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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 h 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 h of incubation, while counts of the microbiota strains ranged from 5.5 to 9.0 log CFU/mL. Prolonged incubation up to 48 h 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.

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 (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

Abbreviations: CV, crystal violet; LAB, lactic acid bacteria; *Lm*, *Listeria monocytogenes*; MM, mushroom medium.

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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.

2. Materials and methods

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 3M™ petrifilm™ aerobic count plates (3M company), and petrifilms were incubated at 30 °C for 72 h. 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 h at 30 °C. Single colonies of non-pure cultures were restreaked on TSAYE plates followed by incubation for 24 to 48 h 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 h 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 h 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 h at 30 °C followed by another streak on BHI agar plates incubated for 24 h at 30 °C to obtain pure isolates. A single colony was transferred to BHI broth that was cultured statically for 17 h at 30 °C, and −80 °C stock cultures were prepared with a final concentration of 25 % glycerol (Sigma-Aldrich).

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 h 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 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) 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 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. Then, 5 μL of PCR product was mixed with 1 μL 6× loading dye (TriTrack, Thermo Scientific) and the mixture was loaded and examined in a 1 % agarose (SeaKem LE agarose, Lonza) gel containing 1× TAE buffer (Bio-Rad) and DNA safe stain (SYBR Safe DNA

Gel Stain, Invitrogen). Gels were run in a 1× 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* (supplementary Table 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 mixtures contained 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) 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 the microbiota strains 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 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min. 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) (supplementary Table 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 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.

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 (supplementary Table 2). These strains belonged to the highly represented genera isolated during mushroom processing and after C&D procedures. Each selected genera was at least three times isolated from a minimum of two sampling spots and multiple strains were selected from the four most highly represented genera that covered over 77 % of all strains (supplementary Table 2). In addition, three *Listeria* spp. strains, namely one strain of *L. innocua*, *L. grayi* and *L. seeligeri* were selected (supplementary Table 2). Moreover, three *L. monocytogenes* strains that differ in serogroup type and clonal complex type were selected for further characterization (supplementary Table 2).

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 h. 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 h. The cultures were centrifuged for 2 min at 16,000 ×*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 g. Portions of 500 g 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 min at 230 rpm. Obtained mushroom medium was centrifuged for 5 min at 15,000 ×*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 min at 15,000 ×*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 h 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 h 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 h 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.

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 2.4). The filter-sterilized mushroom medium (Section 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 (supplementary Table 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 h 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 h at 30 °C and ALOA plates were incubated for 24 h at 37 °C. Two to four

biologically independent reproductions were performed on different days for each of the strain combinations.

2.6. Spent medium experiments

The filter-sterilized mushroom medium (Section 2.4) was inoculated 1:100 (vol/vol%) with the working culture of the microbiota strain or the *Listeria* spp. strain (Section 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 h 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 h at 30 °C. The culture was extracted from the wells plate after 72 h of incubation by combining the volumes of identically treated wells. The culture was centrifuged for 5 min at 15,000 ×*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 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 h 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 h at 30 °C. Two or three biologically independent reproductions were performed on different days for each of the strain combinations.

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 h or 72 h at 20 °C. Prior to pH measurements of the medium incubated with (a) bacterial strain(s), cultures were centrifuged for 2 min at 16,000 ×*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.

2.8. Biofilm formation

Strains were grown in single cultures (Section 2.4), in co-cultures (Section 2.5) and in spent medium (Section 2.6) and the biofilm forming capacity of the strains was determined after 48 h 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 h of incubation, wells were washed twice with 300 μL PBS, and the biofilm was stained for 30 min 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 min 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.

2.9. HPLC analysis

Cultures of the growth experiments were centrifuged at 16,000 ×*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 h 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 ×*g* for 5 min 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 × 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-hour incubated non-inoculated filter-sterilized mushroom medium.

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 $p = 0.05$.

3. Results

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 1 and supplementary Table 3).

Microbiota identification demonstrated the prominent presence of *Acinetobacter* (26.8 %), *Enterobacteriaceae* (22.2 %), *Lactococcus* (18.4 %) and *Pseudomonas* (9.6 %) (Table 1, supplementary Table 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 (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 1, supplementary Tables 2 and 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*) (see supplementary Table 4 for 16S sequences and taxonomical identification of the 18 selected strains).

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 h at 20 °C (Fig. 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 h 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 h 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 h of incubation, respectively. The final counts of this mixture were comparable to the counts of the two high-performer strains *Ewingella* and *Raoultella* ($p = 0.15$) (Fig. 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.

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 h incubation at 20 °C, with the exception of *Chryseobacterium* which showed no significant CV staining (Fig. 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 (Fig. 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 (Fig. 2).

Compound analysis of non-inoculated mushroom medium that was statically incubated for 48 h 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 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 h of incubation (supplementary Table 5). Compound analysis of the medium after culturing the microbiota strains showed that both *Raoultella* and *Ewingella* consumed mannitol in large extents (Table 2) and interestingly, these two strains showed the highest growth and biofilm counts (see Figs. 1 and 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 (Fig. 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 2).

Table 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

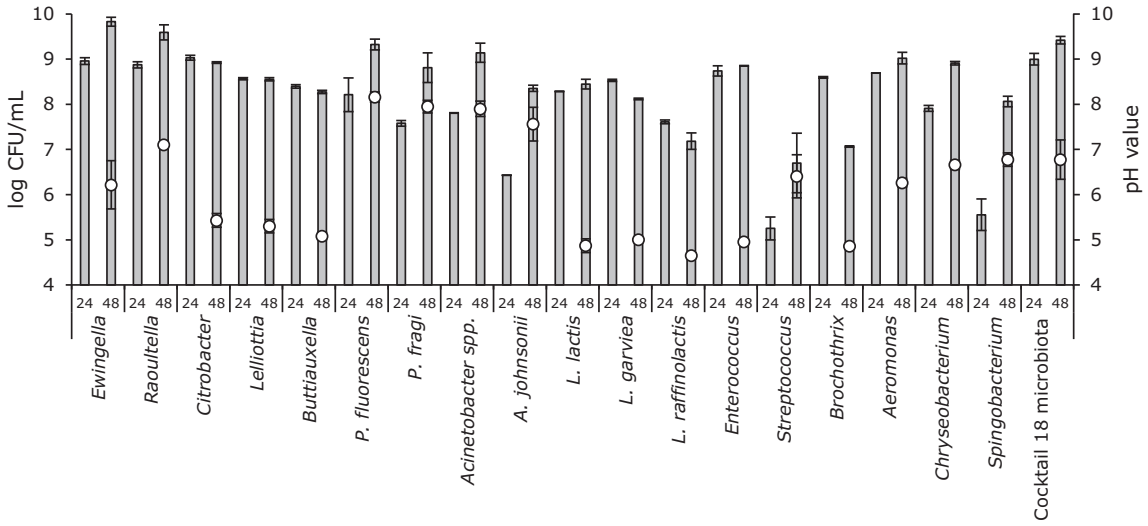

Fig. 1. Growth performance of the 18 microbiota strains in single culture and as cocktail during static incubation in filter-sterilized mushroom medium for 48 h at 20 °C. Inoculum levels of single microbiota cultures and the cocktail were approximately 5 log CFU/mL. Grey bars represent the microbiota counts after 24 h (24) and 48 h (48) of incubation. The initial pH of the medium is around 6.8 and the pH after 48 h of incubation is represented with the white circles. The error bars represent the standard deviation of two biological reproductions.

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 (Fig. 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 h 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 (Fig. 3). The counts of *L. monocytogenes* decreased during the following up 24 h 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 h of co-incubation with the *Pseudomonas* and *Acinetobacter* strains reaching final counts of 9.1 to 9.2 log CFU/mL (Fig. 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 h of co-incubation with the *Lactococcus* strains and final counts were 5.8 to 7.7 log CFU/mL, with the lowest counts in the co-culture with the *Lactococcus raffinolactis* strain, which also had the lowest final pH value (Fig. 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.

All co-incubations showed the development of a biofilm consisting of both the *L. monocytogenes* strain 636 and the microbiota strain after 48 h 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 (Fig. 4). Biofilms formed in co-cultures with *Raoultella* and *Ewingella* contained 8.9 and 8.4 log CFU/mL microbiota counts, respectively (Fig. 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 (Figs. 3 and 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 (Figs. 3 and 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).

The medium composition after the co-culture experiments with *L. monocytogenes* (supplementary Table 6) was compared to the mono-culture experiments of the microbiota strains (Table 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 lactic acid 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.

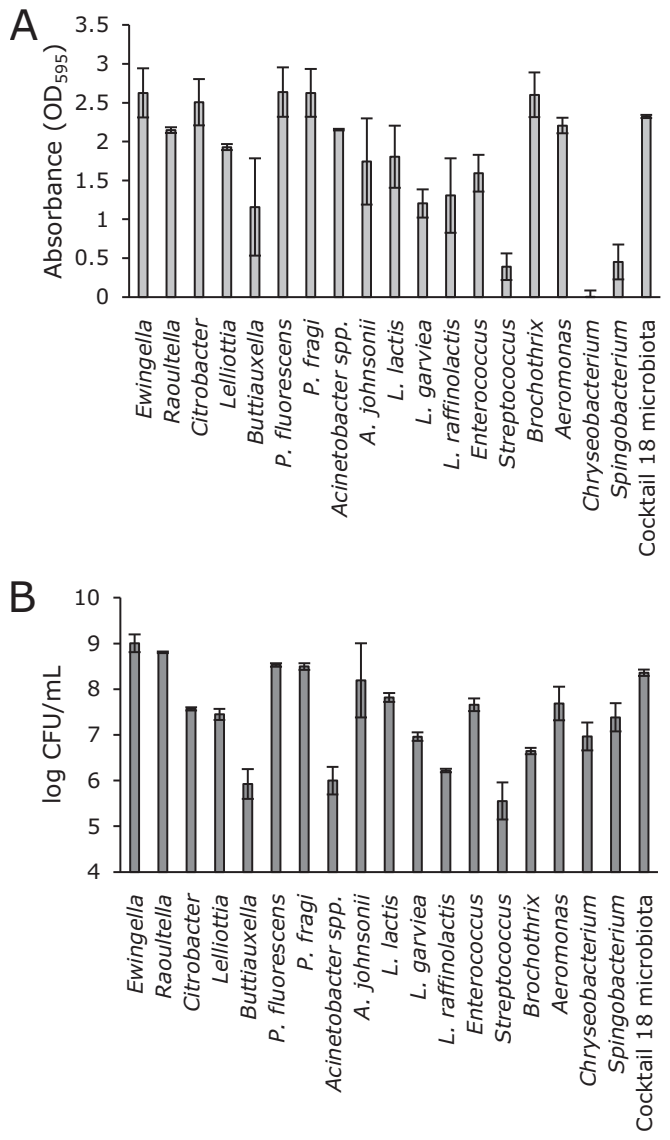

Fig. 2. Biofilm determination of the 18 microbiota strains in single culture and as cocktail after static incubation in filter-sterilized mushroom medium for 48 h at 20 °C. Inoculum levels of single microbiota cultures and the cocktail were approximately 5 log CFU/mL. (A) Staining assessed with the crystal 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.

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-hour cultures of microbiota strains (supplementary Fig. 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 h at 20 °C depended on the microbiota strain used in the preculturing (Fig. 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 (supplementary Fig. 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 (Fig. 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 (Fig. 5). As expected, the log increases in the pH-adjusted and the non-pH adjusted spent medium of the other microbiota strains were comparable (Fig. 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 (supplementary Table 7).

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.

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 h 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 to 4.8 was observed (Fig. 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 (Fig. 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 (Fig. 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 (supplementary Table 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-h cultures of the *Listeria* spp. strains (supplementary Fig. 2). Incubation of *L. monocytogenes* for 48 h 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 (Fig. 7). Incubation of the other *L. monocytogenes* strains in spent medium of the *Listeria* spp.

Table 2
Compound analysis of mushroom medium after 48 h 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.

Culture	Component							
	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)

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

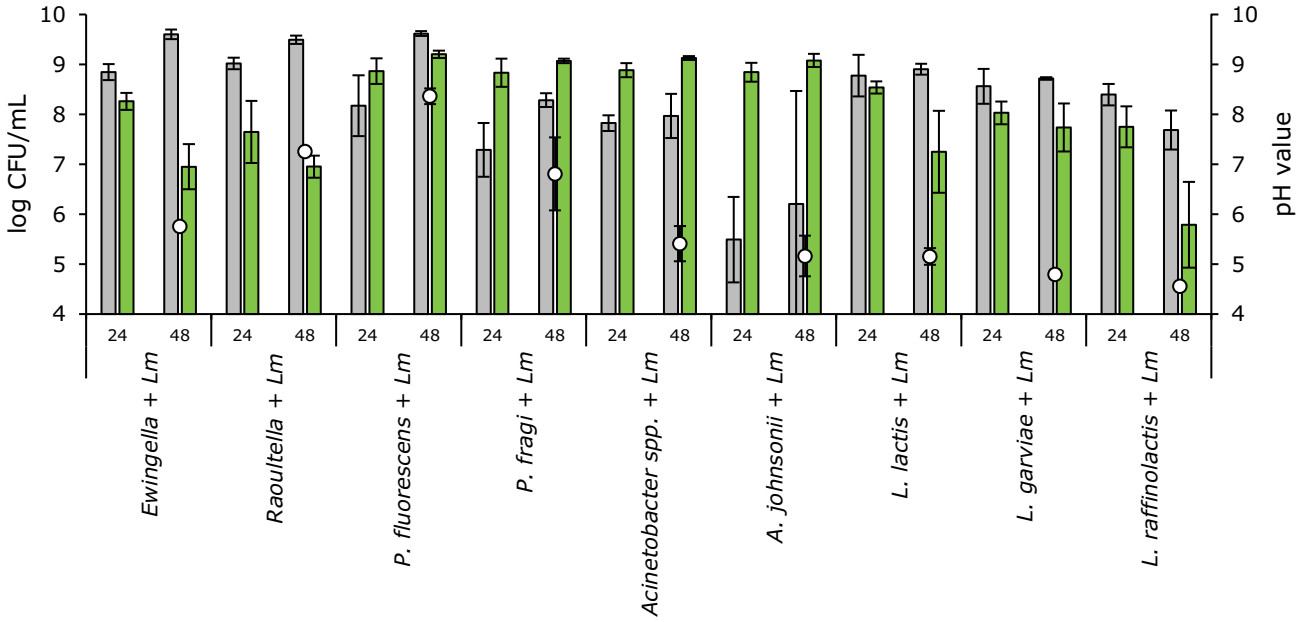

Fig. 3. Co-incubation of nine selected microbiota strains with *L. monocytogenes* (*Lm*) strain 636 in mushroom medium for 48 h at 20 °C. Inoculum levels of the co-cultures were approximately 5 log CFU/mL for each of the strains. Grey bars represent the microbiota counts and green bars represent the *L. monocytogenes* counts after 24 h (24) and 48 h (48) of incubation. The initial pH of the medium is around 6.9 and the pH after 48 h of co-incubation is represented with the white circles. The error bars represent the standard deviation of four biological reproductions.

strains showed similar trends (data not shown). The significant log increase in spent medium resulted in only a small decrease in the pH value (Fig. 7) and minor differences in compounds in the spent medium before and after growth of *L. monocytogenes* (supplementary Table 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*.

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;

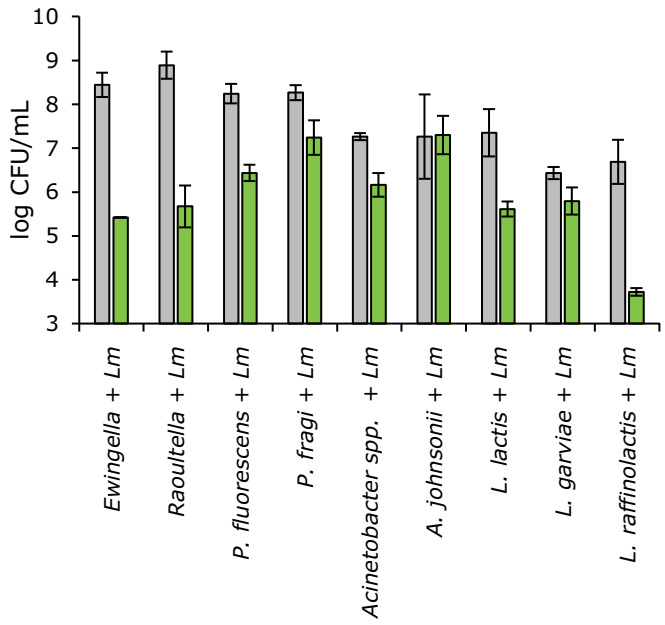

Fig. 4. Biofilm cell counts of nine selected microbiota strains in co-culture with *L. monocytogenes* (*Lm*) strain 636 after 48 h of incubation at 20 °C in mushroom medium. Inoculum levels of the co-cultures were approximately 5 log CFU/mL for each of the strains. Biofilm cell counts are expressed in 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.

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, mushroom medium was used in the current study 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

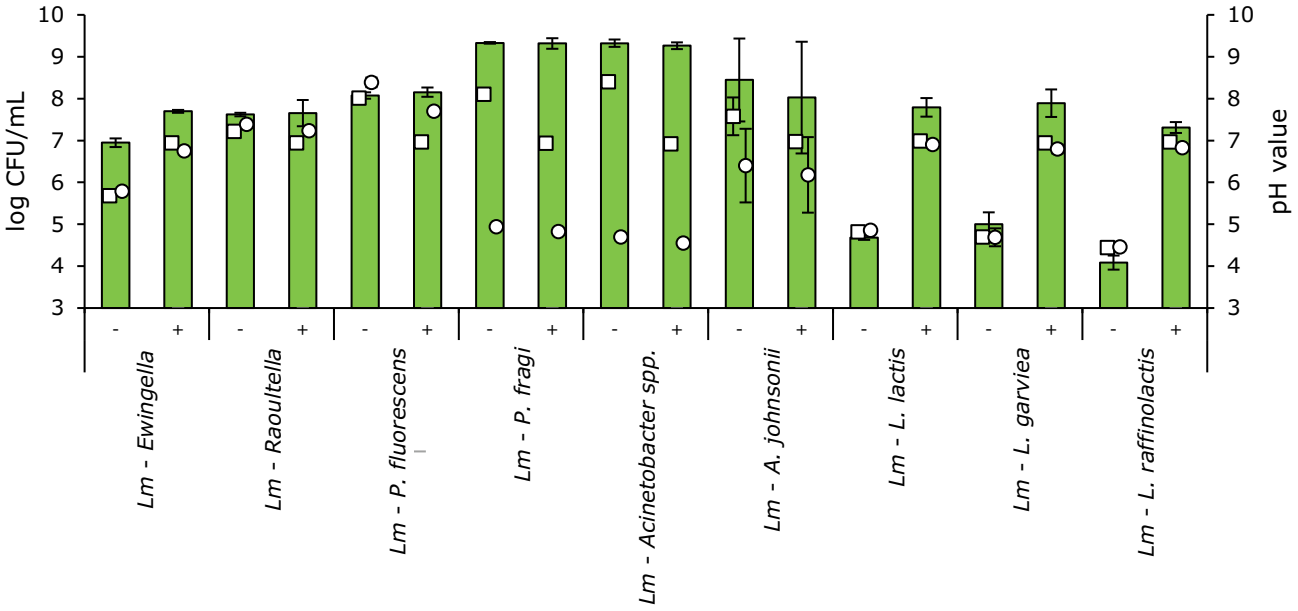

Fig. 5. Growth of *L. monocytogenes* (*Lm*) strain 636 during static incubation at 20 °C in non-pH adjusted and pH-adjusted spent mushroom medium obtained from a 72-hour microbiota strain culture. Inoculum levels of *L. monocytogenes* were approximately 5 log CFU/mL. Green bars represent the *L. monocytogenes* counts after 48 h 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 h in the spent media, respectively. The error bars represent the standard deviations of two or three biological reproductions.

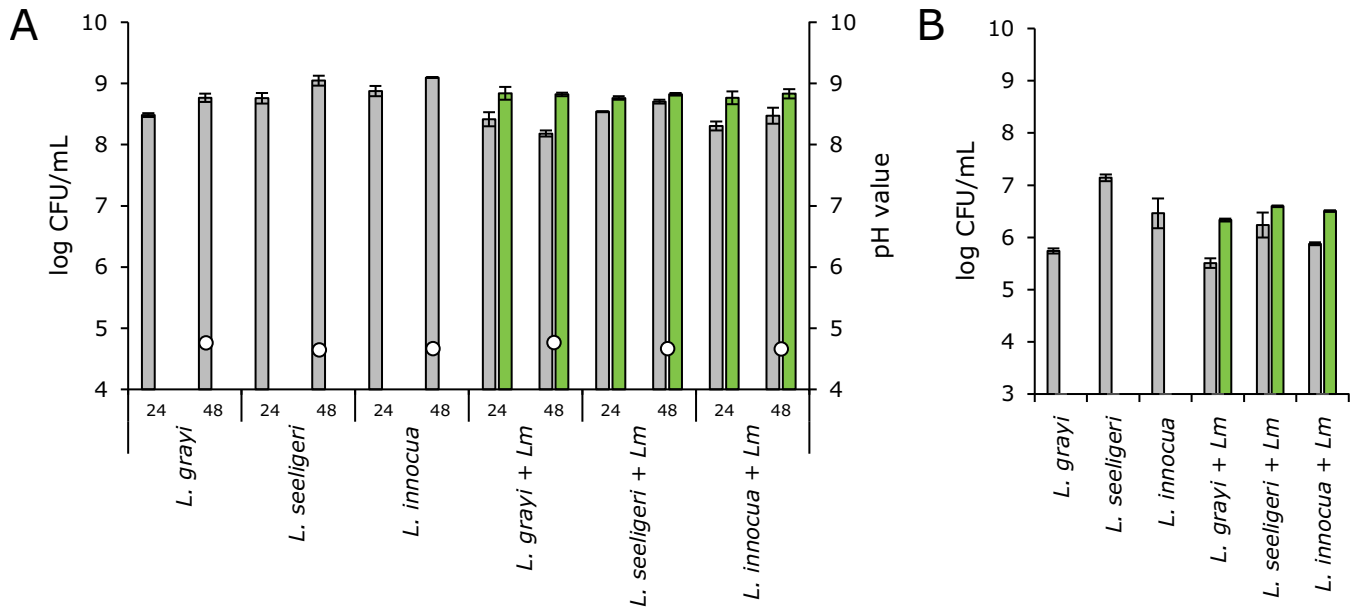

Fig. 6. Growth performance of three *Listeria* spp. strains in mono-culture and in co-cultures with *L. monocytogenes* (*Lm*) strain 636 during static incubation in filter-sterilized mushroom medium for 48 h at 20 °C. Inoculum levels of the individual strains were approximately 5 log CFU/mL. (A) Growth 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 h (24) and 48 h (48) of incubation. The initial pH of the medium is 6.9 and the pH after 48 h 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 h 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.

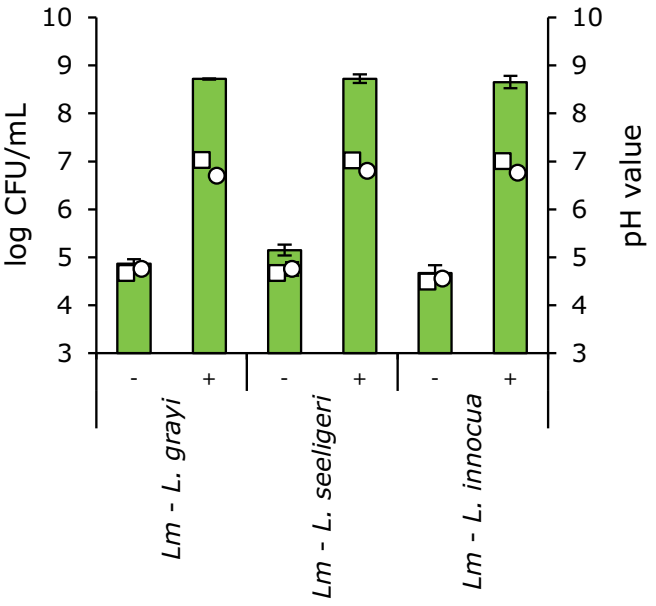

Fig. 7. Growth of *L. monocytogenes* (*Lm*) strain 636 during static incubation at 20 °C in non-pH adjusted and pH-adjusted spent mushroom medium obtained from a 72-hour culture of a *Listeria* spp. strain. Inoculum levels of *L. monocytogenes* were approximately 5 log CFU/mL. Green bars represent the *L. monocytogenes* counts after 48 h 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 h in the spent media, respectively. The error bars represent the standard deviations of two or three biological reproductions.

observed somewhat higher *L. monocytogenes* biofilm counts than *P. fluorescens* biofilm counts after 48 h 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 including 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* 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 h 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 h 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 h and 48 h of incubation, with a slight decline of *L. monocytogenes* after 48 h 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.

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CRediT authorship contribution statement

Frank B. Lake: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Data curation, Validation, Investigation, Formal analysis. **Leo S. van Overbeek:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition, Project administration, Validation. **Johan J.P. Baars:** Conceptualization, Methodology, Writing – review & editing, Supervision, Resources. **Tjakko Abee:** Conceptualization, Methodology, Writing – review & editing, Supervision, Validation, Formal analysis. **Heidy M.W. den Besten:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Validation, Formal analysis, Resources.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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