxonomy

The first reported human infections with Gram-positive rods, which are retrospectively assigned to the today known *L. monocytogenes* was by Hayem in France in 1891 and by Henle in Germany in 1893 (Gray and Killinger 1966). Moreover, Hülphers described clinical infections in rabbits in 1911 (Hülphers 1911) and Atkinson (Atkinson 1917), Dick (Dick 1920) and Dumont and Cotoni (Dumont and Cotoni 1921) all mentioned cases of human meningitis, which are presumably linked to *L. monocytogenes*. But in 1926, Murray et al. first described and named the bacillus *Bacterium monocytogenes,* today known as *L. monocytogenes,* in predominantly young rabbits (Murray et al. 1926). Pirie isolated the bacterium *Listerella hepatolytica* from livers of African jumping mice the following year (Pirie 1940b). Murrays and Piries isolates were identical (Gray and Killinger 1966). After noticing that the name *Listerella* was already allocated to a slime mould, Pirie submitted the genus *Listeria* in 1940 in honour of Lord Lister (Parte et al. 2020; Pirie 1940a).

The family *Listeriaceae* includes the genus *Listeria* and this, in turn, contains 30 species so far. Furthermore, eight subspecies are described. Table 1 shows the 30 species, which are classified according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al. 2020).

Table 1. List of published *Listeria* spp. according to LPSN (last accessed in March 2023).

No.	Name	Year	Reference
1	*Listeria monocytogenes*	1940	(Pirie 1940b)
2	*Listeria denitrificans*	1961	(Prévot 1961)
3	*Listeria grayi*	1966	(Larsen H E, Seeliger H P R 1966)
4	*Listeria murrayi*	1971	(Welshimer and Meredith 1971)
5	*Listeria innocua*	1983	(Seeliger 1981)
6	*Listeria seeligeri*	1983	(Rocourt and Grimont 1983)
7	*Listeria welshimeri*	1983	(Rocourt and Grimont 1983)
8	*Listeria ivanovii*	1984	(Seeliger et al. 1984)
9	*Listeria marthii*	2010	(Graves et al. 2010)
10	*Listeria rocourtiae*	2010	(Leclercq et al. 2010)
11	*Listeria fleischmanii*	2013	(Bertsch et al. 2013)
12	*Listeria weihenstephanensis*	2013	(Lang Halter et al. 2013)
13	*Listeria aquatica*	2014	(Bakker et al. 2014)
14	*Listeria cornellensis*	2014	(Bakker et al. 2014)

Table 1. Continued

No.	Name	Year	Reference
15	*Listeria floridensis*	2014	(Bakker et al. 2014)
16	*Listeria grandensis*	2014	(Bakker et al. 2014)
17	*Listeria riparia*	2014	(Bakker et al. 2014)
18	*Listeria booriae*	2015	(Weller et al. 2015a)
19	*Listeria newyorkensis*	2015	(Weller et al. 2015a)
20	*Listeria costaricensis*	2018	(Núñez-Montero et al. 2018)
21	*Listeria goaensis*	2018	(Doijad et al. 2018)
22	*Listeria thailandensis*	2019	(Leclercq et al. 2019)
23	*Listeria valentina*	2020	(Quereda et al. 2020)
24	*Listeria cossartiae*	2021	(Carlin et al. 2021)
25	*Listeria farberi*	2021	(Carlin et al. 2021)
26	*Listeria immobilis*	2021	(Carlin et al. 2021)
27	*Listeria portnoyi*	2021	(Carlin et al. 2021)
28	*Listeria rustica*	2021	(Carlin et al. 2021)
29	*Listeria ilorinensis*	2022	(Raufu et al. 2022)
30	*Listeria swaminathanii*	2022	(Carlin et al. 2022)

Chiara et al. divided the genus *Listeria* into two groups, namely *Listeria* sensu stricto and *Listeria* sensu lato. *Listeria* sensu stricto contains the species *L. monocytogenes, L. innocua*, *L. ivanovii*, *L. marthii*, *L. seeligeri*, and *L. welshimeri* (Chiara et al. 2015). The group *Listeria* sensu lato includes all of the other species, which are described from 2009 until 2015 (*L. rocourtiae*, *L. fleischmanii*, *L. weihenstephanensis*, *L. aquatica*, *L. cornellensis*, *L. floridensis*, *L grandensis*, *L. riparia*, *L. booriae*, *L. newyorkensis*) and *L. grayi*, which was first described in 1966 (Orsi and Wiedmann 2016). In addition, Orsi and Wiedmann proposed new genera, particularly *Murraya*, *Mesolisteria* and *Paenilisteria*, in which the *Listeria* sensu lato group would arrange in order (Orsi and Wiedmann 2016). The recommended genus *Mesolisteria* is already mentioned at the LPSN but not yet validly published (Parte et al. 2020).

1.2 Occurrence of *Listeria monocytogenes* – general information

The genus *Listeria* is classified as a Gram-positive, facultative anaerobic and non-spore forming rod bacterium between 1 µm–1.5 µm in length and 0.5 µm in width (Matle et al. 2020; Farber and Peterkin 1991). *L. monocytogenes* presents the relevant pathogenic species for animals and humans (Bille et al. 1992). It is motile, catalase positive, shows hemolysis, is Voges-Proskauer positive, unable to reduce nitrate to nitrite and it is able to grow at refrigerator temperatures (4 °C) (Orsi and Wiedmann 2016). The optimum growth condition is 37 °C, but

the temperature range expand from - 0.4 °C to 45 °C (Matle et al. 2020). Besides, *L. monocytogenes* reveals further abilities to evade the hosts immune response, for example, the tolerance of low pH conditions (down to pH 3.5) or the adaptation to osmotic stress (Begley et al. 2002; O'Driscoll et al. 1996).

In 1979, Seeliger and Höhne described a serotyping method for *L. monocytogenes* based on O – and H–antigens (Seeliger and Höhne 1979). As a result, the allocation of different serotypes as phenotypical characterization was performed (Gorski 2008). Moreover, Piffaretti et al. first described that *L. monocytogenes* can be divided into two different lineages (Piffaretti et al. 1989). Furthermore, Rasmussen et al. added one more evolutionary line (Rasmussen et al. 1995) and in 2008, Ward et al. reported lineage IV (Ward et al. 2008). Lineage I includes serotypes 1/2b, 3b, 4b, 4d, 4e, and 7, lineage II encompasses serotypes 1/2a, 1/2c, 3a, 3c, and 4h, lineage III consists of serotypes 4a, atypical 4b, and 4c and lineage IV counts serotypes 4a and 4c (Lakicevic et al. 2021).

1.2.1 *Listeria monocytogenes* in food of animal origin

Farm animals are one of the sources of food of animal origin (FAO) contamination. Gill confirmed the first case of listeriosis, at that time circling disease, in sheep caused by *L. monocytogenes* (Gill 1937). It is assumed that farm animals play a key role as carriers of *Listeria* spp., usually without showing clinical symptoms. Esteban et al. reported that *L. monocytogenes* was isolated from healthy farm animals in Northern Spain, like dairy cattle (46.3%), beef cattle (30.6%), and sheep herds (14.2%), but not from swine (Esteban et al. 2009). Other studies stated that bovine animals and the agricultural environment are affected more than other parts of livestock farming as well (Terentjeva et al. 2021; Hurtado et al. 2017). Nevertheless, swine must not be underestimated as a potential carrier for *L. monocytogenes* (Yokoyama et al. 2005) especially when the tonsils of healthy swine are contaminated (Oswaldi et al. 2022; Hellström et al. 2010; Bunčić 1991). Furthermore, cattle and swine feces, as well as small ruminant excretions, are also potential habitats (Terentjeva et al. 2021; Schoder et al. 2011; Yokoyama et al. 2005).

The contamination pathway of FAO is very complex and there are various scenarios for infection. Figure 2 shows different transmission paths for *L. monocytogenes* infections to animals and humans.

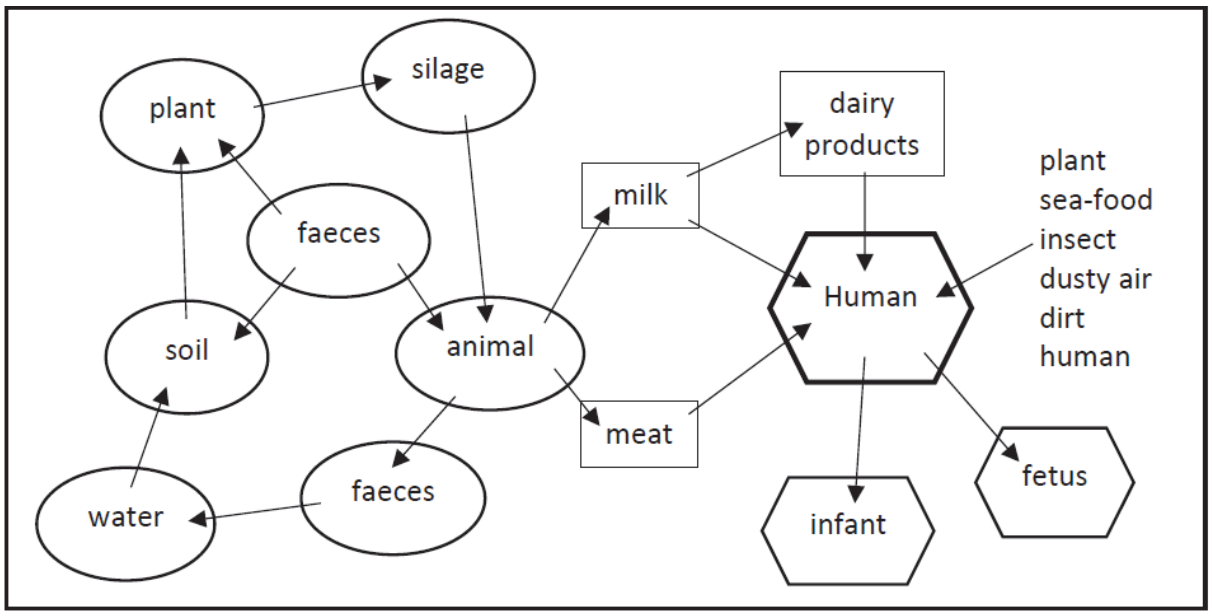

Figure 2. Different transmission paths for *L. monocytogenes* infections to animals and humans. Adapted from Axelsson and Sorin 1998 out of Rip Diane (Rip Diane 2011).

With regard to Figure 2, birds should be included, particularly with respect to wild bird feces that contain human pathogen microorganisms, like *L. monocytogenes* (Sauvala et al. 2021; Hellström et al. 2008). The contamination probability of water, plant, silage or feed at farm level due to birds should be considered.

Furthermore, silage as a potential feed of animal feeding plays an important role in *L. monocytogenes* infections in farm animals. The correct manufacturing process is crucial to avoid faulty fermentation that creates silage of low quality (Dunière et al. 2013). Several studies verify that silage is a reservoir for *Listeria* spp. and *L. monocytogenes* (Stein et al. 2018; Nucera et al. 2016; Schocken-Iturrino et al. 2005; Grønstøl 1979). On the one hand, the presumption is that farm animals fed by silage represent more *L. monocytogenes* isolates in feces (Schoder et al. 2011; Fenlon et al. 1996). On the other hand, Yokoyama et al. reported an increase of *L. monocytogenes* in the substance of swine cecum samples and all swine were fed only manufactured dry feed in this study (Yokoyama et al. 2005). Besides, a study on cattle farms in Latvia reported a smaller contamination rate of silage compared to mixed feed (Terentjeva et al. 2021). In conclusion, the amount of articles confirms silage contamination with *L. monocytogenes* and as a common cause for listeriosis infections in animals (Stein et al. 2018; Nucera et al. 2016; Schocken-Iturrino et al. 2005; Grønstøl 1979). Nonetheless, all forms of feed

should be kept in mind when considering *L. monocytogenes* prevalence at livestock environment.

When using contaminated manure for fertilization, therefore the contaminated forage continues the contamination circuit of *L. monocytogenes* in FAO (Queiroz et al. 2018; Schoder et al. 2011). Regarding Figure 2, pathogens can enter the transmission path through soil, water, the feed or contaminated feces (Rip Diane 2011). Heavy rain and storms can transfer contaminated soil to the part of the plant, which is used for feed.

In the 1980s, the presumption increases that *L. monocytogenes* is transmitted via food (Farber and Losos 1988; Seeliger 1988). Hellström et al. described the possibility of contamination from farm to fork (Hellström et al. 2008). Abattoirs are, conceivably, contamination areas: soiled skin surfaces or hooves, lacking hygiene at the slaughter process flow, especially at evisceration steps, coworkers, drains or facility equipment are possible sources of contamination (Matle et al. 2020; Hellström et al. 2010). As well as Hellström et al., the results from Muhterem-Uyar et al. confirmed that *L. monocytogenes* affects the food processing companies through the environment or in particular the contaminated raw materials (Muhterem-Uyar et al. 2015; Hellström et al. 2010). Relevant food products are raw foodstuffs originating from animals like minced meat (tatar, carpaccio), spreadable short matured raw sausages (Teewurst, Mettwurst), raw milk, raw milk products, unpasteurized cheese (particularly soft cheese) and crustaceans, raw fish products (sushi, oysters) and smoked fish products like cured salmon and graved salmon (Federal Institute for Risk Assessment 2008). With regard to lineage classification I to IV, it is described that lineage I is often associated with dairy products and *L. monocytogenes* isolated from meat and fish products is related to lineage II (Lakicevic et al. 2021).

One of the first associated foodborne outbreaks causing listeriosis was reported from 1949 until 1957 in Halle, Germany and unpasteurized milk was assumed the causative vehicle (Farber and Losos 1988). After that, further reports were documented. In the summer of 1983 in Massachusetts, 49 cases of listeriosis, affecting individuals included fetuses, infants and immunocompromised people were noted. Thereof 14 (29%) patients died. The contaminated pasteurized milk was from dairy farms with confirmed listeriosis, most likely due to cross-contamination (Fleming et al. 1985). Two years later, in 1985, a Mexican-style cheese caused listeriosis outbreaks in California (Centers for Disease Control 1985). The soft cheese was contaminated with raw milk and caused 142 infections, with 48 (34%) reported deaths (Linnan et al. 1988). The *L. monocytogenes* serotype 4b was the prevalent serotype of the foodborne outbreaks from 1983 and 1985 and supports the notion, that lineage I is often

associated with dairy products (Lakicevic et al. 2021; Farber and Losos 1988). Furthermore, Nicolas et al. (1986) documented *L. monocytogenes* in different raw meat and meat products as well as in unheated ready-to-eat (RTE) sausages (Seeliger 1988). Since then, various *L. monocytogenes* strains were detected in FAO.

In summary, there are many transmission routes for FAO contamination (Figure 2). Above all, silage of poor quality, feces of healthy farm animals, following farm and processing surrounding, environmental equipment, farmers, coworker and unhygienic environmental conditions pose a threat for *L. monocytogenes* to enter the food chain (Queiroz et al. 2018; Stein et al. 2018). In this context, hygienic handling from farm to fork plays a key role in decreasing the prevalence of *L. monocytogenes* and the hazard of cross-contamination from environment to food products.

1.2.2 *Listeria monocytogenes* in food of non-animal origin

In 1979, the first documented foodborne *Listeria* outbreak linked to FNAO was reported. Raw celery, lettuce and tomatoes were assumed to have caused 23 cases of listeriosis in the Boston area, USA (Ho 1986). Four years later, Schlech described 41 listeriosis outbreaks, in mostly pregnant women and infants, who had been infected by contaminated coleslaw (Schlech et al. 1983; Seeliger 1988). The transmission pathway was caused by a cabbage field, which had been fertilized with compost and raw manure from sheep infected with listeriosis (Farber and Losos 1988). Throughout the following years, more FNAO was linked to the foodborne pathogen *L. monocytogenes.* In 1997 in Italy, a cold salad of corn and tuna infected students and staff of primary schools and a university, with the same caterer. Experimental contamination of the corn samples showed growth of *L. monocytogenes* which confirmed corn instead of tuna as the causative food (Aureli et al. 2000). In 2010, diced-celery caused 10 listeriosis patients and 5 deaths in Texas (Gaul et al. 2013). Furthermore, cantaloupes, whole apples, soy products, sprouts, stone fruit, packaged salads, frozen vegetables, and, in particular, frozen corn were contaminated with *L. monocytogenes* inducing listeriosis (Centers for Disease Control and Prevention 2022b; EFSA 2018b; Angelo et al. 2017; Jackson et al. 2015). The listeriosis outbreak caused by cantaloupes in 2011 was one of the largest outbreaks in the USA (Centers for Disease Control and Prevention 2011). In 28 states, 147 cases, which included 143 hospitalizations, 33 deaths, and a 22% fatality rate were reported (Centers for Disease Control and Prevention 2022b). It was the first extensive plant based listeriosis outbreak in the USA (Centers for Disease Control and Prevention 2011). It underlines the potential of FNAO as causative vehicle for *L. monocytogenes* infections, especially the fact that most

of FNAO do not undergo further processing or heating steps. The melons were probably contaminated by the facility environment, which accentuates the need for appropriate cleaning and disinfection intervals (Centers for Disease Control and Prevention 2011). Public attention will focus more and more on the issue of *L. monocytogenes* and FNAO, because of the fact that FNAO is mostly eaten raw (Strawn et al. 2013).

Similar to FAO, different transmission pathways exist in how FNAO is contaminated with *L. monocytogenes*. Figure 3 shows that soil and water play a key role in fresh produce contamination (Zhu et al. 2017).

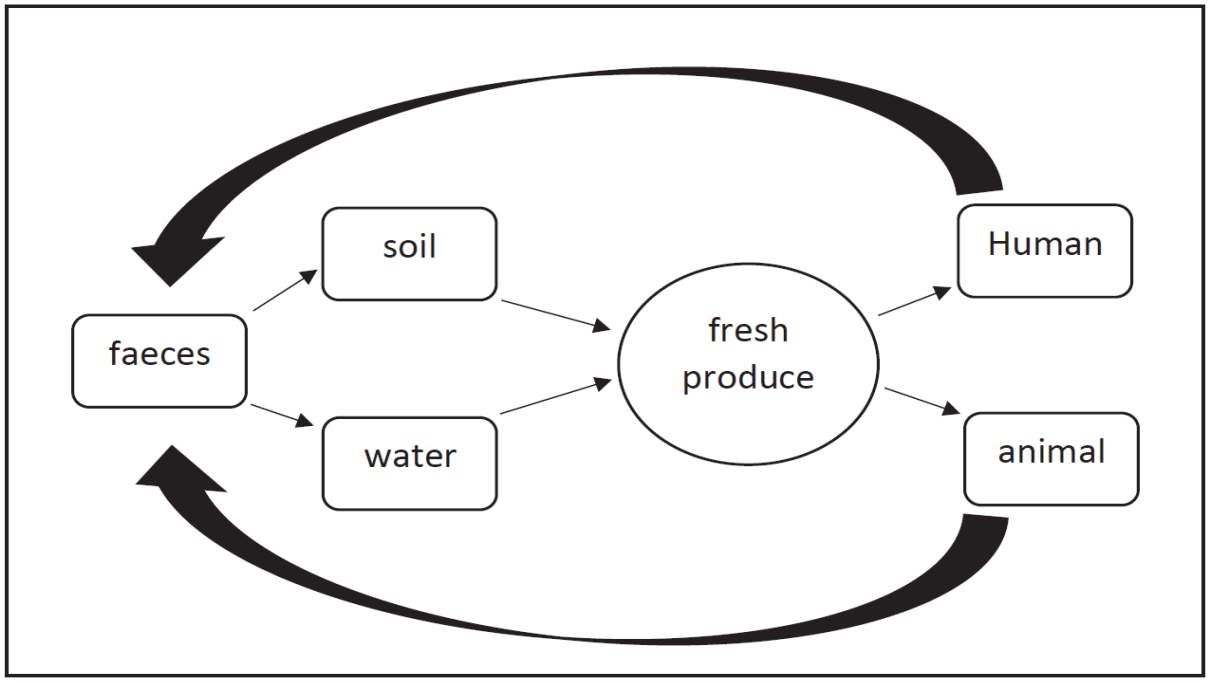

Figure 3. Different transmission paths to human *L. monocytogenes* infection via fresh produce. According to Zhu et al. (Zhu et al. 2017).

L. monocytogenes is a common ubiquitous bacterium, which is frequently found in soil (Strawn et al. 2013). Besides soil, water is another potential source to fresh produce contamination (Zhu et al. 2017). In 2014, irrigation water was responsible for contaminating sprouts (Gartley et al. 2022; U.S. Food and Drug Administration 2014). Bernagozzi et al. and Strawn et al. measured a high prevalence of *L. monocytogenes* in water samples, such as in surface water (Strawn et al. 2013; Bernagozzi et al. 1994). Weller et al. examined the connection between rain and irrigation events to *L. monocytogenes* contamination. Higher *L. monocytogenes* prevalence after < 24 hours rain or irrigation period has been confirmed in spinach fields (Weller et al. 2015c). Furthermore, the spatial proximity of FNAO fields to water sources and roads increases the isolation of *L. monocytogenes* from soil samples (Weller et al. 2015c). Next to river, brackish, steam or pond water, Geuenich et al. identified

L. monocytogenes (92.5%) in municipal waste water as well (Geuenich et al. 1985). Gião and Keevil found out that *L. monocytogenes* is able to survive on stainless steel and consequently colonize tap water in a viable but non-cultivable (VBNC) state (Gião and Keevil 2014). On the one hand, pre-harvest contamination of fruit and vegetables due to irrigation water is increasing (Strawn et al. 2013), but on the other hand, post-harvest contamination due to facility environment, equipment and the processing of water might be even more problematic. Many studies reported that FNAO was contaminated post-harvest (Angelo et al. 2017; Garner and Kathariou 2016; Jackson et al. 2015; McCollum et al. 2013). It underlines the importance of good hygienic practice at processing facilities and the importance of environment and food product investigations on the spot.

As Figure 3 shows, the cycle of contamination and human and animal infection will continue if fresh produce is contaminated via soil or water during primary production or during processing steps at facility plants. The circuit is complete because animal feces, as well as human feces, are potential carriers of the pathogen *L. monocytogenes* (Hafner et al. 2021; Esteban et al. 2009; Olier et al. 2002). It is suggested that between 2% and 10% of the human intestinal tracts worldwide carry *L. monocytogenes* without being clinically infected (FAO/WHO 2000). Animal manure, when used as fertilizer on fields can transmit *L. monocytogenes* back into the soil and the cycle of contamination is repeated (Schoder et al. 2011).

The Codex Alimentarius, International Food Standards, defines RTE food as “any food (including beverages) which is normally consumed in its raw state or any food handled, processed, mixed, cooked, or otherwise prepared into a form in which it is normally consumed without further processing.” (FAO 1999). *L. monocytogenes* is an increasing issue in fresh produce. Figure 4 measures the increasing threat of *L. monocytogenes* in RTE fruits and vegetables (EFSA 2021). The sampling units rose by more than fourfold in the EU from 2017 to 2020 and therefore, the verification rate increased as well. This demonstrates that the prevalence and hazard of *L. monocytogenes* in fresh produce should not be disregarded.

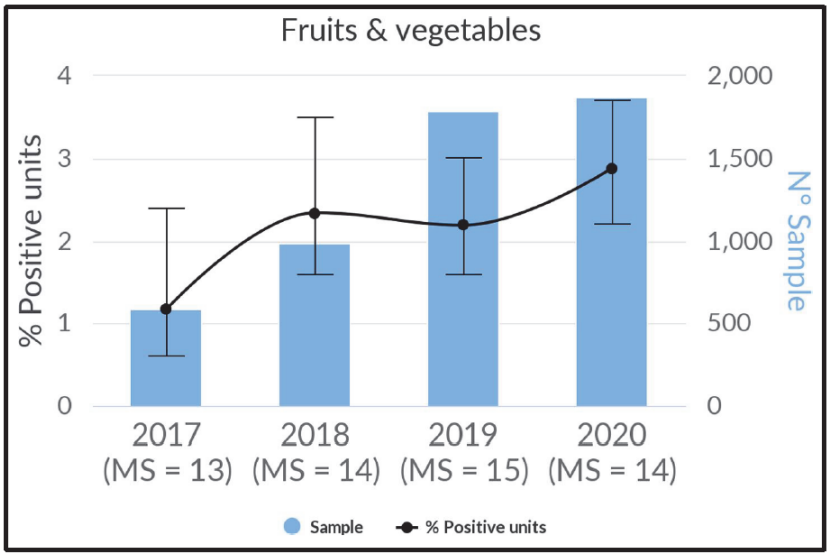

Figure 4. Distribution of *L. monocytogenes* positive sampling units in fruits and vegetables in the EU from 2017 until 2020. According to the European Food Safety Authority (EFSA) (EFSA 2021). MS = Member States

Experimental studies measured the growth of *L. monocytogenes* in RTE salads and vegetables (Culliney and Schmalenberger 2020; Marras et al. 2019). If RTE salads are contaminated with *L. monocytogenes*, the rate of growth is supported by different storage conditions (store temperatures at 4 °C, 8 °C, 25 °C, and 37 °C) (Marras et al. 2019). Raw vegetables like lettuce and salad in modified atmosphere packaging, spinach and rocket leaves, as well as RTE salads, support the growth and the survival of *L. monocytogenes* (Łepecka et al. 2022). Spinach showed the most potential growth of 2.40 $\log_{10}$ CFU/g^{-1} (colony forming unit/gramm) over seven days (Culliney and Schmalenberger 2020). Furthermore, Table 2 shows different frequencies of *L. monocytogenes* in FNAO. The range goes from 0.06 up to 53.3%.

Table 2. Prevalence *of L. monocytogenes* in FNAO published between 2010 and 2022 (last accessed in March 2023).

FNAO Product(s)	Country	Prevalence % (*n*)	Reference
Raw green beans	USA	4.2 (3/72)	(Magdovitz et al. 2021)
Raw green peas	USA	6.3 (6/96)	(Magdovitz et al. 2021)
Raw corn	USA	13.6 (8/59)	(Magdovitz et al. 2021)
Fresh and frozen vegetables	Poland	0.56 (49/8712)	(Maćkiw et al. 2021)
Fresh-cut vegetables	Canada	0.24 (13/5379)	(Zhang et al. 2020b)
Fresh-cut fruit	Canada	0.51 (24/4691)	(Zhang et al. 2020b)
Frozen fruit	England	0.9 (3/351)	(Willis et al. 2020)
Frozen vegetables	England	10.3 (69/673)	(Willis et al. 2020)
Frozen vegetable mix	England	9.3 (14/150)	(Willis et al. 2020)
Frozen fruit and vegetable mix	England	23.0 (6/26)	(Willis et al. 2020)
Fresh vegetables	China	5.49 (23/419)	(Chen et al. 2019)
Iceberg lettuce	USA	0.06 (2/3310)	(Zhang et al. 2018)
Romaine lettuce	USA	0.14 (8/5548)	(Zhang et al. 2018)
Spinach	USA	0.09 (5/5325)	(Zhang et al. 2018)
Cucumber	USA	0.43 (5/1160)	(Zhang et al. 2018)
Mango	USA	0.26 (3/1176)	(Zhang et al. 2018)
Farmers market spinach	USA	3.9 (2/52)	(Roth et al. 2018)
Leafy greens	USA	2.6 (2/77)	(Roth et al. 2018)
Cut green leaves or salads	EU	2.1 (2/97)	(Moravkova et al. 2017)
Frozen vegetables	EU	20.9 (9/43)	(Moravkova et al. 2017)
Sprouts	EU	2.9 (1/35)	(Moravkova et al. 2017)
RTE salads	EU	2.0 (20/1042)	(EFSA 2017)
Spinach	USA	2.1 (1/46)	(Scheinberg et al. 2017)
Stone fruits	USA	53.3 (56/105)	(Chen et al. 2016)
RTE salads	Brazil	5.56 (3/54)	(Vasconcelos Byrne et al. 2016)
Raw lettuce	Brazil	2.22 (1/45)	(Vasconcelos Byrne et al. 2016)
Tomatoes	Nigeria	19.67 (48/244)	(Ajayeoba et al. 2016)

Table 2. Continued

FNAO Product(s)	Country	Prevalence % (*n*)	Reference
Lettuce	Nigeria	19.67 (48/244)	(Ajayeoba et al. 2016)
Cucumber	Nigeria	23.36 (57/244)	(Ajayeoba et al. 2016)
Cabbage	Nigeria	28.28 (69/244)	(Ajayeoba et al. 2016)
Carrot	Nigeria	9.02 (22/244)	(Ajayeoba et al. 2016)
RTE salads	Sweden	1.4 (2/141)	(Söderqvist et al. 2016)
RTE salads	Turkey	6.0 (15/261)	(Gurler et al. 2015)
Parsley	Poland	5.0 (10/210)	(Szymczak et al. 2014)
Potatoes	Poland	15.0 (N.A.)	(Szymczak et al. 2014)
Strawberries	Poland	10.0 (N.A.)	(Szymczak et al. 2014)
Raw mixed salads	Estonia	18.5 (N.A.)	(Kramarenko et al. 2013)
Cucumber	Iran	14.0 (7/50)	(Hossein et al. 2013)
Lettuce	Iran	10.0 (5/50)	(Hossein et al. 2013)
Cabbage	Iran	6.0 (3/50)	(Hossein et al. 2013)
Cut red cabbage	Croatia	1.0 (1/100)	(Kovačević et al. 2013)
RTE vegetables	Brazil	3.1 (16/512)	(Sant'Ana et al. 2012)
Bunch of parsley and spring onion	Brazil	4.5 (1/22)	(Aparecida de Oliveira et al. 2010)
Collard green	Brazil	3.3 (1/30)	(Aparecida de Oliveira et al. 2010)
Yardlong bean	Malaysia	27.2 (83/306)	(Ponniah et al. 2010)
Japanese Parsley	Malaysia	31.3 (96/306)	(Ponniah et al. 2010)

N.A. = not applicable

Other foods, like parsley, can be contaminated with *L. monocytogenes* as well (Szymczak et al. 2014; Ponniah et al. 2010). Some other studies found *L. monocytogenes* in 3.2% of lettuce, parsley and watercress specimens (Aparecida de Oliveira et al. 2010). Soft fruit (such as strawberries) are, as well as vegetables, a possible source of contamination. *L. monocytogenes* is able to remain on strawberries under cooling conditions (Yin et al. 2022; Szymczak et al. 2014). Detecting *L. monocytogenes* in FNAO is one thing, but connecting human related listeriosis cases to contaminated FNAO is another challenge. The EFSA journal published a multi-country outbreak of listeriosis linked to frozen corn and other possibly frozen vegetables in 2018 (EFSA 2018b). This was the only reported large human listeriosis outbreak caused by FNAO in Europe to date. The Centers for Disease Control and Prevention (CDC) in the USA reported human listeriosis cases linked to FNAO, like packaged salads, frozen vegetables, prepackaged caramel apples, bean sprouts or cantaloupes. (Centers for Disease Control and Prevention 2022b). Processing fruits or

vegetables before consumption, e.g. with extended washing steps, minimize the risk of infection (Zhu et al. 2017; Nastou et al. 2012).

In conclusion, it is important to know the occurrence and concentration of *L. monocytogenes* in each step from production to consumption in RTE food to detect the pathogen microorganism at every level (FAO/WHO 2000).

1.2.3 Human related *Listeria monocytogenes*

It is assumed, that Dumont and Cotoni cultured one of the first isolates from a human with meningitis (Dumont and Cotoni 1921) and Seeliger described it as one of the oldest authentical strains of *Listeria* spp. (Seeliger 1988). Nyfeldt first described a listeriosis infection in humans in 1929 and three patients had a mononucleosis-like illness (Gray and Killinger 1966). Animal experiments showed anomalies, which was a large mononuclear leukocytosis and therefore the bacterium's name, *L. monocytogenes*, originated from these laboratory results (Murray et al. 1926).

Listeriosis in humans is caused by invasive *L. monocytogenes* strains (Zhu et al. 2017). The vulnerable group for this disease are immunocompromised people, elderly people (>65 years), pregnant women, their unborn babies and the infants (Zhu et al. 2017; FAO/WHO 2000). *L. monocytogenes* enters via the gastrointestinal tract and can cause two different disease varieties with an incubation time spanning from a few days up to three months (Zhu et al. 2017; FAO/WHO 2000; Seeliger 1988). Influenza-like symptoms or mild febrile gastroenteritis occur in otherwise healthy people (EFSA 2021; FAO/WHO 2000). More severe symptoms are reported from the susceptible group of YOPIs (young, old, pregnant, immunosuppressed) (Federal Institute for Risk Assessment 2011). Common, but not limited, symptoms of severe listeriosis are meningitis, encephalitis, meningoencephalitis, septicemia or endocarditis (FAO/WHO 2000). *L. monocytogenes* infection during pregnancy can result in abortion or stillbirth, but preterm birth is more common (Lamont et al. 2011). Furthermore, neonatal listeriosis is often severe, and may cause neonatal meningitis or ends fatally (Lamont et al. 2011; Becroft et al. 1971). One of the largest human listeriosis outbreaks worldwide was reported in South Africa from 2017 to 2018 (WHO 2018). A total of 1060 cases were reported, women (55%) and neonates (43%) were the most affected group and the outbreak had a fatality rate of 27%. The probable causative vehicle was a RTE processed meat product called polony (comparable to bologna sausage) (Smith et al. 2019).

Early understanding of human listeriosis cases assumed transmission occurred via direct contact from contagious animals. The infection of exposed persons, like veterinarians or slaughterhouse workers, was often reported (Seeliger 1988;

Becroft et al. 1971). However, the common transmission path is via contaminated food, in particular RTE products (EFSA 2021; Lakicevic et al. 2021; Garner and Kathariou 2016; FAO/WHO 2000; Charpentier and Courvalin 1999). Studies on the number of infectious doses are difficult to perform because of different factors related to the food types, the complex disease development or differences in human susceptibility (McLauchlin et al. 2004). However, previous estimates resulted in 10^5 to 10^7 CFU in YOPIs and 10^7 to 10^9 CFU in healthy individuals (Quereda et al. 2021).

If the suspected diagnosis is listeriosis, the isolation of the microorganism is from blood, joint or spinal fluid, other sterile spots or vaginal swab samples in pregnant women (Schlech 2019; Temple and Nahata 2000). Culture based diagnosis or detection of PCR products are applied (Schlech 2019). Beta-lactam antibiotics like amoxicillin, ampicillin or penicillin are the most commonly used drugs for treating listeriosis (Temple and Nahata 2000). Cephalosporins are inactive against the intracellular pathogen (Hof 1991). The current antibiotics of choice are penicillin, ampicillin alone, or the combination of ampicillin and gentamicin (Schlech 2019; Noll et al. 2018; Jones and MacGowan 1995).

Besides *L. monocytogenes*, *L. ivanovii* has pathogenic potential as well (Lakicevic et al. 2021). Most commonly, bovines like cattle and sheep can be infected by *L. ivanovii* (EFSA 2021). In comparison, there are rare reported cases about human *L. ivanovii* infections (Guillet et al. 2010). Furthermore, a human meningitis case caused by *L. seeligeri* was reported as well (Rocourt et al. 1987). But these reports are exceptional cases.

The European member states report human zoonotic cases via The European Surveillance System TESSy, which is maintained by the European Centre for Disease Prevention and Control (ECDC) (EFSA 2021). Table 3 shows numbers of confirmed human listeriosis cases from 2010 to 2021 in the EU.

Table 3. Reported human listeriosis cases in the EU from 2010 until 2021 according to EFSA (last accessed in March 2023).

Year	Reporting Member States	Number of Confirmed Cases	Cases per 100,000 Population	Deaths/Fatality Rate in %	Reference
2021	27	2,183	0.49	196/13.7	(EFSA 2022)
2020	27	1,876	0.42	167/13.0	(EFSA 2021)
2019	28	2,621	0.46	300/17.6	(EFSA 2021)
2018	28	2,549	0.47	229/15.6	(EFSA 2019c)
2017	28	2,480	0.48	225/13.8	(EFSA 2018c)
2016	28	2,536	0.47	247/16.2	(EFSA 2017)
2015	28	2,206	0.46	270/17.7	(EFSA 2016)
2014	27	2,161	0.52	210/15.0	(EFSA 2015b)
2013	27	1,763	0.44	191/15.6	(EFSA 2015a)
2012	?	1,642	0.41	198/17.8	(EFSA 2014)
2011	26	1,476	0.32	134/12.7	(EFSA 2013)
2010	26	1,601	0.35	N.A./17.0	(EFSA 2012)

Table 3 demonstrates a continuous increase of human listeriosis cases in the EU. A possible cause of this is the fact, that most of the RTE products are eaten raw (Strawn et al. 2013).

Since the development of new technologies like whole-genome sequencing (WGS), multi-country *L. monocytogenes* outbreaks were discovered in Europe. Table 4 shows human multi-country outbreaks in FAO and FNAO in Europe which were detected by WGS.

Table 4. Multi-country outbreaks of human listeriosis cases caused by FAO and FNAO in Europe detected by WGS (last accessed in March 2023).

Year	Countries	ST/CC	Food	Cases/ Deaths (Fatality Rate in %)	Reference
2017-19	NED, BEL	ST 6	Sliced RTE meat products	21/3 (14)	(EFSA 2019b)
2015-18	AUT, DEN, FIN, SWE, UK	ST 6	Frozen corn and frozen vegetable mixes (corn, spinach, green beans)	47/9 (19)	(EFSA 2018b)
Since 2015	DEN, GER, FR	ST 8	Cold – smoked salmon Marinated salmon	12/4 (33)	(EFSA 2018a)
Since 2014	DEN, EST, FIN, FR, SWE	ST 1247/ CC 8	Cold – smoked fish products	22/5 (23)	(EFSA 2019a)

ST = sequence type; CC = clonal complex; AUT = Austria; BEL = Belgium; DEN = Denmark; EST = Estonia; FIN = Finland; FR = France; GER = Germany; NED = The Netherlands; SWE = Sweden; UK = United Kingdom.

In comparison to the EU, the CDC in the USA reports the food that cause listeriosis. Table 5 shows different listeria outbreaks according to different food from 2011 to 2021 in the USA (Centers for Disease Control and Prevention 2022b).

Table 5. Multistate outbreaks of human listeriosis cases caused by FAO and FNAO in the USA from 2011 to 2022 according to CDC (Centers for Disease Control and Prevention 2022b) (last accessed in March 2023).

Year	Involved States (*n*)	Involved Food	Cases/Deaths (Fatality Rate in %)
2022	2	Enoki Mushrooms	2/0 (0)
2022	6	Deli Meat and Cheese	16/1 (6)
2022	6	Brie and Camembert Cheese	6/0 (0)

Table 5. Continued

Year	Involved States (*n*)	Involved Food	Cases/Deaths (Fatality Rate in %)
2022	11	Ice Cream	28/1 (4)
2021	13	Dole Packaged Salads	18/3 (16)
2021	8	Fresh Express Packaged Salads	10/1 (10)
2021	2	Fully Cooked Chicken	3/1 (33)
2021	4	Queso Fresco (soft cheese)	13/1 (8)
2020	4	Deli Meats	12/1 (8)
2020	17	Enoki Mushrooms	36/4 (11)
2019	5	Hard-boiled Eggs	8/1 (13)
2019	5	Deli-Sliced Meats and Cheeses	10/1 (10)
2018	4	Pork Products	4/0 (0)
2018	2	Deli Ham	4/1 (25)
2017	4	Vulto Creamery Soft Raw Milk Cheese	8/2 (25)
2016	4	Frozen Vegetables	9/3 (33)
2016	2	Raw Milk	2/1 (50)
2016	9	Packaged Salads	19/1 (5)
2015	10	Soft Cheeses	30/3 (10)
2015	4	Ice Cream	10/3 (30)
2014	12	Commercially Produced, Prepackaged Caramel Apples	35/7 (20)
2014	2	Bean Sprouts	5/2 (40)
2014	4	Cheese	5/1 (20)
2014	2	Dairy Products	8/1 (13)
2013	5	Cheese	6/1 (17)
2012	14	Ricotta Salata Cheese	22/4 (18)
2011	28	Cantaloupes	147/33 (22)

Listeriosis is one of the most severe foodborne diseases with the highest case fatality rate (EFSA 2021). The incident rate increased over the last ten years (EFSA 2022). However, *L. monocytogenes* is an ubiquitous bacterium and once it has entered the food chain, there is a high threat of it persisting around the farm or processing environment (Lakicevic et al. 2021). Therefore, persisting *L. monocytogenes* strains pave the way for more human related listeriosis cases.

1.3 Virulence factors of *Listeria monocytogenes*

The detection of the microorganism *Bacterium monocytogenes* by Murray et al. in 1926 shows that thinking about a monocytosis-producing agent and its virulence had already started at that time (Murray et al. 1926). Throughout the following years, more potential virulence components, like a lipopolysaccharide-like material or hemolysins had been described (Farber and Losos 1988). Toledo-Arana et al. detected the potential of *L. monocytogenes* to transform from saprophytism to virulence depending on environmental context. (Toledo-Arana et al. 2009).

Furthermore, *L. monocytogenes* is known as a foodborne pathogen (EFSA 2021; Lakicevic et al. 2021; Garner and Kathariou 2016; FAO/WHO 2000; Charpentier and Courvalin 1999) and oral and gastrointestinal contamination has many challenges to overcome. The ability to grow at refrigerating temperatures (as low as 4 °C), the potential of resisting low pH ranges, high salt concentration and osmolarity confers high robustness to *L. monocytogenes* (Gaballa et al. 2019; Orsi and Wiedmann 2016; Becker et al. 1998). In 1998, Becker et al. and Wiedmann et al. described an alternative sigma factor (σ^B) in *L. monocytogenes* and the coherent contexts for the acid and osmotolerance abilities (Becker et al. 1998; Wiedmann et al. 1998). Four alternative sigma factors are described in *L. monocytogenes* so far (σ^B, σ^C, σ^H, and σ^L) (Liu et al. 2017). In general, alternative sigma factors are important regulatory mechanisms that confer modification to specific environment conditions and manage the general stress response (GSR) (Guerreiro et al. 2020; Liu et al. 2017). In short, σ^B is responsible for the GSR gene transcription (Gaballa et al. 2019). Additionally, *L. monocytogenes* disposes a positive regulatory factor A (PrfA) that regulates the production of virulence factors that are crucial for pathogenesis (Guerreiro et al. 2020). In the last few years, a crosstalk between σ^B and PrfA has been surmised (Gaballa et al. 2019; Tiensuu et al. 2019; McGann et al. 2007).

Among other things, σ^B is needed for survival in the gastrointestinal tract (Gaballa et al. 2019). Figure 5 gives a schematic overview of the intestinal colonization of *L. monocytogenes* and the participation of important factors (Tiensuu et al. 2019). It illustrates the interaction of σ^B and PrfA.

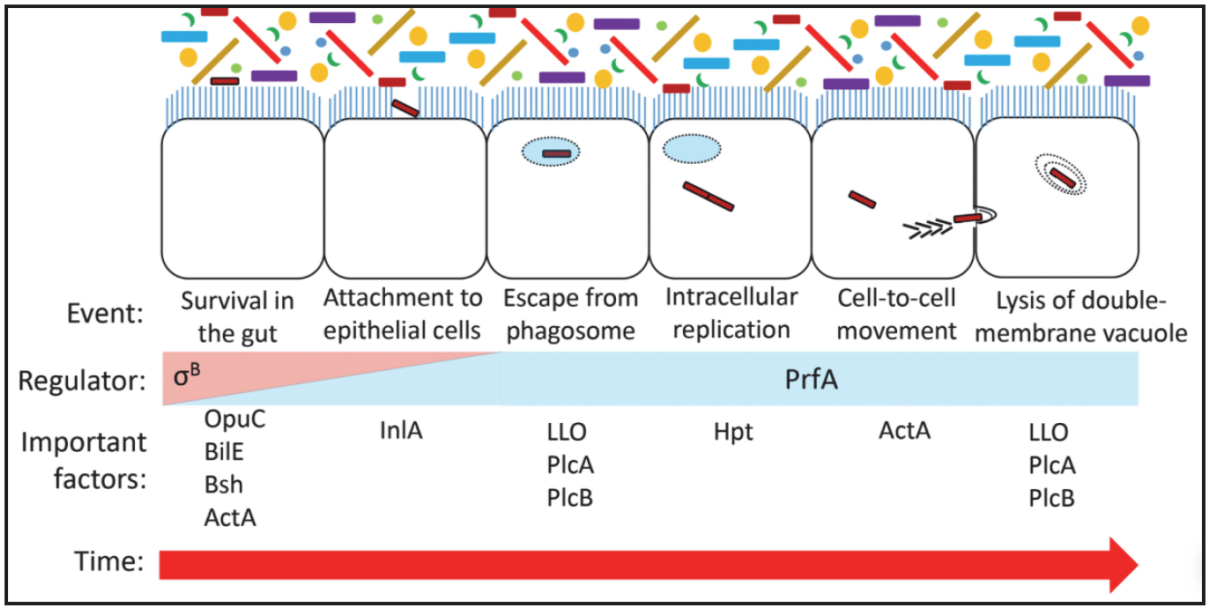

Figure 5. Encoding virulence factors of σ^B and PrfA for *L. monocytogenes* colonization of the intestinal tract. According to Tiensuu et al. (Tiensuu et al. 2019).

The regulator σ^B reduces the PrfA regulon expression when active PrfA is produced and present (Ollinger et al. 2009). Furthermore, it is most likely that different σ^B–dependent glutathione transcriptions and following binding of glutathione (GSH) to PrfA affects PrfA-regulated gene expression (Gaballa et al. 2019). Virulence factors are encoded in *Listeria* pathogenicity islands (LIPI) LIPI-1, LIPI-2, LIPI-3, and LIPI-4 (Lakicevic et al. 2021; Quereda et al. 2021). All PrfA encoding genes are clustered in LIPI-1. PrfA encodes 10 to 15 genes, which are involved to the pathogenic pathway of *L. monocytogenes* (Gaballa et al. 2019). Figure 5 shows the most important encoded genes and proteins of PrfA. *ActA* encodes the protein actA, the *inlAB* operon stands for the surface proteins internalin A (InlA) and internalin B (InlB). *Hly* encodes listeriolysin O (LLO), the *plcA* gene is for the phosphatidylinositol-specific phospholipase C (PI-PLC or PLC-A), *plcB* encodes the nonspecific phosphotidylcholine phospholipase C (PC-PLC or PLC-B) and *hpt* mediated the hexose phosphates (HP) inclusion (Matereke and Okoh 2020; Chico-Calero et al. 2002).

After surviving the acid gastric environment, *L. monocytogenes* enters the intestinal tract. Jensen et al. described the interaction between the invasive *L. monocytogenes* and the Peyer Patches and M cells as initiating the infection of a host (Jensen et al. 1998). InlA and InlB, encoded by the *inlAB* operon, are proteins for a *L. monocytogenes* invasion whereas InlA infect epithelial cells and InlB is responsible for invading hepatocytes (Dramsi et al. 1995; Gaillard et al. 1991). Two types of receptors on the host cell surface are interacting with InlA and InlB for cell invasion. E-Cadherin was identified as the ligand for InlA

(Mengaud et al. 1996) and Met, a receptor tyrosine kinase, for InlB-mediated access (Shen et al. 2000). A study by Harter et al. detected new types of internalins, like internalin-like protein 1 (InlP), InlPq, InlP4 and the InlP3 that were in conjunction with a severe listeriosis outbreak in Austria, the Czech Republic and Germany in 2009/2010. The internalin expression increased under gastric stress and the bacterial colonization in the liver and the spleen point to an enhanced pathogenicity (Harter et al. 2019). After entering the host cell, *L. monocytogenes* is surrounded by a vacuole (Figure 5) (Tiensuu et al. 2019). The significant virulence factor for lysing the phagosome is LLO. First, Harvey and Faber determined the presence of a hemolysin by *L. monocytogenes* in 1941 (Harvey and Faber 1941). Several research studies were performed (Vázquez-Boland et al. 2001) until Geoffroy et al. identified and named the hemolysin LLO in 1987 (Geoffroy et al. 1987). Following this, the gene *hly* was encoded for LLO (Mengaud et al. 1987). The toxins activity was shown at a pH range from 4.5 to 6.5 (optimum 5.5), but showed no growth at pH 7.0 (Geoffroy et al. 1987). In the acid phagosome environment, LLO causes pores to the macrophage phagosome membrane and facilitates the entry of phospholipases whereas PLC-A and PLC-B are specific for *L. monocytogenes* (Vázquez-Boland et al. 2001). Phospholipases support the escape from primary and double membrane vacuoles, especially PLC-B is able to assume the disruption of the vacuole if LLO is absent, depending on cell types (Marquis et al. 1995). After escaping the vacuole, the pathogenicity pathway continues. For replication, the HP is encoded by the *hpt* gene and HP is used by *L. monocytogenes* as a source of carbon and energy to fuel fast intracellular growth (Chico-Calero et al. 2002). The distribution within the host takes place by the protein actA, which induces an actin-based intracellular motility. The protein actA recruits actin on the bacterial surface by actin polymerization and enables the pathogen to move through the host cell cytoplasm and infect neighbor cells (Vázquez-Boland et al. 2001). Furthermore, as Figure 5 demonstrates, it is assumed that *actA* participates in the add-on and access of *L. monocytogenes* in the host cell (Alvarez-Domínguez et al. 1997). At least, the gene *mpl* (not shown in Figure 5) stands for a zinc metalloproteinase, that stimulates on the one hand the PLC-B (Poimenidou et al. 2018) and supports actA processing and protrusion on the other (Alvarez and Agaisse 2016). After cell-to-cell spread LLO, PLC-A and PLC-B are required to evade the double-membrane vacuole to continue a new cell infection (Vázquez-Boland et al. 2001; Gedde et al. 2000).

1.4 Antimicrobial resistance of *Listeria monocytogenes*

In 1988, the first antibiotic resistant *L. monocytogenes* strain was documented and resistance to erythromycin, streptomycin, chloramphenicol and tetracycline was expressed by a 37-kb plasmid (Poyart-Salmeron 1990). Charpentier and Courvalin summarized conjugative transfers of antibiotic resistance genes from other bacteria like streptococci and enterococci to *Listeria* spp. via different kinds of plasmids (Charpentier and Courvalin 1999). For example, the streptococcal plasmid pIP501 has resistance to erythromycin and chloramphenicol (Evans and Macrina 1983). Furthermore, the probability of genetic information exchange from streptococci, enterococci and *L. monocytogenes* in the human and animal intestinal tract is given (Charpentier and Courvalin 1999). As mentioned above, this thesis is supported by revealing the human and animal digestive tract as a *L monocytogenes* harbor (Hafner et al. 2021; Terentjeva et al. 2021; Schoder et al. 2011; Esteban et al. 2009; Yokoyama et al. 2005; Olier et al. 2002). Plasmid transmission and efflux pumps contribute to antibiotic resistance of *L. monocytogenes*. The *lde* gene (*Listeria* drug efflux) is responsible for resistance to fluoroquinolones (Godreuil et al. 2003). Furthermore, Jiang et al. also examined that the efflux pump gene *lde* is able to cause a ciprofloxacin resistance in *L. monocytogenes* (Jiang et al. 2018). However, penicillin, ampicillin, amoxicillin alone or the combination of ampicillin and gentamicin are the antibiotics of choice when treating human listeriosis (Schlech 2019; Noll et al. 2018; Robert Koch-Institute 2010; Jones and MacGowan 1995). Whereas, Park et al. confirmed that the combination of ampicillin and gentamicin is not symbiotic and not more potent than penicillin-based monotherapy. The same study reported that licorice extract synergistically increased the antimicrobial activity of gentamicin, but more research is needed to detect the mechanism behind it (Park et al. 2022). Alternatively, cotrimoxazole is recommended (Robert Koch-Institute 2010). Furthermore, antibiotic resistance to penicillin has already been reported (Vasconcelos Byrne et al. 2016). Besides synthetic antibiotics for disease treatment, natural antimicrobials can be applied as preservation at facility environment or in processed food as well (Lakicevic et al. 2021). For example, nisin is presently used as an authorized food additive in the EU (Younes et al. 2017). The preventative potential of nisin against *L. monocytogenes* is demonstrated by the Bower et al. study (Bower et al. 1995).

Due to increasing antimicrobial resistances, alternative control measures are developing. Current research topics for foodborne pathogen prevention are for example bacteriophages (Hagens and Loessner 2007) or enzymes, like deoxyribonuclease (DNase) or Proteinase K (Nguyen and Burrows 2014) and are designed to both increase detection probability and decrease biofilm formation.

1.5 Sanitizer tolerance and cross-resistance to antibiotics

Next to the evasion of the immune system or variable pH values and osmotic stress in the human or animal gastrointestinal tract, *L. monocytogenes* has to challenge the industrial plant environment like disinfectants (Gaballa et al. 2019). Sublethal concentrations of disinfectants in processing facilities result in consequences, such as reducing the effectiveness of disinfectant. Details like incorrect dosages, inadequate cleaning before disinfection or disinfection of wet surfaces strengthen the issue (Martínez-Suárez et al. 2016). These factors contribute to the growth of persistent *L. monocytogenes* strains, especially in unhygienic company environments (Carpentier and Cerf 2011).

Sanitizers, particularly quaternary ammonium compounds (QACs) are used in the food industry because of its noncorrosive qualities (Kovacevic et al. 2016). The assumption is that a hydrophobic alkyl side chain interpolates into the lipid bilayer of the microorganisms cellular membrane leading to problems in osmoregularity of the bacterium and the leaking of cell contents (Bland et al. 2021). For example, efflux pumps encoded by *bcrABC* (Elhanafi et al. 2010), *mdrl* (Romanova et al. 2006), *emrC* (Kremer et al. 2017), *emrE* (Kovacevic et al. 2016) or *qacH* (Müller et al. 2013) show tolerance to QACs. Besides genetic modification, physiochemical and morphological adaptations are important when discussing *L. monocytogenes* and QAC tolerance. Especially, changes in size, roughness and the fatty acid surface profile suggest a higher tolerance to QACs. Whereas adaptability of surface hydrophobicity measures no appropriate difference (To et al. 2002).

The experimental environment is no comparison to actual facility surroundings with niches, biofilm formation or concentration dilution due to wet surfaces. The study of Bland et al. warned against cross-resistance to clinically relevant antibiotics (Bland et al. 2021). Few studies investigated the phenomenon of cross-resistance from QACs to antibiotics with different approaches and each study measured an adaptation to antibiotics by QAC-adapted *L. monocytogenes* strains (Kode et al. 2021; Bansal et al. 2018; Kovacevic et al. 2013; Rakic-Martinez et al. 2011). Biocides or QACs are needed to meet the hygienic requirements in the food industry. Non-ideal conditions like accumulation of water in niches, unhygienic plant circumstances, sublethal concentrations of QACs or biofilm formation are hardly avoidable and will accelerate the process. It therefore remains to be seen how fast *L. monocytogenes* strains align to these novel conditions and which medical and treatment consequences have to be drawn.

Chapter 2: Task Formulation and Key Objective

L. monocytogenes is well known in FAO. But the cases of *L. monocytogenes* and FNAO are increasing recently (EFSA 2021). In this context, food products, the environment, irrigation and processing water from 39 FNAO producing and processing companies and 123 samples of fresh soft fruit and frozen soft fruit were investigated for the presence of *Listeria* spp. and *L. monocytogenes*.

The aim of this work was

- to detect the prevalence of *Listeria* spp. and *L. monocytogenes* in FNAO producing and processing plants,
- to put special attention to the FNAO facility environment and irrigation and processing water as potential contamination source,
- to characterize *Listeria* spp. isolates, including the pathogenic *L. monocytogenes*, like genotype and serotype detection, genotypic and phenotypic antimicrobial resistances (AMR) and virulence markers in FNAO products and FNAO primary production and processing companies,
- to detect the distribution and genetic relation of *Listeria* spp. and *L. monocytogenes* in FNAO products and producing and processing facilities.

Chapter 3: Presence of *Listeria* at primary production and processing of food of non-animal origin (FNAO) in Bavaria, Germany

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Stefanie Huber	Co Author	Participation water laboratory experiments
Iris Kraemer	Co Author	Participation laboratory reports of the Bavarian Health and Food Safety Authority
Thomas Alter	Co Author	Writing-review and editing, critical revision of the manuscript
Ute Messelhäußer	Co Author	Supervision, participation conceptualization, writing-review and editing, critical revision of the manuscript

3.1 Summary Chapter 3

L. monocytogenes is responsible for a high mortality rate (20–30%) in vulnerable population groups called YOPIs (Federal Institute for Risk Assessment 2011; Newell et al. 2010). The disease causing listeriosis is transmitted via contaminated food, particularly RTE food (Lakicevic et al. 2021; EFSA 2021; Garner and Kathariou 2016; FAO/WHO 2000). Common known food products causing listeriosis are fish/fish products, mixed meat products and cheese (Robert Koch-Institute 2010). However, the sampling and therefore the positive listeriosis cases caused by fruits and vegetables are increasing in recent years (EFSA 2021).

For this reason, we conducted a study that investigated the following topics:

- The prevalence of *Listeria* spp. and *L. monocytogenes* in FNAO producing and processing plants in Bavaria, Germany at different parts of the company, like the environment, food and irrigation and processing water.
- The potential hazard of *L. monocytogenes* at these companies with special attention to irrigation, processing water and environmental sampling.

The study showed that *Listeria* spp. and *L. monocytogenes* are present at FNAO companies in Bavaria, whereas no food was contaminated with the pathogenic *L. monocytogenes*. Good hygienic practice and environmental monitoring are important to minimize the hazard of exposure.

3.2 Abstract

Several foodborne outbreaks associated with food of non-animal origin (FNAO) were reported within the last years. In recent years, *Listeria monocytogenes* has been associated with such outbreaks. For this reason, different producers of FNAO at the primary production and processing level in Bavaria, Germany, were inspected from July 2020 to June 2021. Environmental and food sampling as well as the sampling of irrigation and processing water was performed to investigate the prevalence of *Listeria* spp., including *L. monocytogenes* at facilities that produce ready-to-eat FNAO. Altogether, 39 producers of soft fruit, vegetables, ready-to-eat raw fruits, and vegetables/fresh cut were inspected. In addition to the on-spot inspections, 407 samples were taken in total, among them, 229 were swab samples from food contact material and the environment, 59 food samples (including soft fruit, vegetables and ready-to-eat vegetables), and 119 samples of irrigation and processing water. Samples were analyzed using methods according to ISO 11290-1:2017. Furthermore, the samples of irrigation and processing water were also quantitatively tested for the number of *Escherichia coli* (ISO 9308-2:2014-06), enterococci (ISO 7899-2:2000-11), and *Pseudomonas aeruginosa* (ISO 16266:2008-05). No contamination with *E. coli*, enterococci, and *P. aeruginosa* could be detected in most of the samples. Overall, in 12.53% of the samples, *Listeria* spp. were detected. *L. monocytogenes* was identified in 1.72% of the environmental and processing water samples, whereas *L. monocytogenes* was not detected in food samples.

In addition to water sources and quality, this study demonstrates that irrigation regime, cultivation, hygienic handling, and maintenance protocols are highly important to reduce the potential contamination of ready-to-eat soft fruits and vegetables with *Listeria* spp.

3.3 Introduction

Listeria monocytogenes is an important foodborne pathogen, transmitted by a broad range of contaminated foods (Farber and Losos, 1988, Farber and Peterkin, 1991, Gray and Killinger, 1966, Centers, 2021). Listeriosis, the resulting disease, manifests in particular during pregnancy, in neonates, immunocompromised, and older people (Farber and Peterkin, 1991, Lamont et al., 2011, Schlech, 2019). In Germany, an incidence of 0.70 per 100000 inhabitants in 2020 was recorded by the Robert Koch-Institute (RKI, Germany) (Koch-Institut, 2022). In comparison, the Centers for Disease Control and Prevention (CDC, USA) documented an incidence of 0.21 per 100000 population in 2020 in the USA (U.S. Centers for Disease Control and Prevention, 2022). Of major concern is the high mortality rate related to invasive *Listeria* infections in vulnerable population groups (U.S. Centers for Disease Control and Prevention., 2021, U.S. Centers for Disease Control and Prevention., 2022).

In recent years, the role of *Listeria* transmission by the food of non-animal origin (FNAO) has come into focus. The first suspected vegetable-associated *L. monocytogenes* outbreak was recorded in 1979 in Boston, USA, contaminated raw celery, tomatoes, and lettuce were assumed to cause that outbreak (Ho, 1986). Since then, a number of *L. monocytogenes* outbreaks associated with FNAO were reported, with a wide range of food types affected, e.g., coleslaw, cantaloupes, packaged salads, diced celery, prepackaged caramel apples, frozen corn, and vegetables (Angelo et al., 2017, European Food Safety Authority et al., 2018, Garner and Kathariou, 2016, Gaul et al., 2013, Koutsoumanis et al., 2020, McCollum et al., 2013, Schlech, 2019, Schlech et al., 1983, U.S. Centers for Disease Control and Prevention., 2012, U.S. Centers for Disease Control and Prevention., 2016, U.S. Centers for Disease Control and Prevention, 2016, U.S. Centers for Disease Control and Prevention., 2017). Due to this risk, companies producing and/or processing FNAO should be regularly monitored for the presence of *Listeria* spp.

The aim of the project was to ascertain the prevalence of *Listeria* spp. at different parts of FNAO primary production and processing facilities and to identify potential hazards of *L. monocytogenes* at these companies with special attention to irrigation, processing water, and environmental sampling.

3.4 Materials and Methods

3.4.1 Companies

FNAO companies were selected by using the German national surveillance software BALVI iP (Wollowski, 2019). Keywords for selection were “producers

of soft fruit", "producers of vegetables", and "producers of ready-to-eat raw fruits and vegetables/fresh cut". Producers of the first two categories (soft fruit and vegetables) were inspected at farm and primary production levels. The third category (ready-to-eat raw fruits and vegetables/fresh cut) was monitored at processing level. The sections "producers of soft fruit" and "producers of ready-to-eat raw fruits and vegetables/fresh cut" were chosen by random selection. The category "producers of vegetables" was selected based on plausibility checks. After the assessment of each regional government, 14 producers of soft fruit, 16 producers of vegetables, and nine producers of ready-to-eat raw fruits and vegetables/fresh cut were selected and located in the seven administrative areas in Bavaria. Each company was controlled once. The visiting period extended from July 2020 to June 2021, regardless of weather conditions.

3.4.2 Checklists

Every official inspection followed a predefined protocol. Companies were inspected using two different checklists, adjusted to the nature of business (primary production or processing). Checklists were created by applying the technical report of the European Food Safety Authority (EFSA) (Ana Allende et al., 2018). The checklists contained questions about company structure, staff, range of products, delivery of food, and possible private certification schemes. A second section included information on production and processing hygiene, with special attention to water supply and staff hygiene. The third section was related to self-monitoring, including hazard analysis critical control point (HACCP), traceability, cleaning and disinfection work, chemical self-monitoring, incoming goods, outgoing goods control, and microbiological self-monitoring with a special focus on *Listeria* spp.

3.4.3 Sampling

Altogether, 407 food, water, and environmental samples were taken at the companies. Swab samples (n = 229) from food contact material (n = 119) and the environment (n = 110) were taken in accordance to ISO/FDIS 18593:2018 (International Organization International Organizational for Standardization, 2018). MASTASWAB MD 508 with Amies-Medium (Mast Group, Bootle, UK) was used.

Furthermore, 59 food samples (soft fruit, vegetables, ready-to-eat vegetables, and other food samples) were taken. The category "soft fruit" included blackberries (n = 5), blueberries (n = 4), currants (n = 1), raspberries (n = 5), and strawberries (n = 5). Cultivation was exclusively open land growing, except

for strawberries. Strawberries were grown on open land or hydroponic systems. The category “vegetables” included salads (n = 10), tomatoes (n = 4), carrots (n = 5), cucumbers (n = 1), dried vegetables (n = 1), and frozen mushrooms (n = 1). Salads, carrots, cucumbers, and tomatoes were grown on open land or in greenhouses. The category “ready-to-eat vegetables” contained machine-cut salads (n = 8), machine-cut carrots (n = 3), and machine-cut peppers (n = 1). Other food samples (n = 5) were rice, panna cotta with fruit toppings, rice pudding with fruit toppings, and two smoothies, one based on different fruits and the other based on vegetables.

Finally, 119 samples of irrigation and processing water were investigated. For sampling, wide-neck bottles with 20 mg/L sodium thiosulfate were used (VWR International, Darmstadt, Germany). Water samples were taken from wells (n = 18), surface water (n = 7), water tanks filled with rainwater (n = 4), public water supplies (n = 11), and different spots along water pipelines including intermediate pieces (n = 19) and the end of the water pipeline (n = 31). Furthermore, water specimens from water installations for vegetables including nonsliced and sliced lettuce (n = 18), carrots (n = 4), tomatoes (n = 1), leek (n = 3), radish (n = 1), fennel (n = 1), and red cabbage (n = 1) were taken. Water samples containing more than 300 mL underwent further testing on the amount of *Escherichia coli*, enterococci, and *Pseudomonas aeruginosa.* In the course of this, 111 water samples were investigated. The 111 water samples were divided into irrigation water, which encompassed 75 samples and the processing water contained 36 samples. Eight water samples were not analyzed because of less than <300 mL of water sample.

All samples were analyzed at the accredited laboratory of the Bavarian Health and Food Safety Authority in Oberschleißheim, Germany. After samples were taken, they were transported refrigerated at temperatures of maximum 10°C. Upon arrival at the laboratory, the samples were stored at refrigeration temperature and examination started the following day.

3.4.4 Detection and isolation of *Listeria* spp.

For the detection and isolation of *L. monocytogenes* and *Listeria* spp., ISO 11290-1:2017 was used with the following modifications (International Organization International Organizational for Standardization., 2017). In the beginning, food (25 g in 225 mL), water (unfiltered 25 mL in 225 mL), and swab samples (swab in 10 mL) were enriched in Half Fraser broth as primary enrichment. Secondly, 0.1 mL of each Half Fraser broth was added to 10 mL of Full Fraser broth as secondary enrichment. Incubation times and temperature indications were applied following ISO instructions. The Half Fraser broth and

the Full Fraser broth were plated on two selective solid media, *Listeria* Selective Oxford agar (Oxoid Deutschland, Wesel, Germany) and PALCAM agar (Merck, Darmstadt, Germany). After both plates were incubated for 48 h at 37°C, the plates were checked for suspected *Listeria* spp. colonies. A maximum of five presumptive *Listeria* spp. colonies were picked from each plate and subsequently plated on tryptone soya yeast extract agar (TSYEA, Merck). After 24 h ± 2 h incubation at 37°C on TSYEA, suspected colonies were tested with the Henry illumination test (Wild Heerbrugg M8 Stereomicroscope, Wild Heerbrugg, Heerbrugg, Switzerland). Furthermore, 0.5 mL of the Full Fraser broth as secondary enrichment was used for the VIDAS screening (BioMérieux, Lyon, France) following the manufacturer's instructions.

In general, all 407 food, water, and environmental samples were tested for *L. monocytogenes* and *Listeria* spp. as described above. In addition, all 59 food samples underwent a quantitative test as well. For that, the nonincubated Half Fraser broth was inoculated on three Rapid *Listeria monocytogenes* agar plates (Bio-Rad, Hercules, CA, US) in 1:10 dilution steps. After 48 h ± 2 h and 37°C of incubation, characteristic colonies were counted (Anonymous, 2022).

3.4.5 Identification and differentiation of *Listeria* spp.

If the Henry illumination test showed a positive result, corresponding colonies were streaked out on blood agar (Oxoid Deutschland). Plates were incubated at 37°C for 24 h ± 2 h. *L. monocytogenes* was identified by β-hemolysis activity. Species confirmation was performed by using VITEK 2 Compact System (BioMérieux). By this, it was possible to differentiate *L. monocytogenes, L. grayi, L. innocua, L. ivanovii, L. ivanovii* ssp*. ivanovii, L. ivanovii* ssp*. londoniensis, L. seeligeri,* and *L. welshimeri*. If VITEK data were nonconclusive, MALDI-TOF MS (Maldi Biotyper MBTsmart, Bruker, Billerica, MA, US) was applied. As the second confirmation step, a multiplex real-time PCR was used to identify and differentiate *L. monocytogenes, L. innocua, L. ivanovii,* and *L. welshimeri*. For DNA extraction, a single colony from a blood agar (Oxoid Deutschland) was placed in 300 µL 0.1 × Tris-EDTA buffer (Merck). The suspension was heated at 95°C for 15 min. After one centrifugation step, 5 µL of the supernatant was used for the RT−PCR. The sequences of primers and probes are constituted in Table 1. The Brillant Multiplex QPCR Mastermix (Agilent Technologies, Cedar Creek, TX, US) was applied. The real-time thermal cycler Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, US) was used. The cycling conditions were 95°C for 10 min for the initial denaturation, followed by 95°C for 15 s for denaturation and 55°C for 1 min for annealing and synthesis.

Table 1. Primer and probe sequences for *L. monocytogenes* (modified according to Rodríguez-Lazaro et al. (2004), *L. innocua*, *L. ivanovii* and *L. welshimeri*

Name	**sequence (5´ - 3´)**	**amplicon size**	**gene (accession number)**
L. monocytogenes forward	aat ctg tta gcg caa ctt ggt taa		
L. monocytogenes reverse	cac ctt tga tkg acg taa taa trc tgt t	146 bp	AY174681
L. monocytogenes probe	[b]caa cac cag cgc cas tac gga cg[d]		
L. innocua forward	ttg gcg tta gct tgc ttc tt		
L. innocua reverse	ggg agg cta ctg gag tcg tt	120 bp	AL596172.1
L. innocua probe	[a]aag gtt tta atg cgc gag tg[d]		
L ivanovii forward	atg cgt acg atg gaa gca ac		
L ivanovii reverse	gcc ttt ata gcg tgc gtt gt	117 bp	AY542872.1
L ivanovii probe	[a]ccg tgt tta tcc tag ctg gc[d]		
L welshimeri forward	aat ttg gtt ggc gta caa gg		
L welshimeri reverse	tcc atc act cgc aga ttt tg	120 bp	AJ293985.1
L welshimeri probe	[f]ttc aaa aag gtg ttt gca cg[d]		

bp: base pair, [a] FAM: 6-carboxyfluorescein, [b] HEX: 5´-Hexachloro-Fluorescein, [d] Dark-Quencher, [f] Cy5, grey markers: degenerated/wobble bases

3.4.6 Additional microbiological analyses

Altogether, 119 water samples were collected out of 39 controlled companies. From these, 111 irrigation and processing water samples contained more than 300 mL and underwent further testing on the amount of *E. coli*, enterococci, and *P. aeruginosa*. ISO 9308-2:2014-06 was used for *E. coli* (International Organization International Organizational for Standardization., 2014), ISO 7899-2:2000-11 for enterococci (International Organization International Organizational for Standardization., 2000), and ISO 16266:2008-05 for *P. aeruginosa* (International Organization International Organizational for Standardization., 2008).

3.5 Results

3.5.1 General survey information and microbiological self-monitoring

During the project, official controls were performed at 39 plants in the sector of FNAO production. Table 2 summarizes general survey information of the companies involved in this study, like product range, distribution area, water sources of irrigation and processing water, further processing steps, and the distribution of completed food products. Wells are the most used water source for irrigation water at the farm level. Producers of ready-to-eat (RTE) raw soft fruit and vegetables/fresh cut, exclusively, used public water supply for washing processed food. Washing, cutting, and delivering RTE food products were common in processing companies. Nonetheless, five primary producers delivered RTE vegetables as well. Furthermore, Table 3 reviews microbiological water self-monitoring. At the primary production level (n = 30), the usage of irrigation systems, drip, and overhead irrigation are included. Besides, 20% of primary producers reuse irrigation water. Five of them use decontamination strategies like thermal treatment of water prior to use or UV light application. One facility reused irrigation water without any retreatment.

Table 2. General survey information: Different aspects of primary (n = 30) and processing (n = 9) production information specified by the food business operators concerning the product range, the distribution area, the water source of irrigation and processing water, further processing steps and the distribution of completed food products

	Primary production % (n)	Processing % (n)
Product range		
Only FNAO	86.67 (26/30)	66.67 (6/9)
FNAO + FAO	n/a	33.33 (3/9)
Additional animal farming	13.33 (4/30)	n/a
Distribution area		
worldwide	0.00 (0/30)	11.11 (1/9)
Germany-wide	20.00 (6/30)	11.11 (1/9)
Bavaria	26.67 (8/30)	11.11 (1/9)
Local and regional	53.33 (16/30)	66.67 (6/9)
Water source		
Wells	53.33 (16/30)	n/a
Wells and rain water combined	13.33 (4/30)	n/a
Surface water	10.00 (3/30)	n/a
Public water supply	23.33 (7/30)	100.00 (9/9)
Processing food products		
No processing step	43.33 (13/30)	22.22 (2/9)
Only washing	40.00 (12/30)	0.00 (0/9)
Washing and cutting	16.67 (5/30)	77.78 (7/9)
Distribution food products		
Only distribution	60.00 (18/30)	11.11 (1/9)
Delivering packaged products	23.33 (7/30)	11.11 (1/9)
Delivering RTE	16.67 (5/30)	77.78 (7/9)

n/a = not applicable, RTE = ready-to-eat

Table 3. Microbiological self-monitoring of water examinations by company surveys on the spot. Primary production referred to 30 companies and processing production referred to nine facilities, which were controlled by on-spot inspections

	Primary production		Processing production
	Drip irrigation % (n)	Overhead irrigation % (n)	Processing water % (n)
Microbiological water examination			
No examination	26.67 (8/30)	36.67 (11/30)	33.33 (3/9)
Water examination	20.00 (6/30)	16.67 (5/30)	66.67 (6/9)
Total bacteria count	0.00 (0/6)	20.00 (1/5)	50.00 (3/6)
Colony count 22 °C and 36 °C	83.33 (5/6)	80.00 (4/5)	0.00 (0/6)
Coliform/ *Enterobacteriaceae*	83.33 (5/6)	100.00 (5/5)	83.33 (5/6)
E. coli	66.67 (4/6)	80.00 (4/5)	83.33 (5/6)
Salmonella spp.	16.67 (1/6)	20.00 (1/5)	0.00 (0/6)
Listeria spp.	0.00 (0/6)	0.00 (0/5)	0.00 (0/6)

3.5.2 Microbiological investigation of the samples

A total of 407 samples were analyzed, comprising of 229 swab samples, 59 food samples, and 119 samples of irrigation and processing water. Table 4 summarizes the detection of *Listeria* spp. in the tested samples. A total of 12.53% (51/407) of the samples were *Listeria* spp. positive. *L. seeligeri* was the most identified species, followed by *L. innocua*, *L. monocytogenes,* and *L. ivanovii*. *Listeria* spp. non-typeable were detected as genus *Listeria,* but no further species differentiation was clearly possible.

Table 4. Prevalence of *Listeria* spp. in different sample types

	Swabs % (n)	Food % (n)	Irrigation and processing water % (n)	Total % (n)
L. monocytogenes	2.62 (6/229)	0.00 (0/59)	0.84 (1/119)	1.72 (7/407)
L. innocua	2.18 (5/229)	3.39 (2/59)	0.84 (1/119)	1.97 (8/407)
L. ivanovii	0.87 (2/229)	1.69 (1/59)	1.68 (2/119)	1.23 (5/407)
L. seeligeri	5.24 (12/229)	3.39 (2/59)	4.20 (5/119)	4.67 (19/407)
Listeria spp. non-typeable	2.62 (6/229)	3.39 (2/59)	3.36 (4/119)	2.95 (12/407)
Total *Listeria* prevalence	13.54 (31/229)	11.86 (7/59)	10.92 (13/119)	12.53 (51/407)

When comparing the prevalence of *Listeria* between the sample types, swab samples (especially gullies and drains) showed the highest prevalence of *Listeria* spp. (31/229, 13.54%). Irrigation and processing water samples (13/119, 10.92%; drip and overhead irrigation, intermediate pieces of water pipelines, processing water) and food samples (7/59, 11.86%; lettuce, machine-cut salad, frozen mushrooms) showed lower prevalence. More than one type of *Listeria* spp. was found in twelve samples.

Six swab samples, which were positive with the pathogenic *L. monocytogenes,* extended from the accumulation of condensation water from a cooling unit in a storage room for ready-to-eat soft fruit to different gullies and drains at storage rooms and packaging areas at producers of vegetables and producers of ready-to-eat raw soft fruit and vegetables/fresh cut. Furthermore, one processing water for a salad washing system was tested *L. monocytogenes* positive. Seven *L. monocytogenes* isolates were detected in six different facilities, three at the primary production level, and four at processing plants.

3.5.3 Microbiological analysis of water samples

Figures 1 and 2 show the summarized results of *E. coli* and enterococci water analysis compared to *Listeria* spp. contamination. With increasing contamination level of *E. coli* and enterococci, the detection of *Listeria* spp. increases in irrigation water and processing water. Furthermore,

Figures 3 and 4 present the water analysis results for *P. aeruginosa* and *Listeria* spp. contamination in comparison. Figure 3 shows the increased detection of *Listeria* spp. with increasing *P. aeruginosa* contamination in irrigation water. Processing water was less contaminated with *P. aeruginosa*, nevertheless *Listeria* spp. contamination was detected.

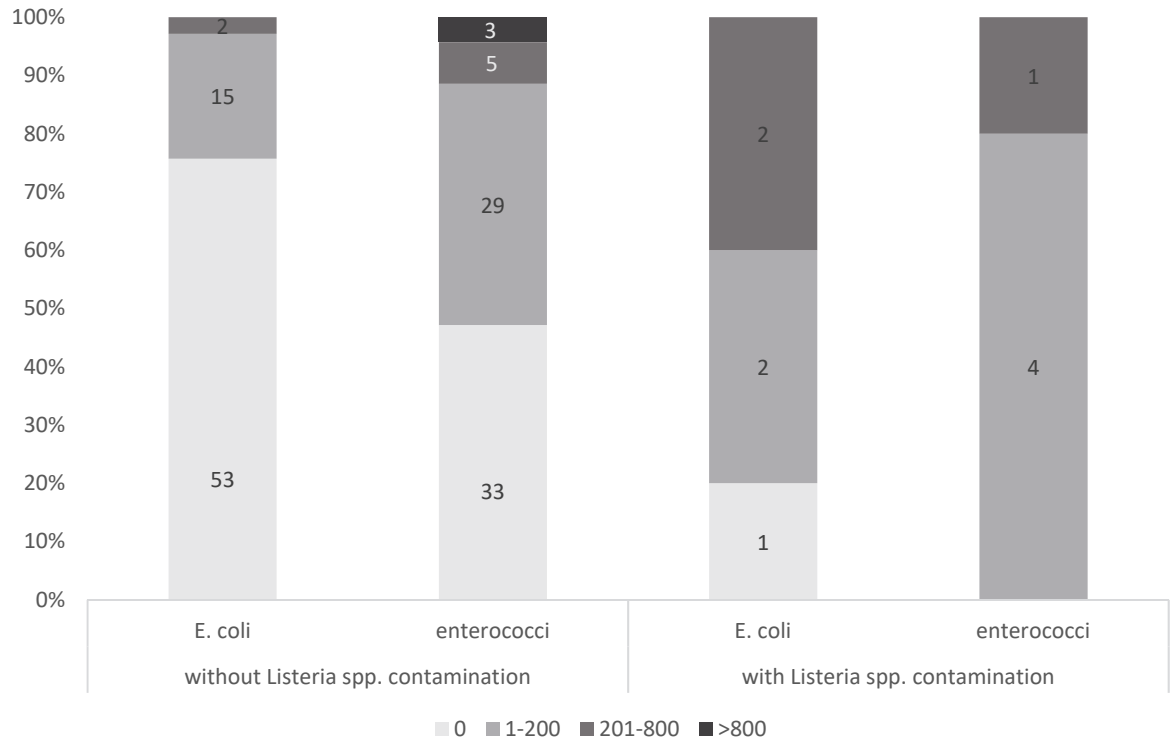

Figure 1. Irrigation water analysis of fecal indicator microorganisms (*E. coli* and enterococci) compared to *Listeria* spp. contamination.

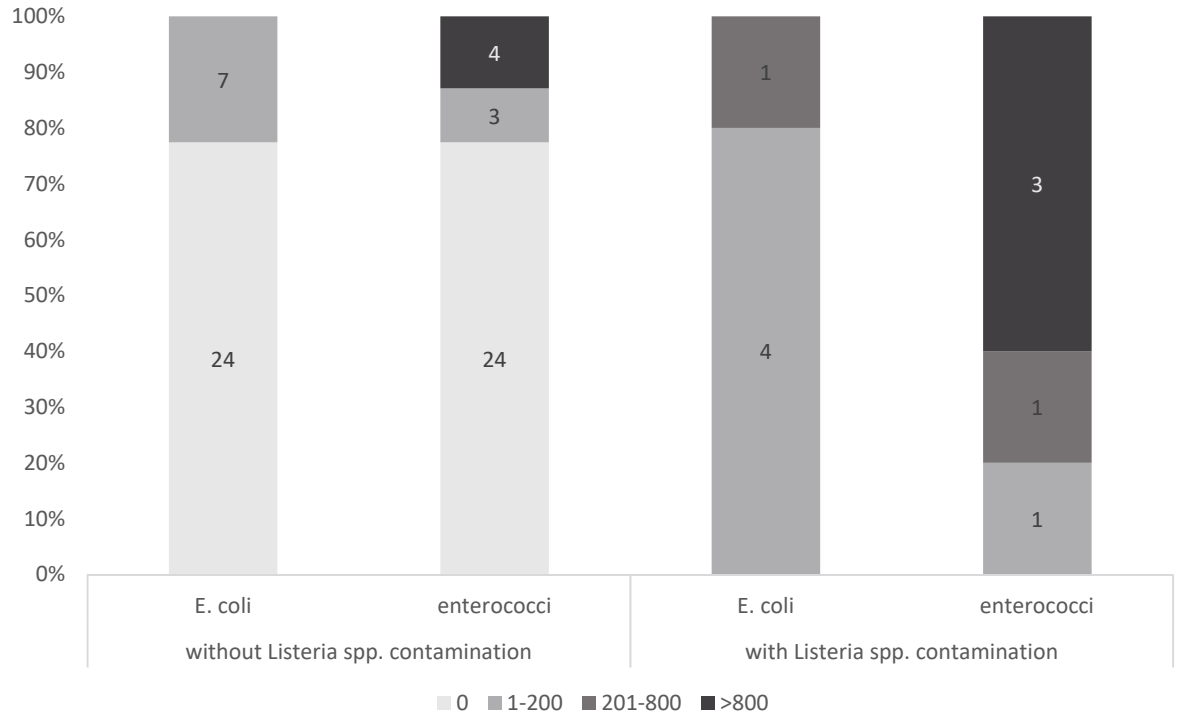

Figure 2. Processing water analysis of fecal indicator microorganisms (*E. coli* and enterococci) compared to *Listeria* spp. contamination.

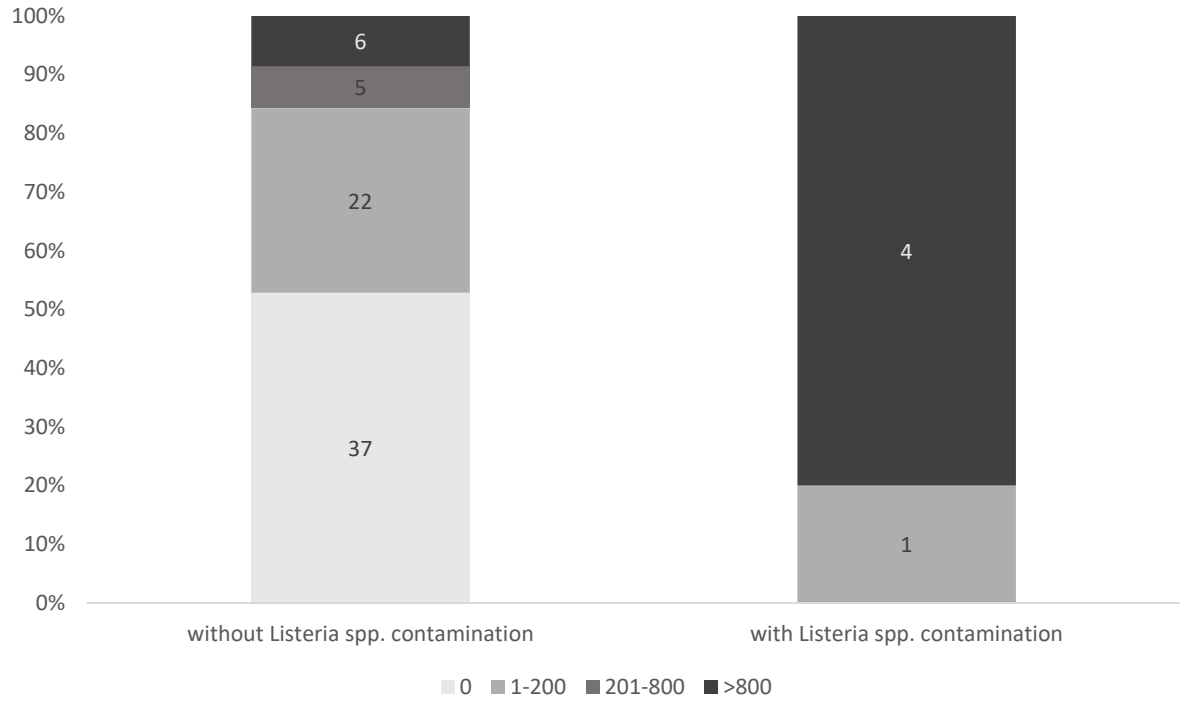

Figure 3. Irrigation water analysis of *P. aeruginosa* compared to *Listeria* spp. contamination.

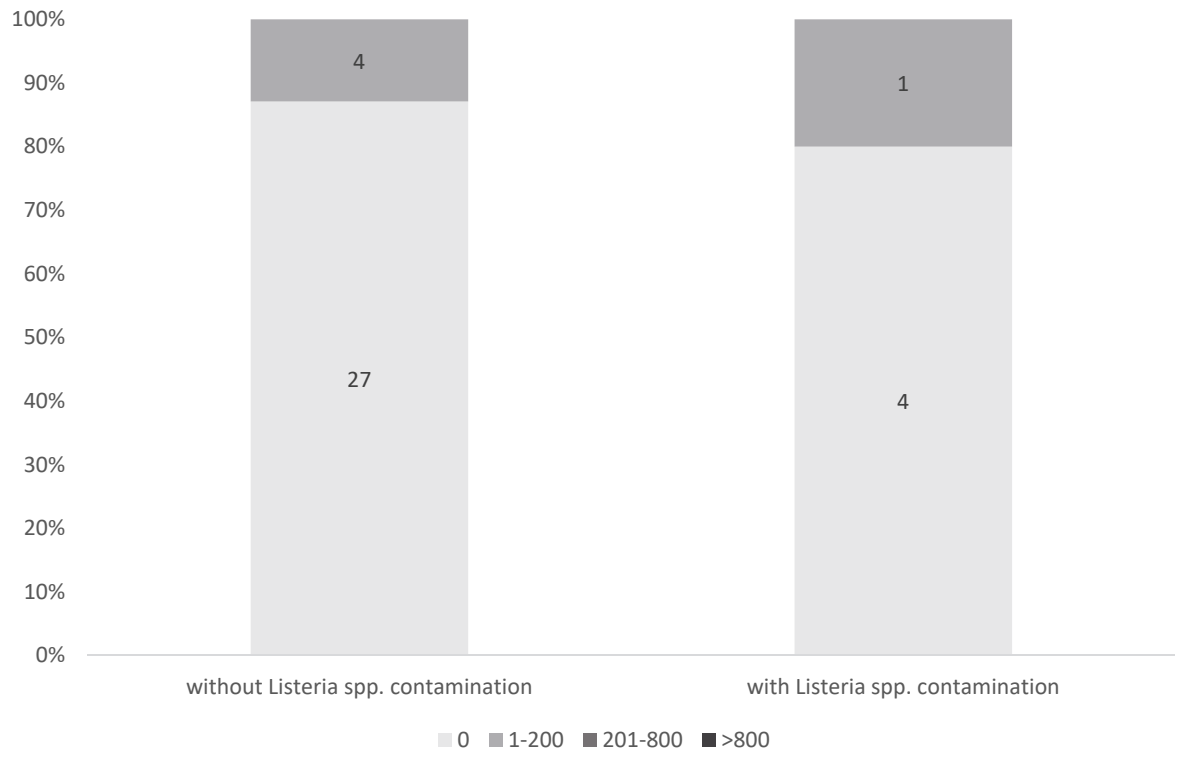

Figure 4. Processing water analysis of *P. aeruginosa* compared to *Listeria* spp. contamination.

In irrigation water samples, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *Listeria* spp. non-typeable were detected. In processing water, *L. monocytogenes*, *L. seeligeri,* and *Listeria* spp. non-typeable were analyzed. Five irrigation water samples and five processing water samples were contaminated with *Listeria* spp. Furthermore, seven *Listeria* spp. were detected in five samples of irrigation water and processing water samples. Three water samples showed more than one detected *Listeria* spp. isolate. All data on water sample contamination with *E. coli*, enterococci, and *P. aeruginosa* and *Listeria* spp. are shown in the supplemental material.

Table 5 and Table 6 specify the results of the microbiological analysis of water samples (irrigation water and processing water) with *E. coli*, enterococci, and *P. aeruginosa* in relation to the verification of *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri,* and *Listeria* spp. non-typeable. A total of 111 out of 119 water samples were analyzed for *E. coli*, enterococci, and *P. aeruginosa* because of containing more than 300 mL. The 111 samples are divided into 75 samples of irrigation water and 36 samples of processing water. Furthermore, the detected *Listeria* spp. isolates refer to the *Listeria* spp. prevalence of 51 (Table 4).

Table 5. Water analysis (irrigation water (75/111), processing water (36/111)) of fecal indicator microorganisms (*E. coli* and enterococci) in relation to *Listeria* spp. (51/407)

	Irrigation water % (n)		*Listeria* spp. % (n)					
CFU/100 mL	*E. coli*	enterococci	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*Listeria* spp. non-typeable	Total *Listeria* spp.
0	72.00 (54/75)	44.00 (33/75)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
1 - 200	22.67 (17/75)	44.00 (33/75)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)	1.96 (1/51)
201 - 800	5.33 (4/75)	8.00 (6/75)	0.00 (0/51)	3.92 (2/51)	1.96 (1/51)	3.92 (2/51)	1.96 (1/51)	11.76 (6/51)
> 800	0.00 (0/75)	4.00 (3/75)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
Total irrigation water	100.00 (75/75)	100.00 (75/75)	0.00 (0/51)	3.92 (2/51)	1.96 (1/51)	3.92 (2/51)	3.92 (2/51)	13.73 (7/51)
	Processing water % (n)		*Listeria* spp. % (n)					
CFU/100 mL	*E. coli*	enterococci	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*Listeria* spp. non-typeable	Total *Listeria* spp.
0	66.67 (24/36)	66.67 (24/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
1 - 200	30.56 (11/36)	11.11 (4/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)	1.96 (1/51)
201 - 800	2.78 (1/36)	2.78 (1/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)	1.96 (1/51)
> 800	0.00 (0/36)	19.44 (7/36)	1.96 (1/51)	0.00 (0/51)	0.00 (0/51)	5.88 (3/51)	1.96 (1/51)	9.80 (5/51)
Total processing water	100.00 (36/36)	100.00 (36/36)	1.96 (1/51)	0.00 (0/51)	0.00 (0/51)	5.88 (3/51)	5.88 (3/51)	13.73 (7/51)

Table 6. Water analysis (irrigation water (75/111), processing water (36/111)) of *P. aeruginosa* in relation to *Listeria* spp. (51/407)

	Irrigation water % (n)	*Listeria* spp. % (n)					
CFU/100 mL	*P. aeruginosa*	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*Listeria* spp. non-typeable	Total *Listeria* spp.
0	49.33 (37/75)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
1 - 200	30.67 (23/75)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)
201 - 800	6.67 (5/75)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
> 800	13.33 (10/75)	0.00 (0/51)	3.92 (2/51)	0.00 (0/51)	3.92 (2/51)	3.92 (2/51)	11.76 (6/51)
Total irrigation water	100 (75/75)	0.00 (0/51)	3.92 (2/51)	1.96 (1/51)	3.92 (2/51)	3.92 (2/51)	13.73 (7/51)
	Processing water % (n)	*Listeria* spp. % (n)					
CFU/100 mL	*P. aeruginosa*	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*Listeria* spp. non-typeable	Total *Listeria* spp.
0	86.11 (31/36)	1.96 (1/51)	0.00 (0/51)	0.00 (0/51)	5.88 (3/51)	3.92 (2/51)	11.76 (6/51)
1 - 200	13.89 (5/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	1.96 (1/51)	1.96 (1/51)
201 - 800	0.00 (0/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
> 800	0.00 (0/36)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)	0.00 (0/51)
Total processing water	100 (36/36)	1.96 (1/51)	0.00 (0/51)	0.00 (0/51)	5.88 (3/51)	5.88 (3/51)	13.73 (7/51)

3.6 Discussion

With regard to FNAO, *L. monocytogenes* has been responsible for a number of outbreaks (European Food Safety Authority et al., 2018, Garner and Kathariou, 2016, Koutsoumanis et al., 2020, McCollum et al., 2013). Thus, significant attention has to be paid to *L. monocytogenes* in FNAO. Hence, the aim of this work was to (i) detect *Listeria* spp. and *L. monocytogenes* at different areas of FNAO production: at primary production and processing facilities of ready-to-eat soft fruit and vegetables/fresh cut and (ii) to identify potential hazards for the FNAO food chain-like irrigation and processing water.

Overall, *Listeria* spp. were detected in 12.53% of samples from 39 FNAO companies. The majority of *Listeria* spp. isolates were detected in environmental samples, including swabs, irrigation, and processing water. In our study, 1.72% of the samples were *L. monocytogenes* positive. The common isolation area of *L. monocytogenes* was the facility surroundings, especially in different gullies and drains. Weller et al. support our findings that the environment is a common reservoir for *L. monocytogenes* in processing plants (Weller et al., 2015). These data highlight that environmental monitoring is one of the most important control measures for *Listeria* in food-producing and processing companies (Zhu et al., 2012).

To avoid contamination of soft fruit, vegetables, or ready-to-eat fresh produce, the quality of irrigation and processing water plays a decisive role (Banach and van der Fels-Klerx, 2020, Uyttendaele et al., 2015). In general, 10.92% of irrigation and processing water samples were positive for *Listeria* spp., but *Listeria* spp. were not detected when wells (n=18) were the primary water source. That suggests that water contamination occurs during water transport in different water pipelines. Furthermore, one processing water sample of a lettuce washing system was tested positive to *L. monocytogenes* at the primary production level. *L. monocytogenes* is able to colonize different environmental niches at processing facilities and as a result, irrigation and processing water can pose a risk for FNAO contamination when passing water pipelines (Gião and Keevil, 2014). This underlines our results that the primary water sources (wells, public water supply) were negative for *Listeria* spp., but water samples at different pipeline sections were tested positive. Some studies show that different water sources represent different risks of contamination (Uyttendaele et al., 2015, van der Linden et al., 2014). In addition, the irrigation system has different effects on *L. monocytogenes* detection. Banach and van der Fels-Klerx confirmed that irrigation water can become a serious hazard for fresh produce (Banach and van der Fels-Klerx, 2020). In this study, producers of soft fruit use drip irrigation systems in particular. This procedure mitigates microbiological contamination because of avoiding direct water contact to the edible portion of

ready-to-eat soft fruit and vegetables (Banach and van der Fels-Klerx, 2020, Gil et al., 2013, Qadir, 2008). On the other hand, sprinkler irrigation was common among the producers of vegetables. Our study showed no relation between positive microbiological findings and the usage of specific irrigation systems. Using overhead spraying should not be dismissed at all but delaying harvesting after irrigation is suggested to avoid contamination with *L. monocytogenes* (Banach and van der Fels-Klerx, 2020, Weller et al., 2015). The awareness of probable cross-contamination of irrigation and processing water was partially anchored by growers and processors.

Within our study, water sample analyses were examined on *E. coli*, enterococci, and *P. aeruginosa* as well as besides *Listeria* spp. Table 5, Figures 1 and 2 show the results of water contamination according to the prevalence of fecal contaminants (*E. coli* and enterococci) and *Listeria* spp. Statistical testing was not performed due to low positive samples. More research is needed when following the approach that contaminated irrigation or processing water with fecal indicator microorganisms, like *E. coli*, might indicate a contamination with *L. monocytogenes*.

The food samples in our study showed the presence of *Listeria* spp., like *L. innocua*, *L. ivanovii*, and *L. seeligeri*. *L. monocytogenes* was not detected in food samples, although it was verified in the companies' environment. The detection of different *Listeria* spp. within the same company suggests that *Listeria* spp. may become an issue at FNAO primary production and processing companies, if hygienic environmental handling is neglected. For instance, Pennone et al. described cross-contamination between production series at a mushroom production company and because of recontamination after several disinfection steps, the good hygienic practice has to be adopted (Pennone et al., 2020). In conclusion, appropriate monitoring is recommended to decrease contamination and cross-contamination during processing procedures (Mazaheri et al., 2021).

While processing ready-to-eat fresh produce, 33.33% of the processing facilities handled food of animal origin (FAO) as well (Table 2). FAO contaminated with *L. monocytogenes* can pose a source of cross-contamination to FNAO plants. D'Arrigo et al. demonstrated the prevalence of *L. monocytogenes* in ham-processing facilities (D'Arrigo et al., 2020). The threat of cross-contamination and the hazard of introducing *Listeria* strains to FNAO plants increases by processing meat products in ready-to-eat raw fruits and vegetable companies. In our study, *L. monocytogenes* was found in a drain where ready-to-eat salad with FAO components was processed, highlighting the need to comply with good manufacturing and hygienic practice, especially when producers of FNAO process FAO at the same premises.

Our study shows that *Listeria* spp. and *L. monocytogenes* are regularly present at FNAO primary production and processing level. Water sources, water quality, irrigation regime, cultivation, hygienic handling, and maintenance protocols are highly important to reduce the potential exposure to ready-to-eat soft fruit and vegetables (Banach and van der Fels-Klerx, 2020).

3.7 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

3.8 Acknowledgment

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Chapter 4: Genetic Characterization of Listeria from Food of Non-Animal Origin Products and Producing and Processing Companies in Bavaria, Germany

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4.1 Summary Chapter 4

In the last few years, WGS became more and more important in characterizing and connecting different isolates of *L. monocytogenes* to the wide range of food production (Moura et al. 2016).

For this reason, we conducted a study that investigated the following topics:

- Characterization of 64 *Listeria* spp isolates, including the pathogenic *L. monocytogenes*, to retrieve data on the serotype, genotypic and phenotypic AMR and virulence markers in FNAO products and FNAO primary production and processing companies.
- Distribution and genetic relation of *Listeria* spp. and *L. monocytogenes* in FNAO products and producing and processing facilities.

The study showed that the genetic differences of *Listeria* spp. and *L. monocytogenes* suggested external introduction and plant-specific distribution in FNAO producing and processing plants. Some detected *L. monocytogenes* isolates showed virulence markers and antibiotic resistances. *L. monocytogenes* in relation to FNAO should be focused increasingly.

4.2 Abstract

Reported cases of listeriosis from food of non-animal origin (FNAO) are increasing. In order to assess the risk of exposure to *Listeria monocytogenes* from FNAO, the genetic characterization of the pathogen in FNAO products and in primary production and processing plants needs to be investigated. For this, 123 samples of fresh and frozen soft fruit and 407 samples of 39 plants in Bavaria, Germany that produce and process FNAO were investigated for *Listeria* contamination. As a result, 64 *Listeria* spp. isolates were detected using ISO 11290-1:2017. Environmental swabs and water and food samples were investigated. *L. seeligeri* (36/64, 56.25%) was the most frequently identified species, followed by *L. monocytogenes* (8/64, 12.50%), *L. innocua* (8/64, 12.50%), *L. ivanovii* (6/64, 9.38%), *L. newyorkensis* (5/64, 7.81%), and *L. grayi* (1/64, 1.56%). Those isolates were subsequently sequenced by whole-genome sequencing and subjected to pangenome analysis to retrieve data on the genotype, serotype, antimicrobial resistance (AMR), and virulence markers. Eight out of sixty-four *Listeria* spp. isolates were identified as *L. monocytogenes*. The serogroup analysis detected that 62.5% of the *L. monocytogenes* isolates belonged to serogroup IIa (1/2a and 3a) and 37.5% to serogroup IVb (4b, 4d, and 4e). Furthermore, the MLST (multilocus sequence typing) analysis of the eight detected *L. monocytogenes* isolates identified seven different sequence types (STs) and clonal complexes (CCs), i.e., ST1/CC1, ST2/CC2, ST6/CC6, ST7/CC7, ST21/CC21, ST504/CC475, and ST1413/CC739. The core genome MLST analysis also showed high allelic differences and suggests plant-specific isolates. Regarding the AMR, we detected phenotypic resistance against benzylpenicillin, fosfomycin, and moxifloxacin in all eight *L. monocytogenes* isolates. Moreover, virulence factors, such as *prfA*, *hly*, *plcA*, *plcB*, *hpt*, *actA*, *inlA*, *inlB*, and *mpl*, were identified in pathogenic and nonpathogenic *Listeria* species. The significance of *L. monocytogenes* in FNAO is growing and should receive increasing levels of attention.

4.3 Introduction

The public health sector has had to increasingly focus on foodborne illnesses over the last decade [1]. Microbiologically contaminated food, in particular, plays a decisive role. Next to *Escherichia coli*, *Salmonella* spp., and *Campylobacter*, *Listeria monocytogenes* has caused severe foodborne diseases [2]. *L. monocytogenes* records one of the highest mortality rates in humans (20–30%) with regard to invasive listeriosis in vulnerable population groups [3]. Food of animal origin (FAO), such as meat, milk, and fish products, is commonly known as a vehicle for *L. monocytogenes* [4], but the listeriosis cases associated with food of non-animal origin (FNAO) have been increasing in recent years [5,6,7,8,9,10,11]. Diced celery, packaged salads, stone fruit, sprouts, soy products, whole apples, cantaloupes, frozen vegetables, and, in particular, frozen corn were found to have been contaminated with *L. monocytogenes* inducing listeriosis [5,6,7,8,9,10,11,12]. One of the largest and first widespread plant-based listeriosis outbreaks in the United States was caused by cantaloupes in 2011. During this outbreak, 147 cases including 143 hospitalizations and 33 deaths were reported in 28 different states [5,13]. The melons were most likely contaminated by the company's environment [13]. This underlines the risk associated with FNAO, especially ready-to-eat (RTE) products that do not undergo further heating or processing steps. Furthermore, frozen corn and probably other frozen vegetables (vegetables mixes, green beans, and spinach) were responsible for 47 human listeriosis cases and a 19% fatality rate in the European Union up to June 2018 [8]. As with the *L. monocytogenes* isolates found in cantaloupes, *L. monocytogenes* isolates from frozen corn and vegetables have also been found in the facilities' environments [8,13]. This fact accentuates the need for appropriate disinfection and cleaning interventions in FNAO-producing companies. Furthermore, the annual European Union One Health Zoonoses Report showed *L. monocytogenes* in fruits and vegetables as a more pervasive issue [14,15]. From 2017 to 2021, the sampling units and, therefore, the prevalence rate increased as well [15]. Therefore, the topic of FNAO and *L. monocytogenes* will continue to gain importance in the sector.

In order to assess the risk of exposure to *L. monocytogenes* from FNAO, the occurrence and further characteristics, such as genotype and serotype detection, antimicrobial resistances (AMR), and virulence markers of the pathogen in FNAO products and primary production and processing plants, needs to be investigated. Up to now, only a few studies examined the presence and further characteristics of *L. monocytogenes* in different areas of FNAO production and processing companies [16,17,18]. In addition, the more isolates from agricultural environments, FNAO fresh produce, and RTE food and processing environments that are collected and characterized, the greater the

understanding of foodborne-associated outbreaks related to *L. monocytogenes* [19].

The linkage between human isolates and food as the transmission vehicle was revolutionized through the development of next-generation sequencing technologies [20]. Over the last few years, whole-genome sequencing (WGS) has become more important in characterizing and connecting different isolates of *L. monocytogenes* in a wide range of food production [19,21,22]. To obtain more knowledge on the relationship of *Listeria* spp., especially *L. monocytogenes*, isolates, and FNAO, 64 *Listeria* spp. isolates were detected from FNAO products and the companies that produce and process these products. The isolates were sequenced by WGS and subjected to pangenome analysis.

4.4 Materials and Methods

The sampling scheme, number of samples, and *Listeria* spp. detection methods are already described by Wartha et al. [23]. In addition to the 407 samples from 39 primary production and processing companies already described [23], 123 samples of fresh fruit and frozen berries from supermarkets were collected and analyzed. The study of Wartha et al. [23] was furthermore extended with WGS results of 64 detected *Listeria* spp. and *L. monocytogenes* isolates from FNAO products from supermarkets and FNAO-producing and -processing plants.

4.4.1 Origin of Isolates from Supermarkets

In this study, 123 samples of fresh soft fruit and frozen berries were collected randomly from supermarkets across the south of Bavaria, Germany. The sampling period was between January 2021 and April 2021 and the samples came from different countries (including Canada, Chile, Greece, Mexico, Morocco, Peru, Poland, Portugal, Serbia, Spain, The Netherlands, and the USA). Blueberries, raspberries, strawberries, and blackberries were included in the 63 fresh soft fruit samples. Furthermore, 60 samples of frozen raspberries, blueberries, blackberries, strawberries, currants, cherries, and cranberries (partially mixed together) were included among the frozen berries category. The frozen samples were prepared in three different ways before examination; the first group was thawed for 3 h at room temperature, the second group was defrosted for 16 h in the refrigerator, and the third group was tested frozen. The samples were analyzed using methods described in ISO 11290-1:2017 [24].

4.4.2 Origin of Isolates from FNAO-Producing and -Processing Plants

During the on-the-spot verification visits, 407 samples were taken from 39 FNAO primary producing and processing companies in Bavaria, Germany from July 2020 until June 2021. Thirty plants were categorized as primary production (i.e., farm and primary production level) and nine companies as processing companies (i.e., processing level). The 407 samples included 229 swab samples (food contact material and environment), 59 food samples (soft fruit, vegetables, RTE vegetables, and other food samples), and 119 irrigation and processing water samples [23]. For the detection of *Listeria* spp. and *L. monocytogenes*, ISO 11290–1:2017 [24] was used as described [23].

4.4.3 WGS

For sequencing, the DNA was extracted using the Invitrogen PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA), which is suitable for Gram-positive bacteria, with slight modifications. Particularly, blood agar plates (Oxoid Deutschland GmbH, Wesel, Germany) were incubated at 37 °C for 24 ± 2 h. One third of the bacterial colony overgrown on the blood agar plate was diluted in 200 µL 1× phosphate-buffered saline (PBS; Biochrom AG, Berlin, Germany). After the first centrifugation step (20,000× g for 5 min), the supernatant was removed. A second centrifugation step (20,000× g again briefly) was performed to remove all of the supernatant. The pellet was used for further DNA extraction steps. Furthermore, instead of the PureLink Genomic Elution Buffer, the EDTA free elution buffer from the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) was used. After purification, the DNA purity was measured with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). The DNA concentration was determined either with a Quantus Fluorometer (Promega Corporation, Madison, WI, USA) with the QuantiFluor ONE dsDNA System kit (Promega Corporation) or a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) with the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific). Lambda DNA (Promega Corporation) was used as the standard. Before preparing the dual-indexed paired-end libraries, all samples were diluted to 24 ng [25]. In preparing the libraries, Illumina DNA Prep (Illumina Inc., San Diego, CA, USA) was used with a few modifications. Instead of Sample Purification Beads (SPB; Illumina Inc.), Agencourt AMPure XP Beads (Beckman Coulter GmbH, Krefeld, Germany) were used during the final clean-up step. After the concentration measurements with the Quantus Fluorometer and the associated QuantiFluor ONE dsDNA System kit (Promega Corporation), the libraries' quality and fragment size distribution were checked on a 5200 Advanced Analytical Fragment Analyzer (Agilent Technologies Inc., Santa Clara, CA, USA) using the HS NGS High Sensitivity 474 kit (Agilent

Technologies Inc.) as instructed by the manufacturer. The Fragment Analyzer data were edited using the ProSize Data analysis software, version 4.0.2.7 (Agilent Technologies Inc.). Library normalization was performed using the Biomek i7 Automated Workstation (Beckman Coulter GmbH). After pooling the libraries, the denaturation and dilution steps were performed following the NextSeq System Denature and Dilute Libraries Guide Protocol A (Illumina Inc.), as the manual instructions required. The denaturated library solution was diluted to 1.3 pM. The sequencing was performed on a NextSeq 550 using a Mid Output Reagent Cartridge v2 for 300 cycles (Illumina Inc.). The read length was 2 × 149 bp, and PhiX (1%, Illumina Inc.) was used as the sequencing control.

The raw sequencing data were uploaded to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/sra accessed on 28 February 2023) under the project number PRJNA935401.

4.4.4 Data Analysis

The AQUAMIS pipeline v1.2.0 (Bundesinstitut für Risikobewertung, BfR, Berlin, Germany) was used for the trimming of the sequencing reads, read assembly, and overall quality analysis [26]. The generated assemblies were annotated using Prokka v1.14.6 [27]. Subsequently, the pangenome was calculated with PIRATE v1.0.3 [28]. The dendrogram was generated using PIRATE v.1.0.3 with the default parameters based on the core-genome alignments [29]. Metadata such as the holdings of origin were added with iTOL [30]. Moreover, the assemblies were screened for the presence of virulence and AMR genes with abricate v1.0.1 [31], utilizing the data from the Comprehensive Antibiotic Resistance Database (CARD) [32] and the virulence factor database (VFDB) [33]. For the serogroup detection, a serogroup scheme (v1.0) of five loci [34] was used, and for the MLST (multilocus sequence typing) a seven loci MLST scheme (v1.0) [35] was used, both integrated in the Ridom SeqSphere+ Software (v 8.3.1) [36]. The used scheme for serogrouping allowed the classification into the following serogroups: IIa (1/2a and 3a), IIb (1/2b), IIc (1/2c and 3c), and IVb (4b, 4d and 4e) [37]. The core genome MLST (cgMLST) was performed for the *L. monoyctogenes* isolates using the 1701 loci scheme [38] (v 2.1) integrated in the Ridom SeqSphere+ Software (v 8.3.1) [37]. The cgMLST minimum spanning tree (MST) was generated based on the core genome targets integrated in the Ridom SeqSphere+ Software (v 8.3.1) [36].

4.4.5 Phenotypic AMR Testing

For the phenotypic characterization, the isolates were incubated at 37 °C for 24 ± 2 h on blood agar (Oxoid Deutschland GmbH). For the AMR testing, the BD Phoenix System (Becton Dickinson, Franklin Lakes, NJ, USA) with the PMIC/ID 88 panel was used, according to the manufacturer's guidance for Gram-positive bacteria. The phenotypic resistance of 22 *Listeria* spp. isolates against benzylpenicillin, erythromycin, trimethoprim-sulfamethoxazole, gentamicin, tetracycline, fosfomycin, ciprofloxacin, and moxifloxacin was tested according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The EUCAST breakpoints for *L. monocytogenes* were used for erythromycin and trimethoprim–sulfamethoxazole [39], whereas, for the other tested antibiotics (i.e., benzylpenicillin, gentamicin, tetracycline, fosfomycin, ciprofloxacin, and moxifloxacin), the EUCAST *Staphylococcus* spp. breakpoints were applied as a substitute, as described in previous studies [40,41].

4.5 Results

In summary, this study examined 64 *Listeria* spp. isolates in total (63 isolates from 39 FNAO-producing and -processing plants and one isolate from 123 FNAO samples from supermarkets). *L. seeligeri* (36/64, 56.25%) was the most frequently identified species, followed by *L. monocytogenes* (8/64, 12.50%), *L. innocua* (8/64, 12.50%), *L. ivanovii* (6/64, 9.38%), *L. newyorkensis* (5/64, 7.81%), and *L. grayi* (1/64, 1.56%).

4.5.1 Occurrence of *Listeria* spp. in FNAO from Supermarkets

A total of 123 samples from fresh and frozen soft fruit taken from randomly chosen supermarkets in the south of Bavaria were investigated. *L. grayi* (1/123, 0.81%) was detected in a frozen blackberry package. All of the other 122 samples showed no presence of *Listeria* spp., including *L. monocytogenes*. Accompanying bacterial and fungal flora, such as moulds, yeasts, enterococci, and bacilli, were noticed. Furthermore, the analytical approach of preparing the frozen soft fruit samples in three different ways before examination (frozen, refrigerator, or room temperature) showed no differences in the results and revealed no impact on detecting *Listeria* spp. or *L. monocytogenes* in this study.

4.5.2 Occurrence of *Listeria* spp. in FNAO-Producing and Processing Plants

Overall, 407 samples from 39 FNAO primary production and processing facilities were investigated, as described previously [23]. Of the companies visited, 48.72% (19/39) of the samples tested positive for *Listeria* spp. In 42.11% (8/19) of the samples from these facilities, only one *Listeria* spp. was detected. In comparison, more than one *Listeria* spp. was identified in 57.89% (11/19) of the samples from FNAO plants. Eight *L. monocytogenes* isolates were identified in six environmental swabs and two processing water samples. No food sample tested positive for *L. monocytogenes*.

4.5.3 Genetic Relation of 64 *Listeria* Isolates Sequenced by WGS

Overall, 64 *Listeria* isolates were sequenced by WGS and subjected to pangenome analysis. The coverage depth of the sequencing ranged from a minimum of 30.6-fold to a maximum coverage of 107-fold. In the 64 assemblies, a total of 8475 gene families were found, with 1588 of these presenting in at least 95% of the assemblies.

The different isolates were grouped according to their species. However, the isolates within the same species and from the same company differed from each other. For example, the *L. seeligeri* isolates (SWB9, SWA9, and SWC4) were isolated from processing water for a salad washing system, and SWC4 differed from SWB9 and SWA9. Moreover, the *L. ivanovii* isolates (SWC3 and SWC7) from a drain differed from each other, whereas the isolate SWD3 from a delight mixed salad from company A was more similar to the isolate SWC7. The dendrogram, which depicts the genetic relationships and phylogeny among the 64 *Listeria* spp. isolates, is provided in the Supplemental Materials (Figure S1).

4.5.4 Serogroup Determination of *L. monocytogenes*

Eight isolates of *L. monocytogenes* were detected in six various plants, with four at the processing level and four at the production level. Table 1 shows that the *L. monocytogenes* isolates belonged to lineages I and II, with 62.5% (5/8) of the isolates belonging to serogroup IIa (comprising serovars 1/2a and 3a) and 37.5% (3/8) to serogroup IVb (serovars 4b, 4d, and 4e).

Table 1. Characterization of the *L. monocytogenes* isolates from FNAO plants.

Sample ID	Plant (PP or PC)	Sample	Serogroup and Serotype (In Silico)	Sequence Type (ST) (MLST)	Clonal Complex (CC) (MLST)	Complex Type (CT) (cgMLST)
SWH3	K (PC)	Drain—delivery raw goods (S)	IVb (4b, 4d, and 4e)	ST1	CC1	16888
SWH8	I (PP)	Drain—cooling area lettuce (S)	IVb (4b, 4d, and 4e)	ST2	CC2	16889
SWC6	M (PP)	Condensate ponding of a cooling unit (S)	IVb (4b, 4d, and 4e)	ST6	CC6	7504
SWA5	N (PC)	Drain—frozen food packaging (S)	IIa (1/2a and 3a)	ST7	CC7	16884
SWF3	G (PC)	Drain—packaging area lettuce (S)	IIa (1/2a and 3a)	ST21	CC21	16887
SWD4	I (PP)	Processing water for a salad washing system (PW)	IIa (1/2a and 3a)	ST504	CC475	16886
SWH4	I (PP)	Processing water for a salad washing system (PW)	IIa (1/2a and 3a)	ST504	CC475	16886
SWC5	E (PC)	Drain—pre-washing area (S)	IIa (1/2a and 3a)	ST1413	CC739	16885

L. monocytogenes isolates were detected in six different plants (N, E, M, G, K, and I). The sample types were swabs (S) and processing water (PW). Four *L. monocytogenes* isolates were found at processing companies (PCs, n = 9) and four *L. monocytogenes* isolates at primary production plants (PPs, n = 30). The isolates SWD4 and SWH4 were from the same sample.

4.5.5 MLST Analysis of *L. monocytogenes*

Seven different sequence types (STs) and clonal complexes (CCs) were identified, namely, ST1/CC1, ST2/CC2, ST6/CC6, ST7/CC7, ST21/CC21, ST504/CC457, and ST1413/CC739 (Table 1). The isolates SWD4 and SWH4 (a single processing water sample for a salad washing system) showed the same ST and CC combination.

4.5.6 cgMLST Analysis of *L. monocytogenes*

Figure 1 shows the cgMLST MST for the eight detected *L. monocytogenes* isolates. With the exception of the two isolates SWD4 and SWH4, the *L. monocytogenes* isolates differed by 1058 to 1648 alleles. Moreover, seven different complex types (CTs) were identified (Table 1). The isolates SWH8 (CT16889), SWD4 (CT16886), and SWH4 (CT16886) originated from the same primary production plant, but isolate SWH8 showed a different CT compared to the isolates SWD4 and SWH4. However, SWD4 and SWH4 were isolated from the same sample.

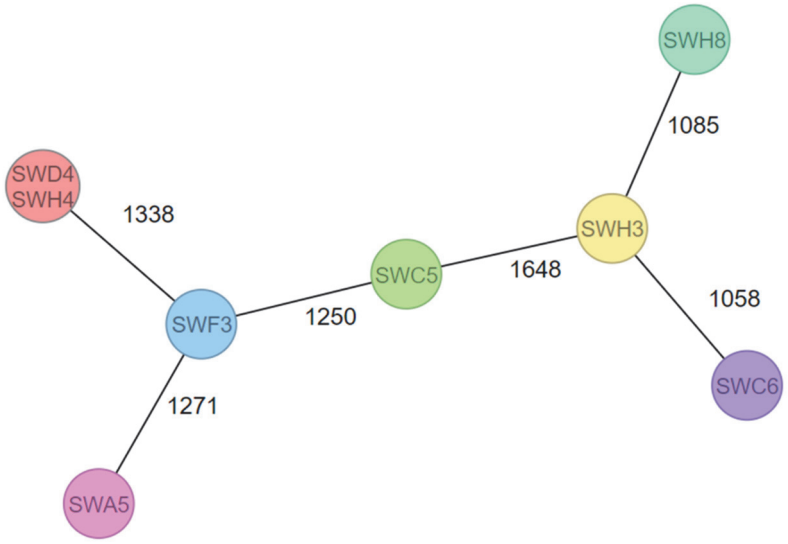

Figure 1. cgMLST MST of eight *L. monocytogenes* isolates from drains (SWH8, SWC6, SWH3, SWC5, SWF3, and SWA5) and from processing water for a salad-washing system (SWD4 and SWH4). The allele distances are indicated. Identical isolates are grouped within a common circle.

4.5.7 Prevalence of Genetic and Phenotypic AMRs in *Listeria* spp.

Sixty-four created assemblies were used for further investigations, namely, AMR and virulence gene analysis. In 17 isolates, five AMR genes (antibiotic class) were detected, namely, *lin* (lincomycin), *norB* (fluoroquinolone),

fosX (fosfomycin), *tetM* (tetracycline), and *ANT(6) - Ia* (aminoglycosides). Additionally, *mprF*, a gene encoding an integral membrane protein with resistance to cationic peptides that disrupt the cell membrane, including defensins, was detected [33]. The presence of the AMR genes is shown in Table 2.

Table 2. AMR genes and phenotypically detected resistances of 17 *Listeria* isolates.

No.	Isolate	*Listeria* spp.	Sample	Resistance Gene(s)	Resistance	Phenotypical Resistance
1	SWA5	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin, norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, MOX
2	SWC5	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, CIP, MOX
3	SWC6	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, GEN, FOS, MOX
4	SWH8	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, CIP, MOX
5	SWH3	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, CIP, MOX
6	SWF3	*L. monocytogenes*	Swab	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, CIP, MOX
7	SWD4	*L. monocytogenes*	Water	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, MOX
8	SWH4	*L. monocytogenes*	Water	*fosX*, *mprF*, *lin*, *norB*	Fosfomycin, cationic peptide, lincomycin, fluoroquinolones	PEN, FOS, CIP, MOX
9	SWA11	*L. innocua*	Swab	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
10	SWB4	*L. innocua*	Swab	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
11	SWB11	*L. innocua*	Swab	*norB*	Fluoroquinolones	PEN, Trim-sulfa, FOS, CIP, MOX

Table 2. Continued.

No.	Isolate	*Listeria* spp.	Sample	Resistance Gene(s)	Resistance	Phenotypical Resistance
12	SWA1	*L. innocua*	Swab	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
13	SWF1	*L. innocua*	Swab	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
14	SWF5	*L. innocua*	Food	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
15	SWG7	*L. innocua*	Food	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX
16	SWH6	*L. innocua*	Swab	*norB*, *tetM*, *ANT(6)-Ia*	Fluoroquinolones, tetracycline, aminoglycoside nucleotidyltransferase	PEN, Trim-sulfa, GEN, TET, FOS, CIP, MOX
17	SWG3	*L. seeligeri*	Swab	*norB*	Fluoroquinolones	PEN, FOS, CIP, MOX

Lin = *L monocytogenes* EGD-e line gene for the lincomycin resistance ABC-F-type ribosomal protection protein of complete CDS; *norB* = a multidrug efflux pump in *Staphylococcus aureus* that confers resistance to fluoroquinolones and other structurally unrelated antibiotics such as tetracycline; *fosX* = an enzyme used to confer resistance to fosfomycin, which is dependent on the cofactor manganese (II) and uses water to generate a vicinal diol; *mprF* = an integral membrane protein that modifies the negatively charged phosphatidylglycerol on the membrane surface. This confers resistance to cationic peptides that disrupt the cell membrane including defensins; *tetM* = a ribosomal protection protein that confers tetracycline resistance. It is found on transposable DNA elements, and its horizontal transfer between bacterial species has been documented; *ANT(6)-Ia* = an aminoglycoside nucleotidyltransferase gene encoded by plasmids and chromosomes in *Staphylococcus epidermidis, E. faecium, Streptococcus suis, S. aureus, E. faecalis,* and *Streptococcus mitis*. All gene descriptions were defined by CARD [33]; PEN = benzylpenicillin; GEN = gentamicin; FOS = fosfomycin; CIP = ciprofloxacin; MOX = moxifloxacin; Trim-sulfa = trimethoprim-sulfamethoxazole; TET = tetracycline. The five *L. newyorkensis* isolates are not included in this table.

AMR genes were detected in eight *L. monocytogenes* isolates, eight *L. innocua,* and one *L. seeligeri* isolate. The phenotypic characterization was performed with 22 *Listeria* spp. isolates: 17 isolates harboring AMR genes (Table 2) and 5 *L. newyorkensis* isolates to obtain more information on the antimicrobial susceptibility of this species (Table 3). The associated minimum inhibitory concentration (MIC) results are shown in the Supplemental Materials (Table S1). All *L. newyorkensis* isolates were from swabs (drains) and water samples (a single processing water sample for lettuce and an irrigation water sample for vegetables). Furthermore, the genetic AMR results of *L. monocytogenes* differed from the phenotypical AMR results. More phenotypical AMR findings were detected in the *L. innocua* and *L. seeligeri* isolates than genetic resistances identified (Table 2).

Table 3. Phenotypical characterization of 22 *Listeria* isolates.

Antibiotics	MIC Breakpoints (mg/L)		Number of Resistant Isolates (*n*)			
	S ≤ [4]	R > [4]	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. newyorkensis*
PEN [2]	0.125	0.125	8/8	8/8	1/1	5/5
ERY [1]	1	1	0/8	0/8	0/1	5/5
Trim-sulfa [1, 3]	0.06	0.06	0/8	2/8	0/1	0/5
GEN [2]	2	2	1/8	1/8	0/1	0/5
TET [2]	1	2	0/8	1/8	0/1	0/5
FOS [2]	32	32	8/8	8/8	1/1	5/5
CIP [2]	0.001	1	5/8	8/8	1/1	0/5
MOX [2]	0.25	0.25	8/8	8/8	1/1	0/5

Seventeen isolates harbored AMR genes (Table 2), and five *L. newyorkensis* isolates showed no AMR genes. Five *L. newyorkensis* isolates were checked for phenotypic AMR to obtain more information on the antimicrobial susceptibility of this species. Breakpoints were evaluated using the EUCAST v 12.0 clinical breakpoints table [32]. PEN = benzylpenicillin; ERY = erythromycin; Trim-sulfa = trimethoprim-sulfamethoxazole; GEN = gentamicin; TET = tetracycline; FOS = fosfomycin; CIP = ciprofloxacin; MOX = moxifloxacin; [1] Minimal inhibitory concentration (MIC) breakpoints (mg/L) for *L. monocytogenes*; [2] MIC breakpoints (mg/L) for *Staphylococcus* spp.; [3] Trimethoprim–sulfamethoxazole in the ratio 1:19. The breakpoints are expressed as the trimethoprim concentration; [4] S = susceptible, standard dosing regimen; [4] R = resistant [39].

4.5.8 Prevalence of Virulence Genes in *Listeria* spp.

Table 4 demonstrates the presence of different virulence genes. All eight *L. monocytogenes* isolates carried *prfA*, which regulates the production of virulence factors [42]. Consequently, the eight sequenced *L. monocytogenes* isolates carried the virulence genes, which are regulated by *prfA*: *hly*, *plcA*, *plcB*, *hpt*, *actA*, *inlA,* and *inlB* (Table 4) [43]. The *llsX* gene belonging to LIPI (*Listeria* pathogenicity island)-3 was detected in two *L. monocytogenes* isolates belonging to lineage I (SWC6 and SWH3).

Table 4. Prevalence of virulence genes in 64 *Listeria* isolates.

	prfA % (*n*)	*hly* % (*n*)	*plcA* % (*n*)	*plcB* % (*n*)	*hpt* % (*n*)	*actA* % (*n*)	*inlA* % (*n*)	*inlB* % (*n*)	*mpl* % (*n*)
L. monocytogenes	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)
L. innocua	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)	0.00 (0/8)
L. ivanovii	66.67 (4/6)	66.67 (4/6)	0.00 (0/6)	0.00 (0/6)	100 (6/6)	0.00 (0/6)	66.67 (4/6)	66.67 (4/6)	0.00 (0/6)
L. seeligeri	91.67 (33/36)	91.67 (33/36)	91.67 (33/36)	91.67 (33/36)	100 (36/36)	0.00 (0/36)	0.00 (0/36)	0.00 (0/36)	91.67 (33/36)
L. newyorkensis	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)
L. grayi	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)	0.00 (0/1)

% (*n*) = percentage (number of isolates detected with virulence gene/total number of isolates of a designated species); *prfA* = positive regulatory factor A [44]; *hly* = listeriolysin O, LLO [45]; *plcA* = phosphatidylinositol-specific phospholipase C, PI-PLC or PLC-A [45]; *plcB* = nonspecific phosphotidylcholine phospholipase C, PC-PLC or PLC-B [45]; *hpt* = hexose phosphates, HP [46]; *actA* = protein actA [45]; *inlA* = internalin A [45]; *inlB* = internalin B [45]; *mpl* = zinc metalloproteinase [47].

Furthermore, the *prfA*, *hly*, *plcA*, *plcB,* and *mpl* genes were detected in 33 *L. seeligeri* isolates (every isolate except SWE1, SWH7, and SWH10). The *hpt* gene was detected in every *L. seeligeri* isolate, as well as in every *L. ivanovii* isolate. Four isolates of *L. ivanovii* (SWA3, SWC7, SWD3, and SWG2) carried the genes *prfA*, *hly*, *inlA,* and *inlB*. *L. innocua*, *L. newyorkensis,* and *L. grayi* did not show any of the selected virulence factor genes.

4.6 Discussion

4.6.1 Distribution and Genetic Relation of *Listeria* spp. in FNAO from Supermarkets and FNAO-Producing and Processing Plants

The study showed that *Listeria* spp. were spread among 48.72% (19/39) of the FNAO facilities in Bavaria. The distribution and genetic heterogeneity of the isolates within the species and the companies (Figure S1) suggests different contamination pathways of *Listeria* spp. and the introduction from the environment or irrigation and processing water. As the genus *Listeria* is ubiquitous in the environment [48], incoming raw goods, irrigation and processing water, coworkers, or working utensils are potential sources of contamination [48,49]. In particular, incoming raw material is described as a key determinant [49]. Less similar isolates are less likely to share a recent common ancestor [50]. The different values of *L. monocytogenes* CTs from the same company (Table 1) support the hypothesis of *L. monocytogenes* introduction from the environment. Nevertheless, once *L. monocytogenes* and other *Listeria* spp. have entered the processing plant, the likelihood of persisting increases [51,52]. In addition to good hygienic practice, periodical environmental sampling helps to obtain an overview of the prevalence and persistence of *Listeria* spp. and *L. monocytogenes* within a plant [49].

4.6.2 Serogroup and MLST Analysis of *L. monocytogenes*

From 2007 to 2015, the EFSA reported an 87% prevalence of serogroup IIa and IVb in the EU [26]. This is confirmed by our results, as the *L. monocytogenes* isolates in this study were classified into serogroups IIa (1/2a and 3a) (62.5%, 5/8) and IVb (4b, 4d, and 4e) (37.5%, 3/8). They were sampled from the environments of the FNAO companies (Table 1) [53]. An isolate detected in the condensate ponding of a cooling unit (SWC6) belonged to serogroup IVb and ST6 (Table 1). *L. monocytogenes* of serogroup IVb and ST6 was already responsible for an outbreak that impacted five different countries. As of June 2018, the case fatality rate was 19% with 47 reported cases, and the causative food was frozen corn and frozen vegetables [8].

Furthermore, the *L. monocytogenes* isolates with ST1/CC1, ST2/CC2, ST7/CC7, and ST21/CC21 identified in our study were detected in various drains at FNAO primary production and processing plants [54,55,56,57]. ST1 and ST2 were reported as hypervirulent by Mafuna et al. and well-adapted to persist in food processing environments in South Africa [54]. ST7/CC7 was reported as one of the most relevant agents of cattle abortions in Latvia [55]. Furthermore, ST21 was associated with *L. monocytogenes* isolated from vegetables by Cabal et al. [56]. ST21/CC21 was identified exclusively in 2011 and 2014 from environmental swab samples in a small meat processing facility in Montenegro [57]. This demonstrates that the detected isolates in our study were already described and showed diverse distribution.

4.6.3 cgMLST Analysis of *L. monocytogenes*

The created MST (Figure 1) shows high allelic differences between the eight detected *L. monocytogenes* isolates from the environment of FNAO-producing and -processing companies. These results suggest plant-specific *Listeria* isolates with sporadic introduction. However, due to the low number of positive samples and missing data of periodical sampling, it is difficult to generalize and extrapolate the statement. In 2011, contaminated cantaloupes were responsible for a listeriosis outbreak in 28 different states in the US. One single company was the starting point for this listeriosis outbreak [58]. Furthermore, investigations into the context of a listeriosis outbreak caused by ice cream products in the United States revealed operation-related food product contamination as well. The human *L. monocytogenes* isolates matched with the food product (ice cream) isolated from a production line in a specific company [59]. A comparison of ice cream products from another producing facility operated by the same brand showed no accordance with the listeriosis outbreak [60]. In comparison, other studies detected related *L. monocytogenes* isolates from meat (e.g., poultry or pork) in geographically independent producing plants [61,62], which suggests food matrix-dependent and persisting *L. monocytogenes* isolates in the meat sector. The finding of no genetically related *L. monocytogenes* isolates in the environment of the FNAO-producing and -processing companies suggested that there was no connection among the plants. Isolates that were linked to the producing and processing company and geographically dependent isolates in the FNAO sector were suggested. No transfer of *L. monocytogenes* among Bavarian FNAO-producing and -processing companies' environment to food products has taken place.

4.6.4 Genetic and Phenotypic AMRs of *Listeria* spp.

AMR has already become a global issue [63]. In 1988, AMR against erythromycin, tetracycline, chloramphenicol, and streptomycin was already reported in *L. monocytogenes* [64]. The detection of the genes *fosX*, *lin*, *norB*, and *mprF* in *L. monocytogenes* isolates was identical with the results of Parra-Flores et al. from RTE food in Chile [65]. The lincomycin resistance, as indicated by the presence of the *lin* gene, was not possible to confirm phenotypically, because the BD-Phoenix-System PMIC/ID 88 panel for *Staphylococcus* spp. did not include the lincomycin antibiotic. Furthermore, neither the EUCAST *L. monocytogenes* nor the *Staphylococcus* spp. breakpoints show MIC breakpoints for lincomycin [39]. In our study, every *L. monocytogenes* isolate showed phenotypic resistance to benzylpenicillin (Table 3). Tîrziu et al. detected *L. monocytogenes* isolates out of FAO, all of which showed benzylpenicillin resistance [66]. Furthermore, benzylpenicillin resistance in isolates from RTE vegetables was documented [67]. Penicillin is used as an antibiotic against listeriosis [41], and a 100% AMR detection rate in our study is a cause of concern. However, our results showed a phenotypically detected benzylpenicillin resistance without an underlying antibiotic gene. The lack of suitable breakpoints may explain the discrepancies between the phenotypic and genotypic antimicrobial susceptibility data [68].

Genotypic and phenotypic resistances to fluoroquinolones (here, ciprofloxacin and moxifloxacin were tested) were shown (Table 2), and ciprofloxacin resistance was reported in other studies [41,66,69]. We detected the *norB* gene that confers resistance to fluoroquinolones with an antibiotic efflux mechanism [33]. Other studies described *lde* as an underlying antibiotic gene for AMR to fluoroquinolones in *L. monocytogenes* [70,71]. Developing acquired AMR is rarely described in *L. monocytogenes* isolates, but Morvan et al. showed AMR of *L. monocytogenes* to fluoroquinolones, which suggests acquired AMR even if remaining low [71]. Acquired AMR to tetracycline was shown as well [71].

Moreover, the gene *fosX* and resistance to fosfomycin was detected (Table 2). Scortti et al. described that, despite *fosX* gene expression, *L. monocytogenes* isolates were susceptible to the antibiotic fosfomycin due to epistasis, but only during infection [72]. On the other hand, a natural in vitro resistance of *L. monocytogenes* to fosfomycin has been reported as well [41].

L. innocua, *L. seeligeri*, and *L. newyorkensis* showed AMR as well (Table 3), which suggests *Listeria* spp. as a habitat of AMR genes [73]. Different studies detected *L. innocua* as the species that is less susceptible to antibiotics compared to other *Listeria* spp. and reduced sensitivity against benzylpenicillin, tetracycline, fosfomycin, and ciprofloxacin, as our results confirmed [73,74,75].

Potential resistance gene transfer in *Listeria* spp. was discussed and increases the risk of emerging AMR in *L. monocytogenes* [73,75].

In general, the comparability of the AMR data is limited due to the different methods used [69] and the small number of isolates tested in our study. Furthermore, this study showed that the genotypic AMR results differed from the phenotypic AMR results. Under the heading of intrinsic antibiotic resistome, it is possible that phenotypical characteristics were influenced by bacterial metabolism, i.e., inactivation of genes, which changes the bacterial efficacy to antibiotics [76]. On the other hand, revisions of the threshold values were recommended to avoid misclassifying susceptibilities [68], as this may explain the differences between the genotypic and phenotypic susceptibility data. Gygli et al. described that clinical concentrations of antimicrobial susceptibility testing of *Mycobacterium tuberculosis* were defined too high and, thus, misclassifying and discrepancies between the genotypic and phenotypic data occurred [68]. The linkage of genotypic and phenotypic and a continuous surveillance of AMR could improve the understanding of AMR expressions [68,71].

Until now, *L. newyorkensis* was isolated from a milk processing company in the state of New York and from river water in Japan [77,78]. In our study, *L. newyorkensis* was isolated from swabs (drains) and water samples (a single processing water sample for lettuce and an irrigation water sample for vegetables). To the best of our knowledge, there is no literature concerning AMR in *L. newyorkensis* so far. Our results showed phenotypic resistances against benzylpenicillin, erythromycin, and fosfomycin according to the EUCAST *Staphylococcus* spp. breakpoints. However, no underlying genetic resistances were detected in the five *L. newyorkensis* isolates.

4.6.5 Prevalence of Virulence Genes in *Listeria* spp.

The presence of virulence genes is presented in Table 4. *L. monocytogenes* isolates exhibited the *prfA* gene, which is responsible for further virulence gene expression [79]. However, premature stop codon mutations in *inlA* in lineage II *L. monocytogenes* strains [53,80] were identified and suggest less invasiveness in 62.5% (5/8) of our detected *L. monocytogenes* isolates. Quereda et al. described that LIPI-3 is present in 50% of *L. monocytogenes* lineage I isolates, and LIPI-4 was present in CC4 isolates [81]. The *llsX* gene, belonging to LIPI-3, was detected in two *L. monocytogenes* isolates. A strong relationship between *llsX* and the invasiveness of *L. monocytogenes* is discussed [82]. LIPI-4 has been detected in CC4 isolates [81], which were not identified in this study. However, the *L. monocytogenes* singleton ST382 was responsible for multistate

outbreaks linked to FNAO (caramel apples, stone fruit, and packaged leafy green salad) and carried LIPI-4 [83]. Furthermore, Disson et al. described LIPI-4 in *L monocytogenes* CC87, as well as in *L. innocua* isolates [84].

In addition to *L. monocytogenes*, *L. ivanovii* was reported as a pathogenic species of *Listeria* [51,85,86]. Gouin et al. described that *L. ivanovii* and *L. seeligeri* carried genes of the virulence gene cluster of *L. monocytogenes* (i.e., *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) [86]. Our results showed that every *L. ivanovii* isolate carried the *hpt* gene, and four out of six isolated *L. ivanovii* isolates carried the *prfA*, *hly*, *inlA*, and *inlB* genes (Table 4). The *inlA* gene encoding for the *inlA* protein is necessary for entering the host cell [87] and was present in four *L. ivanovii* isolates. However, experimental assays showed that the *L. ivanovii* isolates that possessed the protein *inlA* showed less invasion in human cells compared to *L. monocytogenes*, and human listeriosis cases caused by *L. ivanovii* were rare as well [85]. Additionally, the absence of *plcA*, *mpl*, *actA*, and *plcB* suggested a missing virulence potential [88].

Furthermore, 56.25% (36/64) of the detected *Listeria* spp. were assigned to *L. seeligeri*. The gene *hpt* was present in every *L. seeligeri* isolate (Table 4). Our results showed that 91.67% of the detected *L. seeligeri* isolates were verified, with five virulence genes, namely, *prfA*, *hly*, *pclA*, *plcB*, and *mpl*. However, the *actA*, *inlA*, and *inlB* genes were not present. On the basis of the WGS analysis and because of the temporal and spatial dependency of virulence gene regulation, it is unlikely that the *L. seeligeri* isolates in our study had virulent potential [89]. Nevertheless, despite experimental studies [90,91] concluding that the species *L. seeligeri* is nonpathogenic, a single human meningitis case caused by *L. seeligeri* was reported in Switzerland [92].

4.7 Conclusions

In conclusion, this study showed that *Listeria* spp., including the pathogenic *L. monocytogenes*, were present in FNAO plants in Bavaria, Germany, but no food sample tested positive for *L. monocytogenes*. The genetic differences suggested external introduction, diverse ancestors, and plant-specific distribution of *L. monocytogenes* in FNAO-producing and -processing plants. One identified *L. monocytogenes* isolate in our study belonged to serogroup IVb and ST6. *L. monocytogenes* serogroup IVb and ST6 isolated from frozen corn and other frozen vegetables was already responsible for a multi-country outbreak. Furthermore, isolates with virulence markers and antibiotic resistances were identified, which should not be underestimated. However, further periodical sampling could provide more insights into persisting isolates.

4.8 Supplementary Materials

The following supporting information can be downloaded at: **https://www.mdpi.com/article/10.3390/foods12061120/s1**, Figure S1: Dendrogram of *Listeria* isolates and their holding of origin based on the PIRATE v1.0.3 presence/absence analysis. Only one isolate of the species *L. grayi* (SWG4) was detected out of 123 samples of fresh and frozen soft fruit samples spread across supermarkets in the south of Bavaria, Germany. All other isolates were received from 39 FNAO-producing and -processing facilities. The holdings of origin are abbreviated randomly from A until the alphabetical letter T. The letters A, D, E, G, K, N, and O encode the processing companies (processing level), and the remaining letters stand for the primary production plants (farm and primary production level). The letter T represents a grocery. Table S1: MIC results of *Listeria* spp.

4.9 Author Contributions

Conceptualization, S.W., T.A. and U.M.; data curation, S.W. and U.M.; formal analysis, S.W., N.B. and B.H.; investigation, S.W., N.B. and B.H.; methodology, U.M.; project administration, U.M.; resources, A.D., B.H., S.H., I.H., L.M., M.P., A.S., M.W. and U.M.; software, N.B.; supervision, T.A. and U.M.; validation, S.W., N.B, B.H., T.A. and U.M.; visualization, S.W., T.A. and U.M.; writing—original draft, S.W.; writing—review and editing, N.B., A.D., B.H., S.H., I.H., L.M., M.P., A.S., M.W., T.A. and U.M. All authors have read and agreed to the published version of the manuscript.

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4.11 Data Availability Statement

The raw sequencing data were uploaded to the SRA of the NCBI database (**https://www.ncbi.nlm.nih.gov/sra** accessed on 28 February 2023) under the project number PRJNA935401.

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4.13 Conflicts of Interest

The authors declare no conflict of interest.

4.14 References

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Chapter 5: General Discussion

L. monocytogenes is a zoonotic hazard for humans, esp. vulnerable population groups called YOPIs (Centers for Disease Control and Prevention 2022a; Federal Institute for Risk Assessment 2011). As well as fish/fish products, meat/meat products and milk/milk products, fruits and vegetables are posing an increasing risk for listeriosis infections (EFSA 2021).

Different studies evaluated the presence of *L. monocytogenes* in FNAO production and processing plants (Truchado et al. 2022; Lake et al. 2021; Pennone et al. 2020). In our studies, 39 FNAO producers of soft fruit, producers of vegetables and producers of RTE vegetables/soft fruit were investigated to detect the prevalence of *Listeria* spp. and *L. monocytogenes*. Furthermore, 123 samples of fresh soft fruit and frozen berries were tested for the presence of *Listeria* spp. and *L. monocytogenes*. Food samples showed no presence of the pathogenic *L. monocytogenes*. However, *L. monocytogenes* was present in the environment tested in our study and in irrigation and processing water of FNAO primary production and processing companies.

Using WGS, the genetic relation between *Listeria* spp. and *L. monocytogenes* from different FNAO facilities were shown. High allelic differences suggested various ways of *Listeria* introduction into producing plants and operation-dependent isolates. Next-generation sequencing (NGS) technologies offer new possibilities for detecting human listeriosis outbreaks (Wheeler et al. 2008). The combination of sequencing data of human and food isolates and epidemiological background are key determinants for solving listeriosis outbreaks (Brown et al. 2019).

5.1 Prevalence of *Listeria monocytogenes* in food of non-animal origin

The EFSA publishes the European Union One Health Zoonoses Report annually. In this context, various zoonotic pathogens were reported, including *L. monocytogenes* among other things. In recent years, the number of sampling and therefore positive units of RTE fruits and vegetables increased (EFSA 2021). In the Zoonoses Report of 2017, the overall prevalence of *L. monocytogenes* in RTE fruit and vegetables was 0.5% in 1,772 samples (EFSA 2017) compared to 3.0% in 1,407 samples in 2021 (EFSA 2022). In our studies, 59 food samples were taken from 39 FNAO primary production and processing plants. Furthermore, 123 samples of fresh soft fruit and frozen fruit mixes were tested for the presence of *L. monocytogenes*. None of the food samples were tested positive for the pathogenic *L. monocytogenes*. However, *Listeria* spp., such as *L. seeligeri* (3.39%, 2/59), *L. innocua* (3.39%, 2/59),

L. ivanovii (1.69%, 1/59), *Listeria* spp. non-typeable (3.39%, 2/59) and *L. grayi* (0.81%, 1/123) were detected in our food samples. Different studies showed different prevalences on FNAO (Maćkiw et al. 2021; Magdovitz et al. 2021; Willis et al. 2020; Zhang et al. 2020b; Chen et al. 2019; Roth et al. 2018; Zhang et al. 2018; Moravkova et al. 2017; Scheinberg et al. 2017; Chen et al. 2016). The *L. monocytogenes* prevalence varied from 0.06% to 53.3% (Zhang et al. 2018; Chen et al. 2016). Intact stone fruits showed the highest occurrence of *L. monocytogenes* (Chen et al. 2016). The temperature and product surface structures influenced the growth and persistence of *L. monocytogenes* on intact fruit and vegetables (Marik et al. 2020). Furthermore, Table 2 (Chapter 1.2.2) showed the prevalence of *L. monocytogenes* in different FNAO products. As mentioned above, *L. monocytogenes* was not detectable in 59 tested food samples in our study, but *Listeria* spp. were isolated in vegetable food. Lettuce, RTE salad and a frozen mushroom package were positive for *Listeria* spp. Willis et al. examined frozen fruit and vegetables and *L. monocytogenes* was detected in 0.9% (3/351) frozen fruit samples (Willis et al. 2020). Frozen vegetables have a high prevalence for carrying *L. monocytogenes* (Truchado et al. 2022; Willis et al. 2020). Moravkova et al. described a significant higher prevalence of *L. monocytogenes* in frozen vegetables than in RTE vegetables (Moravkova et al. 2017). Furthermore, frozen corn was responsible for a multi-country listeriosis outbreak from 2015 to 2018 in five countries (EFSA 2018b). Particularly, blanched frozen vegetables pose a high risk of *L. monocytogenes* contamination (Koutsoumanis et al. 2020) and the consumption of thawed uncooked vegetables increases the possibility of infection (Willis et al. 2020). Nevertheless, our results showed no *L. monocytogenes* in frozen samples, but *L. seeligeri* and *L. innocua* grew in a frozen mushroom package. Furthermore, *L. grayi* was detected in a frozen blackberry package. Accompanying bacterial and fungal flora like yeasts, moulds, bacilli and enterococci were detected in this analysis approach (Chapter 4). Errebo Larsen described that bacilli and moulds could inhibit the development of *L. monocytogenes* (Grønstøl 1979; Larsen 1969).

Although our results showed no presence of *L. monocytogenes* in food, it is important to mention that various studies investigated the prevalence of *Listeria* spp. and *L. monocytogenes* in FNAO producing plants (Truchado et al. 2022; Lake et al. 2021; Pennone et al. 2020). Different scenarios for *Listeria* introduction were discussed (Barnett-Neefs et al. 2022; Strawn et al. 2013). The occurrence of *L. monocytogenes* in the food producing and processing environment and irrigation and processing water as a potential hazard for contamination are discussed in the following section.

5.2 Occurrence of *Listeria monocytogenes* in food production and processing environment

L. monocytogenes is known as a saprophyte and ubiquitous microorganism in nature (Strawn et al. 2013; Bernagozzi et al. 1994; Geuenich et al. 1985). Different introduction pathways of *L. monocytogenes* into production and processing facilities include incoming raw goods, coworkers, irrigation and processing water or various working utensils (Barnett-Neefs et al. 2022; Muhterem-Uyar et al. 2015; Uyttendaele et al. 2015). A variety of *Listeria* spp. was detected in 48.72% (19/39) of on-the-spot visited FNAO primary production and processing plants in our study. Food contamination is possible at various junctures in the supply chain (Banach and van der Fels-Klerx 2020). In our study, eight *L. monocytogenes* isolates were detected in six different companies, with four in primary production and four in processing plants. Six isolates were detected in the facility surrounding. The core genome multilocus sequence typing (cgMLST) analysis showed high allelic differences between *L. monocytogenes* isolates, which suggested different ancestors, external entrance and operation-dependent isolates (Chapter 4). Pennone et al. described that low genetic relation of *L. monocytogenes* strains within a processing plant is a result of efficient hygienic practices but recontamination is common (Pennone et al. 2018). In order to assess whether recontamination happened at the 39 controlled FNAO primary production and processing companies, long period sampling would be preferable. Once *L. monocytogenes* entered the food plants, the risk of persistence is high (Mazaheri et al. 2021). Therefore, cross-contamination is one of the most concerning threats with regards to foodborne diseases (Londero et al. 2019). *L. monocytogenes* is commonly detected in the plant environments (Lake et al. 2021; Dzieciol et al. 2016; Weller et al. 2015b). In our study, six (2.26%, 6/229) *L. monocytogenes* isolates were found in the facility surrounding, esp. in swab samples of drains and a condensate ponding of a cooling unit (Chapter 3). Studies assumed that the surrounding in food-processing companies constitutes a high risk of contamination with *L. monocytogenes* (Lee et al. 2019). However, Barnett Neefs et al. described that detecting *Listeria* spp. and *L. monocytogenes* in the food-processing environment cannot be equated with the rate of growth (Barnett-Neefs et al. 2022). The presence of *Listeria* in FNAO primary production and processing companies that produce RTE food should not be underestimated for food safety. A contamination within niches that are difficult to clean, are of great concern (Tompkin 2002).

Moreover, drains were a common reservoir for *Listeria* spp. and *L. monocytogenes*, as our results confirmed (Estrada et al. 2020; Dzieciol et al. 2016). We could not detect any *L. monocytogenes* isolates in food samples during sampling period, but FNAO companies containing *L. monocytogenes* in

the production environment is a reason of concern and a potential risk for consumers. One of the largest listeriosis outbreaks in the USA caused by cantaloupes was revealed in 2011 (Centers for Disease Control and Prevention 2012). Food and surrounding investigations confirmed that environmental cross-contamination was responsible for the cantaloupe listeriosis outbreak due to unhygienic production conditions (McCollum et al. 2013). In general, post-harvest contamination of FNAO has been reported repeatedly (Angelo et al. 2017). Regular cleaning and disinfection steps are crucial to reduce the risk of persisting pathogenic microorganisms and therefore the risk of cross-contamination of RTE food (Mazaheri et al. 2021; Pennone et al. 2020; Tompkin 2002).

5.3 Occurrence of *Listeria monocytogenes* in irrigation and processing water

The quality of irrigation water and processing water is important for safe food production (Banach and van der Fels-Klerx 2020). Some studies reported the contamination of FNAO with transmission of pathogenic microorganisms via irrigation water (Gibbs et al. 2009; Greene et al. 2008; California Department of Health Services and U.S.Food And Drug Administration 2007). Moreover, *L. monocytogenes* was isolated from irrigation water, which was possibly used for RTE vegetable and fruit products (Gartley et al. 2022). Different spots along the water supply were investigated in 39 FNAO producing companies in our study. Water samples were taken from wells, public water supplies, surface water and water tanks filled with rainwater. Furthermore, other various spots along the water pipeline were examined, namely intermediate pieces, the end of the water pipes and installations for washing non-sliced and sliced vegetables. In general, we detected a total *Listeria* prevalence of 10.92% (13/119) in irrigation and processing water samples, whereas *L. monocytogenes* was detected in a processing water for a salad washing system at primary production level. Other water samples showed an occurrence of *L. seeligeri* (4.20%, 5/119), *L. ivanovii* (1.68%, 2/119), *Listeria* spp. non-typeable (3.36%, 4/119), and *L. innocua* (0.84%, 1/119) (Chapter 3). Wells were sampled as a primary water source of the water supply. None of the sampled wells were positive for *Listeria* spp. or *L. monocytogenes*. However, testing different spots throughout the water pipelines showed positive results for *Listeria* spp. The ability to form biofilms should be acknowledged in this context (Lee et al. 2019; Gião and Keevil 2014; Møretrø and Langsrud 2004). Gião and Keevil concluded that *L. monocytogenes* was able to persist on stainless steel in tap water at temperatures of 4, 22, 33, and 37 °C, respectively (Gião and Keevil 2014). Our results suggested that water contamination with

Listeria spp. appeared during water transport through different pipelines. Sporadic cleaning of water pipelines support the threat of contamination, persisting and its entrance to the food chain (Mazaheri et al. 2021). Furthermore, different environmental conditions contributed to the forming of VBNC states and complicated the detection and evaluation of *Listeria* spp. and *L. monocytogenes* in food processing plants (Gião and Keevil 2014).

Drip and overhead irrigation were two common irrigation regimes at primary production. Producers of soft fruit preferred drip irrigation and producers of vegetables used both regimes, while overhead irrigation was more common (Chapter 3). In addition to conserving water, drip irrigation reduces the risk of contamination with pathogenic microorganisms because it waters only the root zone and not the eatable portion of the plant. However, experimental studies showed that *L. monocytogenes* was able to colonize roots of plants and vegetables and was capable of entering the food chain, respectively (Marinho et al. 2020). Overhead irrigation is more critical because the crop surface is fully wetted (Uyttendaele et al. 2015). Furthermore, there is a correlation between *L. monocytogenes* detection and an irrigation regime within three days of sampling (Weller et al. 2015b). Therefore, delaying harvesting after irrigation is recommended (Banach and van der Fels-Klerx 2020; Weller et al. 2015b).

Furthermore, every water sample containing more than 300 mL underwent further investigations of the amount of *Escherichia* (*E.) coli*, enterococci and *Pseudomonas* (*P.) aeruginosa* (Chapter 3). *E. coli* and enterococci are known as fecal indicator microorganisms (Environmental Protection Agency 2022; Khan and Gupta 2020). Besides, *P. aeruginosa* is noted as an opportunistic bacteria, which is able to cause a variety of diseases (Januário et al. 2019). The prevalence of *Listeria* spp. and *L. monocytogenes* in water samples was compared to the results of the occurrence of *E. coli*, enterococci and *P. aeruginosa*. The presence of fecal indicator microorganisms enhanced the probability of the presence of other disease-causing agents like bacteria or viruses (Environmental Protection Agency 2022). Our study indicated that water contamination with *Listeria* spp. and/or *L. monocytogenes* showed a higher contamination of *E. coli* and enterococci. However, due to low positive samples, no statistical testing was performed and more research is needed to corroborate this statement.

5.4 Whole-genome sequencing of *Listeria* spp. and *Listeria monocytogenes*

The starting point for deoxyribonucleic acid (DNA) sequencing was in the 1970s and the subsequent NGS technologies revolutionized the food safety and consumer protection sector at the beginning of the 21st century (Immy Mobley 2021). Collecting, characterizing and comparing various microorganism isolates from humans, food and the environment of food processing plants at a genetic level could give important context with epidemiological background which, in turn, can provide a crucial advantage for resolving food-causing outbreaks (Brown et al. 2019; Jackson et al. 2016). The success depends on scientific communication and the exchange of data. In recent years, WGS has contributed to the detection of foodborne listeriosis outbreaks (EFSA 2019a; EFSA 2019b; Smith et al. 2019; EFSA 2018a; EFSA 2018b; Jackson et al. 2016). It is reported that the detection of foodborne outbreaks increased by introducing WGS technologies in the USA (Jackson et al. 2016).

5.4.1 Serotyping of *Listeria monocytogenes*

Serotyping methods deepened the knowledge about phenotypic characteristics of the pathogenic *L. monocytogenes* (Seeliger and Höhne 1979). The categorization into serogroups facilitated the classification of *L. monocytogenes* and their specific occurrence (Doumith et al. 2004). In this study, the following scheme was used: IIa (1/2a and 3a), IIb (1/2b), IIc (1/2c and 3c), and IVb (4b, 4d, and 4e) (Ricci et al. 2017). Our study showed 62.5% (5/8) detected *L. monocytogenes* isolates allocated to serogroup IIa (1/2a and 3a) and 37.5% (3/8) were characterized into serogroup IVb (4b, 4d, and 4e) (Chapter 4). Our results confirmed that 87% of the stated serogroups consisted to serogroup IIa and IVb in the EU (Ricci et al. 2017). Furthermore, various studies described IIa and IVb as the dominating serogroups (Mafuna et al. 2021; Zhang et al. 2020a; Halbedel et al. 2018; Jennison et al. 2017), whereas serovar 1/2a strains were mostly isolated in food. Serovar 4b was commonly documented in human listeriosis cases (Orsi et al. 2011; Doumith et al. 2004). Moreover, Tompkin summarized that serotype 1/2a and 4b showed a trend of persisting in food-processing environments (Tompkin 2002). In our study, *L. monocytogenes* isolates were detected in the food-processing surrounding, namely in different drains, in a condensate ponding of a cooling unit and a processing water sample for a salad washing system. Several studies confirmed the food-processing environment as a reservoir for *Listeria* spp. and *L. monocytogenes* and as a threat of cross-contamination (Barnett-Neefs et al. 2022; Estrada et al. 2020; Jordan et al. 2018; Muhterem-Uyar et al. 2015; O'Connor et al. 2010).

5.4.2 MLST and cgMLST of *Listeria monocytogenes*

The dendrogram of 64 detected *Listeria* spp. and *L. monocytogenes* isolates shows genetic heterogeneity, which suggests the hypothesis of environmental introduction (Chapter 4). Several scenarios of pathogenic microorganisms entering producing and processing companies have previously been discussed (Barnett-Neefs et al. 2022; Strawn et al. 2013). Barnett-Neefs et al. detected incoming raw material as one of the key determinants for introducing *L. monocytogenes* into FNAO facilities (Barnett-Neefs et al. 2022). Irrigation water, coworkers or equipment were further contamination pathways (Barnett-Neefs et al. 2022; Uyttendaele et al. 2015). Moreover, Tompkin debunked the statement that air is a source of contamination (Tompkin 2002). In general, if contamination vehicles are known, good hygienic practice is able to reduce the amount of pathogenic microorganisms (Carpentier and Cerf 2011).

The eight detected *L. monocytogenes* isolates showed seven different STs, CCs and complex types (CTs), namely ST1/CC1/CT16888, ST2/CC2/CT16889, ST6/CC6/CT7504, ST7/CC7/CT16884, ST21/CC21/CT16887, ST504/CC475/CT16886 and ST1413/CC739/CT16885. Eight *L. monocytogenes* isolates were detected from six different companies. Three isolates originated from the same primary production plant (SWD4, SWH4, and SWH8). Two of them showed the same ST/CC values (SWD4 and SWH4). The third isolate (SWH8) differed in STs, CCs and CTs (Chapter 4). SWD4 and SWH4 had ST504/CC475 and CT16886, whereas SWH8 showed a combination of ST2/CC2 and CT16889. This indicated an external introduction to FNAO producing and processing facilities. Diverse STs and CCs showed different occurrences (Mafuna et al. 2021; Šteingolde et al. 2021; Zuber et al. 2019). The swab sample SWC6 from a condensate ponding of a cooling unit from a primary production plant presented serogroup IVb and ST6. Up to 2015, a multi-country outbreak of listeriosis due to *L. monocytogenes* serogroup IVb and ST6 was attributed to frozen corn and probably other frozen vegetables (spinach, green beans, and vegetables mixes). Furthermore, the isolates SWH3 and SWH8 showed ST1/CC1 and ST2/CC2, respectively (Chapter 4), which are described as well adapted food-processing environment isolates (Mafuna et al. 2021).

Furthermore, the cgMLST minimum spanning tree (MST) in Chapter 4 suggests plant-specific and geographically dependent isolates of *L. monocytogenes* with sporadic introduction. With exception of the isolates SWD4 and SWH4, allelic differences ranged from 1058 to 1648 alleles and consequently no genetic relationship was shown. Allelic differences ≤ 10 indicate closely linked genotypes (Ruppitsch et al. 2015). Operation-dependent *L. monocytogenes* isolates were described in FNAO (McCollum et al. 2013), whereas

L. monocytogenes isolates from FAO companies showed geographical independent producing facilities (Chasseignaux et al. 2001).

5.5 Antimicrobial resistance of *Listeria monocytogenes*

Human listeriosis, caused by *L. monocytogenes*, is treated with ampicillin, amoxicillin or penicillin alone or in combination with an aminoglycoside like gentamicin (Schlech 2019; Noll et al. 2018; Robert Koch-Institute 2010). Alternatively, cotrimoxazole is also an option (Robert Koch-Institute 2010). In our study, genotypic and phenotypic AMR testing was performed. In this context, 64 *Listeria* spp. isolates were analyzed for the presence of AMR genes (Chapter 4). In general, in 17 *Listeria* spp. isolates, including eight *L. monocytogenes*, eight *L. innocua* and one *L. seeligeri* isolate, AMR genes were detected. Every *L. monocytogenes* isolate showed genotypic resistance to fosfomycin, fluoroquinolones, lincomycin and cationic peptides. *L. innocua* had resistance genes against fluoroquinolones, tetracycline and an aminoglycoside-nucleotidyltransferase and *L. seeligeri* showed fluoroquinolones resistance genes. Furthermore, phenotypic AMR testing was performed with the 17 isolates containing AMR genes. In addition, five *L. newyorkensis* isolates were investigated for phenotypic AMR to get more data on the antimicrobial susceptibility of this species. Generally, resistance was tested against benzylpenicillin, erythromycin, fosfomycin, gentamicin, ciprofloxacin, moxifloxacin, tetracycline and trimethoprim-sulfamethoxazole. Phenotypic characterization revealed eight *L. monocytogenes* isolates with resistance against benzylpenicillin, fosfomycin and moxifloxacin. Furthermore, five *L. monocytogenes* isolates showed reduced susceptibility to ciprofloxacin and one isolate was resistant against gentamicin. Resistances against benzylpenicillin were reported in FAO as well as in FNAO (Tîrziu et al. 2022; Vasconcelos Byrne et al. 2016). It is a cause of concern detecting a 100% resistance rate against one of the main therapeutic antibiotics (Noll et al. 2018). Furthermore, efflux pumps were reported for being responsible for fluoroquinolone resistance, which our findings of genotypic and phenotypic fluoroquinolone resistances in *L. monocytogenes* confirmed (Jiang et al. 2018; Godreuil et al. 2003). Due to natural *in vitro* reduced susceptibility against fosfomycin, this antibiotic is not used as therapeutic agent (Noll et al. 2018). However, Scortti et al. described that during listeriosis infection, *L. monocytogenes* isolates were susceptible to fosfomycin due to epistasis. In this context, they evidenced that it is possible to apply fosfomycin as a treating antibiotic (Scortti et al. 2018). Genotypic and phenotypic AMR to fluoroquinolones were shown (Chapter 4). AMR to fluoroquinolones and tetracycline were reported to be acquired ones (Morvan et al. 2010).

AMR was detected in *Listeria* spp., namely *L. innocua*, *L. seeligeri* and *L. newyorkensis*. *L. innocua* showed phenotypic resistance against benzylpenicillin, trimethoprim-sulfamethoxazole, gentamicin, tetracycline, fosfomycin, ciprofloxacin and moxifloxacin and the results confirmed that *L. innocua* showed increased AMR compared to other *Listeria* spp. (Da Rocha et al. 2012; Li et al. 2007). If various *Listeria* spp. and *L. monocytogenes* harbour the same primary or processing plant, horizontal gene transfer within *Listeria* spp. pose a threat for developing AMR (Da Rocha et al. 2012). In general, it is reported that antimicrobial plasmid transfer between other pathogenic bacterial species and within *L. monocytogenes* isolates was possible as well (Biavasco et al. 1996; Flamm et al. 1984). Enterococci are microbial intestinal inhabitants and Doucet-Populaire et al. measured genetic exchange from enterococci to *L. monocytogenes* in the digestive tract of gnotobiotic mice (Doucet-Populaire et al. 1991). Moreover, enterococci were reported with increased gentamicin resistance (Rice et al. 1991).

In conclusion, this study showed that *Listeria* spp. and *L. monocytogenes* were present in FNAO primary production and processing plants, whereas *L. monocytogenes* were present in small numbers. No food samples were tested positive for *L. monocytogenes*. The facilities surrounding, esp. drains, irrigation and processing water were particular habitats of the pathogenic *L. monocytogenes*. Furthermore, the presence of *L. monocytogenes* in the surrounding of RTE FNAO-processing companies has to be monitored to avoid cross-contamination (Barnett-Neefs et al. 2022; Estrada et al. 2020; Jordan et al. 2018; Muhterem-Uyar et al. 2015; O'Connor et al. 2010). The category of RTE food pose a risk for vulnerable population groups because no further processing step like heating is included (EFSA 2021; Federal Institute for Risk Assessment 2011). In this context, good hygienic practice is more important than ever to avoid severe listeriosis outbreaks (Mazaheri et al. 2021; Pennone et al. 2020; Tompkin 2002). Moreover, using NGS technologies like WGS improved food security and the protection of consumers (Brown et al. 2019). Our results showed that external introduction and operation-dependent isolates of *Listeria* spp. and *L. monocytogenes* were suggested. RTE FNAO food business operators have to be aware of the threat of *L. monocytogenes* in FNAO. *Listeria* monitoring, continuous sampling of the environment and the food, support this acknowledgement and can shorten the process of identifying the cause of a potential listeriosis outbreak (Tompkin 2002).

Chapter 6: Zusammenfassung

Listeria monocytogenes bei Lebensmitteln pflanzlichen Ursprungs

Das grampositive pathogene Bakterium *L. monocytogenes* verursacht eine durch Lebensmittel übertragbare Krankheit namens Listeriose, die vor allem empfindliche Personengruppen wie YOPIs betrifft. Im Vergleich zu anderen lebensmittelassoziierten Mikroorganismen ist vor allem die hohe Sterblichkeit von 20–30% der schweren Listeriose ein Grund zur Besorgnis. Listeriosefälle, die durch *L. monocytogenes* in Lebensmitteln pflanzlichen Ursprungs verursacht wurden, nahmen in den letzten Jahren zu. Vor allem, verzehrfertige pflanzliche Lebensmittel, die keine weiteren Erhitzungsprozesse durchlaufen, stellen ein Risiko dar.

In diesem Zusammenhang wurden im Rahmen dieser Studie 39 Primärproduzenten und weiterverarbeitende Betriebe in Bayern, Deutschland von Juli 2020 bis Juni 2021 kontrolliert, die Lebensmittel pflanzlichen Ursprungs produzieren und weiterverarbeiten (Chapter 3). Die Kategorien waren „Hersteller von Obst“, „Hersteller von Gemüse“ und „Hersteller von Rohkost/Fresh Cut“. Das Ziel der Studie war das Vorkommen von *Listeria* spp. und *L. monocytogenes* in FNAO produzierenden Betrieben zu detektieren. Insgesamt wurden 407 Proben gezogen, aufgeteilt auf das Prozessumfeld (229 Proben), Lebensmittel (59 Proben) und Bewässerungs- und Prozesswasser (119 Proben) und nach ISO 11290 - 1:2017 untersucht. 12,53% der untersuchten Proben waren mit *Listeria* spp. und *L. monocytogenes* kontaminiert. *L. monocytogenes* Isolate wurden in der Betriebsumgebung, vor allem in Abflussrinnen und in einer Prozesswasserprobe festgestellt. Die Lebensmittelproben hingegen waren nicht positiv auf die pathogene Spezies *L. monocytogenes*. Bewässerungs- und Prozesswasser stellen eine mögliche Kontaminationsquelle für Lebensmittel pflanzlichen Ursprungs dar. Unzureichende Hygienemaßnahmen in Wasserleitungen sind ein erhöhtes Risiko für Kreuzkontaminationen.

Desweiteren wurden die isolierten *Listeria* spp. und *L. monocytogenes* Stämme mittels WGS sequenziert (Chapter 4). Die Fragestellung bezog sich auf genetische Verwandtschaft der Isolate innerhalb eines Betriebes bzw. zwischen den untersuchten Firmen. Aufgrund des geringen festgestellten Verwandtschaftsgrades von *Listeria* spp. und *L. monocytogenes* innerhalb und zwischen den Betrieben, wird ein Eintritt von außen und betriebsspezifische Isolate in den lebensmittelproduzierenden und –weiterverarbeitenden Firmen vermutet. Angelieferte Rohware wird als Eintrittsquelle diskutiert, aber Bewässerungs- und Prozesswasser kommen ebenso als mögliche Eintritts- und Kontaminationsquelle in Frage. Zudem wurden die Isolate auf Virulenz- und

Antibiotikaresistenzgene untersucht und eine phänotypische Antibiotikaresistenzcharakterisierung durchgeführt. Virulenzgene, wie *prfA*, *hly*, *plcA*, *plcB*, *hpt*, *actA*, *inlA*, *inlB* und *mpl* wurden sowohl in pathogenen als auch in nicht-pathogenen *Listeria* spp. nachgewiesen. Phänotypische Resistenzen gegen Benzylpenicillin, Fosfomycin und Moxifloxacin wurden bei den acht identifizierten *L. monocytogenes* Isolaten nachgewiesen. Resistenznachweise von Benzylpenicillin sind Grund zur Sorge, da das Antibiotikum als Therapeutikum bei Listeriose eingesetzt wird. Innerhalb *Listeria* spp. zeigten in dieser Studie die *L. innocua* Isolate die meisten Antibiotikaresistenzen im Vergleich zu *L. seeligeri* und *L. newyorkensis* auf.

Zusätzlich wurden 123 frische Beeren und Tiefkühlbeeren randomisiert aus Supermärkten im südlichen Bayern, Deutschland gesammelt und untersucht. Lediglich ein *L. grayi* Stamm konnte aus einer Tiefkühlbrombeerenverpackung isoliert werden, alle anderen Proben waren negativ auf *Listeria* spp. und *L. monocytogenes*.

Zusammenfassend lässt sich sagen, dass *Listeria* spp. und *L. monocytogenes* in FNAO produzierenden und weiterverarbeitenden Betrieben in Bayern, Deutschland vorhanden sind. Umgebungs-, Bewässerungs- und Prozesswasserproben zeigten ein hohes Vorkommen an *Listeria* spp. und *L. monocytogenes*. Ein externer Eintritt durch angelieferte Rohware und Bewässerungs- und Prozesswasser ist möglich. Zudem werden betriebsspezifische *L. monocytogenes* Isolate in FNAO Betrieben diskutiert. Daher ist gute hygienische Praxis unabdingbar um Kreuzkontaminationen zu vermeiden. Die Daten dieser Studie zeigen, dass FNAO produzierende und weiterverarbeitende Betriebe einen Schwerpunkt auf *L. monocytogenes* legen sollten. *Listeria* Monitoring, d.h. kontinuierliches Beproben der Betriebsumgebung, des Bewässerungs- und Prozesswassers und der Lebensmittel selbst stärkt das Bewusstsein und stellt ein wichtiges Instrument zur Ausbruchsaufklärung dar.

Chapter 7: Summary

Listeria monocytogenes in food of non-animal origin

L. monocytogenes is a Gram-positive pathogenic bacterium causing the foodborne disease listeriosis, esp. in vulnerable population groups called YOPIs. Compared to other foodborne pathogenic microorganisms, the fatality rate of 20–30% of severe listeriosis is a serious concern. Listeriosis cases associated with FNAO have been increasing in recent years. In particular, RTE FNAO without any further processing steps like heating is of great concern in this context.

This study investigated 39 FNAO primary producers and processing companies that produce and process FNAO products in Bavaria, Germany from July 2020 until June 2021 (Chapter 3). The categories were “producers of soft fruit”, “producers of vegetables” and “producers of RTE raw fruits and vegetables/fresh cut”. The aim of this study was to detect the prevalence of *Listeria* spp. and *L. monocytogenes* in FNAO producing and processing plants. In general, 407 samples from the processing environment (229 samples), the food (59 samples) and irrigation and processing water (119 samples) were taken and investigated using ISO 11290 - 1:2017. Overall, in 12.53% of the samples *Listeria* spp. and *L. monocytogenes* were detected. *L. monocytogenes* isolates were identified in the FNAO surrounding, especially drains, and in a sample of processing water. Whereas no food was contaminated with the pathogenic *L. monocytogenes*. Irrigation and processing water are probable contamination sources of FNAO. Unhygienic circumstances of water pipelines pose a risk of cross-contamination.

Furthermore, the detected *Listeria* spp. and *L. monocytogenes* isolates were sequenced with WGS (Chapter 4). It poses the question about genetic relationship within and between companies. Due to low genetic relationship of *Listeria* spp. and *L. monocytogenes* within and between companies, external introduction and plant-specific *Listeria* in FNAO facilities were suggested. In particular, incoming raw material is discussed but irrigation and processing water is possibly both an entrance and contamination pathway. Moreover, virulence and AMR genes were detected and phenotypic characterization was performed, additionally. Virulence factors such as *prfA*, *hly*, *plcA*, *plcB*, *hpt*, *actA*, *inlA*, *inlB*, and *mpl* were identified in pathogenic and nonpathogenic *Listeria* species. Besides, phenotypic resistance was detected against benzylpenicillin, fosfomycin and moxifloxacin in all eight *L. monocytogenes* isolates. Benzylpenicillin is a therapeutic agent against listeriosis and detected AMR is a cause of concern. *L. innocua* was the most resistant *Listeria* spp. next to *L. seeligeri* and *L. newyorkensis* in this study.

In addition, 123 samples of fresh soft fruit and frozen berries were collected randomly from supermarkets across the south of Bavaria, Germany. Only one *L. grayi* isolate was detected in frozen blackberries, while all other samples were negative for *Listeria* spp. and *L. monocytogenes*.

In conclusion, *Listeria* spp. and *L. monocytogenes* were present in FNAO primary production and processing companies in Bavaria, Germany. Environmental, irrigation and processing water samples showed high prevalences of *Listeria* spp. and *L. monocytogenes*. An external introduction by incoming raw material or irrigation and processing water is probable. Furthermore, plant-specific *L. monocytogenes* isolates in FNAO facilities are discussed. Good hygienic practice is crucial to avoid cross-contamination. The data determined in this study indicate that FNAO primary production and processing plants should be focusing more on *L. monocytogenes*. *Listeria* monitoring means continuous sampling of the environment, irrigation/processing water and food, and represents an important mechanism to resolve foodborne outbreaks.

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Food Microbiol, 79, 116–122
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List of Publications

Publications

Simone Wartha, Stefanie Huber, Iris Kraemer, Thomas Alter, Ute Messelhäußer (2022 / Research Note): Presence of *Listeria* at primary production and processing of food of non-animal origin (FNAO) in Bavaria, Germany, *Journal of Food Protection*, Volume 86, Issue 1, January 2023, 100015, https://doi.org/10.1016/j.jfp.2022.11.007

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Posters

Simone Wartha, Petra Kaiser, Sabine Kremnitz, Beatrix Gutmann, Miriam Endres, Karl Zettl, Dr. Mareike Wenning, Dr. Albert Rampp, Dr. Ute Messelhäußer, Prof. Dr. Thomas Alter. (2021). *Listeria monocytogenes* bei Lebensmitteln pflanzlichen Ursprungs – ein relevantes Problem im Rahmen des gesundheitlichen Verbraucherschutzes? (Poster/Abstract). 9. Bayerischer Kongress für den Öffentlichen Gesundheitsdienst, Online, 09/2021.

Speeches

Simone Wartha (2021). Handling of *Listeria monocytogenes* at primary production and processing of Food of Non-animal origin (FNAO). Cost Huplant control meeting, Wageningen University & Research, Online, 06/2021.

Simone Wartha (2022). Vorkommen von *Listeria monocytogenes* bei Lebensmitteln pflanzlichen Ursprungs. Forschungskolloquium, Bavarian Health and Food Safety Authority, Online, 07/2022.

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Declaration of Independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only sources and aids indicated.

Marktoberdorf, 19th September 2023

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