Ecophysiological behaviour of *Listeria monocytogenes* in *Agaricus bisporus* mushroom processing environments

Frank B. Lake

Propositions

1. Complete elimination of *Listeria monocytogenes* from mushroom processing environments is not possible. (this thesis)

2. Pathogen laboratory experiments should be performed using a food-derived medium to enable the translation of results to the respective food processing environments. (this thesis)

3. As organic agriculture leads to lower yields compared to conventional agriculture (Seufert et al. (2012) Nature Vol 485, pp. 229-232), replacing conventional agricultural farming systems by organic agricultural farming systems negatively impacts food security.

4. Open access to peer-reviewed publications in scientific journals and scientific data should be the new standard.

5. The quality of food safety measures determines the life expectancy of a food company.

6. The mirror of self-reflection is needed to push yourself in the right direction.

Propositions belonging to the thesis, entitled

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Wageningen, 10 October 2023

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Thesis

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Table of contents

1

General introduction

1.1 Food safety management

Food safety is the assurance that food will not cause adverse health effects to the consumer when it is prepared and/or eaten according to its intended use (FAO and WHO, 2020). Hence ensuring food safety is a public health priority and an essential step to achieve food security (FAO, 2023a). The application of effective control systems in food safety, food hygiene and food quality are of high importance to avoid the adverse human health and economic consequences of foodborne illness and food spoilage. This will safeguard the health and well-being of people and foster economic developments (CAC, 2003; FAO, 2023a). National food control systems ensure that food which is available within a country is safe, wholesome, fit for human consumption and conforms to food safety and quality requirements. Food control systems protect the health and safety of consumers and help assuring the safety and quality of foods being traded both nationally and internationally (FAO, 2023b). Governments, producers and consumers have a shared responsibility towards food safety and all parties play a role to ensure that consumed foods are safe and healthy to help ensure stronger food systems (WHO, 2023; Wu et al., 2021).

Food businesses have to address food safety risks by implementing a food safety management system (Kirezieva, 2015). Food safety management systems are seen as a means to ensure that all potential food hazards are properly identified, assessed and controlled so as to not pose a risk to the health of consumers (Stoyanova et al., 2022). Mandating the implementation of integrated food safety management systems has been seen by many as one of the most certain ways of assuring food safety (Mensah and Julien, 2011), because food safety management is seen as an effective tool to ensure compliance against requirements (Stoyanova et al., 2022). The food safety management systems are based on various standards and guidelines (Kirezieva, 2015) and these standards aim to promote and enhance food safety (FAO and WHO, 2020; Mensah and Julien, 2011). For the European Union, the EU General Food Law Regulation contains the general principles and requirements of food law (EC (European Commission), 2023). In addition, the European Food Safety Authority (EFSA) will give transparent, independent and trustworthy scientific advice with respect to safe food and sustainable food systems (EFSA, 2023). Moreover, the Codex Alimentarius is a collection of internationally adopted food standards and relating texts that aim to protect consumer's health (FAO, 2023c). Besides, the international ISO 22000:2018 describes the requirements for a food safety management system for organisations

involved in the food production chain. It describes what organizations should do to control food safety hazards and to ensure that its products are safe for consumption (International Organization for Standardization, 2018).

A food safety management system is a set of programs and procedures. It includes, at the food processing level, Good Manufacturing Practice (GMP) and the principles of the Hazard Analysis and Critical Control Point (HACCP) system (Kirezieva et al., 2013; Stoyanova et al., 2022). The basis of the food safety system to be applied in the food industry, consists of a combination of GMP and the HACCP system together with the sanitation standard operating procedures (SSOP) (De Oliveira et al., 2016). GMP and SSOP are prerequisite programs for HACCP that deal with the good housekeeping issues in the facility and may prevent a hazard from occurring (De Oliveira et al., 2016). The GMP aims at the production of high-quality foodstuffs and foods that are safe for consumer's health. The SSOP on the other hand are written procedures developed and implemented in a facility to prevent direct contamination or adulteration of food products (De Oliveira et al., 2016). HACCP is a management system for food safety. It is addressed as an approach for the identification, evaluation, and control of food safety hazards during raw material production, food processing, manufacturing, distribution, retail and consumption of the finished product (FDA, 2022a; FDA, 2022b; Sanders, 1999). The primary production activities may have potential effects on the safety and suitability of food and should therefore always be considered. This includes particularly the identification of particular points in the production activities where high contamination probabilities exist and therefore, these probabilities should be minimized by taking specific measures. The HACCP approach assists in taking actions to enhance the food safety (CAC, 2003) and in this way, the HACCP system ensures food safety and quality. The HACCP system can be considered as an efficient tool for the food industry in controlling foodborne diseases as this system addresses and controls all significant hazards associated with a particular food (Varzakas, 2016). Food hazard analysis can be applied to all sectors of the food chain from primary production through the point of consumption. The strength of it is that it focuses on the identification of the main risks and tackling them (Sanders, 1999). Risks may be observed during monitoring and appropriate and immediate precautions should be taken to correct the deviations when results indeed show that criteria are not met or loss of control is observed. This could be applied with corrective actions if critical limits are exceeded (Dalgiç and Belibaǧli, 2008; Doménech et al., 2008). The corrective actions that might be applied include cleaning and disinfection, which are an essential

part of the HACCP system (Mazaheri et al., 2021). Application of corrective actions will re-establish control in a timely manner so as to assure that potentially hazardous products do not reach the consumer (Dalgiç and Belibağli, 2008; Doménech et al., 2008). Among the more significant risks is the acceptation of food supplies contaminated with pathogenic micro-organisms (Stoyanova et al., 2022). The bacterial pathogens are nowadays the most common microbial food safety hazards, which include among others the foodborne pathogen *Listeria monocytogenes* (Rivera et al., 2018). This foodborne pathogen constitutes a big challenge for food processing companies (Shamloo et al., 2019). Therefore, many scientific research studies have been focussed on characterizing and understanding the ecology and physiology of *L. monocytogenes* (amongst others, (Aryani et al., 2015a; Buchanan et al., 2017; Chasseignaux et al., 2002; Di Bonaventura et al., 2008; Ebner et al., 2015; Fagerlund et al., 2017; Fox et al., 2011; Gray et al., 2021a; Gray et al., 2021b; Kadam et al., 2013; Lee et al., 2019; Miettinen and Wirtanen, 2006; Norton et al., 2001)). A better understanding of the characteristics of *L. monocytogenes* is necessary for enhancing control measures to reduce the incidence of foodborne disease (Buchanan et al., 2017).

1.2 The human foodborne pathogen *Listeria monocytogenes*

The species *Listeria monocytogenes* has been recognized as an important opportunistic human foodborne pathogen and is classified within the *Listeria* genus (Liu, 2006). The species within the *Listeria* genus are described as Gram-positive, non-spore forming, facultative anaerobic and rod shaped bacteria (Chlebicz and Śliżewska, 2018; Collins et al., 1991; Liu, 2006; Orsi and Wiedmann, 2016). The number of recognized species that are belonging to the *Listeria* genus has been increased in the last decennium. This has resulted in 28 validly published and correctly named *Listeria* species described and determined until January 2023 (https://lpsn.dsmz.de/genus/listeria, last accessed 15 February 2023) (Carlin et al., 2022b; Chlebicz and Śliżewska, 2018; Nwaiwu, 2020; Orsi and Wiedmann, 2016; Parte et al., 2020) in which the newly described *Listeria* species could be found in recent literature (Carlin et al., 2021; Carlin et al., 2022a; Quereda et al., 2020; Raufu et al., 2022). Most of the *Listeria* subspecies are non-pathogenic, but the human foodborne pathogen *L. monocytogenes* is able to cause infections in humans called listeriosis (Orsi and Wiedmann, 2016; Radoshevich and Cossart, 2018). This bacterium is usually transmitted to humans via the consumption of food products contaminated with *L. monocytogenes* (Cabal et al., 2019; Holley and

Cordeiro, 2014). The susceptibility of the human host after the exposure to *L. monocytogenes* plays an important role in the outcome of the disease (Vázquez-Boland et al., 2001). *L. monocytogenes* could be established as a course of acute, self-limited, febrile gastroenteritis in healthy persons and common symptoms may include headache, stomach ache, nausea, articular pain and vomiting (Chlebicz and Śliżewska, 2018; Ooi and Lorber, 2005). People at risk for listeriosis include neonates, pregnant women, elderly, immunocompromised or debilitated adults with underlying diseases and recipients of organ transplants. Thus especially the people from these groups that are exposed to *L. monocytogenes* may develop life-threatening bacteraemia or meningoencephalitis (Chlebicz and Śliżewska, 2018; Ooi and Lorber, 2005; Vázquez-Boland et al., 2001). Although the incidence rate of listeriosis is relatively low, the mortality rate is high. This is shown by a mortality rate between 13.0% and 17.7% in the EU from 2014 to 2021 (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015; 2016; 2017; 2018; 2019; 2021a; 2021b; 2022) (table 1.1) and was between 13.3% and 23.1% in the United States from 2014 to 2020 (CDC and FoodNetFast, website: https://wwwn.cdc.gov/FoodNetFast/PathogenSurveillance/AnnualSummary, last accessed 24 March 2023) (table 1.2).

Table 1.1. Number of reported confirmed cases of human listeriosis and the corresponding hospitalization rates and case fatality rates in the EU between 2014 and 2021. The table was constructed based on the yearly scientific reports of EFSA and ECDC focussing on human zoonoses (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015; 2016; 2017; 2018; 2019; 2021a; 2021b; 2022)

Year	Number of confirmed human cases	Hospitalization			Deaths		
		Outcome available (% and n)*	Reported hospitalized cases (n)	Proportion hospitalized (%)	Outcome available (% and n)*	Reported deaths (n)	Case fatality (%)
2014	2,161	38.0 - 821	812	98.9	64.8 – 1,400	210	15.0
2015	2,206	44.9 - 990	964	97.4	69.1 – 1,524	270	17.7
2016	2,536	38.8 - 974	962	97.7	60.1 – 1,524	247	16.2
2017	2,480	40.4 - 1,091	988	98.6	65.8 – 1,632	225	13.8
2018	2,549	42.4 - 1,081	1,049	97.0	57.6 – 1,468	229	15.6
2019	2,621	51.1 - 1,339	1,234	92.1	65.1 – 1,706	300	17.6
2020	1,876	42.8 - 803	780	97.1	68.4 – 1,283	167	13.0
2021	2,183	43.8 - 956	923	96.5	65.4 - 1,427	196	13.7

* Data is not available for all confirmed human cases. Only cases in which the outcome is available are incorporated for determining the proportion hospitalized and case fatality.

Table 1.2. Number of reported confirmed cases of human listeriosis and the corresponding hospitalization rates and case fatality rates in the USA between 2014 and 2020. The table was constructed based on the yearly scientific data of Centers for Disease Control and Prevention, published on FoodNetFast (website: https://wwwn.cdc.gov/FoodNetFast/PathogenSurveillance/AnnualSummary)

Year	Number of confirmed human cases *	Incidence rate**	Hospitalization		Deaths	
			Reported hospitalized cases (n)	Proportion hospitalized (%)	Reported deaths (n)	Case fatality (%)
2014	115	0.24	106	92.2	17	14.8
2015	120	0.25	113	94.2	16	13.3
2016	127	0.26	123	96.9	17	13.4
2017	162	0.33	155	95.7	33	20.4
2018	131	0.26	126	96.2	28	21.4
2019	135	0.27	132	97.8	21	15.6
2020	108	0.21	104	96.3	25	23.1

* Data collected in Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, and selected counties in California, Colorado, and New York.
** per 100,000 persons.

The severe outcomes of listeriosis makes it necessary to apply adequate antibiotic treatments. The standard therapy of severe *L. monocytogenes* infections is based on amoxicillin, ampicillin or penicillin G, combined with an aminoglycoside, which is classically gentamicin. The combination of trimethoprim with a sulfonamide, as sulfamethoxazole in co-trimoxazole, is considered to be a second-choice therapy. Furthermore, vancomycin may be used in nonmeningeal infections, erythromycin may be used during pregnancy related listeriosis and other antibiotics such as rifampicin or linezolid may be applied as well in therapies with severe infections (Baquero et al., 2020; Charpentier and Courvalin, 1999). *L. monocytogenes* may however be resistant to certain antibiotics that are mainly used during treatment of *L. monocytogenes* infected patients, which might cause problems (Baquero et al., 2020). The resistance to antibiotics, such as ampicillin, gentamycin, erythromycin, trimethoprim and vancomycin is however low and described as very uncommon and infrequent. On the other hand, resistance to tetracycline is the most frequent resistance trait in *L. monocytogenes* isolated from humans. So, *L. monocytogenes* strains are in general susceptible to a wide range of antibiotics except for cephalosporins, fosfomycin and nalidixic acid (Baquero et al., 2020; Charpentier and Courvalin, 1999; Hof, 2004).

1.3 *Listeria monocytogenes* detection and typing

A good detection methodology for *L. monocytogenes* is necessary in an environmental monitoring program to determine the prevalence and potential sources of *L. monocytogenes* contamination in food processing environments. Besides, it may also monitor the effectiveness of control measures against *L. monocytogenes* and identify places for improved control. The results of the environmental monitoring program can be used to take the necessary actions to prevent contamination (Gupta and Adhikari, 2022). For the detection of this pathogen, standard culture procedures are used as reference methods. The two most widely used culturing-based reference methods for the detection of *L. monocytogenes* include the FDA bacteriological and analytical method (BAM) and the International Organization of Standardization (ISO) 11290 method (Gasanov et al., 2005). The ISO 11290 describes two protocols, with part 1 the detection protocol, and part 2 the enumeration protocol (International Organization for Standardization, 2017a; 2017b). Presumptive *L. monocytogenes* cultures obtained with the ISO methods should be plated on selective solid media including Agar Listeria according to Ottaviani and Agosti (ALOA) (International Organization for Standardization, 2017a). Here, the chromogenic agar medium ALOA can differentiate *L. monocytogenes* from other *Listeria* species allowing direct enumeration of *L. monocytogenes* from the plated sample (Becker et al., 2006). Since the chromogenic agar media does not differentiate between *L. monocytogenes* and *L. ivanovii*, the differentiation of these species is accomplished by sugar fermentations (Gasanov et al., 2005). The identification of *Listeria* spp. using these culturing-based methods on selective enrichment and plating, followed by further characterization including sugar fermentations and also haemolytic properties is considered as the golden standard method (Gasanov et al., 2005). On the other hand, the polymerase chain reaction (PCR) may be applied following the selective culturing enrichment procedures. This PCR method will identify *Listeria* spp. and differentiate the various *Listeria* subspecies, including *L. monocytogenes*. This may be performed by multiplex PCR in which multiple primer sets are incorporated, allowing multiple detections in one reaction that will reduce time, reagents and labour costs for determinations (Gasanov et al., 2005).

The isolated *L. monocytogenes* species may be grouped in four evolutionary lineages, namely, lineages I, II, III and IV. Moreover, at least 13 serotypes of *L. monocytogenes* are described and include serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Chlebicz and Śliżewska, 2018; Orsi et al., 2011; Seeliger and Höhne, 1979).

Most of the *L. monocytogenes* strains are attributed to lineage I and II, which include the serotypes 1/2b, 3b, 4b, 4d, 4e, 7 and 1/2a, 1/2c, 3a, 3c, respectively. The other serotypes are associated with lineage III (4a, 4c and atypical 4b) and lineage IV (4a, 4c and atypical 4b). Among these 13 serotypes, *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c and 4b are described as the four major *L. monocytogenes* serotypes isolated from foods and patients (Chen et al., 2016; Chlebicz and Śliżewska, 2018; Doumith et al., 2004; Gianfranceschi et al., 2009; Gilbreth et al., 2005; Orsi et al., 2011; Ragon et al., 2008; Ward et al., 2008). More specifically, serotype 1/2a, 1/2b, and 1/2c are most frequently isolated from food or the food production environment, while serotype 1/2a, 1/2b and 4b are more commonly associated with human clinical cases and outbreaks (Duze et al., 2021; Orsi et al., 2011; Swaminathan and Gerner-Smidt, 2007). This was also illustrated previously as more than 95% of human *L. monocytogenes* infections are caused by these three serotypes (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007).

A common way to classify newly isolated *L. monocytogenes* strains is by the serotyping technique. This technique is based on the variation in the somatic (O) and flagellar (H) antigens, antigenic determinants located on the cellular surface. The serotyping technique is based on the use of antisera and is a universally accepted subtyping method for *L. monocytogenes* (Borucki and Call, 2003; Chlebicz and Śliżewska, 2018; Gasanov et al., 2005; Seeliger and Höhne, 1979). This serotyping technique is described as a gold standard for *L. monocytogenes* serotype differentiation in the past (Borucki and Call, 2003), but this classical serotyping technique is laborious. Therefore, a more rapid and practical alternative serogrouping method is developed based on multiplex PCR. This method divides the four major serotypes of *L. monocytogenes* strains (1/2a, 1/2b, 1/2c and 4b) in four PCR serogroups: 1/2a-3a, 1/2c-3c, 4b-4d-4e and 1/2b-3b-7 (Doumith et al., 2004). The serogroups 1/2a-3a and 1/2c-3c, that are also mentioned with their respective serogroup name IIa and IIc, belong to lineage II. On the other hand, the serogroups 4b-4d-4e and 1/2b-3b-7 that are mentioned with their respective serogroup name IVb and IIb, belong to lineage I (Duze et al., 2021; Orsi et al., 2011).

Other genetic typing methods have been developed to further differentiate *L. monocytogenes* strains. These methods use data of the core genome, which is the collection of genes found among all strains of a particular species. This core genome can be applied for the identification of particular genomic characteristics of the

particular strain (den Bakker et al., 2013). Therefore, broader genetic relationships could be more appropriately studied by the use of multiple sequences of the core genome as the multi-locus sequence typing (MLST) method (Haase et al., 2014). This MLST method is considered a gold standard for bacterial typing (Larsen et al., 2012). It discriminates the *L. monocytogenes* strains in clonal complexes (CCs) and sequence types (STs) based on the sequence analysis of seven housekeeping genes (Ragon et al., 2008). The MLST uses these variations within the sequences of each of the sequenced housekeeping genes. Based on this variation, different alleles were attributed to these genes in which the combination of alleles determines the ST (Gasanov et al., 2005; Larsen et al., 2012). However, the traditional MLST typing method is expensive and time consuming, but MLST data could now be derived from whole genome sequencing (WGS) data (Larsen et al., 2012). The obtained WGS data can be applied in the MLST typing determination by substituting the classic MLST approach (Larsen et al., 2012). This WGS of bacterial genomes has been described with a high discriminatory power and information content (Leopold et al., 2014). With respect to bacterial strain typing, WGS is currently the method with the highest possible discriminatory power that could be used for the typing of pathogens (Rossen et al., 2018). Besides, the obtained WGS data can also be applied for determining the core genome MLST (cgMLST). This cgMLST uses the sequence variation in the core genome and provides high-resolution data across a group of related isolates (Maiden et al., 2013). The application of WGS (and the analytical post-sequencing approaches) can be used to compare a relationship between isolates. Moreover, it can also be used in identifying a link between clinical *L. monocytogenes* isolates and environmental samples or contaminated food products (Brown et al., 2019).

Data of WGS analyses may also be applied for determining gene presence/absence in bacterial genomes, such as genes associated with antimicrobial resistance or virulence and pathogenicity (Rossen et al., 2018). Determination of virulence genes via WGS is an approach for the characterization of the virulence profile of the isolated *L. monocytogenes* strains (Schiavano et al., 2022). This is important, as *L. monocytogenes* has a versatile arsenal of virulence factors to infect, survive, and replicate in a variety of host cell types (Cossart, 2011). Virulence factors responsible for key steps of *L. monocytogenes* intracellular parasitism are located on a genetic cluster named as Listeria Pathogenicity Island 1 (LIPI-1) (Vázquez-Boland et al., 2001). Another set of important virulence genes are internalin A (*inlA*) and internalin B (*inlB*). These are two major internalins of *L. monocytogenes* that are involved in

binding to and bacterial entry of the host cells (Cossart, 2011; Hamon et al., 2006). Also other internalins (virulence proteins containing leucine-rich repeats) identified in *L. monocytogenes* play an important role in the infection process (Bierne et al., 2007). The Listeria Pathogenicity Islands LIPI-3 and LIPI-4 are other virulence factors that also contribute to the pathogenicity of *L. monocytogenes*. However, the presence of LIPI-3 is described for only a subset of the Lineage I strains (Cotter et al., 2008). On the other hand, LIPI-4 is only present in particular clonal complexes of Lineage I strains (Kim et al., 2018; Maury et al., 2016). Variation in virulence-associated genes (absence/presence) could account, at least for a part, for differences in virulence among *L. monocytogenes* strains (Maury et al., 2016). Besides the full length presence of the virulence genes in the *L. monocytogenes* strains, the virulence genes could also be truncated or interrupted (internal deletion) (Maury et al., 2016). These non-intact virulence genes (as truncations or premature stop codons (PMSC) in *inlA*) are associated with loss or reduction of virulence (Maury et al., 2016; Nightingale et al., 2005).

1.4 Prevalence and persistence of *L. monocytogenes*

A thorough understanding of the ecology and diversity of *L. monocytogenes* is needed for effective and improved prevention of foodborne infections related to *L. monocytogenes* (Orsi et al., 2011; Sauders et al., 2006). Previous surveys have shown that *L. monocytogenes* is present in different environments, such as natural environments, urban environments (soil, vegetation, surface water, animal faeces, wastewater and other environmental locations) (Linke et al., 2014; Lyautey et al., 2007a; Lyautey et al., 2007b; Paillard et al., 2005; Sauders et al., 2006; Sauders et al., 2012; Vivant et al., 2013; Weis and Seeliger, 1975) and food processing environments (Belias et al., 2022; Ferreira et al., 2014; Jordan et al., 2018; Møretrø and Langsrud, 2004; Zoellner et al., 2018a). *L. monocytogenes* may enter food processing environments through contaminated raw materials, but also other ways are described such as via personnel, water and packaging supplies (Smith et al., 2018; Spanu and Jordan, 2020). *L. monocytogenes* from contaminated materials may subsequently spread throughout the food system (Fagerlund et al., 2022). This may lead to the contamination of the food processing environment after which *L. monocytogenes* may spread further throughout the facility after colonization (Ricci et al., 2018). In the food processing environment, *L. monocytogenes* may occupy the so-

called harbourage sites on materials, in which water and organic components can accumulate (Carpentier and Cerf, 2011). The most problematic sites and major contamination sources for *L. monocytogenes* in food processing environments are equipment, conveyor belts and other transport systems, trays, floors and drains (Belias et al., 2022; Gudbjörnsdóttir et al., 2004). Bacteria can shelter in these harbourage sites and may cause cross-contamination during food processing (Fagerlund et al., 2017), resulting in an increase of contamination during production and processing (Gudbjörnsdóttir et al., 2004; Gudmundsdóttir et al., 2005). Various studies indicated that raw materials were not the major source of food product contamination with *L. monocytogenes*, but that the major source of *L. monocytogenes* food contamination is the food processing environment (Di Ciccio et al., 2012b; Fallah et al., 2013; Jami et al., 2014; Vogel et al., 2001). A schematic scheme of the transmission route of *L. monocytogenes* during food production is visualized in figure 1.1.

Figure 1.1. A schematic scheme of the transmission route of *L. monocytogenes*. *L. monocytogenes* may be transferred from natural environments via agricultural materials to the food processing environment, leading to contamination of food products and which may eventually lead to foodborne diseases.

L. monocytogenes is a robust bacterial pathogen as it is able to cope with different stresses. Therefore, this pathogen is a major challenge for the food processing industry (Shamloo et al., 2019). It has the ability to adapt, survive and grow in environmental stress conditions that may be present in food processing environments (Osek et al., 2022). *L. monocytogenes* is able to grow at a wide temperature range with values between -1.5 °C and 45 °C (Lado and Yousef, 2007) in which temperatures between 30 °C and 37 °C are within the optimal growth temperature range (Allerberger, 2003; Lado and Yousef, 2007). *L. monocytogenes* is not able to grow at temperatures below -1.5 °C, but it can survive temperatures that are much lower. This is illustrated by a *Listeria* population that had a decrease of <1 log during a storage time of three months at temperatures of -18 °C to -20 °C (Lado and Yousef, 2007). Also, *L. monocytogenes* is able to grow in a wide pH range (4.0-9.6), at low water activity (a_w) (a_w as low as 0.90) and high salt concentrations (up to 10% NaCl) (Ferreira et al., 2014; Lado and Yousef, 2007; Mcclure et al., 1989). However, the actual growth of *L. monocytogenes*

depends on the combination of various environmental factors (Mcclure et al., 1989; Van der Veen et al., 2008).

The food processing environment may lead to the selection of particular *L. monocytogenes* strains, which may subsequently lead to the persistence of these strains in the food processing environments (Ferreira et al., 2014; Nowak et al., 2017; Wang et al., 2015). This can lead to *L. monocytogenes* strain persistence for months to years and even decades in food processing environments (Ferreira et al., 2014). Although *L. monocytogenes* persistence in food processing environments has not a clear consensus definition (Ferreira et al., 2014; Taylor and Stasiewicz, 2019; Unrath et al., 2021), the persistence of an *L. monocytogenes* strain generally refers to the repeated isolation of genetically related strains across a given time period in a given food processing environment (Daeschel et al., 2022). Persistence of *L. monocytogenes* has previously been described for various food processing environments. This includes among others swine slaughterhouses, fish processing plants, a poultry processing facility, a cheese production environment and also in a commercial fresh mushroom production facility (Cherifi et al., 2020; Holch et al., 2013; Melero et al., 2019; Nüesch-Inderbinen et al., 2021; Sun et al., 2021). Persistent *L. monocytogenes* isolates found in food processing environments are often isolated from surfaces of food equipment (machines, conveyor belts, cutting boards) or food environments (walls, floors, drains) (Møretrø and Langsrud, 2004). The key contributors to persistence have been identified as environmental factors, such as facilities and equipment that are difficult to clean (Ferreira et al., 2014). Besides, it was described that no single genetic marker was universally responsible for persistence of a strain in the food production environment of their isolation (Palaiodimou et al., 2021). Moreover, persistent *L. monocytogenes* isolates are probably not persistent based on strain specific phenotypic adaptations, but persistent strains more likely rely on a combination of environmental conditions and factors (Taylor and Stasiewicz, 2019). The WGS approach may help to decipher the persistence potential of *L. monocytogenes* strains in food processing environments (Unrath et al., 2021). Here, the subtyping of *L. monocytogenes* isolates by WGS is necessary to differentiate if the isolates belong to the persistent strain types (Koutsoumanis et al., 2020). The persistence of *L. monocytogenes* in food processing environments is a major threat to the food producing companies or food associated environments due to its continued presence, and this may lead to repeated contamination of food products (Belias et al., 2022; Cherifi et al., 2020; Taylor and Stasiewicz, 2019).

A wide range of food products has previously been observed to be contaminated with *L. monocytogenes* (Gandhi and Chikindas, 2007; Leong et al., 2014). This includes *L. monocytogenes* contamination of meat (Gómez et al., 2015; Kurpas et al., 2020; Leong et al., 2017; Muhterem-Uyar et al., 2015; Uyttendaele et al., 2009; Vitas et al., 2004; Wu et al., 2015), seafood and smoked fish (Leong et al., 2017; Uyttendaele et al., 2009; Vitas et al., 2004; Wu et al., 2015), milk, cheese and other dairy products (Greenwood et al., 1991; Leong et al., 2017; Muhterem-Uyar et al., 2015; Pyz-łukasik et al., 2021), fresh produce as fruits and vegetables (Balali et al., 2020; Leong et al., 2017; Maćkiw et al., 2021; Wu et al., 2015; Zhu et al., 2017) and frozen fruits and vegetables (Aguado et al., 2004; Maćkiw et al., 2021; Vitas et al., 2004; Willis et al., 2020). Several of these food products that have been contaminated with *L. monocytogenes* have also been implicated in listeriosis outbreaks (Buchanan et al., 2017; Cartwright et al., 2013; CDC, 2016; Jordan and McAuliffe, 2018; Lopez-Valladares et al., 2018; Martinez-Rios and Dalgaard, 2018; Raheem, 2016; Zhu et al., 2017). Following *L. monocytogenes* strain isolation from food and from food processing environments, these isolated strains can be subtyped to determine the strain diversity in that particular environment. Strain diversity determination has shown a low genomic diversity of *L. monocytogenes* strains that were isolated from conveyor belt surfaces in a swine slaughterhouse (Shedleur-Bourguignon et al., 2021). This is however in contradiction to other food environments or food products in which isolated *L. monocytogenes* strains showed a high genomic diversity. This high genomic diversity was previously observed for retail pork samples from various slaughterhouses, milk equipment from dairies, mushroom processing facilities, dairy farms and various farm products (Borucki et al., 2005; Elsayed et al., 2022; Kim et al., 2018; Pennone et al., 2018; Suo et al., 2022; Wang et al., 2021). Also, a large set of *L. monocytogenes* strains isolated from various ready-to-eat (RTE) food products and food processing environments were categorized in multiple serotypes (Acciari et al., 2022). Moreover, multiple serogroups have been observed after isolation of *L. monocytogenes* from RTE foods and pasteurized milk (Chen et al., 2020; Shen et al., 2006).

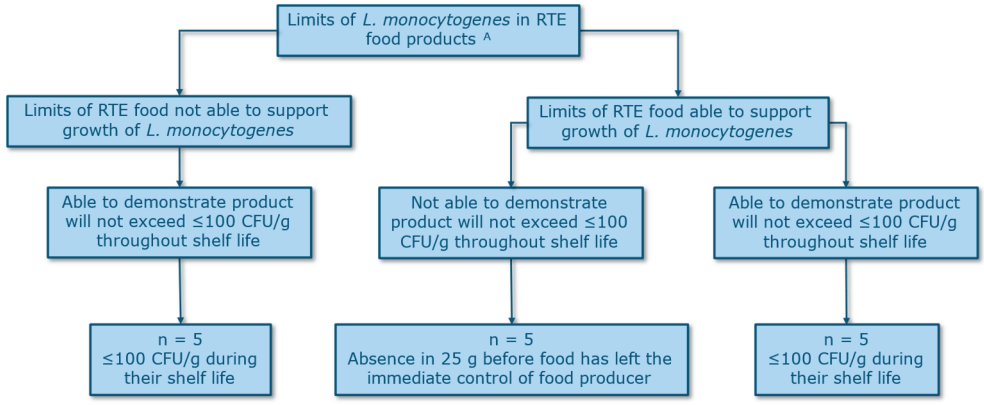
L. monocytogenes has also been isolated from fresh mushrooms (Balali et al., 2020; Chen et al., 2018; Wu et al., 2015), from frozen mushrooms (Willis et al., 2020) and in mushroom processing environments (Sun et al., 2021; Xu et al., 2023). More specifically, *L. monocytogenes* has been isolated from the white button mushroom (*Agaricus bisporus*) as has been reported during multiple recalls (Anonymous, 2012;

2016; 2021a; 2021b). Moreover, *L. monocytogenes* has been isolated in *A. bisporus* mushroom processing environments and multiple serogroups have been observed after isolation of *L. monocytogenes* from these mushroom processing environments (Murugesan et al., 2015; Pennone et al., 2018; Viswanath et al., 2013). As *L. monocytogenes* is a severe human pathogen, this may pose a possible health threat to consumers. In addition, the recalls of contaminated *A. bisporus* mushrooms may also lead to a negative impact for the mushroom industry. Such contamination may then lead to large economic losses, as high social and economic costs were observed for other food products that were contaminated with *L. monocytogenes* (Olanya et al., 2019; Thomas et al., 2015). Notably, to date, no listeriosis cases have been reported due to consumption of *A. bisporus* mushrooms, although another mushroom species (enoki mushrooms) has been reported in multiple listeriosis outbreaks (CDC, 2023; FDA, 2020). The absence of *L. monocytogenes* infection cases in relation to *A. bisporus* mushrooms may be attributed to the fact that *A. bisporus* mushrooms are generally processed (cooked, baked or stir-fried) before consumption (Borgdorff, 2012). Such a processing step will lower the risk of an infection with *L. monocytogenes*. Nevertheless, high attention should be paid to the presence of *L. monocytogenes* on *A. bisporus* mushrooms. This is important, as the *A. bisporus* mushroom is one of the most cultivated mushroom species worldwide. More specifically, it is the most widely cultivated mushroom species in the United States, Europe, New Zealand and Australia (Sonnenberg et al., 2011). The Netherlands is the fourth largest producer worldwide, after China, the United States and Poland (Robinson et al., 2019; Royse et al., 2017). A large portion (90%) of these *A. bisporus* mushrooms that are produced in the Netherlands is exported (Royse et al., 2017). This all showed that the *A. bisporus* mushroom type has an important agricultural value for the Netherlands. Therefore, it is important to determine and understand the presence and behaviour of *L. monocytogenes* in *A. bisporus* mushroom processing conditions.

1.5 *L. monocytogenes* food legislation

Food products contaminated with *L. monocytogenes* are an important food safety issue. Therefore, high attention must be paid to the presence of *L. monocytogenes* in food products and especially the RTE food products. This, as RTE food products are foods intended by the producer for direct human consumption without the need for cooking or any other processing step for elimination or reduction of pathogens to an

acceptable level of concern (Bergis et al., 2021; EC (European Commission), 2005). Strict regulations apply with respect to *L. monocytogenes* in RTE food products and recalls may be performed if the RTE food products do not comply with the legislation. For the United States, the 'zero tolerance policy' of the US Food and Drug Administration (FDA) and US Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) for RTE food products is applied. This means the limit of detection for approved methods and includes the absence of *L. monocytogenes* in 2x or 1x 25 gram of food product, respectively (Archer, 2018; Farber et al., 2021; Shank et al., 1996). RTE food products sold in the European Union should also comply with the legislations. These criteria are however different for food products that support growth of *L. monocytogenes* compared to food products that do not support *L. monocytogenes* growth (EC (European Commission), 2005) (see figure 1.2 for legislation). This makes it important to determine the growth characteristics of *L. monocytogenes* on food products. Such growth characterization tests were previously reported for many different types of food products including RTE foods (Alegbeleye and Sant'Ana, 2022; Culliney and Schmalenberger, 2020; Flessa et al., 2005; Salazar et al., 2017; Salazar et al., 2022; Ziegler et al., 2018; Ziegler et al., 2019). It has been mentioned previously in several cases that mushrooms may also be considered as a RTE food product (Food Safety Authority of Ireland (FSAI), 2006; Health Canada, 2022). Therefore, it is also important to perform growth tests on *A. bisporus* mushrooms to determine if this mushroom species is able to support the growth of *L. monocytogenes*. Such tests were previously executed by various authors, but obtained results were however contradictory. Some studies reported growth of *L. monocytogenes* on whole and sliced *A. bisporus* mushrooms (Chikthimmah et al., 2007; Leong et al., 2013), while another study reported no growth of *L. monocytogenes* on the whole *A. bisporus* mushrooms (Leong et al., 2015). Moreover, one study only reported growth of *L. monocytogenes* on whole mushroom products during the lag phase of microbiota communities associated with *A. bisporus* (González-Fandos et al., 2001). Such an effect was however not reported in the other studies, although it has been reported that background microbiota that are present on the *A. bisporus* mushrooms have high counts (7.3 - 10.3 log CFU/gram) (Donzellini et al., 2018; Venturini et al., 2011) and are highly diverse (Leff and Fierer, 2013). These microbial communities on mushrooms were reported to contain relatively high abundances of Pseudomonadaceae, whereas abundances of *Enterobacteriaceae* are relatively low compared to other fruits and vegetables (Leff and Fierer, 2013).



[A] RTE food product not intended for infants and special medical purposes.

Figure 1.2. Definitions of the microbiological criteria for *L. monocytogenes* in ready-to-eat (RTE) foods others than those intended for infants and special medical purposes. Described by EU regulations in EC No 2073/2005 and adapted from EC No 2073/2005.

Besides the characterization of *L. monocytogenes* on food products, determination of growth parameters of *L. monocytogenes* are however mainly executed in laboratory medium (Aryani et al., 2015a; Aryani et al., 2015b; Capita et al., 2019; Taylor and Stasiewicz, 2019). This is despite the suggestions that nutrient sources used in laboratory conditions should resemble the conditions of the food processing environment as closely as possible (Overney et al., 2016). Therefore, as this thesis aims to assess the growth of *L. monocytogenes* in the specific *A. bisporus* mushroom processing environmental conditions, this should comprise the appropriate nutrient source. For that reason, this should include mushroom-derived medium that mimics the mushroom-derived liquid that is abundantly present during mushroom harvesting and processing. The mushroom-derived medium may contain various nutrient source components as the *A. bisporus* mushroom species contains among others sugar components, fatty acids, proteins, various amino acids, minerals and vitamins (Mattila et al., 2001; Mattila et al., 2002; Reis et al., 2012). The assumed nutrient richness of the *A. bisporus* mushrooms may suggest that the mushroom-derived medium is a potential good nutrient source for growth of *L. monocytogenes*, and this hypothesis will be tested in this thesis.

1.6 *Agaricus bisporus* mushroom production and processing chain

The production and the processing of the *A. bisporus* mushrooms involves many different steps at multiple companies. The process starts at the composting company that produces compost, the nutrient source in which the *A. bisporus* mycelium grows. For the Dutch composting companies, the main compost component is (wheat-) straw-bedded horse manure that consists, based on dry matter, of approximately 54% straw and 46% horse droppings. The compost mixture consists of approximately 88.5% of this straw-bedded horse manure. Other compost components are broiler chicken manure that functions as nitrogen source (9%) and gypsum that functions as a calcium sulphate source for pH buffering and for minimizing greasiness (2.5%). These compost components are mixed thoroughly together with water (approximately 300-900 litre of water per 1,130 kg of compost components) after which the mixture is placed in special aerated tunnels. The temperatures in the tunnels rises to 80 °C after the addition of oxygen. The mixture is turned around after 2-3 days and incubated for another 2-3 days at 80 °C. This is the phase I composting step and usually takes around 1 week. Then, the phase I compost is transported to other tunnels for the phase II composting step. The phase I compost is mixed with small amounts (0.5%) of the previous batch of phase II compost to allow transfer of micro-organisms to the final phase I compost. This compost mixture is heated for 8 hours at 56 °C-60 °C followed by incubation for 5 days at 45 °C-50 °C for obtaining the phase II compost. This composting step decreases the amount of ammonia in the process air during this 5 day incubation. This is important as free ammonia in the air and in compost is toxic to the mushroom mycelium. Following the phase II composting step, the compost is ready for the growth of mycelium. For this, the phase II compost will be inoculated with sterilised rye grains that are overgrown by the *A. bisporus* fungus (also known as spawn) (phase III). Then the fungus is able to grow through the compost for approximately 17-19 days at 25 °C. The different temperatures during all these phase I, phase II and phase III steps are carefully monitored by the composting company. After the fungus has fully grown through the compost, the compost is subsequently transported to the locations that produce mushrooms, i.e. the mushroom growers. Here, the substrate is filled on long shelves in climate chambers, while simultaneously being covered with casing soil. This casing soil is produced at another company, and is a mixture that is made of black peat (pH=4.5) and some sugar beet lime, a by-product of the sugar beet production, which is added to the black peat so the pH of the final casing soil increases (pH=7.2). This casing soil mixture creates a microclimate

for the growth of mushrooms i.e. the presence of micro-organisms within the casing soil initiates the fruiting body formation (mushrooms growth) on the surface. During the filling the climate chamber, the first step that is applied by the mushroom growers nowadays is the technique called 'caccing'. This means that a little bit of compost is pulled through the casing soil layer. This allows a faster growth of the mycelium in the casing soil than without the 'caccing' technique. The mycelium is then able to grow through the casing soil during 5 days of incubation. Following this 5-day incubation, the climate rooms are ventilated, after which the mycelium initiates fruiting body formation on the top surface of the casing soil. The first flush can be harvested 10 days after ventilation, so the mushroom growers harvest the first mushrooms approximately 15 days after covering the substrate with the casing soil (information by communication with mushroom companies, see also (Anonymous, 2023; Royse et al., 2017; Royse and Beelman, 2023; Straatsma et al., 2000; Van Griensven, 1988; Van Griensven and Van Roestel, 2004)).

The mushroom harvest can be performed in two ways; with machines or by hand. During machine harvesting, all mushrooms are harvested at once after which it will take around 8 days for the next mushroom harvest. This way of mushroom harvesting is usually performed 2 or 3 times from the same substrate and casing soil combination. Mushroom harvesting by hand-picking is usually done 2-3 weeks from the same substrate and casing soil combination (information by communication with mushroom companies, see also (Anonymous, 2023)). The hand-picked and machine-harvested *A. bisporus* mushrooms supply different markets. The hand-picked mushrooms are not processed and are intended for the fresh market, while the machine-harvested mushrooms are processed by the mushroom processing industry (Smit et al., 2013). Approximately 2/3 of the total Dutch mushroom production is intended for the processing industry, while 1/3 of the total mushroom production is intended for the fresh market (Boon, 2017). Mushrooms intended for the processing industry are mainly processed into frozen sliced mushrooms or they are preserved by canning (Boon, 2015; Boon, 2017) (A schematic drawing of mushroom harvesting and processing into frozen sliced mushrooms is displayed in figure 1.3). The canned mushrooms first undergo a blanching step followed by a bacterial elimination step by means of sterilization (Diamantopoulou and Antonios, 2015). The frozen mushroom on the other hand, are produced without the application of a thermal treatment (Baars, 2006). Freezing mushrooms provides storage stability and offers the consumers a product with high nutritional value (Diamantopoulou and Antonios, 2015). Moreover, the

freezing of mushrooms is described as the best processing method for preserving the natural taste and aroma (Bernaś et al., 2006). The duration of frozen storage may however affect the nutrients present in the mushroom product and the sensory quality (Jaworska et al., 2008). The temperature at which frozen mushrooms are stored also has an effect on the practical storage life. This is indicated with a lower practical storage life at -12 °C (2 months), and a higher practical storage life at lower temperatures (8 months when stored at -18 °C, and >24 months when stored at -24 °C) (Zaritzky, 2008). The shelf life of frozen stored food products is mostly limited by adverse chemical (enzymatic and oxidative) and physical (freezer burn) changes, rather than it is affected by microbiological concerns (Golden and Arroyo-Gallyoun, 1997). Freezing food products however results in the conservation of bacteria on the food product, and thawing the food product may result in the growth of the bacteria (USDA-FSIS, 2023). Hence, frozen contaminated food products may pose a potential safety risk for *L. monocytogenes* infection.

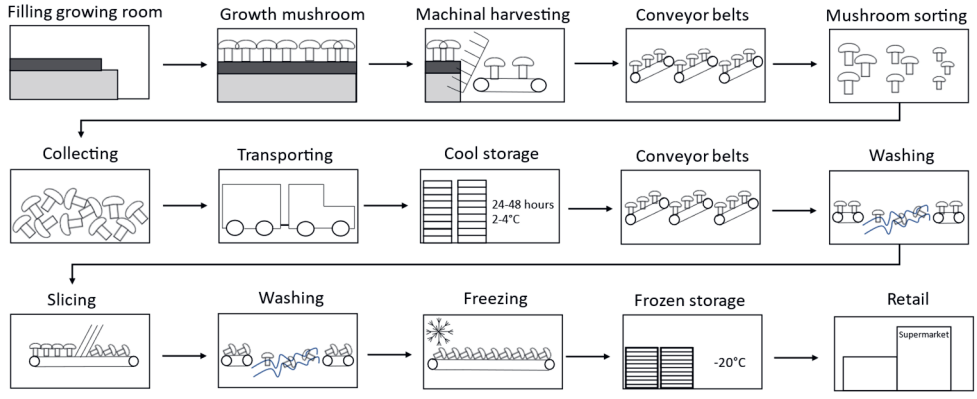


Figure 1.3. Schematic drawing of the (most important) processes during the production and processing of frozen sliced mushrooms.

1.7 Biofilm formation *L. monocytogenes*

Bacterial cells present in food processing environments are most commonly found as an aggregation of micro-organisms attached to and growing on surfaces and embedded in an extracellular matrix, which are generally named as biofilms (Branda et al., 2005; Mazaheri et al., 2021; Møretrø and Langsrud, 2004; Srey et al., 2013). Biofilms are complex communities of micro-organisms in which these micro-organisms are held together by the biofilm matrix components, like polysaccharides, secreted proteins and

extracellular DNA (Muhammad et al., 2020). The ability of *L. monocytogenes* to form biofilm structures on various surfaces present in food processing environments represents a serious concern to food safety, since biofilms serve as a potential contamination source (Colagiorgi et al., 2017). The biofilm formation is described by multiple steps in a dynamical process (Colagiorgi et al., 2017; Srey et al., 2013; Zhang et al., 2021). It starts with the initial attachment to the surface after which the attachment becomes irreversible and an early structure of the biofilm begins to develop (Srey et al., 2013). This is followed by maturation of the biofilm in which it develops an organized structure after which the biofilm cells may disperse into the environment (Srey et al., 2013). A schematic overview of biofilm formation is displayed in figure 1.4. The ability to form biofilm varies between bacterial species, serotypes and strains of a particular bacterial species (Srey et al., 2013). Therefore, a lot of research has been performed to determine the biofilm formation of (pathogenic) bacteria from the food industry (Carrascosa et al., 2021; Galié et al., 2018; Van Houdt and Michiels, 2010), including biofilm formation of the foodborne pathogen *L. monocytogenes* (Di Ciccio et al., 2012a; Mazaheri et al., 2021; Møretrø and Langsrud, 2004). The different species can form different biofilm structures such as flat or mushroom shaped structures (Rumbaugh and Sauer, 2020; Srey et al., 2013). In case of *L. monocytogenes* biofilms, strains formed an uniform distribution of cells on the surface that leads to single or several layers of cells that covered the surface as a uniform flat biofilm (Balsa-Canto et al., 2020; Kalmokoff et al., 2001; Rieu et al., 2008). The majority of naturally formed biofilms consist of multiple bacterial species (Yang et al., 2011). The maturation of these biofilms in general leads to structures that may appear as a pyramid or mushroom-shaped multicellular structure on the surface (Dunne, 2002). The bacterial cells may be detached from these biofilm structures when food is

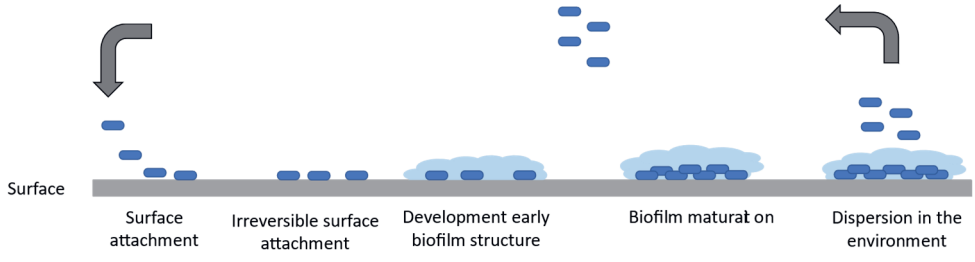


Figure 1.4. Schematic presentation of the different stages of biofilm formation. Five stages of biofilm formation are described and include the (1) the reversible attachment to the surface, (2) the irreversible attachment, (3) the early formation of a biofilm structure, (4) the formation of a multidimensional biofilm structure and (5) dispersion of biofilm cells in the environment.

processed on contaminated food contact surfaces, and this may lead to cross-contamination of food products (Giaouris et al., 2014; Gudbjörnsdóttir et al., 2004).

The bacterial compositions and interactions within biofilms and the growth of *L. monocytogenes* are affected by several environmental factors. These factors include among others raw materials, nutrient availability, surface material, temperature, pH, humidity and cleaning and disinfection (Fagerlund et al., 2021). Of these multiple factors, the biggest effects were attributed to temperature and medium/nutrient availability (Dygico et al., 2020; Kadam et al., 2013; Nowak et al., 2015; Tomiĉić et al., 2016). The type of medium is relevant for *L. monocytogenes* biofilm formation as the amount of biofilm that is formed varied between various nutrient conditions (Kadam et al., 2013; Nowak et al., 2015; Stepanović et al., 2004). Here, the nutrient richness of the applied media influenced the biofilm formation of *L. monocytogenes* (Lee et al., 2019; Nowak et al., 2015; Stepanović et al., 2004). Moreover, *L. monocytogenes* is able to adhere within a short contact time to a wide variety of surfaces types (Beresford et al., 2001). Following adherence, *L. monocytogenes* is able to form biofilms on these various surface types, including surface types present in food processing environments such as stainless steel and plastics as polystyrene and polypropylene (Di Bonaventura et al., 2008; Dygico et al., 2020; Mazaheri et al., 2021; Møretrø and Langsrud, 2004; Poimenidou et al., 2016; Ramires et al., 2021; Skowron et al., 2018). The type of surface material is an important factor for the attachment and biofilm formation of *L. monocytogenes* as biofilm formation differs on material surface (Di Bonaventura et al., 2008; Di Ciccio et al., 2012a; Luo et al., 2022; Møretrø and Langsrud, 2004; Skowron et al., 2018; Srey et al., 2013). The biofilm forming ability of *L. monocytogenes* was influenced by the surface roughness and this was found to be positively correlated with the level of biofilm formation (Chaturongkasumrit et al., 2011; Dygico et al., 2020).

Biofilm formation has been shown to be different between various *L. monocytogenes* strains (Chae and Schraft, 2000). Possible correlations between biofilm formation and lineage type, serotype or source of isolation were non-conclusive amongst the different studies (Folsom et al., 2006; Guilbaud et al., 2015; Kadam et al., 2013; Lee et al., 2019). Some studies observed that biofilm formation differed between serotypes (Di Bonaventura et al., 2008; Kadam et al., 2013), while other studies did not find a correlation between biofilm formation and serotype (Borucki et al., 2003; Doijad et al., 2015), mentioning that biofilm formation of individual *L. monocytogenes* strains is

strain-dependent (Doijad et al., 2015). Besides, higher biofilm formation was observed for lineage II as compared to lineage I (Borucki et al., 2003; Combrouse et al., 2013), but also the other way around (Djordjevic et al., 2002; Takahashi et al., 2009). This indicates that particular environmental conditions may lead to specific outcomes in the biofilm formation of *L. monocytogenes* strains.

Biofilms may consist of a single microbial species, but may also consist of a combination of different microbial species forming multispecies biofilms (Liu et al., 2016; Muhammad et al., 2020). This is important, since a major factor influencing the growth behaviour of *L. monocytogenes* in food processing environments is the accompanying microbiota (Fagerlund et al., 2017; Heir et al., 2018; Langsrud et al., 2016). These accompanying microbiota are constantly introduced in the food processing environment via raw materials, water, equipment and staff (Bjørge Thomassen et al., 2023). The presence of various types of microbiota has been documented for many food processing environments including food processing plants that produce fresh produce (Bagge-Ravn et al., 2003; Gudbjörnsdóttir et al., 2005; Kaneko et al., 1999; Lehto et al., 2011; Liu et al., 2013). Particular accompanying microbiota present in food processing environments may be present as "core microbiota" (Cobo-Díaz et al., 2021) or as "in house microbiota" (Bokulich and Mills, 2013). The main groups/genera of background microbiota present in food processing environments include *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae*, spore-forming bacteria, *Staphylococcus* spp., and lactic acid bacteria (Møretrø and Langsrud, 2017). The accompanying microbiota present on food processing environmental surfaces in the food industry have however a high diversity (Møretrø and Langsrud, 2017). This was more specifically documented for the microbial diversity in the food processing environments of salmon, meat, dairy and vegetables (Bjørge Thomassen et al., 2023; Cherif-Antar et al., 2016; Einson et al., 2018; Fagerlund et al., 2017).

The microbiota present in food processing environments may influence the growth of *L. monocytogenes* in multispecies biofilms in different ways (Fagerlund et al., 2021). The development of these multispecies biofilms is especially thought as a process of neutral, cooperative and competitive events (Carpentier and Chassaing, 2004; Fagerlund et al., 2021; Liu et al., 2016; Rodríguez-López et al., 2015). Usually, the counts of *L. monocytogenes* in multispecies biofilms were lower than in *L. monocytogenes* monospecies biofilms (Carpentier and Cerf, 2011; Fagerlund et al., 2021). These lower counts of *L. monocytogenes* in multispecies biofilms may be

explained by the competition for nutrients (Fagerlund et al., 2021). Various studies also attribute the negative impact of *L. monocytogenes* fitness to the production of antimicrobial substances, such as bacteriocins, that can be produced by lactic acid bacteria (Zilelidou and Skandamis, 2018). Moreover, also the drop in pH may affect the growth of *L. monocytogenes* during incubation with microbiota (Guerrieri et al., 2009). This highlights the importance of co-culturing *L. monocytogenes* and microbiota originating from food processing environments when characterizing the growth of *L. monocytogenes* in complex microbiota mixtures present in these environments (Sinclair et al., 2022).

1.8 Effect of cleaning and disinfection

Bacterial cells that are present in biofilms possess higher resistance towards disinfectants than bacteria present in the planktonic culture/loosely adherent cells (Aarnisalo et al., 2000; Azizoglu et al., 2015; Norwood and Gilmour, 2000; Van Houdt and Michiels, 2010; Wirtanen and Salo, 2003). Comparing these results of biofilm and planktonic cultures clearly showed the protection of the biofilm against disinfectants (Wirtanen and Salo, 2003). Therefore, hygiene measurements comprising cleaning and disinfection (C&D) are important for the safety processes in food processing environment (González-Rivas et al., 2018). The C&D practices aim to reduce the presence of surface attached viable microbes and therefore reduce the risk of *L. monocytogenes* exposure in food production environments (Hu et al., 2023; Wirtanen and Salo, 2003). First, the cleaning is performed in which visible or invisible dirt on the surface is eliminated. The chosen cleaning agents are based among others on the processed food product and most of the cleaning agents in the food industry are alkaline compounds (González-Rivas et al., 2018). After cleaning, disinfection treatment is applied to surfaces and equipment in order to totally remove micro-organisms or reduce the level of micro-organisms to an acceptable level (González-Rivas et al., 2018; Mazaheri et al., 2021). Various kinds of disinfection agents are approved for their use in food processing environments. The use of a particular disinfectant depends on the used material and the attached micro-organisms (González-Rivas et al., 2018; Wirtanen and Salo, 2003), but the formation of undesirable by-products residues should also be taken into account (Gil et al., 2009). Removal of *L. monocytogenes* biofilms from surfaces has been shown to vary among different kinds of disinfectants (Skowron et al., 2018). Moreover, disinfectant

effectiveness towards *L. monocytogenes* biofilms was affected by the surface material on which the biofilms have been formed (Di Ciccio et al., 2012a; Poimenidou et al., 2016; Skowron et al., 2018).

Several disinfectants are used in the mushroom industry, and peracetic acid is among one of the frequently used disinfectants (O'Neill et al., 2015). The important advantages of the peracetic acid disinfectant over other compounds are its strong oxidizing capabilities, its environmental friendliness and that it has no known cytotoxic effects (Zoellner et al., 2018b). Another advantage of the peracetic acid disinfectant is that it degrades in no harmful by products, leaving no residues behind (Chhetri et al., 2014; Zoellner et al., 2018b). Organic matter may however impair the efficiency of disinfectants (Bridier et al., 2011; Nyati et al., 2012; Srey et al., 2013). This was also observed for the peracetic acid solution as the main antimicrobial compound in this disinfectant is very unstable and reacts easily with organic material (Zoellner et al., 2018b). For this reason, higher disinfectant concentrations are needed to receive the same amount of reduction for dirty conditions compared to clean conditions (González-Rivas et al., 2018). Therefore, it is important to establish and maintain a good cleaning process prior to the application of disinfection in the food industry. This removal of the organic matter before disinfectant application maximizes the disinfectant efficacy (Korany et al., 2018). However, conventional or routine C&D procedures are often ineffective in the removal and elimination of micro-organisms present on surfaces in the biofilm structure (Mazaheri et al., 2021; Møretrø and Langsrud, 2004). Also *Listeria* spp. (including *L. monocytogenes*) were not completely eliminated from the food processing environments by conventional cleaning methods (Gudmundsdóttir et al., 2005). This resulted in the survival of *L. monocytogenes* in various food processing facilities after the application of C&D regimes (Brauge et al., 2020; Conficoni et al., 2016; Fagerlund et al., 2017). The disinfection resistance of biofilm cells may be attributed to bacterial adaptation, stress responses or presence of subpopulations that may be especially resistant to C&D (Langsrud et al., 2003; Mazaheri et al., 2021; Wulff et al., 2006). Other studies mentioned that the disinfection susceptibility of individual *L. monocytogenes* strains is strain-dependent (Pricope et al., 2013; Rodríguez-Campos et al., 2019; Skowron et al., 2019). Moreover, the biofilm structure and the components that are present in the biofilm structure may cause a restricted diffusion of disinfectants in the biofilms, leading to biofilm cell survival (Bridier et al., 2011). Presence of *L. monocytogenes* on food contact surfaces after disinfection indeed indicates insufficient disinfection procedures (Gudbjörnsdóttir et al., 2004), although

L. monocytogenes counts declined after C&D treatments compared to the sampling before C&D (Conficoni et al., 2016). This showed that conventional C&D procedures may be insufficient in the removal of micro-organisms (including *L. monocytogenes*) from surfaces. Therefore, it is important to determine the effect of C&D regimes applied in mushroom food processing environments.

1.9 Objective and outline of this thesis

The aim of this study was to obtain a better understanding of the eco-physiological behaviour of *L. monocytogenes* in the production and processing chain of frozen sliced *A. bisporus* mushrooms.
An outline of this thesis is presented in figure 1.5.

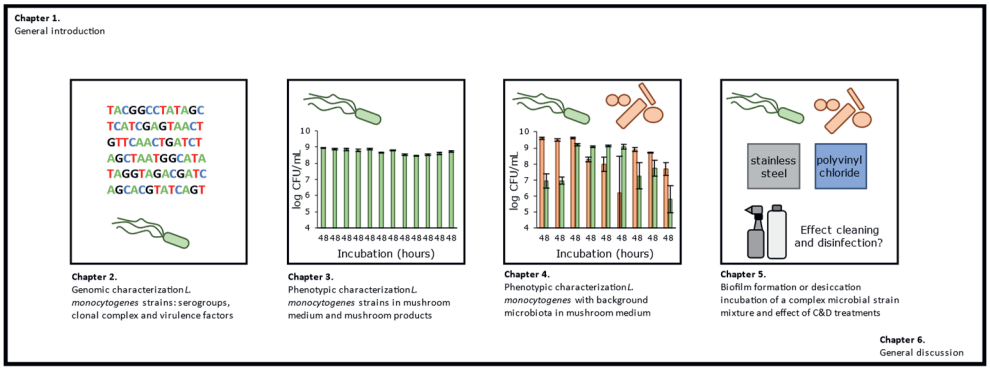


Figure 1.5. Graphical presentation of the thesis outline.

Chapter 1 gives an overview of the presence and possible transmission routes of *L. monocytogenes* in mushroom production and processing environments, the physiology of *L. monocytogenes* and the current knowledge of cleaning and disinfection treatments applied in food processing environments.

Chapter 2 focuses on the diversity of *L. monocytogenes* strains present in the production and processing chain of frozen sliced mushrooms. Multiple methods were applied for genetic and phenotypic characterization of the isolated *L. monocytogenes* strains. This revealed new insights in diversity, virulence repertoire and antibiotic resistance of *L. monocytogenes* strains isolated from frozen sliced mushroom processing facilities.

Chapter 3 focusses on strain variability of *L. monocytogenes* in conditions that mimicked the mushroom processing environment. The growth and biofilm characteristics were determined for *L. monocytogenes* strains isolated from mushroom processing environments and for strains isolated from other food product (environments) and human (clinical) patients to compare phenotypic characteristics. Moreover, growth performance test of *L. monocytogenes* mushroom strains were executed on mushroom products to determine *L. monocytogenes* growth potential in the presence of product-associated microbiota.

Chapter 4 focusses on the effect of background microbiota on the phenotypic behaviour of *L. monocytogenes*. Growth and biofilm formation of *L. monocytogenes* was assessed in dual-culture experiments and spent media incubations involving highly abundant background microbiota strains. Also closely related *Listeria* species were included to determine their effect on *L. monocytogenes* behaviour. The pH and organic acids of the mushroom media were determined to evaluate possible inhibiting effects and the nutrient availability of this medium.

Chapter 5 focusses on *L. monocytogenes*' behaviour in complex microbial biofilms formed on mushroom processing surfaces, namely, polyvinyl chloride and stainless steel. The biofilms formed on these materials were repeatedly exposed to cleaning and disinfection regimes followed by re-incubation in fresh medium. This allowed the determination of the survival, presence, abundance, diversity and regrowth capabilities of the *L. monocytogenes* strains in the monospecies mixture and the multispecies mixture conditions. Moreover, presence, abundance and diversity of *L. monocytogenes* was determined in desiccated suspensions of planktonic cultures of *L. monocytogenes* monospecies and multispecies conditions on polyvinyl chloride and stainless steel. These suspensions were also exposed to cleaning and disinfection agents for determining the efficacy of these agents towards desiccated cells.

Chapter 6 provides a general discussion of the results from this thesis, including the evaluation of the possible transmission routes and growth characteristics of *L. monocytogenes* in mushroom processing environmental conditions, the effect of associated microbiota on *L. monocytogenes* growth, and a quantitative model to predict *L. monocytogenes'* growth along the different steps of the mushroom processing chain. Moreover, possible strategies to reduce establishment and to prevent persistence of *L. monocytogenes* in food processing environments are discussed.

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2

Genomic characteristics of *Listeria monocytogenes* isolated during mushroom (*Agaricus bisporus*) production and processing

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Abstract

Listeria monocytogenes is a foodborne pathogen ubiquitously found in nature and which has been isolated from food and food processing environments. This study aimed to characterize *L. monocytogenes* strains isolated from the production and processing environments of frozen sliced mushrooms (*Agaricus bisporus*). An analysis was executed along the mushroom processing chain including one mushroom grower and two mushroom processing factories. A total of 153 *L. monocytogenes* strains were isolated, which could be grouped in three PCR serogroups, namely, serogroup 1/2a-3a (39.2%), serogroup 1/2b-3b-7 (34.0%) and serogroup 4b-4d-4e (26.8%). A selection of 44 *L. monocytogenes* strains isolated from the processing environment after cleaning and disinfection (C&D) and from frozen sliced mushrooms were genotyped by whole genome sequencing (WGS), because these strains pose a potential risk for product contamination after C&D and for human consumption. Multi Locus Sequence Typing (MLST) revealed 11 Clonal Complexes (CCs), with strains belonging to CC1, CC4, CC37 and CC87 being detected in both processing factories. Comparative WGS analysis of the 44 strains showed the presence of Listeria pathogenicity island-1 (LIPI-1) with a disrupted version of *actA* in all CC1, CC4, CC5, CC59 strains, and all but one CC224 strains. Notably, both *inlA* and *inlB* were detected as full-length loci in every strain, except for *inlA* in a CC6 strain that harbored a three amino acid deletion. LIPI-3 was detected in all CC1, CC4, CC6 and CC224 strains, while LIPI-4 was detected in all CC4 and CC87 strains. In addition, antibiotic susceptibility tests showed susceptibility towards fourteen antibiotics tested. The *bcrABC* operon was found in one CC5 strain, that showed a higher tolerance towards benzalkonium chloride than any other strain tested with confluent growth till 12.5 µg/mL for the CC5 strain compared to 2.5 µg/mL for the other strains. This study highlights that the ecology of *L. monocytogenes* in the frozen sliced mushroom production chain is highly diverse, and shows the importance of hygienic measures to control *L. monocytogenes* along the frozen sliced mushroom production chain.

2.1. Introduction

Listeria monocytogenes is a major foodborne pathogen that can cause listeriosis in humans. This disease is especially relevant for sensitive population groups (the elderly, immunocompromised persons, pregnant women and infants). Infection with *L. monocytogenes* could lead to spontaneous abortion in pregnant women, septicemia or meningitis and infections have led to a high case fatality rate of 17.6% in the EU in 2019 (EFSA and ECDC, 2021).

L. monocytogenes has been isolated in food products such as ready-to-eat (RTE) vegetables and frozen vegetables (Moravkova et al., 2017). Presence of *L. monocytogenes* on food products may be attributed to its ubiquity in nature (Sauders et al., 2006; Sauders et al., 2012) and its robustness to cope with different stresses as *L. monocytogenes* is able to grow at low pH, high salt concentrations and at refrigeration temperatures (Van der Veen et al., 2008; Walker et al., 1990). These characteristics could be of serious concern for the RTE foods industry, as RTE foods are intended by the producer for direct consumption without the need for cooking or other type of processing (European Comission (EC), 2014). This concerns the mushroom industry, as the white button mushroom (*Agaricus bisporus*) can be classified as a RTE food. This includes both hand-picked mushrooms that are sold fresh and machine-harvested mushrooms that are sliced and sold frozen, although the frozen mushrooms are usually not classified and sold as RTE food by the producer.

L. monocytogenes has been detected in the *A. bisporus* mushroom production environment (Pennone et al., 2018), on a variety of edible mushrooms (*A. bisporus* not included) (Chen et al., 2018) and on frozen mushrooms (Willis et al., 2020). *L. monocytogenes* strains isolated from *A. bisporus* mushroom production environments were demonstrated to form biofilms on materials used in the mushroom processing industry (Dygico et al., 2020). Detection of *L. monocytogenes* has led to a limited number of mushroom recalls, including recalls of fresh enoki mushrooms in Europe (Pennone et al., 2018) and fresh sliced white mushrooms in Canada (Anonymous, 2012). To date, in spite of no reports of listeriosis cases associated with the consumption of *A. bisporus* mushrooms, a listeriosis case associated with the consumption of fresh enoki mushrooms has been reported in a multistate outbreak in the USA that led to 36 diseased individuals of which 31 were hospitalized and four died (Anonymous, 2020).

Various typing methods can be applied to characterize and group different *L. monocytogenes* strains. The multiplex polymerase chain reaction (PCR) serogrouping technique discriminates between groups of serotypes, namely, serotypes 1/2a and 3a, serotypes 1/2b, 3b and 7, serotypes 1/2c and 3c, and serotypes 4b, 4d and 4e (Doumith et al., 2004). Most *L. monocytogenes* strains have been described to belong to lineages I and II, which contain serotypes 1/2b, 3b, 4b, 4d, 4e and serotypes 1/2a, 1/2c, 3a, 3c, respectively. Most listeriosis cases to date have been attributed to serotypes 1/2a, 1/2b and 4b (Orsi et al., 2011). Further strain discrimination could be applied with PFGE analysis, but whole genome sequencing (WGS) has demonstrated higher discriminatory power (Pietzka et al., 2019) enabling the detection of a common cause of a listeriosis outbreak (Pettengill et al., 2020). WGS data can be applied for Multi-Locus-Sequence-Typing (MLST) that has been used to cluster *L. monocytogenes* strains into clonal complexes (Ragon et al., 2008). WGS data could also be used for the core genome MLST (cg-MLST) technique, which is a method with a high discriminatory power that uses the sequence variation of 1,748 core genome genes of *L. monocytogenes* (Moura et al., 2016). Analyzing large amounts of *L. monocytogenes* genomic data has led to the identification of hypervirulent and hypovirulent clonal complex types, that were overrepresented among clinical and food isolates, respectively (Maury et al., 2016), as well as grouping of CCs into hypovirulent, medium virulent and hypervirulent clonal complex groups based on clinical frequency (Fritsch et al., 2018).

A wide variety of *L. monocytogenes* virulence genes have been described so far, including the Listeria pathogenicity island 1 (LIPI-1). LIPI-1 genes encode for virulence factors that are involved in important processes in the intracellular life cycle of *L. monocytogenes*. This includes *actA*, which was shown to be involved in actin recruitment for intracellular movement and intercellular spreading (Vázquez-Boland et al., 2001). LIPI-3 encodes a cytotoxic and a hemolytic factor that have been shown to contribute to the virulence of *L. monocytogenes* (Cotter et al., 2008) and LIPI-4 is associated to infection of the central nervous systems and to maternal neonatal infections (Maury et al., 2016). In addition, internalins have been identified in *L. monocytogenes* with roles in pathogenicity, except for the InlI protein (Bierne et al., 2007). An important internalin is the surface protein InlA of *L. monocytogenes* that plays a key role in the epithelial cell entry (Nikitas et al., 2011). Mutations in *inlA* could

lead to a pre-mature stop codon (PMSC) and it has been shown that PMSCs in *inlA* are associated with attenuated virulence (Nightingale et al., 2005).

Various *L. monocytogenes* stress defense and survival strategies have been described including resistance to environmental stresses, disinfectants and antibiotics. Genes encoded on stress survival islet 1 (SSI-1) have been shown to enhance growth at low pH and high salt concentrations (Ryan et al., 2010), while SSI-2 encoded functions were shown to enhance survival of *L. monocytogenes* in alkaline and oxidative stress conditions (Harter et al., 2017). Presence of the *bcrABC* resistance gene cassette in *L. monocytogenes* has been attributed for growth at elevated levels of benzalkonium chloride (Elhanafi et al., 2010). In addition, resistance to antibiotics that are commonly used for treatment of *L. monocytogenes* infections, such as ampicillin and penicillin, has been reported and this has raised concerns since antibiotic treatments could become less effective in case multiple antibiotic resistant strains arise (Olaimat et al., 2018). Additionally, an ongoing discussion concerns the possible persistence of pathogens to cleaning and disinfectants used in food industry. Particularly because the resistance mechanisms involved in survival to industrial disinfectants may provide cross resistance to antibiotics used in clinical settings (Donaghy et al., 2019).

To date, no chain-wide analysis has been performed that characterized *L. monocytogenes* strains present in mushrooms from the growing farms up to the frozen sliced mushrooms in mushroom processing factories. Therefore, this study aims at characterizing *L. monocytogenes* strains isolated from industrial equipment surfaces after cleaning and disinfection, as well as isolates from frozen sliced mushrooms. The complementary genetic and phenotypic typing approaches gave new insights into the presence, diversity and virulence repertoire of the *L. monocytogenes* strains from frozen sliced mushroom production facilities.

2.2. Materials and methods

2.2.1. *Listeria monocytogenes* isolation

Isolates of *L. monocytogenes* were collected during an analysis in the spring of 2018 in the Netherlands. Samples were taken in a chronological order following one particular batch of mushrooms, from the filling of the growing room at the mushroom

grower's facility to the frozen sliced mushrooms at the mushroom processing factory (supplemental table 2.1). Casing soil was sampled at the grower's facility. This was followed by sampling mushrooms and underlying casing soil during the first and second harvest, and by swabbing harvest equipment during the second harvest and after cleaning and disinfection (C&D). Mushrooms were transported to the factory and were sampled both before processing and after processing as frozen sliced mushrooms. Moreover, processing equipment in the factory was swabbed during mushroom processing and after C&D treatment. In addition, frozen sliced mushrooms were sampled before and after the aforementioned batch of grower 1 and included mushrooms from multiple growers . Also, in spring of 2018, samples were taken from the frozen sliced mushrooms of processing factory 2, which uses mushrooms from multiple growers. Lastly, samples were taken of fresh mushrooms at factory 1 during previous years (2016 and 2017) including mushrooms from multiple growers. Surface sampling was executed using cotton swabs (CLASSIQSwabs, 165KS01, Copan) moisturized in 10 mL half Fraser broth containing 0.1% Tween80.They were used to swab a surface area of 100 cm2 after which the swab was resuspended in the half Fraser broth suspension. All casing soil samples and mushroom samples were diluted 1:10 in half Fraser broth by adding 25 gram of sample in 225 mL half Fraser broth.

Samples of casing soil, mushrooms and surface swabs were analyzed for presence of *L. monocytogenes* following the ISO protocol NEN-EN-ISO 11290-1:2017 (International Organization for Standardization, 2017). After incubation, a loopful of the half Fraser broth and the full Fraser broth were streaked on Agar Listeria according to Ottaviani-Agosti (ALOA) plates (Biomérieux) and plates were incubated for 24-48 hours at 37 °C. Presumptive *L. monocytogenes* colonies, blue-green colonies with an opaque halo (one colony per positive sample), were purified by restreaking on ALOA and incubated for 24 hours at 37 °C. Subsequently, a single colony was restreaked on Brain Heart Infusion agar (BHI) (Becton Dickinson and Company, Difco) supplemented with 1.5% agar (Oxoid) and incubated for 24 hours at 30 °C followed by another streak on BHI agar plates incubated for 24 hours at 30 °C to ensure isolate purity. Pure isolates were cultured in BHI broth incubated statically for 17 hours at 30 °C for preparing -80 °C stock cultures with a final concentration of 25% glycerol (Sigma-Aldrich).

2.2.2. *L. monocytogenes* confirmation

Each isolate was streaked on BHI agar followed by incubation for 24 hours at 30 °C. Presumptive *L. monocytogenes* isolates were confirmed by heamolysis tests using blood (defibrinated sheep blood, Biotrading) agar. For that purpose, colony material from BHI agar plates was streaked on 6% (v/v%) blood agar plates and incubated for 24 hours at 37 °C. Carbohydrate utilization was tested by taking colony material from BHI agar plates and inoculating three tubes, each containing carbohydrate utilization medium with 0.5% of carbohydrate (mannitol, xylose or rhamnose). Tubes were incubated for 24-48 hours at 37 °C and *L. monocytogenes* was confirmed when a color change had occurred for rhamnose only (NEN-EN-ISO 11290-1:2017) (International Organization for Standardization, 2017).

Further confirmation of the *L. monocytogenes* isolates was executed using multiplex PCR analysis with *Listeria* spp. specific primers (*prs* primer set targeting *prs* gene) (Doumith et al., 2004) and *L. monocytogenes* specific primers (*isp* primer set targeting *isp* gene) (Rawool et al., 2016). Several colonies per isolate were transferred from the BHI agar plate and were resuspended in 100 µL InstaGene Matrix (Bio-Rad) and the manufacturer's protocol was followed for DNA extraction. The PCR reaction mixture contained 0.5 µL genomic DNA, 0.2 µM of *prs* primer set for *Listeria* spp. determination (Doumith et al., 2004), 0.2 µM of *isp* primer set for *L. monocytogenes* determination (Rawool et al., 2016), 2.5 µL of 10x Taq buffer (including 20 mM $MgCl_2$, Thermo Scientific), 0.2 mM dNTP mix (Thermo Scientific), 0.6 U Dreamtaq DNA polymerase (Thermo Scientific), in a total volume of 25 µL. The PCR cycle was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and included an initial denaturation step at 94 °C for 10 minutes, followed by 35 cycles of denaturation at 94 °C for 0.40 minutes, annealing at 56 °C for 1.15 minutes and extension at 72 °C for 2 minutes followed by final extension step at 72 °C for 10 minutes. Five microliter of PCR product was mixed with 1 µL 6x DNA loading dye (TriTrack, Thermo Scientific) and samples were examined in a 1% agarose (SeaKem LE agarose, Lonza) gel containing 1x TAE buffer (Bio-Rad) and DNA safe stain (SYBR Safe DNA Gel Stain, Invitrogen). Gels were run in 1x TAE buffer and DNA bands were visualized with ultra violet light (Uvitec, Cambridge). Isolates confirmed to be *L. monocytogenes* were used in further analyses and an overview is listed in supplemental table 2.1.

2.2.3. *L. monocytogenes* serogroup determination

Confirmed *L. monocytogenes* isolates were PCR serogrouped using the multiplex PCR protocol (Doumith et al., 2004) with some modifications. The components of the reaction included 0.5 µL genomic DNA, 0.4 µM of three primer sets (lmo0737, orf2819, orf2110) and 0.6 µM of one primer set (lmo1118), 2.5 µL 10x Taq buffer, 0.2 mM dNTPs, 0.6 U Dreamtaq DNA polymerase, in a total volume of 25 µL. The PCR cycle was adapted from Doumith et al. (2004) and PCR products were visualized as described in section 2.2.2.

2.2.4. Genomic DNA isolation, library preparation, sequencing and genome annotation

A selection of 44 strains isolated from frozen sliced mushrooms and from swab samples taken after C&D was used for whole genome sequencing. These locations were selected because strains in the frozen sliced mushrooms pose a higher risk due to probability of consumption and strains surviving on processing equipment after C&D practicing pose a potential risk for product contamination during processing. Selection of the strains was based on the serogroup abundance in a location and the number of selected strains per serogroup reflected the relative abundance of that serogroup. If a particular serogroup was more abundant, a higher percentage of strains of this serogroup was selected for WGS. If a particular strain was less abundant, a lower percentage of this serogroup was selected for WGS. This strain selection included 31 strains from factory 1, namely 11 strains from frozen sliced mushrooms that were supplied by multiple growers , 12 strains from frozen sliced mushrooms when mushrooms of grower 1 were processed, and eight strains from processing equipment after C&D. Moreover, 13 strains were selected from the frozen sliced mushrooms processed at factory 2.

Extraction of genomic DNA was performed using the DNeasy Blood & Tissue kit (Qiagen), according to the protocol provided by the manufacturer with some modifications. Strains were grown from frozen stock cultures by streaking on BHI agar and plates were incubated at 30 °C for 24 hours. Colonies were collected and resuspended in 10 mL BHI broth and grown statically for 17 hours at 30 °C. Two milliliter overnight cultures were centrifuged for 2 minutes at 16,000 x *g* and pellets were washed in one mL Peptone Physiological Salt (PPS, Tritium Microbiologie) and

resuspended in one mL enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% (w/v) Triton-X-100, 5 mg/mL lysozyme, pH 8.0). Then, solutions were incubated at 37 °C for one hour after which 10 µL RNAse was added followed by incubation at room temperature for 30 minutes. Subsequently, 62.5 µL proteinase K and 500 µL AL buffer were added and the mixtures were incubated at 56 °C for one hour. Five hundred microliter ethanol (96% ethanol, Merck) was added to the mixtures and the solutions were transferred to the spin columns provided by the kit for DNA isolation. The mixtures were left on the columns for 10 minutes and centrifuged for 1 minute at 6,000 x *g*. Five hundred microliter ethanol (96%) was added to the columns, left for 10 minutes and centrifuged for 1 minute at 6,000 x *g* and this step was repeated one time. Additionally, the columns were washed two times with 500 µL AW1 and two times with 500 µL AW2 by centrifuging 1 minute at 6,000 x *g* and unloaded columns were centrifuged for 3 minutes at 16,000 x *g* to dry the membranes. DNA was eluted by adding two times 50 µL MilliQ followed by incubation at room temperature for 10 minutes and centrifugation for 1 minute at 6,000 x *g*. DNA concentrations and quality were determined using a nanodrop (ND-1000 Spectrophotometer).

Library preparation and paired-end 2 x 150bp short-reads were generated using the INVIEW resequencing of bacteria service from Eurofins GmbH (Constance, Germany) using Illumina Mi-seq chemistry. Read quality control was performed using FastQC 0.11.5, after which reads were de novo assembled via SPAdes 3.13.1 (Bankevich et al., 2012) with the careful option and k-mer values of 21, 33, 55, 77. Assemblies were checked using QUAST (Mikheenko et al., 2018). Annotation of genomes was done using Prokka (Seemann, 2014). The raw sequences of all strains were deposited in the Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/sra under BioProject PRJNA726944.

2.2.5. MLST and cgMLST determination and tree construction

MLST was performed using the sequences of seven housekeeping genes to assign strains to sequence types (STs) and clonal complexes (CCs) (Ragon et al., 2008). This was determined using the analysis platform of Institut Pasteur (bigsdb-Lm; https://bigsdb.pasteur.fr/listeria/listeria.html, last accessed 22 January 2021) (Moura et al., 2016). In addition, a phylogenetic tree was constructed using the 44 strains based on the concatenated DNA sequences of the seven MLST genes. The tree was

constructed using Clustal W - Simple Phylogeny (Madeira et al., 2019) with default setting and using UPGMA clustering method. The tree was visualized using the Interactive Tree Of Life (iTOL) version 5 (https://itol.embl.de, last accessed 22 January 2021) (Letunic and Bork, 2019).

In addition, the core genome MLST (cgMLST) of all 44 strains was performed using 1,748 conserved loci and cgMLST profiles were determined using the analysis platform of Institut Pasteur (bigsdb-Lm; https://bigsdb.pasteur.fr/listeria/listeria.html, last accessed 22 January 2021) (Moura et al., 2016). The genome sequencing approach was not able to cover the whole genome and to assign numbers to all 1,748 loci of every strain. Unidentified alleles were marked as NA and excluded from every strain. This yielded a total of 1,463 cgMLST alleles, out of 1,748 cgMLST alleles, for which allele numbers were assigned that were used to construct the minimum spanning tree. A minimum spanning tree of the 44 strains was created using Phlyloviz online (http://online2.phyloviz.net/index, last accessed 22 January 2021) (Nascimento et al., 2017; Ribeiro-Gonçalves et al., 2016). Supplemental table 2.2 provides a detailed list of the characteristics of the 44 strains.

2.2.6. Genetic characterization of *L. monocytogenes* strains

Assembled genomes were screened for the presence of virulence factors, stress survival islets, cleaning and disinfectant resistance markers, biofilm genes and suppantibiotic resistance genes. Genes of interest were extracted and mapped to every strain for gene visualization, after which presence, absence and gene modifications were assessed. Alignments were performed against the *L. monocytogenes* EGD-e genome (RefSeq = NC_003210.1, NCBI), except for alignment of LIPI-3, LIPI-4, and *bcrABC* for which the genomes of *L. monocytogenes* PNUSAL000019 (RefSeq = NZ_CP054040.1), *L. monocytogenes* Clip80459 (RefSeq = (NC_012488.1) and *L. monocytogenes* N1-011A (RefSeq = NC_022045.1, derived from the plasmid) were used, respectively.

2.2.7. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using a range of antibiotics that have been reported in treatment of listeriosis patients (Temple and Nahata, 2000). Antimicrobial susceptibility testing of the selected *L. monocytogenes* strains using the disc diffusion method was performed by streaking the stock cultures on BHI agar followed by incubation for 24 hours at 30 °C. After incubation, several colonies were picked and resuspended in a tube containing nine mL PPS. The homogenized suspension was streaked with a sterile cotton swab (CLASSIQSwabs, 165KS01, Copan) on Mueller-Hinton agar (MH) (Oxoid). The antibiotic susceptibility discs (Oxoid) were placed on top of the agar surface and plates were incubated at 37 °C for 24 hours. Different antibiotics (disc content) were tested, namely, ampicillin (10 µg), gentamycin (10 µg), sulfamethoxazole-trimethoprim (23.75 µg + 1.25 µg), erythromycin (15 µg), penicillin G (10 units), streptomycin (10 µg), tetracycline (30 µg), vancomycin (30 µg), rifampin (5 µg), imipenem (10 µg), ciprofloxacin (5 µg), amoxicillin-clavulanic acid (20 µg + 10 µg), linezolid (30 µg) and chloramphenicol (30 µg). Nalidixic acid (30 µg) was included as a positive control. Inhibition zones of the disk diffusion method were measured after incubation and compared with the inhibition zones of *L. monocytogenes* (EUCAST, 2020) and of Staphylococci spp., recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI M100-S22, 2012), since not all tested antibiotics had breakpoints available for *L. monocytogenes*. The inhibition zones for two antibiotics, streptomycin and vancomycin, have not been reported for Staphylococci, but were taken from *Enterobacteriaceae* and Enterococcus spp, respectively. Experiments were performed with two biological replicates.

2.2.8. Resistance determination according to benzalkonium chloride

Benzalkonium chloride resistance was determined according to a previously published procedure (Elhanafi et al., 2010) with slight modifications. Briefly, strains were grown on BHI plates as described before, after which colonies were resuspended in 200 µL MH broth. Five microliter of suspension was spotted in quintuplicate onto MH agar plates supplemented with benzalkonium chloride (Acros Organics) in different concentrations, namely, 0, 2.5, 5, 7.5, 10, 12.5 and 15 µg/mL. Plates were incubated for four days at 25 °C and bacterial growth was scored as 'no growth', 'growth of

individual colonies' and 'confluent growth'. Experiments were performed with two biological replicates.

2.3. Results

2.3.1. Prevalence and serogrouping of *Listeria monocytogenes* isolates

Samples were taken in a chronological order following one particular batch of mushrooms from the grower's facility up to the processing factory, resulting in the collection of 133 *L. monocytogenes* isolates. Sampling started with raw materials at the mushroom grower's facility (grower 1) up to the final frozen mushrooms, i.e. frozen sliced mushrooms at mushroom processing factory 1. In addition, 13 isolates were collected from frozen sliced mushrooms at mushroom processing factory 2, resulting in a total of 146 *L. monocytogenes* isolates. Sampling at grower 1 resulted in 49 *L. monocytogenes* isolates. None of the 100 casing soil samples were found to be positive for *L. monocytogenes* during filling of the growing room. Although, six out of 60 casing soil samples were found positive at the time of harvest, no *L. monocytogenes* was detected in the mushroom samples taken above the sampled casing soil. After mechanical harvesting and transporting the mushrooms over the distribution line at grower 1, *L. monocytogenes* was detected on the processing equipment during processing (11 out of 44 samples) on the processing equipment after C&D (seven out of 31 samples) and on mushrooms transported to factory 1 (25 out of 50 samples). Sampling in the processing factory resulted in 84 isolates. *L. monocytogenes* was detected on processing equipment during processing mushrooms of grower 1 (five positive samples) and on the frozen sliced mushrooms originating from grower 1 (29 out of 99 samples). Furthermore, *L. monocytogenes* was detected on the frozen sliced mushrooms that were sampled before and after processing the mushrooms of grower 1 (34 positive samples). *L. monocytogenes* was also detected on processing equipment of factory 1 after C&D (16 out of 74 samples). Seven additional isolates were included, which were isolated in 2016 and 2017 from fresh mushrooms after mechanically harvesting, resulting in 153 isolates in total.

Serogroup typing of the 153 isolates revealed that 39.2% belonged to group 1/2a-3a, 34% belonged to group 1/2b-3b-7 and 26.8% belonged to group 4b-4d-4e. Serogroup 1/2a-3a and serogroup 4b-4d-4e were detected at the grower's facility and also in

factory 1 in fresh mushrooms, frozen sliced mushrooms and in environmental samples. Serogroup 1/2b-3b-7 was first detected in fresh mushrooms from grower 1 at factory 1 with a prevalence of 4%. The prevalence of this serogroup increased through the production chain, reaching a maximum of 72% on frozen sliced mushrooms. The same three serogroups were also detected in the frozen sliced mushrooms of factory 2. Additionally, the isolates from fresh mushrooms of previous years belonged to serogroup 1/2a-3a and 4b-4d-4e (supplemental table 2.1).

2.3.2. Characterization of *L. monocytogenes* strains by MLST analysis

The 44 selected *L. monocytogenes* strains, isolated from frozen sliced mushrooms and from processing equipment after C&D, were included in MLST analysis to further characterize the strains that survived C&D regimes, and strains that were present in the frozen sliced mushrooms that could reach the consumer phase. The MLST analysis revealed 11 sequence types (STs) and 11 clonal complexes (CCs) (figure 2.1). Six CCs (CC5, CC6, CC7, CC29, CC59, CC451) were isolated from one of the four places of isolation, once or twice. The other five CCs were isolated from multiple places and included CC1, CC4, CC37, CC87 and CC224. CC224 was the most frequently found CC among the samples with a total of 15 out of 44 samples. CC87 appeared to be the only CC observed in all four places (figure 2.1). The strains were grouped in lineage I and II, with a higher number of strains belonging to lineage I (84%) than to lineage II (16%) (figure 2.1).

Following the identification of hypervirulent, medium virulent and hypovirulent clones (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019), 38 out of 44 strains (86%) and six out of 44 (14%) were hypervirulent and medium virulent, respectively. The CCs of the groups CC1, CC4, CC6, CC7, CC87, CC224, CC451 are considered to be hypervirulent and CC5, CC29, CC37, CC59 are considered to be medium virulent (highlighted in red and orange respectively, figure 2.1). Notably, CC1, CC4 and CC87 types were observed in the frozen sliced mushrooms at both factories, while CC224 type was found in a high abundance in frozen sliced mushrooms of factory 1, while it was not observed in frozen sliced mushrooms of the factory 2.

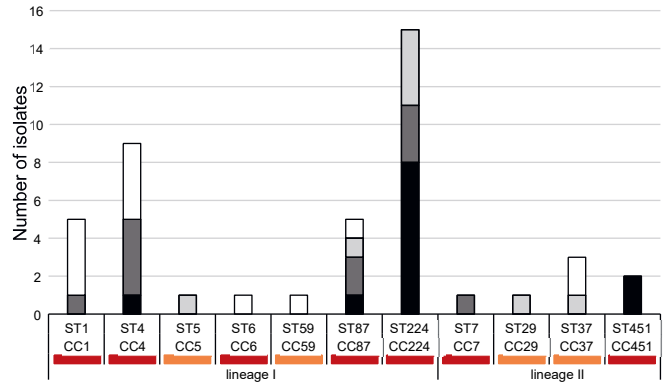

Figure 2.1. Distribution of clonal complexes and sequence types among the 44 *L. monocytogenes* strains. Strains were isolated from four locations in the mushroom production and processing chain (frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (black bars, 12 samples), frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (dark grey bars, 11 samples), surface samples after C&D at factory 1 (light grey bars, eight samples) and frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (white bars, 13 samples)). The colored bars in the figure indicate the identification of hypervirulent clones (red) and medium virulent clones (orange), according to the classification used by (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019).

2.3.3. Distribution of the *L. monocytogenes* strains

The minimum spanning tree constructed using all 44 *L. monocytogenes* strains (figure 2.2) revealed that strains were distributed in three serogroups (figure 2.2a) and eleven clonal complexes (figure 2.2b). Strains belonging to the same serogroup clustered together based on their cgMLST profile in which the lineages are clearly separated from each other (figure 2.2a). In addition, strains comprising the same clonal complex based on MLST are closely linked to each other (figure 2.2b).

The cgMLST profiles of the selected strains were obtained by including all available loci per strain, leading to a total of 18 cgMLST profiles. The 31 strains of factory 1 were assigned to ten cgMLST profiles and the 13 strains of factory 2 were assigned to ten cgMLST profiles as well. The two factories had two overlapping cgMLST profiles and these included strains belonging to CC87 and CC4. CCs comprising of multiple strains displayed either one cgMLST profile (CC451 and CC87), or two to four cgMLST profiles (CC1, CC4, CC37 and CC224). High heterogeneity was observed in cgMLST profiles for CC1 and CC4 of factory 2, yielding both three cgMLST profiles for four *L.*

monocytogenes strains. Not all similar named cgMLST profiles had an unique cgMLST loci numbering and were therefore displayed by multiple circles instead of one circle (18 cgMLST profiles and 21 unique loci numbering profiles) (figure 2.2). This is due to one locus mismatch (two cgMLST profiles of CC4) or two loci mismatches (one cgMLST profile of CC37) between similar named cgMLST profiles, leading to 21 unique loci numbering profiles in total. The highest representative cgMLST profile belonged to the CC224 strains, in which 14 out of 15 CC224 strains belonged to the same cgMLST profile.

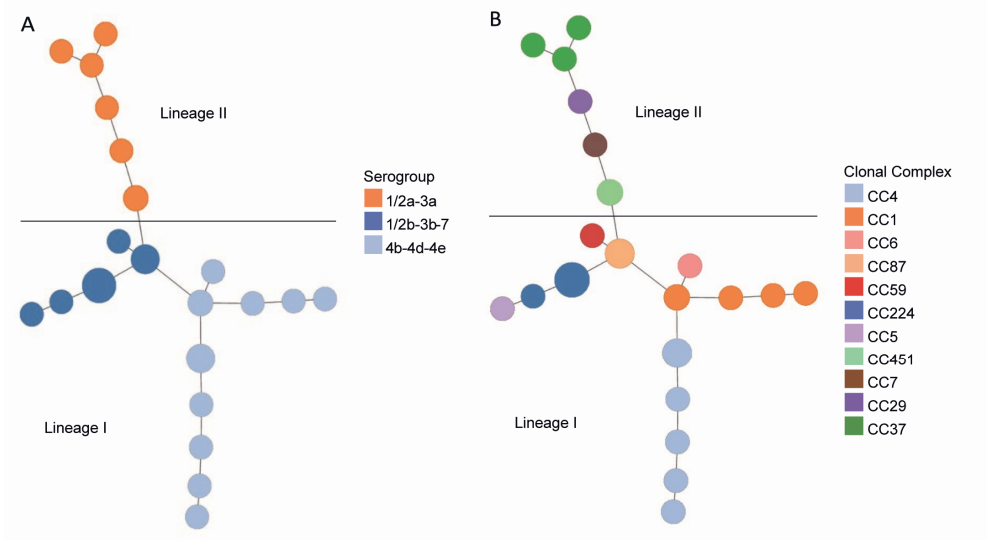

Figure 2.2. Minimum spanning tree based on the allelic profiles of the cgMLST of the 44 *L. monocytogenes* strains. Strains were isolated from four locations in the mushroom production and processing chain (frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (12 samples), frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (11 samples), surface samples after C&D at factory 1 (eight samples) and frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (13 samples)).

Allelic profiles are based on a total of 1,463 (out of 1,748) cgMLST genes. The size of the circles is proportional to the number of strains and the distances between circles indicate the allelic relationship. The colors of the circles represents (a) the three serogroups and (b) the eleven clonal complexes. The line separates the lineage I from the lineage II strains.

2.3.4. Presence of virulence genes and biofilm genetic markers in *L. monocytogenes* strains

The WGS sequencing results showed the presence of Listeria pathogenicity islands 1 (LIPI-1), LIPI-3, and LIPI-4 in 100%, 68% and 32% of the 44 strains, respectively (figure 2.3). The virulence genes of LIPI-1, consisting of *prfA*, *hly*, *mpl*, *actA*, *plcA*, *plcB* and *lmo0206* were present at full length in all 44 strains, except for *actA*. Although this gene was present in every strain, 14 out of 44 strains harbored a full-length gene, 18 out of 44 harbored a 35 amino acid (AA) internal deletion and 12 out of 44 had a large truncation. Genes of the LIPI-3 cluster were found in all 4b-4d-4e strains containing CC1, CC4 and CC6, and in all CC224 strains belonging to serogroup 1/2b-3b-7. The LIPI-4 cluster was observed in all CC4 (serogroup 4b-4d-4e) and CC87 (serogroup 1/2b-3b-7) strains.

A total of 11 internalin genes were observed among the strains. Internalin A (*inlA*) and B (*inlB*) were present in full length among all the tested strains, with the exception of *inlA* in the CC6 strain that harbored a three AA deletion within the sequence, but which did not result in an *inlA* PMSC. Also other members of the internalin gene family were present in the strains, with *inlC*, *inlE*, *inlF*, *inlH*, *inlI*, *inlJ*, *inlK*, *inlP* being present in all tested strains, while *inlG* was present in the lineage II strains and in the CC6 strain of lineage I (figure 2.3).

In addition, WGS-analysis revealed the presence of several biofilm associated genes. This included *prfA*, (virulence factor and regulator of flagella biosynthesis), *secA2*, (involved in cell aggregation, biofilm formation and biofilm structure), *luxS* (involved in the inhibition of biofilm formation with the involvement of autoinducer 2), *agrABCD* (involved in adherence and regulation of early stages of biofilm formation), *relA* and *hpt* (regulators of the starvation response), *yneA* (involved in SOS response) and *degU* (response regulator for the activation of biofilm formation) (Abee et al., 2011; Kocot and Olszewska, 2017). All these genes were present in full length in all tested strains. On the other hand, *bapL*, encoding biofilm associated protein required for cell attachment (Abee et al., 2011; Kocot and Olszewska, 2017) was not found in any of the tested strains (figure 2.3).

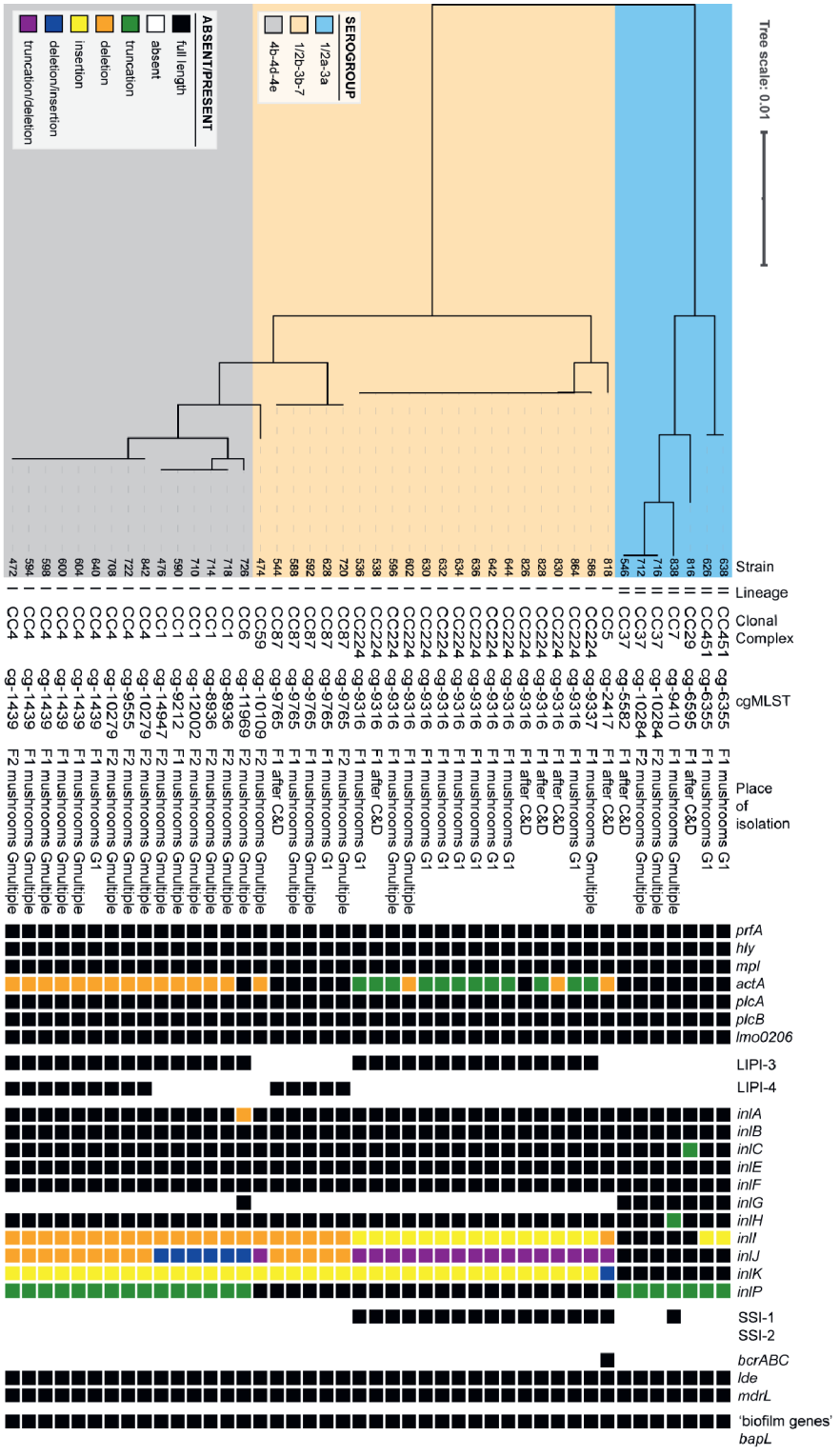
Tree scale: 0.01
SEROGROUP
1/2a-3a
1/2b-3b-7
4b-4d-4e
ABSENT/PRESENT
full length
absent
truncation
deletion
insertion
deletion/insertion
truncation/deletion
Strain
Lineage
Clonal Complex
cgMLST
Place of isolation
prfA
hly
mpl
actA
plcA
plcB
lmo0206
LIPI-3
LIPI-4
inlA
inlB
inlC
inlE
inlF
inlG
inlH
inlI
inlJ
inlK
inlP
SSI-1
SSI-2
bcrABC
lde
mdrL
'biofilm genes'
bapL

Figure 2.3. Phylogenetic tree of the 44 *L. monocytogenes* strains determined with concatenated sequences of seven housekeeping genes derived from MLST. The serogroups are highlighted by color; serogroup 1/2a-3a is highlighted in blue, serogroup 1/2b-3b-7 is highlighted in orange and serogroup 4b-4d-4e is highlighted in grey. The strains were isolated from two mushroom processing companies, indicated by factory 1 (F1) and factory 2 (F2). Strains were isolated from four locations in the mushroom production and processing chain 'F1 mushrooms G1' are frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (12 samples), 'F1 mushrooms Gmultiple' are frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (11 samples), 'F1 after C&D' are surface samples after C&D at factory 1 (eight samples) and 'F2 mushrooms Gmultiple' are frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (13 samples). Gene products including virulence factors, disinfectants resistance mechanisms and biofilm genes were screened for their presence. In case of presence, the matrix shows the full length (black box), truncations (green box), deletions (orange box), insertions (yellow box), deletion/insertion (blue box) and truncation/deletion (purple box). The box of the 'biofilm genes' are presumed biofilm genes and include *agrABCD*, *luxS*, *relA*, *yneA*, *hpt*, *prfA*, *secA2*, *degU*.

2.3.5. Presence of resistance markers in *L. monocytogenes* strains

The stress survival islet-1 (SSI-1) has been associated with higher tolerance to low pH and high salt concentrations (Ryan et al., 2010) and was observed in 17 out of 44 strains (39%). The islet was observed in the CC7 strain (serogroup 1/2a-3a), and all CC5 and CC224 strains (serogroup 1/2b-3b-7). The SSI-2 was not observed in any of the strains.

2.3.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests showed that all 14 antibiotics were effective against the 44 *L. monocytogenes* strains tested. All the strains showed clear inhibition zones for every antibiotic tested, and the inhibition zones were above the sensitivity threshold except for one antibiotic. For penicillin G, the inhibition zones of 15 strains were one mm below the breakpoint for sensitivity classification for only one of the two replicates. For two strains, the inhibition zone was one mm below the breakpoint for both of the replicates. Due to the clear inhibition zones, small differences and lack of exact *L. monocytogenes* breakpoints, the strains were also classified being sensitive to penicillin G. On the other hand, WGS analysis showed presence of the antimicrobial resistance genes *fosX*, *sul*, *norB* and *lin* in all sequenced strains, possibly conveying resistance towards fosfomycin (inhibition of peptidoglycan biosynthesis), sulfonamides

(inhibition of folic acid synthesis), quinolones (inhibition of DNA synthesis) and lincosamides (inhibition of protein synthesis) (Peach et al., 2013).

2.3.7. Benzalkonium chloride determination

The *bcrABC* resistance cassette, in which all three genes are essential to provide resistance to benzalkonium chloride (Elhanafi et al., 2010), was found in one strain belonging to CC5 (strain 818, serogroup 1/2b-3b-7). Indeed, this strain was found to be more resistant to benzalkonium chloride compared to all other strains that lacked this cassette. Confluent growth was observed up to 12.5 µg/mL benzalkonium chloride for strain 818. The strains lacking this *bcrABC* cassette showed confluent growth at 2.5 µg/mL, and single colony growth at 5.0 µg/mL. Depending on the strain, single colonies or no colonies were observed with 7.5 µg/mL benzalkonium chloride, and no growth was observed at values of 10 µg/mL benzalkonium chloride and higher (table 2.1). Other efflux pump systems, namely *lde* and *mdrL*, were present in all 44 sequenced strains.

Table 2.1. Growth of the 44 *L. monocytogenes* strains exposed to various concentrations of benzalkonium chloride using the agar spot plating method. Experiment was performed in duplicate for 44 strains and each experiment was performed with quintuplicate spots on the agar. The *L. monocytogenes* strains are indicated with their number (No.), their clonal complex and the presence/absence (+/-) of the *bcrABC* cassette. Growth on the agar plate is indicated as confluent growth (dark gray), single colony growth (light grey) and no colonies (white boxes). Confluent growth was defined as continuous growth of *L. monocytogenes* within the originally spotted droplet. Single colony growth was defined when at least one colony of *L. monocytogenes* was observed in one of the spots in one replicate

No.	CC type	*bcrABC* cassette	Benzalkonium chloride (µg/mL) 0.0	2.5	5.0	7.5	10.0	12.5	15.0
626	CC451	-							
638	CC451	-							
816	CC29	-							
838	CC7	-							
716	CC37	-							
546	CC37	-							
712	CC37	-							
818	CC5	+							
586	CC224	-							
864	CC224	-							
830	CC224	-							
828	CC224	-							
826	CC224	-							
644	CC224	-							
642	CC224	-							
636	CC224	-							
634	CC224	-							
632	CC224	-							
630	CC224	-							
602	CC224	-							
596	CC224	-							
536	CC224	-							
538	CC224	-							
720	CC87	-							
628	CC87	-							
592	CC87	-							
544	CC87	-							
588	CC87	-							
474	CC59	-							
726	CC6	-							
718	CC1	-							
714	CC1	-							
710	CC1	-							
476	CC1	-							
590	CC1	-							
842	CC4	-							
722	CC4	-							
708	CC4	-							
640	CC4	-							
604	CC4	-							
600	CC4	-							
598	CC4	-							
472	CC4	-							
594	CC4	-							

2.4. Discussion

This study evidenced the high diversity of *L. monocytogenes* within the mushroom production chain, from the grower to frozen sliced mushrooms. Remarkably, two of the three serogroups (1/2a-3a and 4b-4d-4e) were already detected at the grower's facility, indicating that the high diversity originates already at the first stage of the production chain. Three serogroups (1/2a-3a and 1/2b-3b-7 and 4b-4d-4e) were detected in the frozen sliced mushrooms at both factories, indicating that the high diversity is conserved at the end of the chain. The relative prevalence of each serogroup of *L. monocytogenes* is comparable to a previous study also focused on *L. monocytogenes* in *A. bisporus* mushrooms in Ireland (Pennone et al., 2018). These authors grouped 30.1% of the isolates to serogroup 1/2b-3b-7, 40.8% to serogroup 1/2a-3a and 29.1% to serogroup 4b-4d-4e. In the current study, 34% of isolates were grouped to serogroup 1/2b-3b-7, 39.2% were grouped to serogroup 1/2a-3a and 26.8% to serogroup 4b-4d-4e. On the other hand, a study that focused on a fresh mushroom processing environment in the United States grouped 3.5% to serogroup 1/2b-3b-7, 2.9% to serogroup 1/2a-3a and 93.6% to serogroup 1/2c-3c (Murugesan et al., 2015), while the last serogroup was not detected in the current study or the study in Ireland. Analysis of the frozen sliced mushrooms showed up to 43% (13 positive out of 30 samples, detection in 25 gram) of the samples to be positive for *L. monocytogenes*. This value was comparable with a previous study done in England where it was found that 50% (five positive out of 10 samples, detection in 25 gram) of the frozen sliced mushrooms were contaminated with *L. monocytogenes* (Willis et al., 2020).

WGS analysis showed the high genetic diversity among the 44 selected strains, isolated either from surface samples after C&D or from the frozen sliced mushrooms, with 11 different CCs/STs identified. The identified CCs of this study have been reported in other studies that focused on other types of processed foods. Studies showed presence of CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC451 on frozen vegetables and fruits (Willis et al., 2020), CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC59, CC87, CC224, CC451 in milk and milking equipment (Kim et al., 2018), CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC59, CC451 from various food products (Ebner et al., 2015), indicating a wide spread of the CCs in food production environments. High genetic diversity was also observed when the cgMLST profiles of the 44 strains were determined, which led to a total of 18 cgMLST profiles. For CC4 and CC87, the same cgMLST profiles in the frozen sliced mushrooms was observed in both factories, which may be the result of

introduction events by raw materials from a common source. On the other hand, 14 of the 15 CC224 strains isolated at factory 1 displayed a single cgMLST profile. These CC224 strains were isolated from different batches of the frozen sliced mushrooms and of surface samples after C&D. Also CC87 was present in multiple sampling locations with the same cgMLST profile across these locations. This could suggest a better biofilm forming capability of these possibly persistent CC87 and CC224 strains. However, the biofilm formation capacity depends not only on genetic biofilm markers, but also on the type of surface (Magalhães et al., 2017). Therefore the different types of surfaces in the mushroom industry will also influence the biofilm forming capacity of *L. monocytogenes*. Various biofilm genes were identified in the isolated strains, a factor that may contribute to the presence and prevalence of *L. monocytogenes* in mushroom production environments. Whether this high genetic diversity among the isolated strains affects biofilm forming capacity on different surface materials remains to be elucidated.

A high percentage (86%) of the strains could be classified as hypervirulent strains using the CC virulence association (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019). These hypervirulent CC types have previously been found in other food types, such as milk and milking equipment (Kim et al., 2018) and RTE food samples (Chen et al., 2020). Every strain showed full length genes of the LIPI-1 cluster, except for *actA* in some strains, which is involved in actin recruitment and intercellular spreading (Vázquez-Boland et al., 2001). The *actA* sequence showed an internal deletion of 35 AA (AA position 265-299) in all CC1, CC4, CC5, CC59 and two out of 15 CC224 strains. In addition, one out of 15 CC224 strain had a truncation of 282 AA and 11 out of 15 CC224 strains had a truncation of 317 AA. Disrupted versions of *actA* were previously described in the aforementioned CCs (Maury et al., 2016) and disrupted versions of this gene could have an effect on the intracellular motility. The amino-terminal region (AA position 128-151) has been found to be essential for *actA* in actin filament recruitment, while the proline rich repeats (AA position 265-390) are involved in higher efficiency for the recruitment of filamentous actin (Pistor et al., 1995). Effects of mutations in *actA* on *L. monocytogenes* virulence and pathogenicity in humans is not fully understood, because disrupted versions of *actA* are also present in hypervirulent strains. LIPI-3 was absent in all lineage II strains, but was present in a subset of the lineage I strains involving CC1, CC4, CC6 and CC224 and presence of LIPI-3 in these CCs had already been reported previously (Kim et al., 2018). These results are in accordance with a previous study mentioning the presence of LIPI-3 in a

subset of the lineage I strains, but absence in lineage II and lineage III *L. monocytogenes* strains (Cotter et al., 2008). LIPI-4 was observed in all CC4 and CC87 strains of this study, which is in accordance with other studies, which reported the presence of the LIPI-4 cluster for CC4 strains (Maury et al., 2016) and for both the clonal complexes (Kim et al., 2018).

All 44 *L. monocytogenes* strains sequenced in this study had a full length *inlA* and *inlB* coding sequence, except for the CC6 strain that had a nine nucleotide deletion in the *inlA* sequence at position 2212 to 2220. This resulted in a three AA loss in the protein sequence. The same deletion is previously mentioned as characteristic for CC6 strains with the deletion located at the pre anchor region of *inlA* and it is not expected that this deletion would affect the *inlA* mediated action (Cantinelli et al., 2013). On the contrary, other studies described *inlA* PMSCs in 10.8% to 20.8% of the *L. monocytogenes* strains that were isolated from a variety of food products and from meat and meat processing environments (Chen et al., 2020; Kurpas et al., 2020). Clonal complexes containing an *inlA* PMSC included CC5 among others, although not all CC5 strains harbored a PMSC in *inlA* (Chen et al., 2020; Kurpas et al., 2020). Other internalins detected in every strain analyzed in this study included *inlC*, *inlE*, *inlF*, *inlH*, *inlJ*, *inlK*, *inlP*, which had already been detected previously in strains from lineage I and lineage II of refrigerated RTE food samples, except *inlF* in one CC121 strain (Chen et al., 2020). Gene *inlF* was identified in all lineage I and II strains in this study, in accordance with previous study reporting its presence in the majority of strains belonging to lineage I and lineage II (Chen et al., 2020; Kurpas et al., 2020). Gene *inlG* was identified in all lineage II strains in this study and in the CC6 strain belonging to lineage I, which is in accordance with previous research in which *inlG* was observed in the majority of the lineage II strains and not in lineage I, except for CC6 strains that were isolated from RTE meat and meat processing environments (Kurpas et al., 2020) and refrigerated RTE food (Chen et al., 2020). The high abundance of virulence factors in the *L. monocytogenes* strains isolated from the mushroom production and processing chain indicate the virulence potential of these strains.

The SSI-1 cluster was present in 39% of the 44 *L. monocytogenes* strains, while SSI-2 cluster was absent in every strain. The SSI-2 cluster was not observed in this study, possibly due to the absence of CC121 strains isolated, as this CC was previously identified as the main group containing this islet (Harter et al., 2017). On the other hand, our results showed the presence of the SSI-1 cluster in the CC7 strain of lineage

II and in CC5 and all CC224 strains of lineage I, in line with previous data on *L. monocytogenes* strains obtained from food and food processing environments and from sporadic human listeriosis (Hingston et al., 2017). Functions encoded by this islet have previously been shown to enhance growth at suboptimal conditions/environmental stresses, as low pH and high salt concentrations (Ryan et al., 2010), but contradictory results have been reported on stress tolerance related to SSI-1 (Hingston et al., 2017). However, the presence of SSI-1 in 39% of our strain selection may point to a selective advantage for growth and prevalence in conditions encountered in the production of frozen sliced mushrooms.

All selected strains were classified as sensitive against the antibiotics tested, including those commonly used in clinical settings for listeriosis patients. Additional analysis showed no inhibition zones upon exposure to nalidixic acid, in line with the previously reported results of natural resistance of *L. monocytogenes* to this compound (Olaimat et al., 2018). Hence, although *L. monocytogenes* could be found in the mushroom production and processing chain, no multidrug resistance types were found in our study.

Both the presence and high genomic diversity of the *L. monocytogenes* strains isolated in this study underlines the importance of hygienic measures to control *L. monocytogenes* along the whole mushroom production and processing chain. Resistance toward the disinfectant benzalkonium chloride (BC) is linked to the *bcrABC* resistance cassette (Elhanafi et al., 2010; Minarovičová et al., 2018). Our results support this hypothesis, as the CC5 strain harboring this cassette had a higher tolerance to BC than the rest. Indeed, the presence of the *bcrABC* cluster in *L. monocytogenes* strains has been described and associated with CC5 strains (Meier et al., 2017). On the other hand, strains that did not harbor the *bcrABC* genes showed single colony growth at 5 µg/mL of BC and a subset of strains also at 7.5 µg/mL BC, which might indicate natural resistance to low concentrations of BC. This could be due to the presence of additional efflux pumps. Efflux pumps *mdrL* and *lde* were present in all tested strains in which *mdrL* is partly responsible for BC resistance, but *lde* is not (Romanova et al., 2006). Other studies show higher percentages of BC resistance in *L. monocytogenes* strains isolated from food production, which could be derived either from *bcrABC* or *qacH*. BC resistance was reported for 18% of the strains isolated from food matrices in Switzerland (Ebner et al., 2015) and 22% of the strains from a meat processing facility in Slovakia (Minarovičová et al., 2018). The fact that only one out of 44 *L. monocytogenes* strains contained the *bcrABC* resistance cassette could be due

to the lack of selection pressure for BC resistance since quaternary ammonium compounds are not applied in cleaning and disinfection regimes at the selected mushroom production and processing companies.

2.5. Conclusion

Our analysis demonstrated that *L. monocytogenes* is present in the mushroom production and processing chain, with low prevalence at the grower's facility and higher prevalence in the frozen sliced mushrooms. Genotypic analysis showed high diversity in serogroups, CCs and cgMLST types among the strains. Two of the three serogroups (1/2a-3a and 4b-4d-4e) were already detected at the grower's facility, indicating a rather high diversity already in the first stage of the mushroom production chain. The diversity increased further in the end product i.e. the frozen sliced mushroom products. The majority of strains (86%) isolated from the processing environment and the frozen sliced mushrooms were assigned hypervirulent CCs and a wide diversity of virulence genes was present among the strains, yet all were sensitive towards a wide range of antibiotics.

Abbreviations

RTE, Ready-to-Eat; ST, sequence type; CC, clonal complex; BC, benzalkonium chloride; LIPI, Listeria Pathogenicity Island; inl, internalin; SSI, Stress Survival Islet; WGS, Whole Genome Sequenced

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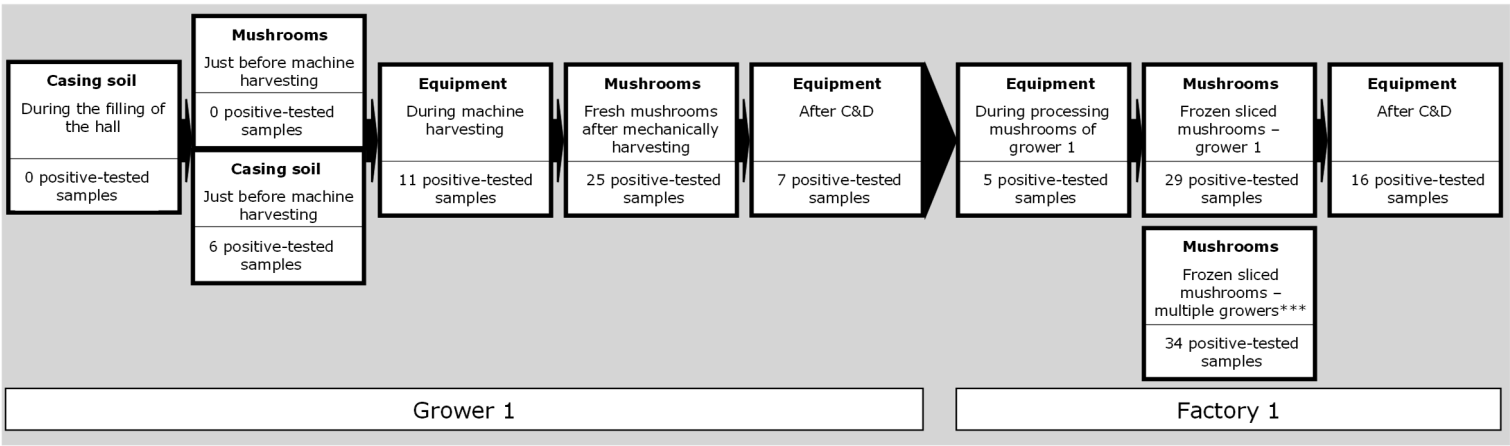
Supplemental information

Supplemental tables

Supplemental table 2.1. Overview of the prevalence of *L. monocytogenes* at different sampling stages, serogroup determination and strains taken for WGS analysis

Company	Sampled product / sampled location (extra explanation)	# positive-tested samples*	# strains in a serogroup (% percentage of strains in serogroup) 1/2a-3a	1/2b-3b-7	4b-4d-4e	WGS sequenced **
Results chronological analysis - following a particular batch of mushrooms						
Grower1	Casing soil (during the filling of the hall)	0	-	-	-	
Grower1	Mushrooms (just before machine harvesting)	0	-	-	-	
Grower1	Casing soil (just before machine harvesting)	6	-	-	6 (100%)	
Grower1	Equipment (during machine harvesting)	11	8 (73%)	-	3 (27%)	
Grower1	Mushrooms (fresh mushrooms after mechanically harvesting)	25	23 (92%)	1 (4%)	1 (4%)	
Grower1	Equipment (after C&D)	7	5 (71%)	-	2 (29%)	
Factory1	Equipment (during processing mushrooms grower 1)	5	2 (40%)	1 (20%)	2 (40%)	
Factory1	Mushrooms*** (frozen sliced mushrooms - multiple growers)	34	5 (15%)	15 (44%)	14 (41%)	11
Factory1	Mushrooms (frozen sliced mushrooms - grower 1)	29	6 (21%)	21 (72%)	2 (7%)	12
Factory1	Equipment (after C&D)	16	4 (25%)	12 (75%)	-	8
	Total All products / locations of chronological analysis	133	53 (40%)	50 (38%)	30 (22%)	31
Factory2	Mushrooms (frozen sliced mushrooms - multiple growers)	13	2 (15%)	2 (15%)	9 (70%)	13
Factory1	Mushrooms (fresh mushrooms after mechanically - multiple growers)	7****	5 (71%)	-	2 (29%)	
	Total All products / locations	153	60 (39%)	52 (34%)	41 (27%)	44



Notes
* One strain was selected from a positive-tested sample; ** More information about these strains is provided in supplementary information 2; *** Frozen sliced mushrooms that were sampled before and after processing mushrooms of grower 1; **** Sampled in previous sampling plan in 2016 and 2017.

Supplemental table 2.2. Overview of strains and their corresponding genomic characteristics and place of isolation. All the characterized strains were isolated in 2018 from two factories that produce frozen sliced mushrooms

Isolate*	Sampled product / sampled location (extra explanation)	Factory	PCR-serogroup	Lineage	ST (MLST)	CC (MLST)	cgMLST	Missing Loci (cgMLST)
472	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST4	CC4	cg1439	38
474	Mushrooms (frozen sliced mushrooms - multiple growers)	2	1/2b-3b-7	I	ST59	CC59	cg10109	14
476	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST1	CC1	cg14947	3
536	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	31
538	Equipment (after C&D)	1	1/2b-3b-7	I	ST224	CC224	cg9316	30
544	Equipment (after C&D)	1	1/2b-3b-7	I	ST87	CC87	cg9765	19
546	Equipment (after C&D)	1	1/2a-3a	II	ST37	CC37	cg5582	10
586	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2b-3b-7	I	ST224	CC224	cg9337	23
588	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2b-3b-7	I	ST87	CC87	cg9765	18
590	Mushrooms (frozen sliced mushrooms - multiple growers)	1	4b-4d-4e	I	ST1	CC1	cg9212	16
592	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2b-3b-7	I	ST87	CC87	cg9765	18
594	Mushrooms (frozen sliced mushrooms - multiple growers)	1	4b-4d-4e	I	ST4	CC4	cg1439	37
596	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2b-3b-7	I	ST224	CC224	cg9316	32
598	Mushrooms (frozen sliced mushrooms - multiple growers)	1	4b-4d-4e	I	ST4	CC4	cg1439	36

Supplemental table 2.2 continued

Isolate*	Sampled product / sampled location (extra explanation)	Factory	PCR-serogroup	Lineage	ST (MLST)	CC (MLST)	cgMLST	Missing Loci (cgMLST)
600	Mushrooms (frozen sliced mushrooms - multiple growers)	1	4b-4d-4e	I	ST4	CC4	cg1439	37
602	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2b-3b-7	I	ST224	CC224	cg9316	34
604	Mushrooms (frozen sliced mushrooms - multiple growers)	1	4b-4d-4e	I	ST4	CC4	cg1439	36
626	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2a-3a	II	ST451	CC451	cg6355	28
628	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST87	CC87	cg9765	18
630	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	31
632	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	32
634	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	30
636	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	31
638	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2a-3a	II	ST451	CC451	cg6355	27
640	Mushrooms (frozen sliced mushrooms - grower 1)	1	4b-4d-4e	I	ST4	CC4	cg1439	36
642	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	30
644	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	31
708	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST4	CC4	cg10279	9
710	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST1	CC1	cg12002	11
712	Mushrooms (frozen sliced mushrooms - multiple growers)	2	1/2a-3a	II	ST37	CC37	cg10284	5

Supplemental table 2.2 continued

Isolate*	Sampled product / sampled location (extra explanation)	Factory	PCR-serogroup	Lineage	ST (MLST)	CC (MLST)	cgMLST	Missing Loci (cgMLST)
714	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST1	CC1	cg8936	14
716	Mushrooms (frozen sliced mushrooms - multiple growers)	2	1/2a-3a	II	ST37	CC37	cg10284	2
718	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST1	CC1	cg8936	11
720	Mushrooms (frozen sliced mushrooms - multiple growers)	2	1/2b-3b-7	I	ST87	CC87	cg9765	18
722	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST4	CC4	cg9555	13
726	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST6	CC6	cg11969	9
816	Equipment (after C&D)	1	1/2a-3a	II	ST29	CC29	cg6595	15
818	Equipment (after C&D)	1	1/2b-3b-7	I	ST5	CC5	cg2417	6
826	Equipment (after C&D)	1	1/2b-3b-7	I	ST224	CC224	cg9316	29
828	Equipment (after C&D)	1	1/2b-3b-7	I	ST224	CC224	cg9316	31
830	Equipment (after C&D)	1	1/2b-3b-7	I	ST224	CC224	cg9316	28
838	Mushrooms (frozen sliced mushrooms - multiple growers)	1	1/2a-3a	II	ST7	CC7	cg9410	24
842	Mushrooms (frozen sliced mushrooms - multiple growers)	2	4b-4d-4e	I	ST4	CC4	cg10279	5
864	Mushrooms (frozen sliced mushrooms - grower 1)	1	1/2b-3b-7	I	ST224	CC224	cg9316	30

* The 44 strains were isolated from four locations in the mushroom production and processing chain 'Mushrooms (frozen sliced mushrooms - grower 1)' are frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (12 samples), 'Mushrooms (frozen sliced mushrooms - multiple growers)' are frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (11 samples), 'Equipment (after C&D)' are surface samples after C&D at factory 1 (eight samples) and 'Mushrooms (frozen sliced mushrooms - multiple growers)' are frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (13 samples).

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3

Variability in growth and biofilm formation of *Listeria monocytogenes* in *Agaricus bisporus* mushroom products

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Abstract

Foods and food production environments can be contaminated with *Listeria monocytogenes* and may support growth of this foodborne pathogen. This study aims to characterize the growth and biofilm formation of sixteen *L. monocytogenes* strains, isolated from mushroom production and processing environments, in filter-sterilized mushroom medium. Strain performance was compared to twelve *L. monocytogenes* strains isolated from other sources including food and human isolates. All twenty-eight *L. monocytogenes* strains showed rather similar growth performance at 20 °C in mushroom medium, and also significant biofilm formation was observed for all strains. HPLC analysis revealed the presence of mannitol, trehalose, glucose, fructose and glycerol, that were all metabolized by *L. monocytogenes*, except mannitol, in line with the inability of *L. monocytogenes* to metabolize this carbohydrate. Additionally, the growing behaviour of *L. monocytogenes* was tested on whole, sliced and smashed mushroom products to quantify performance in the presence of product-associated microbiota. A significant increase of *L. monocytogenes* was observed with higher increase of counts when the mushroom products were more damaged, even with the presence of high background microbiota counts. This study demonstrated that *L. monocytogenes* grows well in mushroom products, even when the background microbiota is high, highlighting the importance to control (re)contamination of mushrooms.

3.1. Introduction

Listeria monocytogenes is an important foodborne pathogen and the causative agent of listeriosis. Risk groups for listeriosis are the elderly, pregnant women, children and immunocompromised individuals (Radoshevich and Cossart, 2018). The incidence rates of listeriosis are relatively low, but listeriosis has a high case fatality rate, which was 13% in the EU in 2020 (EFSA and ECDC, 2021). *L. monocytogenes* is widespread in the environment and has been isolated from soil, water and plant samples (Sauders et al., 2006), but it is also frequently present and widely distributed in food processing environments (Ferreira et al., 2014).

Fresh and frozen vegetables are among the ready-to-eat (RTE) food products that may be contaminated by *L. monocytogenes* (Montero et al., 2015). This is of concern for consumers, since previous *L. monocytogenes* outbreaks have been related to the consumption of contaminated processed vegetables such as celery and frozen corn (EFSA and ECDC, 2019; Gaul et al., 2013). Previously, *L. monocytogenes* has been isolated from the white button mushroom (*Agaricus bisporus*) processing environments (Lake et al., 2021; Murugesan et al., 2015; Pennone et al., 2018) and from fresh-sliced and frozen *A. bisporus* mushrooms (Anonymous, 2021; Lake et al., 2021). Most consumers cook, stir-fry or bake *A. bisporus* mushrooms before consumption (Borgdorff, 2012), thereby reducing the exposure risk. The hand-picked *A. bisporus* mushrooms that are intended for the fresh market may also be eaten raw, while the machine-harvested *A. bisporus* mushrooms that are sliced and frozen by the producers are usually not intended and sold as RTE food by the mushroom industry. Although presence of *L. monocytogenes* on fresh sliced *A. bisporus* mushrooms has resulted in several recalls (Anonymous, 2021), to date no listeriosis cases have been associated with the consumption of *A. bisporus* mushrooms, while other mushroom types such as Enoki mushrooms have been involved in outbreaks (Anonymous, 2020).

L. monocytogenes is capable of forming biofilms on different surfaces of food processing environments (Doijad et al., 2015; Møretrø and Langsrud, 2004) and on surfaces relevant to the mushroom processing industry, such as stainless steel, rubber and different types of tarpaulins (Dygico et al., 2020). The ability of *L. monocytogenes* to adhere to surfaces and form biofilms is essential for its survival and persistence, and could lead to food contamination (Melo et al., 2015). When frozen food products get contaminated, then *L. monocytogenes* may survive during frozen storage (Liu et

al., 2016; Miladi et al., 2008) and growth could occur after thawing, posing a risk for foodborne illness (Kataoka et al., 2017).

Characterization of the growth and biofilm forming behaviour of *L. monocytogenes* food and food environmental isolates has been done in various studies using non-diluted and diluted nutrient-rich laboratory media. These studies demonstrated that medium composition has an influence on the planktonic growth and biofilm formation of *L. monocytogenes* (Dygico et al., 2020; Kadam et al., 2013; Lee et al., 2019; Nowak et al., 2015). Therefore, it is recommended to use food-derived media, instead of laboratory media, to approach field conditions as close as possible (Overney et al., 2016). The medium composition does not solely affect the growth of *L. monocytogenes*, since significant differences between strains were observed when twenty *L. monocytogenes* strains were characterized in Brain Heart Infusion (BHI) medium (Aryani et al., 2015). This variability is described as strain variability and is an inherent property of microorganisms that cannot be reduced when strains of the same microorganism are identically treated using the same specified conditions (Whiting and Golden, 2002). This strain variability is defined by differences among strains of the same species, while differences between independently reproduced experiments of the same strain is known as the reproduction variability (Aryani et al., 2015). Quantifying the strain variability and the reproduction variability allows to compare the impact of both variability factors in order to evaluate the significance of differences in growth characteristics of (particular groups of) *L. monocytogenes* strains.

We recently isolated a genomic-diverse set of *L. monocytogenes* strains from the frozen sliced mushroom production and processing environment (Lake et al., 2021). As a following-up, a selection was made of these *L. monocytogenes* strains to evaluate the impact of strain variability on growth performance and biofilm formation. The performance of mushroom isolates was compared to strains isolated from other foods and clinical isolates in order to evaluate whether mushroom isolates perform better than non-mushroom isolates. In contrast to many other research investigations that use nutrient-rich laboratory media for strain characterization, this research used mushroom medium to mimic the nutrient availability that *L. monocytogenes* may encounter in mushroom processing environments. In addition, the growth performance of a subset of *L. monocytogenes* mushroom strains was characterized on whole, sliced and smashed mushroom products to determine *L. monocytogenes* growth potential in the presence of natural microbiota.

3.2. Materials and methods

3.2.1. Selection of *L. monocytogenes* strains

Sixteen *L. monocytogenes* strains were selected that were isolated at different steps of the mushroom production and processing chain during an extensive sampling survey in April 2018 at a mushroom growing facility and two mushroom processing factories (supplemental table 3.1) (Lake et al., 2021). These mushroom strains were selected based on place of isolation and PCR serogroup, covering strains of different PCR serogroups and from different places of isolation (including strains isolated from fresh mushrooms, frozen sliced mushrooms, equipment during mushroom processing and equipment after cleaning and disinfection (C&D)). The clonal complex and sequence type numbers of ten mushroom strains were already determined by Lake et al. (2021), and in the current study, six additional mushroom strains were sequenced and genotypically characterized following the same procedure (Lake et al., 2021). Briefly, by using the analysis platform of Institut Pasteur (bigsdb-Lm; https://bigsdb.pasteur.fr/listeria/, last accessed 11 April 2022) (Moura et al., 2016) the sequence types (STs) and clonal complexes (CCs) were assigned to the strains by using the sequences of the seven housekeeping genes (Ragon et al., 2008). Moreover, 12 non-mushroom strains were selected including four human clinical strains, seven food strains and one animal strain (Aryani et al., 2015; Yin et al., 2015). The strains of which the in-house whole genome sequencing data was available were processed in the analysis platform of Institut Pasteur as described above to determine clonal complex and sequence type numbers, or this information was taken from Yin et al. (2015). Detailed information of all strains is presented in supplemental table 3.1.

3.2.2. Preparation of strains

Strains of *L. monocytogenes* were stored in Brain Heart Infusion (BHI) broth (Becton Dickinson and Company, Difco) containing 25% glycerol (Sigma-Aldrich) at -80 °C. Stationary phase cultures of the strains were obtained by inoculating 10 mL BHI broth with the stock culture, followed by static incubation at 30 °C for 18 hours. After incubation, one milliliter of culture was centrifuged for 2 minutes at 16,000 x *g*, after which the supernatant was discarded and the cell pellet was washed in Phosphate Buffered Saline (PBS) buffer. PBS buffer was prepared according to the ISO protocol

NEN-EN-ISO 11290-1:2017 (International Organization for Standardization, 2017), containing 8.98 gram di-sodium hydrogen phosphate dihydrate, 2.71 gram sodium dihydrogen phosphate and 8.5 gram sodium chloride dissolved in 1 L demineralized water (pH 7.2). The washing step was repeated and cells were resuspended in 1 mL PBS buffer and subsequently diluted 1:100 (volume/volume) in PBS buffer to obtain the working culture with approximately 7 log CFU/mL for each strain.

3.2.3. Preparation of mushroom medium

Mushroom medium was prepared to mimic nutrient availability in mushroom processing environments, and this medium was used for the characterization of the growth (section 3.2.4), biofilm formation (section 3.2.5) and nutrient consumption (section 3.2.6) of the *L. monocytogenes* strains. For this, mushrooms (*Agaricus bisporus*) were harvested at a mushroom growing facility, transported to the laboratory and stored refrigerated for maximum 3 days. Upon processing, the mushrooms were cut into pieces and divided in amounts of 500 grams. Portions of 500 grams of mushrooms and 200 mL of non-sterilized demineralized water were added to a stomacher filter bag (Antonides) and homogenized using a stomacher (Stomacher 400 circulator, Seward) for 1 minute at 230 rpm. Obtained mushroom medium was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific). Supernatants of different portions that were prepared on the same day were collected and pooled in a flask and the suspension was shaken for obtaining one homogenized mushroom medium batch. Four additional mushroom medium batches were prepared with mushrooms that were harvested at other times of the year to determine whether phenotypic behaviour of *L. monocytogenes* was different between mushroom batches. Each mushroom medium batch was stored for a maximum of six months at -20 °C upon use. Upon use, the mushroom medium was thawed and centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific). The collected supernatant was filter-sterilized with a 0.45 µm filter (Minisart® syringe filter, Sartorius) followed by filtration using a 0.22 µm filter (Minisart® syringe filter, Sartorius), after which the sterilized mushroom medium was ready-to-use.

3.2.4. Growth of *Listeria monocytogenes* in mushroom medium

The working cultures (section 3.2.2) were inoculated 1:100 (volume/volume) in filter-sterilized mushroom medium (section 3.2.3) to start with approximately 5 log CFU/mL for each strain. The individual cultures of *L. monocytogenes* were subsequently added in 300 µL aliquots into a polystyrene 96-well plate (Greiner Bio-One) of which the wells at the edge of the plate were filled with sterile PBS buffer. Plates were incubated in a static incubator at 20 °C for 48 hours. Counts of the individual *L. monocytogenes* cultures were determined at 0, 24 and 48 hours of incubation by preparing decimal dilutions in PBS followed by plating on Brain Heart Infusion agar (BHI) (Becton Dickinson and Company, Difco), supplemented with 1.5% agar (Oxoid) and incubation at 30 °C for 24 hours. In addition, the pH of the mushroom medium was determined at the start of each experiment (fresh sterile mushroom medium), after 48 hours of incubation with *L. monocytogenes*, and also after 48 hours of incubation of non-inoculated medium. Prior to pH measurements of the medium incubated with *L. monocytogenes*, cultures were centrifuged to remove cells (2 minutes at 16,000 x *g*) and the supernatant was collected. The pH of the samples was determined using a microelectrode (Inlab Ultra Micro-ISM, Mettler Toledo) coupled with a pH meter instrument (PHM240 pH/ion meter, Meterlab, Radiometer Analytical). Growth characterization was done in up to five mushroom medium batches and was performed with two biologically independent replicates on different days for each of the strains.

3.2.5. Crystal violet staining and biofilm CFU counting

Strains were grown statically as described in section 3.2.4 and the biofilm forming capacity of the strains was determined after 48 hours of incubation using the same mushroom medium batch as used for planktonic growth determination. Biofilm quantification was performed using the crystal violet (CV) assay, that has been proven as a useful biofilm determinator (Wilson et al., 2017). The biofilm formation was determined using the CV assay adapted from Fernández Ramírez et al. (2015), with some modifications. In short, wells were washed twice with 300 µL PBS, and the biofilm was stained for 30 minutes with 300 µL 0.1% (weight/volume) CV (Merck). The solution was removed and wells were washed twice with 300 µL PBS. The bound CV was dissolved in 96% ethanol for 15 minutes and the biofilm was quantified by measuring the absorbance at 595 nm with the Spectramax M2 plate reader (Molecular

Devices). Experiments were performed with two biological independent replicates obtained on different days, each consisting of two technical replicates. Not inoculated mushroom medium was included as a control. In parallel, the CFU counts were determined using the plate count method since living cells cannot be quantified using the CV staining assay as both living and dead cells will be stained (Kadam et al., 2013). Therefore, the CFU counting technique was applied for biofilm cell estimation, with the advantage that only culturable cells will be counted that form colonies on the plate (Wilson et al., 2017). Briefly, wells were washed twice with 300 µL PBS and filled with 300 µL PBS. The attached biofilm was detached from the wells by rigorously scraping the wells with a 200 µL pipet tip (Greiner Bio-One) and single cells were obtained by subsequent rigorous pipetting using the same pipet tip. Decimal dilutions were prepared in PBS followed by plating on BHI agar. Biofilm CFU count experiments were performed with two biologically independent replicates obtained on different days.

3.2.6. HPLC and UPLC analysis

The mushroom medium batch characterized by High Performance Liquid Chromatography (HPLC) and Ultra-high Performance Liquid Chromatography (UPLC) analysis was the same as used for growth and biofilm experiments and samples of the mushroom medium were taken after 48 hours of incubation with *L. monocytogenes*. Prior to measurements, cultures were centrifuged for 2 minutes at 16,000 x *g* to pellet the cells. Supernatants of samples were stored at -20 °C upon further analysis of extracellular metabolites by HPLC and UPLC analyses. In addition, non-inoculated mushroom medium samples were taken before and after 48 hours of incubation at 20 °C and samples were also stored at -20 °C. Protocols for HPLC and UPLC analyses were slightly adapted from Lanzl et al. (2022). HPLC analyses were performed for detection and quantification of trehalose, glucose, mannitol, fructose, glycerol, lactate, acetate and acetoin. Briefly, samples were deproteinated by mixing two volumes of sample with one volume of cold Carrez A (0.1 M potassium ferrocyanide trihydrate) after which one volume of cold Carrez B (0.2 M zinc sulphate heptahydrate) was added and mixed. Samples were centrifuged at 16,000 x *g* for 5 minutes and the supernatant was collected. A total volume of 10 µL of the sample was injected on an Ultimate 3000 (Dionex, Germany) equipped with an Aminex HPX-87H column (300 x 7.8 mm) with guard-column (Bio-Rad, USA). The temperature of the column oven was kept at 60 °C and 0.01 N H_2SO_4 was used as a mobile phase with a flow rate of 0.6 mL per minute.

Compound detection was performed using a refractive index detector (RefractoMax 520) together with using UV measurements at 220, 250, 280 nm for peak identification followed by quantification. Calibration curves were prepared for trehalose (Merck), glucose (Merck), mannitol (Acros Organics), fructose (Merck), glycerol (Sigma-Aldrich), lactate (Sigma-Aldrich), acetate (Merck) and acetoin (Sigma-Aldrich).

UPLC analyses were performed for detection and quantification of amino acids (histidine, asparagine, serine, glutamine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine, tryptophan) and ammonium. Briefly, samples were subjected to protein removal by mixing 40 µL of sample with 50 µL of 0.1 M HCl containing 250 µM norvaline internal standard. The sample was mixed with 10 µL of 300 mg/mL sulfosalicylic acid (SSA) followed by centrifugation at 17,000 x *g* for 10 minutes at 4 °C. Amino acids and ammonium were subsequently derivatized using the AccQ·Tag Ultra Derivatization Kit (Waters Corporation, USA). First, the pH of the AccQ·Tag Ultra Borate buffer was increased by adding 75 µL of 4 M NaOH to 5 mL of borate buffer for neutralization of the SSA addition. Then, 60 µL of the borate/NaOH buffer was mixed with 20 µL deproteinated sample in glass vials. In parallel, 60 µL borate buffer without NaOH was mixed with a standard amino acid mixture in glass vials to prepare a calibration curve for each amino acid. In each sample, 20 µL of AccQ·Tag Ultra Derivatization Reagent (reagent powder dissolved in 2.0 mL AccQ·Tag Ultra Reagent Diluent) was added, after which samples were immediately capped, vortexed for 10 seconds and heated at 55 °C in a heat block for 10 minutes. To quantify amino acids by UPLC, a total volume of 1 µL of the sample was injected on an Ultimate 3000 (Dionex, Germany) equipped with an AccQ·Tag Ultra BEH C18 column (150 mm x 2.1 mm, 1.7 µm) (Waters Corporation, USA) and a BEH C18 guard column (5 mm x 2.1 mm, 1.7 µm) (Waters Corporation, USA). The temperature of the column oven was set at 55 °C and the mobile phase had a flow rate of 0.7 mL per minute. Eluent A was 5% AccQ·Tag Ultra concentrate solvent A and eluent B was the 100% AccQ·Tag Ultra solvent B. The separation gradient of the system was 0-0.04 minute 99.9% A, 5.24 minute 90.9% A, 7.24 minute 78.8% A, 8.54 minute 57.8% A, 8.55-10.14 minute 10% A, 10.23-17 minute 99% A. Compounds were detected by UV measurement at 260 nm. Glutamine and arginine could not be separated in the UPLC analysis. HPLC or UPLC analyses were executed with at least two biologically independent replicates using the same mushroom medium batch.

3.2.7. *L. monocytogenes* growth determination on mushroom products

A selection of *L. monocytogenes* strains with differences in PCR serogroup, clonal complex and place of isolation (supplemental table 3.1) was used to determine growth performance on whole mushrooms, sliced mushrooms and smashed mushrooms. Stationary phase cultures were prepared as described in section 3.2.2 and 100 µL of the culture was transferred into 10 mL of fresh BHI and cultures were statically incubated for 24 hours at 20 °C. Working cultures were prepared in PBS as described in section 3.2.2, except that the concentration of these working cultures were approximately 4 log CFU/mL for each strain. Whole mushrooms were bought in a local supermarket and stored refrigerated for further use for maximum 1 day. Each mushroom batch was tested for natural contamination with *L. monocytogenes* by mixing mushrooms 1:3 (weight/volume) with sterile PBS buffer in stomacher bags (Antonides) following by homogenization for 1 minute at 230 rpm using a stomacher device (Stomacher 400 circulator, Seward). One milliliter was plated on Agar Listeria according to Ottaviani-Agosti (ALOA) plates (Biomérieux) using the spread plate method and plates were incubated for 24-48 hours at 37 °C. When count on ALOA plates were below the limit of enumeration of 0.5 log CFU/gram, then the mushroom batch was used for further experiments using artificial inoculation. Smashed mushrooms were obtained by adding equal portions of mushroom and sterilized demineralized water (weight/volume) into a stomacher bag (Antonides) followed by processing in the stomacher device (Stomacher 400 circulator, Seward) for 1 minute at 230 rpm. Sliced mushrooms were obtained by cutting the mushrooms in half, while whole mushrooms were not processed. Whole mushrooms, sliced mushrooms and smashed mushrooms were subsequently transferred to sterile polystyrene containers (Greiner Bio-One). A working culture of *L. monocytogenes* was inoculated 1:100 (volume/weight) on these mushroom products, aiming for a start inoculum of approximately 2 log CFU/gram to mimic realistic contamination levels. Whole mushrooms were inoculated on the cap, sliced mushrooms on the damaged mushroom tissue and the smashed mushrooms in the mushroom product. Whole and sliced mushrooms were dried in the laminar flow after spreading the droplets with a sterile loop on the surface. All polystyrene containers were closed and incubated statically in a 20 °C incubator having extra polystyrene containers of water to obtain a humid environment that mimicked the humid environment present in the mushroom production and processing environments. The growth potential of *L. monocytogenes* and groups of microbiota naturally present on mushroom products were determined at

the start and after 1, 2 and 6 days of incubation. As a control, natural microbiota was also determined on non-inoculated mushroom products at the start and after 1, 2 and 6 days of incubation. For CFU count determination, whole, sliced and smashed mushrooms were diluted 1:10 (weight/volume) with sterile PBS buffer in a stomacher bag. Products were homogenized for 1 minute at 230 rpm using the stomacher device and decimal dilutions were prepared using Peptone Physiological Salt (PPS) (Tritium Microbiologie). *L. monocytogenes* CFU counts were determined on ALOA plates (Biomérieux) using the spread plate method and plates were incubated for 24-48 hours at 37 °C. Next to *L. monocytogenes* determination, CFU counts of mesophilic and psychrotrophic microorganisms were determined by spread-plating 100 µL on Plate Count Agar (PCA) (Oxoid), followed by incubation of plates for 2-3 days at 30 °C (NEN-EN-ISO 4833-2:2013 (International Organization for Standardization, 2013)) and 10 days at 7 °C (NEN-EN-ISO 17410:2019 (International Organization for Standardization, 2019)), respectively. *Pseudomonas* spp. were determined by spread-plating on Pseudomonas agar base (Oxoid) supplemented with CFC (Cephalothin, Fucidin, Cetrimide) supplement (Oxoid), followed by incubation of plates for 48-72 hours at 25 °C. Lactic acid bacteria were determined by plating on DeMan, Rogosa and Sharpe (MRS) (Merck) supplemented with 1.5 % agar (Oxoid) executed with the pour plate method followed with an overlay of the same medium and plates were incubated for 72 hours at 30 °C. *Enterobacteriaceae* were determined by plating on violet red bile glucose (VRBG) agar (VWR) executed with the pour plate method followed with an overlay with the same medium and plates were incubated for 24 hours at 37 °C. The pH and the HPLC analyses of the smashed mushrooms were done using liquids obtained at the start and after 48 hours of incubation following the approaches described in section 3.2.4 and section 3.2.6, respectively. Experiments were performed with two biologically independent replicates obtained on different days, each consisting of two technical replicates.

3.2.8. Quantifying reproduction and strain variability

The reproduction variability and strain variability were quantified for all strains, and for different PCR groups of strains (e.g. mushroom strains, non-mushrooms strains, and strains of the same serogroup) using the following equations (1) (2) that were adopted from Aryani et al. (2015). The reproduction variability and strain variability were determined for the CFU count increase in planktonic growth during 24 and 48

hours incubation, and for both the CV staining and the CFU counts of the biofilm cells after 48 hours incubation.

Reproduction variability:

$$MSE_{Reproduction} = \frac{RSS}{df} = \frac{\sum_{S=1}^{i} \sum_{R=1}^{j} (X_{RS} - X_S)^2}{n-p} \quad (1)$$

In which MSE is the mean square error,

RSS is the residual sum of squares,

i is the number of strains in a group (ranging from 3 strains in PCR serogroup clusters and 28 strains when all strains are combined),

j is the number of biological reproductions per strain (at least 2),

X_{RS} is the growth capacity (log CFU/mL increase) or CV value of each biological reproduction "R" of strain "S",

X_S is the average growth capacity or CV value of X_{RS} for strain "S",

df is the degrees of freedom with n the number of biologically independent reproductions of all strains (n = i * j) and,

p the number of parameters, which are the number of strains.

Strain variability:

$$MSE_{Strain} = \frac{RSS}{df} = \frac{\sum_{S=1}^{i} (X_S - X)^2}{n-p} \quad (2)$$

In which i is the number of strains in a group (ranging from 3 strains in PCR serogroup clusters and 28 strains when all strains are combined),

X_S is the average growth capacity (log CFU/mL increase) or CV value of strain "S",

X is the average growth capacity or CV value of a particular group of strains (PCR serogroups, mushroom strains, non-mushroom strains, all strains),

df is the degrees of freedom with n the number of strains and,

p the number of parameters, which equals one.

The strain variability and the reproduction variability were compared using the F-test:

$$F = \frac{MSE_1}{MSE_2} \quad (3)$$

Where MSE_1 is the mean square error of variability factor 1 and MSE_2 is the mean square error of variability factor 2. The F-test was used to compare reproduction and strain variabilities, but also variabilities between the groups. Significance was considered at a p-value of 0.01 or lower.

3.2.9. Statistical analysis

A minimum of two biological replicates were obtained for all experiments, and average values and standard deviations (stdev) were determined for each strain per experiment using Microsoft Excel. Two-tailed Student's t-tests were performed using Microsoft Excel to evaluate whether differences between groups of strains were significant, using a significance value of p = 0.05. In addition, two-tailed Student's t-tests were performed to evaluate the significance of differences in nutrient compounds of mushroom medium incubated without and with *L. monocytogenes* for 48 hours at 20 °C, using a significance value of p = 0.05.

3.3. Results

3.3.1. Growth of *L. monocytogenes* in mushroom medium

A well-defined subset of *L. monocytogenes* strains from the mushroom production and processing chain as well as other foodborne strains, human clinical strains and an animal strain (supplemental table 3.1) were characterized for their growth potential in filter-sterilized mushroom medium. This mushroom medium was used for *L. monocytogenes* growth characterization since this medium mimics the nutrient availability in the mushroom processing environment. The results for one mushroom medium batch are shown in figure 3.1, figure 3.2, table 3.1, supplemental figure 3.2B, yet similar trends were observed in other mushroom medium batches (see supplemental figure 3.1, supplemental figure 3.2A and supplemental table 3.2). Starting with approximately 5 log CFU/mL, the *L. monocytogenes* CFU counts increased

till 8.1 to 8.7 log CFU/mL after 24 hours of incubation and till 8.4 to 9.1 log CFU/mL after 48 hours of incubation (figure 3.1), resulting in an increase of 2.9 to 3.6 log units for 24-hours cultures and an increase of 3.3 to 3.7 log units for 48-hours cultures, respectively. More specifically, the CFU counts of mushroom strains increased till 8.3 to 8.7 log CFU/mL and till 8.5 to 8.9 log CFU/mL after 24 hours and 48 hours of incubation, respectively, resulting in an increase of 3.2 to 3.6 log units for 24-hours cultures and 3.3 to 3.7 log units for 48-hours cultures, respectively. Differences between mushroom and non-mushroom strains were not significant after 24 hours and 48 hours of incubation, because non-mushroom strains showed an increase of 2.9 to 3.5 log units for 24-hours cultures and 3.3 to 3.7 log units for 48-hours cultures, respectively. In addition, no significant differences ($p > 0.05$) were observed between PCR serogroups when increases of CFU counts were compared after 24 hours of incubation. Following incubation of the mushroom medium for 2 days at 20 °C, the pH of the non-inoculated mushroom medium remained comparable at 6.7, while the pH of the mushroom medium inoculated with individual *L. monocytogenes* strains decreased from 6.7 till 5.0 to 5.3 (figure 3.1).

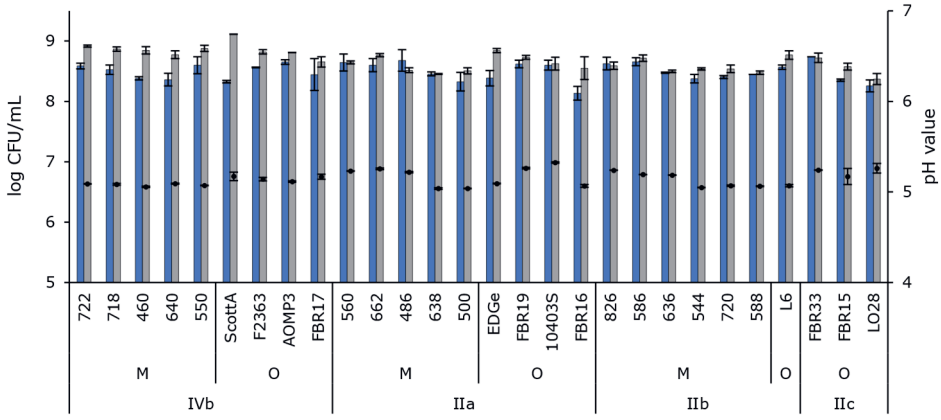

Figure 3.1. Growth performance of *L. monocytogenes* strains during static incubation at 20 °C in filter-sterilized mushroom medium. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in sterile mushroom medium and CFU counts were determined after 24 hours (blue bars) and 48 hours (grey bars) of incubation. Strains were clustered based on PCR serogroup followed by clustering based on mushroom strains "M" and non-mushroom strains "O" (including human clinical strains, other food strains and an animal strain). The black dots represent the pH values after 48 hours of growth. Error bars indicate the standard deviation of the biological replicates (n=2). replicates of all strains were executed in the same mushroom medium batch.

Interestingly, after 24 hours and 48 hours of incubation, the strain variability of the log increase was not significantly higher than the reproduction variability when calculated for all strains, or when calculated for the mushroom strains or the non-mushroom strains. This indicates that strain differences in growth performance were rather low. Also, the strain variabilities in log increase after 24 hours and 48 hours were not significantly different between the mushroom strains and the non-mushroom strains and between the four PCR serogroups. This points to rather comparable behaviour between strains and between groups of strains.

3.3.2. Biofilm formation of *L. monocytogenes* using mushroom medium

As the planktonic growth counts of most of the strains further increased after 24 hours, biofilm formation was determined after 48 hours. All strains formed biofilms, and the average OD_{595} values of bound CV for all but one strain was between 1-2 (figure 3.2A). Comparable CV staining values were observed between technical replicates, but variations were observed in the CV staining values for biological replicates of the analyzed strains explaining the standard deviations (figure 3.2A). Such variations in CV values between biological replicates were also observed in other mushroom medium batches (data not shown). The biofilm CFU counting technique was applied to quantify the counts of culturable cells present in the biofilm, and average CFU counts ranged between 5.5 and 7.5 log CFU/mL per strain (figure 3.2B). Although variations exist in biofilm forming capabilities between strains, grouping the mushroom strains and the non-mushroom strains showed rather similar average values of the biofilm CFU counts of 6.9 and 6.6 log CFU/mL, respectively. In addition, the grouping of the *L. monocytogenes* strains per PCR serogroup revealed rather similar average values of the biofilm CFU counts since PCR serogroup IVb, IIa and IIb showed average values of 7.0, 6.7, and 6.8 log CFU/mL, respectively. However, the PCR serogroup IIc, that included a low number of strains (n=3), had a lower average biofilm CFU count of 6.2 log CFU/mL, which was due to the low biofilm formation of *L. monocytogenes* strain LO28. In addition, the CV staining values were not directly correlated with the CFU counts since for some lower CV values rather high CFU values were observed as shown for Lm460 and AOMP3.

The non-mushroom strains showed more variation in biofilm formation than the mushroom strains based on CFU counts, demonstrated by the ranges of 6.6 - 7.3 log

CFU/mL for the mushroom strains and 5.5 – 7.5 log CFU/mL for the non-mushroom strains, respectively. Indeed, also the strain variability of the non-mushroom strains was significantly higher than the mushroom strains based on the biofilm CFU counts ($p < 0.01$), though this was not the case when comparing the CV values ($p = 0.50$). Especially the non-mushroom strains FBR17 and LO28 showed rather low biofilm CFU counts, while the CV values of these strains were rather comparable to other non-mushroom and mushroom strains.

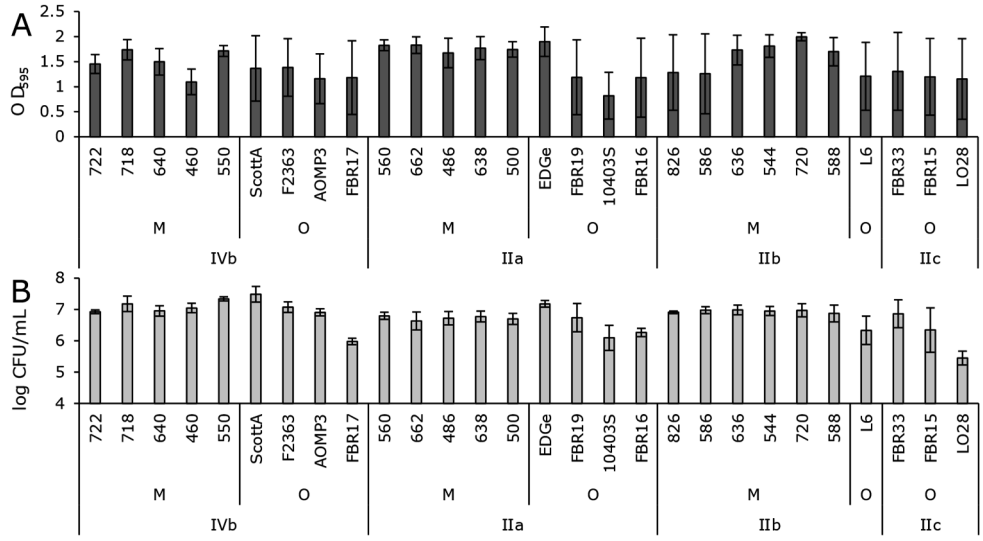

Figure 3.2. Biofilm formation of *L. monocytogenes* strains after incubation for 48 hours at 20 °C in polystyrene plates. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in filter-sterilized mushroom medium and incubated statically. (A) CV staining is shown as optical density at OD_{595} in which the OD_{595} value of the blank is subtracted by the OD_{595} value of the sample. (B) Culturable biofilm cells are expressed in log CFU/mL. Strains were clustered based on PCR serogroup followed by clustering based on mushroom strains "M" and non-mushroom strains "O" (including human clinical strains, other food strains and an animal strain). Error bars indicate the standard deviation of the biological replicates (n=2) of the same mushroom medium batch.

3.3.3. Compound analysis of mushroom medium

HPLC analysis of the mushroom medium showed high concentrations of mannitol (46.71 mM) and significant levels of other compounds, namely, glucose, glycerol and fructose with concentrations of 2.21 mM, 4.88 mM and 6.41 mM, and with trehalose present at trace levels (table 3.1). Similar trends in compound composition were

observed for other mushroom medium batches (supplemental table 3.2). Following 48 hours of static incubation of *L. monocytogenes* in the mushroom medium, the concentration of mannitol remained constant, while concentrations of the other substrates decreased. Metabolism of these substrates resulted in the formation of lactate, acetate and acetoin as the main products (table 3.1). The formation of acetic acid and lactate possibly contributes to the decrease in the pH, while the other major product acetoin is a neutral component and does not influence the pH decrease. The individually tested *L. monocytogenes* strains incubated at static conditions showed similar patterns of compound utilization and product formation when cultured in the same mushroom medium batch and were therefore averaged (table 3.1). Similar trends of compound utilization and product formation were also observed in the other mushroom medium batches (data not shown).

Table 3.1. Extracellular metabolite composition of filter-sterilized mushroom medium without inoculation of *L. monocytogenes* and with inoculation of *L. monocytogenes* followed by static incubation for 48 hours at 20 °C using the same mushroom medium batch as in figure 3.1. Compound analysis of non-inoculated mushroom medium was performed with 8 technical replicates and the standard deviation represents variations among technical replicates. Compound analysis after incubation with *L. monocytogenes* was determined for 28 strains, the same strains that are depicted in figure 3.1, namely nine strains of PCR serogroup IVb, nine strains of PCR serogroup IIa, seven strains of PCR serogroup IIb and three strains of PCR serogroup IIc. Compound analysis was performed with two biological replicates for each strain. The standard deviation represents variations among the 28 strains using the mean of the biological replicates

	Without *L. monocytogenes* growth in mM (stdev)	With *L. monocytogenes* growth in mM (stdev)
trehalose	0.04 (0.03)	0.00** (0.00)*
glucose	2.21 (0.13)	0.31 (0.02)*
fructose	6.41 (0.32)	0.00 (0.00)*
glycerol	4.88 (0.58)	2.26 (0.90)*
mannitol	46.71 (1.52)	45.52 (0.81)
lactate	0.34 (0.43)	10.22 (0.66)*
acetate	0.54 (0.73)	4.44 (0.84)*
acetoin	1.04 (0.22)	1.63 (0.18)*

* Significant difference ($p < 0.05$) in extracellular metabolite concentration in mushroom medium with *L. monocytogenes* growth compared to filter-sterilized mushroom medium without *L. monocytogenes* growth (not inoculated mushroom medium).

** Values of 0.00 represent values that are below the detection limit (detection limit of 0.01 mM).

3.3.4. Amino acid analysis of mushroom medium

Analysis of nitrogen compounds in fresh sterile mushroom medium showed the presence of 20 amino acids (histidine, asparagine, serine, glutamine/arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine, tryptophan) and ammonium in all the tested mushroom medium batches. The concentrations of the amino acids differed between the batches, but the relative proportions of amino acids in a particular batch was comparable with the other mushroom medium batches tested. Amino acids present in the highest amounts in the mushroom medium were asparagine, alanine and glutamine/arginine, while amino acids present in the lowest amounts in the mushroom media batches were cysteine, tyrosine and lysine. The average amino acid concentrations of five mushroom medium batches is shown in supplemental figure 3.2A.

The amino acid concentrations in sterile mushroom medium incubated for 48 hours at 20 °C without inoculation of *L. monocytogenes* were higher compared to the initial concentrations in the fresh sterile mushroom medium at the start of the incubation (supplemental figure 3.2B), pointing to proteolytic activity in the sterile mushroom medium. Interestingly, an increase of amino acids in mushroom medium was also observed when the medium was inoculated with *L. monocytogenes* and tested after incubation for 48 hours at 20 °C. Only the amino acids glutamic acid and cysteine slightly decreased during sterile mushroom medium incubation and during *L. monocytogenes* incubation for most of the strains tested. This indicated that the mushroom medium did not lack critical amino acids for *L. monocytogenes* growth, as also reflected in the high cell numbers reached in the growth experiments.

3.3.5. *L. monocytogenes* growth on mushroom products

Static incubation at 20 °C led to comparable growth increases among five genetically different *L. monocytogenes* strains (supplemental table 3.1) at day 2 and day 6 for each of the three mushroom products using different mushroom batches. This highlights comparable behaviour among *L. monocytogenes* strains in the presence of natural microbiota. The CFU counts of *L. monocytogenes* at day 6 was the lowest for whole mushrooms with average final numbers of 4.6 log CFU/gram, while sliced mushrooms had average final numbers of 5.5 log CFU/gram, and the highest numbers

were observed for smashed mushrooms with average final numbers of 8.5 log CFU/gram (supplemental figure 3.3).

One of these five *L. monocytogenes* mushroom strains (namely Lm636) was randomly selected for complementary growth experiments, in which also the CFU counts of the natural microbiota was determined. At the start of incubation, the accompanying microbiota showed relative high *Pseudomonas* counts and relative low counts of lactic acid bacteria and *Enterobacteriaceae*. The initial counts of *Pseudomonas* of each analyzed mushroom sample was comparable with the mesophilic counts and the psychrotrophic counts and were between 6.3 and 7.6 log CFU/gram (figure 3.3A, 3.3B, 3.3C). Also during the six days of incubation, the mesophilic counts, psychrotrophic counts and *Pseudomonas* counts were comparable and increased over time (figure 3.3A, 3.3B, 3.3C), with the average final mesophilic counts of 8.8, 9.5 and 9.6 log CFU/gram for whole, sliced and smashed mushroom products, respectively. Also the counts of lactic acid bacteria and *Enterobacteriaceae* increased during the incubation with average final counts of lactic acid bacteria and *Enterobacteriaceae* of 3.0 and 4.5 log CFU/gram for whole mushrooms and 3.6 and 5.9 log CFU/gram for sliced mushrooms, respectively. The highest increase in CFU counts of lactic acid bacteria and *Enterobacteriaceae* was observed during incubation of smashed mushrooms, with values reaching approximately 8.7 log CFU/gram and 8.1 log CFU/gram, respectively (figure 3.3A, 3.3B, 3.3C). The *L. monocytogenes* mushroom strain Lm636 showed growth on all three mushroom products (figure 3.3A, 3.3B, 3.3C), indicating little interference by high CFU counts of competitive microbiota present on the different mushroom products. Growth of the accompanying microbiota on the three mushroom products was not affected by the addition of the *L. monocytogenes* strain Lm636 since incubation without *L. monocytogenes* resulted in comparable growth behaviour of the accompanying microbiota (supplemental figure 3.4).

To determine the impact of the natural microbiota of mushrooms on substrate utilization and product formation, compounds were measured in incubated smashed mushrooms without and with the presence of *L. monocytogenes* strain Lm636. Following 2 days of incubation, trehalose, glucose and fructose were not detected or decreased to low detectable levels, while residual levels of glycerol were relatively high (supplemental table 3.3). Interestingly, the concentration of mannitol decreased after 2 days of incubation, while at the same time, an increase was observed for the acidic components lactate and acetate, although the concentration of especially lactate varied

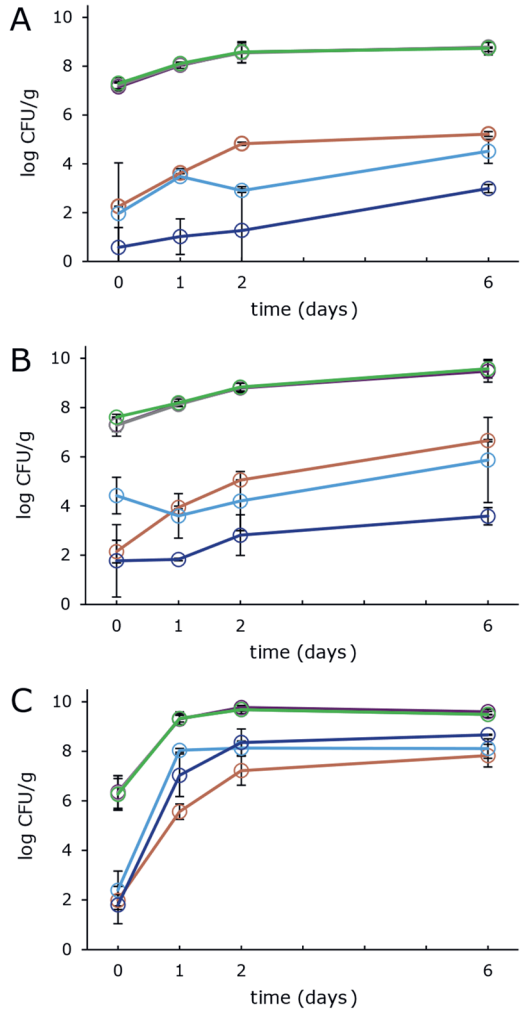

Figure 3.3. Growth performance testing of *L. monocytogenes* strain Lm636 and accompanying microbiota on mushroom products. *L. monocytogenes* strain Lm636 was inoculated (2 log CFU/gram) on three mushroom products; (A) whole mushrooms, (B) sliced mushrooms and (C) smashed mushrooms and statically incubated at 20 °C for a maximum of 6 days. *L. monocytogenes* and groups of accompanying microbiota were determined in log CFU/gram (log CFU/g) at day 0, 1, 2 and 6. The dark red line represents the *L. monocytogenes* counts, the purple line the mesophilic counts, the grey line the psychrotrophic counts, the green line the *Pseudomonas* counts, the light blue line the counts of *Enterobacteriaceae* and the dark blue line the counts of the lactic acid bacteria. Experiments were executed in at least two biological replicates in two mushroom batches, each consisting of two technical replicates. Error bars indicate the standard deviation of the biological replicates.

from batch to batch (supplemental table 3.3). Smashed mushroom products that were not inoculated with *L. monocytogenes* showed comparable decreases of the substrates and an increase of the acidic products and a comparable decrease in pH was observed (supplemental table 3.3). These results indicate that *L. monocytogenes* is able to grow well in the tested mushroom products (figure 3.3) (supplemental figure 3.3), despite extensive growth of natural microbiota.

3.4. Discussion

This study demonstrated that *L. monocytogenes* is able to grow in conditions conceivably encountered in the mushroom production and processing industry environment. The diverse selection of mushroom strains and non-mushroom strains, that covered different PCR serogroups and clonal complexes, showed significant growth in the mushroom medium. This mushroom medium was used in this study to mimic the nutrient availability in the mushroom processing environments. Although *L. monocytogenes* strains have previously been isolated from mushroom processing environments (Lake et al., 2021; Murugesan et al., 2015; Pennone et al., 2018) knowledge on growth and biofilm formation of *L. monocytogenes* in relation to the industrial mushroom production and processing conditions is limited. A previous study performed a large scale biofilm experiment with mushroom isolates on relevant mushroom production surfaces, but the nutrient-rich laboratory medium broth BHIYE was used (Dygico et al., 2020), which does not reflect the nutrient availability during mushroom processing. Another study that determined the growth of *L. monocytogenes* mushroom isolates used unsterilized mushroom broth, but the authors included only a small number of mushroom isolates (i.e. four) (Murugesan et al., 2016).

The strain variability in growth performance was not significantly higher than reproduction variability, pointing to relatively little variation in growth performance between strains. Strain type CC224, that was previously multiple times isolated in a mushroom factory, and strain type CC87, that also was isolated during multiple occasions in two factories, belong both to PCR serogroup IIb (Lake et al., 2021) and did not show a better performance in terms of growth in mushroom medium. Besides, the growth performance of the non-mushroom strains was rather comparable to the mushroom strains, highlighting that mushroom strains did not perform better than non-mushroom strains. This underlined that mushroom medium is a rich nutrient

source for *L. monocytogenes* and may explain the high genetic diversity between the *L. monocytogenes* strains that were previously isolated from the mushroom production and processing environment (Lake et al., 2021). These results are in line with results that suggested that strain specific phenotypes in energy source utilization are probably not involved in the persistence of the persistent strains (Taylor and Stasiewicz, 2019). On the contrary, another study stated that persistent strains may possibly better adapt than non-persistent strains in stressful conditions (Magalhães et al., 2016). It was suggested that the persistence of *L. monocytogenes* may be attributed to other factors such as the resistance to cleaning and disinfection (Wulff et al., 2006), but whether the abundant strain types CC87 and CC224 have a higher resistance to cleaning and disinfection remains to be elucidated.

Biofilm formation was observed for all 28 tested *L. monocytogenes* strains, This is in agreement with another study which stated that the strain origin was not a significant factor in influencing biofilm production (Kadam et al., 2013), although other types of media were used compared to the presented study. Both methods showed variations between the strains in their ability to form biofilm, which is in agreement with another study that showed that biofilm formation is strain dependent and not associated with a serotype and in which no differences were observed between lineage I and II strains (Rodríguez-Campos et al., 2019). Indeed, the highly abundant strain types CC224 and CC87 grouped in PCR serogroup IIb did not show a better biofilm performance compared to other *L. monocytogenes* strains. No correlation was observed between persistence and higher biofilm formation of *L. monocytogenes* strains in a study using polystyrene microtiter plates (Magalhães et al., 2017), while other studies observed increased biofilm formation for persistent strains compared to sporadic strains (Borucki et al., 2003; Rodríguez-Campos et al., 2019). These studies are however difficult to compare as the studies used different media, temperatures and incubation times for biofilm development and none of them used mushroom related medium. For future studies it is relevant to also evaluate biofilm formation on surfaces like stainless steel and polyvinyl chloride, as those materials are used in mushroom processing environments.

Five genetically different *L. monocytogenes* strains showed similar growth characteristics on each of the three mushroom products, i.e. whole, sliced and smashed mushrooms. A higher increase in *L. monocytogenes* CFU counts was observed when the product was more damaged, probably caused by the release of higher levels

of suitable substrates that supporting *L. monocytogenes* growth. Growth of *L. monocytogenes* on whole and sliced mushroom products was demonstrated before (Chikthimmah et al., 2007; González-Fandos et al., 2001; Leong et al., 2013) with higher maximum CFU counts for the sliced mushrooms compared to whole mushrooms, which was also ascribed to increased available nutrients (Chikthimmah et al., 2007; Leong et al., 2013) that led to higher specific growth rates for sliced mushrooms (figure 3.4). One study described *L. monocytogenes* growth on whole mushrooms during the lag phase of the competitors, and no growth or limited growth of *L. monocytogenes* when growth of competitors was evident (González-Fandos et al., 2001). Leong et al. (2015) did not report an increase of *L. monocytogenes* incubated on whole mushrooms, although the same handling steps were applied as in this study. These studies are in contrast with our research that showed *L. monocytogenes* growth in the presence of high microbiota CFU numbers, underlining the competitive fitness of *L. monocytogenes* on mushroom products.

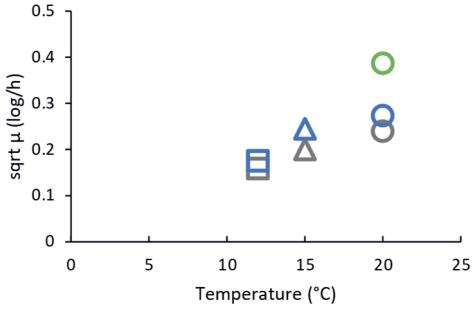

Figure 3.4. Specific growth rate of *L. monocytogenes* on mushroom products as function of temperature. The (highest estimated) values of the specific growth rate (μ) were determined by the log increase in CFU/gram divided by the time in hours (h) necessary for this log increase of the *L. monocytogenes* strains: squares are values adapted from Chikthimmah et al. (2007), triangles are values adapted from Leong et al. (2013) and circles are values adapted from this paper in which the specific growth rates were determined after one day of incubation. The growth rates of González-Fandos et al. (2001) are excluded since growth of *L. monocytogenes* was reported to be influenced by competitors, which was not observed/reported in this study or the other studies. Grey, blue and green color corresponds to whole mushrooms, sliced mushrooms and smashed mushrooms, respectively.

Fresh raw mushrooms contain naturally present microbiota with an average total viable counts (TVC) of 7.0 log CFU/gram. Other studies determined similar or higher TVC on raw mushrooms (7 to 8 log CFU/gram) (González-Fandos et al., 2001; Reyes et al., 2004; Venturini et al., 2011) and in agreement with these studies, we observed that

the total viable count were comparable to the counts of *Pseudomonas*. On the other hand, another study presented lower average TVC (3.8 log CFU/gram) (Leong et al., 2015). The TVC present on raw mushrooms could possibly have a competitive effect on the growth performance of *L. monocytogenes* (González-Fandos et al., 2001). However, our study demonstrated not such effect, despite the high starting numbers of microbiota. Moreover, CFU counts of *L. monocytogenes* still increased in smashed mushroom products when CFU counts of other microbiota, i.e. *Pseudomonas*, were as high as 9.6 log CFU/gram. A factor that may explain these results is the presence of enzymes in the mushroom tissue, as protease activity has been described before in *A. bisporus* mushrooms (Burton et al., 1994). Proteolytic activity was also observed in the current study (supplemental figure 3.2B) showing higher amino acid concentrations in the sterile mushroom medium after incubation for 2 days at 20 °C compared to fresh sterile mushroom medium. Enzyme activity could lead to increased nutrient availability, next to the availability of several carbohydrates and amino acids, and this may support the good growth of *L. monocytogenes* in the presence of other microorganisms.

3.5. Conclusion

This research showed that mushroom strains and non-mushroom strains grew well in filter-sterilized mushroom medium and also formed biofilms. The variability in growth performance of *L. monocytogenes* mushroom strains was rather low, and comparable between PCR serogroups. Also *L. monocytogenes* strains with clonal complex type that were isolated more often in mushroom production facilities did not show any better performance compared to clonal complex types that were less frequently isolated. *L. monocytogenes* CFU counts increased on whole, sliced and smashed mushroom products despite the presence of high numbers of background microbiota, highlighting that mushroom products are a suitable nutrient source for the growth of *L. monocytogenes*.

Abbreviations

RTE, ready-to-eat; CV, crystal violet; *Lm*, *Listeria monocytogenes*

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Supplemental information

Supplemental tables

Supplemental table 3.1. Strains used in growth performance tests in filter-sterilized mushroom medium, with a selection of strains used in growth performance tests on three different mushroom products, i.e. whole mushrooms, sliced mushrooms and smashed mushrooms, with their corresponding information

Supplemental table 3.1 part A. *Listeria monocytogenes* strains isolated from different steps of the mushroom production and processing chain during an extensive sampling survey in April 2018 at a mushroom growing facility and two mushroom processing factories in the Netherlands (Lake et al., 2021)

Strain number	Source	Isolation type	Type of plant	Plant	PCR-serogroup	Lineage	ST (MLST)	CC (MLST)
460	Casing soil during second harvest	Casing soil	Grower	1	IVb	I	ST6	CC6
486	Equipment during machine harvesting second harvest	Swabs during processing	Grower	1	IIa	II	ST37	CC37
500*	Equipment after C&D	Swab after C&D	Grower	1	IIa	II	ST451	CC451
544	Equipment after C&D	Swab after C&D	Factory	1	IIb	I	ST87	CC87
550	Raw material after mechanically harvesting	Fresh mushroom	Grower	1	IVb	I	ST1	CC1
560*	Raw material after mechanically harvesting	Fresh mushroom	Grower	1	IIa	II	ST37	CC37

Supplemental table 3.1 part A continued

Strain number	Source	Isolation type	Type of plant	Plant	PCR-serogroup	Lineage	ST (MLST)	CC (MLST)
586	Final product - mix growers	Frozen sliced mushroom	Factory	1	IIb	I	ST224	CC224
588	Final product - mix growers	Frozen sliced mushroom	Factory	1	IIb	I	ST87	CC87
636*	Final product - grower 1	Frozen sliced mushroom	Factory	1	IIb	I	ST224	CC224
638	Final product - grower 1	Frozen sliced mushroom	Factory	1	IIa	II	ST451	CC451
640*	Final product - grower 1	Frozen sliced mushroom	Factory	1	IVb	I	ST4	CC4
662	Equipment during mushroom processing - grower 1	Swabs during processing	Factory	1	IIa	II	ST207	CC207
718	Final product - mix growers	Frozen sliced mushroom	Factory	2	IVb	I	ST1	CC1
720*	Final product - mix growers	Frozen sliced mushroom	Factory	2	IIb	I	ST87	CC87
722	Final product - mix growers	Frozen sliced mushroom	Factory	2	IVb	I	ST4	CC4
826	Equipment after C&D	Swab after C&D	Factory	1	IIb	I	ST224	CC224

* Strain used for growth performance test on whole, sliced and smashed mushroom products.

Supplemental table 3.1 Part B. *Listeria monocytogenes* strains including human clinical strains, other food strains and an animal strain. Descriptions derived from in-house whole genome sequencing data, Aryani et al., 2015 and Yin et al., 2015

Strain	Source	Isolation type	Serotype	Lineage	ST (MLST)	CC (MLST)
ScottA	Human isolate from Massachusetts milk outbreak	Human isolate	4b	I	ST2	CC2
F2365	Jalisco cheese	Food isolate	4b	I	ST1	CC1
AOMP3	Human isolate	Human isolate	4b	I	ST2	CC2
EGDe	Rabbit	Animal isolate	1/2a	II	ST35	CC9
10403S	Human	Human isolate	1/2a	II	ST85	CC7
L6	Milk	Food isolate	1/2b	I	ST3	CC3
LO28	Healty pregnant carrier	Human isolate	1/2c	II	ST9	CC9
FBR15	Ice cream packaging machine	Food isolate	1/2c	II	ST122	CC9
FBR16	Ham (after cutting machine)	Food isolate	1/2a	II	ST121	CC121
FBR17	Frozen fried rice	Food isolate	4d	I	ST1	CC1
FBR19	Frozen meat	Food isolate	1/2a	II	ST391	CC89
FBR33	Pancake	Food isolate	1/2c	II	ST9	CC9

Supplemental table 3.2. HPLC compound analysis of extracellular metabolites in four filter-sterilized mushroom medium batches. Presence of extracellular metabolites is shown as average value of two technical replicates per mushroom medium batch. Values are the average values in mM with range of the two technical replicates between brackets. Mushroom medium batches presented here are other mushroom medium batches than presented in the main text

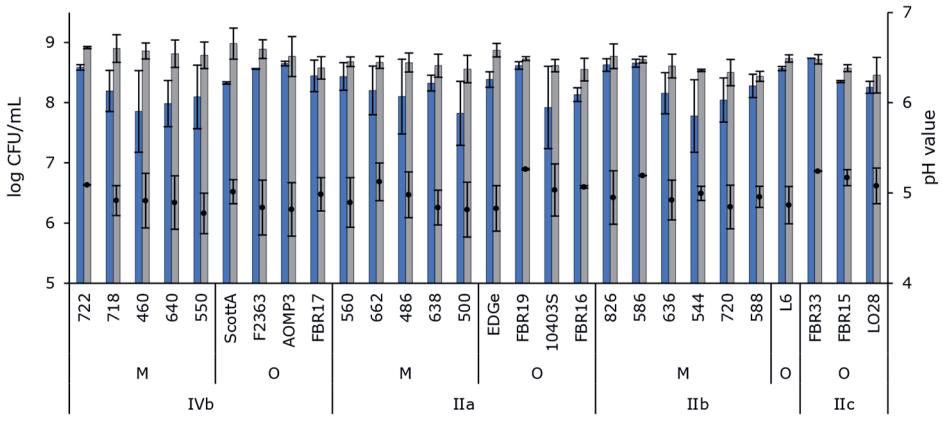
	Batch 1	Batch 2	Batch 3	Batch 4
trehalose	0.19 (0.18-0.19)	0.53 (0.52-0.54)	0.04 (0.03-0.04)	0.03 (0.03-0.03)
glucose	4.57 (4.55-4.59)	4.19 (4.17-4.21)	1.56 (1.56-1.57)	2.98 (2.98-2.98)
fructose	4.10 (4.02-4.17)	3.17 (3.17-3.17)	2.78 (2.67-2.89)	4.84 (4.84-4.85)
glycerol	1.44 (1.41-1.47)	2.12 (2.12-2.12)	2.90 (2.90-2.90)	2.04 (1.98-2.10)
mannitol	53.77 (53.68-53.85)	74.52 (74.51-74.51)	49.55 (49.47-49.63)	75.78 (75.74-75.80)
pH	6.74	6.7	6.73	6.83

Supplemental table 3.3. HPLC compound analysis of extracellular metabolites in two batches of smashed mushrooms with and without *L. monocytogenes* strain Lm636 inoculation onto the product and determined both before and after 48 hours of static incubation at 20 °C. Presence of extracellular metabolites is shown as average value of at least 3 technical replicates per smashed mushroom batch together with the standard deviation values between brackets

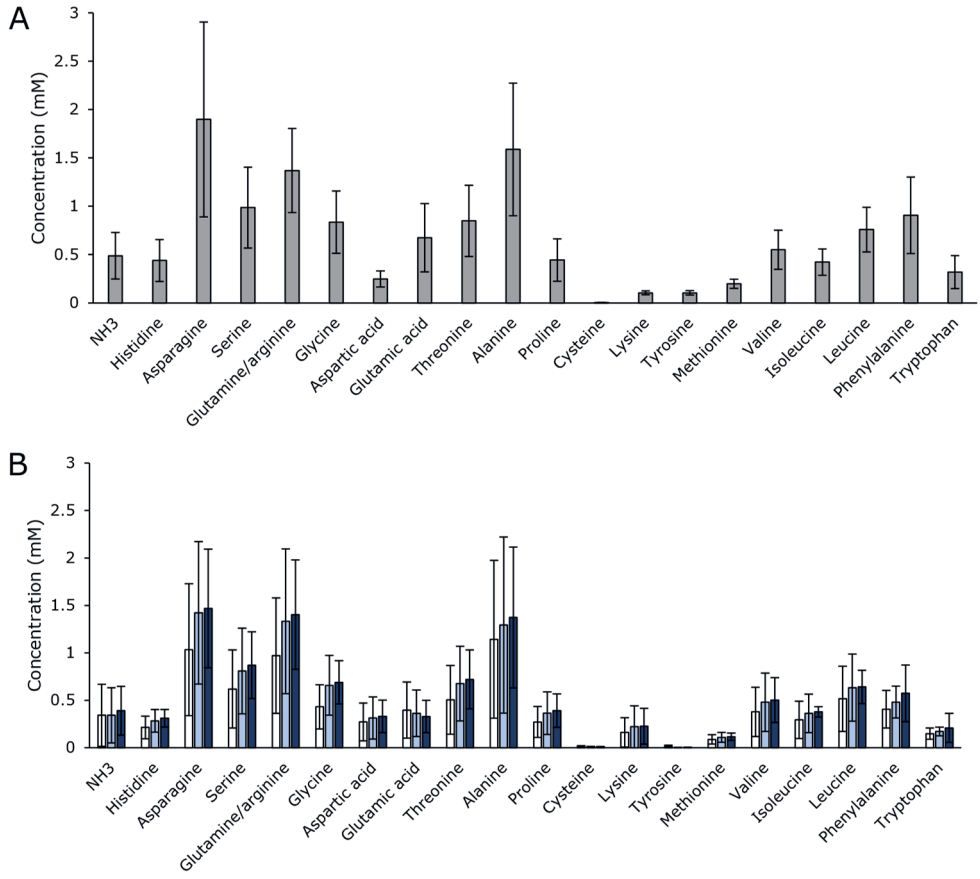
	Before incubation in mM (stdev)		After 2 day incubation with *L. monocytogenes* addition in mM (stdev)		After 2 day incubation without *L. monocytogenes* addition in mM (stdev)	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
trehalose	0.28 (0.12)	1.05 (0.06)	0.01 (0.01)	0.18 (0.06)	0.01 (0.01)	0.24 (0.15)
glucose	0.67 (0.27)	2.58 (0.46)	0.18 (0.08)	0.49 (0.31)	0.14 (0.11)	0.66 (0.71)
fructose	0.36 (0.11)	0.72 (0.19)	0.00* (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
mannitol	35.94 (9.14)	72.30 (8.19)	22.64 (3.98)	57.24 (7.10)	17.72 (7.43)	51.40 (9.34)
glycerol	1.48 (0.37)	1.41 (0.08)	0.37 (0.27)	1.08 (0.23)	0.13 (0.05)	1.04 (0.09)
lactate	0.00 (0.00)	0.01 (0.01)	11.94 (5.41)	37.52 (4.49)	6.42 (0.65)	41.83 (2.55)
actetate	0.12 (0.26)	0.00 (0.00)	8.33 (2.50)	5.72 (0.61)	7.24 (0.84)	6.76 (0.63)
acetoin	0.54 (0.66)	0.72 (0.73)	1.48 (0.84)	1.38 (0.12)	1.49 (0.76)	1.18 (0.88)
pH	6.71 (0.07)	6.64 (0.06)	5.84 (0.32)	4.63 (0.12)	6.22 (0.15)	4.61 (0.15)

* Values of 0.00 represent values that are below the detection limit (detection limit of 0.01 mM).

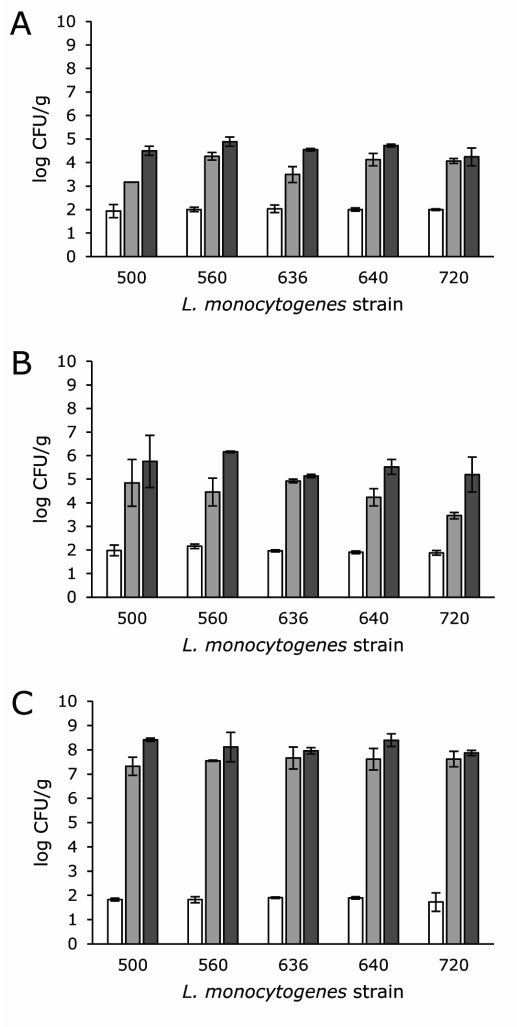
Supplemental figures



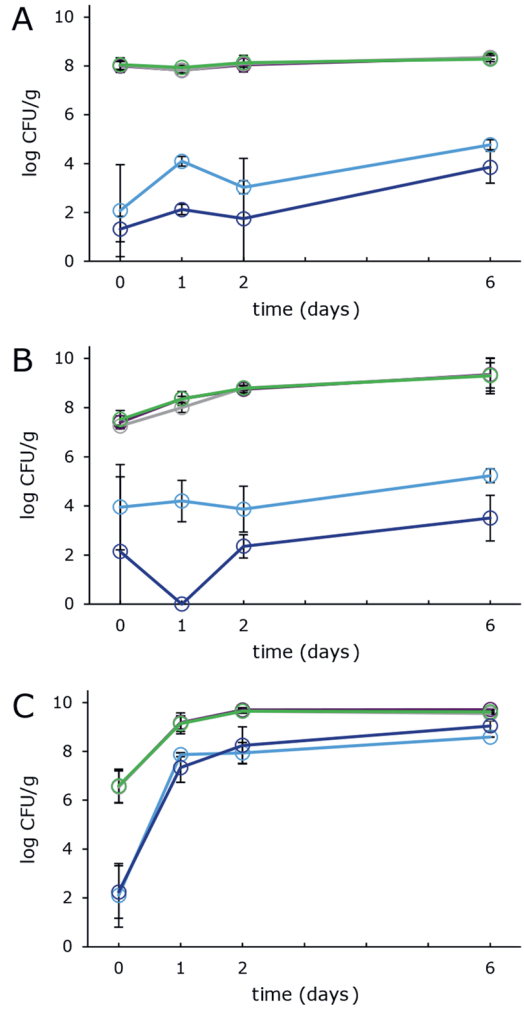
Supplemental figure 3.1. Growth performance of *L. monocytogenes* strains during static incubation at 20 °C in filter-sterilized mushroom medium with data of up to five mushroom medium batches combined. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in sterile mushroom medium and CFU counts were determined after 24 hours (blue bars) and 48 hours (grey bars) of incubation. Strains were clustered based on PCR serogroup followed by clustering based on mushroom strains "M" and non-mushroom strains "O" (including human clinical strains, other food strains and an animal strain). The black dots represent the values for pH after 48 hours of growth. Error bars indicate the standard deviation of the biological replicates (n between 2-8).



Supplemental figure 3.2. (A) Average amino acid concentrations of five fresh sterile mushroom medium batches including the mushroom medium batch analyzed in B. Error bars indicate the standard deviation when the technical replicates of the five mushroom medium batches were combined. (B) Average amino acid concentrations of one mushroom medium batch including fresh sterile mushroom medium (white bars), filter-sterilized mushroom medium after 48 hours of static incubation at 20 °C (light blue bars) and filter-sterilized mushroom medium inoculated with *L. monocytogenes* after 48 hours of static incubation at 20 °C (dark blue bars). The individually tested *L. monocytogenes* strains incubated at static conditions showed similar patterns during amino acid determination within the analyzed mushroom medium batch and were therefore averaged. The analyzed mushroom medium batch was the same as described in figure 3.1, figure 3.2 and table 3.1. Error bars of the fresh sterile mushroom medium at the start and the sterile mushroom medium after 48 hours of incubation indicate the standard deviation of the biological replicates (n=2) each consisting of two technical replicates. Error bars of the mushroom medium inoculated with *L. monocytogenes* followed by incubation for 48 hours at 20 °C indicate the standard deviation when the biological replicates (n=2) of all strains (n=28) are combined. Replicates of all strains were executed in the same mushroom medium batch.



Supplemental figure 3.3. Growth performance testing of five *L. monocytogenes* strains on three different mushroom products; (A) whole mushrooms, (B) sliced mushrooms and (C) smashed mushrooms. Each mushroom product was inoculated with an individual *L. monocytogenes* strain (2 log CFU/gram) followed by static incubation at 20 °C for a maximum of 6 days. CFU counts were determined in log CFU/gram (log CFU/g) at the start (white bars), after 2 days (light grey bars) and after 6 days (dark grey bars) of incubation. Experiments were executed in at least two biological replicates in two mushroom batches, each consisting of two technical replicates. Error bars indicate the standard deviation of the biological replicates.



Supplemental figure 3.4. Growth performance testing of accompanying microbiota on three different mushroom products; (A) whole mushrooms, (B) sliced mushrooms and (C) smashed mushrooms without *L. monocytogenes* strain Lm636 inoculation onto the product. Mushroom products were statically incubated at 20 °C for a maximum of 6 days and groups of accompanying microbiota were determined in log CFU/gram (log CFU/g) at day 0, 1, 2 and 6. The purple line represents the mesophilic counts, the grey line the psychrotrophic counts, the green line the *Pseudomonas* counts, the light blue line the counts of *Enterobacteriaceae* and the dark blue line the counts of the lactic acid bacteria. Experiments were executed in at least two biological replicates in two mushroom batches, each consisting of two technical replicates. Error bars indicate the standard deviation of the biological replicates.

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4

Growth performance of *Listeria monocytogenes* and background microbiota from mushroom processing environments

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Abstract

Interaction between *Listeria monocytogenes* and resident background microbiota may occur in food processing environments and may influence the survival of this pathogen in a factory environment. Therefore the aim of this study was to characterize the growth performance of microbiota isolated from the processing environments of frozen sliced mushrooms, and to investigate the competitive performance of *L. monocytogenes* when co-cultured with accompanying environmental microbiota. *Acinetobacter*, *Enterobacteriaceae*, *Lactococcus* and *Pseudomonas* were the most prominent background microbiota isolated from the processing environment of frozen sliced mushrooms. All individual microbiota strains were able to grow and form biofilm in filter-sterilized mushroom medium, with the mannitol-consumers *Raoultella* and *Ewingella* as top performers, reaching up to 9.6 and 9.8 log CFU/mL after 48 hours incubation at room temperature. When *L. monocytogenes* mushroom isolates were co-cultured with the microbiota strains, *L. monocytogenes* counts ranged from 7.6 to 8.9 log CFU/mL after 24 hours of incubation, while counts of the microbiota strains ranged from 5.5 to 9.0 log CFU/mL. Prolonged incubation up to 48 hours resulted in further increase of *L. monocytogenes* counts when co-cultured with non-acidifying species *Pseudomonas* and *Acinetobacter* reaching 9.1 to 9.2 log CFU/mL, while a decrease of *L. monocytogenes* counts reaching 5.8 to 7.7 log CFU/mL was observed in co-culture with *Enterobacteriaceae* and acidifying *Lactococcus* representatives. In addition, *L. monocytogenes* grew also in spent mushroom media of the microbiota strains, except in acidified spent media of *Lactococcus* strains. These results highlight the competitive ability of *L. monocytogenes* during co-incubation with microbiota in fresh and in spent mushroom medium, indicative of its invasion and persistence capacity in food processing factory environments.

4.1. Introduction

Listeria monocytogenes is an important human foodborne pathogen that can cause listeriosis mainly in the susceptible population, including infants, elderly, pregnant women and immunocompromised people. It is ranked in the top five of human zoonoses in the EU in 2021 having a relatively high case fatality rate of 13.7% (EFSA and ECDC, 2022). This foodborne pathogen has been isolated from natural environmental niches, farm environmental niches (Fox et al., 2009; Terentjeva et al., 2021; Weis and Seeliger, 1975; Weller et al., 2015b) and from food and food processing environments (Ferreira et al., 2014; Jordan et al., 2018).

The frequent presence of *L. monocytogenes* in different food processing environments together with its ability to adapt and survive under stressful conditions makes the control of *L. monocytogenes* in food processing environments challenging (Ferreira et al., 2014). *L. monocytogenes* is a robust organism and can cope with a variety of stresses, because it is able to grow in low temperature conditions till around 1 °C (Junttila et al., 1988), high salt concentrations up to 10.5 % (Shahamat et al., 1980) and at low pH conditions down to pH 4.5 (Parish and Higgens, 1989). Also, *L. monocytogenes* can persist in food processing environments (Carpentier and Cerf, 2011; Møretrø and Langsrud, 2004) where food processing equipment can act as a reservoir of *L. monocytogenes* (Møretrø and Langsrud, 2004; Vogel et al., 2001). Food products can get contaminated when the raw ingredients are contaminated or during food processing when *L. monocytogenes* is transferred from food contact surfaces to the food product (Vogel et al., 2001).

The presence and the robustness of *L. monocytogenes* is a concern for the food industry and especially for the ready-to-eat (RTE) food industry, since RTE food products lack a bacterial inactivation step before consumption (Bergis et al., 2021). Various RTE food products such as fish, meat, cheese and vegetables have been reported to be contaminated with *L. monocytogenes* (EFSA and ECDC, 2018; Szymczak et al., 2020). The presence of *L. monocytogenes* was also described on fresh and frozen white button mushrooms (*Agaricus bisporus*) and in the processing environments of the *A. bisporus* mushroom species (Lake et al., 2021; Murugesan et al., 2015; Pennone et al., 2018). Although mushrooms may be considered as RTE foods as described in some documentation (Health Canada, 2022) (FSAI, 2006) (Jiang et al., 2018), a consumer research showed that consumers usually bake, stir-fry or

cook the mushrooms before consumption (Borgdorff, 2012), and this will reduce the possible risk of exposure.

Survival and growth of *L. monocytogenes* in food processing environments could be affected by resident background microbiota. Sampling of food processing factories has shown that the microbial composition of resident factory microbiota is diverse, while some microbial groups can be dominant including *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae* and lactic acid bacteria (LAB) (Møretrø and Langsrud, 2017). *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae* and/or LAB have been isolated from meat, fish, shrimp, and vegetable processing facility environments (Fagerlund et al., 2017; Gudbjörnsdóttir et al., 2005; Langsrud et al., 2016; Stellato et al., 2016; Xu et al., 2022), and the presence of these background microbiota can affect the growth and biofilm formation of *L. monocytogenes*. Indeed, inhibitory effects, stimulating effects as well as no effects have been reported in laboratory mixed-culture experiments (Carpentier and Cerf, 2011; Carpentier and Chassaing, 2004; Dygico et al., 2019; Haddad et al., 2021; Heir et al., 2018; Martín et al., 2022; Mellefont et al., 2008; Saraoui et al., 2016). Additionally, growth performance and biofilm formation capacity of *L. monocytogenes* was shown to be affected by the media composition used in the experiments (Kadam et al., 2013; Nowak et al., 2015). Up to now, application of model food media representing specific foods and food processing environments has been very limited, while this will approach field conditions as closely as possible to enhance understanding of (competitive) behaviour of microorganisms in food-related environments (Overney et al., 2016).

L. monocytogenes can also be introduced to factory environmental niches that are already occupied by other microorganisms, where *L. monocytogenes* conceivably has to cope with reduced nutrient availability and/or growth inhibitory compounds produced by competitive microbiota. Such conditions can be mimicked in laboratory settings by quantifying growth in so-called spent media. Previous studies that use spent broth media following growth of LAB reported growth inhibition of *L. monocytogenes*, which is conceivably due to the acidification of the spent medium, the production of antibacterial compounds or a combination of both (Bungenstock et al., 2020; Hartmann et al., 2011; Mariam et al., 2014; Milillo et al., 2013). The behaviour of *L. monocytogenes* was also determined in spent broth media following growth of non-LAB strains isolated from meat and salmon industry, including *Pseudomonas*

fluorescens and *Serratia liquefaciens*, and here growth of *L. monocytogenes* was not inhibited (Heir et al., 2018).

To date, microbiota strains isolated from the mushroom processing factory environments have not been characterized, as well as their interaction with *L. monocytogenes* strains. Therefore, this study aims to determine the growth and biofilm formation of microbiota strains in mushroom medium, and in coculture with *L. monocytogenes* strains previously isolated from mushroom processing environments (Lake et al., 2021). Growth performance of selected *L. monocytogenes* isolates will also be assessed in spent mushroom medium following growth of selected microbiota strains. These results will give insights in competitive growth and survival potential of *L. monocytogenes* in conditions mimicking mushroom processing environments.

4.2. Materials and methods

4.2.1. Isolation of bacteria in mushroom processing environments

A factory that produces frozen sliced mushrooms (*Agaricus bisporus*) was visited in the spring of 2019 in the Netherlands and samples were taken from different spots along the whole mushroom processing line. Surface samples from the processing equipment were taken during mushroom processing and after the cleaning and disinfection (C&D) procedures with 3MTM petrifilmTM aerobic count plates (3M company), and petrifilms were incubated at 30 °C for 72 hours. A maximum of up to ten colonies were picked from a randomly chosen side of the petrifilm, and colony selection was done based on relative abundance of the different morphology types of the colonies. The colonies were individually streaked on Tryptone Soya Agar (TSA) (Oxoid) plates supplemented with 0.6% Yeast Extract (YE) (Oxoid), and the TSAYE plates were incubated for 24 to 48 hours at 30 °C. Single colonies of non-pure cultures were restreaked on TSAYE plates followed by incubation for 24 to 48 hours at 30 °C. Single colonies were picked from the pure cultures and inoculated in 10 mL Tryptone Soya Broth (TSB) (Oxoid) medium supplemented with 0.6% Yeast Extract (YE) (Oxoid). Cultures were grown statically for 24 hours at 30 °C in TSBYE, and -80 °C stock cultures were prepared with a final concentration of 25% glycerol (Sigma-Aldrich). In addition, targeted sampling was done for *L. monocytogenes* and *Listeria* spp. strains in the spring of 2018 in the Netherlands (Lake et al., 2021). During this survey, presumptive *L. monocytogenes*

and *Listeria* spp. strains were obtained from Agar Listeria according to Ottaviani-Agosti (ALOA) plates (Biomérieux), with *L. monocytogenes* having blue-green colonies with an opaque halo, and *Listeria* spp. having blue-green colonies without a halo. Presumptive *L. monocytogenes* colonies and *Listeria* spp. colonies (one colony per positive sample), were restreaked on ALOA plates and incubated for 24 hours at 37 °C. A single colony was subsequently restreaked on Brain Heart Infusion agar (BHI) (Becton Dickinson and Company, Difco) plates supplemented with 1.5% agar (Oxoid) and incubated for 24 hours at 30 °C followed by another streak on BHI agar plates incubated for 24 hours at 30 °C to obtain pure isolates. A single colony was transferred to BHI broth that was cultured statically for 17 hours at 30 °C, and -80 °C stock cultures were prepared with a final concentration of 25% glycerol (Sigma-Aldrich).

4.2.2. Identification of microbiota

Strain identification of the microbiota was performed by 16S rRNA gene sequencing. For that, a loopful of the stock culture of each strain was streaked on TSAYE agar plates followed by incubation for 24 to 48 hours at 30 °C. Several colonies per strain were transferred to 100 µL InstaGene Matrix (Bio-Rad) and the manufacturer's protocol was followed for DNA isolation. The 16S rRNA gene was amplified using the universal 16S rRNA gene primers with forward primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards et al., 1989) and reverse primer p6 ('5-CTACGGCTACCTTGTTACGA-3') (Di Cello et al., 1997). The polymerase chain reaction (PCR) mixture contained 0.5 µL genomic DNA, 2.5 µL of 10x Taq buffer (including 20 mM $MgCl_2$, Thermo Scientific), 0.2 mM dNTP mix (Thermo Scientific), 0.6 U Dreamtaq DNA polymerase (Thermo Scientific) and 0.6 µM of each primer in a total volume of 25 µL. The PCR cycle was executed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and included an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 1 minute and extension at 72 °C for 2 minutes, followed by a final extension at 72 °C for 7 minutes. Then, 5 µL of PCR product was mixed with 1 µL 6x loading dye (TriTrack, Thermo Scientific) and the mixture was loaded and examined in a 1% agarose (SeaKem LE agarose, Lonza) gel containing 1x TAE buffer (Bio-Rad) and DNA safe stain (SYBR Safe DNA Gel Stain, Invitrogen). Gels were run in a 1x TAE buffer solution and visualized using ultra violet light (Uvitec, Cambridge). Correct DNA fragments (~1500 base pairs) were purified with the MinElute PCR purification kit (Qiagen) according to the manufactures protocol and the

16S rRNA gene fragments were sent for Sanger sequencing. Taxonomical strain identification was executed using the nucleotide BLAST function on the NCBI website (http://blast.ncbi.nlm.nih.gov) (Boratyn et al., 2013).

The presumed *Listeria* spp. strains were confirmed using a *Listeria* spp. specific primer set targeting the *prs* gene (Doumith et al., 2004) and the species identification was performed with species targeted primers for *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (supplemental table 4.1) using a multiplex PCR reaction (Ryu et al., 2013). Two PCR reaction mixtures were created each having the *Listeria* spp. specific primer set, and reaction mixture 1 contained the species targeted primers of *L. grayi*, *L. ivanovii* and *L. seeligeri*, and reaction mixture 2 contained the species targeted primers of *L. innocua* and *L. welshimeri*. Both PCR reaction mixtures contained 2.5 µL of 10x Taq buffer (including 20 mM $MgCl_2$, Thermo Scientific), 0.2 mM dNTP mix (Thermo Scientific), 0.6 U Dreamtaq DNA polymerase (Thermo Scientific) and the primer sets with concentrations that were previously described (Doumith et al., 2004; Ryu et al., 2013), namely prs primer set of 0.2 µM, *L. grayi* primer set of 0.2 µM, *L. ivanovii* primer set of 0.6 µM, *L. innocua* primer set of 1.2 µM, *L. seeligeri* primer set of 1.4 µM and *L. welshimeri* primer set of 1.0 µM. DNA isolation of the *Listeria* spp. strains was performed with InstaGene Matrix (Bio-Rad) as described above for microbiota and 0.5 µL genomic DNA of each strain was added to both of the reaction mixtures in a total volume of 25 µL. The PCR cycle was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and was adapted (Ryu et al., 2013) with some modifications. Briefly, the PCR cycle contained an initial denaturation step at 94 °C for 10 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute followed by a final extension step at 72 °C for 10 minutes. PCR products were examined as described above and confirmed *Listeria* spp. strains were classified into species.

The presumed *L. monocytogenes* strains were confirmed using a *Listeria* spp. specific primer set targeting the *prs* gene (Doumith et al., 2004) and the *L. monocytogenes* specific primer set targeting the *isp* gene (Rawool et al., 2016) (supplemental table 4.1) using a multiplex PCR reaction (Lake et al., 2021). DNA isolation of the *L. monocytogenes* strains was performed with InstaGene Matrix (Bio-Rad) as described above. A PCR master mixture was constructed containing 0.5 µL genomic DNA, 0.2 µM of *prs* primer set, 0.2 µM of *isp* primer set, 2.5 µL of 10× Taq buffer (including 20 mM $MgCl_2$, Thermo Scientific), 0.2 mM dNTP mix (Thermo Scientific), 0.6 U Dreamtaq DNA

polymerase (Thermo Scientific), in a total volume of 25 µL. The PCR cycle was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and contained an initial denaturation step at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 0.40 min, annealing at 56 °C for 1.15 min and extension at 72 °C for 2 min followed by final extension step at 72 °C for 10 min. PCR products were examined as described above and confirmed *L. monocytogenes* strains were used in further analysis.

4.2.3. Strain selection of background microbiota strains, *Listeria* spp. and *L. monocytogenes*

A total of 18 background microbiota strains that were isolated from equipment of the mushroom factory after the C&D procedures were selected (supplemental table 4.2). These strains belonged to the highly represented genera isolated during both the mushroom processing and after the C&D procedures. Each selected genera was at least three times isolated from a minimal of two sampling spots and multiple strains were selected from the four most highly represented genera that covered over 77% of all strains (supplemental table 4.2). In addition, three *Listeria* spp. strains, namely one strain of *L. innocua*, *L. grayi* and *L. seeligeri* were selected (supplemental table 4.2). Moreover, three *L. monocytogenes* strains that differ in serogroup type and clonal complex type were selected for further characterization (supplemental table 4.2).

4.2.4. Growth of planktonic cultures and mushroom medium preparation

A loopful of the stock cultures of the microbiota strains were inoculated in 10 mL TSBYE medium followed by static incubation at 30 °C for 24 hours. The stock cultures of the *Listeria* spp. strains and the *L. monocytogenes* strains were inoculated in 10 mL BHI medium followed by static incubation at 30 °C for 18 hours. The cultures were centrifuged for 2 minutes at 16,000 x *g*, after which supernatant was discarded and the pellet was dissolved in phosphate buffered saline (PBS) buffer (International Organization for Standardization, 2017). These steps were repeated once and pellets were dissolved and diluted in PBS buffer to obtain a working culture of approximately 10^7 CFU/mL.

Mushroom medium was prepared as before (Lake et al., 2023) by harvesting the mushrooms (*Agaricus bisporus*) at a mushroom grower, transporting the mushrooms to the laboratory followed by refrigerated storage for maximum three days. Upon processing, the mushrooms were cut into pieces and divided in quantities of 500 grams. Portions of 500 grams of mushrooms and 200 mL of non-sterilized demineralized water were added to a stomacher filter bag (Antonides) and homogenized using a stomacher (Stomacher 400 circulator, Seward) for 1 minute at 230 rpm. Obtained mushroom medium was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific). Supernatants of different portions prepared on the same day were collected and combined in a big flask and homogenized by shaking to obtain a mushroom medium batch. Multiple mushroom media batches were prepared on different days and each mushroom medium batch was stored for a maximum of six months at -20 °C upon use.

Upon use, the thawed mushroom medium was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific) and the supernatant was filter-sterilized using a 0.45 µm filter (Minisart® syringe filter, Sartorius) followed by a 0.22 µm filter (Minisart® syringe filter, Sartorius). This fresh filter-sterilized non-inoculated mushroom medium was used as a control in each of the experiments together with non-inoculated mushroom media that was incubated for 48 hours at 20 °C. For individual microbiota strain characterization, filter-sterilized mushroom medium was inoculated 1:100 (vol/vol%) with the working culture of a microbiota strain, reaching approximately 10^5 CFU/mL. In addition, a cocktail of strains was prepared by mixing in equal quantities the working cultures of each of the 18 microbiota strains (each approximately 10^7 CFU/mL), after which the filter-sterilized mushroom medium was inoculated 1:100 (vol/vol%) with this cocktail, reaching approximately 10^5 CFU/mL. Inoculated mushroom medium was added into polystyrene 96-wells plates (Greiner Bio-One) and incubated statically for 48 hours at 20 °C. Samples were taken every day followed by preparing decimal dilutions and plating on TSAYE plates, which were incubated for 24 to 48 hours at 30 °C. Two to three biologically independent reproductions were performed on different days for each of the strains, and also for the cocktail of strains.

4.2.5. Co-culture growth experiments

The microbiota, *Listeria* spp. and *L. monocytogenes* strains were individually cultured and a working culture was prepared as described above (section 4.2.4). The filter-sterilized mushroom medium (section 4.2.4) was inoculated 1:100 (vol/vol%) with both the working culture of one of the *L. monocytogenes* strains and one of the microbiota or *Listeria* spp. strains (supplemental table 4.2) to obtain an initial concentration of approximately 10^5 CFU/mL for both strains. Inoculated mushroom medium was added into polystyrene 96-wells plates (Greiner Bio-One) and incubated statically for 48 hours at 20 °C. Samples were taken every day followed by preparing decimal dilutions and plating on TSAYE plates and on ALOA plates. TSAYE plates were incubated for 24 to 48 hours at 30 °C and ALOA plates were incubated for 24 hours at 37 °C. Two to four biologically independent reproductions were performed on different days for each of the strain combinations.

4.2.6. Spent medium experiments

The filter-sterilized mushroom medium (section 4.2.4) was inoculated 1:100 (vol/vol%) with the working culture of the microbiota strain or the *Listeria* spp. strain (section 4.2.4) to obtain an initial concentration of approximately 10^5 CFU/mL. Inoculated mushroom medium was added into polystyrene 96-wells plates (Greiner Bio-One) and incubated statically for 72 hours at 20 °C. Samples were taken every day followed by preparing decimal dilutions and plating on TSAYE plates, which were incubated for 24 to 48 hours at 30 °C. The culture was extracted from the wells plate after 72 hours of incubation by combining the volumes of identically treated wells. The culture was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific) and the supernatant was collected, while the pelleted cells were discarded. The supernatant was equally divided in two 15 mL tubes (Greiner Bio-One) and the pH of the spent medium in one of the tubes was not adjusted, while the pH in the other tube was adjusted to 6.9 using 2.5 M and 0.25 M NaOH or 2.5 M and 0.25 M HCl to obtain a similar pH as fresh mushroom medium. Before use, the spent media were sterilized by using a 0.45 µm filter (Minisart® syringe filter, Sartorius) followed by a 0.22 µm filter (Minisart® syringe filter, Sartorius).

The *L. monocytogenes* strains were cultured in BHI medium and a working culture with a concentration of approximately 10^7 CFU/mL was prepared as described above (section 4.2.4). Each *L. monocytogenes* strain was individually inoculated 1:100 (vol/vol%) in spent media (pH-adjusted and non-pH adjusted) to obtain an initial concentration of approximately 10^5 CFU/mL. Inoculated spent mushroom medium was added into polystyrene 96-wells plates (Greiner Bio-One) and incubated statically for 48 hours at 20 °C. The counts of *L. monocytogenes* strains were monitored every day followed by plating decimal dilutions on TSAYE plates and plates were incubated for 24 to 48 hours at 30 °C. Two or three biologically independent reproductions were performed on different days for each of the strain combinations.

4.2.7. pH measurements

The pH measurements were performed before and after all growth experiments. The pH was also measured of the non-inoculated mushroom medium statically incubated for 48 hours or 72 hours at 20 °C. Prior to pH measurements of the medium incubated with (a) bacterial strain(s), cultures were centrifuged for 2 minutes at 16,000 x *g* to remove cells, and the supernatant was collected. The pH measurements were executed using a microelectrode (Inlab Ultra Micro-ISM, Mettler Toledo) coupled with a pH meter instrument (PHM240 pH/ion meter, Meterlab, Radiometer Analytical) and the device was calibrated before each series of measurements.

4.2.8. Biofilm formation

Strains were grown in single cultures (section 4.2.4), in co-cultures (section 4.2.5) and in spent medium (section 4.2.6) and the biofilm forming capacity of the strains was determined after 48 hours of incubation in polystyrene 96-wells plates (Greiner Bio-One). Quantifying biofilm formation is frequently applied with the crystal violet (CV) assay (Kadam et al., 2013) to determine total biomass and this method was adapted from Fernández Ramírez et al. (2015) and Lake et al. (2023). In short, after 48 hours of incubation, wells were washed twice with 300 µL PBS, and the biofilm was stained for 30 minutes with 300 µL 0.1% (w/vol) CV (Merck). After the solution was removed, wells were washed twice with 300 µL PBS to remove unbound CV and 300 µL 96% ethanol was added to the wells and incubated for 15 minutes to dissolve the bound CV.

The biofilm was quantified by measuring the absorbance at 595 nm with the Spectramax M2 plate reader (Molecular Devices). Experiments were performed with two technical reproductions and two biologically independent reproductions obtained on different days. Crystal violet stains non-viable cells, extracellular matrix components, and viable cells and is therefore an indicator of total attached biomass (Kadam et al., 2013; Pitts et al., 2003). Crystal violet staining is not always correlated with the amount of viable cells in the biofilm, and therefore biofilm CFU plate counting is a good addition for live cell determination (Kadam et al., 2013) and this method was adapted from Lake et al. (2023). In short, wells were washed twice with 300 µL PBS to remove unbound cells and filled with 300 µL PBS. The attached biofilm was dissolved in PBS by rigorously scraping the wells with a 200 µL pipet tip (Greiner Bio-One) and subsequent rigorous pipetting with the same tip to obtain single cells. Decimal dilutions were prepared in PBS followed by plating on TSAYE plates for single culture experiments, or TSAYE plates and ALOA plates for co-culture experiments. Experiments were performed with two or three biologically independent reproductions obtained on different days.

4.2.9. HPLC analysis

Cultures of the growth experiments were centrifuged at 16,000 x *g*, after which the pellet was discarded and the supernatant samples were stored at -20 °C upon further analysis of extracellular metabolites by High Performance Liquid Chromatography (HPLC). Moreover, the fresh filter-sterilized mushroom medium, and the non-inoculated filter-sterilized mushroom medium that was kept for 48 hours at 20 °C were also included for analysis. HPLC compound analysis was performed for detection and quantification of trehalose, glucose, fructose, mannitol, glycerol, lactate, acetate and acetoin and was adapted from Lake et al. (2023). Shortly, samples were deproteinated by mixing two volumes of sample with one volume of cold Carrez A (0.1 M potassium ferrocyanide trihydrate) after which one volume of cold Carrez B (0.2 M zinc sulphate heptahydrate) was added and mixed. Samples were centrifuged at 16,000 x *g* for 5 minutes and supernatant was collected. A total volume of 10 µL of the sample was injected on an Ultimate 3000 (Dionex, Germany) equipped with an Aminex HPX-87H column (300 x 7.8 mm) with guard-column (Bio-Rad, USA). Temperature of the column oven was kept at 60 °C and 0.01 N H_2SO_4 was used as a mobile phase with a flow rate of 0.6 mL/min. Compound detection was performed using a refractive index detector

(RefractoMax 520) together with using UV measurements at 220, 250, 280 nm for peak identification followed by quantification. Experiments were performed with two to four biologically independent reproductions obtained on different days for each of the single and co-cultures, and with multiple technical reproductions for the fresh filter-sterilized mushroom medium and 48-hours incubated non-inoculated filter-sterilized mushroom medium.

4.2.10. Statistical analysis

The mean values and the standard deviations of the biological reproductions were calculated for the phenotypic experiments using Microsoft Excel. The technical replicates of the biofilm experiments were first averaged, after which the mean and standard deviations of the biological reproductions were calculated. Statistical significance was determined by performing the Student's t-tests using a significance value of $p = 0.05$.

4.3. Results

4.3.1. Identification of microbiota in mushroom processing environments

In total, 239 bacterial strains were isolated during the survey and the strains were grouped in 26 genera and the *Enterobacteriaceae* family. The strains belonging to the *Enterobacteriaceae* family were grouped together, since strain characterization based on 16S rRNA gene sequences resulted in imperfect genus characterization for most of the *Enterobacteriaceae* strains. More specifically, 103 bacterial strains (from the *Enterobacteriaceae* family and 14 genera) were isolated during mushroom processing and 136 bacterial strains (from the *Enterobacteriaceae* family and 20 genera) were isolated after the cleaning and disinfection (C&D) procedures. Overall, the *Enterobacteriaceae* family and eight genera were present on mushroom processing equipment during mushroom processing and after the C&D procedures (table 4.1 and supplemental table 4.3).

Microbiota identification demonstrated the prominent presence of *Acinetobacter* (26.8%), *Enterobacteriaceae* (22.2%), *Lactococcus* (18.4%) and *Pseudomonas* (9.6%)

(table 4.1, supplemental table 4.3). Most of the sampling spots during mushroom processing were dominated by *Acinetobacter*, while the dominance of the four groups mentioned above was more heterogeneous in the sampling spots after C&D procedures

Table 4.1. Represented microbiota and number of strains from mushroom processing sites during mushroom processing and after the C&D procedures

Group	During processing	After C&D	Total
Acinetobacter	43	21	64
Lactococcus	14	30	44
Enterobacteriaceae	20	33	53
Pseudomonas	6	17	23
Chryseobacterium	5	3	8
Brochothrix	2	3	5
Serratia		4	4
Streptoccoccus		4	4
Enterococcus		4	4
Aeromonas	1	3	4
Sphingobacterium	2	2	4
Comamonas	3	1	4
Thrichococcus		2	2
Staphylococcus		2	2
Mycetocola	2		2
Microbacterium		1	1
Hafnia		1	1
Brevibacterium		1	1
Plantibacter		1	1
Micrococcus		1	1
Vagococcus		1	1
Delftia		1	1
Leuconostoc	1		1
Shewanella	1		1
Paenarthrobacter	1		1
Rothia	1		1
Stenotrophomonas	1		1
Total strains	103	136	239

(data not shown). A total of 18 strains were selected for phenotypic characterization and represented a group of strains/genus that was at least three times isolated on at least two sampling spots (table 4.1, supplemental table 4.2 and supplemental table 4.3). Multiple selected strains were included of *Lactococcus*, *Pseudomonas*, *Acinetobacter* and *Enterobacteriaceae* species, namely three *Lactococcus* strains (*L. lactis*, *L. raffinolactis* and *L. garvieae*), two *Pseudomonas* strains (*P. fluorescens* and *P. fragi*), two *Acinetobacter* strains (*A. johnsonii*, and *Acinetobacter* spp.), and five *Enterobacteriaceae* strains (*Raoultella*, *Citrobacter*, *Ewingella*, *Buttiauxella* and *Lelliottia* strain) (see supplemental table 4.4 for 16S sequences and taxonomical identification of the 18 selected strains).

4.3.2. Growth and biofilm formation of microbiota strains in mushroom medium

All 18 selected microbiota strains showed good growth performance in the mushroom medium during incubation for 48 hours at 20 °C (figure 4.1). The highest log increase was observed for the *Raoultella* and *Ewingella* strains, both belonging to the *Enterobacteriaceae* family, with final concentrations of 9.6 and 9.8 log CFU/mL, respectively. Other strains with a relatively high log increase included the strains of the *Pseudomonas* group and one strain of the *Acinetobacter* group, with final concentrations ranging from 8.8 to 9.3 log CFU/mL and with final pH values of 7.6 to 8.2, with the highest pH value observed for *P. fluorescens*. The *Lactococcus* spp. strains that belong to the group of lactic acid bacteria and the *Brochothrix* strain had, especially after 48 hours of incubation, a lower log increase and final concentrations ranged from 7.1 to 8.4 log CFU/mL, and this coincided with a final pH of 5.0 or lower. The final concentrations of the *Raoultella* and the *Ewingella* strains were significantly higher ($p < 0.05$) than the reported final concentrations of a diverse collection of *L. monocytogenes* strains that reached on average 8.7 log CFU/mL when cultured for 48 hours in mushroom medium (Lake et al., 2023). On the other hand, the final concentrations of the *Lactococcus* strains were significantly lower ($p < 0.05$) compared to *L. monocytogenes*, but the final average pH value of 4.9 of the *Lactococcus* strains was comparable to the final average pH value of 5.1 of *L. monocytogenes* (Lake et al., 2023). When all 18 microbiota strains were co-cultured, this cocktail of strains resulted in average counts of 9.0 and 9.4 log CFU/mL after 24 and 48 hours of incubation, respectively. The final counts of this mixture were comparable to the counts of the two

high-performer strains *Ewingella* and *Raoultella* (p = 0.15) (figure 4.1). The final pH of the cocktail was approximately 6.8, which is close to the original pH, conceivable due to the mixture of acidifying and alkalizing bacteria.

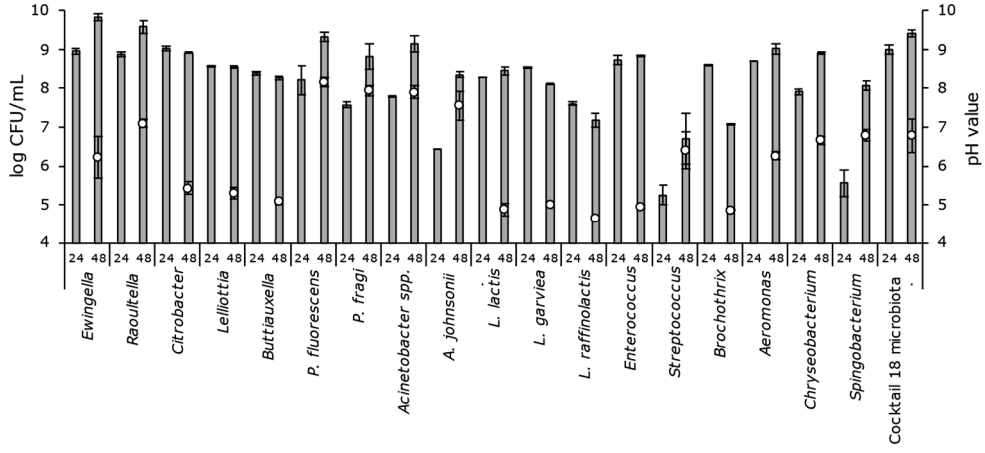

Figure 4.1. Growth performance of the 18 microbiota strains in single culture and as cocktail during static incubation in filter-sterilized mushroom medium for 48 hours at 20 °C. Inoculum levels of single microbiota cultures and the cocktail were approximately 5 log CFU/mL. Grey bars represent the microbiota counts after 24 hours (24) and 48 hours (48) of incubation. The initial pH of the medium is around 6.8 and the pH after 48 hours of incubation is represented with the white circles. The error bars represent the standard deviation of two biological reproductions.

Total biofilm formation determined by CV staining for total biomass quantification and biofilm cell counts showed that all microbiota strains were able to form biofilm in mushroom medium during 48 hours incubation at 20 °C, with the exception of *Chryseobacterium* which showed no significant CV staining (figure 4.2A). The CV staining values of the other strains ranged from 0.4 to 2.6. Microbiota biofilm cell counts ranged from 5.6 to 9.0 log CFU/mL (figure 4.2B), with highest values of 8.8 and 9.0 log CFU/mL for the *Enterobacteriaceae* species *Raoultella* and *Ewingella*, respectively. Also the two *Pseudomonas* species, *P. fragi* and *P. fluorescens*, and the *Acinetobacter johnsonii* strain developed high biofilm counts with values of 8.5, 8.5 and 8.2 log CFU/mL, respectively. Biofilm counts of the *Lactococcus* spp. were lower and ranged from 6.2 to 7.8, and the lowest biofilm cell counts were observed for the *Streptococcus*, *Buttiauxella* and the *Acinetobacter* spp. strains, with values of 5.6, 6.0 and 6.0 log CFU/mL, respectively. An apparent correlation between CV staining and

biofilm cell counts was not observed in all cases, since low CV staining and high viable biofilm counts were observed for the *Chryseobacterium* strain and vice versa for the *Acinetobacter* spp. strain (figure 4.2).

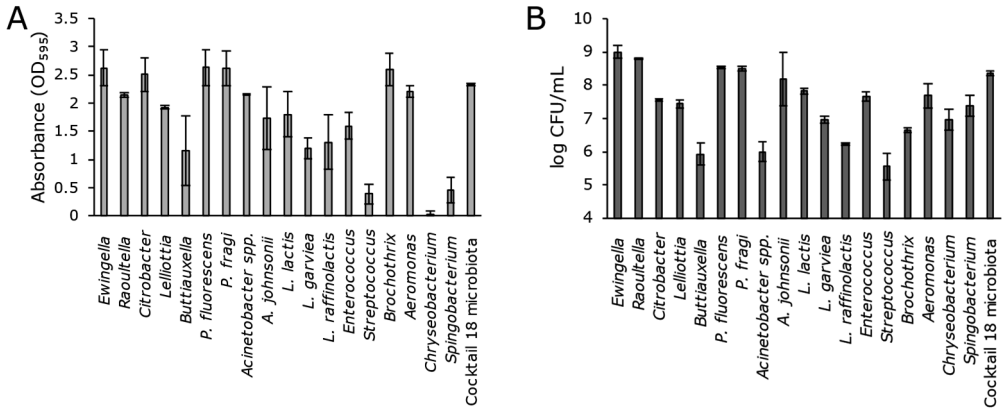

Figure 4.2. Biofilm determination of the 18 microbiota strains in single culture and as cocktail after static incubation in filter-sterilized mushroom medium for 48 hours at 20 °C. Inoculum levels of single microbiota cultures and the cocktail were approximately 5 log CFU/mL. (A) Staining assessed with the crystal violet assay is expressed as optical density at OD_{595}. (B) Biofilm cell counts are expressed in log CFU/mL. The error bars represent the standard deviation of two biological reproductions.

Compound analysis of non-inoculated mushroom medium that was statically incubated for 48 hours at 20 °C showed relatively high concentrations of mannitol (approximately 66.2 mM), glucose (8.4 mM), fructose (7.7 mM) and glycerol (2.5 mM) and low levels of trehalose (below 0.5 mM) (table 4.2). Notably, for all mushroom medium batches, the compound concentrations were not static during the incubation, because the mannitol concentration decreased and the fructose concentration increased after 48 hours of incubation (supplemental table 4.5). Compound analysis of the medium after culturing the microbiota strains showed that both *Raoultella* and *Ewingella* consumed mannitol, with no mannitol detection after 48 hours for the *Raoultella* strain (table 4.2) and interestingly, these two strains showed the highest growth and biofilm counts (see figures 4.1 and 4.2). A smaller decrease in mannitol was observed for Citrobacter, *Lelliottia* and *Aeoromonas*, while mannitol concentrations for the other microbiota were comparable to non-inoculated mushroom medium. Especially the *Enterobacteriaceae* strains produced a relatively high amount of acetate, while the lactic acid bacteria and the *Brochothrix* strain produced a relatively high amount of lactate, which can explain

the low pH after incubation (figure 4.1). On the other hand, the *Pseudomonas* and the *Acinetobacter* strains produced small amounts of these organic acids and this may point to further respiratory degradation of the sugars. Incubation of the cocktail of strains in the mushroom medium resulted in a large decrease of all sugar compounds and production of lactate, acetate and acetoin (table 4.2).

4.3.3. Co-incubation of microbiota and *L. monocytogenes*

Nine strains belonging to the top four frequently isolated genera or family, namely, *Lactococcus*, *Acinetobacter*, *Pseudomonas* and *Enterobacteriaceae*, were selected for co-incubation with *L. monocytogenes* strain 636 (figure 4.3). This strain was chosen, because of the relatively high abundance of this clonal complex (CC224) in a previous *L. monocytogenes* sampling survey in the frozen sliced mushroom production and processing environment (Lake et al., 2021), and co-incubation screening of two other genetically different *L. monocytogenes* strains showed similar counts (data not shown). All co-incubations started with approximately 5 log CFU/mL for both strains, and after 24 hours of incubation *L. monocytogenes* grew to 7.6 to 8.9 log CFU/mL, while counts of the microbiota strains ranged from 5.5 to 9.0 (figure 4.3). The counts of *L. monocytogenes* decreased during the following up 24 hours of co-incubation with the two high performance strains *Raoultella* and *Ewingella*, resulting in final *L. monocytogenes* counts of 7.0 log CFU/mL. On the other hand, the counts of *L. monocytogenes* increased further during the following up 24 hours of co-incubation with the *Pseudomonas* and *Acinetobacter* strains reaching final counts of 9.1 to 9.2 log CFU/mL (figure 4.3). These counts were significantly higher than those of a diverse collection of *L. monocytogenes* strains ($p < 0.05$) when mono-cultured in mushroom medium with average counts of 8.7 log CFU/mL (Lake et al., 2023). However, *L. monocytogenes* counts decreased in the second 24 hours of co-incubation with the *Lactococcus* strains and final counts were 5.8 to 7.7 log CFU/mL, with the lowest counts

Table 4.2. Compound analysis of mushroom medium after 48 hours of static incubation at 20 °C. Microbiota strains were inoculated individually in the medium, and as cocktail of 18 strains, and the medium was incubated without bacterial inoculum (MM48). The error bars represent the standard deviation of three biological reproductions

	Organic acid component							
Culture	trehalose mM (stdev)	glucose mM (stdev)	fructose mM (stdev)	mannitol mM (stdev)	glycerol mM (stdev)	lactate mM (stdev)	acetate mM (stdev)	acetoin mM (stdev)
MM48	0.15 (0.14)	8.55 (1.43)	7.46 (0.66)	68.09 (7.63)	2.54 (0.28)	0.34 (0.54)	0.46 (0.28)	0.65 (0.30)
Ewingella	<0.01*	0.16 (0.05)	<0.01	6.34 (10.12)	1.22 (1.06)	10.72 (4.83)	10.54 (0.67)	15.58 (4.73)
Raoultella	<0.01	0.23 (0.04)	0.05 (0.09)	0.15 (0.04)	0.08 (0.03)	6.01 (0.92)	16.86 (7.91)	2.04 (0.76)
Citrobacter	0.01 (0.01)	0.25 (0.02)	<0.01	54.65 (9.06)	0.51 (0.19)	4.72 (0.96)	26.81 (4.17)	5.20 (0.43)
Lelliottia	<0.01	0.33 (0.02)	<0.01	56.48 (7.69)	1.01 (0.16)	15.48 (3.54)	22.82 (0.52)	4.37 (2.72)
Buttiauxella	0.02 (0.01)	0.32 (0.02)	<0.01	61.65 (6.99)	2.46 (0.48)	15.85 (1.83)	18.72 (0.49)	3.90 (2.94)
P. fluorescens	0.11 (0.07)	0.39 (0.28)	2.94 (0.61)	60.06 (8.13)	0.42 (0.46)	0.83 (0.53)	5.87 (1.32)	3.19 (2.31)
P. fragi	0.71 (0.12)	0.33 (0.21)	5.63 (0.21)	66.89 (8.76)	0.12 (0.04)	1.73 (0.13)	3.61 (0.29)	4.78 (0.57)
Acinetobacter spp.	0.43 (0.11)	6.95 (1.58)	7.96 (1.05)	65.08 (6.54)	0.89 (0.24)	0.14 (0.20)	0.92 (0.69)	1.07 (0.54)
A. johnsonii	0.28 (0.33)	7.22 (1.44)	8.11 (1.61)	65.65 (6.43)	1.42 (0.83)	0.13 (0.15)	1.32 (0.07)	0.36 (0.40)
L. lactis	<0.01	0.99 (0.13)	<0.01	58.85 (9.82)	0.42 (0.14)	23.31 (2.36)	7.26 (0.22)	0.95 (0.05)
L. garviea	0.04 (0.01)	0.76 (0.35)	<0.01	62.75 (7.14)	1.35 (0.06)	28.78 (2.90)	5.25 (0.47)	3.47 (1.98)
L. raffinolactis	0.05 (0.00)	1.12 (0.10)	<0.01	63.30 (6.61)	1.85 (0.31)	33.17 (1.32)	3.78 (0.62)	1.15 (0.80)
Enterococcus	<0.01	1.13 (0.13)	<0.01	63.36 (6.77)	1.18 (0.35)	29.63 (2.07)	4.25 (0.30)	2.28 (0.77)
Streptococcus	0.18 (0.13)	6.38 (2.20)	6.88 (0.57)	64.81 (6.84)	2.43 (0.23)	4.23 (5.78)	0.96 (0.38)	0.79 (0.54)
Brochothrix	0.04 (0.01)	1.11 (0.12)	<0.01	60.54 (8.90)	1.68 (0.39)	20.24 (2.08)	2.65 (0.84)	4.03 (2.81)
Aeromonas	<0.01	0.28 (0.01)	<0.01	47.62 (18.37)	0.31 (0.04)	4.65 (0.83)	16.06 (3.48)	0.49 (0.32)
Chryseobacterium	0.37 (0.18)	5.14 (1.74)	6.43 (0.47)	65.79 (7.63)	1.40 (0.09)	0.17 (0.13)	5.72 (3.20)	0.98 (1.03)
Sphingobacterium	0.39 (0.20)	6.58 (1.30)	7.08 (0.37)	65.07 (6.09)	2.26 (0.33)	0.12 (0.03)	0.92 (0.70)	1.20 (0.76)
Cocktail 18 microbiota	<0.01	0.15 (0.01)	<0.01	9.31 (11.34)	2.38 (1.88)	15.38 (9.79)	13.37 (1.49)	1.97 (1.71)

in the co-culture with the *Lactococcus raffinolactis* strain, which also had the lowest final pH value (figure 4.3). Notably, although some co-incubations resulted in a decrease of *L. monocytogenes* counts after an initial increase, final *L. monocytogenes* counts were still at least 0.5 log CFU/mL higher than the inoculum concentration.

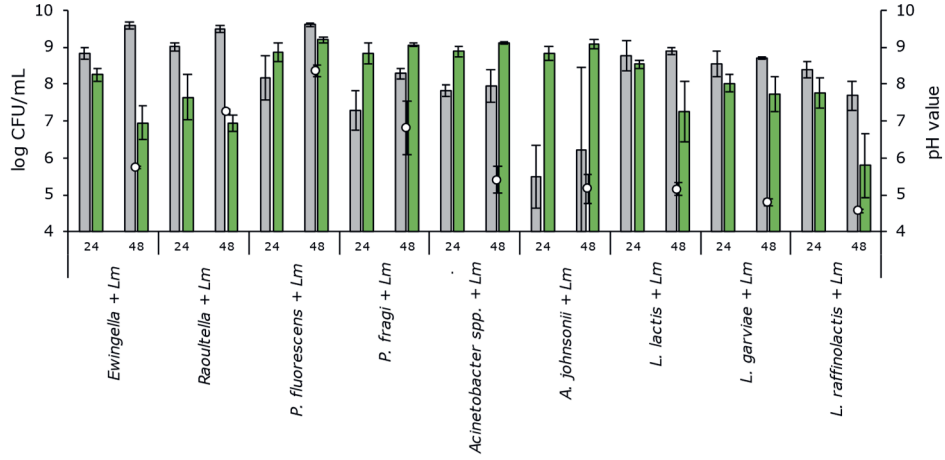

Figure 4.3. Co-incubation of nine selected microbiota strains with *L. monocytogenes* (*Lm*) strain 636 in mushroom medium for 48 hours at 20 °C. Inoculum levels of the co-cultures were approximately 5 log CFU/mL for each of the strains. Grey bars represent the microbiota counts and green bars represent the *L. monocytogenes* counts after 24 hours (24) and 48 hours (48) of incubation. The initial pH of the medium is around 6.9 and the pH after 48 hours of co-incubation is represented with the white circles. The error bars represent the standard deviation of four biological reproductions.

All co-incubations showed the development of a biofilm consisting of both the *L. monocytogenes* strain 636 and the microbiota strain after 48 hours of incubation. Screening of the other *L. monocytogenes* strains demonstrated similar *L. monocytogenes* biofilm counts when co-cultured with the microbiota strains (data not shown). The biofilm cell counts of the microbiota strains were higher than the *L. monocytogenes* strain in most of the combinations, ranging from 6.4 to 8.9 log CFU/mL and from 3.7 to 7.3 log CFU/mL for the microbiota strains and *L. monocytogenes*, respectively (figure 4.4). Biofilms formed in co-cultures with *Raoultella* and *Ewingella* contained 8.9 and 8.4 log CFU/mL microbiota counts, respectively (figure 4.4), while respective *L. monocytogenes* counts reached 5.7 and 5.4 log CFU/mL. On the other hand, *L. monocytogenes* biofilm counts in co-cultures with the *Pseudomonas* and *Acinetobacter* strains were higher, with counts ranging from 6.2 to 7.3 log CFU/mL and with microbiota counts ranging from 7.3 to 8.3 log CFU/mL. More specifically, the *L. monocytogenes* counts in co-culture with *P. fragi* and *A. johnsonii* were 7.2 and 7.3

log CFU/mL, respectively. This is slightly higher than *the L. monocytogenes* biofilm counts of a diverse collection of *L. monocytogenes* strains when mono-cultured in mushroom medium, when average biofilm counts of 6.8 log CFU/mL were reached with 7.0 log CFU/mL for *L. monocytogenes* strain 636 (Lake et al., 2023). The co-cultures with the *Lactococcus* strains showed the lowest total biofilm counts compared to the other three strain groups in which the *L. monocytogenes* biofilm counts were among the lowest, and this coincided with the acidification of the medium (figures 4.3 and 4.4). In particular, in co-culture with *L. raffinolactis* when a pH value of 4.6 was reached after incubation, the biofilm cell counts of *L. monocytogenes* were 3-log lower than those of *L. raffinolactis*, with biofilm cell counts of 6.7 and 3.7 log CFU/mL for *L. raffinolactis* and *L. monocytogenes*, respectively (figures 4.3 and 4.4). This was in line with the CV staining values of the co-incubations where the co-incubations with the three *Lactococcus* strains had the lowest CV-values ranging from 0.6 to 1.1 (data not shown).

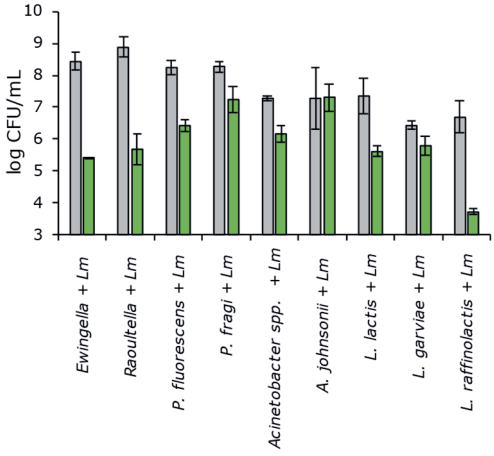

Figure 4.4. Biofilm cell counts of nine selected microbiota strains in co-culture with *L. monocytogenes* (*Lm*) strain 636 after 48 hours of incubation at 20 °C in mushroom medium. Inoculum levels of the co-cultures were approximately 5 log CFU/mL for each of the strains. Biofilm cell counts are expressed in log CFU/mL in which the grey bars represent the biofilm cell counts of the microbiota and the green bars represent the biofilm cell counts of *L. monocytogenes*. The error bars represent the standard deviation of three biological reproductions.

The medium composition after the co-culture experiments with *L. monocytogenes* (supplemental table 4.6) was compared to the mono-culture experiments of the microbiota strains (table 4.2). Comparable trends were observed for the *Raoultella* and *Ewingella* strains as also in co-culture a large decrease in mannitol was observed. In

addition, comparable trends were observed for the *Lactococcus* strains in co-cultures and monocultures in which high concentrations of lactate were formed in both conditions. Patterns were however different for the co-cultures of *L. monocytogenes* with *Pseudomonas fragi* and the *Acinetobacter* strains, with single cultures of these microbiota showing substantial amounts of sugars still present and with no or little product formation, while the co-cultures had a substantial decrease in sugars (except mannitol) and increase in product formation. The observed substrate consumption and product formation (i.e. lactate) matched that of single *L. monocytogenes* incubations including the observed acidification of the medium (Lake et al., 2023), and this points to a dominant role of *L. monocytogenes* in these mixed cultures.

4.3.4. Growth of *L. monocytogenes* in spent medium of microbiota strains

To assess whether *L. monocytogenes* is capable to establish itself in environments where another bacterial strain has been dominating, culturing experiments were performed in spent mushroom medium that was obtained from 72-hours cultures of microbiota strains (supplemental figure 4.1). To exclude additional pH effects, also pH-adjusted spent medium was used of which the pH was set at 6.9 that corresponds to the pH of fresh mushroom medium. *L. monocytogenes* strain 636 was selected for growth in the spent media and the screening of other *L. monocytogenes* strains showed similar growth trends (data not shown). The growth of *L. monocytogenes* in the non-pH adjusted spent mushroom media after 48 hours at 20 °C depended on the microbiota strain used in the preculturing (figure 4.5). *L. monocytogenes* counts increased with 2.3 and 1.8 log CFU/mL in spent media of the *Raoultella* and *Ewingella* strains, respectively, despite the high counts that these microbiota strains had achieved during pre-culturing (supplemental figure 4.1). The highest increase in *L. monocytogenes* counts were observed in the spent media of the microbiota strains that increased the pH during culturing, namely the *Pseudomonas* and *Acinetobacter* strains. The log increase of *L. monocytogenes* in these spent media ranged from 2.8 to 4.1 log CFU/mL leading to *L. monocytogenes* counts as high as 9.3 log CFU/mL in the spent media of the *P. fragi* and the *Acinetobacter* spp. strains. On the other hand, *L. monocytogenes* counts remained comparable or slightly decreased during incubation in the acidified spent media of the *Lactococcus* strains (figure 4.5). However, when the pH of the spent mushroom media of those strains was adjusted to 6.9, also an increase of 2.2 to 2.7 log CFU/mL was observed (figure 4.5). As expected, the log increases in

the pH-adjusted and the non-pH adjusted spent medium of the other microbiota strains were comparable (figure 4.5). The significant growth of *L. monocytogenes* in the spent mushroom media underlines the nutrient richness of these spent media, which was also reflected in metabolism of the remaining sugars (except mannitol) during *L. monocytogenes* incubation in these spent mushroom media (supplemental table 4.7).

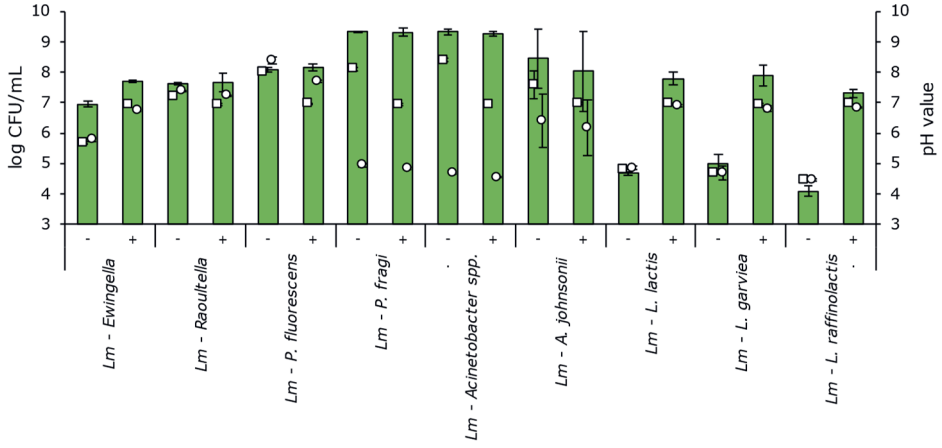

Figure 5. Growth of *L. monocytogenes* (*Lm*) strain 636 during static incubation at 20 °C in non-pH adjusted and pH-adjusted spent mushroom medium obtained from a 72-hours microbiota strain culture. Inoculum levels of *L. monocytogenes* were approximately 5 log CFU/mL. Green bars represent the *L. monocytogenes* counts after 48 hours of incubation at 20 °C in which non-pH adjusted spent mushroom medium is indicated by a minus (-) and pH-adjusted spent mushroom medium is indicated by a plus (+). The white squares and the circles represent the pH value of the medium at the start and after culturing *L. monocytogenes* for 48 hours in the spent media, respectively. The error bars represent the standard deviations of two or three biological reproductions.

In addition, *L. monocytogenes* was also able to form biofilm in all non-pH adjusted and pH-adjusted spent media with counts that ranged from 2.8 to 7.0 log CFU/mL. The highest *L. monocytogenes* biofilm counts were observed in the non-pH adjusted and pH-adjusted spent media of the *P. fragi* and the *Acinetobacter* spp. strains where biofilm counts reached 6.7 and 6.6 log CFU/mL and 6.9 and 7.0 log CFU/mL, respectively. On the other hand, the lowest biofilm cell counts of *L. monocytogenes* were observed in the non-pH adjusted spent medium of the three *Lactococcus* strains with counts ranging from 2.8 to 3.6 log CFU/mL. However, when the pH was adjusted, the biofilm counts in these spent media clearly increased (4.6 to 5.2 log CFU/mL),

indicated again that the pH and not the nutrient richness was the limiting factor for biofilm formation in the spent media.

4.3.5. Growth and biofilm formation of *Listeria* spp. mushroom isolates in single and in co-culture with *L. monocytogenes*

Sampling mushrooms and the mushroom processing environment resulted in the identification of other *Listeria* species, namely *L. grayi*, *L. seeligeri*, and *L. innocua*. When these *Listeria* species were cultured in mushroom medium for 48 hours at 20 °C, the final counts were 8.8 to 9.1 log CFU/mL and a pH drop of the medium to pH of 4.6

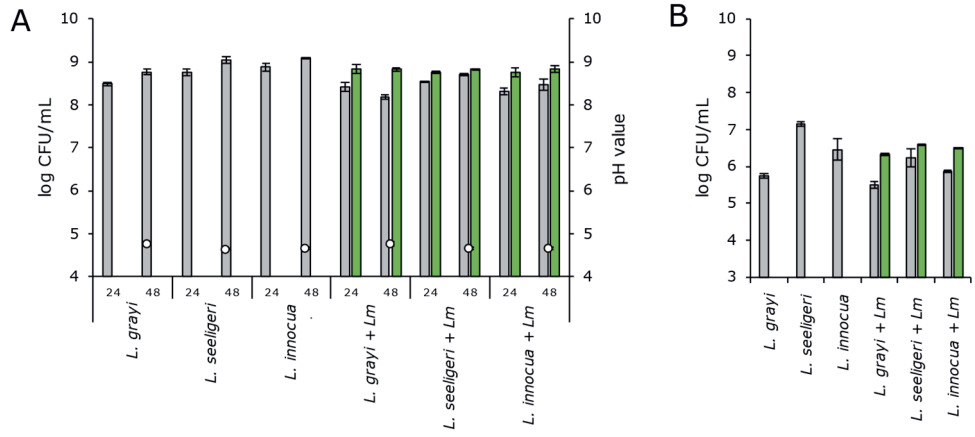

Figure 4.6. Growth performance of three *Listeria* spp. strains in mono-culture and in co-cultures with *L. monocytogenes* (*Lm*) strain 636 during static incubation in filter-sterilized mushroom medium for 48 hours at 20 °C. Inoculum levels of the individual strains were approximately 5 log CFU/mL. (A) Growth of *Listeria* spp. strains in mono-cultures and in co-cultures with *L. monocytogenes* strain 636. Grey bars represent the *Listeria* spp. counts and green bars represent the *L. monocytogenes* counts after 24 hours (24) and 48 hours (48) of incubation. The initial pH of the medium is 6.9 and the pH after 48 hours of incubation is represented by the white circles. The error bars represent the standard deviation of two biological reproductions. (B) Biofilms cell counts of *Listeria* spp. strains formed in mono-cultures and in co-cultures with *L. monocytogenes* strain 636 after 48 hours of static incubation at 20 °C. Biofilm cell counts are expressed in log CFU/mL in which the grey bars represent the biofilm cell counts of the *Listeria* spp. strains and the green bars represent the biofilm cell counts of *L. monocytogenes*. The error bars represent the standard deviation of two biological reproductions.

to 4.8 was observed (figure 4.6A). This growth performance was comparable to reported growth of *L. monocytogenes* strains using similar incubation conditions, where final *L. monocytogenes* counts ranged from 8.4 to 9.1 log CFU/mL and average pH values were 5.1 (Lake et al., 2023). When *L. monocytogenes* strain 636 was co-cultured with one of these *Listeria* strains, a substantial increase of cell counts of both strains was observed as *Listeria* spp. reached counts between 8.2 and 8.7 log CFU/mL and *L. monocytogenes* reached counts of 8.8 log CFU/mL (figure 4.6A), indicating no growth inhibition of *L. monocytogenes*. A similar growth trend was observed for the other *L. monocytogenes* strains tested (data not shown). Analysis of biofilm formation under these conditions showed that all strains established biofilm cell counts in co-culture, with *Listeria* species counts ranging from 5.5 to 6.2 log CFU/mL and *L. monocytogenes* counts ranging from 6.3 to 6.6 log CFU/mL (figure 4.6B). Compound analysis of the medium after mono-culture incubation of the *Listeria* spp. strains or after co-incubations with *L. monocytogenes* showed similar trends in sugar decreases and product formation (supplemental table 4.8), and these patterns were similar to that of *L. monocytogenes* in mono-culture (Lake et al., 2023).

The growth of *L. monocytogenes* strain 636 was also assessed in spent mushroom media that was obtained from 72-hours cultures of the *Listeria* spp. strains (supplemental figure 4.2). Incubation of *L. monocytogenes* for 48 hours at 20 °C in these non-pH adjusted spent media resulted in no increase or a slightly decrease in counts, while incubation in pH-adjusted spent media showed a significant increase in cell counts, i.e. 3.5 log CFU/mL, resulting in final counts of 8.7 log CFU/mL (figure 4.7). Incubation of the other *L. monocytogenes* strains in spent medium of the *Listeria* spp. strains showed similar trends (data not shown). The significant log increase in spent medium resulting in only a small decrease in the pH value (figure 4.7) and minor differences in compounds in the spent medium before and after growth of *L. monocytogenes* (supplemental table 4.9). The observation that pH-adjusted spent mushroom media of closely related *Listeria* spp. supported growth of *L. monocytogenes* is another indication that mushroom medium is a nutrient-rich medium, and it also demonstrates that the tested *Listeria* spp. strains did not produce compounds that inhibited the growth of *L. monocytogenes*.

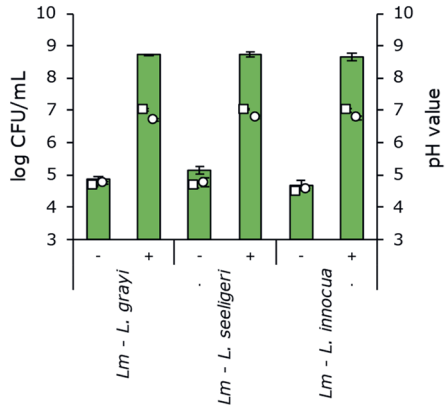

Figure 4.7. Growth of *L. monocytogenes* (*Lm*) strain 636 during static incubation at 20 °C in non-pH adjusted and pH-adjusted spent mushroom medium obtained from a 72-hours culture of a *Listeria* spp. strain. Inoculum levels of *L. monocytogenes* were approximately 5 log CFU/mL. Green bars represent the *L. monocytogenes* counts after 48 hours of incubation at 20 °C in which non-pH adjusted spent mushroom medium is indicated by a minus (-) and pH-adjusted spent mushroom medium is indicated by a plus (+). The white squares and the circles represent the pH value of the medium at the start and after culturing *L. monocytogenes* for 48 hours in the spent media, respectively. The error bars represent the standard deviations of two or three biological reproductions.

4.4. Discussion

In this study, we isolated microbiota strains from the mushroom processing environments during mushroom processing and after the C&D procedure. The most dominant strains at both sampling times were representatives from four groups, namely *Acinetobacter*, *Lactococcus*, *Enterobacteriaceae* and *Pseudomonas*. The dominant presence of these groups has previously been reported during both food processing and after the C&D procedure in other food processing environments, including vegetable, meat and cold smoked salmon processing companies (Bagge-Ravn et al., 2003; Cobo-Díaz et al., 2021; Einson et al., 2018; Fagerlund et al., 2017; Kable et al., 2019; Langsrud et al., 2016; Møretrø and Langsrud, 2017; Møretrø et al., 2013; Xu et al., 2022; Zwirzitz et al., 2021).

Although the microbiota strains that were isolated from mushroom processing environments showed variable growth and biofilm formation in mushroom-derived medium, *L. monocytogenes* demonstrated to be very capable to establish itself in co-

culture with these microbiota in mushroom medium, unless the pH decreased during incubation as this resulted in a reduction of counts over time. Most previous studies that assessed the growth of *L. monocytogenes* and microbiota food factory isolates used laboratory media such as BHI, TSBYE or BHI supplemented with Yeast Extract (BHIYE) for strain characterization (Dygico et al., 2019; Fagerlund et al., 2017; Heir et al., 2018; Mellefont et al., 2008; Van der Veen and Abee, 2011). However, translation of outcomes obtained with laboratory media to performance in food factory environmental conditions may be challenging as exemplified with challenge tests, that showed different outcomes for the same tested strain in foods and in the tested laboratory medium (Bungenstock et al., 2020; 2021). Therefore in the current study mushroom medium was used to approach availability of substrates as closely as possible to obtain a better understanding of the behaviour of microorganisms in relevant food (processing) conditions.

All selected microbiota strains that were individually incubated in mushroom medium showed significant growth and biofilm formation, of which the *Ewingella* and *Raoultella* strains were the best performers. *Ewingella* is a mushroom spoilage organisms, and can cause internal stipe necrosis, a browning disease in *A. bisporus* mushrooms (Inglis and Peberdy, 1996; Inglis et al., 1996; Lee et al., 2009; Reyes et al., 2004). *Raoultella* is ubiquitous in nature and has been isolated from plants, water and soil (Appel et al., 2021). These two *Enterobacteriaceae* family members are able to consume mannitol, which conceivably provides a growth advantage, as this is the main sugar constituent in the mushroom medium. Co-culturing of *L. monocytogenes* with *P. fragi* and A*. johnsonii* resulted in higher planktonic and biofilm counts of *L. monocytogenes* compared to mono-culturing of *L. monocytogenes*. Such positive effects were also observed for *L. monocytogenes* when cultured in a biofilm with a *P. fluorescens* strain/*Pseudomonas* genus mixture (Haddad et al., 2021; Papaioannou et al., 2018; Puga et al., 2018), but not in the broth cultures (Haddad et al., 2021) and after extended time (Papaioannou et al., 2018). On the contrary, other studies showed a negative effect for *L. monocytogenes* during co-culture with *P. fluorescens* (Carpentier and Chassaing, 2004; Mohan et al., 2020). During the co-incubations in this study, higher biofilm counts of *Pseudomonas* over *L. monocytogenes* were seen and this was also observed during co-incubation in fish medium (Papaioannou et al., 2018). This is contradictory to other studies that observed somewhat higher *L. monocytogenes* biofilm counts than *P. fluorescens* biofilm counts after 48 hours of incubation in a meat slurry medium and Tryptone Soya Broth (TSB) medium, respectively (Haddad et al.,

2021; Puga et al., 2018). These results suggest that phenotypical differences in *L. monocytogenes* growth and biofilm behaviour during co-incubations are complex and depend on multiple factors among others the type of medium. In addition, also the inoculation levels of the strains influence their maximum population densities (Mellefont et al., 2008), and therefore the initial counts of the strains used in co-culture experiments were similar in all experiments in the current study. The co-cultures of *L. monocytogenes* with *Lactococcus* and *Enterobacteriaceae* strains showed growth and biofilm formation of *L. monocytogenes*, although prolonged incubation showed a reduction of *L. monocytogenes* (biofilm) cell counts. Such negative effects for *L. monocytogenes* in both planktonic and biofilm counts were also shown for co-culture of *L. monocytogenes* with *Lactobacillus plantarum* in a meat slurry medium (Haddad et al., 2021) and for different biofilm co-cultures on stainless steel surfaces in TSB (Rodríguez-López et al., 2015). However, our study and the other studies also demonstrated that despite the inhibitory effects of the microbiota strains, *L. monocytogenes* can still obtain significant viable counts in co-cultures (Carpentier and Chassaing, 2004; Haddad et al., 2021). The growth inhibitory effect by lactic acid bacteria observed in our study was low pH related, and this is in line with other studies that also used spent media from lactic acid bacteria (Bungenstock et al., 2020; Mariam et al., 2014). In addition, other studies described the production of antimicrobial compounds (bacteriocins) by lactic acid bacteria that inhibit the growth of *L. monocytogenes*, and suggested the possibility of applying these strains as a biocontrol for *L. monocytogenes* (Dygico et al., 2019; Martín et al., 2022). However, the lactic acid bacteria tested in the current study did not show this anti-listerial behaviour, highlighting that production of antimicrobial compounds is strain-dependent.

Co-incubation of *L. monocytogenes* with other *Listeria* species isolated from mushroom processing environments, i.e. *L. innocua*, *L. seeligeri* and *L. grayi*, showed no competition advantage for one of the strains. These results are in line with some studies with co-cultures of *L. monocytogenes* and *L. innocua* (Heir et al., 2018; Langsrud et al., 2016; Petran and Swanson, 1993; Yokoyama et al., 1998), while other studies reported competitive interactions in co-cultures of *L. monocytogenes* and *L. innocua* (Heir et al., 2018; Petran and Swanson, 1993; Yokoyama et al., 1998). A direct comparison between these studies cannot be made, because the studies used different *L. innocua* strains, broth media and/or incubation temperatures (Heir et al., 2018; Langsrud et al., 2016; Petran and Swanson, 1993; Yokoyama et al., 1998). In the current study, also no competition advantage was shown for the *L. grayi* strain in

co-culture with *L. monocytogenes*, although *L. grayi* is reported to be mannitol-positive (Weller et al., 2015a). Notably, the *L. grayi* strain was able to consume mannitol in mannitol-enriched carbohydrate utilization medium (NEN-EN-ISO 11290-1:2017) (International Organization for Standardization, 2017) (data not shown), however, in mushroom medium the *L. grayi* strain did not consume mannitol in single and co-culture incubations, which is conceivably due to the availability of other preferred substrates, i.e., glucose, fructose, trehalose and glycerol.

Growth of *L. monocytogenes* occurred in all co-incubations and pH-adjusted spent mushroom media, including spent media of the lactic acid bacteria and the *Listeria* spp. strains. So nutrient competition in mushroom media was not apparent and this points to excess availability of carbon and nitrogen sources and other growth factors. It should be noted that the composition of the filter-sterilized mushroom medium is not completely static in absence of bacterial inoculation. This is exemplified by an increase of amino acid concentrations in control incubations (non-inoculated mushroom medium) after 48 hours incubation at room temperature compared to fresh filter-sterilized mushroom medium, conceivably due to proteolytic enzyme activity (Lake et al., 2023). In addition, also a change in sugar composition was observed in control incubations compared to fresh filter-sterilized mushroom medium in which a decrease in mannitol and an increase in fructose was observed after 48 hours incubation at room temperature. This was probably also due to mushroom-derived enzyme activity since this change in sugar composition was observed in non-inoculated mushroom medium incubated under both ambient air as well as in anaerobic conditions. This dynamic and complex nature of the mushroom medium results in a surplus of nutrients for *L. monocytogenes*, and may have contributed to its good performance in this nutrient-rich medium.

In conclusion, the current study demonstrated that microbiota strains isolated from the mushroom processing environment grow and form biofilm in mushroom medium, with the highest growth and biofilm counts for the mannitol-consuming *Enterobacteriaceae* strains *Ewingella* and *Raoultella*. Co-incubations of the frequently isolated microbiota with *L. monocytogenes* in mushroom medium showed competitive growth of *L. monocytogenes* and high *L. monocytogenes* cell counts were reached after 24 hours and 48 hours of incubation, with a slight decline of *L. monocytogenes* after 48 hours in co-cultures with acidifying *Lactococcus* spp. microbiota. Also, *L. monocytogenes* grew in spent media of all tested microbiota strains, except for

acidified spent media of *Lactococcus* spp. and *Listeria* spp., but also here growth was restored after the pH was increased. Altogether, this shows the competitiveness of *L. monocytogenes* during mixed culture incubations and indicates that a pH reduction and not the nutrient availability or antimicrobials is the main growth limiting factor for *L. monocytogenes* growth in mixed cultures in mushroom medium.

Abbreviations

CV, crystal violet; LAB, lactic acid bacteria; *Lm*, *Listeria monocytogenes*; mm, mushroom medium

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Supplemental information

Supplemental tables

Supplemental table 4.1. List of the species primers for *Listeria* strain determination. Nucleotide sequences of primer sets used in this study

Species specificity	Gene target	Primer sequence (5'-3')	Product size (bp)	Primer concentration (µM)	Reference
All *Listeria* species	*prs*	prs-F: GCTGAAGAGATTGCGAAAGAAG prs-R: CAAAGAAACCTTGGATTTGCGG	370	0.2	Doumith et al., 2004
L. monocytogenes	*isp*	isp-F: TGCAGCGAATGCTCTTAGTG isp-R: AGCCAAGCACGGCTACTTTA	713	0.2	Rawool et al., 2016
L. innocua	*lin0464*	lin0464-F: CGCATTTATCGCCAAAACTC lin0464-R: TCGTGACATAGACGCGATTG	749	1.2	Ryu et al., 2013
L. ivanovii	*namA*	liv22-228-F: CGAATTCCTTATTCACTTGAGC liv22-228-R: GGTGCTGCGAACTTAACTCA	463	0.6	Ryu et al., 2013
L. seeligeri	*lmo0333*	lseelin-F: GTACCTGCTGGGAGTACATA lseelin-R: CTGTCTCCATATCCGTACAG	673	1.4	Ryu et al., 2013
L. welshimeri	*scrA*	lwe1801-F: CGTGGCACAATAGCAATCTG lwe1801-R: GACATGCCTGCTGAACTAGA	281	1.0	Ryu et al., 2013
L. grayi	*Oxido-reductase*	JOgrayi-F: GCGGATAAAGGTGTTCGGGTCAA JOgrayi-R: ATTTGCTATCGTCCGAGGCTAGG	201	0.2	Ryu et al., 2013

Supplemental table 4.2. List of strains used in this study, including metadata of the strains.

Supplemental table 4.2 part A. List of microbiota and *Listeria* spp. strains

Bacterial strain	Source of isolation	Type of plant	Isolation year
*Ewingella**	Swab after C&D	Factory	2019
*Raoultella**	Swab after C&D	Factory	2019
Citrobacter	Swab after C&D	Factory	2019
Lelliottia	Swab after C&D	Factory	2019
Buttiauxella	Swab after C&D	Factory	2019
*Pseudomonas fluorescens**	Swab after C&D	Factory	2019
*Pseudomonas fragi**	Swab after C&D	Factory	2019
Acinetobacter spp.*	Swab after C&D	Factory	2019
*Acinetobacter johnsonii**	Swab after C&D	Factory	2019
*Lactococcus lactis**	Swab after C&D	Factory	2019
*Lactococcus garviea**	Swab after C&D	Factory	2019
*Lactococcus raffinolactis**	Swab after C&D	Factory	2019
Enterococcus	Swab after C&D	Factory	2019
Streptococcus	Swab after C&D	Factory	2019
Brochothrix	Swab after C&D	Factory	2019
Aeromonas	Swab after C&D	Factory	2019
Chryseobacterium	Swab after C&D	Factory	2019
Spingobacterium	Swab after C&D	Factory	2019
*Listeria grayi**	Fresh mushroom after mechanical harvesting	Grower	2018
*Listeria seeligeri**	Casing soil during second harvest	Grower	2018
*Listeria innocua**	Swab after C&D	Factory	2018

* Strain selected for co-culture experiments and spent medium experiments besides mono-culture experiments

Supplemental table 4.2 part B. List of *L. monocytogenes* strains

Listeria monocytogenes strain number	Type of plant	Isolation year	Serogroup	Lineage	ST (MLST)	CC (MLST)
636** ***	Factory	2018	1/2b-3b-7	I	ST224	CC224
638** ***	Factory	2018	1/2a-3a	II	ST451	CC451
640** ***	Factory	2018	4b-4d-4e	I	ST4	CC4

** Strains derived from Lake et al., 2021. (Lake, F.B., van Overbeek, L.S., Baars, J.J.P., Koomen, J., Abee, T., den Besten, H.M.W., 2021. Genomic characteristics of *Listeria monocytogenes* isolated during mushroom (*Agaricus bisporus*) production and processing. Int. J. Food Microbiol. 360, 109438. https://doi.org/10.1016/j.ijfoodmicro.2021.109438)

*** Strains were isolated from frozen sliced mushroom - final product.

Supplemental table 4.3. List of microbiota strains isolated during mushroom processing and after C&D procedures including *Enterobacteriaceae* discrimination

Supplemental table 4.3 part A. Represented microbiota and number of strains (percentages) isolated from the mushroom processing line during mushroom processing and after the C&D procedures

Group	During mushroom processing, number of strains (percentages)	After C&D procedures, number of strains (percentages)	Total amount of isolated strains, number of strains (percentages)
*Acinetobacter**	43 (41.7%)	21 (15.4%)	64 (26.8%)
*Lactococcus**	14 (13.6%)	30 (22.1%)	44 (18.4%)
*Enterobacteriaceae** **	20 (19.4%)	33 (24.3%)	53 (22.2%)
*Pseudomonas**	6 (5.8%)	17 (12.5%)	23 (9.6%)
*Chryseobacterium****	5 (4.9%)	3 (2.2%)	8 (3.3%)
*Brochothrix****	2 (1.9%)	3 (2.2%)	5 (2.1%)
Serratia		4 (2.9%)	4 (1.7%)
*Streptococcus****		4 (2.9%)	4 (1.7%)
*Enterococcus****		4 (2.9%)	4 (1.7%)
*Aeromonas****	1 (1.0%)	3 (2.2%)	4 (1.7%)
*Sphingobacterium****	2 (1.9%)	2 (1.5%)	4 (1.7%)
Comamonas	3 (2.9%)	1 (0.7%)	4 (1.7%)
Thrichococcus		2 (1.5%)	2 (0.8%)
Staphyloccous		2 (1.5%)	2 (0.8%)
Mycetocola	2 (1.9%)		2 (0.8%)
Microbacterium		1 (0.7%)	1 (0.4%)
Hafnia		1 (0.7%)	1 (0.4%)
Brevibacterium		1 (0.7%)	1 (0.4%)
Plantibacter		1 (0.7%)	1 (0.4%)
Micrococcus		1 (0.7%)	1 (0.4%)
Vagococcus		1 (0.7%)	1 (0.4%)
Delftia		1 (0.7%)	1 (0.4%)
Leuconostoc	1 (1.0%)		1 (0.4%)
Shewwanella	1 (1.0%)		1 (0.4%)
Paenarthrobacter	1 (1.0%)		1 (0.4%)
Rothia	1 (1.0%)		1 (0.4%)
Stenotrophomonas	1 (1.0%)		1 (0.4%)
Total number of strains	103	136	239

* Multiple strains of this group are used in mono-culture experiments, co-culture experiments and spent medium experiments

** *Enterobacteriaceae* strain determination based on 16S rRNA is further elaborated in the table below

*** One strain of this group is used in mono-culture experiments

Supplemental table 4.3 part B. Classification of the *Enterobacteriaceae* strains isolated from the mushroom processing line during mushroom processing and after C&D procedures

Enterobacteriaceae group****	During mushroom processing, number of strains (percentages)	After C&D procedures, number of strains (percentages)	Total amount of *Enterobacteriaceae* strains, number of strains (percentages)
Raoultella - Klebsiella	6 (30%)	2 (6.1%)	8 (15.1%)
*Raoultella******	3 (15%)	3 (9.1%)	6 (11.3%)
Lelliottia - Enterobacter		6 (18.2%)	6 (11.3%)
*Ewingella*****	3 (15%)	2 (6.1%)	5 (9.4%)
Enterobacteriaceae		4 (12.1%)	4 (7.5%)
Kluyvera	2 (10%)	2 (6.1%)	4 (7.5%)
Cedecea - Enterobacter	3 (15%)	1 (3.0%)	4 (7.5%)
*Lelliottia******		3 (9.1%)	3 (5.7%)
*Buttiauxella******	2 (10%)	1 (3.0%)	3 (5.7%)
*Citrobacter******	1 (5%)	2 (6.1%)	3 (5.7%)
Yersinia		2 (6.1%)	2 (3.8%)
Rahnella - Ewingella		1 (3.0%)	1 (1.9%)
Rahnella - Serratia		1 (3.0%)	1 (1.9%)
Enterobacter		1 (3.0%)	1 (1.9%)
Kluyvera - Citrobacter		1 (3.0%)	1 (1.9%)
Kluyvera - Lelliottia		1 (3.0%)	1 (1.9%)
Total number of strains	20	33	53

**** Although the 16S rRNA method resulted in imperfect genus characterization of the *Enterobacteriaceae* strains, the best hit or the two best hits based on the sequence were described in this table

***** One strain of this group is used in mono-culture experiments, co-culture experiments and spent medium experiments

****** One strain of this group is used in mono-culture experiments

Supplemental table 4.4. List of the 16S rRNA gene sequences (~1.5 kb) for strain determination (taxonomical identification) of the 18 selected microbiota strains

Bacterial strain and the corresponding 16S rRNA sequence

Ewingella

CAAGTCGAGCGGCAGCGGGAAGTAGCTTGCTACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG
CCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACCTCGAAAGAGCAAAGTGGGGGACCTTCG
GGCCTCACGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCG
AGGAGGAAGGCGTTAAGGTTAATAACCTTAGCGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA
GATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGG
TTGTGGGCTTGACCCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACT
CTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCGTKATGGRGGG
AACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGG
GCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGA
GGGCGCTACCA

Raoultella

GCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAA
CGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATG
GGATTAGCTAGTARGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
CATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGYGWTRWGGTTAATAACCK
YRKYKATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG
AACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG
ATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGG
AGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAG
ATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA
CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATAC
AAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTC
CGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence
Citrobacter
TGCAAGTCGAACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAAC
TGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTT
CGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCT
AGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAG
CGAGGAGGAAGGTGTTGTGGTTAATAACCGCAGCRATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAG
TCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAG
AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACT
GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTG
GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC
CTACTCTTGACATCCAGAGAAGTTTGCAGAGATGCGAASGTGCCTTCGGGAACTSTGAGACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTC
GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGT
AGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTC
GTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTC
GGGAGGGCGCTTACCAC
Lelliottia
GCAGTCGAGCGGTAGCACAGGAGAGCTTGCTCTCYGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG
CCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAAYGTCGCAAGACCAAAGAGGGGGACCTTCG
GGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG
AGGAGGAAGGCRTTAAGGTTAATAACCTTAGTGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTC
GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAAT
TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGG
AGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCT
ACTCTTGACATCCAGAGAACTTWSCAGAGATGSWTTGGTGCCTTCGGGAACTSTGAGACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGG
GAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAG
GGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGT
AGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGG
GAGGGCGCTACC

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence
Buttiauxella
TTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACRATCCCTAGCTGGTC
TGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAARAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGG
AAGGCATTGTGGTTAATAACCGCAGTGATTGACGTTACTCGCAGAARAARCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTCAAGTCGGATGT
GAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTT
CCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGGAGTACGGCCGCAAGGTTAAAACTCAA
ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTT
GACATCCACAGAATTCGGTAGAGATACCTTAGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACT
CAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTA
CACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCC
GG
Pseudomonas fluorescens
GCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGG
TAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCT
TGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAG
GAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGC
GGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTAGTTAAGTTGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGT
GTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAG
CCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGAC
ATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAA
GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACA
CGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGT
T

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence

Pseudomonas fragi

CAAGTCGAGCGGTAGAGAGGTGCTTGCACCTCTTGAGAGCGGCGGACGGGTGAGTAATACCTAGGAATCTGCCTGAT
AGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTT
GCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACTGGTC
TGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGG
AAGGGCATTAACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG
GTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAATGTGA
AATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTAGTGGAATTTCCTGTG
TAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGATACTGACACTGAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGT
CTTGAACTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACA
TCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACA
CGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCG

Acinetobacter spp.

CTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTCCGAAAGGAATGCTAATACC
GCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCAGATTAGCTAGTTG
GTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTG
TGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTAGATTAATACTCTAGGATAGTGGACGTT
ACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTAC
TGGGCGTAAAGCGTGCGTAGGCGGCTTCTTAAGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATAC
TGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC
GATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTA
GACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCG
GGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTTTCCTTATTTGCCAGCGGGTTAAGCCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGG
ACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTA
GCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
CTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAATT
TGTTGCACCAGAAGTAGGTAGTCTAACCGCA

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence

Acinetobacter johnsonii

TGCAGTCGAGCGGGGAWGGTAGCTTGCTACCTWWCCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTAT
TAGTGGGGGACAACATTCCGAAAGGAATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTT
GCGCTAATAGATGAGCCTAAGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTC
TGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGG
AGGCTACYTGGATTAATACTCTRGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGC
GGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTYTTAAGTCGGATGTG
AAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGT
GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGG
TACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGC
CTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGAC
ATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAMCGGGTTAAGCCGGGAACTTTAA
GGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA
CGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATT
GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCATGGGAATTTGTTGCACCAGAAGTAGGTAGTCTAACCGCAAGGAGGACGCTA
CCA

Lactococcus lactis

GCAGTTGAGCGATGAAGATTGGTGCTTGCACCAATTTGAAGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGC
CTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGCATAACAACTTTAAACATAAGTTTTAAGTTTGAAAGAT
GCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACAT
AGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGG
TAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAG
TCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGAC
ACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATG
TAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCA
CTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTC
GGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCCTAACCGCAAGGAGG
GCGCTC

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence
Lactococcus garviea
GCAAGTCGAGCGATGATTAAAGATAGCTTGCTATTTTTATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCT
GCCGAGTAGCGGGGGACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTAAAA
GAAGCAATTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATA
CATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGT
TGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTT
AAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTG
GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACT
GACACTGAGGCTCGAAAGCGTGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGC
TGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATACTCGTGCTATCCTTAGAGATAAGGAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGG
CACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGATGGTACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGT
TCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTAGGTTGCCTAACCGCAAGGAG
GGCGCTCCT
Lactococcus raffinolactis
TAATGCAGTTGACGCTTGATTTTCACCGAAGCTTGCTTCACCGAAAATCAAGAGTAGCGAACGGGTGAGTAACGCGTG
GGTAACCTACCTTTCAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAAYAATGTTGGATGCATATTCGAC
ATTTGAAAGTACCAATTGGTACACTGAGAGATGGACCCGCGTTGTATTAGCTAGTTGGTAGTGTAATGGACTACCAAGG
CGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAA
AACTCTGTTGTTAGAGAAGAAYGTTGCATAGAGTGGAAAATTATGCAAGTGACGGTATCTAACCAGAAAGGGACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGT
GGTTTAATAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGTTAGACTTGAGTGCAGTAGAG
GAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGA
CTGTCACTGACACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAGTTGTTTGGGGCTATCCAGCCCTAAGTGACGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCG
CAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCA
TTAAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAAGGCAGTGATGTCAAGCTAATCTCTTAAAGC
CAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAATACCCAAAGCCGGTGAGCTAA
CCTTTTAGGAGGCAGCCGTCTAAA

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence

Enterococcus

GCAGTCGAACGCTTCTTTTCCCACCGGAGCTTGCTCCACCGGGRAAAGAGGAGTGGCGAACGGGTGAGTAACACGTG
GGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTCG
TTTTGAAAGGCGCTTTACGGTGCCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATC
GTAAAACTCTGTTGTTAGAGAAGAACAAGGGTGAGAGTAACTGTTCACCCCTTGACGGTATCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA
AGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTC
TGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
ACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTRGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCC
ATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCYAAGTCGCGAGGCTAAGCTAATCTCTTA
AAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGC
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGG
TAACCTTTTGGAGCCAGCCG

Streptococcus

CTATACATGCAAGTAGAACGCTGAAGACTGGTGCTTGCACTAGTCAGATGAGTTGCGAACGGGTGAGTAACGCGTAGG
TAACCTACCTCATAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATGACAATTAAGTACTCATGTACTAAATT
TAAAAGGAGCAATTGCTTCACTATGAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCA
CGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAG
CTCTGTTGTTAGAGAAGAACGGTAATGGGAGTGGAAAATCCATTACGTGACGGTAACTAACCAGAAAGGGACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTCTCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGYAGGCG
GTTATTTAAGTCTGAAGTTAAAGGCCGTGGCTCAACCATGGTTCGCTTTGGAAACTGGATAACTTGAGTGCAGAAGGGG
AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCT
GTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC
AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCCCTCTGACCGTCCTAGAGATAGGACTTTCCTTCGGGACAGAGGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCA
TTAAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCAAGCCGGTGACGGCAAGCTAATCTCTTAAAG
CCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTA
ACCTATTAGGAGCCAG

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence

Brochothrix

GCAGTCGACGAACGGATAARGAGCTTGCTCTTTTGAAGTTAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
CACAGCTGGGGATAACATCGAGAAATCGATGCTAATACCGAATGTGCTGAACATCATAAGATGTTCAAGTGAAAGACG
GTTTCGGCTGTCACTGTGAGATGGACCCGCGCTGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATCC
ATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTT
GTTAGAGAAGAACATGGGTGAGAGTAACTGTTCACCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTA
AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGGACAGAAGAGGAGAGT
GGAATTCCAAGTGTAGCGGTGAAATGCGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTTAC
TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTAGGGGTTTCCGCCCCTTAGTGCTGCAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTTTGACCATTCTGGAGACAGAACTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTGCCAGCATTTAGTTG
GGCACTCTAAAGTGACTGCCGGTGTAAGCCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGATAATACAAAGGGTCGCGAAGCCGCGAGGTGGAGCCAATCCCATAAAATTATTCTCA
GTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCAAAGCCGGTTTGGTAACCTTCGGG
AGCTAGCCG

Aeromonas

CGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCC
AGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGC
CTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTG
GTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAG
GAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAG
ATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTC
CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGC
TCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGC
TGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC
AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCC
TTGACATGTCTGGAATCCTGRAGAGATYCGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGA
ACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
CTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAG
TCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGA
GGGCGTTA

Supplemental table 4.4 continued

Bacterial strain and the corresponding 16S rRNA sequence

Chryseobacterium

TGCAAGCCGAGCGGTAGAGATTCTTCGGAATCTTGAGAGCGGCGCACGGGTGCGGAACACGTGTGCAACCTGCCTTTA
TCAGGGGAATAGCCTTTCGAAAGGAAGATTAATGCCCCATAATATATCATATGGCATCATTTGATATTGAAAACTCCGGT
GGATAAAGATGGGCACGCGCAGGATTAGATAGTTGGTAGGGTAACGGCCTACCAAGTCAGCGATCCTTAGGGGGCCT
GAGAGGGTGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACA
ATGGGTGAGAGCCTGATCCAGCCATCCCGCGTGAAGGACGACGGCCCTATGGGTTGTAAACTTCTTTTGTATAGGGAT
AAACCTACCCTCGTGAGGGTAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCTGATGTGTAAGTCAGTGGTGAAATCT
CACAGCTTAACTGTGAAACTGCCATTGATACTGCATGTCTTGAGTGTTGTTGAAGTAGCTGGAATAAGTAGTGTAGCGG
TGAAATGCATAGATATTACTTAGAACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGACGCTGATGGACGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCTAACTCGTTTTTGGGCTTTCGGGTTC
AGAGACTAAGCGAAAGTGATAAGTTAGCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGATTATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGGGAAATGACA
GGCTTAGAAATAGGCTTTTCTTCGGACATTTTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTAGG
TTAAGTCCTGCAACGAGCGCAACCCCTGTCACTAGTTGCCATCATTCAGTTGGGGACTCTAGTGAGACTGCCTACGCAA
GTAGAGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCCACACACGTAATACAATGGCCGGT
ACAGAGGGCAGCTACACAGCGATGTGATGCAAATCTCGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGACT
CTATGAAGCTGGAATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCAAGCCATGGAAGTCTGGGGTACCTGAAGTCGGTGACCGTAACAGG

Spingobacterium

AAGTCGAACGAGATTATCCAGCTTGCTGGATATGAAAGTGGCGCACGGGTGCGTAACGCGTGAGCAACCTACCTCTGT
CAGGGGGATAGCCCGGCGAAAGTCGGATTAACACCGCATGACATTATTGATGTGGCATCACATTATAATCAAATATTTA
TAGGACAGAGATGGGCTCGCGTGACATTAGCTAGTTGGAGAGGTAACGGCTCACCAAGGCAACGATGTCTAGGGGCT
CTGAGAGGAGAATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTAAGGAATATTGGTC
AATGGAGGGAACTCTGAACCAGCCATGCCGCGTGCAGGATGACTGCCCTATGGGTTGTAAACTGCTTTTGTCGGGGAA
TAAACCTACGTTTGCGAACGTAGCTGAATGTACCCGAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGATCCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGTTCTTTAAGTCAGAGGTGAAAGA
CGGCAGCTTAACTGTCGCAGTGCCTTTGATACTGAAGAACTTGAATTGGGTTGAGGAATGCGGAATGAGACAAGTAGC
GGTGAAATGCATAGATATGTCTCAGAACCCCGATTGCGAAGGCAGCATTCCAAGCCTATATTGACGCTGATGCACGAAA
GCGTGGGGATCGAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATAACTCGATGTTGGCGATAGACAG
TCAGCGTCCCAGCGAAAGCGTTAAGTTATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGAGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCCGGGCTTGAAAGTTAGTGAA
TGATCCAGAGACGGATCAGTCCTTCGGGACACGAAACTAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCCTATGTTTAGTTGCCAGCATGTAATGATGGGGACTCTAAACAGACTGCCT
GCGCAAGCAGAGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGTCCGGGGCTACACACGTGCTACAAT
GGTCGGTACAGCGGGCAGCTACACAGTAATGTGATGCCAATCTCTGAAAGCCGATCACAGTTCGGATTGAGGTCTGCA
ACTCGACCTCATGAAGTTGGATTCGCTAGTAATCGCGTATCAGCAATGACGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCMKCCGTCAAGCCATGAAAGTTGGGGGTGCCTAAAGCATGTAACCGCAAGGAGCGTTT

Supplemental table 4.5. Compound analysis of fresh filter-sterilized mushroom medium at the start of incubation ("Mushroom medium 0 hours") and non-inoculated filter-sterilized mushroom medium incubated statically for 48 hours at 20 °C ("Mushroom medium 48 hours"). Batch 1 was used for the individual strain characterization of the 18 microbiota strains + the all-strain mixture (mixture of the 18 microbiota strains). Batch 2 was used for the strain characterization during the co-incubations, the spent media incubations and the incubations with the *Listeria* spp. strains

Batch 1

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 0 hours*	0.03 (0.00)***	8.62 (0.18)	2.74 (0.07)	79.06 (1.05)	2.50 (0.04)	0.11 (0.01)	0.48 (0.05)	0.60 (0.00)
Mushroom medium 48 hours**	0.15 (0.14)	8.55 (1.43)	7.46 (0.66)	68.09 (7.93)	2.54 (0.28)	0.34 (0.54)	0.46 (0.28)	0.65 (0.30)

Batch 2

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 0 hours*	0.20 (0.21)	5.69 (0.45)	3.40 (1.34)	78.38 (4.63)	2.38 (0.25)	0.09 (0.08)	0.23 (0.34)	< 0.01****
Mushroom medium 48 hours **	0.41 (0.16)	5.78 (0.35)	17.89 (0.55)	65.67 (3.19)	3.46 (0.16)	0.19 (0.07)	0.71 (0.25)	0.14 (0.26)

* Mushroom medium at the start of incubation

** Mushroom medium after 48 hours of static incubation at 20 °C

*** Values are derived from two to four technical replicates

**** Value below detection limit (detection limit of 0.01 mM)

Supplemental table 4.6. Compound analysis of mushroom medium during co-incubation experiment. Compound analysis of mushroom medium without bacterial inoculation after 48 hours of static incubation at 20 °C ("Mushroom medium 48 hours") and after co-incubations of nine microbiota strains with *L. monocytogenes* (*Lm*) strain 636 for 48 hours at 20 °C ("*Lm* + microbiota")

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 48 hours	0.41 (0.16)*	5.78 (0.35)	17.89 (0.55)	65.67 (3.19)	3.46 (0.16)	0.19 (0.07)	0.71 (0.25)	0.14 (0.26)
Lm + Ewingella	0.05 (0.03)	0.16 (0.16)	<0.01	30.23 (0.65)	0.59 (0.81)	11.74 (0.50)	10.12 (0.55)	14.29 (0.69)
Lm + Raoultella	< 0.01**	0.16 (0.16)	<0.01	0.32 (0.04)	0.19 (0.10)	6.00 (0.35)	12.67 (0.77)	2.58 (0.39)
Lm + P. fluorescens	<0.01	0.22 (0.22)	<0.01	62.03 (3.79)	0.21 (0.04)	4.59 (1.16)	6.17 (0.92)	0.07 (0.07)
Lm + P. fragi	<0.01	0.27 (0.28)	<0.01	64.92 (4.83)	0.46 (0.17)	24.80 (2.22)	2.75 (0.90)	1.55 (0.56)
Lm + Acinetobacter spp.	<0.01	1.05 (0.03)	<0.01	65.82 (3.37)	0.92 (0.03)	23.64 (0.93)	3.87 (0.72)	2.07 (0.34)
Lm + A. johnsonii	0.03 (0.03)	1.08 (0.05)	<0.01	65.31 (4.13)	0.88 (0.17)	22.47 (2.38)	3.72 (1.23)	3.42 (0.62)
Lm + L. lactis	<0.01	1.13 (0.06)	<0.01	64.98 (4.20)	0.96 (0.06)	26.98 (1.82)	6.91 (0.18)	1.22 (0.32)
Lm + L. garviea	0.07 (0.07)	1.11 (0.07)	<0.01	66.25 (4.47)	1.44 (0.20)	34.04 (1.62)	5.33 (0.84)	2.47 (0.46)
Lm + L. raffinolactis	0.06 (0.02)	1.18 (0.15)	<0.01	65.15 (4.93)	1.56 (0.11)	36.99 (0.98)	4.1 (1.29)	0.71 (0.28)

* Values are derived from four biological replicates

** Value below detection limit (detection limit of 0.01 mM)

Supplemental table 4.7. Compound analysis of mushroom medium during spent mushroom medium experiment. Compound analysis of mushroom medium without bacterial inoculation after 72 hours of static incubation at 20 °C ("Mushroom medium 72 hours"), after incubation with a microbiota strain for 72 hours at 20 °C ("spent microbiota"), and after subsequent incubation with the *L. monocytogenes* (*Lm*) strain 636 in the non-pH adjusted spent media ("*Lm* in non-pH adjusted spent microbiota") and pH-adjusted spent media ("*Lm* in pH-adjusted spent microbiota") after 48 hours at 20 °C

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 72 hours	0.41 (0.16)*	5.78 (0.35)	17.89 (0.55)	65.67 (3.19)	3.46 (0.16)	0.19 (0.07)	0.71 (0.25)	0.14 (0.26)
Spent *Ewingella*	0.07 (0.01)	0.13 (0.13)	0.10 (0.01)	2.01 (0.24)	0.52 (0.52)	12.47 (0.23)	10.36 (0.84)	14.82 (0.26)
Lm in non-pH adjusted spent *Ewingella*	< 0.01**	0.14 (0.14)	0.15 (0.00)	2.08 (0.27)	0.14 (0.04)	12.80 (0.29)	10.51 (0.72)	11.82 (0.17)
Lm in pH-adjusted spent *Ewingella*	<0.01	0.17 (0.17)	0.06 (0.06)	1.91 (0.28)	0.25 (0.04)	12.58 (0.37)	10.92 (0.75)	12.63 (0.06)
Spent *Raoultella*	<0.01	0.13 (0.13)	0.07 (0.07)	0.29 (0.03)	0.18 (0.07)	6.82 (0.10)	20.24 (2.54)	1.86 (0.19)
Lm in non-pH adjusted spent *Raoultella*	<0.01	0.16 (0.16)	<0.01	0.27 (0.00)	0.24 (0.08)	6.83 (0.03)	20.63 (2.50)	1.76 (0.14)
Lm in pH-adjusted spent *Raoultella*	<0.01	0.14 (0.14)	<0.01	0.27 (0.01)	0.23 (0.05)	6.84 (0.04)	20.60 (2.38)	1.94 (0.22)
Spent *P. fluorescens*	0.13 (0.00)	0.59 (0.00)	3.74 (0.08)	48.10 (3.33)	0.55 (0.04)	0.38 (0.04)	1.05 (0.24)	<0.01
Lm in non-pH adjusted spent *P. fluorescens*	<0.01	0.22 (0.22)	2.15 (0.10)	47.16 (2.73)	0.40 (0.02)	4.00 (0.41)	1.40 (0.26)	0.06 (0.06)
Lm in pH-adjusted spent *P. fluorescens*	<0.01	0.24 (0.24)	1.11 (0.34)	45.14 (2.21)	0.81 (0.03)	5.40 (0.21)	1.35 (0.06)	<0.01
Spent *P. fragi*	0.71 (0.03)	1.45 (0.15)	17.21 (0.30)	61.69 (2.60)	0.49 (0.14)	1.55 (0.04)	3.53 (0.17)	<0.01
Lm in non-pH adjusted spent *P. fragi*	0.04 (0.05)	0.22 (0.31)	4.43 (1.14)	54.00 (2.22)	0.29 (0.01)	28.88 (1.18)	12.44 (1.17)	2.10 (0.34)
Lm in pH-adjusted spent *P. fragi*	0.10 (0.02)	0.20 (0.29)	4.92 (0.51)	54.09 (1.91)	0.66 (0.20)	26.25 (0.92)	10.67 (2.21)	1.19 (0.72)
Spent *Acinetobacter* spp.	0.75 (0.01)	4.82 (0.07)	19.23 (0.61)	60.46 (4.14)	1.26 (0.09)	0.24 (0.24)	0.97 (0.49)	0.07 (0.07)
Lm in non-pH adjusted spent *Acinetobacter* spp.	0.05 (0.05)	1.24 (0.08)	6.74 (0.12)	55.94 (5.84)	1.12 (0.09)	27.68 (0.72)	6.94 (0.40)	3.76 (0.31)

Supplemental table 4.7 continued

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Lm in pH-adjusted spent *Acinetobacter* spp.	<0.01	1.26 (0.02)	6.91 (0.15)	55.52 (4.23)	1.12 (0.01)	23.55 (0.60)	5.85 (0.83)	3.53 (0.54)
Spent *A. johnsonii*	0.45 (0.07)	3.72 (0.13)	11.96 (0.92)	65.30 (2.27)	2.56 (0.22)	11.45 (0.09)	1.93 (0.29)	0.81 (0.13)
Lm in non-pH adjusted spent *A. johnsonii*	0.45 (0.28)	2.64 (1.72)	14.75 (3.90)	60.25 (3.38)	2.79 (0.41)	13.58 (10.21)	2.86 (1.28)	1.38 (0.74)
Lm in pH-adjusted spent *A. johnsonii*	0.52 (0.28)	2.85 (1.63)	15.49 (3.96)	59.03 (3.81)	3.10 (0.18)	9.21 (9.01)	2.24 (1.21)	1.20 (0.57)
Spent *L. lactis*	<0.01	1.05 (0.03)	<0.01	63.37 (0.46)	0.98 (0.05)	27.26 (1.04)	6.45 (0.45)	0.91 (0.07)
Lm in non-pH adjusted spent *L. lactis*	<0.01	1.20 (0.03)	<0.01	64.26 (1.28)	1.08 (0.02)	27.66 (1.35)	6.13 (0.42)	0.70 (0.07)
Lm in pH-adjusted spent *L. lactis*	<0.01	1.07 (0.03)	<0.01	61.50 (1.15)	1.14 (0.05)	27.58 (1.25)	7.05 (0.68)	0.87 (0.12)
Spent *L. garviae*	0.26 (0.05)	1.28 (0.05)	5.65 (0.00)	65.05 (2.15)	1.44 (0.06)	24.72 (1.02)	4.29 (0.26)	1.33 (0.07)
Lm in non-pH adjusted spent *L. garviae*	0.05 (0.04)	1.17 (0.04)	<0.01	67.27 (1.60)	1.96 (0.04)	36.35 (1.35)	4.49 (0.30)	1.68 (0.18)
Lm in pH-adjusted spent *L. garviae*	<0.01	0.42 (0.59)	<0.01	65.22 (1.84)	1.99 (0.15)	36.64 (1.68)	5.61 (0.39)	2.01 (0.11)
Spent *L. raffinolactis*	0.08 (0.00)	1.23 (0.04)	<0.01	65.38 (1.14)	1.89 (0.26)	39.12 (1.77)	3.39 (0.06)	0.48 (0.10)
Lm in non-pH adjusted spent *L. raffinolactis*	0.07 (0.00)	1.26 (0.04)	<0.01	65.53 (1.34)	1.84 (0.21)	39.05 (1.71)	2.91 (0.27)	0.52 (0.05)
Lm in pH-adjusted spent *L. raffinolactis*	<0.01	0.55 (0.55)	<0.01	63.37 (1.43)	2.10 (0.05)	39.03 (1.92)	3.49 (0.02)	0.40 (0.04)

* Values are derived from two or three biological replicates

** Value below detection limit (detection limit of 0.01 mM)

Supplemental table 4.8. Compound analysis of mushroom medium during monoculture experiments and co-culture experiments of *Listeria* spp. strains. Compound analysis of mushroom medium without bacterial inoculation after 48 hours of static incubation at 20 °C ("Mushroom medium 48 hours"), after incubation with a single *Listeria* strain after 48 hours at 20 °C ("*Listeria* spp."), and after co-incubation with the *L. monocytogenes* (*Lm*) strain 636 and the *Listeria* species ("*Lm* + *Listeria* spp.") after 48 hours at 20 °C

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 48 hours	0.41 (0.16)*	5.78 0.35)	17.89 (0.55)	65.67 (3.19)	3.46 (0.16)	0.19 (0.07)	0.71 (0.25)	0.14 (0.26)
L. grayi	< 0.01**	1.11 (0.03)	2.61 (0.36)	68.93 (0.55)	1.07 (0.04)	21.52 (0.28)	5.43 (0.19)	2.41 (0.07)
L. seeligeri	<0.01	1.12 (0.02)	1.63 (0.54)	69.88 (1.69)	1.05 (0.04)	22.31 (0.49)	5.50 (0.31)	3.00 (0.23)
L. innocua	<0.01	1.11 (0.02)	1.87 (0.14)	68.62 (1.54)	1.00 (0.05)	22.25 (0.14)	5.38 (0.07)	3.06 (0.01)
Lm + L. grayi	<0.01	1.07 (0.00)	<0.01	66.18 (1.18)	0.97 (0.02)	29.46 (0.10)	5.19 (0.25)	3.66 (0.10)
Lm + L. seeligeri	0.02 (0.02)	1.08 (0.02)	0.91 (0.04)	66.92 (1.38)	1.03 (0.02)	24.71 (0.02)	5.27 (0.10)	2.61 (0.18)
Lm + L. innocua	<0.01	1.09 (0.01)	1.00 (0.19)	67.16 (0.63)	1.07 (0.02)	23.97 (0.27)	5.57 (0.03)	2.67 (0.12)

* Values are derived from three biological replicates

** Value below detection limit (detection limit of 0.01 mM)

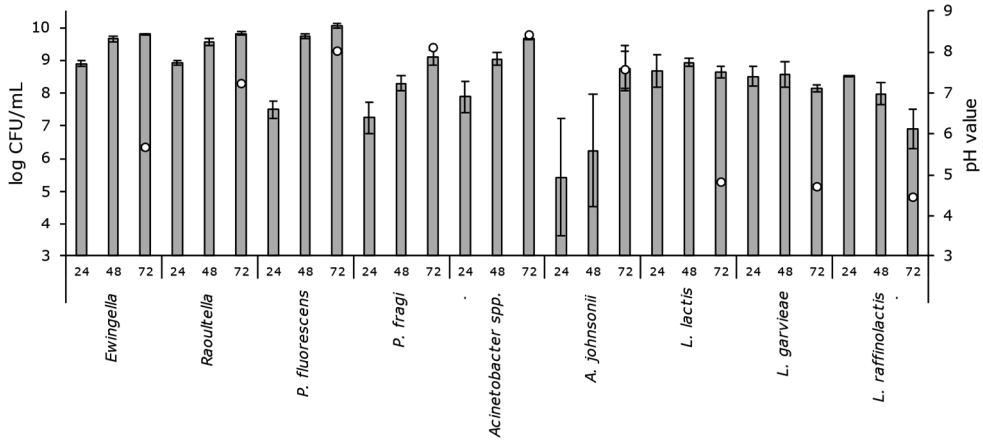
Supplemental table 4.9. Compound analysis of mushroom medium during spent mushroom medium experiment *Listeria* spp. strains. Compound analysis of mushroom medium without bacterial inoculation after 72 hours of static incubation at 20 °C ("Mushroom medium 72 hours"), after incubation with a *Listeria* spp. strains for 72 hours at 20 °C ("spent *Listeria* spp."), and after subsequent incubation with the *L. monocytogenes* (*Lm*) strain 636 in the non-pH adjusted spent media ("*Lm* in non-pH adjusted spent *Listeria* spp.") and pH-adjusted spent media ("*Lm* in pH-adjusted spent *Listeria* spp.") of the microbiota after 48 hours at 20 °C

	Trehalose mM (stdev)	Glucose mM (stdev)	Fructose mM (stdev)	Mannitol mM (stdev)	Glycerol mM (stdev)	Lactate mM (stdev)	Acetate mM (stdev)	Acetoin mM (stdev)
Mushroom medium 72 hours	0.41 (0.16)*	5.78 (0.35)	17.89 (0.55)	65.67 (3.19)	3.46 (0.16)	0.19 (0.07)	0.71 (0.25)	0.14 (0.26)
Spent *L. grayi*	< 0.01**	0.97 (0.04)	<0.01	65.63 (1.34)	0.90 (0.07)	29.89 (0.52)	4.51 (0.34)	4.44 (0.20)
Lm in non-pH adjusted spent *L. grayi*	<0.01	1.08 (0.01)	<0.01	62.43 (0.05)	1.13 (0.02)	31.21 (0.06)	4.98 (0.20)	3.72 (0.09)
Lm in pH-adjusted spent *L. grayi*	<0.01	1.00 (0.00)	<0.01	60.63 (1.04)	0.29 (0.29)	31.37 (0.52)	7.31 (0.20)	4.08 (0.11)
Spent *L. seeligeri*	0.07 (0.00)	1.11 (0.03)	<0.01	67.36 (0.93)	1.01 (0.06)	26.87 (0.20)	5.72 (0.35)	3.28 (0.04)
Lm in non-pH adjusted spent *L. seeligeri*	0.07 (0.00)	1.10 (0.02)	<0.01	61.44 (0.05)	1.03 (0.00)	26.53 (0.72)	5.97 (0.17)	2.72 (0.06)
Lm in pH-adjusted spent *L. seeligeri*	<0.01	0.53 (0.53)	<0.01	59.92 (1.30)	0.65 (0.24)	27.47 (0.14)	7.62 (0.27)	3.54 (0.05)
Spent *L. innocua*	0.06 (0.00)	1.05 (0.00)	0.98 (0.01)	67.57 (0.48)	1.01 (0.01)	25.26 (0.07)	5.71 (0.11)	2.46 (0.01)
Lm in non-pH adjusted spent *L. innocua*	0.07 (0.00)	1.13 (0.00)	0.35 (0.35)	62.52 (0.87)	1.10 (0.00)	25.30 (0.07)	6.10 (0.23)	2.50 (0.15)
Lm in pH-adjusted spent *L. innocua*	<0.01	0.53 (0.53)	<0.01	59.25 (0.46)	1.13 (0.04)	27.01 (0.09)	7.37 (0.16)	3.37 (0.08)

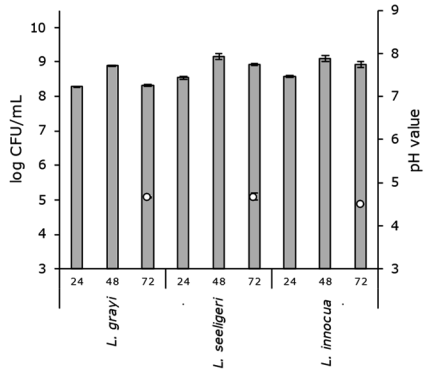
* Values are derived from at least three biological replicates

** Value below detection limit (detection limit of 0.01 mM)

Supplemental figures



Supplemental figure 4.1. Growth performance of the microbiota strains in fresh filter-sterilized mushroom medium. Single microbiota strains were inoculated with approximately 5 log CFU/mL in fresh filter-sterilized mushroom medium and cultures were statically incubated for 72 hours at 20 °C. Grey bars represent the microbiota counts after 24 hours (24), 48 hours (48) and 72 hours (72) of incubation. The initial pH of the medium is around 7.0 and the pH after 72 hours of incubation is represented by the white circles. The error bars represent the standard deviation of at least two biological reproductions.



Supplemental figure 4.2. Growth performance of the Listeria spp. strains in fresh filter-sterilized mushroom medium. Single *Listeria* spp. strains were inoculated with approximately 5 log CFU/mL in fresh filter-sterilized mushroom medium and cultures were statically incubated for 72 hours at 20 °C. Grey bars represent the microbiota counts after 24 hours (24), 48 hours (48) and 72 hours (72) of incubation. The initial pH of the medium is around 7.0 and the pH after 72 hours of incubation is represented by the white circles. The error bars represent the standard deviation of at least two biological reproductions.

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5

Biofilm formation and desiccation survival of *Listeria monocytogenes* with microbiota on mushroom processing surfaces and the effect of cleaning and disinfection

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Under review

Abstract

Listeria monocytogenes and resident background microbiota could be established as microbial multispecies communities on materials used in food processing environments. The presence, abundance and diversity of the individual strains within this community may be affected by interactions and competition between the microorganisms, but also due to differences in resistance towards regular cleaning and disinfection (C&D) procedures. Therefore, this study aimed to characterize the growth and diversity of a *L. monocytogenes* strain cocktail (n=6) during biofilm formation on polyvinyl chloride (PVC) and stainless steel (SS) without and with the presence of a diverse set of background microbiota (n=18) in simulated mushroom processing environmental conditions. The *L. monocytogenes* strains during monospecies incubation formed biofilms on PVC and SS, and C&D treatments every second day resulted in effective removal of biofilms from SS (reduction of 4.5 log CFU/cm^2 or less, resulting in counts below detection limit of 1.5 log CFU/cm^2 after every C&D treatment), while C&D treatments on biofilms formed on PVC resulted in limited reductions (reductions between 1.2 and 2.4 log CFU/cm^2). Co-incubation of the *L. monocytogenes* strains with the microbiota resulted in establishment of *L. monocytogenes* with high strain diversity in the multispecies biofilm on SS and PVC. C&D treatments removed *L. monocytogenes* from multispecies biofilm communities on SS (reduction of 3.5 log CFU/cm^2 or less, resulting in counts below detection limit of 1.5 log CFU/cm^2 after every C&D treatment), with varying dominance of microbiota species during different C&D cycles. However, repeated C&D treatments of multispecies biofilm on PVC resulted in lower reductions of *L. monocytogenes* (between 0.2 and 2.4 log CFU/cm^2) compared to single species biofilm, and subsequent regrowth of *L. monocytogenes* and stable dominance of *Enterobacteriaceae* and *Pseudomonas*. In addition, a higher reduction of *L. monocytogenes* was observed on SS compared to PVC when *L. monocytogenes* was exposed to desiccation stress on dry surfaces without and with the presence of background microbiota and with a high *L. monocytogenes* strain diversity. C&D treatment of desiccated cells on SS and PVC resulted in high cell count reductions (reduction of 5.9 log CFU/coupon or less, resulting in counts below detection limit of 1.7 log CFU/coupon). This study shows that *L. monocytogenes* is able to form single and multispecies biofilms on PVC with high strain diversity following C&D treatments. This highlights the needs for improved C&D treatments to remove biofilm cells from food processing surfaces.

5.1. Introduction

Listeria monocytogenes is a Gram-positive, facultative anaerobic and rod shaped bacterium that is isolated from a variety of environmental sources and different foods and food processing environments (Farber and Peterkin, 1991; Ferreira et al., 2014; Liu, 2006; Vázquez-Boland et al., 2001). *L. monocytogenes* is a foodborne pathogen that can cause listeriosis and particular groups at risk for listeriosis are pregnant women, neonates, the elderly and immunocompromised individuals (Ferreira et al., 2014; Liu, 2006; Vázquez-Boland et al., 2001).

L. monocytogenes is robust towards various stresses (van der Veen et al., 2008) and is able to survive cleaning and disinfection regimes (Fagerlund et al., 2017), which makes this foodborne pathogen a major concern for the food processing industry. *L. monocytogenes* has the ability to colonize and survive on food processing surfaces (di Bonaventura et al., 2008; Møretrø and Langsrud, 2004) and the transfer of *L. monocytogenes* from food contact surfaces to processed foods has been documented (Lin et al., 2006; Pang and Yuk, 2019). *L. monocytogenes* has been isolated from various food processing environments including the white button mushroom (*Agaricus bisporus*) processing environment (Murugesan et al., 2015; Pennone et al., 2018; Lake et al., 2021) and from fresh and frozen *A. bisporus* mushrooms (Lake et al., 2021). There have been no outbreaks of listeriosis associated with *A. bisporus* mushrooms, although some recalls have been reported for contaminated *A. bisporus* mushrooms (Anonymous, 2021).

Bacterial attachment and biofilm formation is affected by multiple variables, such as surface material type, environmental factors (e.g. pH and temperature), the type of medium, and cleaning and disinfection regimes (Donlan, 2002; Dunne, 2002; Fagerlund et al., 2021; Skowron et al., 2019). Single cultures or cocktails of *L. monocytogenes* strains are able to adhere and form biofilms on surfaces used in food production environments, such as stainless steel, polystyrene, polyvinyl chloride, glass surfaces (di Bonaventura et al., 2008; Dygico et al., 2020; Fagerlund et al., 2017; Heir et al., 2018; Rodríguez-López et al., 2015) and various surfaces related to the mushroom processing environment (Dygico et al., 2020). Microbiota present on surfaces in food processing facilities are commonly reported as having a high diversity (Møretrø and Langsrud, 2017) and these multispecies biofilm communities may affect the growth behaviour of *L. monocytogenes* (Fagerlund et al., 2021). The growth of *L.*

monocytogenes in multispecies biofilms may either led to enhanced, comparable or reduced viable cell numbers of *L. monocytogenes* (Carpentier and Chassaing, 2004; Fagerlund et al., 2021; Fox et al., 2014; Rodríguez-López et al., 2015), but biofilm formation of *L. monocytogenes* was in general lower in multispecies biofilms compared to single species biofilms (Fagerlund et al., 2021; Pang et al., 2019). The taxonomic composition of microbial communities can be determined with amplicon sequencing in which species-specific marker-genes are amplified using the polymerase chain reaction (PCR) technique, sequenced and aligned to a reference database. This high-throughput technique enables the identification of a high number of unique microbial communities (Bokulich et al., 2016). The resident background microbiota may play a role in protecting *L. monocytogenes* towards biocides used in disinfection regimes (van der Veen and Abee, 2011; Saá Ibusquiza et al., 2012). Among the disinfection agents approved for use in the food industry are oxidants (e.g. peracetic acid, hydrogen peroxide) (Wirtanen and Salo, 2003) in which peracetic acid is, in contrary to some other disinfectants, biodegradable as it decomposes into safe and environmental friendly residues in food (Dagher et al., 2017; Srey et al., 2013). Peracetic acid has a strong oxidizing capacity (Srey et al., 2013) and is found as an efficient disinfectant against biofilms (Skowron et al., 2018). In contrast to wet biofilms, *L. monocytogenes* can be exposed to desiccation stress when cells are attached to a surface followed by surface dehydration. Previous studies reported a significant decrease of *L. monocytogenes* counts during the first stage of dehydration, while a fraction of the population showed survival after extended incubation times, especially in the presence of food debris (Lim et al., 2020; Takahashi et al., 2011; Vogel et al., 2010).

To date, the competitive ability of surface-attached *L. monocytogenes* has not been characterized in conditions resembling the mushroom processing environment. Therefore, this study aims to characterize the biofilm forming capability and the desiccation survival of a genetically diverse set of *L. monocytogenes* strains in the presence of microbiota using simulated mushroom processing conditions. Abundance and diversity tests were included to determine the competitive fitness of different *L. monocytogenes* strains. In addition, this study also determined the effect of cleaning and disinfection on *L. monocytogenes*' ability to survive and regrow after cleaning and disinfection treatment. This approach gave new insights in the competitive behaviour of *L. monocytogenes* in complex communities that resembles mushroom processing environmental conditions.

5.2. Materials and methods

5.2.1. Selection of *L. monocytogenes* strains

Six *L. monocytogenes* strains were used in the biofilm and desiccation experiments. These *L. monocytogenes* strains were isolated during an extensive sampling plan in the frozen sliced mushroom production and processing chain (Lake et al., 2021). The six strains were isolated from different steps in the chain and belonged to different clonal complexes (CC), namely, three strains (CC4, CC7, CC224) isolated from the final frozen mushroom products (i.e. strains that could reach the consumer stage) and three strains (CC5, CC37, CC87) isolated from equipment after cleaning and disinfection (C&D) (i.e. strains that survived C&D and may regrow causing possible product contamination during processing) (table 5.1). The selected *L. monocytogenes* strains could be genetically differentiated by amplicon sequencing (sections 5.2.9 and 5.2.10).

Table 5.1. *Listeria monocytogenes* strains used in biofilm and desiccation experiments

Strain	Serogroup type	Sequence Type	Clonal Complex	Place of isolation
Lm544	1/2b-3b-7	ST87	CC87	Swab after C&D
Lm546	1/2a-3a	ST37	CC37	Swab after C&D
Lm818	1/2b-3b-7	ST5	CC5	Swab after C&D
Lm636	1/2b-3b-7	ST224	CC224	Frozen sliced mushrooms
Lm640	4b-4d, 4e	ST4	CC4	Frozen sliced mushrooms
Lm838	1/2a-3a	ST7	CC7	Frozen sliced mushrooms

5.2.2. Selection of background microbiota strains

A total of 18 microbiota strains were used in the biofilm and desiccation experiments. These microbiota strains were obtained during a sampling plan in the same factory where also the *L. monocytogenes* strains were isolated (Lake et al., 2023a). The 18 microbiota strains that were selected belonged to a genus or bacterial group that was minimal three times isolated from at least two different sampling spots. Multiple strains were selected of the four genera/bacterial groups that were highly abundant (i.e. *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter* and *Lactococcus*) (77% of total isolated microbiota) and included five *Enterobacteriaceae* strains (*Lelliottia*, *Raoultella*, *Citrobacter*, *Buttiauxella* and *Ewingella*), two *Pseudomonas* strains (*P. fluorescence*

and *P. fragi*), two *Acinetobacter* strains (*A. johnsonii*, and *Acinetobacter* spp. (no species determination possible)) and three *Lactococcus* strains (*L. lactis*, *L. raffinolactis* and *L. garvieae*). Also, one strain was selected of each of the less frequently isolated genera, namely, *Enterococcus*, *Sphingobacterium*, *Chryseobacterium*, *Aeromonas*, *Streptococcus* and *Brochothrix*. The selected microbiota strains could be genetically differentiated based on 16S rRNA sequencing (Lake et al., 2023a) and (sections 5.2.9 and 5.2.10), except for some strains, namely the *Raoultella* and *Citrobacter* strains (grouped as *Enterobacteriaceae*) and *A. johnsonii*, and *Acinetobacter* spp. (grouped as *Acinetobacter* spp.).

5.2.3. Preparation of *L. monocytogenes* and microbiota strains and mushroom medium

Stock cultures of *L. monocytogenes* were stored frozen at -80 °C in Brain Heart Infusion (BHI) medium (Becton Dickinson and Company, Difco) containing 25% glycerol (Sigma-Aldrich). Liquid cultures of the *L. monocytogenes* strains were prepared by inoculating 10 mL BHI medium with the stock culture followed by static incubation at 30 °C for 18 hours. Stock cultures of background microbiota were stored frozen at -80 °C in Tryptone Soya Broth (TSB) medium (Oxoid) supplemented with 0.6% Yeast Extract (YE) (Oxoid) in 25% glycerol (Sigma-Aldrich). Liquid cultures of the background microbiota strains were prepared by inoculating 10 mL TSBYE medium with the stock culture followed by static incubation at 30 °C for 24 hours. Cultures were individually centrifuged for 2 minutes at 16,000 x *g*, after which the supernatant was discarded and the pellet was dissolved in Phosphate Buffered Saline (PBS) buffer. PBS buffer was prepared according to NEN-EN-ISO 11290-1:2017 (International Organization for Standardization, 2017), containing 8.98 gram di-sodium hydrogen phosphate dihydrate, 2.71 gram sodium dihydrogen phosphate and 8.5 gram sodium chloride dissolved in 1 L demineralized water (pH 7.2). The washing step was repeated once and the obtained pellets were subsequently dissolved in PBS buffer and diluted with PBS buffer to obtain a working culture with a concentration of $\sim 10^7$ CFU/mL for each individual strain.

Mushroom medium was used as experimental growth medium (Lake et al., 2023b) to represent growth conditions in the mushroom processing environments. To prepare the medium, mushrooms (*Agaricus bisporus*) were harvested at a mushroom grower,

transported to the laboratory and stored refrigerated for a maximum of three days until processing. Upon processing, the mushrooms were cut into pieces and divided in quantities of 500 grams. Portions of 500 grams of mushrooms and 200 mL of non-sterilized demineralized water were added in a stomacher filter bag (Antonides) and homogenized for 1 minute at 230 rpm using a stomacher device (Stomacher 400 circulator, Seward). The mushroom medium was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific) and the obtained supernatant of different portions prepared on the same day were collected and combined in a big flask and mixed by shaking to obtain a mushroom medium batch. Multiple batches of mushroom medium were prepared on different days and each batch of mushroom medium was stored for a maximum of six months at -20 °C upon use. Upon use, the thawed mushroom medium was centrifuged for 5 minutes at 15,000 x *g* (Sorvall Legend XTR centrifuge, Thermo Scientific) and the supernatant was filter-sterilized using a 0.45 µm filter (Minisart® syringe filter, Sartorius) followed by a 0.22 µm filter (Minisart® syringe filter, Sartorius) after which the filter-sterilized mushroom medium was ready-to-use.

5.2.4. Cleaning and disinfection agents

The cleaning and disinfection agents applied in this study are typically used in the frozen mushroom production and processing environment. The cleaning agent EnduroPlus (Diversey) is a high chlorinated alkaline cleaning agent with a recommended concentration between 2-10%, depending on the type and degree of food soil attached to the surface. The minimum recommended concentration by the producer was applied in this study (2%) together with the recommended contact time of 10 minutes. The disinfection agent P3-topactive® DES (Ecolab) with the major compounds peracetic acid and hydrogen peroxide (H_2O_2) has a recommended concentration of 1-3%. The maximum recommended concentration (3%) was used together with the recommended contact time of 5 minutes. In addition, a ten times diluted concentration of the maximum recommended concentration (i.e. 0.3%) was applied to simulate exposure to diluted disinfectants. The cleaning and disinfection agents were individually mixed with sterile demineralized water to achieve the appropriate concentrations and mixtures were prepared just before application.

5.2.5. Biofilm experiments with C&D cycles

5.2.5.1. Strain inoculation and coupon preparations

Two different start inocula were prepared for the biofilm experiment, namely an inoculum of six equally mixed *L. monocytogenes* strains (i.e. monospecies cocktail) (table 5.1) and an inoculum of equally mixed strains containing six *L. monocytogenes* strains and eighteen microbiota strains (i.e. multispecies cocktail) (table 5.1 and section 5.2.2). Both inocula were subsequently added 1:100 (vol/vol%) in filter-sterilized mushroom medium reaching an initial concentration of ~10^5 CFU/mL. Aliquots of 3-mL inoculated mushroom medium were added in polystyrene 12-wells plates (Greiner Bio-One) and sterilized coupons of either polyvinyl chloride (PVC) or stainless steel (SS) were placed vertically in each well in a tilted position so the coupons were partly submerged in the mushroom medium. PVC and SS were chosen as surface materials since these are the two most frequently used food contact materials in the mushroom processing industry. PVC coupons were made of new conveyor belt material and had a surface area of 15 mm by 21 mm. SS coupons were made of new SS material type AISI 304 with a surface area of 15 mm to 21 mm. PVC coupons were sterilized by autoclaving at 121 °C for 15 minutes, and the SS coupons were sterilized by the method adapted from (Castelijn et al., 2013) with some changes. Shortly, the SS coupons were soaked for 30 minutes in 1 M NaOH (Sigma-Aldrich) in a 50 °C water bath followed by rinsing the coupons under running tap water. Coupons were subsequently placed in acetone and incubated for 15 minutes at room temperature followed by rinsing the coupons four times with demineralized water. Cleaned coupons were sterilized by autoclaving at 121 °C for 15 minutes. Plates with the coupons were incubated statically for 48 hours at 20 °C to mimic mushroom processing temperatures followed by the first cleaning and disinfection cycle.

5.2.5.2. Cleaning and disinfection procedure

After incubation, coupons were gently rinsed by pipetting three times 10 mL sterile demineralized water on both sides of the coupons to remove the non-attached cells from the surface. The coupons were transferred to a 12-wells plate prefilled with cleaning agent (4 mL/well, 2.0% concentration) and coupons were incubated for 10 minutes at room temperature. The coupons were rinsed again by gently pipetting three times 10 mL sterile demineralized water on both sides of the coupons. The coupons

were subsequently transferred to another 12-wells plate prefilled with disinfection agent (4 mL/well, 3.0% or 0.3% final concentration) and coupons were incubated for 5 minutes at room temperature. The coupons were rinsed again by gently pipetting three times 10 mL sterile demineralized water on both sides of the coupons. The C&D treated coupons were subsequently transferred to a 12-wells plate containing fresh filter-sterilized mushroom medium. The plates with the coupons were statically incubated for another 48 hours at 20 °C and the C&D procedures and following incubations of C&D treated coupons in fresh filter-sterilized mushroom medium was repeated every 48 hours up to four C&D cycles and eight days of incubation. The coupons treated with either 3% or 0.3% disinfection agent in the first cycle were treated with the same concentration of disinfection agent in the following C&D cycles. A subset of the coupons was removed during each cycle to determine the biofilm formation and strain diversity. A schematic drawing of the experimental approach is visualized in supplemental figure 5.1.

5.2.6. CFU counts of planktonic and biofilm cultures

Samples from planktonic and biofilm cultures were collected at every sampling day (i.e. day 2, 4, 6 and 8). Planktonic cell counts were determined by taking samples of the suspension surrounding the coupon, in which the planktonic culture was carefully pipetted up and down for homogenization before sampling. To determine biofilm cell counts before C&D, coupons were removed from the mushroom medium and were gently rinsed by pipetting three times 10 mL sterile demineralized water on both sides of the coupons to remove the non-attached cells followed by count determination. To determine biofilm cell counts after C&D, C&D-treated coupons (section 5.2.5) were used for count determination immediately after the last rinsing step. The biofilm cells were harvested by transferring the rinsed or C&D-treated coupons into a 50 mL tube (Greiner Bio-One) containing 4.5 mL sterilized PBS and 2 gram sterilized glass beads (425-600 microns) (Sigma-Aldrich). Tubes were vortexed for 30 seconds followed by sonication for 10 minutes (Branson 5510 ultrasonic cleaner) and tubes were subsequent vortexed for 30 seconds.

Parts of the planktonic and detached biofilm cell suspensions were used for determination of the CFU counts. Decimal dilutions obtained from the monospecies cocktail (both biofilm and planktonic cultures) were plated on Tryptone Soya Agar (TSA)

(Oxoid) supplemented with 0.6% Yeast Extract (YE) (Oxoid) for cell count determination. Decimal dilutions obtained from the multispecies cocktail (both biofilm and planktonic cultures) were plated on TSAYE plates to determine the total microbiota cell counts, on Agar Listeria according to Ottaviani-Agosti (ALOA) plates (Biomerieux) to determine the *L. monocytogenes* counts and on *Pseudomonas* Agar Base (Oxoid) plates supplemented with Cephalothin, Fucidin and Cetrimide (CFC) supplements (Oxoid) to determine the *Pseudomonas* spp. counts. *Pseudomonas* spp. counts were determined in both the planktonic culture and the biofilm on PVC coupons, since *Pseudomonas* spp. were previously reported as highly present on mushroom products and were able to reach high counts in mushroom medium (Lake et al., 2023a; Lake et al., 2023b) TSAYE plates were incubated for 24-48 hours at 30 °C, ALOA plates were incubated for 24 hours at 37 °C and *Pseudomonas* agar plates were incubated for 48-72 days at 25 °C. The viable cell count determination of the biofilm experiments (planktonic cells, biofilm cells before and after C&D treatment) was performed with two biological replicates having minimal two technical replicates.

5.2.7. Collecting samples for strain identification

After cell enumeration of the planktonic and detached biofilm cell suspensions (section 5.2.6), the remaining part of the cell suspensions was pelleted by centrifugation (5 minutes at 16,000 x *g*) using 2-mL tubes (Greiner Bio-One). Two identical samples were collected each time from all three pelleted cell suspensions (planktonic cells, detached biofilm cells before and after C&D) at each specific time point for individual strain identification. One sample was stored immediately at -20 °C upon further use for strain identification, while the other sample received cell viability treatment for exploring strain diversity of intact cells only.

The cell viability treatment was performed using the photo-reactive dye PMAxx™ (20 mM in H_2O) (Biotium) as a DNA-binding dye. PMAxx™ is cell membrane-impermeant, so only cells with damaged membranes are susceptible for covalent attachment of the dye to the DNA to inhibit PCR amplification of compromised cells. Pellets were dissolved in 500 µL sterile PBS buffer followed by the addition of PMAxx™ to the mixture to obtain a final concentration of 20 µM PMAxx™. Samples were incubated in the dark for 10 minutes with mixing at regular intervals. Samples were subsequently incubated for 15 minutes in the PMA-Lite™ LED Photolysis device (Biotium) with mixing at regular

intervals. The incubated samples were subsequently centrifuged for 2 minutes at 16,000 x *g* after which supernatant was discarded. One mL PBS was added to the sample and sample was vortexed and centrifuged at 16,000 x *g*, after which the supernatant was discarded. The PMAxx™ treated cell pellets were stored at -20 °C upon further use.

Strain diversity was also determined from colonies harvested from plates to determine the strain ratio based on colony formation ability. For this, colonies from the monospecies PVC biofilm experiment (both planktonic and biofilm cultures) that were grown on TSAYE plates (usually between 30 – 300 colonies, ideally >100) were dissolved in PBS buffer and the colony pellet was collected in a 2-mL tube (Greiner Bio-One) by centrifugation (2 minutes at 16,000 x *g*) and the pellets were stored at -20 °C upon further use.

5.2.8. Survival and disinfection treatment of desiccated cells

Two cocktails (i.e. monospecies cocktail and multispecies cocktail) were prepared from the individual working cultures, the individual working cultures of having an initial concentration of approximately 10^9 CFU/mL for each of the cocktails. Cocktails were added 1:10 (vol/vol%) in filter-sterilized mushroom medium for obtaining a starting concentration of approximately 10^8 CFU/mL of which 100 µL aliquots were spotted on SS and PVC coupons to determine the survival during desiccation. PVC coupons were inoculated on both sides, since these coupons had two different surface finishes, namely, a smooth and a rough finished polyvinyl chloride top layer. The PVC coupon counts of both sides were averaged to come to an overall coupon count. The spotted cell suspensions were dried at room temperature following either a fast drying step using a high air flow (~2 hours needed for drying) or a slow drying step using a low air flow (~6 hours needed for drying). Dried coupons were incubated statically at 20 °C to mimic the temperature of the mushroom processing environment. The cell counts were determined at the start and on regular days up to 2 weeks of incubation to monitor the cell survival over time. Parallel incubated coupons were C&D treated as described before (section 5.2.5.2) to determine the effect of C&D on desiccated cells. C&D-treated and untreated SS and PVC coupons were transferred into sterile 50 mL tubes containing 4.5 mL sterile PBS buffer and 2 gram glass beads. The same procedure was followed as before to detach cells from the coupons and to determine

the viable cell counts by plating (section 5.2.6). After incubation of the agar plates, colonies were also harvested from plates (section 5.2.7) and cell pellets were stored at -20 °C upon further use. The viable cell determination of the desiccation experiment was performed with two or three biological replicates.

5.2.9. Primer design and primer validation for amplicon sequencing

Two primer sets were designed for strain or species differentiation using amplicon sequencing, namely, a primer set to identify the individual *L. monocytogenes* strains in the monospecies cocktail (table 5.1) and a primer set to identify the individual microbiota strains in the complex multispecies cocktail (including *L. monocytogenes*). None of the primers introduced mismatches or other ways of incorrect binding to their DNA target strand.

The *L. monocytogenes* primer set targeted the LMOf2365_0321 / lmo0300 gene of *L. monocytogenes*, which is located within the LMO region that was described as the most informative sequenced region (Ducey et al., 2007) (Ward et al., 2008). This primer set included the forward primer with the 10-bp tag (5'-(tag)TGAACAATACCAATTTGCCC-3') and the reverse primer (5'-CAAACCTTGGCATAATGCTC-3'). Obtained PCR products had a total amplicon length of 243 bp including the 10-bp tag attached to the forward primer. See supplemental table 5.1 for the LMOf2365_0321 / lmo0300 gene sequences of the six *L. monocytogenes* amplicon fragments (233 bp each).

The microbiota primer set targeted the V4 region of the 16S rRNA gene. This primer set included the forward primer with the 10-bp tag (5'-(tag)CAGCAGCCGCGGTAATAC-3') and the reverse primer (5'-ACAGGATTAGATACCCTGGTAGTC-3'). Obtained PCR products had a total amplicon length of approximately 297 bp including the 10-bp tag attached to the forward primer. See supplemental table 5.2 for the V4 gene sequences of the 18 microbiota and the *L. monocytogenes* species amplicon fragments (approximately 287 bp each).

The *L. monocytogenes* primer set and the primer set for species identification using amplicon sequencing were validated for good and equal amplification of each of the strains by qPCR. For this, individual cultures of all strains (6 *L. monocytogenes* and 18 microbiota strains) were prepared as described before (section 5.2.3) and two milliliter

of the individual overnight cultures were subjected to DNA isolation using the DNeasy blood and tissue kit (Qiagen). In short, the cells of the washed pellet were lysed using the enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% (w/v) Triton-X-100, 5 mg/mL lysozyme, pH 8.0) and the lysed cells were processed using the DNeasy blood and tissue kit (Qiagen) following the procedure described before (Lake et al., 2021). DNA concentrations were determined using the nanodrop device (NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer) (Thermo Scientific) and concentrations were equalized and decimally diluted for each strain.

The two qPCR reactions were executed with 12.5 µL of the 2x SYBR® green master mix reaction mixture (Bio-Rad), 0.5 µL of forward primer and 0.5 µL of reverse primer (final concentration each primer of 0.2 µM) and 2.5 µL purified DNA filled with sterile water to a final volume of 25 µL. The PCR mixtures were run on a qPCR device (CFX96 touch Real-Time PCR detection system (Bio-Rad)) including an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. Decimally diluted DNA suspensions of the individual strains tested with both primer sets had overlapping patterns with comparable slopes when the quantification cycles (Cq) were plotted (data not shown). This strongly indicates that there are no (major) differences in amplicon efficiencies between the applied qPCRs.

5.2.10. Preparation of samples for amplicon sequencing

Frozen stored pellets from the monospecies cocktails and the multispecies cocktails from either the biofilm experiment and the desiccation experiment were applied for amplicon sequencing. For this, frozen stored pellets of both PMAxx™ treated and non-PMAxx™ treated cell pellets that were derived from the planktonic cells, the biofilm cells before C&D and after C&D and the dissolved cells from agar plates (biofilm and desiccation experiments) were used. Samples were thawed and DNA was isolated and purified using the DNeasy blood and tissue kit (Qiagen) as mentioned before (section 5.2.9). The concentration and purity of the isolated DNA was determined using the nanodrop device. The isolated DNA was used for amplicon PCR with primers targeting the LMOf2365_0321 / lmo0300 gene region for *L. monocytogenes* discrimination or targeting the V4 region of the 16S rRNA for species discrimination (section 5.2.9).

The amplicon PCR mixture for *L. monocytogenes* strain discrimination contained 12.5 µL 2x KAPA HiFi HotStart ReadyMix (Roche), 0.4 µM forward primer (containing the tag), 0.4 µM reverse primer, 0.5-10.5 µL DNA (depending on the concentration) and filled with sterile water to a total volume of 25 µL (if applicable). The optimized PCR cycle was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and included an initial denaturation step at 95 °C for 4 minutes, followed by 35 cycles of denaturation at 98 °C for 20 seconds, annealing at 65 °C for 15 seconds and extension at 72 °C for 15 seconds followed by final extension step at 72 °C for 2 minutes. The amplicon PCR mixture targeting the 16S rRNA for species discrimination contained the same components and was applied with the same PCR cycle, except different primers were used and the annealing temperature was set to 68 °C.

A 5-µL aliquot of the PCR product was mixed with 1 µL 6x DNA loading dye (TriTrack, Thermo Scientific) and samples were added in a 1% agarose gel (SeaKem LE agarose, Lonza) containing 1x TAE buffer (Bio-Rad) and DNA safe stain (SYBR Safe DNA Gel Stain, Invitrogen). Gels were run in 1x TAE buffer and DNA bands were visualized using ultra violet light (Uvitec, Cambridge). Samples confirmed as pure and correct PCR products (243 bp products when using the *L. monocytogenes* primer set and 297 bp products when using the 16S rRNA primer set) were purified using the MinElute PCR purification kit (Qiagen) according to the manufacturer's instructions, and PCR product concentrations were determined using the nanodrop device. The PCR products were pooled and these pooled samples were sent for sequencing using adapter ligation HiSeq/NovaSeq protocol (Illumina) using 2 x 150 bp pairs.

5.2.11. Bacterial community analysis

The raw sequences were first processed by fastp (v0.23.2) (Chen et al., 2018) to filter low quality sequences and sequences shorter than 145 bp after trimming were discarded. Sequences were then demultiplexed and barcode trimmed using cutadapt (v4.1) (Martin, 2011). Due to mixed-orientation single-end barcode library construction before sequencing, demultiplexing was performed in three steps: 1) all sequences were first demultiplexed and barcode-trimmed in one orientation; 2) all sequences not demultiplexed in first step went through a second round of demultiplexing and trimming in another orientation; 3) up to this step, all sequence that could be demultiplexed would have been sorted into same orientation and tail

primer of these sequences were finally trimmed. Paired-end sequences were merged using FLASH (v1.2.11) (Magoč and Salzberg, 2011) and a "--min-overlap" was set to 4 for the multispecies samples.
Primer-free pair-end joined sequences were imported into QIIME2 q2cli v2022.08 (Bolyen et al., 2019). Sequences were then processed with dada2 "denoise-pyro" (Callahan et al., 2016) to remove PCR chimeras in order to obtain representative ASV sequences and feature table. These representative sequences were subsequently assigned taxonomy using customized "nearest-seed" method; representative sequences were assigned the taxon of its most similar reference sequence using blastn (2.13.0+) (Altschul et al., 1997), if the similarity between a representative sequence and its most similar reference is lower than 99%, it will be annotated as unknown. Combining with the information of feature table, the quantification of each taxon was obtained, which was further used to determine the strain or species ratio in a community.

The strain distribution and abundance based on amplicon sequencing contained two biological replicates (for both the biofilm experiment and the desiccation experiment). Ratios were constructed based on translating the number of reads to percentages. Percentages of biological experiments were averaged to determine the relative abundance of strains in a community.

5.2.12. Statistical analysis

Data analysis was performed using Microsoft Excel. Students *t*-tests were used to determine potential differences between groups and significance was set at $p<0.05$.

5.3. Results

5.3.1. Biofilm formation of *L. monocytogenes* on PVC and SS coupons

Incubation of the *L. monocytogenes* monospecies cocktail for two days in the mushroom medium resulted in an increase of planktonic cell counts from 5.0 to 8.9-9.0 log CFU/mL in all tested conditions (figure 5.1). Biofilm cell counts on SS coupons were significantly lower ($p<0.05$) compared to the PVC coupon (6.0 log CFU/cm^2

versus 6.8 log CFU/cm^2, respectively) (figure 5.1A and 5.1B versus 5.1C and 5.1D). C&D treatment of the biofilms on PVC with either 3% or 0.3% of the disinfection agent resulted in respective reductions of 2.4 and 1.5 log CFU/cm^2, with remaining *L. monocytogenes* counts of 4.4 and 5.3 log CFU/cm^2, respectively (figure 5.1A and 5.1B), while biofilm counts on SS were below the detection limit for both disinfectant concentrations (detection limit of 1.5 log CFU/cm^2) (figure 5.1C and 5.1D).

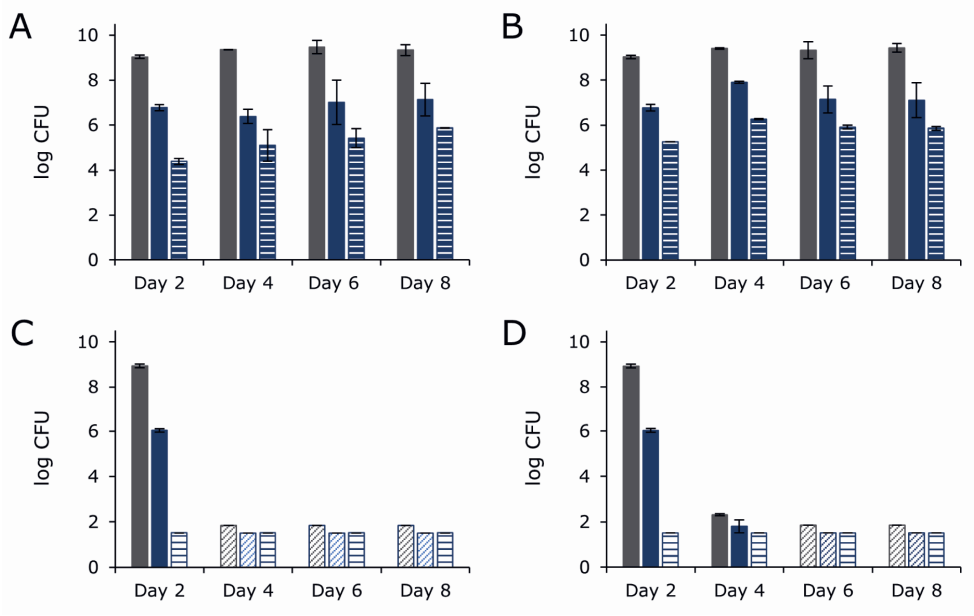

Figure 5.1. Growth performance and biofilm formation of the *L. monocytogenes* monospecies cocktail (n=6). Biofilm formation was performed on PVC coupons (A and B) and on SS coupons (C and D) using periodical C&D treatments with either 3% disinfectant (A and C) or 0.3% disinfectant (B and D). Counts were determined just before (planktonic growth and biofilm counts) and immediately after (only biofilm counts) the C&D procedure. The dark grey bars correspond to the planktonic counts and the dark blue bars correspond to the biofilm counts in which the dark blue filled bars represent the biofilm counts just before C&D treatment and the dark blue filled bars with white horizontal stripes represent the biofilm counts immediately after C&D treatment. Counts below the detection limit are indicated with a white fill and diagonal dark grey stripes (planktonic counts), white fill and diagonal dark blue stripes (biofilm counts before C&D) and white fill with dark blue horizontal stripes (biofilm counts after C&D). The planktonic counts are displayed as log CFU/mL and the biofilm counts are displayed as log CFU/cm^2. The error bars represent the standard deviation of the two biological replicates, each consisting of two technical replicates.

Transferring the C&D treated coupons to fresh mushroom medium followed by another two days of incubation may allow the surviving cells to grow in the biofilm and/or

detach and grow in the medium. Indeed, this led to comparable or even higher planktonic and biofilm counts for the PVC coupons as after the first 2 days of incubation (figure 5.1A and 5.1B) and similar trends were observed upon the following-up C&D cycles (total of 4 cycles of (re-)incubation and C&D treatment, 8 days in total) (a scheme for C&D cycles and re-incubation is presented in supplemental figure 5.1). In contrast, re-incubation of the SS coupons after 3% disinfectant treatments did not result in detectable counts of *L. monocytogenes* in both the planktonic culture and the biofilm for all following-up C&D treatments (figure 5.1C), while for the 0.3% disinfection treatments, planktonic and biofilm counts were only detected before the second treatment on day 4 (figure 5.1D).

5.3.2. Biofilm formation of *L. monocytogenes* and microbiota on PVC and SS coupons

The performance of *L. monocytogenes* was also investigated in a multispecies culture (figure 5.2), which resulted in lower planktonic and biofilm cell counts for *L. monocytogenes* compared to monospecies incubation (figure 5.1). Two days of incubation of the multispecies biofilm resulted in an increase of planktonic *L. monocytogenes* cell counts from 4.4 to 7.2 log CFU/mL in all tested conditions (figure 5.2). The *L. monocytogenes* biofilm cell counts were lower (although not significant ($p=0.15$)) on the SS coupons compared to the PVC coupons (5.0 log CFU/cm^2 versus 5.4 log CFU/cm^2, respectively) (figure 5.2). The total planktonic cell counts increased from 5.0 log CFU/mL to 9.6-9.7 log CFU/mL after two days of incubation, in which the total biofilm cell counts were lower (although not significant ($p=0.16$)) on the SS coupons compared to the PVC coupons (7.6 log CFU/cm^2 versus 7.9 log CFU/cm^2, respectively) (figure 5.2). *Pseudomonas* spp. was a significant contributor to both the planktonic and the biofilm microbiota communities after two days of incubation, because the *Pseudomonas* spp. counts were 9.1 log CFU/mL and 7.1 log CFU/cm^2 for the PVC coupons and 8.9 log CFU/mL and 7.1 log CFU/cm^2 for the SS coupons (supplemental figure 5.2).

C&D treatment of the biofilm on PVC coupons after 2 days of incubation with either the 3% or 0.3% of the disinfection agent resulted in comparable decreases in biofilm counts, with respective reductions of 1.9 and 2.4 log CFU/cm^2 for *L. monocytogenes* and 2.3 and 2.4 log CFU/cm^2 for the total microbiota (figure 5.2A and 5.2B). This

resulted in remaining *L. monocytogenes* counts of 3.5 and 3.0 log CFU/cm^2 and microbiota counts of 5.6 and 5.5 log CFU/cm^2, respectively (figure 5.2A and 5.2B). C&D treatment with either the 3% or 0.3% disinfection agent to biofilms formed on SS coupons after 2 days of incubation resulted in *L. monocytogenes* biofilm counts below the detection limit for both disinfectant concentrations (detection limit of 1.5 log CFU/cm^2) (figure 5.2C and 5.2D), while microbiota counts had respective reductions of 5.4 and 3.5 log CFU/cm^2 resulting in remaining microbiota counts of 2.3 and 4.1 log CFU/cm^2, respectively (figure 5.2C and 5.2D). All conditions showed *Pseudomonas* spp. as an important contributor of the biofilm after C&D treatments (supplemental figure 5.2).

Re-incubation of the C&D treated PVC coupons in fresh mushroom medium for two days resulted in regrowth and comparable planktonic and biofilm cell counts for both *L. monocytogenes* and microbiota as after 2 days of incubation (figure 5.2A and 5.2B), and similar trends were observed upon the following-up C&D cycles. Notably, these regrown biofilm cells were more resistant towards C&D treatments with both disinfectant concentrations, because a smaller reduction of the biofilm counts (both *L. monocytogenes* and microbiota) in all following-up C&D cycles was observed compared to day 2. Especially the *L. monocytogenes* biofilm counts at day 8 only declined 0.2 and 0.3 log CFU/cm^2 after applying the 3% and 0.3% disinfection regime, respectively (figure 5.2A and 5.2B). This increased resistance of biofilm cells when the number of C&D cycles increased was also observed, though to a lesser extent, in monospecies biofilm of *L. monocytogenes*, because in monospecies biofilms the lowest reductions in biofilm counts were 1.3 and 1.2 log CFU/cm^2 after applying the 3% and 0.3% disinfection regime at day 8, respectively (figure 5.1A and 5.1B). Re-incubation of the C&D treated SS coupons for multiple cycles resulted in lower (3% disinfectant treatment) or rather comparable (0.3% disinfectant treatment) planktonic and biofilm microbiota cell counts as determined after 2 days of incubation (figure 5.2C and 5.2D). In contrast, the planktonic and biofilm counts of *L. monocytogenes* remained below the detection limit after the first 3% disinfectant treatment, except for a slight increase in planktonic cells at day 6, while for the 0.3% disinfectant treatment, a slight increase in planktonic cells and biofilm counts was observed at day 4 and 6 (figure 5.2C and 5.2D). Again, the *Pseudomonas* spp. was a significant contributor to both the planktonic and the biofilm (before and after C&D treatment) communities during all count determination steps in both C&D treatments (0.3% and 3% disinfectant) and for both types of surfaces (supplemental figure 5.2).

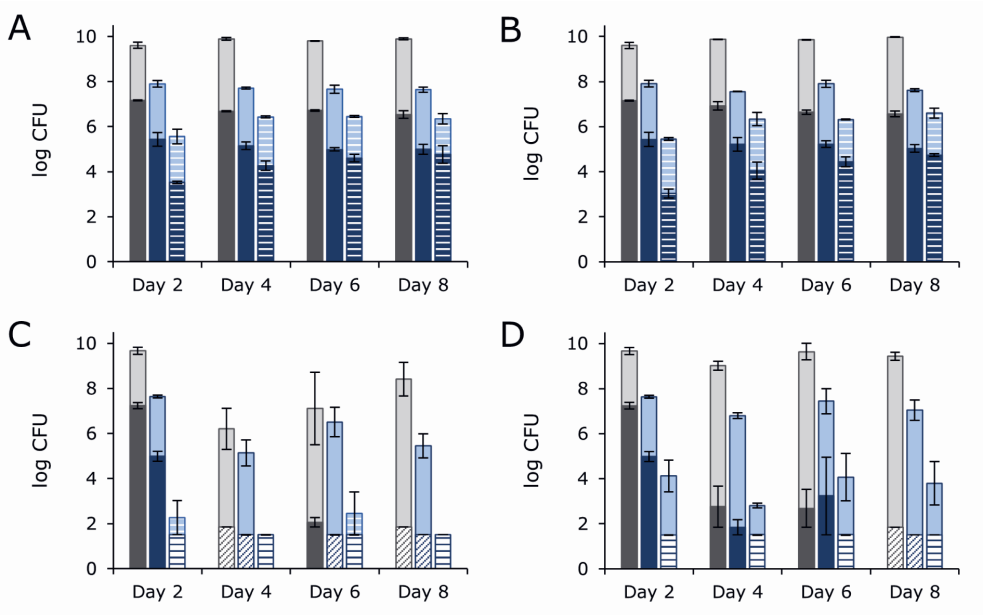

Figure 5.2. Growth performance and biofilm formation of the multispecies cocktail (n=24) (consisting of *L. monocytogenes* strains (n=6) and background microbiota strains (n=18)). Biofilm formation was performed on PVC coupons (A and B) or SS coupons (C and D) using periodical C&D treatments with either 3% disinfectant (A and C) or 0.3% disinfectant (B and D). Counts were determined just before (planktonic growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedure. The grey bars correspond to the planktonic counts and the blue bars correspond to the biofilm counts in which the blue filled bars represent the biofilm counts before C&D treatment and the blue filled bars with white horizontal stripes represent the biofilm counts immediately after C&D treatment. The dark colored bars represent the *L. monocytogenes* counts, while the light colored bars represent the microbiota counts. Counts below the detection limit are indicated with a white fill and diagonal grey stripes (planktonic counts), white fill and diagonal blue stripes (biofilm counts before C&D) and white fill with blue horizontal stripes (biofilm counts after C&D). The planktonic counts are displayed as log CFU/mL and the biofilm counts are displayed as log CFU/cm^2. The error bars represent the standard deviation of the two biological replicates, each consisting of two technical replicates.

5.3.3. Strain diversity in monospecies and multispecies biofilms

Planktonic and biofilm samples were PMAxx™ treated and compared to untreated samples to determine whether the diversity was different based on DNA isolation from only intact cells or all DNA present. Diversity analyses demonstrated comparable ratios of the strains in PMAxx™ and non-PMAxx™ treated samples, indicating that strain ratios among intact cells and lysed cells were rather similar (see supplemental figure 5.3 and

5.4 for monospecies biofilm experiment on PVC, supplemental figure 5.5 for monospecies biofilm experiment on SS, supplemental figure 5.6 and 5.7 for multispecies biofilm experiment on PVC and supplemental figure 5.8 for multispecies biofilm experiment on SS). Strain ratios of the PMAxx™ treated samples disinfected with the highest recommended disinfection concentration (3%) at the last disinfection cycle are shown in figure 5.3 to illustrate the strain diversity among intact cells that survived multiple cycles of C&D treatments, and are thus a possible threat persisting in food environments and contaminate food. Information of intermediate timepoints could be found in supplemental figure 5.3 (monospecies cocktail) and supplemental figure 5.6 (multispecies cocktail).

Strain identification of *L. monocytogenes* planktonic and biofilm cells grown on PVC coupons showed the presence of all six *L. monocytogenes* strains during the eight day monospecies incubation, although the relative abundance of the *L. monocytogenes* strain Lm640 in the monospecies biofilm tended to decrease following C&D cycles. This led to a final percentage between 0.03% and 0.09% of strain Lm640 at day 8 after C&D treatments (either 3% or 0.3% C&D treatment) (figure 5.3A and supplemental figure 5.3 and 5.4), but such decrease was not observed in the presence of the microbiota cocktail (figure 5.3B and supplemental figure 5.6 and 5.7) that resulted in a high *L. monocytogenes* strain diversity following the C&D cycles. Next to *L. monocytogenes*, sixteen different sequences were detected in the multispecies biofilms because *Raoultella* and *Citrobacter* were indistinguishable based on sequence (grouped as *Enterobacteriaceae*) and *Acinetobacter* spp. and *Acinetobacter johnsonii* were also indistinguishable based on sequence (grouped as *Acinetobacter* spp.). The *Enterobacteriaceae* strains (*Raoultella* and *Citrobacter*), *P. fluorescens*, *Brochothrix* and *Acinetobacter* spp. (*Acinetobacter* spp. and *Acinetobacter johnsonii*) were most abundant at day 4 (supplemental figure 5.6 and 5.7), and were high abundant together with the *Lactococcus lactis* strain at day 8 (figure 5.3C and supplemental figure 5.6 and 7). In correspondence with the plate count data (figure 5.2), *L. monocytogenes* represented a minor fraction in the multispecies biofilm.

The strain ratio of *L. monocytogenes* in monospecies biofilms on PVC was also determined based on colonies grown on agar medium (supplemental figure 5.9). This strain ratio was comparable to the ratio determined from the pellets of the planktonic and biofilm culture (supplemental figure 5.3 and 5.4), indicating that intact cells were reproductive and also able to form colonies.

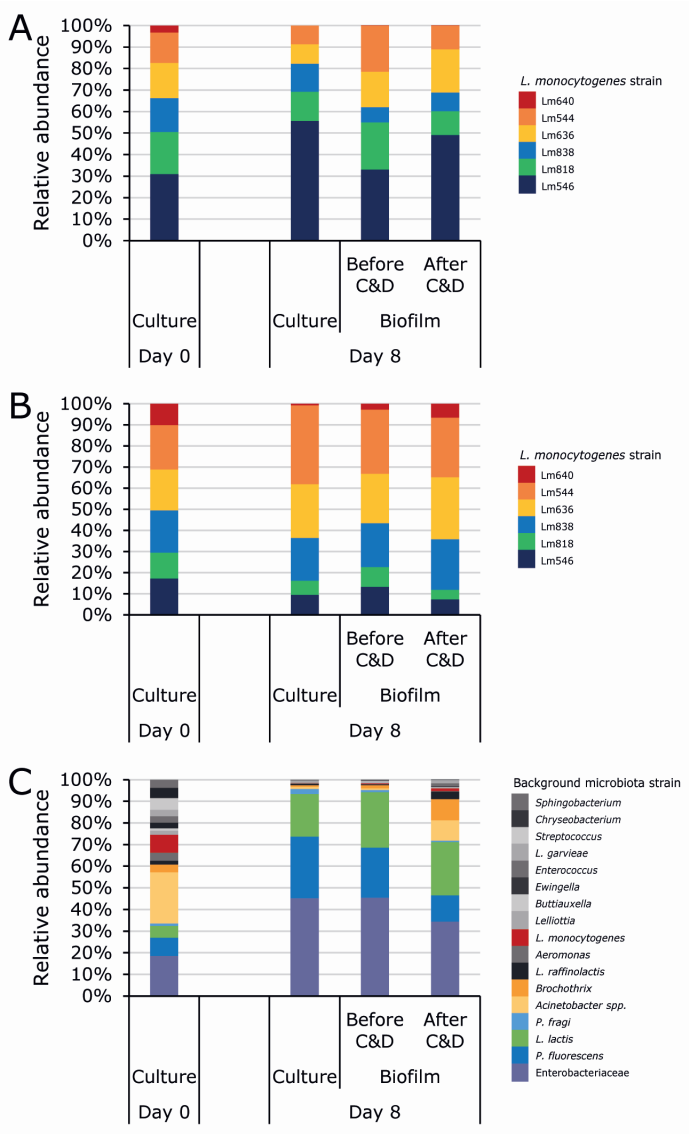

Figure 5.3. Relative abundance of the bacterial strains during the 8-day incubation on PVC coupons before and after the 3% C&D treatment. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) in the monospecies cocktail (A) (linked to figure 5.1), for the individual *L. monocytogenes* strains (n=6) in the multispecies cocktail (B) (linked to figure 5.2) and for the individual background microbiota strains in the multispecies cocktail (C) (linked to figure 5.2). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of PMAxx™ treated samples. Results of replicates (n=2) are pooled and averages are shown.

Similar to results obtained with PVC coupons, also all six *L. monocytogenes* strains were present in the biofilm on SS before the application of the first C&D treatment (day 2), both without and with microbiota addition. During the following C&D cycles on SS coupons (without and with the microbiota and others than day 2), *L. monocytogenes* was absent or showed varying strain ratios (supplemental figure 5.5 and 5.8). Also the species abundance in the microbiota biofilm varied throughout the C&D cycles. The *Enterobacteriaceae* strains (*Raoultella* and *Citrobacter*), *P. fluorescens*, *P. fragi* and *Acinetobacter* spp. were among the most abundant strains after 2 days of incubation, with *Lelliottia* and *Aeromonas* present in lesser extents (data not shown). This shifted to higher abundance of *P. fragi*, *Ewingella* and *Acinetobacter* spp. at day 4 (supplemental figure 5.8) and these strains were also the more abundant strains at day 8 (supplemental figure 5.8).

5.3.4. Desiccation survival of *L. monocytogenes* monospecies cocktail on PVC and SS and the effect of cleaning and disinfection treatments

The effect of drying speed did not significantly affect ($p > 0.05$) the survival of *L. monocytogenes*, because the survival of cells in fast-dried and slow-dried droplets, on either the PVC or SS coupons, was rather comparable (figure 5.4A and 5.4B). In accordance with survival in wet biofilms, *L. monocytogenes* counts were higher on PVC compared to SS after two weeks of desiccation. After two weeks, the viable counts of the fast-dried and slow-dried droplets on the PVC coupons declined with 1.9-1.8 log CFU/coupon to 5.0 and 5.4 log CFU/coupon, while the viable counts on the SS coupons declined with 4.4-4.1 log CFU/coupon to 2.9 and 3.4 log CFU/coupon, respectively (figure 5.4A and 5.4B). Irrespective of the drying method, both the PVC and the SS coupons showed a relative high decline in viable cell counts during the first days of desiccation and minor reduction in the second week of the two-week desiccation period (figure 5.4A and 5.4B). Desiccated cell suspensions on PVC and SS coupons were also subjected to either the 3% and the 0.3% disinfectant treatments, and *L. monocytogenes* cells were reduced to counts below the detection limit of 1.7 log CFU/coupon, regardless of coupon type, disinfectant concentration and sample day (figure 5.4A and 5.4B, horizontal dashed line). Since minor log reductions were observed during the second week of desiccation, the relative abundance of the monospecies cocktail on both PVC and SS coupons were determined after the first week of desiccation (figure 5.4C and 5.4D). Shifts in strain ratio were observed

following desiccation, but all six *L. monocytogenes* strains were still present after 7 or 8 days of incubation (figure 5.4C and 5.4D), and this was also observed after the second week of incubation (supplemental figure 5.10).

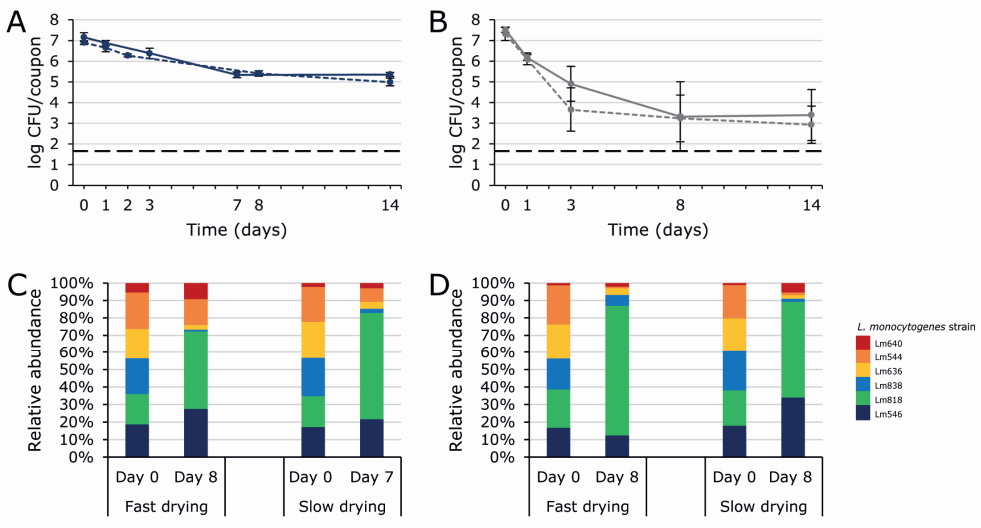

Figure 5.4. Desiccation survival of the *L. monocytogenes* monospecies cocktail on PVC (A) and SS (B) surfaces and the relative abundance of the individual *L. monocytogenes* strains (n=6) during desiccation on PVC (C) and SS (D) coupons. Desiccation survival of *L. monocytogenes* on PVC and SS (panel A and B) was performed following fast drying - 2 hours needed for drying (dashed line), or slow drying - 6 hours needed for drying (full line) for a maximum of two weeks at 20 °C. The dashed horizontal line indicates the detection limit (1.7 log CFU/coupon). The error bars represent the standard deviation of three biological replicates. The relative strain abundance (panel C and D) was determined for the desiccated cells without C&D treatment. Results of replicates (n=3) are pooled and averages are shown.

5.3.5. Desiccation survival of *L. monocytogenes* and microbiota on PVC and SS and the effect of cleaning and disinfection treatments

The microbiota strains were more resistant to desiccation survival than *L. monocytogenes*, especially on SS (figure 5.5A and 5.5B), and fast-dried *L. monocytogenes* cells declined rather fast on SS coupons (figure 5.5B). After two weeks, the viable microbiota counts of the fast-dried and slow-dried droplets on the PVC coupons declined with 1.2-1.4 log CFU/coupon to 5.8 and 6.0 log CFU/coupon, while the viable counts on the SS coupons declined with 1.5-1.7 log CFU/coupon to 5.4 and 5.5 log CFU/coupon, respectively (figure 5.5A and 5.5B). Simultaneously, the viable *L.*

monocytogenes counts of the fast-dried and slow-dried droplets on the PVC coupons declined with 1.9-1.2 log CFU/coupon to 4.4 and 5.2 log CFU/coupon, while the viable counts on the SS coupons declined with 3.8-3.2 log CFU/coupon to 2.4 and 3.5 log CFU/coupon, respectively (figure 5.5A and 5.5B). The presence of microbiota strains

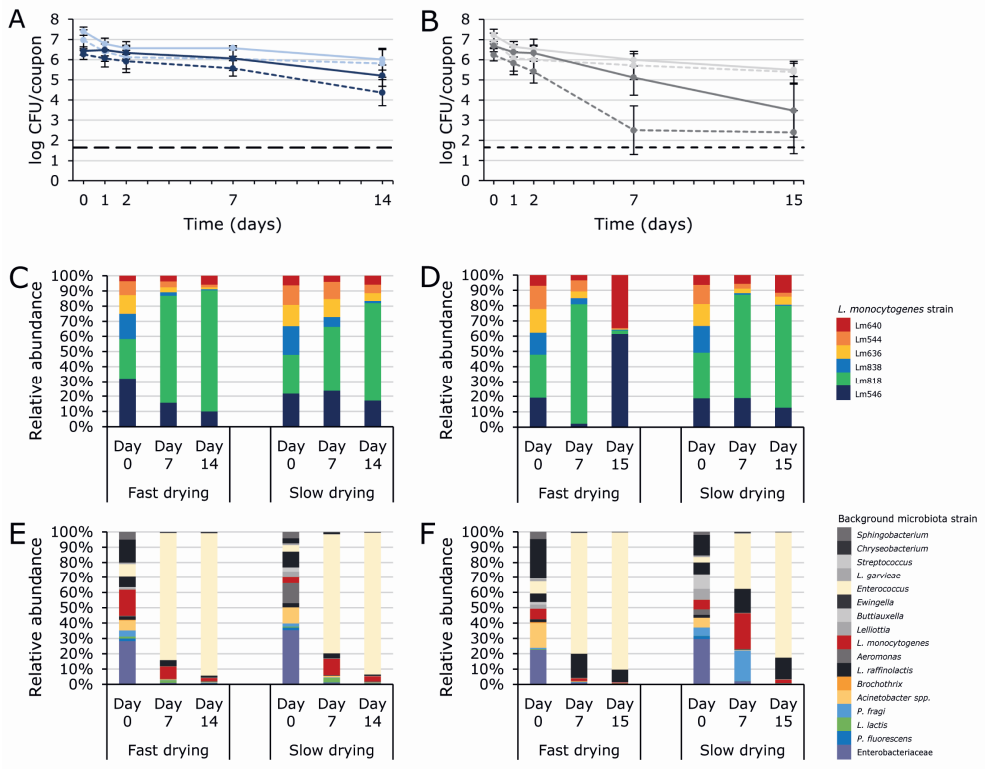

Figure 5.5. Desiccation survival of the multispecies cocktail (consisting of *L. monocytogenes* strains (n=6) and background microbiota strains (n=18)) on PVC (A) and SS (B) surfaces and the relative abundance of the individual *L. monocytogenes* strains and microbiota strains (including *L. monocytogenes*) during desiccation on PVC (panels C and E) and SS (panels D and F), respectively. Desiccation survival of the multispecies cocktail on PVC and SS (A and B) was performed following fast drying - 2 hours needed for drying (dashed line), or slow drying - 6 hours needed for drying (full line) for a maximum of two weeks at 20 °C. The dark colored lines correspond to the *L. monocytogenes* counts and the light colored lines correspond to the total viable counts. The dashed horizontal line indicates the detection limit (1.7 log CFU/coupon). The error bars represent the standard deviation of three biological replicates. The relative strain abundance was determined for the desiccated cells without the C&D treatment on PVC coupons (panels C and E) and SS coupons (panels D and F) for both the *L. monocytogenes* mixture (panels C and D) and the microbiota mixture including *L. monocytogenes* (panels E and F). Results of replicates (n=3) are pooled and averages are shown.

affected the reduction of *L. monocytogenes* over time, because *L. monocytogenes* was slower inactivated during the first days in the presence of microbiota compared to desiccation survival as monospecies (Figure 5.4A and 5.4B versus 5.5A and 5.5B). This indicated that *L. monocytogenes* was more protected in the multispecies community, especially during the incubation on the SS coupons. Cells on PVC and SS coupons were also subjected to either the 3% and the 0.3% disinfectant treatments, and the microbiota and *L. monocytogenes* were inactivated to counts below the detection limit of 1.7 log CFU/coupon, irrespective of coupon type, disinfectant concentration and sample day (figure 5.5A and 5.5B, horizontal dashed line). Since changes in log-reductions during desiccation were different for the microbiota and *L. monocytogenes* on both surfaces, relative abundances were determined after the first and the second week of desiccation. Although shifts in strain distribution were observed during incubation, all six *L. monocytogenes* strains were still present in the multispecies community after two week of desiccation (figure 5.5C and 5.5D), rather comparable as to the *L. monocytogenes* monospecies incubation (figure 5.4C and 5.4D). This highlighted that *L. monocytogenes* maintained a high strain diversity without and with the presence of microbiota on dried surfaces. On the other hand, the species diversity of the microbiota was reduced over time. This resulted in the dominance of the *Enterococcus* strain on both coupons types and for both the fast-dried and slow-dried droplets after 7 days and after 14 days of desiccation (figure 5.5E and 5.5F).

5.4. Discussion

This study demonstrated that *L. monocytogenes* is able to form biofilms as monospecies and also with microbiota on PVC and SS that were soiled with mushroom medium. We used a selection of *L. monocytogenes* strains and representative Gram-negative and Gram-positive microbiota, all isolated from mushroom processing environments. In addition, all experiments were performed at room temperature in mushroom medium to mimic conditions encountered in the mushroom processing environment. Higher biofilm counts of *L. monocytogenes* were observed on the plastic PVC coupons compared to the SS coupons, and this is in correspondence with other studies showing higher *L. monocytogenes* biofilm formation on plastic (polystyrene/polypropylene) compared to stainless steel (Poimenidou et al., 2016; Skowron et al., 2018). Interestingly, reported cell counts differences between these two material types were rather comparable among the different studies although

different incubation conditions were applied compared to the current study. Also in the multispecies biofilm, higher *L. monocytogenes* counts were observed on PVC compared to SS coupons. This corresponds with other studies in which *L. monocytogenes* was able to establish itself in multispecies biofilms (Fagerlund et al., 2017; Heir et al., 2018; Langsrud et al., 2016). The comparable performance in our study using mushroom medium and that in nutrient-rich broth media used in the other studies can be explained by the excess of nutrients in the mushroom medium, supporting efficient growth and biofilm formation of a wide range of *L. monocytogenes* isolates and model strains (Lake et al., 2023a; Lake et al., 2023b).

The C&D treatments applied after every two days of incubation in this study were performed with a disinfectant containing peracetic acid and hydrogen peroxide as active substances. The results of our study indicate no large difference in disinfection efficiency when the maximum recommended disinfection concentration was ten times diluted. On the other hand, other studies previously reported a larger reduction of the *L. monocytogenes* biofilm cell counts with higher disinfectant concentrations on conveyor belt surfaces (polyester urethane) and on SS surfaces (Barroso et al., 2019; Chaturongkasumrit et al., 2011), although the used disinfectants or applied disinfectant concentrations differed as compared to our study. The current study showed that C&D treatments of monospecies and multispecies biofilms resulted in higher *L. monocytogenes* biofilm count reductions for SS coupons than PVC coupons. This is in line with another study that also observed higher reductions for peracetic acid treated *L. monocytogenes* biofilm counts on stainless steel compared to polystyrene (Poimenidou et al., 2016). Indeed, higher efficiencies of sanitizing agents towards *L. monocytogenes* biofilms on nonporous surfaces (stainless steel, glass) compared to the porous surfaces (polypropylene, polyurethane, rubber, polyester, teflon, acetal) has been demonstrated, although different sanitizing procedures were applied (Krysinski et al., 1992; Mafu et al., 1990; Pan et al., 2006; Tolvanen et al., 2007). On the other hand, the peracetic acid treated *L. monocytogenes* biofilm cell counts on both polypropylene and stainless steel in another study had such high log-reductions that it resulted in almost complete elimination of *L. monocytogenes* (Skowron et al., 2018). The ineffective removal of *L. monocytogenes* in mono- and multispecies biofilms on PVC in our study was even more obvious during repeated C&D treatments, with decreased reductions over time. Such presumed increased resistance over time of *L. monocytogenes* biofilms towards a sanitizer has been seen before for *L. monocytogenes* on stainless steel and teflon that were daily treated with a sanitizing

agent (Pan et al., 2006) and for *L. monocytogenes* in mono- and multispecies biofilms on PVC coupons in BHI broth (Fagerlund et al., 2017). Presence of organic material may also have affected the efficacy of the disinfection treatment as this may decrease the access and/or active concentration of sanitizers/disinfectants/antimicrobial additives in different tested surfaces and experimental settings, leading to disinfection failure (Aarnisalo et al., 2000; Cerf et al., 2010; Chaitiemwong et al., 2010; Dagher et al., 2017; Hua et al., 2019; Nyati et al., 2012).

The current study showed high *L. monocytogenes* strain diversity in monospecies and multispecies biofilms on SS and PVC coupons, and also after repetitive C&D cycles on the PVC coupons. This includes strains that belong to frequently isolated subtypes within the mushroom processing environment (CC4, CC87 and CC224) as well as less frequently isolated subtypes (CC5, CC7 and CC37) (Lake et al., 2021). Another study that used *L. monocytogenes* strains isolated from meat processing environments also showed that when strains were applied in a seven-strain cocktail, these strains were equally capable of growth and survival in biofilms on PVC conveyor belts repeatedly exposed to C&D while using BHI as growth medium (Fagerlund et al., 2017). Other studies grouped *L. monocytogenes* strains in persistent and non-persistent strains, and some studies suggested a link between persistence and biofilm forming capacity (Borucki et al., 2003; Nakamura et al., 2013; Nowak et al., 2017), while other studies did not observe such a correlation (Djordjevic et al., 2002; Heir et al., 2018; Lee et al., 2019). The results of the current study showed that the six *L. monocytogenes* strains that were isolated from final frozen mushroom products and from swabs after C&D, were all present following the C&D cycles. This high diversity might correlate to the high diversity of *L. monocytogenes* strains isolated from mushroom processing environments (Lake et al., 2021).

The microbiota in a mushroom processing factory was shown to be rather diverse, and the Gram-negative and Gram-positive bacteria *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae* and *Lactococcus* were the highest abundant genera (Lake et al., 2023a). Biofilm experiments showed that *P. fluorescens* and *Enterobacteriaceae* strains dominated the multispecies biofilms on PVC coupons during the 8-day incubation period with an increase of *L. lactis* on the 8th day, but dominant microbiota species varied somewhat throughout the 8-day incubation period on SS coupons. The high counts after C&D procedures on PVC coupons may maintain the same strains as high contributors in the multispecies biofilm, but the constantly disruption and

construction of new biofilm after each C&D regime on SS coupons might cause some variations in strain abundance after growth. The dominance of *Pseudomonas* strains on PVC was also observed during biofilm formation on PVC conveyor belt repeatedly exposed to C&D (Fagerlund et al., 2017), but the *P. fluorescens* strain was not as dominant as reported in our study. Also *Pseudomonas* strain dominance was observed on (non-C&D treated) stainless steel during a 9-day incubation period in multispecies biofilm communities (Heir et al., 2018; Langsrud et al., 2016) and although *Pseudomonas* strains were also among the more abundant microbiota strains in untreated biofilms on SS coupons in our study, they were not as abundant as mentioned in the previous studies. The *Pseudomonas* strains may shelter *L. monocytogenes* during biofilm formation, providing an extra protection against physical-chemical damages (Puga et al., 2018). This shelter effect of the microbiota might explain the differences in *L. monocytogenes* decreases during repeated C&D cycles in monospecies and multispecies biofilms at which *L. monocytogenes* reductions were smaller during the following-up C&D cycles with multispecies compared to monospecies. Moreover, the lactic acid bacteria or their cell free supernatant showed negative interactions towards *L. monocytogenes* in dual species incubations (Bungenstock et al., 2020; Dygico et al., 2019; Haddad et al., 2021; Lake et al., 2023a; Sinclair et al., 2022), but this study showed stable *L. monocytogenes* presence in multispecies biofilms. Also another study demonstrated inhibitory properties of lactic acid bacteria towards *L. monocytogenes* in a spot inoculation assay, but not in a multispecies cocktail (Sinclair et al., 2022). In addition, food debris may affect inhibitory properties of lactic acid bacteria as inhibitory properties were observed in agar well diffusion tests, but not in challenge tests (Bungenstock et al., 2020; Bungenstock et al., 2021). This incomplete biofilm removal of *L. monocytogenes* formed on food processing surfaces may lead to recontamination of processed products, and must be controlled to reduce cross contamination risks in food processing plants (Mazaheri et al., 2021).

Desiccation of *L. monocytogenes* cells resulted in high reductions in viable cell counts during the first days of desiccation on PVC and SS coupons, after which the loss of viability decreased and stabilized, leading to *L. monocytogenes* survival for over 14 days. Such a trend in desiccation was also shown for desiccated *L. monocytogenes* cells on SS surfaces with constant cell counts at a plateau for weeks after the initial fast decline in viability loss, with survival of up to 60 days of desiccation (Hansen and Vogel, 2011; Takahashi et al., 2011; Vogel et al., 2010). Notably, in our experiments

the *L. monocytogenes* strain characterization in the monospecies and multispecies cocktails demonstrated the presence of all six *L. monocytogenes* strains during the two-weeks desiccation treatment. Another study using *L. monocytogenes* isolated from various sources, showed differences in survival efficacy of strains during desiccation incubation on stainless steel coupons (Takahashi et al., 2011), and strain dependent desiccation resistance has been demonstrating (Takahashi et al., 2011; Zoz et al., 2017). Analysis of microbiota performance showed that *Pseudomonas* spp. strains were rapidly inactivated and represented only a minor fraction of the multispecies cocktail on the dried surfaces, which was in contrast to the wet biofilms on PVC and SS in which *Pseudomonas* spp. strains were highly abundant. The high *Enterococcus* abundance following multispecies desiccation might be ascribed to the high desiccation resistance of *Enterococcus* strains compared to that of other mushroom microbiota species including *Aeromonas*, *Pseudomonas* and *Enterobacteriaceae* family members (Janning and in 't Veld, 1994). The long term desiccation survival of *L. monocytogenes* in this study might be partly attributed to the presence of the mushroom medium components, since several other studies demonstrated that organic material facilitates the long term desiccation of *L. monocytogenes* on stainless steel (Lim et al., 2020; Takahashi et al., 2011; Vogel et al., 2010). Also the incubation temperature has an effect on the *L. monocytogenes* survival on various surfaces showing higher *L. monocytogenes* survival at lower temperatures compared to higher temperatures (Chaitiemwong et al., 2010; di Ciccio et al., 2020). The effects of the soil material and temperature on the desiccation survival of *L. monocytogenes* highlight the significance of conducting desiccation experiments in simulated food processing environmental conditions. As food processing environments are usually soiled and have non-optimal growth temperatures, special attention should be paid by the food industry to implement strategies to prevent *L. monocytogenes* desiccation survival on food processing surfaces.

In conclusion, our study demonstrated that commonly used C&D procedures towards *L. monocytogenes* monospecies and multispecies biofilms were more effective when formed on SS and less effective on PVC. *L. monocytogenes* cells present in monospecies and multispecies biofilms on PVC that survived the C&D treatment were able to regrow with high strain diversity, and were more resistant in sequential C&D cycles. Although C&D treatments of dried surfaces showed significant reduction of *L. monocytogenes* cells, tested *L. monocytogenes* mushroom isolates in monospecies and multispecies incubations were able to survive long desiccation periods on dried

surfaces. This ability of *L. monocytogenes* to survive regular C&D treatments and tolerate desiccation stress is a major problem for food processing plants and therefore, improved measures should be developed to control *L. monocytogenes* in food processing environments.

Abbreviations

Lm, *Listeria monocytogenes*; PVC, polyvinyl chloride; SS, stainless steel; C&D, cleaning and disinfection

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Supplemental information

Supplemental tables

Supplemental table 5.1. Amplicon sequences of a part of the LMOf2365_0321 / lmo0300 gene of 6 *L. monocytogenes* strains. Sequence list of the LMOf2365_0321 / lmo0300 gene sequences of the *L. monocytogenes* strains

***L. monocytogenes* strain and the corresponding LMOf2365_0321 / lmo0300 gene sequence**
***L. monocytogenes* strain 544**
TGAACAATACCAATTTGCCCTGGATAGCCACCTTCTTTAAAAGCTTTGACGCCTAAAGCACTCGCGTACATCACATTATA
AGCGGCAATCATCGTTTTTTGTGTATCTTGATAGCCAGGTGGGTAGTTGCCGATTTTATATCCGTTAGCAACAAACCATT
TCGGCTCGTTAAACGTAGTCCAATTCGTGATTTTATCGCCAAAGTGGTCATAACAAACCTTGGCATAATGCTC
***L. monocytogenes* strain 546**
TGAACAATACCAATTTGCCCAGGATAGCCACCTTCTTTAAAAGCTTTGACACCTAAAGCACTCGCGTACATCACATTATA
AGCAGCAATCATTGTTTTTTGCGTATCTTGATAGCCAGGCGGGTAGTTGCCGATTTTATATCCGTTAGCAACAAACCATT
TTGGCTCGTTAAACGTAGTCCAATTCGTGATTTTATCGCCAAAGTGGTCATAACAAACCTTGGCATAATGCTC
***L. monocytogenes* strain 636**
TGAACAATACCAATTTGCCCTGGGTAGCCACCTTCTTTAAAAGCCTTCACGCCTAAAGCACTCGCGTACATTACATTATA
AGCGGCAATCATCGTTTTATGTGTATCTTGATAGCCAGGCGGGTAGTTGCCAATTTTATATCCATTAGCAACAAACCATT
TTGGCTCGTTAAACGTAGTCCAATTCGTTATTTTATCGCCAAAATGGTCATAACAAACCTTGGCATAATGCTC
***L. monocytogenes* strain 640**
TGAACAATACCAATTTGCCCTGAGTAGCCACCTTCTTTAAAAGCTTTGACGCCTAAAGCACTCGCGTACATCACATTATA
AGCGGCAATCATCGTTTTTTGTGTATCTTGATAGCCGGGCGGGTAGTTGCCGATTTTATATCCATTAGCAACAAACCATT
TTGGCTCGTTAAACGTAGTCCAATTCGTGATTTTATCGCCAAAGTGGTCATAACAAACCTTGGCATAATGCTC
***L. monocytogenes* strain 818**
TGAACAATACCAATTTGCCCTGGGTAGCCACCTTCTTTAAAAGCCTTCACGCCTAAAGCACTCGCGTACATTACATTATA
AGCGGCAATCATCGTTTTTTGTGTATCTTGATAGCCAGGTGGGTAGTTGCCGATTTTATATCCGTTAGCAACAAACCATT
TTGGCTCGTTAAACGTAGTCCAATTCGTGATTTTATCGCCAAAATGGTCATAACAAACCTTGGCATAATGCTC
***L. monocytogenes* strain 838**
TGAACAATACCAATTTGCCCTGGATAGCCACCTTCTTTAAAAACTTTGACGCCTAAAGCACTCGCGTACATCACATTATA
AGCGGCAATCATCGTTTTTTGTGTATCTTGATAGCCGGGCGGGTAGTTGCCGATTTTATATCCGTTAGCAACAAACCATT
TTGGCTCGTTAAAAGTGGTCCAATTCGTGATTTTATCGCCAAAATGGTCATAACAAACCTTGGCATAATGCTC

Supplemental table 5.2. Amplicon sequences of the V4 region of the 16S rRNA gene of 18 microbiota and *L. monocytogenes*. Sequence list of the 16S rRNA sequences of the microbiota, including *L. monocytogenes*

Bacterial strain and the corresponding strain sequence of the V4 region of the 16S rRNA gene
Ewingella
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTA AGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Raoultella
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGT AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGA CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Citrobacter
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGT AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGA CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Lelliottia
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGT AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGA CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Buttiauxella
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTCA AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Pseudomonas fluorescens
CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTAGTTAA GTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGG AATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTG ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC

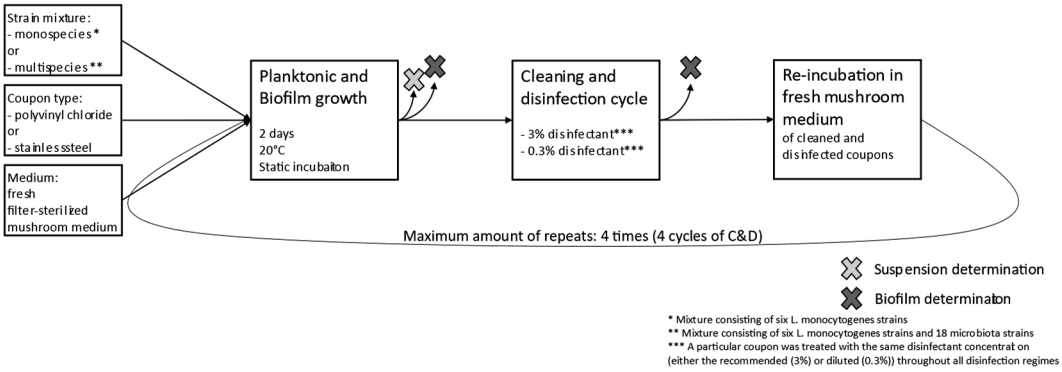
Supplemental table 5.2 continued

Bacterial strain and the corresponding strain sequence of the V4 region of the 16S rRNA gene
Pseudomonas fragi
CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA GTTGAATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTAGTGG AATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGATACTG ACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Acinetobacter spp.
CAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTCTTAA GTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAG AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTG ACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Acinetobacter johnsonii
CAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTYTTAA GTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAG AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTG ACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Lactococcus lactis
CAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAA GTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGA ATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGA CACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Lactococcus garviea
CAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAA GTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGG AATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACTG ACACTGAGGCTCGAAAGCGTGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Lactococcus raffinolactis
CAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTAATAA GTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGTTAGACTTGAGTGCAGTAGAGGAGAGTGGA ATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGACTGTCACTGA CACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC

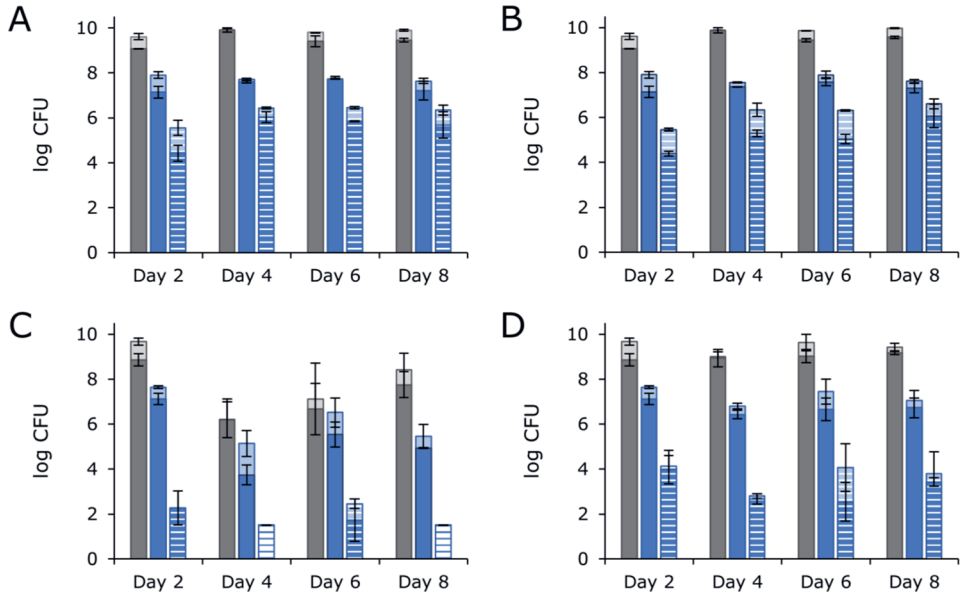
Supplemental table 5.2 continued

Bacterial strain and the corresponding strain sequence of the V4 region of the 16S rRNA gene
Enterococcus
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAA GTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTG GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACT GACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Streptococcus
CAGCAGCCGCGGTAATACGTAGGTCTCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGYAGGCGGTTATTTAA GTCTGAAGTTAAAGGCCGTGGCTCAACCATGGTTCGCTTTGGAAACTGGATAACTTGAGTGCAGAAGGGGAGAGTGGA ATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGA CGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Brochothrix
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTA AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGGACAGAAGAGGAGAGT GGAATTCCAAGTGTAGCGGTGAAATGCGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTTAC TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Aeromonas
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATA AGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
Chryseobacterium
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCTGATGTGTAA GTCAGTGGTGAAATCTCACAGCTTAACTGTGAAACTGCCATTGATACTGCATGTCTTGAGTGTTGTTGAAGTAGCTGGA ATAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGAACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGAC GCTGATGGACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC
Spingobacterium
CAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGTTCTTTAA GTCAGAGGTGAAAGACGGCAGCTTAACTGTCGCAGTGCCTTTGATACTGAAGAACTTGAATTGGGTTGAGGAATGCGG AATGAGACAAGTAGCGGTGAAATGCATAGATATGTCTCAGAACCCCGATTGCGAAGGCAGCATTCCAAGCCTATATTGA CGCTGATGCACGAAAGCGTGGGGATCGAACAGGATTAGATACCCTGGTAGTC
Listeria monocytogenes
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTTTTAA GTCTGATGTGAAAGCCCCCGGCTTAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTG GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACT GACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC

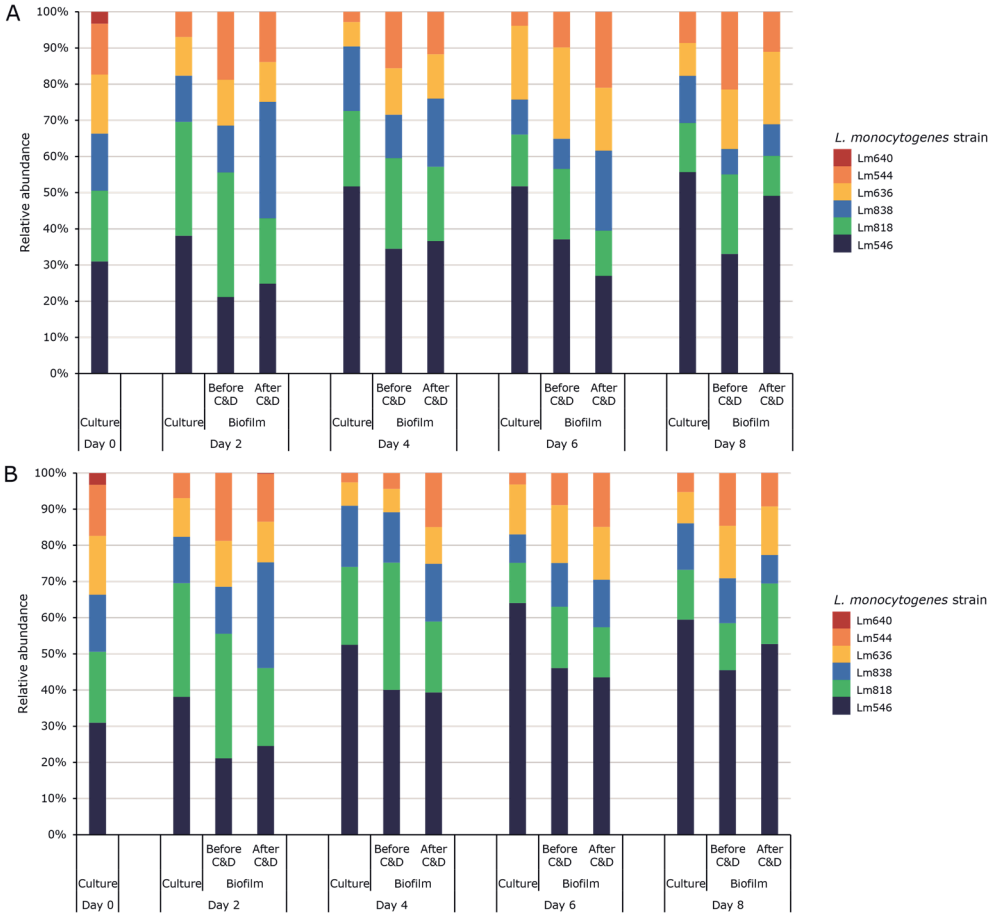
Supplemental figures



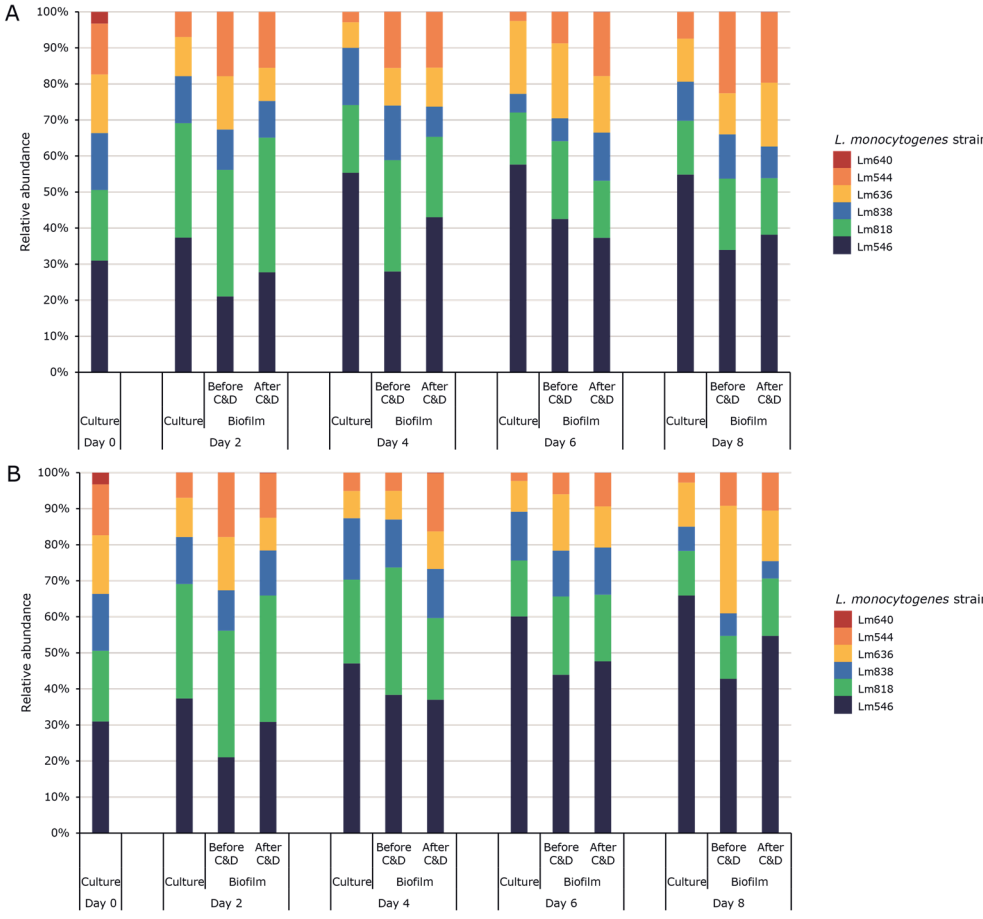
Supplemental figure 5.1. Schematic representation of the biofilm growth experiment and C&D treatments. Strains for both the monospecies and multispecies cocktail were individually precultured and diluted till ~7 log CFU/mL in PBS buffer. Individual strains were mixed in equal quantities to achieve a concentration of ~7 log CFU/mL for either the monospecies or the multispecies mixture, and both mixtures were subsequently diluted 1:100 (vol/vol) in fresh mushroom medium (start concentration of ~5 log CFU/mL). The mixtures were subsequently transferred in a 12-wells plate and coupons (PVC or SS) were submerged in the medium. Cultures were statically incubated at 20 °C for 2 days after which the coupons were subjected to a C&D treatment (either with the 0.3% or the 3% disinfection concentration). The C&D treated coupons were subsequently transferred to a new 12-wells plate prefilled with fresh filter-sterilized mushroom medium and the plate was statically re-incubated for 2 days at 20 °C allowing possible re-growth of cells that survived the C&D treatment. The C&D procedure and re-incubation of the coupons was repeated every second day until 8 days of incubation in which a particular coupon was either treated with 0.3% disinfectant or 3% disinfectant. Every second day, planktonic growth was determined just before C&D treatment and designated PVC and SS coupons were extracted for biofilm count determination (just before the C&D treatment and immediately after the C&D treatment).



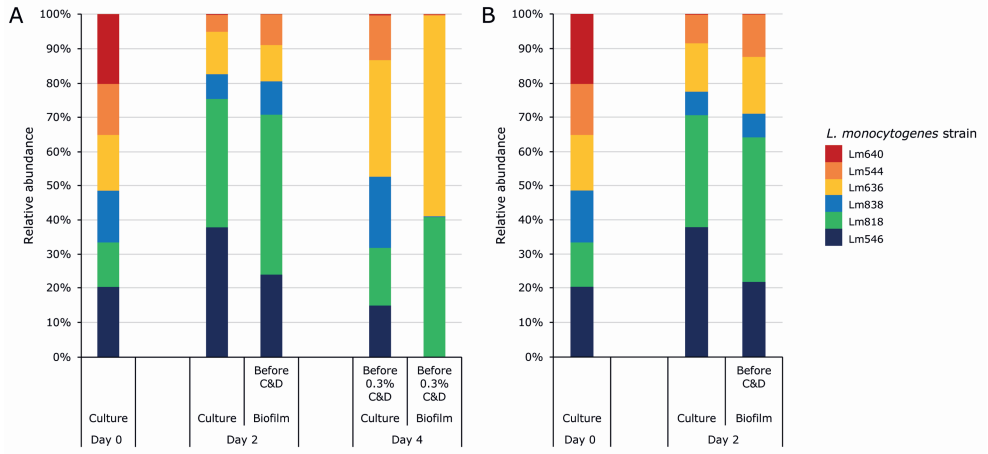
Supplemental figure 5.2. Growth performance and biofilm formation of the multispecies cocktail (n=24) (consisting of *L. monocytogenes* strains (n=6) and background microbiota strains (n=18), including *Pseudomonas* species). Biofilm formation was performed on PVC coupons (A and B) or SS coupons (C and D) using periodical C&D treatments with either 3% disinfectant (A and C) or 0.3% disinfectant (B and D). Counts were determined just before (planktonic growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedure. The grey bars correspond to the planktonic counts and the blue bars correspond to the biofilm counts in which the blue filled bars represent the biofilm counts before C&D treatment and the blue filled bars with white horizontal stripes represent the biofilm counts immediately after C&D treatment. The dark colored bars represent the *Pseudomonas* counts, while the light colored bars represent the microbiota counts. Counts below the detection limit are indicated with a white fill with blue horizontal lines (biofilm counts after C&D). The planktonic counts are displayed as log CFU/mL and the biofilm counts are displayed as log CFU/cm^2. The error bars represent the standard deviation of the two biological replicates, each consisting of two technical replicates.



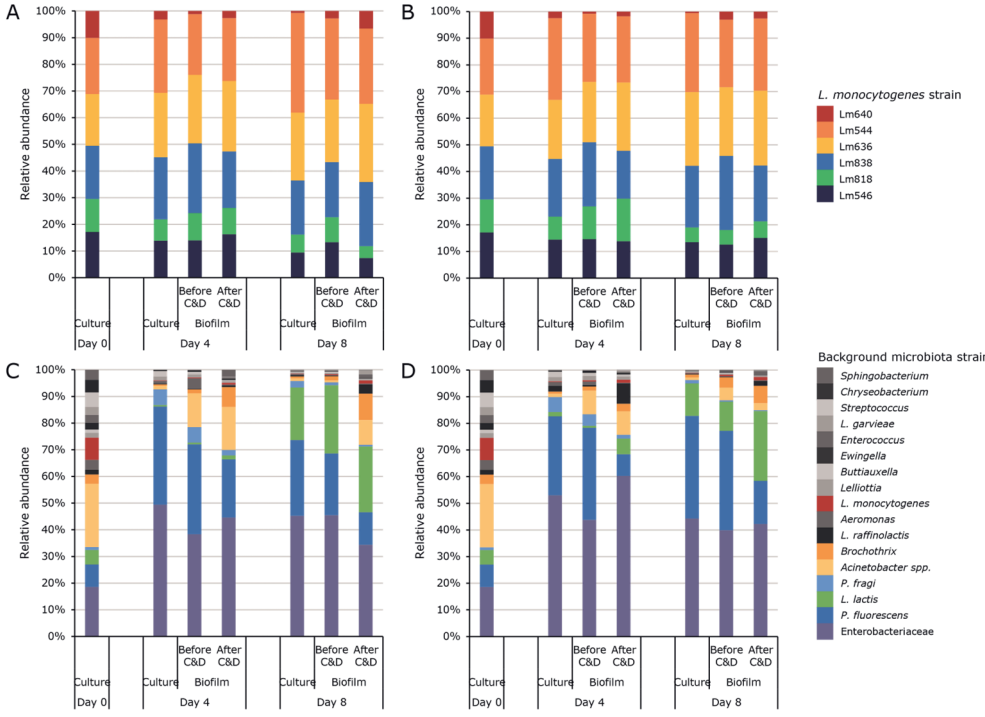
Supplemental figure 5.3. Relative abundance of *L. monocytogenes* strains during the 8-day monospecies incubation on PVC coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the 3% C&D treatment (A) and the 0.3% C&D treatment (B). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of PMAxx™ treated samples. Results of replicates (n=2) are pooled and averages are shown.



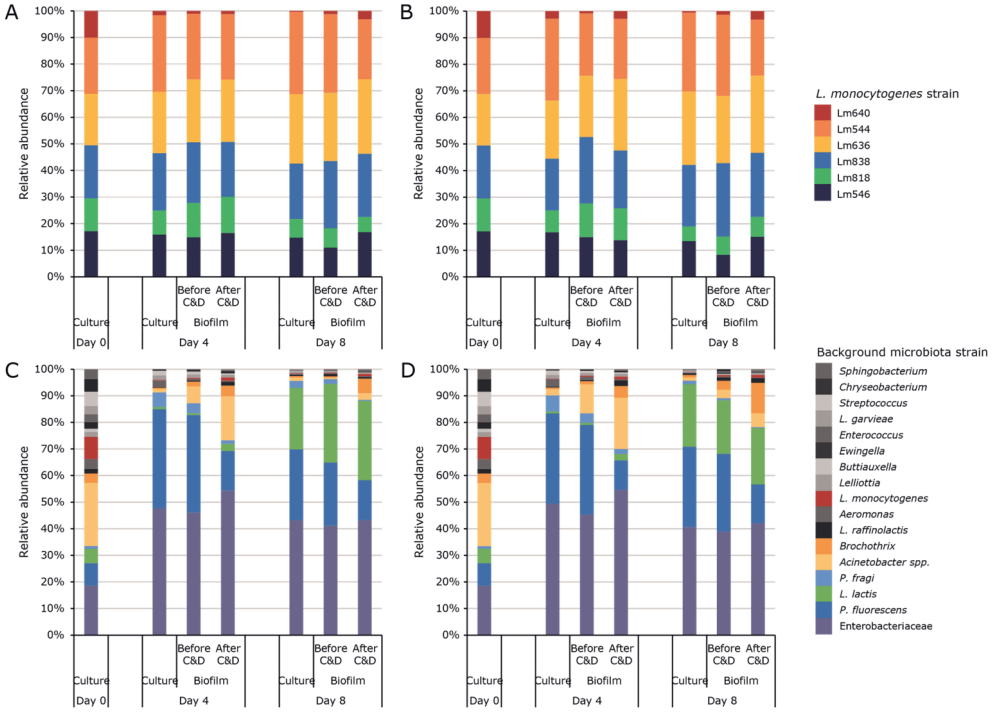
Supplemental figure 5.4. Relative abundance of *L. monocytogenes* strains during the 8-day monospecies incubation on PVC coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the 3% C&D treatment (A) and the 0.3% C&D treatment (B). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of non-PMAxx™ treated samples. Results of separate experiments are pooled (n=2) and averages are shown.



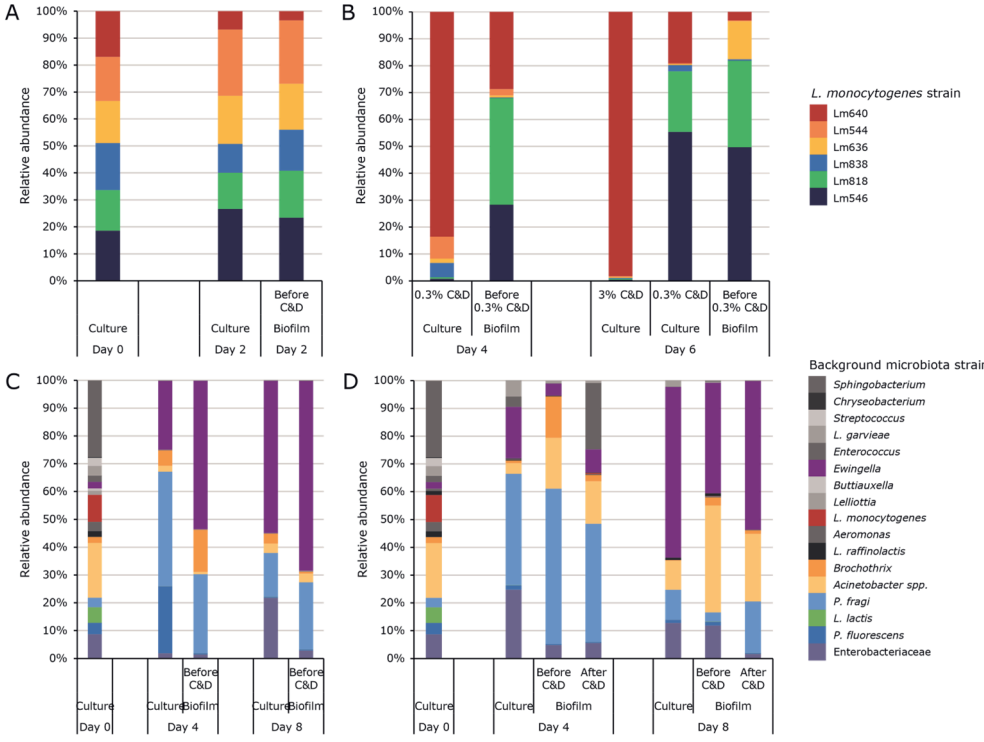
Supplemental figure 5.5. Relative abundance of *L. monocytogenes* strains during the 8-day monospecies incubation on SS coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the PMAxx™ treated samples (A) and the non-PMAxx™ treated samples (B). Relative abundances were determined just before (growth and biofilm formation) the C&D procedure, while relative abundances immediately after (only biofilm formation) the C&D procedure was not feasible as *L. monocytogenes* was absent from those samples. Results of individual experiments or replicates (n=1 or n=2 and replicates are pooled) are shown by the average abundance.



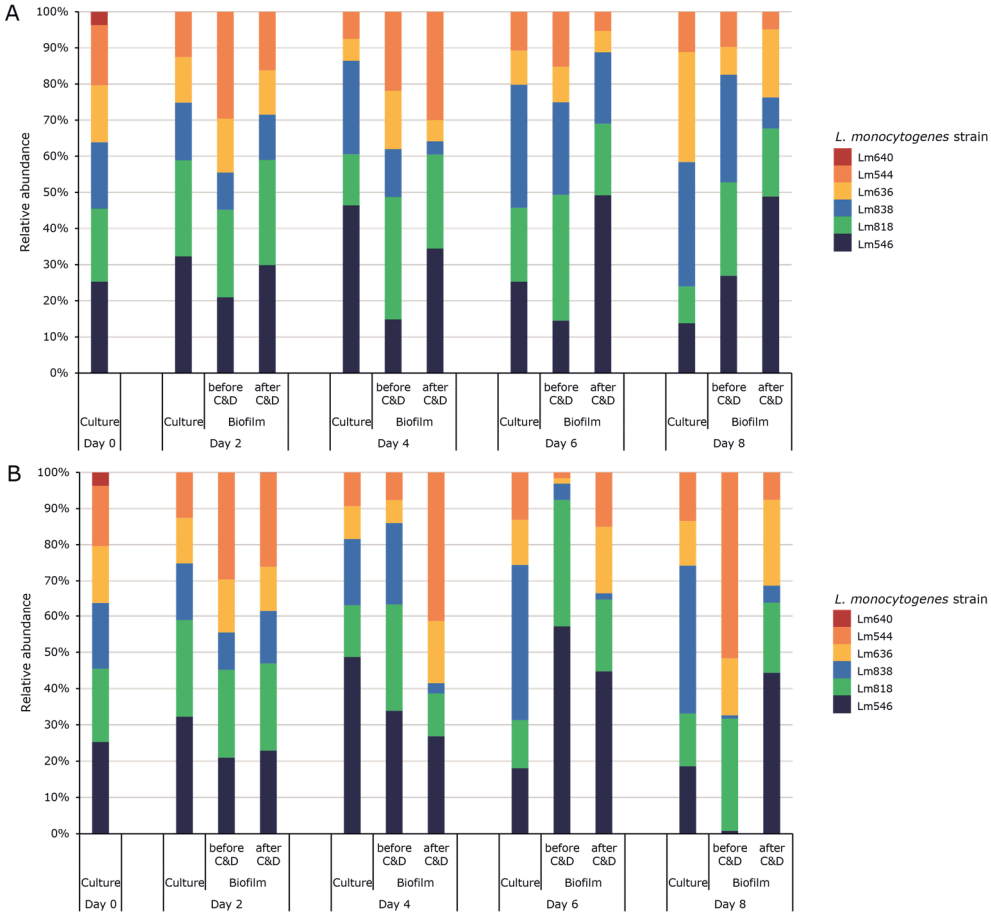
Supplemental figure 5.6. Relative abundance of *L. monocytogenes* strains and background microbiota strains during the 8-day multispecies incubation on PVC coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the 3% C&D treatment (A) and the 0.3% C&D treatment (B) and for the individual background microbiota strains, including *L. monocytogenes* strains, (n=18 + n=6, total n=24) for the 3% C&D treatment (C) and the 0.3% C&D treatment (D). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of PMAxx™ treated samples. Results of replicates (n=2) are pooled and averages are shown.



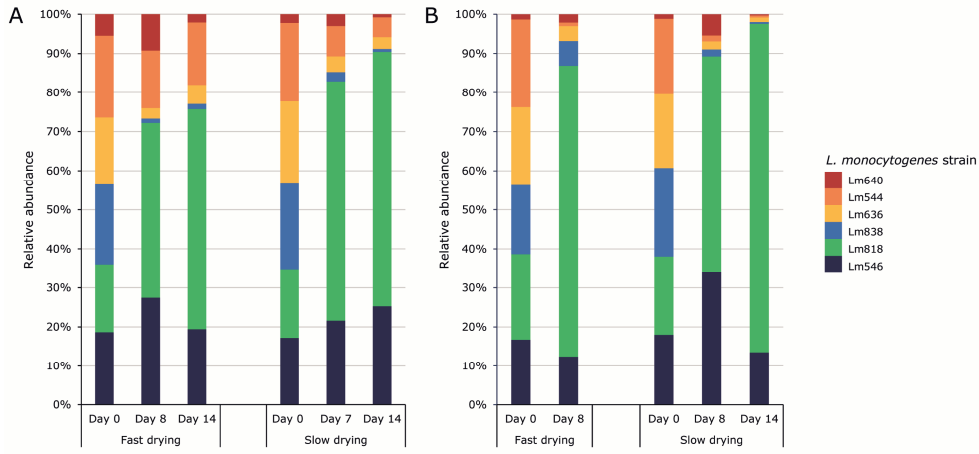
Supplemental figure 5.7. Relative abundance of *L. monocytogenes* strains and background microbiota strains during the 8-day multispecies incubation on PVC coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the 3% C&D treatment (A) and the 0.3% C&D treatment (B) and for the individual background microbiota strains, including *L. monocytogenes* strains, (n=18 + n=6, total n=24) for the 3% C&D treatment (C) and the 0.3% C&D treatment (D). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of non-PMAxx™ treated samples. Results of replicates (n=2) are pooled and averages are shown.



Supplemental figure 5.8. Relative abundance of *L. monocytogenes* strains and background microbiota strains during the 8-day multispecies incubation on SS coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) before the first C&D treatment (A) and some cases after the first C&D treatment in which *L. monocytogenes* was detected (B) and for the individual background microbiota strains, including *L. monocytogenes* strains, (n=18 + n=6, total n=24) for the 3% C&D treatment (C) and the 0.3% C&D treatment (D). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures of PMAxx™ treated and non-PMAxx™ treated samples. Both treatments were combined here since comparable values were obtained for both treatments (applied to all duplicate samples). Results of individual experiments or replicates (n=1 or n=2 and replicates are pooled) are shown by the average abundance.



Supplemental figure 5.9. Relative abundance of *L. monocytogenes* during the 8-day monospecies incubation experiment on PVC coupons. Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) for the 3% C&D treatment (A) and the 0.3% C&D treatment (B). Relative abundances were determined just before (growth and biofilm formation) and immediately after (only biofilm formation) the C&D procedures. For this, *L. monocytogenes* was harvested from the culture (growth) or the PVC coupons (biofilm, just before and immediately after C&D treatment) and plated on TSAYE plates. Following incubation, all colonies of the countable plate (between 30 and 300, ideally >100) were harvested at once and *L. monocytogenes* relative abundance was determined. Result of technical replicates (n=2-6) are pooled and averages are shown.



Supplemental figure 5.10. Relative abundance of *L. monocytogenes* during the 14-day monospecies desiccation experiment on PVC coupons (A) and SS coupons (B). Relative abundance was determined for the individual *L. monocytogenes* strains (n=6) from either the "fast dried" coupons - 2 hours of drying or "slow dried" coupons - 6 hours of drying. For this, *L. monocytogenes* was harvested from the coupons and plated on TSAYE plates after which all colonies of the countable plate (between 30 and 300, ideally >100) were harvested at once and *L. monocytogenes* relative abundance was determined. Results of replicates (n=2) are pooled and averages are shown. No sufficient samples could be extracted from the "Fast drying" samples of the SS coupons at day 14 and therefore this timepoint was omitted.

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6

General discussion

6.1 Introduction

This thesis describes the ecology and physiological behaviour of *Listeria monocytogenes* along the whole production and processing chain of the frozen and sliced white button mushroom, *Agaricus bisporus*. *L. monocytogenes* is a robust pathogen and well equipped to adapt to different environmental niches and this makes it a difficult pathogen to eliminate from food processing environments. **Chapter 2** describes the presence and diversity of *L. monocytogenes* along the whole production and processing chain of frozen sliced mushrooms by sampling all relevant locations at the mushroom grower and the mushroom processing companies. The sampling survey demonstrated that the prevalence of *L. monocytogenes* increased towards the final stage in the processing factories. The *L. monocytogenes* strains isolated along the different stages of the mushroom production and processing chain showed high genomic diversity in serogroups and clonal complexes, and this disproves the hypothesis that the mushroom environment selects for specific serogroups of clonal complexes. To phenotypically assess this genetically highly diverse set of *L. monocytogenes* strains, a well-balanced strain selection was further characterized in conditions that mimicked mushroom post harvesting handling and processing environments (**Chapter 3**). This revealed that mushrooms are a rich nutrient source for *L. monocytogenes*. Performance of strains that originate from the mushroom value chain was compared to human isolates and isolates from other foods, and the observed strain variability (with respect to growth characteristics) was remarkably low. Different mushroom products (whole, sliced and smashed mushrooms) supported the growth of *L. monocytogenes*, despite the presence of high counts of microbiota. Here, the increase of *L. monocytogenes* was higher when mushroom products were more damaged, indicating that increased release of suitable substrates supported the growth of *L. monocytogenes*. Growth performance of *L. monocytogenes* was also assessed in co-culture with various background microbiota strains, and in spent media of the microbiota strains (**Chapter 4**). These co-incubations showed competitive growth of *L. monocytogenes* and extensive growth in spent media, except in conditions with a low pH. Growth of *L. monocytogenes* could however be restored when the pH was increased, showing that pH is an important inhibiting factor for *L. monocytogenes* and confirming the high nutrient availability of the mushroom medium. The behaviour of *L. monocytogenes* was further characterized with complex background microbiota cocktails in biofilms on stainless steel and on the conveyor belt material polyvinyl chloride (**Chapter 5**). Complex microbial biofilms were formed on both surface

materials, but regular exposure to cleaning and disinfection regimes was more efficient on stainless steel than on polyvinyl chloride. *L. monocytogenes* survival in the complex biofilm after the regular cleaning and disinfection treatments resulted in the maintenance of high *L. monocytogenes* strain diversity. The current chapter (**Chapter 6**) describes additional results and discusses the findings of this research. The discussion has a focus on *L. monocytogenes* contamination in the mushroom post harvesting handling and processing environments, growth of *L. monocytogenes* in non-static environments, the modelling of *L. monocytogenes* growth in mushroom post harvesting handling and processing environments, and will discuss the current and emerging cleaning and disinfection strategies to combat *L. monocytogenes* in food processing environments. A graphical overview of the main findings obtained in this thesis is represented in figure 6.1.

6.2 *Listeria monocytogenes* contamination in the *A. bisporus* mushroom post harvesting handling and processing environments

L. monocytogenes may enter environments that handle food products via various routes (Duze et al., 2021). Previous studies have suggested that contamination could come from raw materials (Bolocan et al., 2015; Saludes et al., 2015), including soil as a pre-harvest *L. monocytogenes* contamination source (Beuchat, 2002). No *L. monocytogenes* contamination was however assumed for the substrate, although this substrate contains horse and chicken manure that are both known sources of *L. monocytogenes* (Chemaly et al., 2008; Gudmundsdottir et al., 2004; Rothrock et al., 2017; Wesley, 2007). This was supposed since substrate production involves the tight control of high temperatures (temperatures of up to 80 °C) and these temperatures are detrimental for *L. monocytogenes*. Besides, in this study, *L. monocytogenes* was not detected in fresh casing soil (0 out of 100 samples) upon the delivery of casing soil at the mushroom grower. On the other hand, *L. monocytogenes* was detected in the casing soil when sampled at harvest (6 out of 60 samples) (**Chapter 2**). This may suggest a low contamination of *L. monocytogenes* in the starting materials (below the detection limit after enrichment with NEN-EN-ISO 11290-1:2017 enrichment protocol) (International Organization for Standardization, 2017a; 2017b) and that these starting materials play a minor role in *L. monocytogenes* contamination. It may also be suggested that the contamination of the casing soil is derived from other sources. This

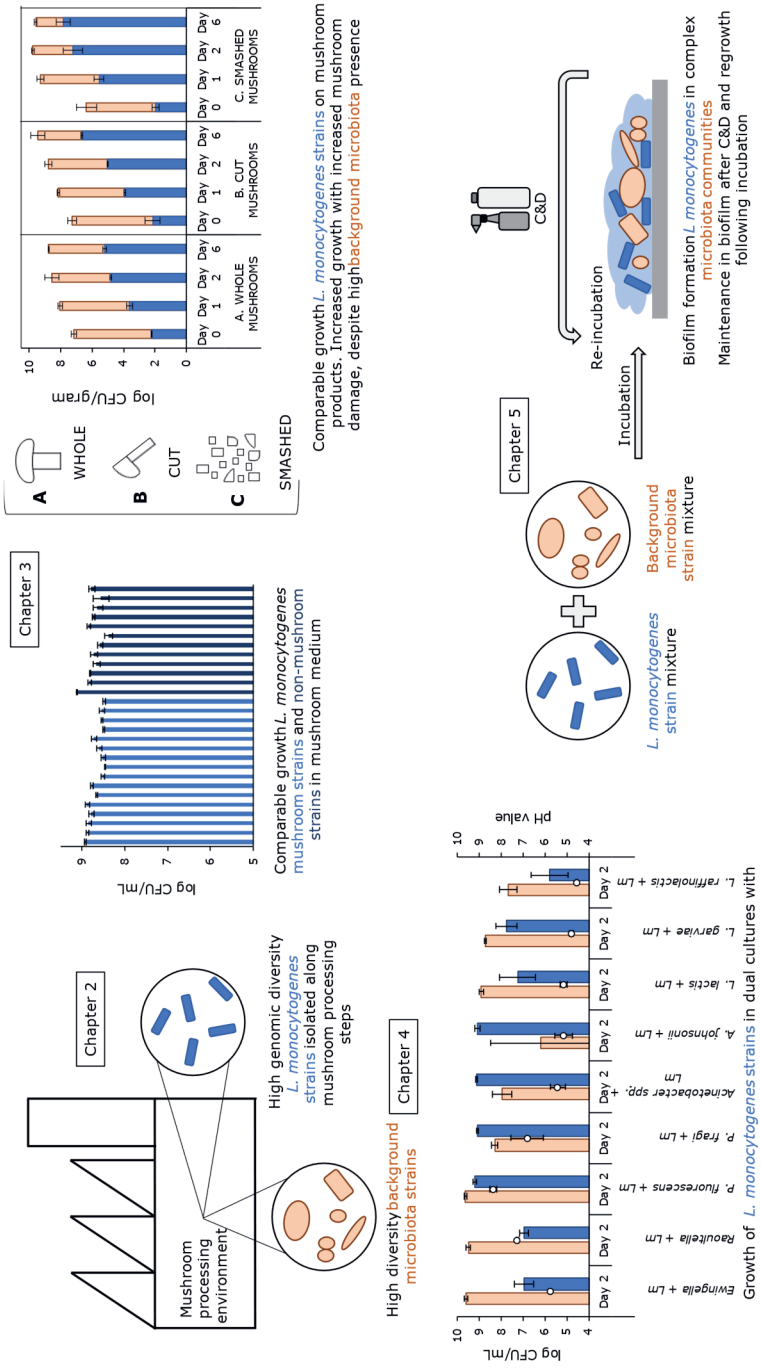


Figure 6.1.

Graphical overview of the main findings of *L. monocytogenes* related to *A. bisporus* mushroom production and processing environments described in this thesis.

could be, among others, due to contaminated soil dust (Matle et al., 2020) as the substrate and casing soil are unloaded in the outside environment and exposed to wind and rain. This outside soil could be contaminated as *L. monocytogenes* has previously been isolated from soil in various studies (Iwu and Okoh, 2020; Linke et al., 2014; Locatelli et al., 2013; Sauders et al., 2006; Strawn et al., 2013a; Strawn et al., 2013b; Weis and Seeliger, 1975; Welshimer and Donker-Voet, 1971). Another possible *L. monocytogenes* contamination source may include irrigation water (Acheamfour et al., 2021; Beuchat, 2002; Gartley et al., 2022). See figure 6.2A for possible *L. monocytogenes* sources and transmission routes for casing soil contamination. However, despite the presence of *L. monocytogenes* in the casing soil at harvest (6 out of 60 samples), *L. monocytogenes* was not recovered from mushrooms before harvesting (0 out of 60 samples) (**Chapter 2**). Therefore, the exact entry point(s) of *L. monocytogenes* in mushroom growing environments remain unclear and will require further examination.

As *L. monocytogenes* was detected on the fresh mushroom after harvesting, several studies suggested that contamination could come from cross-contamination via the production and processing environment (Burnett et al., 2020; Jordan et al., 2018; Sauders and D'Amico, 2016). This contamination of the environment may be derived from water sources that are used in mushroom post harvesting handling and processing environments. Also the floors of mushroom processing companies may contain *L. monocytogenes* (Murugesan et al., 2015; Pennone et al., 2018; Sun et al., 2021; Xu et al., 2023). *L. monocytogenes* may especially reside on wet floors containing mushroom debris and may cross contaminate food contact surfaces (Murugesan et al., 2015; Pennone et al., 2018). A prevention strategy may therefore aim to prevent establishment of *L. monocytogenes* on floors. This can be performed by a reduction or elimination of moisture on the floors and by revising the spots/equipment from which mushroom debris arises. Another route of *L. monocytogenes* cross-contamination may occur via processing equipment (machine harvesting device and equipment along the processing line). See figure 6.2B for possible *L. monocytogenes* sources and transmission routes for contamination of the mushroom post harvesting handling and processing environments. After cross-contamination events, *L. monocytogenes* may establish itself in niches close to the food contact surfaces (Bolocan et al., 2015; Chiarini et al., 2009; Hoelzer et al., 2011). This may make the food processing equipment a post-harvesting contamination source (Beuchat, 2002). As the prevalence of *L. monocytogenes* on raw products (mushrooms

before harvesting) is conceivably low, cross-contamination via the mushroom harvesting equipment, post harvesting equipment and processing environment appeared to be the main *L. monocytogenes* contamination route (**Chapter 2**). The contamination of food products with *L. monocytogenes* via cross-contamination by the environments that handle food products was also suggested in other studies as the most common route of food product contamination and that raw materials are not the main contamination source (Autio et al., 1999; Ferreira et al., 2014; Jami et al., 2014; Jordan et al., 2018; Keto-Timonen et al., 2007; Lin et al., 2006; Mazaheri et al., 2020; Møretrø and Langsrud, 2004; Zhang et al., 2021), in line with results presented in **Chapter 2**. Cross-contamination and subsequent growth of *L. monocytogenes* may lead to increased cell counts on the (sliced) mushroom products before the freezing step, as temperature can only be regulated to a limited extent during mushroom post harvest handling and processing. Therefore, a modelling tool to determine *L. monocytogenes* growth during the post harvest handling and processing of mushrooms was developed and discussed in section 6.5.

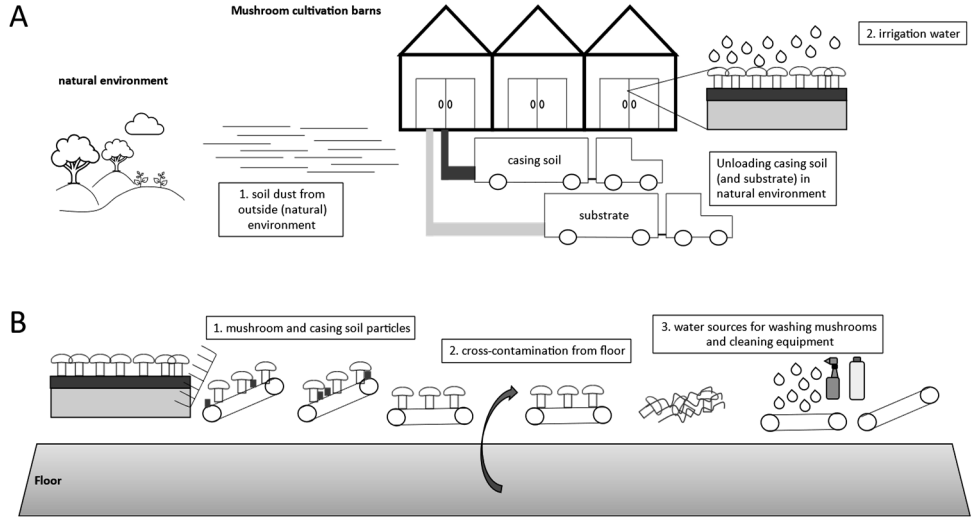


Figure 6.2. Possible transmission routes that may lead to the contamination with *L. monocytogenes*.

(A) Possible contamination routes of the casing soil may be explained by contamination from natural sources, such as contaminated soil dust and irrigation water (see text for details).

(B) Possible contamination routes of the mushroom post harvesting handling and processing environments may include contamination from casing soil particles, cross-contamination from floors and from water sources used for washing mushrooms and cleaning equipment (see text for details).

Both the mushroom harvesting procedure at the mushroom grower and the mushroom processing procedure at the mushroom factory may contribute to contamination as *L. monocytogenes* was isolated from both locations (**Chapter 2**). Moreover, the *L. monocytogenes* strains that were isolated from both environments were genetically diverse (**Chapter 2**). This is in line with data reported for the mushroom processing environment and other food processing environments that also showed diverse *L. monocytogenes* populations (fresh-cut vegetable, frozen vegetables, meat, seafood, dairy) (Kim et al., 2018; Leong et al., 2014; Lomonaco et al., 2009; Martín et al., 2014; Pennone et al., 2018; Truchado et al., 2022; Vongkamjan et al., 2017). The high diversity in *L. monocytogenes* subtypes described in **Chapter 2** could subsequently be explained by the excess availability of compounds present in mushroom (derived medium) that can serve as carbon and energy sources for this pathogen (**Chapter 3** and **4**). The impact of the presumed nutrient richness on *L. monocytogenes* growth performance and (re)contamination during mushroom post harvesting handling and processing is discussed below.

6.3 Nutrient composition *Agaricus bisporus* mushroom products

Mushroom medium was used throughout the thesis (**Chapter 3**, **Chapter 4** and **Chapter 5**) for mimicking closely the nutrient composition present in the mushroom post harvesting handling and processing environments. Control experiments with sterile mushroom medium showed varying concentrations of amino acids and carbohydrates during incubations for 48-hours at room temperature (**Chapter 3** and **Chapter 4**). Fluctuations in the carbohydrate concentrations were also observed during the development of the *A. bisporus* mushrooms (Wannet et al., 2000, Hammond and Nichols, 1976), and after mushroom harvest (Golak-Siwulska et al., 2018, Tseng and Mau, 1999). This resulted in a decrease in fructose and mannitol concentrations during mushroom storage, while contents of the other reducing sugars remained constant (Tseng and Mau, 1999), in line with the results in the current study in which the concentrations of only these two carbohydrates differed during sterile medium incubation (**Chapter 4**). A possible explanation for this phenomenon includes the conversion of mannitol to fructose via the bidirectional *A. bisporus* mannitol dehydrogenase in the tested conditions (Morton et al., 1985). As *L. monocytogenes* is not able to utilize mannitol, but is able to utilize fructose (Weller et al., 2015), the

observed carbohydrate conversions provide additional substrates that support growth of *L. monocytogenes*.

This study (**Chapter 3**) showed considerable growth of *L. monocytogenes* in static cultures in mushroom medium and on whole, sliced and smashed mushroom products. Besides, shaking cultures of *L. monocytogenes* in mushroom medium also led to considerable growth of *L. monocytogenes*. These shaking cultures were similarly prepared, incubated and sampled as the static cultures (**Chapter 3**), except that the shaking cultures were added in honeycomb plates and incubated with continuous shaking. Static and shaking cultures of the selected 28 *L. monocytogenes* strains reached average growth values of 8.7 and 8.6 log CFU/mL after 48 h incubation at room temperature (20 °C), and final pH values of approximately 5.1 and 5.5, respectively (figure 3.1 (**Chapter 3)**, and figure 6.3 below). The difference in pH decrease might be explained by the differences in product formation during these two conditions, with lower lactate production, comparable acetate production and higher acetoin production during shaking incubation compared to static incubation (table 3.1

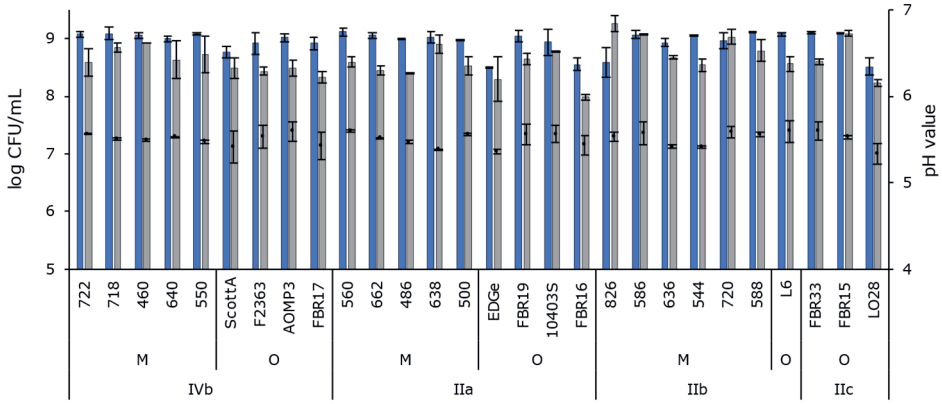


Figure 6.3. Growth performance of *L. monocytogenes* strains during shaking incubation at 20 °C in filter-sterilized mushroom medium. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in sterile mushroom medium and CFU counts were determined after 24 h (blue bars) and 48 h (grey bars) of shaking incubation. Strains were clustered based on PCR serogroup followed by clustering based on mushroom strains "M" and non-mushroom strains "O" (including human clinical strains, other food strains and an animal strain). The black dots represent the pH values after 48 h of growth of the shaking incubated cultures. Error bars indicate the standard deviation of the biological replicates (n = 2). Replicates of all strains were executed in the same mushroom medium batch. The same mushroom medium batch was used for incubating the *L. monocytogenes* strains in both static conditions (figure 3.1) and shaken conditions (this figure).

Table 6.1. Extracellular metabolite composition of filter-sterilized mushroom medium without inoculation of *L. monocytogenes* and with inoculation of *L. monocytogenes* followed by shaking incubation for 48 h at 20 °C using the same mushroom medium batch as in Fig. 6.2 and during static incubation (Fig. 3.1 and table 3.1). Compound analysis of non-inoculated mushroom medium was performed with 8 technical replicates and the standard deviation represents variations among technical replicates. Compound analysis after incubation with *L. monocytogenes* was determined for 28 strains, the same strains that are depicted in Fig. 6.1, namely-nine strains of PCR serogroup IVb, nine strains of PCR serogroup IIa, seven strains of PCR serogroup IIb and three strains of PCR serogroup IIc. Compound analysis was performed with two biological replicates for each strain. The standard deviation represents variations among the 28 strains using the mean of the biological replicates

	Without *L. monocytogenes* growth in mM (stdev)	With *L. monocytogenes* growth in mM (stdev)
trehalose	0.04 (0.03)	0.00** (0.00)*
glucose	2.21 (0.13)	0.32 (0.02)*
fructose	6.41 (0.32)	0.00 (0.00)*
glycerol	4.88 (0.58)	3.81 (0.64)*
mannitol	46.71 (1.52)	45.46 (0.59)
lactate	0.34 (0.43)	7.11 (1.17)*
acetate	0.54 (0.73)	4.02 (0.92)*
acetoin	1.04 (0.22)	3.32 (0.43)*

* Significant difference ($p < 0.05$) in extracellular metabolite concentration in mushroom medium with *L. monocytogenes* growth compared to filter-sterilized mushroom medium without *L. monocytogenes* growth (not inoculated mushroom medium), see also table 3.1.
** Values of 0.00 represent values that are below the detection limit (detection limit of 0.01 mM).

(**Chapter 3)**, and table 6.1 below). This is in accordance with a study that also described a different ratio in product formation by *L. monocytogenes* in conditions with different oxygen availability (aerobic versus anaerobic conditions) (Rivera-Lugo et al., 2022). As the p*K*a of lactate is one unit lower than acetate and since acetoin is a neutral compound, this might explain the higher decrease in pH during static incubation compared to shaking incubated *L. monocytogenes* cultures. These results showed that the facultative anaerobic micro-organism *L. monocytogenes* is able to grow in conditions with both lower and higher oxygen availabilities and both conditions resulted in comparable growth performance, supporting its establishment in mushroom post harvesting handling and processing environments.

The good growth performance of *L. monocytogenes* strains in static and shaking conditions in mushroom medium and also on mushroom products, even in the presence of microbiota, can be ascribed to the high nutrient richness of the *A. bisporus*

mushrooms. This is in contrast to another study in some other food products (cantaloupe, celery and sprouts) that described apparent nutrient limitation at outer surfaces of intact fruits and vegetables that may limit growth of *L. monocytogenes* (Marik et al., 2020). Notably, in contrast to the surface of fruits and vegetables, the surface of mushrooms has an open structure and is without a cuticle to protect them from physical or microbial attack or water loss (Brennan et al., 2000; Sapers et al., 1999). The differences in the surface might therefore explain the better accessibility of the nutrients from the mushrooms compared to other intact fruits and vegetables and may offer an additional explanation for the growth potential of *L. monocytogenes* on mushroom products.

6.4 Background microbiota and competitive microbial species

The high microbial diversity in the mushroom processing environment (**Chapter 4**), corresponds with findings of multiple studies in which many different types of bacteria were isolated from one particular food processing environment (Møretrø and Langsrud, 2017). Of the more abundant isolated microbiota groups, the lactic acid bacteria (LAB) strains were the only group that had clear inhibiting effects towards *L. monocytogenes* in dual culture incubations (**Chapter 4**). The inhibiting effect of the LAB strains on *L. monocytogenes* may be due to various antimicrobial compounds such as organic acids and bacteriocins (Webb et al., 2022). The inhibitory effect based on the low pH due to the production of organic acids was shown as the main inhibiting factor for *L. monocytogenes* growth in this study, as growth was restored in pH-adjusted medium (**Chapter 4**).

It has been proposed that bacteriocins produced by LAB strains may be applied to control *L. monocytogenes* (Miceli and Settanni, 2019), as they could reduce adhesion and biofilm formation by *L. monocytogenes* (Galié et al., 2018; Serna-Cock et al., 2019). LAB strains or their derivatives have already been shown to inhibit growth or decrease counts of *L. monocytogenes* during storage of food products (Aymerich et al., 2019; Gonzales-Barron et al., 2020; Ramos et al., 2020). *L. monocytogenes* was however still detected during the storage period and therefore it was suggested to apply increased bacteriocin concentrations or a combination of inhibiting compounds for total elimination of *L. monocytogenes* on food products (Ramos et al., 2020). Also other limitations of bacteriocins have been mentioned as high purification costs,

restricted antimicrobial spectrum, high dosage requirement and sensitivity to proteolytic enzymes (Garsa et al., 2014; Sidhu and Nehra, 2019). This all makes the use of this application not appropriate and too expensive to apply in mushroom post harvesting handling and processing environments. Moreover, it was mentioned that the combined action of the bacteriocins produced by LAB and the acidifying behaviour of the strains may result in the controlling and/or growth reduction of the *L. monocytogenes* strains (Pisano et al., 2022). However, the presence of a complex microbial community may not necessarily result in a pH decrease of the mushroom medium after incubation (**Chapter 4** and **Chapter 5,** data not shown), but rather showed a good microbial growth environment in terms of pH (close to neutral pH) (**Chapter 4** and **Chapter 5,** data not shown). This suggests that application of LAB strains used as natural control agents towards *L. monocytogenes* in food processing environments as mentioned before (Webb et al., 2022) is probably not feasible for mushroom environments.

6.5 *L. monocytogenes* growth modelling

A growth model was developed to estimate the growth of *L. monocytogenes* on mushroom products as function of temperature. Temperature is the most important extrinsic or intrinsic factor affecting the microbial growth, because other factors such as pH and water activity have limited growth inhibitory effects in fresh mushrooms. The pH values of fresh mushrooms and fresh mushroom medium in our study were predominantly just below pH 7 (**Chapter 3** and **Chapter 4**) and the water activity (aw) of mushrooms was between 0.993 and 0.996 (Leong et al., 2015), hence these values are within the optimal range for the growth of *L. monocytogenes* and were therefore not incorporated in the model ((optimum pH between 6 and 8) (Meloni, 2015)) ((optimum of aw ≥ 0.97) (Lado and Yousef, 2007)). The model was built using growth data derived from this study (**Chapter 3**; whole, sliced and smashed mushrooms, and mushroom medium), from a project partner (anonymous source) and from other studies that determined growth of *L. monocytogenes* on mushroom products (Chikthimmah et al., 2007; Leong et al., 2013). The growth rates obtained in this study were based on the growth increase during the first 24 hours of growth (increase in CFU/mL or CFU/gram during the first 24 hours of incubation), as growth decreased when incubation times were extended. The growth rate was estimated using the following equation:

$$\mu = \ln\left(\frac{N_t}{N_0}\right)/t$$

In which the μ is the specific growth rate (1/h), N_t is the concentration of cells at time t (concentration at 24 hours in this study), N_0 is the initial microbial concentration (concentration at start) and t is the time interval (24 hours in this study).

The square-root relationship proposed by Ratkowsky was used to model the effect of temperature on the growth rate (Ratkowsky et al., 1982) (figure 6.4):

$$\sqrt{\mu} = b(T - T_{min})$$

where b is a constant and T_{min} the theoretical minimum for growth (°C). The fitted parameters derived from the model fit in figure 6.4 were b= 0.0304 sqrt(/h/°C) and T_{min}= -2.260 °C.

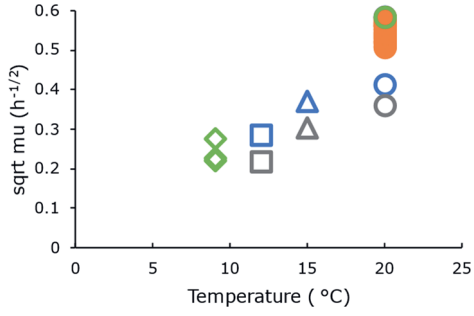


Figure 6.4. Specific growth rate of *L. monocytogenes* on mushroom products as function of temperature. The specific growth rate values (μ) were determined over a particular period of time in hours (h) for the *L. monocytogenes* strains and plotted as sqrt mu. Here, diamonds are data obtained from a project partner (anonymous source), squares are data adapted from Chikthimmah et al. (2007), triangles are data adapted from Leong et al. (2013) and circles are data adapted from this paper in which the specific growth rates were determined after one day of incubation. Grey, blue, green and orange color corresponds to whole mushrooms, sliced mushrooms, smashed mushrooms and mushroom medium, respectively.

The growth model was integrated in a user friendly interface (supplemental figure 6.1). The default input parameter for the initial concentration of *L. monocytogenes* was derived from the presence/absence testing of mushroom samples (**Chapter 2**), see supplemental figure 6.1C. This default input value was determined by incorporating

the amount of mushroom samples (60 fresh mushroom samples of 25 gram before harvesting) and the contamination level on these mushroom samples (no contamination, <1 CFU), resulting in the default input value for the initial contamination level of 0.0006667 CFU/gram (i.e. -3.2 log CFU/gram). The user can modify this input value and increase or decrease the initial concentration to simulate batches that have higher or lower contamination loads, respectively. Furthermore, the user can use the model to simulate the growth in different steps with different temperature/time regimes, and evaluate how changes in temperature and storage/processing times may affect the growth increase.

Frozen sliced mushroom products could be contaminated with *L. monocytogenes* as was shown in **Chapter 2**, but the counts of *L. monocytogenes* on the frozen sliced mushrooms during frozen storage will obviously not alter. However, thawing the frozen sliced mushroom product following by storing this product in the fridge will have an impact on *L. monocytogenes* numbers. This is especially important since this thesis demonstrated that *L. monocytogenes* can grow on mushroom products and mushroom medium (**Chapter 3**, **Chapter 4** and **Chapter 5**). The Information Document 85 published by the Netherlands Food and Consumer Product Safety Authority (NVWA (Nederlandse Voedsel- en Warenautoriteit), 2021), which is a Dutch interpretation document containing explanations of a number of texts from the Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs (EC No 2073/2005), includes a decision tree on control of *L. monocytogenes.* The decision tree mentions that the criterion of 100 CFU/gram should be met for thawed frozen products that have a limited shelf life (up to five days) after thawing. The growth model can be used to estimate the possible increase of *L. monocytogenes* during this shelf life of five days. Assuming a storage temperature of the domestic refrigerator of 7 °C (James et al., 2008; Roccato et al., 2017), a storage time for 5 days will result in a 4.1 log increase. Assuming that the initial concentration of *L. monocytogenes* on the frozen sliced mushroom is 1 CFU/25 gram product (i.e. -1.4 log CFU/gram, the minimum concentration of a positive-tested sample), the final *L. monocytogenes* cell counts could then be 2.7 log CFU/gram, above the limit of 100 CFU/gram at the end of shelf life. This example calculation illustrates the importance that frozen sliced mushrooms are not be sold as ready-to-eat, but should be sold as ready-to-cook. Based on a previous outbreak of *L. monocytogenes* linked to frozen vegetables (frozen corn) (EFSA (European Food Safety Authority), 2018), hygiene guidelines for the control of *L. monocytogenes* in the production of quick-frozen vegetables have been established by

PROFEL (PROFEL (European Association of Fruit and Vegetable Processors), 2020). Also another document provides recommendations with regard to the control of *L. monocytogenes* in frozen fruits and vegetables (Koutsoumanis et al., 2020). This document also describes to standardise the labelling by food producers which will lead to a better consumer understanding (Koutsoumanis et al., 2020). Appropriate labelling of food products on the intended preparation and use is therefore important. Frozen sliced mushroom products should therefore not be incorporated in meals for direct human consumption that do not undergo a bacterial elimination step, such as salads.

The developed model may be restricted to growth prediction in mushrooms since it could be assumed that the nutrient composition of other food products may be different. Therefore, for modelling the growth of *L. monocytogenes* on other food products, it is recommended to experimentally test the growth of *L. monocytogenes* on these food products for model development.

6.6 *Listeria monocytogenes* on surface materials and the cleaning and disinfection practices

Quantification of the biofilm formation by biofilm cell counts in experiments with *L. monocytogenes* mushroom isolates with or without the microbiota showed a large impact of the material surface type; stainless steel and polyvinyl chloride/conveyor belt material. In addition, a large impact of the material surface type was also observed when these biofilms were subjected to cleaning and disinfection agents used in food processing environments. Besides, also the effect of desiccation stress on *L. monocytogenes* survival when suspended in mushroom medium was impacted by the material surface type. This showed that material surface types has an effect during biofilm formation and corresponding C&D treatments, but also during desiccation stress (**Chapter 5**). Because of the differences between materials, it has been suggested that the type of surface material for specific applications or surfaces should be carefully chosen within the available options (Wilks et al., 2006).

The results presented in this study (**Chapter 5**) were obtained with new conveyer belt materials, while these materials in practice are obviously used for extended periods of time. Used conveyor belt material has been shown to have a higher surface roughness value compared to new conveyor belt material. This may lead to increased *L.*

monocytogenes biofilm formation and decreased *L. monocytogenes* susceptibility towards C&D treatments that may be due to increases in the possible hiding places of *L. monocytogenes* (Chaturongkasumrit et al., 2011). As many different materials are used in the mushroom production and processing environment (Dygico et al., 2020), it could be assumed that this phenomenon can also be applied to these other materials. The used materials may therefore provide increased safety issues with respect to pathogen eradication. As it is difficult to substitute materials by other kinds of materials in environments that handle food products, it is important that C&D regimes and the lifetime of the materials used in these environments are critically assessed and that materials are replaced in time. Such an approach may further reduce surface contamination and (mixed) biofilm formation of *L. monocytogenes* on these materials and decrease the possible cross-contamination of the processed food products.

The regimes of repeated C&D treatments in this study resulted in increased resistance of *L. monocytogenes* monospecies and multispecies biofilms (**Chapter 5**), which was also observed before for *L. monocytogenes* biofilms grown in non-selective nutrient-rich medium (Fagerlund et al., 2020; Pan et al., 2006). This could be explained by the protective nature of biofilms towards disinfectants (Spanu and Jordan, 2020) as detached *L. monocytogenes* biofilm cells did not show such an increased resistance (Pan et al., 2006). Therefore, protective effects of the biofilm towards disinfectants may be due to biofilm matrix components and not by intrinsic factors of the individual biofilm cells (Pan et al., 2006; Yun et al., 2012). The lower log reductions over time of the biofilm might be related to a more mature biofilm during prolonged incubation as it has been shown that older biofilms have lower log reductions after C&D procedures compared to newly formed biofilms (Carpentier and Cerf, 2011; Chaturongkasumrit et al., 2011; Yang et al., 2009). As a similar trend was observed in this study (**Chapter 5**), it is therefore more likely that disinfection tolerance is caused by the protective effect of the biofilm matrix rather than intrinsic properties of the individual cells. The survival of adhered bacteria, including pathogenic bacteria, may then cause increased food safety risks (González-Rivas et al., 2018). Therefore, the application of C&D treatment steps in food post harvesting handling and processing environments is essential to provide food products that are microbiologically safe.

However, several studies have shown the inability of C&D agents used in food processing environments to effectively remove laboratory or industrial biofilms from surfaces that consisted of various bacteria (including *L. monocytogenes*) (Bagge-Ravn

et al., 2003; Fagerlund et al., 2017; Fagerlund et al., 2020; Kaneko et al., 1999; Lehto et al., 2011; Mazaheri et al., 2022). It was described that current C&D practices often showed inabilities of effectively removing and eliminating microorganisms that are present in the biofilm (Mazaheri et al., 2021). Promising improvements could be obtained by using higher disinfectant concentrations, disinfectant combinations and increased exposure times compared to regular cleaning and disinfection procedures (Chaturongkasumrit et al., 2011; Fagerlund et al., 2020; Tolvanen et al., 2007). Increasing efficacy of C&D treatments could also be established with an additional drying step following C&D treatments since this resulted in increased CFU reductions (Overney et al., 2017). Other results indicated the need to train personnel for C&D purposes to improve practices in the disinfection operations for minimizing cross-contamination (Agüeria et al., 2021; Charalambous et al., 2015; Coughlan et al., 2016). Also rotation of disinfectants containing different active substances was recommended for avoiding strain resistance and improve C&D efficiency (Meyer, 2006). However, applications of various disinfectants towards *L. monocytogenes* (and *L. innocua*) have shown cross-adaptation, even with unrelated disinfectants (Lundén et al., 2003a; Pan et al., 2006; Rahman et al., 2022). In addition, thorough sanitation in addition to daily sanitation, is negatively associated with *L. monocytogenes* (Aalto-Araneda et al., 2019). Thorough sanitation includes dismantling of complex machinery for making the surface accessible to disinfectants (Aalto-Araneda et al., 2019; Holah, 2014; Ortiz et al., 2016; Wirtanen and Salo, 2003) However, equipment present in the food processing environment is usually complex and difficult or even impossible to dismantle for cleaning (Aarnisalo et al., 2006; Tolvanen et al., 2007). The dismantling and reassembling of the equipment is also time and labour intensive, generating high expenses for the producer (Horigan et al., 2019). Partly dismantling machinery may not be satisfactory as the partly dismantling of a dicing machine has shown that not all parts of the machine are well reached during the C&D treatment and these difficult-to-reach places may potentially contain *L. monocytogenes* (Lundén et al., 2003b). Therefore, as food processing facilities may harbour sites that are difficult or impossible to clean, many authors and official documents have been insisting for the need to improve the hygienic design of equipment within food processing environments (Carpentier and Cerf, 2011). Improvements of the hygienic design of food processing equipment may then lead to decreased harborage spots for *L. monocytogenes* and a better accessibility of the C&D agents towards the remaining harborage spots. Together with the application of the above mentioned improved practices in environments that handle food products (mushrooms), this may lead to the increased

eradication of *L. monocytogenes* from these environments and therefore to decreases in the possibility of food product contamination.

6.7 Emerging disinfection methods in eliminating *L. monocytogenes*

Although disinfectants are at this moment the best method to combat biofilms (Carrascosa et al., 2021), this study (**Chapter 5**) and other studies (Fagerlund et al., 2017; Fagerlund et al., 2020; Mazaheri et al., 2022) also showed the ineffective removal of *L. monocytogenes* from surfaces with C&D agents that are used in food processing environments. This led to the development of novel biocontrol methods for *L. monocytogenes* biofilms (Gray et al., 2018) including application of specific enzymes (Mazaheri et al., 2022). Enzyme-based detergents may be applied to disrupt the biofilm matrix components in which biofilm cells are embedded (Bridier et al., 2011). These enzyme-based detergents may partly degrade the biofilm matrix, making the microorganisms more accessible and therefore more sensitive for the disinfection agent (Bridier et al., 2011; Galié et al., 2018). These enzymes may also lead to the detachment and dispersal of the biofilm structure (Mazaheri et al., 2022). This removal of the biofilm by enzymes could be executed with single enzymes, but some bacterial species require enzyme mixtures (Galié et al., 2018). Hence, commercial enzyme formula contain mixtures of enzymes with different substrate spectra to combat different components of the biofilm (Bridier et al., 2011). Promising results have been obtained with enzymatic treatment tests of mature *L. monocytogenes* biofilms on stainless steel that showed significant reduction of all *L. monocytogenes* strains (Mazaheri et al., 2020). Although various commercial enzyme mixtures are available (Torres et al., 2011), it could be suggested that their reduction capacity may be limited in complex microbial communities containing many different kinds of biofilm matrix components. Moreover, the application of enzymes to control biofilm nowadays is restricted as the costs of disinfectants are less compared to enzymes. Also the usage of the of different enzyme activities are restricted, because of their low commercial accessibility (Simões et al., 2010).

Another strategy to control *L. monocytogenes* in food production systems is the application of bacteriophages, viruses that infect and kill bacteria (Bridier et al., 2011; Carrascosa et al., 2021). Bacteriophages are however highly specific and therefore have a narrow host range, but this may be overcome with phage cocktails that may

be applied for a broader target (Bridier et al., 2011; Kawacka et al., 2020). These phage cocktails have shown high efficacies towards *L. monocytogenes* (Byun et al., 2022; Ganegama Arachchi et al., 2013). Therefore, phage cocktails are mentioned as potential promising candidates in the biocontrol of *L. monocytogenes* (Byun et al., 2022). However, good efficacies of phage cocktails in the mushroom post harvesting handling and processing environments are very doubtful because of the high genetic diversity of *L. monocytogenes* strains in these environments (**Chapter 2**). A bacteriophage mixture may then, because of the narrow host range, not efficiently target all *L. monocytogenes* subtypes present, resulting in an inadequate control strategy for *L. monocytogenes* removal from the mushroom post harvesting handling and processing environments. Another limitation of phages is targeting bacterial cells within biofilm structures. The biofilm structure and the extracellular material form a physical obstacle for phage diffusion (Galié et al., 2018; Ganegama Arachchi et al., 2013). The spatial structure of the biofilm may also affect phage efficacy as resistant cells within the biofilms may protect clusters of susceptible cells (Simmons et al., 2020). In addition, the extracellular material may also include organic matter that may influence the efficacy of phage treatments (Gray et al., 2018) as the target bacteria may be shielded from diffusing phage particles by the food matrix (Guenther et al., 2009; Rossi et al., 2011). Organic matter removal may therefore increase phage treatment efficacies (Ganegama Arachchi et al., 2013), but the high presence of organic material in mushroom post harvesting handling and processing environments may suggest high constraints to the use of phages in this environment. Phages that targeted *L. monocytogenes* within biofilms however showed promising results in successfully removing *L. monocytogenes* from stainless steel and polystyrene surfaces when the concentration of the phage was high enough (Iacumin et al., 2016; Rodríguez-Melcón et al., 2018). This showed that the phage dosage is also an important factor for the success of the bacteriophages in *L. monocytogenes* reduction (EFSA (European Food Safety Authority), 2012; Montañez-Izquierdo et al., 2012). Therefore, the application of bacteriophages to control *L. monocytogenes* could be promising in limited cases, but the use of large phage dosages may be costly (Chaitiemwong et al., 2014). However, for sufficiently controlling *L. monocytogenes* in the large mushroom post harvesting handling and processing environments, large phage dosages are needed. This application will therefore be insufficient and too costly for commercial application in large food post harvesting handling and processing environments, also since regular disinfectants are much cheaper. So, the use of regular cleaning and disinfection agents (as described in **Chapter 5**) applied with improved

practices (as mentioned in section 6.6, **this Chapter**) may currently be suggested as the best way to control *L. monocytogenes* in food post harvesting handling and processing environments.

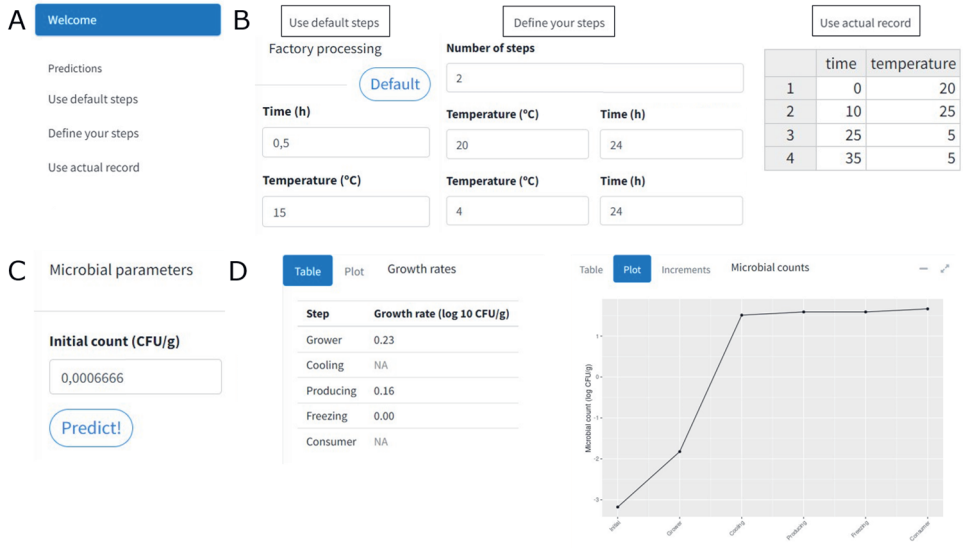
6.8 Main observations, recommendations and future perspectives

The research described in this thesis provided new insights into the ecology and the physiological behaviour of *L. monocytogenes* with respect to the whole production and processing chain of frozen sliced mushrooms. The isolation of *L. monocytogenes* from mushrooms after the first processing step (mechanical harvesting) points to the post harvesting handling and processing environments as main contamination factor. Therefore, *L. monocytogenes* should be especially controlled in these environments by combatting biofilms and preventing biofilm formation. This pathogen is a strong biofilm former and it can maintain itself in biofilms in the presence of background microbiota. Therefore, good cleaning and disinfection operations should be applied to decrease the presence of *L. monocytogenes* in the post harvesting handling and processing environments. Although current C&D treatments are insufficient in the effective removal of *L. monocytogenes* from food processing environments, they are still considered as the best option since novel technologies are limited and/or (still) too expensive for application in large food processing environments. Increased efficacies with conventional C&D agents could be obtained by increasing the current frequency of cleaning and disinfection procedures. Besides, increased efficacies could also be obtained with extensive cleaning procedures before disinfection application that will increase the access of disinfection agents to biofilms on food processing equipment. This can be done with rigorous cleaning for removing the organic material and disrupt biofilm structures that may otherwise inhibit the disinfectant functionality. This also includes the dismantling of the processing equipment for better targeting biofilms in hard-to-reach places if these places could otherwise not easily be accessed. Such thorough cleaning treatments in which biofilms could be more easily accessed by the disinfectant may result in a better control of *L. monocytogenes* in mushroom post harvesting handling and processing environments, lowering the possibility of cross-contamination. As stated by the Information document 85 (NVWA (Nederlandse Voedsel- en Warenautoriteit), 2021), the producer of a particular food product determines whether a food product is a ready-to-eat food product. Although people may consume fresh mushrooms raw, frozen sliced mushroom should be considered

and labelled as ready-to-cook, not ready-to-eat. Future research is recommended to focus on the mushroom post harvesting handling and processing environments as the main *L. monocytogenes* contamination source by carefully monitoring this contamination and identifying the harborage places of *L. monocytogenes*. Such thorough process evaluation at location could further extend the knowledge about the ecology and physiological behaviour of *L. monocytogenes* by incorporating on-site data of *L. monocytogenes*, with the aim to reduce and if possible prevent colonization of this pathogen in food post harvesting handling and processing environments.

Supplemental information

Supplemental figure 6.1.



Supplemental figure 6.1. The interface of the growth modelling tool. (A) For the convenience of the users, the growth modelling tool consists of different tabs for data input for performing modelling to the users preference for determining the growth of *L. monocytogenes* on the mushroom product. (B) In each tab, only the time and temperature need to be altered/entered or uploaded via a datasheet to determine the growth of *L. monocytogenes*. The first tab (Use default steps) includes default steps with the most important steps during mushroom processing in which the user may manually alter the time and temperature to its own preference. The amount of steps with the according time and temperature could be manually entered in the second tab (Define your steps). The third tab (Use actual record) processes data from datasheets containing the time and the corresponding temperature for each step. All tabs relied on the input of time and temperatures data to determine the growth of *L. monocytogenes* on mushrooms during processing. (C) The initial count should be altered/entered from which the model starts to determine the growth increase of *L. monocytogenes* throughout the time/temperature phases of B. (D) The output of the model visualizes the growth of *L. monocytogenes* using the entered values throughout the time/temperature phases. Access tool via: https://foodmicrowur.shinyapps.io/concept_mushroom/.

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Appendix

Summary

Samenvatting

Acknowledgements

About the author

Affiliation of author and co-authors

List of publications

Overview of completed training activities

Summary

Listeria monocytogenes is an important human foodborne pathogen, able to cause severe listeriosis infection, particularly in pregnant women and their newborns, adults aged 65 or older, and people with weakened immune systems. Other people can be infected with this pathogen, but they rarely become seriously ill. This pathogen is ubiquitous in the natural environment and widely distributed in food processing environments. It is a robust micro-organism and able to survive various forms of stress mainly due to its ability to form robust biofilms. The biofilm formation of *L. monocytogenes* in complex microbial communities in food processing environments causes food safety issues, because residing biofilm cells may detach during food production and contaminate food products. This may be harmful to consumers, especially if the food product does not undergo a bacterial elimination step before consumption. Presence of *L. monocytogenes* on food products may also cause recalls, resulting in unexpected costs and brand name damage. Therefore, a good understanding of the ecology and the physiology of *L. monocytogenes* in food processing environments is needed in order to control this pathogen and to prevent the contamination of food products during production and processing. This is why this study aimed to obtain insight in the eco-physiological behaviour of *L. monocytogenes* in mushroom production and processing environments.

The presence, abundance and genetic diversity of *L. monocytogenes* was determined and described along the most important steps of the mushroom production and processing chain in **Chapter 2**, following one mushroom batch from a mushroom grower to the mushroom processing factory. The first *L. monocytogenes* positive samples were isolated from casing soil just before mushroom harvest, while no *L. monocytogenes* could be isolated from the mushrooms on top of this casing soil with the current NEN-EN-ISO-11290 enrichment procedures. Notably, up to 50% of the mushroom samples taken after mechanically harvesting and up to 29% of frozen sliced mushrooms after processing tested positive for *L. monocytogenes*. Additionally, mushroom processing equipment was shown to be contaminated with *L. monocytogenes*, even after cleaning and disinfection (C&D). This suggests that the mushroom processing environment and equipment used are important sources of contamination with *L. monocytogenes*. The isolated *L. monocytogenes* strains showed high genomic diversity, with strains belonging to multiple lineages, serogroups, clonal complexes and sequence types, with all strains harboring the genetic repertoire of

virulence determinants necessary for infection development. Notably, additional experiments provided evidence that all strains were susceptible towards a wide range of antibiotics tested, including those that are currently used to treat *L. monocytogenes* infections in humans.

The growth potential of individual *L. monocytogenes* strains in mushroom medium that mimics the nutrient availability in mushroom processing environments was described in **Chapter 3**. This showed that the genetically highly diverse set of *L. monocytogenes* strains isolated from mushroom processing environments had comparable growth and biofilm characteristics. Moreover, the growth characteristics of the mushroom isolates were also rather comparable to *L. monocytogenes* strains isolated from other sources including foods and patients, pointing to remarkably low strain variability in growth performance, conceivably due to extensive nutrient richness on the model food medium. *L. monocytogenes* isolates also showed growth on whole, sliced and smashed mushrooms, with the highest final cell counts (Colony Forming Units, CFUs per gram of product) reached in the smashed mushrooms, followed by the sliced and the whole mushrooms. Notably, these mushroom products support *L. monocytogenes* growth despite the presence of high numbers of naturally present background microbiota (~7 log CFU/gram) on the fresh mushrooms at the start and which increased during incubation.

The competitive performance of *L. monocytogenes* with background microbiota isolated from mushroom processing environments was described in **Chapter 4**. *L. monocytogenes* was able to grow in dual culture incubations with selected background microbiota strains, including Gram-negative and Gram-positive bacteria. The growth performance of *L. monocytogenes* depended on the type of microbiota strain, with limited increase in cell counts of *L. monocytogenes* in co-culture with acidifying strains. *L. monocytogenes* was also able to grow in the spent media of the non-acidifying background microbiota strains, despite the high (>8.5 log CFU/mL) CFU levels that microbiota strains has achieved during single culture pre-incubation. Growth of *L. monocytogenes* was however not supported in spent media of acidifying strains, but the ability to grow was restored after increasing the pH of the spent media to neutral values. This indicated that the pH of the growth medium in combination with the (weak) acids produced by specific microbiota, is the main growth limiting factor for *L. monocytogenes* in the tested conditions. Combining all results shows that growth performance of *L. monocytogenes* in absence and presence of microbiota is not

hampered by nutrient availability of the mushroom medium and on fresh and processed mushrooms.

Presence of *L. monocytogenes* in complex microbial communities and the effect of cleaning and disinfection treatments were examined in laboratory experiments described in **Chapter 5**. *L. monocytogenes* was able to establish itself in complex microbial communities, both in planktonic state and in biofilm state on selected polyvinyl chloride and stainless steel surface materials that are often used in mushroom growing and processing environments. Biofilms formed on stainless steel coupons were found more susceptible to selected cleaning and disinfection regimes than biofilms formed on polyvinyl chloride coupons, and *L. monocytogenes* counts dropped below detection limit in all tested conditions. Notably, using polyvinyl chloride as surface material showed less efficient removal and/or inactivation of *L. monocytogenes* biofilm cells which led to the outgrowth of *L. monocytogenes* following re-incubation in fresh medium. Repeated cleaning and disinfection treatments followed by re-incubation of these polyvinyl chloride coupons resulted in regrowth of *L. monocytogenes* within the complex microbiological biofilm, which the growth of *L. monocytogenes* to the original population size. Besides, *L. monocytogenes* was also able to maintain itself in complex microbial communities during desiccation on stainless steel and polyvinyl chloride surfaces. The application of regular cleaning and disinfection procedures however resulted in counts below the detection limit in the tested experimental setting.

The results obtained in this thesis (**Chapter 2-5**) are discussed in **Chapter 6** and a model is presented that allows prediction of growth performance of *L. monocytogenes* during mushroom processing and storage, which can be used to determine potentially critical processing steps. The obtained knowledge gives additional directions to control and thus reduce contamination of frozen sliced mushrooms with this notorious human pathogen.

Samenvatting

Listeria monocytogenes is een belangrijke voedselpathogeen die de ernstige ziekte listeriosis kan veroorzaken in vooral zwangere vrouwen en hun pasgeborenen, in volwassenen van 65 jaar en ouder en in mensen met een verzwakt immuunsysteem. Andere mensen kunnen ook geïnfecteerd worden met deze pathogeen, maar zij worden zelden ernstig ziek. *L. monocytogenes* is wijdverspreid in het milieu en ook in omgevingen waar voedsel wordt verwerkt. Daarnaast is het een robuust micro-organisme en in staat om verschillende vormen van stress overleven, mede dankzij het vormen van biofilms op oppervlaktes. *L. monocytogenes* overleeft in biofilms samen met andere micro-organismen in omgevingen waar voedsel wordt verwerkt en deze biofilms kunnen zorgen voor een voedselveiligheidsrisico. De biofilmcellen kunnen namelijk loslaten van een oppervlakte tijdens het productieproces en zo voedselproducten contamineren. Dit kan een gevaar opleveren voor de consument, en dit is vooral het geval als het specifieke voedselproduct niet wordt verhit voor consumptie waarbij de eventuele aanwezige bacteriën dan dus niet worden afgedood. Daarnaast kan de aanwezigheid van *L. monocytogenes* op voedselproducten ook resulteren in terugroepacties, wat kan leiden tot onvoorziene hoge kosten en imagoschade van het product en/of bedrijf. Daarom is het nodig om inzicht te verkrijgen in de ecologie en de fysiologie van *L. monocytogenes* in productieomgevingen van levensmiddelen om zo mogelijk deze pathogeen te bestrijden en te voorkomen dat voedselproducten worden gecontamineerd tijdens het productie- en/of verwerkingsproces. Het hier beschreven onderzoek richt zich op het ecofysiologische gedrag van *L. monocytogenes* in omgevingen waar champignons worden geproduceerd en verwerkt.

De aanwezigheid, de hoeveelheid en de genetische diversiteit van *L. monocytogenes* tijdens de meest belangrijke stappen in de productie- en verwerkingsketen van champignons zijn onderzocht in **Hoofdstuk 2**. Hiervoor werd een bepaalde partij champignons in alle ketenstappen gevolgd en bemonsterd met de NEN-EN-ISO-11290 ophopingsprocedure; monsters werden verzameld bij de champignonteler tot aan het bedrijf dat de champignons verwerkte. De eerste monsters die positief waren getest voor *L. monocytogenes* werden geïsoleerd uit de dekaarde net voor het oogsten van de champignons, terwijl de champignons die waren gegroeid bovenop deze dekaarde allemaal negatief waren voor *L. monocytogenes*. Na het proces van mechanisch oogsten was opvallend genoeg tot 50% van de geteste verse champignonmonsters

positief voor *L. monocytogenes*, en daarnaast was tot 29% van de gesneden en bevroren champignonmonsters positief voor *L. monocytogenes*. Ook de omgeving waar champignons worden verwerkt bleek besmet te zijn met *L. monocytogenes*, zelfs na schoonmaken en desinfecteren. Dit suggereert dat de apparatuur en de omgeving waar de champignons worden verwerkt een belangrijke contaminatiebron zijn. De geïsoleerde *L. monocytogenes* stammen lieten een hoge genetische diversiteit zien, en de stammen konden worden onderverdeeld in meerdere lineages, serogroepen, klonale complexen en sequentietypes, waarbij alle geteste stammen het genetische repertoire van virulentiegenen bevatten die nodig zijn om een infectie te ontwikkelen in mensen. Aanvullende experimenten lieten zien dat alle geteste stammen gevoelig waren voor een breed scala aan antibiotica, inclusief de antibiotica die tegenwoordig worden toegepast om humane *L. monocytogenes* infecties te behandelen.

De groeipotentie van verschillende *L. monocytogenes* stammen is onderzocht in **Hoofdstuk 3**. Deze stammen werden gekweekt in champignonmedium dat qua samenstelling de beschikbare nutriënten in de productieomgeving van champignons nabootst. De genetisch zeer diverse *L. monocytogenes* stammen die waren geïsoleerd uit de verwerkingsomgevingen van champignons lieten vergelijkbare groei en biofilmvorming zien. Daarnaast waren de groeikarakteristieken van deze *L. monocytogenes* stammen vergelijkbaar met *L. monocytogenes* stammen die waren geïsoleerd uit andere omgevingen, waaronder andere voedingsmiddelen en patiënten. Dit wijst op een opvallend lage stamvariabiliteit van de geteste *L. monocytogenes* stammen, wat mogelijk kan worden toegeschreven aan de rijke hoeveelheid nutriënten in het champignonmedium. Daarnaast was *L. monocytogenes* ook in staat om te groeien op hele, gesneden en geplette champignons. Hierbij werden de hoogste cel concentraties (Kolonie Vormende Eenheden, KVE per gram product) bereikt in geplette champignons, gevolgd door gesneden en hele champignons. Opmerkelijk genoeg waren de champignonproducten een rijke voedingsbodem voor groei van *L. monocytogenes* ondanks de aanwezigheid van hoge aantallen achtergrond microbiota op de verse champignons.

De competitie van *L. monocytogenes* met microbiota stammen die waren geïsoleerd uit de verwerkingsomgevingen van champignons is onderzocht in **Hoofdstuk 4**. Het samen opkweken van een *L. monocytogenes* stam met een geselecteerde microbiota stam resulteerde in groei van beide stammen. De mate van groei van *L. monocytogenes* was echter wel afhankelijk van de bacteriesoort, want de groei van *L.*

monocytogenes was beperkt wanneer de microbiota stam het medium verzuurde. *L. monocytogenes* was daarnaast in staat om te groeien in medium waarin eerst een microbiota stam gekweekt was tot hoge concentraties (>8.5 log CFU/mL). Echter, wanneer dit gebruikte kweekmedium verzuurd was door de microbiota stam, werd de groei van *L. monocytogenes* geremd, maar een kunstmatige pH verhoging van het gebruikte kweekmedium herstelde vervolgens de groei van *L. monocytogenes*. Dit laat zien dat de pH van het groeimedium in combinatie met de (zwakke) zuren die worden geproduceerd door specifieke microbiota stammen de belangrijkste factoren zijn voor gereduceerde groei van *L. monocytogenes* in de geteste condities. De resultaten van de co-incubaties en groeiexperimenten in gebruikt kweekmedium laten zien dat *L. monocytogenes* in staat is om te groeien in de afwezigheid en aanwezigheid van microbiota en dat deze groei niet wordt gelimiteerd door de beschikbaarheid van nutriënten in het champignonmedium.

Hoofdstuk 5 beschrijft de competitie van *L. monocytogenes* in biofilms met andere micro-organismen en het effect van schoonmaken en desinfecteren om biofilms van oppervlakten te verwijderen. *L. monocytogenes* was in staat om samen te groeien met andere micro-organismen, zowel in het medium als in biofilms op de oppervlaktematerialen polyvinyl chloride en roestvrijstaal, materialen die veel voorkomen in omgevingen waar champignon worden geproduceerd en verwerkt. De gevormde biofilms op de roestvrijstalen coupons waren gevoeliger voor de schoonmaak- en disinfectiebehandelingen dan de biofilms die gevormd waren op de polyvinyl chloride coupons. Dit resulteerde in *L. monocytogenes* cel concentraties beneden het detectieniveau na de schoonmaak- en desinfectiebehandelingen van de roestvrijstalen coupons. Het gebruik van polyvinyl chloride als oppervlaktemateriaal liet echter een onvoldoende verwijdering en/of inactivatie zien van *L. monocytogenes*, en dit resulteerde in uitgroei van *L. monocytogenes* tijdens de re-incubatie in vers champignonmedium. Het herhalen van deze schoonmaak- en desinfectiebehandelingen en het opnieuw incuberen in vers champignonmedium resulteerde opnieuw in de uitgroei van *L. monocytogenes* op de polyvinyl chloride coupons. Daarnaast was *L. monocytogenes* ook in staat zich samen met microbiota stammen te handhaven tijdens uitdroging op zowel polyvinyl chloride en roestvrijstaal coupons. Het toepassen van schoonmaak- en desinfectiebehandelingen op deze ingedroogde cellen was zeer effectief en resulteerde in verlaging van de cel concentraties beneden het detectieniveau.

De resultaten verkregen in dit proefschrift (**Hoofdstuk 2-5**) zijn bediscussieerd in **Hoofdstuk 6** waarin ook een model wordt gepresenteerd dat gebruikt kan worden om de groei van *L. monocytogenes* te voorspellen tijdens het verwerken en de opslag van champignons. Dit model kan dan worden gebruikt om potentiële kritische stappen in het productieproces van champignons te bepalen. De verkregen kennis in dit onderzoek geeft handvatten om de besmetting van bevroren en gesneden champignons met deze voedselpathogeen te beheersen en te verminderen.

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I had never competed in a running race before and I even didn't have running shoes, but it went amazingly well! I even liked running so much that I continued running after this running event. Thank you for that! **Esther**, you introduced the Thursday running in our department to prepare everybody for the Veluweloop. This was a very good initiative that we also continued after the running event. And thank you for organizing a Veluweloop team for multiple times! **James**, thank you for continuing in doing the Thursday running even during the covid pandemic. We have kept the tradition alive and managed to stay in good shape. Also thank you for caring for me and in pushing me to go home when I was (again) working in the lab during the evening. Thank you for all the nice moments, you are a great person and good friend. **Alberto G**, I want to thank you for the help with the growth model we constructed. This would be impossible without your help and expertise in programming and the visualization of the growth model! In addition, I also want to thank you for playing football together. This was really nice, especially during the tournament when we won the first prize!

I want to especially thank **Xuchuan** and **George** for being my paranymphs. It really means a lot to me that you are standing next to me on this special occasion. **Xuchuan**, you are such a kind person! You developed wonderful skills in programming in which you also helped me during one of my projects for which I want to thank you for that. Your scripts are really useful for the department! I really admire your patience and dedication you have for your project as it took a lot of effort in constructing the right mutants. **George**, thank you for the nice moments during the PhD trip in Germany and Switzerland. Thank you for driving us around safely with the car and for your stories you told us in the car. And I really admire your perseverance and commitment to get the best out of yourself. You have also become an important member of the Thursday running team and I hope you can join our team for the next Veluweloop!

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About the author

Frank Bernardus Lake was born on the 5th of April 1993 in Groningen, the Netherlands. He completed vwo at the Hondsrug College in Emmen after which he started his bachelor studies in Biology at the University of Groningen, because of his broad interest in biology. The first half year consisted of courses dealing with many aspects within the field of biology during which he discovered the topics he likes the most: molecular biology and microbiology! So, after half a year, he chose the major Molecular Biology with a minor in pharmacy. He completed his bachelor with a thesis at the department of Molecular Microbiology.

After his bachelor studies, he continued studying at the University of Groningen with the master Molecular Biology and Biotechnology. During this master, he followed a variety of courses organized by different departments that also involved practical work within the laboratories of these departments. By working with many different techniques and the different focus points of the departments, he developed a strong interest in molecular biology, microbiology and molecular genetics. That is why he performed two theses in two different departments that covered these subjects, namely the department of Molecular Genetics and the department of Medical Microbiology.

In the fall of 2017, he started a PhD project at the Laboratory of Food Microbiology at Wageningen University and Research with the topic entitled "Ecophysiological behaviour of *Listeria monocytogenes* in *Agaricus bisporus* mushroom processing environments", which he completed by defending this thesis.

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List of publications

(This dissertation)

Lake, F. B., van Overbeek, L. S., Baars, J. J. P., Koomen, J., Abee, T. & den Besten, H. M. W. (2021). Genomic characteristics of *Listeria monocytogenes* isolated during mushroom (*Agaricus bisporus*) production and processing. International Journal of Food Microbiology, 360, 109438, https://doi.org/10.1016/j.ijfoodmicro.2021.109438.

Lake, F. B., van Overbeek, L. S., Baars, J. J. P., Abee, T. & den Besten, H. M. W. (2023). Variability in growth and biofilm formation of *Listeria monocytogenes* in *Agaricus bisporus* mushroom products. Food Research International, 165, 112488, https://doi.org/10.1016/j.foodres.2023.112488

Lake, F. B., van Overbeek, L. S., Baars, J. J. P., Abee, T. & den Besten, H. M. W. (2023). Growth performance of *Listeria monocytogenes* and background microbiota from mushroom processing environments. International Journal of Food Microbiology, 395, 110183, https://doi.org/10.1016/j.ijfoodmicro.2023.110183

Lake, F. B., Chen, J., van Overbeek, L. S., Baars, J. J. P., Abee, T. & den Besten, H. M. W. Biofilm formation and desiccation survival of *Listeria monocytogenes* with microbiota on mushroom processing surfaces and the effect of cleaning and disinfection. Under review.

Overview of completed training activities

A. Discipline specific activities

Name of the course/scientific meeting	*Organizers*	*City*	*Year*
Reaction kinetics in food sciences		Wageningen	2018
Congress HUPLANT *		Dubrovnik, Croatia	2019
Symposium MB 6.0 Fall Meeting		Delft	2019
Symposium VLAG Fall Meeting 2019		Wageningen	2019
Symposium Food Science Cluster *		Wageningen	2020
Symposium Fall Meeting KNVM		Online	2020
Symposium on Genomics Epidemiology of Infectious Diseases		Online	2020
LAS-ICMSF webinar		Online	2021
IAFP-Europe Annual Meeting *		Online	2021
Microbiology Society Annual Conference		Online	2021
VLAG online lecture series		Online	2020/ 2021
Congress FEMS		Online	2021
Congress HUPLANT *		Online	2021
IAFP Annual Meeting		Online	2021
Ecophysiology of food-associated micro-organisms: Roles in health and disease		Wageningen	2021

B. General courses

Name of the course/scientific meeting	*Organizers*	*City*	*Year*
VLAG PhD week	VLAG	Baarlo	2018
Statistics: Introduction to R	VLAG	Wageningen	2018
Basic statistics	PE&RC	Wageningen	2018
Teaching and supervising thesis students	WGS	Wageningen	2019
Reviewing a scientific paper	WGS	Wageningen	2019
R-workshop	FHM	Wageningen	2019
Posters and pitching	WGS	Wageningen	2020
The essential of scientific writing and presenting	WGS	Wageningen	2020
Applied statistics	VLAG	Wageningen	2020
Philosophy and ethics of food science and technology	VLAG	Wageningen	2021
Career perspectives	WGS	Wageningen	2021

C. Assisting in teaching and supervision activities

Name of the course/scientific meeting	*Organizers*	*City*	*Year*
FHM-20306 Food Microbiology	FHM	Wageningen	2017-2020
FHM-22306 Advanced food microbiology	FHM	Wageningen	2018-2020
Supervision of 6 MSc students	FHM	Wageningen	2018-2021

D. Other activities

Name of the course/scientific meeting	*Organizers*	*City/Country*	*Year*
Preparation of research proposal	FHM	Wageningen	2017
PhD study tour to China *	FHM	China	2019
Food Microbiology department seminars *	FHM	Wageningen	2017-2022
PhD study tour to Germany/Switzerland *	FHM	Germany/ Switzerland	2022
PhD study tour to Germany/Switzerland - organization	FHM	Wageningen	2022

* Oral Presentation

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