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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 behavior 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.

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,

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

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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 behavior 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.

2. Materials and methods

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 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 strain 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/, accessed April 11, 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 1.

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 h. After incubation, one milliliter of culture was centrifuged for 2 min at 16,000 × *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 g di-sodium hydrogen phosphate dihydrate, 2.71 g sodium dihydrogen phosphate and 8.5 g 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.

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 2.4), biofilm formation (section 2.5) and nutrient consumption (section 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 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 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 behavior 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 min at 15,000 × *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.

2.4. Growth of Listeria monocytogenes in mushroom medium

The working cultures (section 2.2) were inoculated 1:100 (volume/

volume) in filter-sterilized mushroom medium (section 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 h. Counts of the individual *L. monocytogenes* cultures were determined at 0, 24 and 48 h 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 h. In addition, the pH of the mushroom medium was determined at the start of each experiment (fresh sterile mushroom medium), after 48 h of incubation with *L. monocytogenes*, and also after 48 h of incubation of non-inoculated medium. Prior to pH measurements of the medium incubated with *L. monocytogenes*, cultures were centrifuged to remove cells (2 min at 16,000 × *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.

2.5. Crystal violet staining and biofilm CFU counting

Strains were grown statically as described in section 2.4 and the biofilm forming capacity of the strains was determined after 48 h 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 min 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 min 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.

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 h of incubation with *L. monocytogenes*. Prior to measurements, cultures were centrifuged for 2 min at 16,000 × *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 h 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 × *g* for 5 min 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 × 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 × *g* for 10 min 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 s and heated at 55 °C in a heat block for 10 min. 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 × 2.1 mm, 1.7 μm) (Waters Corporation, USA) and a BEH C18 guard column (5 mm × 2.1 mm, 1.7 μm) (Waters Corporation, USA). The temperature of the column oven was set at 55C 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 min 99.9 % A, 5.24 min 90.9 % A, 7.24 min 78.8 % A, 8.54 min 57.8 % A, 8.55–10.14 min 10 % A, 10.23–17 min 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.

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 1) was used to determine growth performance on whole mushrooms, sliced mushrooms and smashed mushrooms. Stationary phase cultures were prepared as described in section 2.2 and 100 μL of the culture was transferred into 10 mL of fresh BHI and cultures were statically incubated for 24 h at 20 °C. Working cultures were prepared in PBS as described in section 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 min 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 h 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 min 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 min 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 h 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 h 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 h 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 h at 37 °C. The pH and the HPLC analyses of the smashed mushrooms were done using liquids obtained at the start and after 48 h of incubation following the approaches described in section 2.4 and section 2.6, respectively. Experiments were performed with two biologically independent replicates obtained on different days, each consisting of two technical replicates.

2.8. Quantifying reproduction and strain variability

The reproduction variability and strain variability were quantified for all strains, and for different groups of strains (e.g. mushroom strains, non-mushrooms strains, and strains of the same PCR 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 h incubation, and for both the CV staining and the CFU counts of the biofilm cells after 48 h 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.

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 h at 20 °C, using a significance value of $p = 0.05$.

3. Results

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 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 Fig. 1, Fig. 2, Table 1, Supplemental Fig. 2B, yet similar trends were observed in other mushroom medium batches (see Supplemental Fig. 1, Supplemental Fig. 2A and Supplemental table 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 h of incubation and till 8.4 to 9.1 log CFU/mL after 48 h of incubation (Fig. 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 h and 48 h 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 h and 48 h 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 h 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 (Fig. 1).

Interestingly, after 24 h and 48 h 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 h and 48 h were not significantly different between the mushroom strains and the non-mushroom strains and between the four PCR serogroups. This points to rather comparable behavior between strains and between groups of strains.

3.2. Biofilm formation of L. monocytogenes using mushroom medium

As the planktonic growth counts of most of the strains further increased after 24 h, biofilm formation was determined after 48 h. All strains formed biofilms, and the average OD_{595} values of bound CV for all but one strain was between 1 and 2 (Fig. 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 (Fig. 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 (Fig. 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.

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 1). Similar trends in compound composition were observed for other mushroom medium batches (Supplemental table 2). Following 48 h 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 1). The formation of acetic acid and lactic acid possibly contributes to the decrease in the pH, while the other major product acetoin

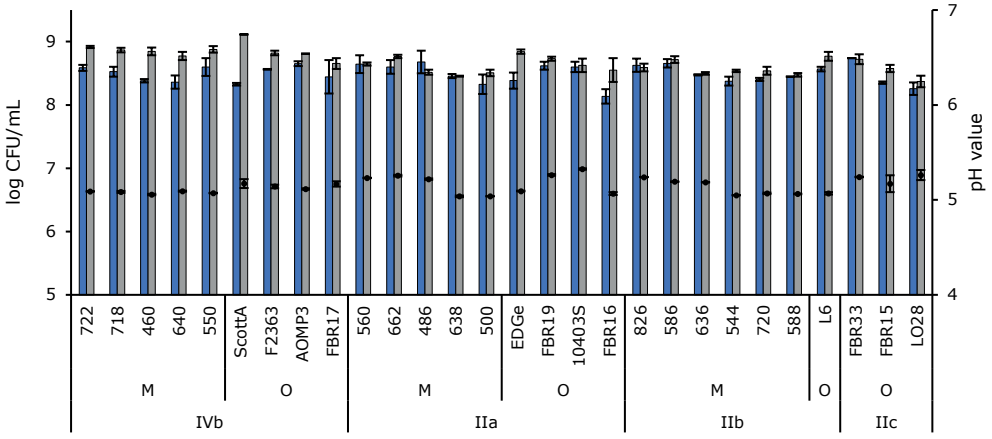

Fig. 1. Growth performance of *L. monocytogenes* strains during static incubation at 20 °C in filter-sterilized mushroom medium. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in sterile mushroom medium and CFU counts were determined after 24 h (blue bars) and 48 h (grey bars) of incubation. 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 h 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.

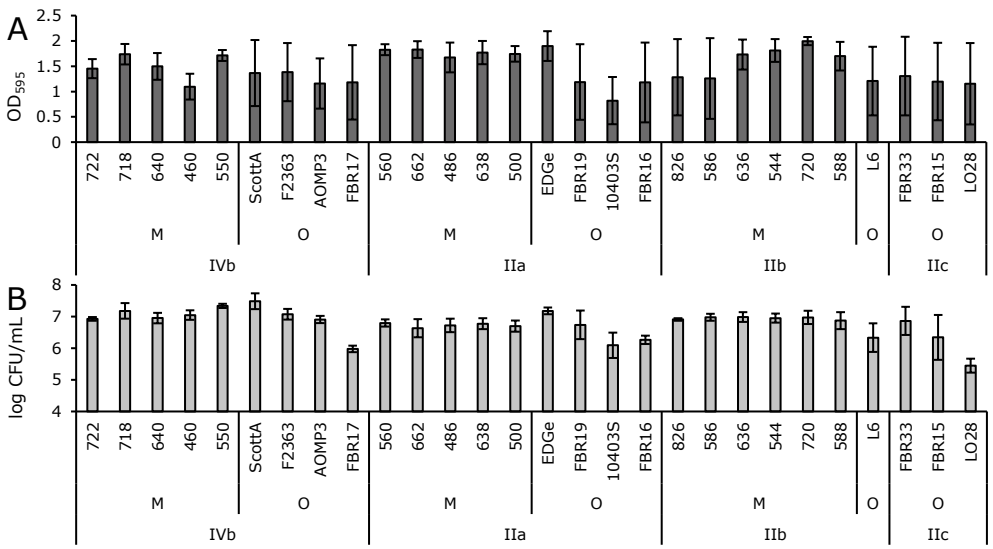

Fig. 2. Biofilm formation of *L. monocytogenes* strains after incubation for 48 h at 20 °C in polystyrene plates. *L. monocytogenes* strains were inoculated (5 log CFU/mL) in filter-sterilized mushroom medium and incubated statically. (A) CV staining is shown as optical density at OD_{595} in which the 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.

Table 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 h at 20 °C using the same mushroom medium batch as in Fig. 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. 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).

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 1). Similar trends of compound utilization and product formation were also observed in the other mushroom medium batches (data not shown).

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 Fig. 2A.

The amino acid concentrations in sterile mushroom medium incubated for 48 h 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 Fig. 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 h 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.

3.5. 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 1) at day 2 and day 6 for each of the three mushroom products using different mushroom batches. This highlights comparable behavior 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 Fig. 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 (Fig. 3A, 3B, 3C). Also during the six days of incubation, the

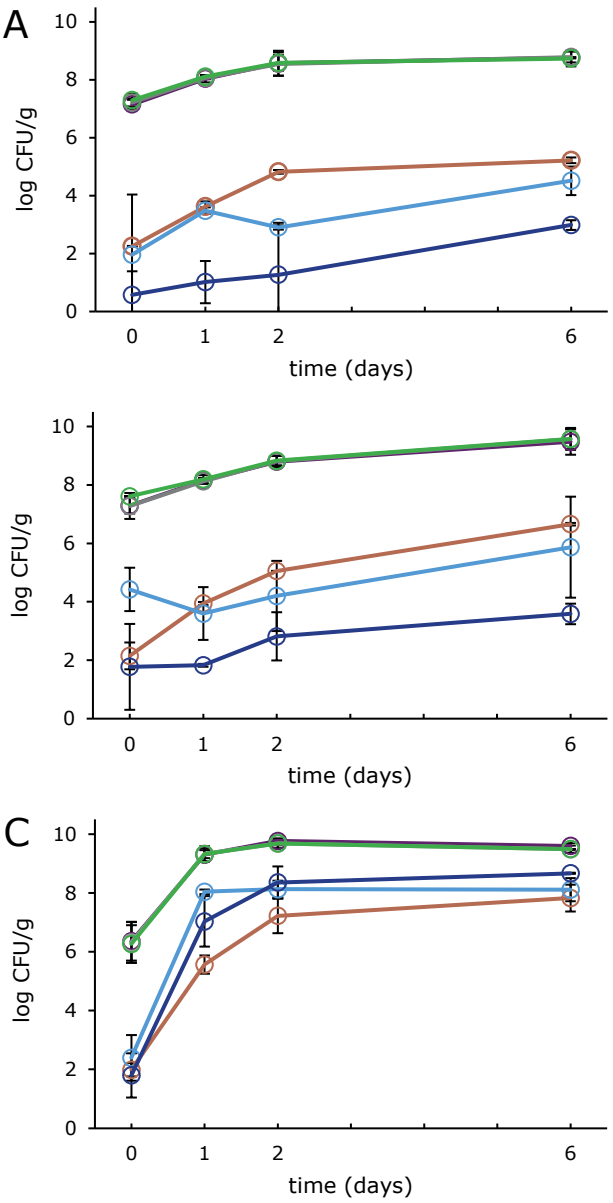

Fig. 3. Growth performance testing of *L. monocytogenes* strain Lm636 and accompanying microbiota on mushroom products. *L. monocytogenes* strain Lm636 was inoculated (2 log CFU/gram) on three mushroom products; (A) whole mushrooms, (B) sliced mushrooms and (C) smashed mushrooms and statically incubated 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.

mesophilic counts, psychrotrophic counts and *Pseudomonas* counts were comparable and increased over time (Fig. 3A, 3B, 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 (Fig. 3A, 3B, 3C). The *L. monocytogenes* mushroom strain Lm636 showed growth on all three mushroom products (Fig. 3A, 3B, 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 behavior of the accompanying microbiota (Supplemental Fig. 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). 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 from batch to batch (Supplemental table 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). These results indicate that *L. monocytogenes* is able to grow well in the tested mushroom products (Fig. 3) (Supplemental Fig. 3), despite extensive growth of natural microbiota.

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 (Fig. 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.

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,

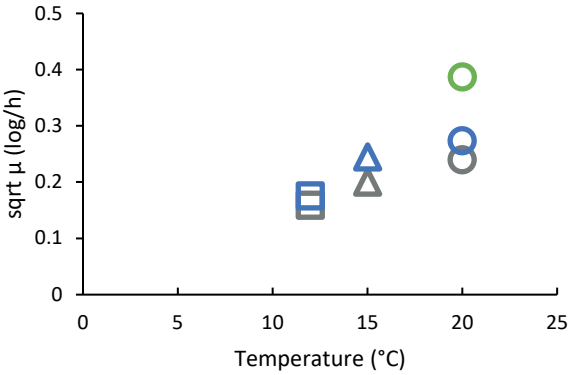

Fig. 4. Specific growth rate of *L. monocytogenes* on mushroom products as function of temperature. The (highest estimated) values of the specific growth rate (μ) were determined by the log increase in CFU/gram divided by the time in hours (h) necessary for this log increase of the *L. monocytogenes* 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.

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 Fig. 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.

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*.

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

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112488.

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