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Listeria monocytogenes strains show large variations in competitive growth in mixed culture biofilms and suspensions with bacteria from food processing environments

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ABSTRACT

Interactions and competition between resident bacteria in food processing environments could affect their ability to survive, grow and persist in microhabitats and niches in the food industry. In this study, the competitive ability of *L. monocytogenes* strains grown together in separate culture mixes with other *L. monocytogenes* (L. mono mix), *L. innocua* (Listeria mix), Gram-negative bacteria (Gram− mix) and with a multigenera mix (Listeria + Gram− mix) was investigated in biofilms on stainless steel and in suspensions at 12 °C. The mixed cultures included resident bacteria from processing surfaces in meat and salmon industry represented by *L. monocytogenes* (n = 6), *L. innocua* (n = 5) and Gram-negative bacteria (n = 6; *Acinetobacter* sp., *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Serratia liquefaciens*, *Stenotrophomonas maltophilia*). Despite hampered in growth in mixed cultures, *L. monocytogenes* established in biofilms with counts at day nine between 7.3 and 9.0 log per coupon with the lowest counts in the Listeria + G− mix that was dominated by *Pseudomonas*. Specific *L. innocua* inhibited growth of *L. monocytogenes* strains differently; inhibition that was further enhanced by the background Gram-negative microbiota. In these multispecies and multibacteria cultures, the growth competitive effects lead to the dominance of a strong competitor *L. monocytogenes* strain that was only slightly inhibited by *L. innocua* and showed strong competitive abilities in mixed cultures with resident Gram-negative bacteria. The results indicates complex patterns of bacterial interactions and *L. monocytogenes* inhibition in the multibacteria cultures that only partially depend on cell contact and likely involve various antagonistic and bacterial tolerance mechanisms. The study indicates large variations among *L. monocytogenes* in their competitiveness under multibacterial culture conditions that should be considered in further studies towards understanding of *L. monocytogenes* persistence in food processing facilities.

1. Introduction

Listeria monocytogenes is among the most serious food safety challenges for the food industry. The ubiquitous nature of *L. monocytogenes*, along with its ability to grow at low temperatures, persist in food processing facilities, contaminate food and cause severe foodborne listeriosis infections, make this bacterium a major food safety threat (Gandhi and Chikindas, 2007). Certain ready-to-eat foods of dairy, meat and fish origin have been categorized as *L. monocytogenes* high-risk products and identified as the implicated food in outbreaks (Buchanan et al., 2017; EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017; Heiman et al., 2016; Jami et al., 2014; Koch et al., 2010; Miettinen et al., 1999; Thevenot et al., 2006; U.S. Food and Drug Administration et al., 2003). In USA, approximately 1600 illnesses and 260 deaths each year are due to listeriosis (Centers for Disease Control and Prevention, 2014). In Europe, an increasing trend of human listeriosis cases was observed in the period 2008–2016 with 2536 cases reported in 2016 of which 97.7% were hospitalized and with an overall case fatality of 16.2% among the 1524 cases with reported outcome (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017).

A number of studies have documented that contamination during processing is a major cause of *L. monocytogenes* in foods (Carpentier and Cerf, 2011; Ferreira et al., 2014; Fonnesbech Vogel et al., 2001; Muhterem-Uyar et al., 2015; Rørvik et al., 1995). Reports have shown *L. monocytogenes* survival and the re-isolation of identical *L. monocytogenes* clones over extended time periods in processing plants

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Table 1
Overview of *L. monocytogenes* and *L. innocua* strains used in this study.

Strain	Source	MLVA[a]	Sequence type (ST)[b]	Reference
L. monocytogenes				
MF3860	Equipment; salmon processing plant	6-10-5-16-6	20 (17-13-3-6-5-7-1)	Møretrø et al., 2017
MF3939	Environment; salmon processing plant	5-8-15-10-6	14 (8-6-13-6-5-2-1)	Langsrud et al., 2016; Møretrø et al., 2017
MF4077	Environment; salmon processing plant	6-9-18-16-6	8 (5-6-2-9-5-3-1)	Fagerlund et al., 2016; Møretrø et al., 2017
MF4562	Equipment; meat processing plant	6-11-15-18-6	9 (6-5-6-4-1-4-1)	Møretrø et al., 2017
MF4627	Environment; meat processing plant	6-9-14-16-6	451 (7-5-10-21-1-4-1)	Møretrø et al., 2017
MF4712	Slicer waste; meat processing plant	7-7-10-10-6	7 (5-8-5-7-6-2-1)	Møretrø et al., 2017
L. innocua				
MF3940	Equipment; salmon processing plant	N.a.[c]	N.a.	Langsrud et al., 2016
MF4052	Environment; salmon processing plant	N.a.	N.a.	This study
MF4386	Environment; salmon processing plant	N.a.	N.a.	This study
MF4401	Environment; salmon processing plant	N.a.	N.a.	This study
MF4608	Environment; salmon processing plant	N.a.	N.a.	This study

[a] Multiple locus variable number tandem-repeats analysis in accordance with Lindstedt et al. (2008).
[b] ST numbers (allelic profiles in parenthesis) refer to Institut Pasteur MLST database (http://bigsdb.web.pasteur.fr/listeria/listeria.html).
[c] Not applicable.

(Carpentier and Cerf, 2011; Fagerlund et al., 2016; Fagerlund et al., 2017; Tompkin, 2002; Vongkamjan et al., 2013; Wulff et al., 2006). Thus, *L. monocytogenes* have the ability to survive, multiply and persist under harsh conditions in food processing environments, and this is a major concern for processors of risk food products (Gandhi and Chikindas, 2007; Carpentier and Cerf, 2011). To understand long-term survival of *L. monocytogenes* in food industry premises, particular focus has been on characterization of strain properties of persistent versus non-persistent strains (see Reviews of (Carpentier and Cerf, 2011; Ferreira et al., 2014)). Hypotheses risen include the ability to adhere to surfaces and form biofilms (Borucki et al., 2003; Norwood and Gilmour, 1999); (Bonsaglia et al., 2014; Borucki et al., 2003; Di Bonaventura et al., 2008; Djordjevic et al., 2002; Doijad et al., 2015; Kadam et al., 2013) and enhanced tolerance or adaptations to processing factors (Holah et al., 2002; Wulff et al., 2006), disinfectants and food associated stresses (Aase et al., 2000; Fagerlund et al., 2017; Heir et al., 2004; Kovacevic et al., 2016; Lunden et al., 2003; Lunden et al., 2008; Magalhaes et al., 2016; Møretrø et al., 2017). However, no genetic determinants or individual trait explaining persistence properties of *L. monocytogenes* have been described (Carpentier and Cerf, 2011; Stasiewicz et al., 2015).

Another factor in the competitiveness and propagation of *L. monocytogenes* under food processing conditions is the role of the accompanying microbiota (Fagerlund et al., 2017; Langsrud et al., 2016; Papaioannou et al., 2018; Røder et al., 2016). Interactions between *L. monocytogenes* and resident background microbiota are likely to occur on processing surfaces between sessile cells, in biofilms and in liquid suspensions. These interactions can provide protection of bacteria to environmental stresses but can also have effects on the growth and survival of the individual members of these microbial consortia (Giaouris et al., 2013; Giaouris et al., 2014; Giaouris et al., 2015; Langsrud et al., 2016; Møretrø and Langsrud, 2017; Papaioannou et al., 2018; Røder et al., 2015; Sanchez-Vizuete et al., 2015; van der Veen and Abee, 2011). Effects on *L. monocytogenes* densities by interactions with background microbiota strains range from no effects to positive and antagonistic effects (Bremer et al., 2001; Carpentier and Chassaing, 2004; Daneshvar Alavi and Truelstrup Hansen, 2013; Fox et al., 2014; Giaouris et al., 2013; Gudmundsdottir et al., 2005; Hassan et al., 2004; Leriche and Carpentier, 2000; Midelet et al., 2006; Norwood and Gilmour, 2000; Papaioannou et al., 2018; Rodriguez-Lopez et al., 2015; Røder et al., 2015; Sasahara and Zottola, 1993; Schirmer et al., 2013). Sampling in salmon and meat processing facilities has shown a diverse microbiota to be present on production surfaces (Bagge-Ravn et al., 2003; Fagerlund et al., 2017; Langsrud et al., 2016; Møretrø and Langsrud, 2017; Møretrø et al., 2013; Møretrø et al., 2016; Røder et al., 2015). Co-residence of different *L. monocytogenes* subtypes and other *Listeria* species has also been described (Alali and Schaffner, 2013; Besse et al., 2005; Langsrud et al., 2016; Vongkamjan et al., 2015; Williams et al., 2011; Zilelidou et al., 2016). Presence and distribution of bacteria on surfaces are influenced by the type of foods processed and environmental conditions (Møretrø and Langsrud, 2017; Røder et al., 2015). After regular cleaning and disinfection the microbiota is likely to include bacteria with a profound ability to survive, grow and form biofilms and thus be of special relevance for studies on interaction effects with *L. monocytogenes*. Future studies will likely unravel the complexity of factors involved when *L. monocytogenes* and background bacteria in food environments interact under relevant conditions.

Few reports exist on the ability of *L. monocytogenes* subtypes to compete when co-cultured under multispecies and multigenera growth conditions in biofilms and suspensions. The aim of this work was to investigate the competitive ability of *L. monocytogenes* strains when these were co-cultured along with other *L. monocytogenes* subtypes, *L. innocua* and accompanying environmental bacteria commonly encountered on food contact surfaces in meat and salmon processing facilities after cleaning and disinfection. The distribution of individual *L. monocytogenes* strains was determined in mixed culture biofilm and broth suspension experiments under industry relevant conditions. Competitive growth effects of background microbiota strains, in combination and individually, on different *L. monocytogenes* strains under co-culture conditions were further studied. The dependence of bacterial cell contact for the observed *L. monocytogenes* growth inhibition was investigated.

2. Materials & methods

2.1. Bacterial strains, cultivation conditions and mixed culture inocula

The *L. monocytogenes* and *L. innocua* strains used are given in Table 1. All strains were collected from salmon and ready-to-eat meat production facilities and isolated and identified as previously described (Møretrø et al., 2017). The species identity was confirmed by partial sequencing of *iap* gene PCR amplicons (Chen and Knabel, 2007). The rationale for selection of bacteria in the mixed culture experiments was to include *L. monocytogenes* from different salmon and meat processing plants having different but commonly occurring MLVA and MLST profiles (Møretrø et al., 2017). The MLVA (Lindstedt et al., 2008) and MLST (Ragon et al., 2008) profiling were performed accordingly. Sequence types (ST) were assigned using the Institute Pasteur *L. monocytogenes* MLST database (http://bigsdb.pasteur.fr/listeria/). The other strains selected were of genera commonly determined from environmental samples after cleaning and disinfection and that contained *L. monocytogenes*. The strains were identified in our previous study using

16S rRNA gene sequencing (Langsrud et al., 2016).

In total, 17 bacterial strains were included in various mixed culture experiments and these included strains of *L. monocytogenes* (n = 6), *L. innocua* (n = 5) and six strains of psychrotrophic Gram-negative bacteria (*Pseudomonas fragi* (MF4987), *Pseudomonas fluorescens* (MF4988), *Serratia liquefaciens* (MF3971), *Stenotrophomonas maltophilia* (MF5364), two *Acinetobacter* sp. (MF4122, MF4124)). Prior to all experiments, bacteria from stock cultures (stored −80 °C in 20% glycerol) were plated on Brain Heart Infusion (BHI) agar and grown for three days at 15 °C. Colonies from individually grown strains were inoculated in 2 ml BHI broth and cultured aerobically for two days at 15 °C. The bacterial cultures were used to generate mixed culture inocula for biofilm (see Section 2.2) and broth culture (see Section 2.3) experiments. Four mixed culture combinations in the biofilm experiments were used. The four mixed culture combinations were termed L. mono mix (six *L. monocytogenes*), Listeria mix (six *L. monocytogenes*, five *L. innocua*), L. mono + G− mix (L. mono mix, six Gram-negative background microbiota bacteria) and Listeria + G− mix (all 17 strains). Certain dual species and mixed culture inocula were included in the broth culture experiment for additional studies on bacterial competition.

2.2. Biofilm formation on stainless steel coupons

The individual strain cultures were added into BHI broth and combined to contain about equal concentrations of each strain with a total cell concentration of approximately 10^7 cfu/ml. The bacterial suspensions (5 ml) were added to 6-well plates containing 2 × 2 cm stainless steel coupons (AISI 304, 2B, Norsk Stål AS, Norway). Bacteria were allowed attaching to the coupons (3 h, 15 °C), the suspension removed and the plates were washed briefly by adding 6 ml dH_2O to the coupon wells followed by swirling of the trays and pipetting to remove non-attached cells. 5 ml of BHI broth were added to each well of the plates and these were incubated at 12 °C (a typical temperature in the production facilities). The BHI broth was replaced with fresh broth daily from day five (weekend at day 3 and 4). Sampling for microbial analyses (two replicate coupons for each time point) was performed after two, five and nine days. The experiment was replicated three times on different days and with freshly prepared solutions and cultures.

2.3. Broth culture experiments

The bacterial cultures were diluted in BHI broth (5 ml) and combined to contain equal cell numbers of each strain, approximately 5×10^4 cfu/ml (if not otherwise stated), verified by plate count determination of the individual cultures The cultures were incubated at 12 °C for up to five days with sampling at day two and five in the incubation period. Two to four experiments were performed for each combination on different days and with freshly prepared solutions and cultures.

2.4. Bacterial quantification

In biofilm experiments, bacterial counts were determined from both the coupons and the culture suspensions surrounding the coupons. In broth culture experiments, bacteria were determined from the suspensions. Number of bacteria attached to coupons was determined after careful rinse of the coupons in 5 ml dH_2O to remove loosely attached cells followed by sonication of the coupons to detach bacteria. Sonication was performed in flat-bottomed glass tubes (diameter 28 mm) with coupons and 6 ml dH_2O. The tubes were sonicated for 10 min at 20 °C in a sonication bath (40 kHz, BRANSON 3510, Bransonic Ultrasonic Corporation, USA). The number of colony forming units from the coupons and the culture suspensions were determined by plating serial dilutions on BHI agar and incubation at 20 °C for 48–72 h. The number of colony forming *L. monocytogenes* and *L. innocua* was determined after serial plating on RAPID′*L. mono* agar (Bio-Rad) incubated at 37 °C for 24 h.

2.5. Genus-specific identification of bacteria by 16S rRNA gene sequencing

To determine the overall distribution of bacteria in the multigenera cultures, all colonies within a zone of the BHI agar plates were picked and identified using 16S rRNA gene sequencing (variable region 3–4). The size of the zones for colony picking was adjusted to have approximately 20 colonies within the zone of BHI agar plates with a total of 30–300 colonies. DNA was isolated from the picked colonies using a microwave oven (Sharp Microwave oven R-5000E) to lyse the cells: A small amount of each colony was transferred to separate wells of a 96-well PCR plate. The wells were covered with a self-adhesive film before microwave treatment for 60 s at 750 W. The lysed cell debris were used directly as template in PCR reactions with universal 16S rRNA gene primers (Nadkarni et al., 2002). The PCR used 5′ hot Master Mix (VWR International AS, Norway) and 0.25 μM each of forward and reverse primer in a reaction volume of 12.5 μl. The cycling conditions were 95 °C 15 min, then 30 cycles of denaturing (94 °C for 30 s), annealing (60 °C for 90 s), extension 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were purified before sequencing, using 0.4 μl of ExoSap-IT (Affymetrix, UK) to 5 μl of PCR product and incubated at 37 °C for 30 min and 80 °C for 15 min. The forward universal 16S rRNA gene primer was used for sequencing. The sequencing reaction contained 0.75× BigDye v1.1 Sequencing Buffer, 1 μl BigDye Terminator v1.1 Cycle Sequencing Kit, 0.32 μM of the forward primer, and 1 μl of purified PCR product in a 10 μl reaction. The sequencing reactions were carried out for 25 cycles of 96 °C, 15 s and 60 °C, 4 min. A BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's recommendations to clean up the sequencing reactions. Sequencing was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The obtained 16S rRNA gene sequences (V3–V4 region) were analysed and taxonomy at genus/species level was identified using the RDP (Ribosomal Database Project) SeqMatch http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp.

2.6. Strain-specific quantification of L. monocytogenes

All six *L. monocytogenes* strains used in this study had different *cat* allele gene sequences that enabled strain identification. The number of colony forming units of individual *L. monocytogenes* strains in mixed cultures was determined by DNA sequencing of the *cat* allele (third gene allele in the MLST protocol) of randomly picked colonies from RAPID′*L. mono* agar plates. Zones of the plates were selected to include 20 colonies and all colonies within the zone were picked. Template DNA was obtained by lysis of picked colony material using microwave heating as described above. The lysed cell debris were used as template in PCR reactions by adding 12 μl of a mix containing 6 μl of 2× Qiagen Multiplex PCR master mix (Qiagen Multiplex PCR kit, Qiagen, Hilden, Germany) and 2.5 pmol of each primer (*cato*F and *cato*R; MLST primers). PCR conditions were initial denaturation (95 °C, 15 min) followed by 35 cycles of the steps denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C, 1 min). Final extension was performed at 72 °C for 10 min. The PCR amplicons were used as templates for DNA sequencing as described above. Obtained *cat* sequences were assigned allele numbers in accordance with the *L. monocytogenes* MLST database (http://bigsdb.pasteur.fr/listeria/).

2.7. Assay for L. monocytogenes growth inhibition by culture supernatants

To determine if *L. innocua* and environmental microbiota strains produced compounds with inhibitory effects on *L. monocytogenes*, supernatants of selected *L. innocua* (MF3940, MF4052, MF 4386) and background microbiota strain cultures (*P. fluorescens* MF4988, *S. liquefaciens* MF3971, *S. maltophilia* MF5364) were added individually to

cultures with the two *L. monocytogenes* strains MF4562 (high competitive) and MF4627 (low competitive) and growth recorded. The supernatants of individual strain cultures were collected by centrifugation (8000*g* for 20 min) and sterile filtration (0.2 µm filter) after aerobic growth at 12 °C for two and five days in BHI broth (inoculum approximately 1×10^5 cfu/ml). Five days old supernatants were also collected from dual co-cultures of *L. innocua* MF3940 and the environmental microbiota strains MF4988 (*P. fluorescens*), MF3971 (*S. liquefaciens*) and MF5364 (*S. maltophilia*) in three individual dual cultures. Growth of *L. monocytogenes* MF4562 and MF4627 (inoculum approximately 1×10^5 cfu/ml) in individual cultures of a 1:1 mix of supernatants and BHI broth incubated at 12 °C for 5 days was determined by plate counts.

2.8. Contact and non-contact dependent growth of L. monocytogenes in co-cultures of environmental bacteria

Two multiculture strain combinations, the *L. innocua* mix (five strains) and the mix combining the *L. innocua* mix and the six background Gram-negative microbiota strains were tested with each of the MF4562 (strong competitor) and the MF4627 (weak competitor) *L. monocytogenes* strains. The different single strain (*L. monocytogenes*) and multiculture suspensions containing approximately 5×10^4 cfu/ml of each strain were prepared by dilution in BHI broth from individual cultures grown for two days, 15 °C (see above). In one set-up, *L. monocytogenes* single strain cultures (2 ml) were added to the wells of a 6-well culture plate (lower chamber). To the wells containing the added *L. monocytogenes* suspensions, polyethylene tetraphthalate (PET) track-etched membrane inserts of 0.4 µm pore size (Thermo Fischer Scientific, Norway) were placed, one per well. The two multiculture suspensions were added (2 ml) into separate membrane inserts (upper chamber). This ensured no contact between the *L. monocytogenes* and the other strains. In a parallel second set-up, the two multiculture suspensions were each added the respective *L. monocytogenes* strains in separate mixes. The bacterial suspensions (2 ml) were added in the lower chamber and with BHI broth (2 ml) added in the upper chamber. This set-up included conditions of non-contact (first set-up) and contact (second set-up) co-culture conditions between *L. monocytogenes* and microbiota strains. The plates were incubated at 12 °C and bacterial counts determined at day 0, 2 and 5. Counts of *L. monocytogenes* were determined in wells added the bacterium. Total counts were determined in wells added environmental microbiota bacteria and *L. innocua*. Bacterial counts in wells not added bacteria were also determined as control.

2.9. Statistical analyses

Estimates for the mean and variance (standard error of mean of two or three biological experiments) for each treatment plotted in the figures were calculated from the average values of log transformed cell numbers of technical replicates using Microsoft Excel®. Analysis of variance (ANOVA function in Minitab® (MINITAB 16.1.1, 2010, www.minitab.com)) was used to calculate the statistical significance main and interaction effects and the Tukeys pairwise comparisons function (Minitab) for testing differences between individual treatment means.

3. Results

3.1. Growth of L. monocytogenes in mixed culture biofilm experiments

Initially, the study investigated how co-culturing with *L. innocua* and Gram-negative bacteria affected growth of *L. monocytogenes* in biofilms and in the surrounding suspensions. The bacterial counts of four different mixed culture combinations during nine days incubation are shown in Fig. 1. The *L. monocytogenes* levels in the surrounding suspensions were higher than on the corresponding coupons (biofilms) in all four mixed cultures (Fig. 1). At Day 2, the levels of *L. monocytogenes* on the coupons reached log 6.4–7.4 being 1–2% of the *L. monocytogenes* in the corresponding suspensions.

Highest levels of *L. monocytogenes* were obtained in the suspensions surrounding the biofilms in the L. mono mix where *L. monocytogenes* reached 9.3 log after two days, further increasing to 10 log at Day 9 (Fig. 1A). In comparison, growth of *L. monocytogenes* was restricted in the Listeria mix (Fig. 1B) and in the L. mono + G− mix (Fig. 1C) where highest *L. monocytogenes* levels reached at Day 5 were 8.8 log and 8.7 log, respectively. Lowest *L. monocytogenes* counts during the incubation period were obtained in mixes with all strains (Listeria + G− mix) where levels in the suspension reached 8.0 log at Day 2 and were maintained at almost the same levels throughout the incubation period (Fig. 1D). Levels of *L. monocytogenes* in the biofilms reached 7.3–9.0 log in the four mixed cultures with highest counts in the L. mono mix. The biofilm counts of *L. monocytogenes*, in contrast to the counts in suspension, slightly increased in all culture combinations during the nine days incubation period. Thus, at Day 9, *L. monocytogenes* counts in the biofilms were at the highest levels and constituted between 8% (L. mono + G− mix; Fig. 1C) and 25% (Listeria + G− mix; Fig. 1D) compared to the planktonic *L. monocytogenes* counts in the respective suspensions.

Total bacterial counts in the multi-genera mixes were 9–10 log in both suspensions and biofilms after two days and remained high indicating an overall good growth and biofilm forming ability of members of the included background bacteria (Fig. 1C, D). The *L. monocytogenes* and *L. innocua* counts reached similar levels in the Listeria mix and the Listeria + G− mix cultures during the incubation period (Fig. 1B, D).

3.2. Distribution of L. monocytogenes strains in mono-species and mixed-culture combinations

According to the observed ability of *L. monocytogenes* to grow and establish in mixed species and multigenera bacterial suspensions and biofilms, studies were performed to assess whether individual strains of *L. monocytogenes* had different ability to compete and grow in such communities. The distribution of the individual *L. monocytogenes* strains after biofilm formation for nine days showed clear differences in the two bacterial communities investigated, represented by the mono-species L. mono mix and the multigenera Listeria + G− mix (Fig. 2). In the L. mono mix cultures, no dominance of a particular strain was observed (Fig. 2A). In the Listeria + G− mix cultures, a dominance of one strain, *L. monocytogenes* MF4562, was evident after nine days incubation (Fig. 2B). The dominance was observed both in biofilms on coupons and in the suspensions surrounding the coupons. In a follow-up study to determine *L. monocytogenes* strain distribution at Day 2 and 5 in the Listeria + G− mix cultures, all 55 picked and isolated colonies were determined to be MF4562 based on partial *cat* gene sequence analyses. The overall bacterial compositions after nine days incubation of the Listeria + G− mix cultures showed a high dominance of *P. fluorescens followed by P. fragi* (Fig. 3).

3.3. Competition among L. monocytogenes strains in dual-species and multibacteria cultures

Further co-culture experiments were done to identify the role of the members of the mixed species microbiota on the inhibition of some, but not all. *L. monocytogenes*. Based on the results in Fig. 2B, three *L. monocytogenes* strains were selected to include both a putative strong competitor strain (MF4562) and potential weak competitor strains (MF4627 and MF4712). Each strain was grown in co-cultures with the *L. innocua* (n = 5) and the Gram− mix (n = 6), separately and together. The three *L. monocytogenes* strains showed highly similar growth pattern when grown alone or together with Gram-negative bacteria. In co-cultures with *L. innocua* mix, MF4562 reached significantly higher cell counts per ml (log 8.4) than MF4627 (log 7.2) and MF4712 (log

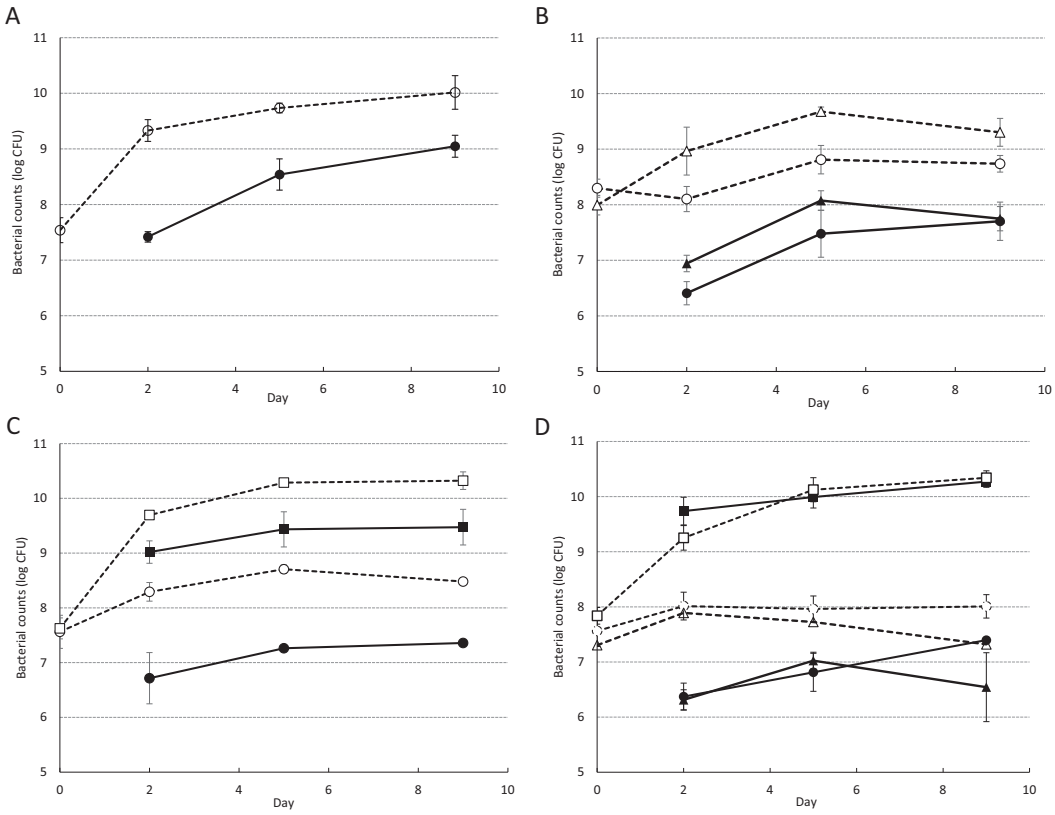

Fig. 1. Bacterial numbers in biofilms and in suspensions surrounding the biofilms. The experiments included *L. monocytogenes* in four different mixed bacterial cultures: (A) L. mono mix (six *L. monocytogenes* strains), (B) Listeria mix (L. mono mix + five *L. innocua* strains), (C) G− mix (L. mono mix + six Gram-negative bacteria) and (D) Listeria + G− mix (Listeria mix + G− mix). The experiments were performed at 12 °C in six-well cell culture plates containing BHI broth and stainless steel coupons as substratum for biofilm formation. Bacterial counts on coupons (log CFU/coupon) shown by filled symbols with continuous lines and in suspensions (log CFU/ml) surrounding the coupons as open symbols with dotted lines. *L. monocytogenes* (circles); *L. innouca* (triangles) and total counts (squares). Mean values of three experiments and standard error of the mean are shown.

7.4) after five days cultivation ($p < 0.01$; Fig. 4). Enhanced cell count differences between the *L. monocytogenes* strains were obtained in the multigenera *L. innocua* + Gram− mix, where the MF4562 counts were about 1000 times higher than the counts of the other two *L. monocytogenes* strains ($p < 0.01$). An additive effect of the *L. innocua* and the Gram− mix on the level of *L. monocytogenes* MF4627 and MF4712 was found (Fig. 4B, C). Mixed-species cultures with and without *L. innocua* provided similar inhibition of MF4562 in the two cultures (Fig. 4A). Corresponding results were obtained in biofilm experiments (incubated 48 h, 12 °C in BHI broth) with higher cell counts of MF4562 on the coupons than the other two *L. monocytogenes* strains under co-culture conditions with *L. innocua* and *L. innocua* + G− mix (Fig. S1, Supplemental material).

New experiments were performed to determine if selective inhibition of some *L. monocytogenes* strains was a general feature of *L. innocua*, or only some strains (Fig. 5). The data showed that individual *L. innocua* strains had different ability to retard growth of the putative weak competitor MF4627 strain while no such differences were observed for the strong competitor MF4562 strain. Two *L. innocua* strains (MF3940 and MF4052) provided lower cell counts of *L. monocytogenes* MF4627 (7.4–7.6 log) after five days co-incubation in separate dual species co-cultures compared to the other three *L. innocua* strains (8.8–9.0 log; $p \leq 0.05$). In follow-up studies with mixed cultures of *L. innocua* MF3940 (selected as a *L. innocua* that provided strain dependent growth inhibition of *L. monocytogenes*) and the individual Gram-negative strains, the *L. monocytogenes* cell counts were significantly reduced for both the strong (MF4562) and weak (MF4627) competitor strain. Respective mean reductions compared to growth in single strain cultures were 1.1 log ($p \leq 0.02$) and 3.2 log ($p \leq 0.02$; Fig. 5). The mix culture with the MF3971 *S. liquefaciens* isolate showed significantly higher abilities to impair the growth of the strong competitor MF4562 (1.9 log reductions; $p < 0.01$) than the other culture mixes. All mixed cultures provided cell count reductions in the range log 2.4–3.7 for the low competitor MF4627 strain, but no significant differences between the cultures in the ability to inhibit growth of this strain were obtained.

The supernatants (in 1:1 mix with BHI broth) of two and five day individual cultures of selected bacteria (*L. innocua* MF3940, *P. fluorescens* MF4988, *S. liquefaciens* MF3971 and *S. maltophilia* MF5364) and three dual strain cultures of MF3940 and these three Gram− bacteria did not influence the cell numbers of the MF4562 and the MF4627 *L. monocytogenes* strains differently. Cell counts of both *L. monocytogenes* strains tested were approximately 9 log after both two and five days incubation in all cultures (data not shown).

3.4. Contact versus non-contact dependent growth inhibition of *L. monocytogenes*

Whether the competitive effect of *L. innocua* and Gram-negative bacteria on *L. monocytogenes* growth was dependent on cell-to-cell contact was tested. The *L. monocytogenes* strains MF4562 and MF4627 were selected due to their different competitive properties in mixed cultures with *L. innocua* and the background microbiota strains (Fig. 5). Experiments performed in culture plates using 0.4 μm PET membrane inserts allowed separation of strains while metabolites produced during growth were able to diffuse through the membrane. Lower cell counts were observed at contact dependent growth compared to non-contact growth (membrane separated *L. monocytogenes* and other bacteria; Fig. S2, Supplemental material). Highest cell count reductions after five

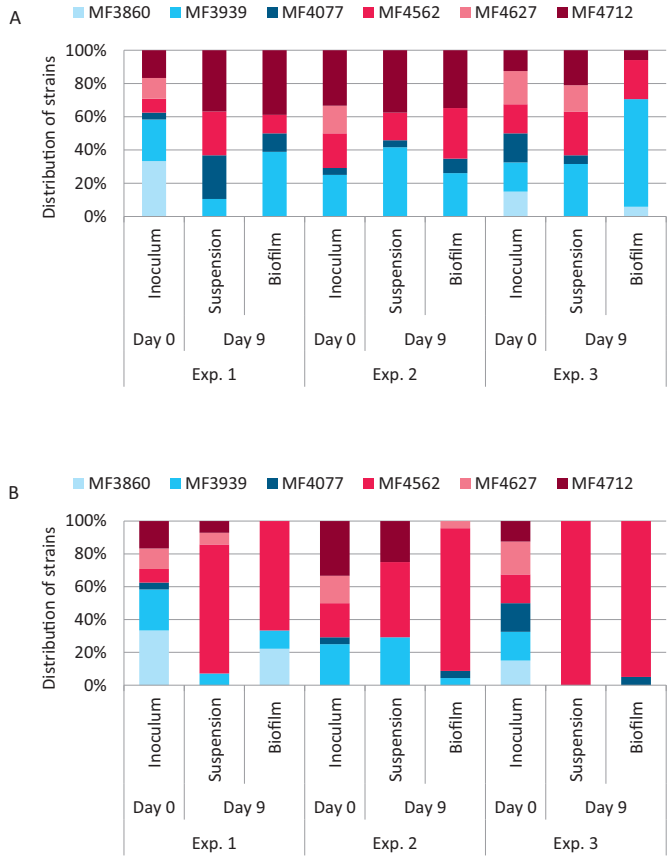

Fig. 2. Distribution of individual *L. monocytogenes* strains (n = 6) at Day 0 (inoculum) and after nine days (Day 9) incubation at 12 °C in biofilms on stainless steel coupons and in the broth suspensions surrounding the biofilms. The experiments included two different mixed culture inocula: (A) L. mono mix, (B) Listeria + G− mix. Data from three individual experiments are shown (Exp. 1–3). Identity and distribution of the individual *L. monocytogenes* strains (MF-numbering) were determined using partial DNA sequencing of the *cat* gene of picked colonies.

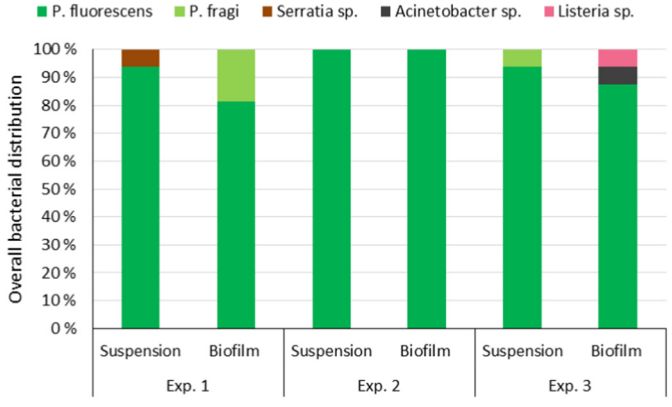

Fig. 3. Overall bacterial compositions in biofilms on stainless steel coupons and in the broth suspensions surrounding the biofilms after nine days incubation. The Listeria + G− mix was used as inoculum. Data are based on partial 16S rRNA gene sequencing of individual colonies obtained after plating from the biofilm and suspension samples. Data from three individual experiments are shown (Exp. 1–3).

days incubation were observed for the MF4627 strain with reductions of 1.4 log (in mix with *L. innocua*) and 1.5 log (in mix with *L. innocua* + G− bacteria) CFU/ml compared to non-contact dependent growth of the same mixes. Minor differences between contact- and non-contact dependent growth was observed for the MF4562 strain. However, the observed differences were not statistically significant (Fig. S2, Supplemental material).

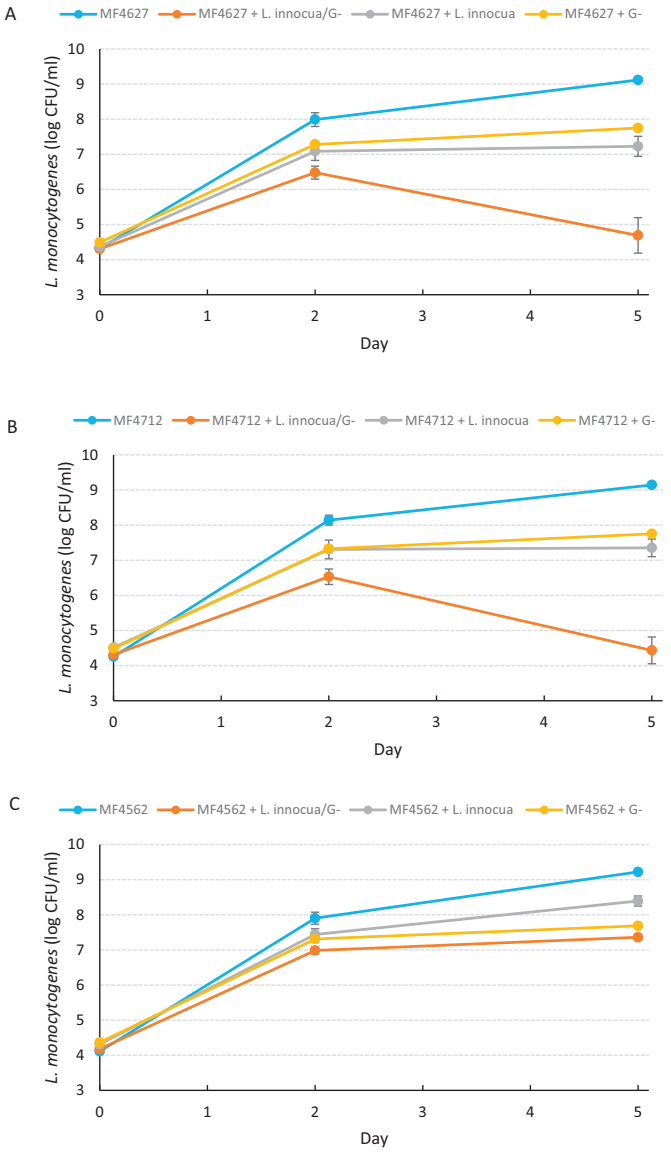

Fig. 4. Growth in suspensions of three strains of *L. monocytogenes* (A) MF4562, (B) MF4627, (C) MF4712 when cultured as single strains and in different mixed culture combinations with *L. innocua* (n = 5) and Gram-negative bacteria (n = 6). *L. monocytogenes* counts of single strains (blue lines), and in mixes with *L. innocua* + G− bacteria (orange lines), *L. innocua* (grey lines) and Gram-negative bacteria (yellow lines) are shown. Cultures were incubated for five days at 12 °C in BHI broth. Mean values of three to five experiments and standard error of the means are shown.

4. Discussion

This study demonstrates that *L. monocytogenes* strains have significant differences in their ability to grow and compete in multi-bacteria cultures and biofilms under food industry relevant conditions. The data show that *L. monocytogenes* establish in different multiculture biofilms although various competition and/or interaction effects occur between members of the bacterial communities that restrict growth of co-cultured *L. monocytogenes*. The strain differences observed indicated the existence of strong and weak competitor *L. monocytogenes* strains. In dual- and multiculture experiments, growth inhibition of certain *L.*

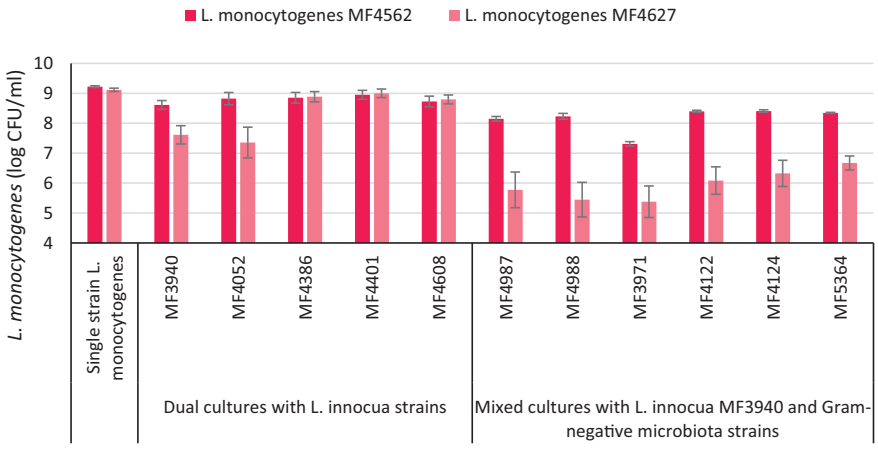

Fig. 5. Counts of two strains of *L. monocytogenes* (MF4562 and MF4627) after five days incubation of different bacterial mixed culture combinations. The *L. monocytogenes* strains were cultured as single strains, in dual cultures with individual strains of *L. innocua* (n = 5) and in multicultures with combinations of *L. innocua* MF3940 and individual strains of Gram-negative bacteria (n = 6). Mean values of three to five experiments and standard error of the means are shown.

monocytogenes by single strains of *L. innocua* was identified, inhibitory effects that were enhanced by Gram-negative bacteria commonly sharing microhabitats with *L. monocytogenes* on surfaces in food processing industry. The study is a contribution to understand the role of intraspecies and multispecies interactions in the ability of *L. monocytogenes* to establish, survive and persist in food processing premises.

When grown in a mixed mono-species culture, no single *L. monocytogenes* strains appeared to dominate in numbers over the other strains. Thus, there was no significant growth reduction of certain *L. monocytogenes* strains in co-cultures with other *L. monocytogenes* as recently reported (Zilelidou et al., 2015). Such differences between studies are commonly found and are likely to be the results of variations in experimental conditions including individual strain properties. This makes comparison of studies difficult, but also emphasise and disclose the complexity of bacterial interactions involving *L. monocytogenes* that require further understanding for improved control of this versatile pathogen (Giaouris et al., 2015). This study indicated that the growth of *L. monocytogenes* in the presence of a mixed Gram-negative microbiota was hampered with no significant variations in inhibition between *L. monocytogenes* strains. However, in co-cultures with *L. innocua*, some *L. monocytogenes* strains appeared to withstand competition from *L. innocua* whereas others did not. This observed difference in competitiveness between *L. monocytogenes* strains was further enhanced when both *L. innocua* and Gram-negative background microbiota were present in the multistrain cultures. Thus, *L. monocytogenes* strains (e.g. MF4627) sensitive to growth inhibition by certain *L. innocua* strains were further inhibited by the accompanying background microbiota. Contrary, *L. monocytogenes* strains (e.g. MF4562) that can withstand such inhibitory interaction effects are likely to have a selective advantage with improved potential for growth, survival and persistence. In accordance with the terminology used by Zilelidou et al. (2015), these strains, having weak and strong growth competitive abilities under the tested conditions, were termed as weak and strong competitors, respectively.

Despite distinct growth inhibition of *L. monocytogenes* co-cultured with other bacteria, *L. monocytogenes* was present in biofilms of all cultures and with increasing relative levels of *L. monocytogenes* during the nine days incubation period. This was generally in line with previous studies where *L. monocytogenes* was able to establish in mixed bacterial biofilms although the growth of *L. monocytogenes* was hampered (Guillier et al., 2008; Langsrud et al., 2016). Studies have shown other bacteria to either increase or decrease surface colonization and biofilm formation of *L. monocytogenes* e.g. (Carpentier and Chassaing, 2004; Daneshvar Alavi and Truelstrup Hansen, 2013; Fox et al., 2014; Hassan et al., 2004). The present study showed the competitive effects to occur in both biofilms and suspensions. This suggests that the competitive interactions between *L. monocytogenes* and the other co-cultured bacteria were not according to specific biofilm-associated mechanisms. Of particular interest was the selective growth inhibition of *L. monocytogenes* strains by certain *L. innocua* isolates, inhibition that was further enhanced in mixed cultures containing Gram-negative bacteria from the background microbiota. Strain-dependent inhibitory interactions of *L. monocytogenes* by *L. innocua* have been reported and include the production of bacteriocin-like agents, quorum sensing molecules, bacteriophages or possession of a prophage rendering infected strains “immune” to a lytic phage (Carvalheira et al., 2010; Kalmokoff et al., 1999; Yokoyama et al., 1998; Yokoyama et al., 2005). Culture supernatants of either *L. innocua* or co-cultures of *L. innocua* and background microbiota strains did not affect the growth of three tested *L. monocytogenes* strains differently. Growth inhibitory mechanisms including e.g. bacteriocin-like substances produced by *L. innocua* strains and their corresponding resistance mechanisms in certain *L. monocytogenes* strains may still be involved. Other studies performed in broth medium and in food and food processing environments have shown inhibition of *L. monocytogenes* by other bacteria to be attributed to competition for nutrients (Cornu et al., 2002; Delignette-Muller et al., 2006; Guillier et al., 2008). The growth inhibitory effects of *L. monocytogenes* under mixed culture conditions are probably a combination of specific and general antagonistic mechanisms where production of antimicrobials, competition for nutrients, growth abilities at low temperature and tolerance mechanisms have a role. This is in agreement with recent studies where the complexity of interactions between *Listeria* strains and species and between *L. monocytogenes* and other bacteria have been identified under multiculture conditions (Fagerlund et al., 2017; Giaouris et al., 2015; Langsrud et al., 2016; Papaioannou et al., 2018; Zilelidou et al., 2015). Additional studies are required for the identification of the exact inhibitory substances and mechanisms involved in the current study.

A role of cell contact on growth inhibition of *L. monocytogenes* in mixed cultures with *L. innocua* and the Gram-negative background bacteria was also indicated. Enhanced growth inhibition was observed when *L. monocytogenes* were co-cultured in direct contact with the *L. innocua* and the Gram-negative background bacteria compared to growth of *L. monocytogenes* and the other strains separated by the membrane. Although further studies are required, the results indicated stronger contact dependent inhibitory effect for the weak competitor MF4627 compared to the strong competitor MF4562 strain. The results are parallel to findings by Zilelidou et al. (2016) who reported similar results in *L. monocytogenes* dual strain co-cultures.

The practical relevance of the presence of strong and weak competitor *L. monocytogenes* strains in food industry premises remains unclear. However, an uneven distribution of *L. monocytogenes* subtypes based on MLVA profiling among isolates from salmon and meat processing plants have been observed (Møretrø et al., 2017). It may therefore be speculated that specific subtypes of *L. monocytogenes* have selective advantages under different environmental conditions (e.g.

nutrient availability or microbial competition) that are present in salmon and/or meat processing industries. The enhanced competitiveness of MF4562 observed in this study could be a factor in the apparent widespread distribution of this subtype in meat plants.

Co-existence and interactions of *L. monocytogenes* with bacteria sharing the same habitats are likely to occur in food processing environments and can exert different effects on *L. monocytogenes* survival, growth and persistence. Experiments were therefore performed under conditions (temperature, humidity, surface material) relatively realistic to those found in food processing industry. The selected background bacteria reflected the survival and overall dominance of certain genera within Gram-negatives (e.g. *Pseudomonas*, *Serratia*, *Acinetobacter*) on surfaces after cleaning in parts of the meat and salmon processing industries (Bagge-Ravn et al., 2003; Brightwell et al., 2006; Langsrud et al., 2016; Møretrø and Langsrud, 2017; Møretrø et al., 2013; Møretrø et al., 2016). Strains of *L. innocua* from food processing surfaces associated with *L. monocytogenes* contamination were also selected. *L. innocua* is in general the most prevalent *Listeria* species, is frequent in food processing environments, and co-existence of *L. innocua* and *L. monocytogenes* in the same microhabitats is not uncommon (Besse et al., 2005; Cornelius et al., 2008; Rørvik et al., 1995). The six *L. monocytogenes* strains included belonged to MLVA- types of variable prevalence in these food environments and had Multi locus sequence types (ST) with worldwide prevalence (Chenal-Francisque et al., 2011; Møretrø et al., 2017). One should be aware that other experimental conditions could have provided other results and conclusions. Interaction networks in microbial consortia are complex, increase with the number of species present and are further complicated by phenotypic and genotypic variations between isolates of the same species. Variations in methodology, experimental conditions and differences in strains applied further enhance the complexity of such studies.

In conclusion, the current study showed that *L. monocytogenes* strains have different ability to grow and compete in multibacteria biofilms and suspension cultures consisting of bacteria common on surfaces in food processing environments. The studies identified *L. innocua* that provided specific growth inhibition of certain *L. monocytogenes* strains while other *L. monocytogenes* withstood this competitive effect. The growth inhibiting effects were further enhanced in multibacteria cultures also containing Gram− bacteria, indicating a combination of specific and more general competitive interactions to be involved. *L. monocytogenes* with competitive growth abilities in environments with a diverse bacterial composition is likely to have a selective advantage in e.g. food environments with improved potential for growth, survival and persistence. Further studies encompassing the multitude of conditions relevant for bacterial habitats in food processing environments would provide improved understanding of competitive interactions and mechanisms involved. This is of particular relevance for conditions and interactions involving *L. monocytogenes*, the most challenging foodborne pathogen to control in many food-processing premises.

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Conflict of interest

The authors declare that there is no conflict of interest regarding publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.03.026.

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