Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid

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ARTICLE INFO

ABSTRACT

We investigated the formation of single and mixed species biofilms of *Listeria monocytogenes* strains EGD-e and LR-991, with *Lactobacillus plantarum* WCFS1 as secondary species, and their resistance to the disinfectants benzalkonium chloride and peracetic acid. Modulation of growth, biofilm formation, and biofilm composition was achieved by addition of manganese sulfate and/or glucose to the BHI medium. Composition analyses of the mixed species biofilms using plate counts and fluorescence microscopy with dual fluorophores showed that mixed species biofilms were formed in BHI (total count, 8-9 $\log_{10}$ cfu/well) and that they contained 1-2 $\log_{10}$ cfu/well more *L. monocytogenes* than *L. plantarum* cells. Addition of manganese sulfate resulted in equal numbers of both species (total count, 8 $\log_{10}$ cfu/well) in the mixed species biofilm, while manganese sulfate in combination with glucose, resulted in 1-2 $\log_{10}$ more *L. plantarum* than *L. monocytogenes* cells (total count, 9 $\log_{10}$ cfu/well). Corresponding single species biofilms of *L. monocytogenes* and *L. plantarum* contained up to 9 $\log_{10}$ cfu/well. Subsequent disinfection treatments showed mixed species biofilms to be more resistant to treatments with the selected disinfectants. In BHI with additional manganese sulfate, both *L. monocytogenes* strains and *L. plantarum* grown in the mixed species biofilm showed less than 2 $\log_{10}$ cfu/well inactivation after exposure for 15 min to 100 µg/ml benzalkonium chloride, while single species biofilms of both *L. monocytogenes* strains showed 4.5 $\log_{10}$ cfu/well inactivation and single species biofilms of *L. plantarum* showed 3.3 $\log_{10}$ cfu/well inactivation. Our results indicate that *L. monocytogenes* and *L. plantarum* mixed species biofilms can be more resistant to disinfection treatments than single species biofilms.

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1. Introduction

The human pathogen *Listeria monocytogenes* is ubiquitously found in the environment, on plant materials and in the soil. As a consequence, raw materials used by the food industry may introduce *L. monocytogenes* into food processing facilities. Several studies have shown that *L. monocytogenes* can be present in food processing environments (Chasseignaux et al., 2002; Pritchard et al., 1995; Tompkin, 2002), and that some strains are persistently present (Keto-Timonen et al., 2007; Lunden et al., 2003; Rorvik et al., 1995; Tompkin, 2002). These resident strains are expected to form biofilms on food processing equipment, on conveyor belts, in pipes, on floors, and in drains. Since biofilms are generally more difficult to eradicate during disinfection treatments (Lewis, 2001; Mah and O'Toole, 2001; Robbins et al., 2005), the capability of *L. monocytogenes* to form biofilms poses a major concern for the food industry. Possible mechanisms involved in the increased resistance of biofilms to antimicrobial agents are the restricted penetration of the biofilm, the slow growth rate of organisms in the biofilm, and the induction of resistance mechanisms in the biofilm (Donlan and Costerton, 2002). Previous studies on the disinfection of *L. monocytogenes* showed that *L. monocytogenes* cells grown in single species biofilms are generally more resistant to disinfectants than planktonic grown cells (Berrang et al., 2008; Folsom and Frank, 2006; Pan et al., 2006; Romanova et al., 2007; Stopforth et al., 2002). However, some studies have also shown that detached biofilm cells and planktonic grown cells are equally sensitive to disinfectants (Kastbjerg and Gram, 2009; Stopforth et al., 2002), indicating that the increased resistance of biofilms against disinfectants might be dependent on the restricted penetration of the biofilm.

Most studies on *L. monocytogenes* biofilm formation focus on the variation between strains and serotypes to form single species biofilms in different conditions and on different types of materials (Borucki et al., 2003; Chae and Schraft, 2000; Chae et al., 2006; Chavant et al., 2002; Kalmokoff et al., 2001). However, in food processing environments, *L. monocytogenes* will most likely grow on surfaces with other microorganisms in a mixed species biofilm (Carpentier and Chassaing, 2004; Habimana et al., 2009). Previous research showed that *L. monocytogenes* is able to form mixed species biofilms with both Gram-positive and Gram-negative species. A broad

study on the effect of 29 bacterial strains isolated from the food processing environment on *L. monocytogenes* biofilm formation on stainless steel showed that co-culture with four strains resulted in increased biofilm formation, while the other strains showed no effect or decreased biofilm formation (Carpentier and Chassaing, 2004). Depending on the secondary species, *L. monocytogenes* cells were gathered around the microcolonies of secondary species, attached as single cells, or attached as separate microcolonies. In a mixed species biofilm of *L. monocytogenes* and *Flavobacterium* spp the number of *L. monocytogenes* cells increased compared with a single species biofilm and more importantly, *L. monocytogenes* was also recoverable for longer incubation periods (Bremer et al., 2001). In contrast, the single species biofilm of *L. monocytogenes* showed higher cell numbers than the mixed species biofilm with *Pseudomonas fragi* (Norwood and Gilmour, 2001). Mixed species biofilm formation experiments of *L. monocytogenes* EGD-e with six different *Staphylococcus aureus* strains showed that, except for one *S. aureus* strain, the number of *L. monocytogenes* cells in the mixed species biofilm was similar to the number of *L. monocytogenes* cells in the single species biofilm (Rieu et al., 2008). In our study we focus on the formation of mixed species biofilms of *L. monocytogenes* in combination with *Lactobacillus plantarum*. This bacterium is encountered in similar niches as *L. monocytogenes* including soil, plant rhizosphere and food-processing environments. Furthermore, *L. monocytogenes* has been isolated together with *L. plantarum* from food products such as green table olives (Caggia et al., 2004) or RTE meat (Campanini et al., 1993). Also, *L. plantarum* is able to form biofilms and cause spoilage of food products (Kubota et al., 2008, 2009).

In this study, we investigated the formation of single and mixed species biofilms of *L. monocytogenes* and *L. plantarum* and the resistance of these single and mixed species biofilms to the disinfectants benzalkonium chloride and peracetic acid. Benzalkonium chloride and peracetic acid are two of the most widely used disinfectants in the food industry (Ceragioli et al., 2010). Benzalkonium chloride is a member of the quaternary ammonium compounds that target cell membranes, while peracetic acid is an oxidizing agent that decomposes into safe waste products. Furthermore, we were able to modulate the contribution of both species to the mixed species biofilm with the addition of manganese sulfate and/or glucose to the growth medium to obtain mixed species biofilms containing equal number of bacteria from both species or mixed species biofilms in which one of the species is dominant. This allowed us to estimate whether a protective effect from one species to the other in the mixed species biofilm is dependent on the number bacteria from each species in the mixed species biofilm. Single and mixed species biofilms were visualized using phase contrast and fluorescence microscopy on cells constitutively expressing the optimized fluorescent proteins EGFP, ECFP, EYFP, or DsRed. The original genes that encode for these proteins contain codons that are optimal for expression in eukaryotic cells, while they are infrequently used by bacteria. Therefore, we modified these genes by replacing the infrequently used codons by codons that are more frequently used by *L. monocytogenes* and *L. plantarum*.

2. Materials and Methods

2.1. Strains, media, and growth conditions

L. monocytogenes strains EGD-e and LR-991 and derivatives thereof (Table 1) were stored in Brain Hearth Infusion (BHI) broth (Becton Dickinson, Le Pont de Claix, France) containing 15% sterile glycerol (Fluka, Buchs, Switserland) at -80 °C. *L. plantarum* WCFS1 and derivatives (Table 1) were stored in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) containing 15% sterile glycerol at -80 °C. BHI or MRS agar plates were streaked with cells from the -80 °C bacterial stocks using an inoculation needle and plates were incubated at 30 °C for 24 h. Single colonies were inoculated in BHI broth, BHI broth with addition of 0.005% manganese sulfate (Merck, Darmstadt, Germany) (BHI-Mn), based on the concentration of the specific *Lactobacillus* medium MRS (de Man et al., 1960), or BHI broth with addition of 0.005% manganese sulfate and 2% glucose (Merck, Darmstadt, Germany) (BHI-Mn-G) and grown for 18 h at 20 °C.

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant genotype or characteristics	Reference
L. monocytogenes		
EGD-e	Wild-type serotype 1/2a strain	(Glaser et al., 2001)
EGD-e:DsRed	EGD-e containing pIMC2-DsRed	This study
EGD-e:Erm[R]	EGD-e containing pSV2	This study
LR-991	Wild-type serotype 1/2b strain	(van der Veen et al., 2008)
LR-991:DsRed	LR-991 containing pIMC2-DsRed	This study
LR-991:EYFP	LR-991 containing pIMC2-EYFP	This study
LR-991:Erm[R]	LR-991 containing pSV2	This study
L. plantarum		
WCFS1	Wild-type strain	(Kleerebezem et al., 2003)
WCFS1:EGFP	Wild-type strain containing pSPAC-EGFP	This study
WCFS1:ECFP	Wild-type strain containing pSPAC-ECFP	This study
Plasmids		
pIMC	Cm[R] ; Site-specific listerial integrative vector	(Monk et al., 2008a)
pIMK2	Kan[R] ; Site-specific listerial integrative vector	(Monk et al., 2008b)
pIMK2-EGFP	Kan[R] ; Site-specific listerial integrative vector containing EGFP behind constitutive Phelp promoter	(van der Veen et al., 2010)
pIMC2-EGFP	Cm[R] ; pIMC derivative containing EGFP behind constitutive Phelp promoter	This study
pIMC2-EYFP	Cm[R] ; pIMC2-EGFP derivative in which EGFP is replaced with EYFP	This study
pIMC2-DsRed	Cm[R] ; pIMC2-EGFP derivative in which EGFP is replaced with DsRed	This study
pSPAC	Cm[R]; Shuttle plasmid containing IPTG-inducible P_{spac} promoter	(Freitag and Jacobs, 1999)
pSPAC-EGFP	Cm[R]; pSPAC derivative containing EGFP behind P_{spac} promoter	This study
pSPAC-ECFP	Cm[R]; pSPAC derivative containing ECFP behind P_{spac} promoter	This study
pSOG30112	Em[R]; Plasmid for random mutagenesis of Gram-positive bacteria	(Hain et al., 2008)
pSV2	Em[R]; pIMK2 derivative in which the Kan[R] gene is replaced with the Erm[R] gene of pSOG30112	This study

2.2. Vector construction

Recombinant DNA techniques were performed according to standard protocols (Sambrook et al., 1989). Sequences of genes expressing the fluorescent proteins EGFP, ECFP, EYFP, and DsRed were optimized to replace codons that are infrequently used by *L. monocytogenes* and *L. plantarum* by codons that are more common (Appendix 1) and synthesized by BaseClear (Leiden, The Netherlands). Vector pIMC2-EGFP, containing the PSA phage integrase system, was constructed by cloning the AatII-PstI digested Phelp-EGFP fragment of vector pIMK2-EGFP in vector pIMC. Vectors pIMC2-EYFP and pIMC2-DsRed were constructed by replacing EGFP from pIMC2-EGFP with EYFP and DsRed, respectively. Vectors pSPAC-ECFP and pSPAC-EGFP were constructed by cloning ECFP and EGFP as a HindIII-PstI fragment in vector pSPAC. Vector pSV2 was constructed by cloning ermC from vector pSOG30112 as a KpnI-NotI (made blunt) fragment in vector pIMK2 cut with KpnI and NdeI (made blunt).

2.3. Biofilm formation

Assessment of biofilm formation was performed using a method based on a previously described protocol (Merritt et al., 2005). 12-well polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 3 ml BHI, BHI-Mn, or BHI-Mn-G and inoculated with 1% of an overnight grown culture (18 h) at 20 °C. The plates were incubated at 20 °C for 24, 48, or 72 h. The medium was removed and the biofilm was washed three times with phosphate buffered saline (PBS) (Merck, Darmstadt, Germany). The biofilm was subsequently resuspended in 1 ml PBS by pipetting rigorously and serial diluted in PBS. To verify complete removal of the biofilm, the wells were stained with 0.1% crystal violet (Merck, Darmstadt, Germany) to detect the presence of any residual biofilm. Wells that contained residual biofilm were omitted. Cells were plated on BHI agar containing 2 µg/ml erythromycin (Sigma-Aldrich, Steinheim, Germany) for specific growth of *L. monocytogenes* strains containing pSV2, which is integrated in the genome due to the presence of a site-specific integrase, and/or MRS agar containing 20 µg/ml kanamycin (Sigma-Aldrich, Steinheim, Germany) for specific growth of *L. plantarum* WCSF1, which is naturally kanamycin resistant. Plates were incubated for 2-3 days at 30 °C and colonies were enumerated. Biofilm formation was assessed in two independent biological experiments using two replicates each.

Phase contrast and fluorescence microscopy experiments were performed to verify the formation of single and mixed species biofilms. Biofilms of *L. monocytogenes* and *L. plantarum* strains containing the pIMC and pSPAC derivatives were grown at 20 °C for 48 h in 6-well polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany) in 5 ml BHI, BHI-Mn, or BHI-Mn-G containing 1 µg/ml chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Steinheim, Germany) using a 1% inoculum of an overnight (18 h) grown culture at 20 °C. The medium was subsequently removed and biofilms were washed three times with PBS. Square cover glasses (18 mm) were placed on the biofilms grown in the 6-well plates and microscopy experiments were performed directly on the biofilms using a BX41 microscope (Olympus, Zoeterwoude, The Netherlands). EGFP and EYFP were visualized using the MNIBA3 filter (Olympus, Zoeterwoude, The Netherlands), ECFP was visualized using the U-MNBV-2 filter (Olympus, Zoeterwoude, The Netherlands), and DsRed was visualized using the U-MWIG3 filter (Olympus, Zoeterwoude, The Netherlands).

2.4. Disinfection treatments

Overnight (18 h) grown cultures at 20 °C were used to inoculate (1%) 12-well polystyrene microtiter plates containing 1 ml BHI, BHI-

Mn, or BHI-Mn-G and incubated at 20 °C for 48 h. Single and mixed species biofilms were washed once with PBS and exposed to 1 ml of 50 or 100 µg/ml benzalkonium chloride (Merck, Darmstadt, Germany) or peracetic acid (Sigma-Aldrich, Steinheim, Germany) up to 15 min at 20 °C. After exposure, the biofilms were washed once with PBS and resuspended in 1 ml PBS by pipetting rigorously. To verify that washing and resuspending in PBS inhibited further inactivation of the cells by residual benzalkonium chloride or peracetic acid, a control experiment was performed in which the cells after standard treatments were incubated in 1 ml PBS for up to 1 h. No further inactivation during this incubation period was observed for both benzalkonium chloride and peracetic acid treatments. The cells were serial diluted in PBS and plated on BHI agar containing 2 µg/ml erythromycin and/or MRS agar containing 20 µg/ml kanamycin. Plates were incubated for 3-5 days at 30 °C and colonies were enumerated.

Overnight (18 h) grown cultures at 20 °C were used to inoculate (1%) 2 ml BHI, BHI-Mn, or BHI-Mn-G in 12 ml polystyrene tubes (Greiner Bio-One, Frickenhausen, Germany). Planktonic cultures were
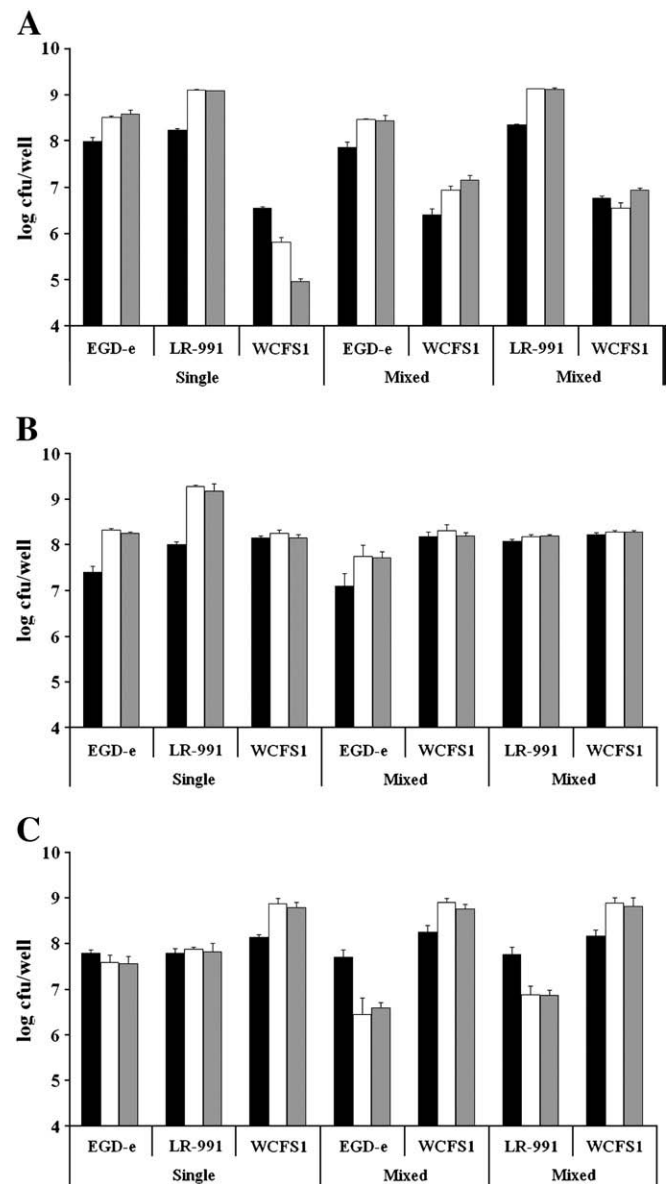
Fig. 1. Single and mixed species biofilm formation of *L. monocytogenes* EGD-e and LR-991 and *L. plantarum* WCFS1. The graphs show the average biofilm formation of two independent biological experiments using two replicates each, after 24 h (black), 48 h (white), and 72 h (grey) at 20 °C in 12-well polystyrene plates in BHI (A), BHI-Mn (B), and BHI-Mn-G (C).

grown statically for 24 h at 20 °C and 1 ml culture was centrifuged (2 min at 5000×g). The pellet was resuspended in 1 ml of 20 or 50 μg/ml benzalkonium chloride or peracetic acid up to 15 min at 20 °C. Samples were serial diluted in PBS and plated on BHI agar or MRS agar and incubated for 3-5 days at 30 °C. All disinfection treatments were performed in two independent biological replicates.

2.5. Data analyses

To determine whether planktonic cells, single species biofilms, and mixed species biofilms showed differences in resistance against benzalkonium chloride and peracetic acid, the inactivation curves were fitted with the reparameterized Gompertz model (Zwietering et al., 1990) using the following equation:

$$log_{10}N_t = log_{10}N_0 + A \cdot exp\left\{-exp\left[\frac{k \cdot e}{-A} \cdot (t_s - t) + 1\right]\right\} \qquad (1)$$

where A is the difference between the surviving population and the initial population (log_{10} cfu/well), k is the maximum specific inactivation rate (log_{10}/min), and t_s is the duration of the shoulder (min). The model was fitted in Microsoft Excel by minimizing the residual sum of squares using the Excel Solver add-in. Statistical significant differences between the average parameter estimates of the inactivation curves were identified using the Student's t-test ($p < 0.05$).

3. Results

3.1. Single and mixed species biofilm formation

The *L. monocytogenes* strain EGD-e, which is one of the most widely studied *L. monocytogenes* strains, and LR-991, which showed highest single and mixed species biofilm formation at 20 °C in BHI in a pre-screen of 143 *L. monocytogenes* strains (results not shown) using a rapid method described previously (Borucki et al., 2003), were used to assess single and mixed species biofilm formation with *L. plantarum* WCFS1 (Fig. 1). In BHI, the single species biofilms of *L. monocytogenes* EGD-e and LR-991 reached 8.5 and 9 log_{10} cfu/well, respectively, after 48-72 h, while the single species biofilm of *L. plantarum* contained 6.5 log_{10} cfu/well after 24 h, which decreased over time resulting in 5 log_{10} cfu/well after 72 h (Fig. 1A). The number of *L. monocytogenes* in the mixed species biofilm in BHI was similar to the single species biofilm and 10-100 fold higher than the number of *L. plantarum*. Interestingly, in the mixed species biofilm, the amount of *L. plantarum* did not decrease over time as was seen in the *L. plantarum* single species biofilm. We were able to modulate the composition of the biofilms with the addition of glucose and/or manganese sulfate to BHI. These components increase the planktonic growth capabilities of *L. plantarum* and not of *L. monocytogenes* (results not shown). Single species biofilm formation of *L. monocytogenes* in BHI-Mn was similar to biofilm formation in BHI (Fig. 1B). However, single species biofilms of *L. plantarum* in BHI-Mn contained 8 log_{10} cfu/well, which did not decrease over time as seen with biofilm formation in BHI. Furthermore, in BHI-Mn, equal numbers of *L. monocytogenes* and *L. plantarum* in the mixed species biofilm were observed (approximately 8 log_{10} cfu/well). In BHI-Mn-G, *L. monocytogenes* single species biofilms contained 7.5-8 log_{10} cfu/well, while *L. plantarum* single species biofilms reached approximately 9 log_{10} cfu/well after 48-72 h (Fig. 1C). The contribution of *L. plantarum* to the mixed species biofilm was also 10-100 times higher than the contribution of *L. monocytogenes*. *L. plantarum* reached approximately 9 log_{10} cfu/well after 48-72 h, while the contribution of *L. monocytogenes* decreased after 48-72 h to 6.5-7 log_{10} cfu/well. The decrease in *L. monocytogenes* viable counts in the mixed species biofilm might be related with the enhanced acidification by *L. plantarum* of the medium containing glucose, which reached approximately pH 3.4 after 48-72 h. In contrast, acidification during *L. monocytogenes* single species biofilm formation in medium containing glucose stopped at approximately
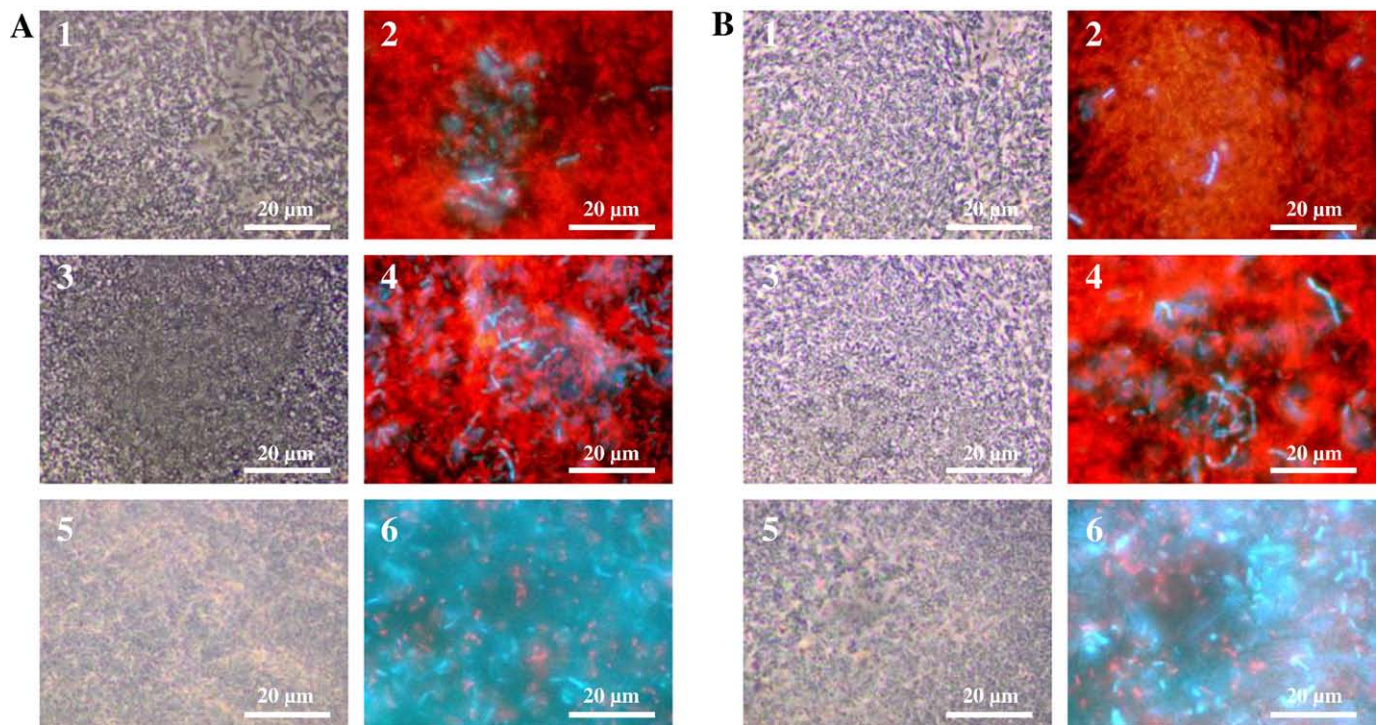
Fig. 2. Mixed species biofilms of *L. monocytogenes* EGD-e and LR-991 and *L. plantarum* WCFS1. The pictures show phase contrast (1, 3, and 5) and fluorescent (2, 4, and 6) images of biofilms grown for 48 h at 20 °C in BHI (1 and 2), BHI-Mn (3 and 4), and BHI-Mn-G (5 and 6). A) Mixed species biofilms of *L. monocytogenes* EGD-e constitutively expressing DsRed and *L. plantarum* WCFS1 constitutively expressing ECFP. B) Mixed species biofilms of *L. monocytogenes* LR-991 constitutively expressing DsRed and *L. plantarum* WCFS1 constitutively expressing ECFP.
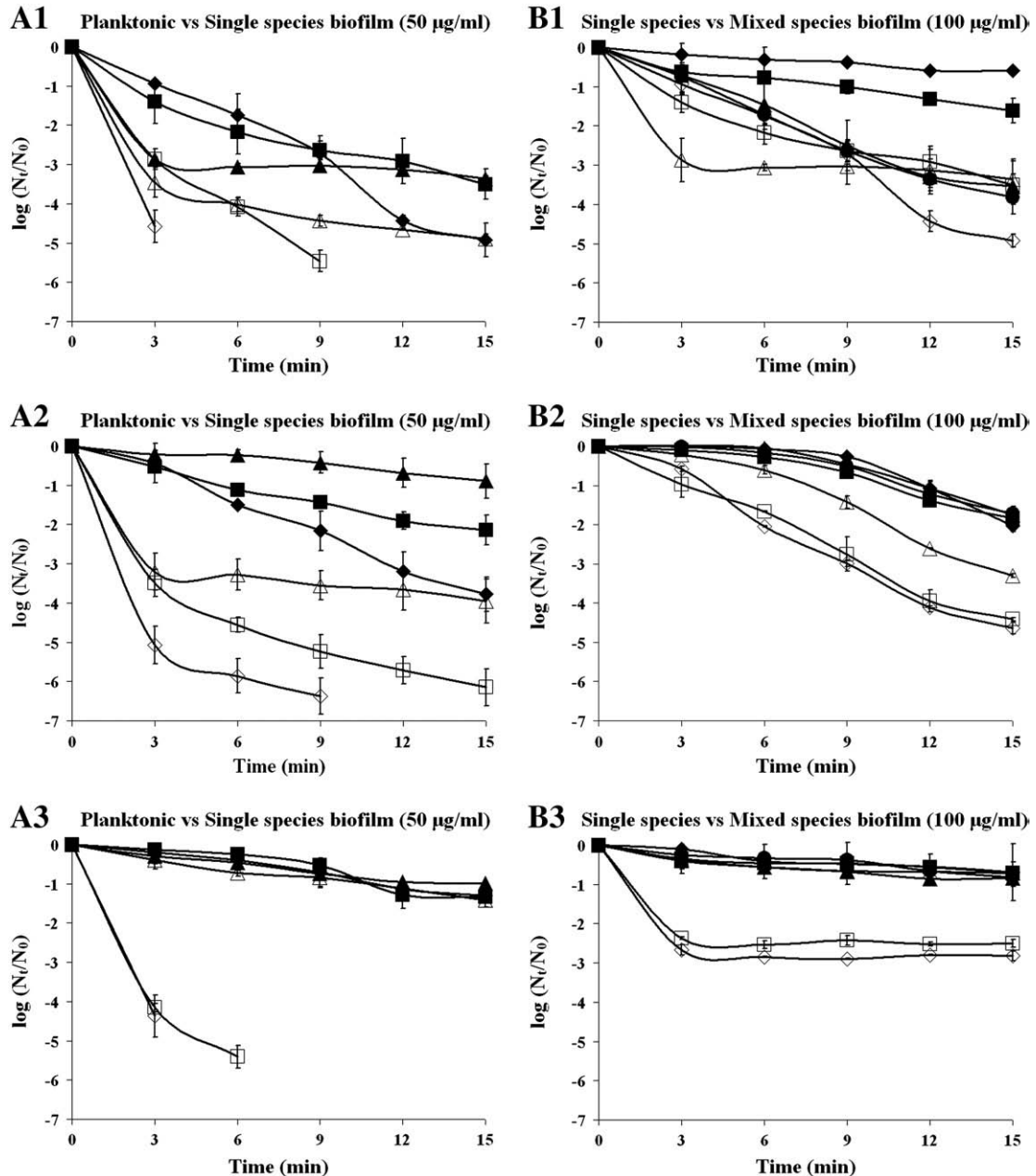
pH 4.3. Single and mixed species biofilm formation in BHI and BHI containing manganese sulfate resulted in a final pH of approximately 5.3-5.5.

The formation of single and mixed species biofilms was microscopically verified using bacteria expressing different fluorescent proteins. The formation of both single (Appendix 2) and mixed species biofilms (Fig. 2) was observed in all conditions. The biofilms of *L. monocytogenes* and *L. plantarum* grown in single and mixed species conditions consisted of a dense structure of multiple heterogeneous layers of cells showing a morphology very similar to planktonic grown cells of the two species. The images of the mixed species biofilms formed under the three growth conditions are in concordance with

the CFU counts of these conditions and furthermore showed that *L. monocytogenes* and *L. plantarum* form a real spatial mixed biofilm.

3.2. Resistance to disinfection treatments

The resistance of planktonic cells and single and mixed species biofilms to the disinfectants benzalkonium chloride and peracetic acid was investigated. The inactivation curves of the various treatments were fitted with the reparameterized Gompertz model and parameter estimates were determined. Differences in resistance can be reflected in differences in the surviving population after disinfectant exposure (A), differences in the maximum specific inactivation rate (k), or

Fig. 3. Disinfection treatment of planktonic cells, single and mixed species biofilms with benzalkonium chloride. The graphs show the average inactivation of two biological independent experiments of planktonic cells (24 h) and biofilms (48 h) grown at 20 °C in 12-well polystyrene plates in BHI (1), BHI-Mn (2), and BHI-Mn-G (3) and treated with the respective concentration of benzalkonium chloride for 15 min. Data points below the detection limit ($\log_{10} (N_t/N_0) \approx -6.5$) are not shown in the graphs. A) Planktonic cells (open symbols) and single species biofilms (closed symbols) of *L. monocytogenes* EGD-e (diamonds), LR-991 (squares), and *L. plantarum* WCFS1 (triangles) treated with 50 µg/ml benzalkonium chloride. B) Single species biofilms (open symbols) and mixed species biofilms (closed symbols) of *L. monocytogenes* EGD-e (diamonds), LR-991 (squares), and *L. plantarum* WCFS1 (triangles: single species biofilms and mixed species biofilms with EGD-e, circles: mixed species biofilms with LR-991) treated with 100 µg/ml benzalkonium chloride.

Table 2
Parameter estimates of the inactivation curves after treatment with benzalkonium chloride using the reparameterized Gompertz model.

Biofilm growth medium	Disinfectant concentration (µg/ml)	Parameter	Planktonic cells			Single species biofilms			Mixed species biofilms			
			EGD-e	LR-991	WCFS1	EGD-e	LR-991	WCFS1	EGD-e	WCFS1	LR-991	WCFS1
BHI	20	A[c]	-5.94 ± 0.39	-1.21 ± 0.33	-2.36 ± 0.37							
		k[d]	0.40 ± 0.05	0.41 ± 0.06	0.35 ± 0.06							
		t_s[e]	0.09 ± 0.43	0.41 ± 0.02	-0.12 ± 0.52							
	50	A	-6.09 ± 0.25	-5.64 ± 0.75	-4.56 ± 0.01	-4.62 ± 0.19[a]	-1.82 ± 0.03[a]	-2.96 ± 0.02[a]	-0.69 ± 0.17[a,b]	-5.86 ± 0.90[b]	-1.56 ± 0.28[a]	-4.38 ± 0.11[b]
		k	2.67 ± 0.22	0.94 ± 0.21	1.41 ± 0.36	0.45 ± 0.07[a]	0.19 ± 0.11[a]	0.81 ± 0.10	0.07 ± 0.01[a,b]	0.32 ± 0.00[a,b]	0.12 ± 0.01[a]	0.32 ± 0.00[a,b]
		t_s	1.08 ± 0.16	0.21 ± 0.15	0.35 ± 0.13	2.26 ± 0.19[a]	-0.06 ± 0.73	0.19 ± 0.12	4.70 ± 0.89[a]	1.71 ± 0.87	-0.73 ± 0.97	1.27 ± 0.34
	100	A				-6.86 ± 0.06	-3.34 ± 0.36	-3.14 ± 0.38	-0.60 ± 0.10[b]	-3.96 ± 0.70	-1.85 ± 0.28[b]	-4.30 ± 0.24
		k				0.42 ± 0.00	0.37 ± 0.06	2.02 ± 0.01	0.14 ± 0.04[b]	0.36 ± 0.01[b]	0.11 ± 0.01[b]	0.36 ± 0.04[b]
		t_s				1.82 ± 0.62	-0.24 ± 0.67	0.99 ± 0.69	3.05 ± 3.40	1.78 ± 1.57	-0.77 ± 0.84	1.28 ± 0.17
BHI-Mn	20	A	-3.60 ± 0.09	-1.90 ± 0.36	-3.31 ± 0.08							
		k	0.48 ± 0.06	0.24 ± 0.08	0.47 ± 0.09							
		t_s	-0.11 ± 0.04	-0.60 ± 1.00	-0.13 ± 0.11							
	50	A	-6.15 ± 0.44	-5.71 ± 0.39	-3.62 ± 0.46	-4.74 ± 0.71	-2.39 ± 0.59[a]	-3.17 ± 0.87	-0.86 ± 0.07[a,b]	-2.39 ± 0.39	-1.39 ± 0.03[a]	-2.59 ± 1.25
		k	2.42 ± 0.29	1.06 ± 0.06	1.62 ± 0.28	0.33 ± 0.02[a]	0.19 ± 0.01[a]	0.09 ± 0.01[a]	0.09 ± 0.01[a,b]	0.13 ± 0.04[a]	0.07 ± 0.03[a,b]	0.21 ± 0.12[a]
		t_s	0.55 ± 0.00	0.02 ± 0.08	0.48 ± 0.00	2.01 ± 0.71	0.56 ± 0.48	5.75 ± 4.23	6.24 ± 4.19	5.69 ± 1.09[a]	3.66 ± 3.03	5.78 ± 1.49[a]
	100	A				-5.24 ± 0.23	-5.78 ± 0.61	-4.86 ± 0.00	-3.65 ± 0.30[b]	-2.99 ± 0.55[b]	-3.05 ± 0.40[b]	-2.50 ± 0.74[b]
		k				0.45 ± 0.00	0.37 ± 0.02	0.34 ± 0.01	0.33 ± 0.01[b]	0.24 ± 0.00[b]	0.20 ± 0.00[b]	0.23 ± 0.01[b]
		t_s				1.87 ± 0.12	1.35 ± 0.61	4.55 ± 0.48	8.83 ± 0.45[b]	7.52 ± 0.84[b]	5.35 ± 0.14[b]	6.68 ± 0.37[b]
BHI-Mn-G	20	A	-5.57 ± 0.23	-4.48 ± 0.71	-0.50 ± 0.01							
		k	2.31 ± 0.02	0.27 ± 0.01	0.06 ± 0.01							
		t_s	1.10 ± 0.10	1.17 ± 0.11	0.79 ± 1.45							
	50	A	-6.06 ± 0.28	-5.53 ± 0.44	-1.73 ± 0.32	-1.78 ± 0.56[a]	-1.89 ± 0.50[a]	-1.22 ± 0.32	-0.34 ± 0.25[a]	-1.70 ± 0.33	-0.52 ± 0.17[a]	-1.37 ± 1.34
		k	2.51 ± 0.35	2.05 ± 0.58	0.10 ± 0.00	0.14 ± 0.02[a]	0.18 ± 0.03[a]	0.30 ± 0.33	0.29 ± 0.34[a]	0.09 ± 0.05	0.19 ± 0.07[a]	0.12 ± 0.04
		t_s	1.08 ± 0.12	0.48 ± 0.73	-0.13 ± 0.59	3.23 ± 3.32	5.44 ± 0.86[a]	1.49 ± 5.45	2.19 ± 0.33[a]	0.52 ± 2.03	0.37 ± 0.02[b]	1.50 ± 1.40
	100	A				-2.84 ± 0.04	-2.50 ± 0.09	-0.71 ± 0.02	-0.63 ± 0.49[b]	-0.96 ± 0.35	-0.60 ± 0.14[b]	-1.65 ± 0.53
		k				1.77 ± 0.43	1.57 ± 0.07	0.20 ± 0.16	0.11 ± 0.12[b]	0.17 ± 0.11	0.17 ± 0.17[b]	0.06 ± 0.00
		t_s				0.57 ± 0.14	0.63 ± 0.13	0.75 ± 1.25	1.25 ± 1.61	0.90 ± 0.43	0.66 ± 0.35	1.39 ± 1.59

[a] Parameter estimate significantly different from planktonic cells ($p < 0.05$; t-test).
[b] Parameter estimate significantly different from single species biofilms ($p < 0.05$; t-test).
[c] Difference between the surviving population and the initial population ($\log_{10}$ cfu/well).
[d] Maximum specific inactivation rate ($\log_{10}$/min).
[e] Duration of the shoulder (min).

differences in the duration of the shoulder (t_s). Differences in disinfectant resistance between planktonic cells and single and mixed species biofilms are considered significant when any of the three parameters A, k, or t_s of the inactivation curve is significantly different. Our results showed that single and mixed species biofilms are more resistant to benzalkonium chloride than planktonic grown cells ($p < 0.05$, t-test) (Fig. 3A and Table 2), except for *L. plantarum* grown in BHI-Mn-G. More importantly, in most conditions mixed species biofilms appeared to be more resistant to benzalkonium chloride than single species biofilms (Fig. 3B and Table 2). Both *L. monocytogenes* strains and *L. plantarum* grown in mixed species biofilms in BHI showed a lower maximum specific inactivation rate

($p < 0.05$, t-test) after exposure for 15 min to 100 μg/ml benzalkonium chloride compared with *L. monocytogenes* and *L. plantarum* grown in single species biofilms and both *L. monocytogenes* strains grown in mixed species biofilms in BHI furthermore showed a higher surviving population ($p < 0.05$, t-test). In BHI-Mn, both *L. monocytogenes* and *L. plantarum* grown in mixed species biofilms showed a higher surviving population ($p < 0.05$, t-test), a lower maximum specific inactivation rate ($p < 0.05$, t-test), and a longer duration of the shoulder ($p < 0.05$, t-test) after exposure for 15 min to 100 μg/ml benzalkonium chloride compared with *L. monocytogenes* and *L. plantarum* grown in single species biofilms. In contrast, in BHI-Mn-G only *L. monocytogenes* grown mixed species biofilms showed a higher surviving population ($p < 0.05$,
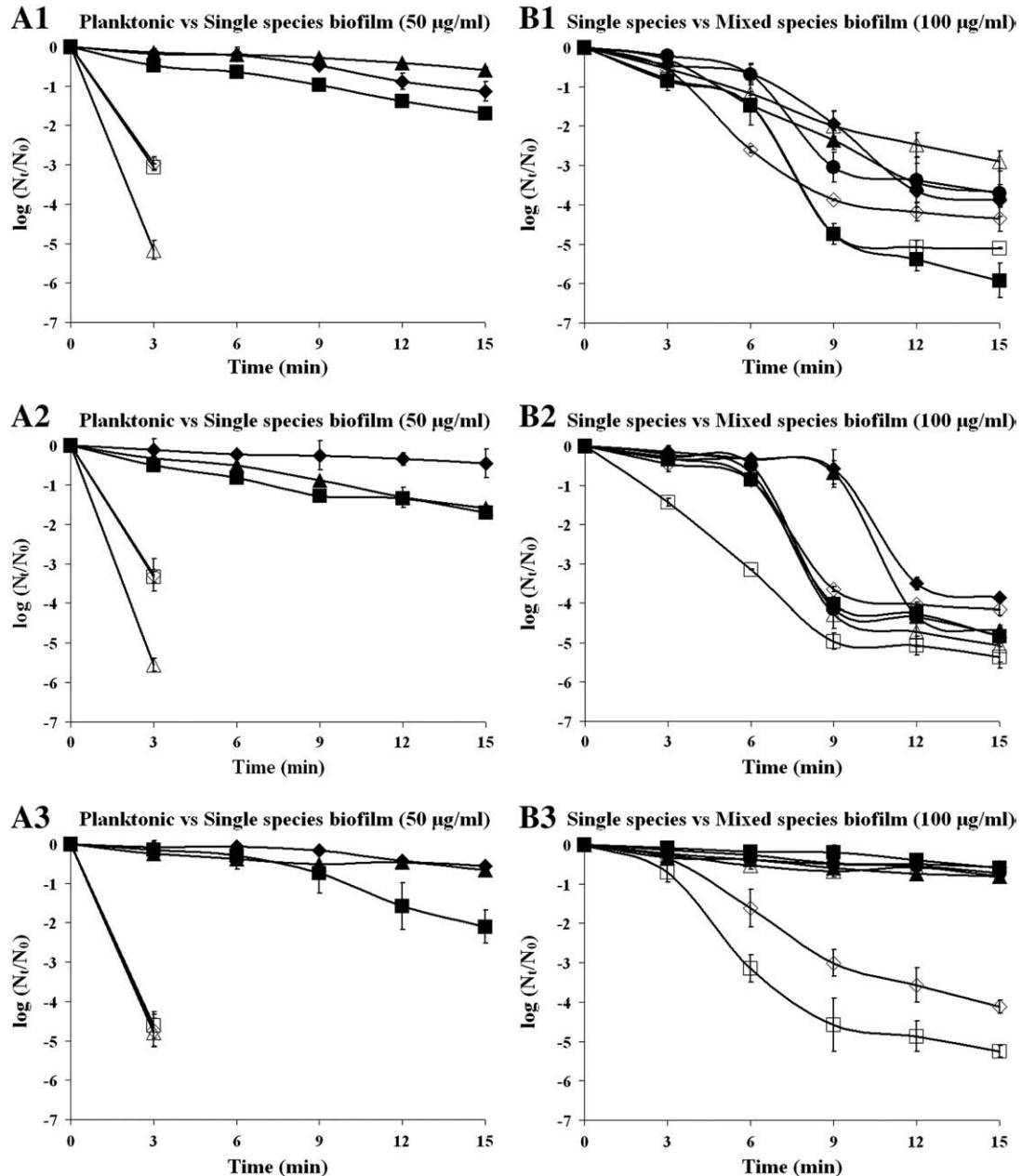
Fig. 4. Disinfection treatment of planktonic cells, single and mixed species biofilms with peracetic acid. The graphs show the average inactivation of two biological independent experiments of planktonic cells (24 h) and biofilms (48 h) grown at 20 °C in 12-well polystyrene plates in BHI (1), BHI-Mn (2), and BHI-Mn-G (3) and treated with the respective concentration of peracetic acid for 15 min. Data points below the detection limit ($\log_{10}(N_t/N_0) \approx -6.5$) are not shown in the graphs. A) Planktonic cells (open symbols) and single species biofilms (closed symbols) of *L. monocytogenes* EGD-e (diamonds), LR-991 (squares), and *L. plantarum* WCFS1 (triangles) treated with 50 μg/ml peracetic acid for 15 min. B) Single species biofilms (open symbols) and mixed species biofilms (closed symbols) of *L. monocytogenes* EGD-e (diamonds), LR-991 (squares), and *L. plantarum* WCFS1 (triangles: single species biofilms and mixed species biofilms with EGD-e, circles: mixed species biofilms with LR-991) treated with 100 μg/ml peracetic acid for 15 min.

Table 3
Parameter estimates of the inactivation curves after treatment with peracetic acid using the reparameterized Gompertz model.

Medium	Disinfectant concentration (µg/ml)	Parameter	Planktonic cells			Single species biofilms			Mixed species biofilms			
			EGD-e	LR-991	WCFS1	EGD-e	LR-991	WCFS1	EGD-e	WCFS1	LR-991	WCFS1
BHI	20	A^{c}	-5.36 ± 0.08	-4.94 ± 0.75	-5.19 ± 0.24							
		k^{d}	1.47 ± 0.19	1.36 ± 0.02	1.08 ± 0.11							
		t_s^{e}	2.23 ± 0.27	2.70 ± 0.64	0.48 ± 0.15							
	50	A	-5.92 ± 0.42	-5.36 ± 0.65	-5.85 ± 0.09	-1.63 ± 0.02^{a}	-2.66 ± 0.43^{a}	-1.16 ± 0.15^{a}	$-0.95 \pm 0.15^{a,b}$	-1.04 ± 0.07^{a}	-1.64 ± 0.75^{a}	-1.44 ± 0.19^{a}
		k	2.23 ± 0.03	1.83 ± 0.09	3.06 ± 0.33	0.13 ± 0.07^{a}	0.12 ± 0.01^{a}	0.05 ± 0.01^{a}	0.07 ± 0.01^{a}	0.08 ± 0.01^{a}	0.09 ± 0.03^{a}	0.10 ± 0.02^{a}
		t_s	1.67 ± 0.09	1.29 ± 0.07	0.83 ± 0.03	4.30 ± 2.83	0.75 ± 0.93	2.13 ± 3.46	3.68 ± 3.36	0.64 ± 1.07	1.20 ± 2.31	1.29 ± 1.27
	100	A				-4.38 ± 0.30	-5.10 ± 0.10	-3.34 ± 0.18	-4.55 ± 0.37	-4.12 ± 0.84	-5.80 ± 0.41	-3.60 ± 0.25
		k				0.74 ± 0.05	1.84 ± 0.40	0.26 ± 0.04	0.52 ± 0.04^{b}	0.40 ± 0.03	1.31 ± 0.02	1.00 ± 0.37
		t_s				2.39 ± 0.24	5.17 ± 0.25	1.39 ± 0.05	4.99 ± 0.80^{b}	2.71 ± 0.79	4.87 ± 0.06	5.23 ± 0.63^{b}
BHI-Mn	20	A	-3.12 ± 0.05	-3.44 ± 0.21	-5.99 ± 0.07							
		k	0.77 ± 0.26	0.56 ± 0.05	1.03 ± 0.04							
		t_s	1.37 ± 0.54	0.73 ± 0.02	0.45 ± 0.12							
	50	A	-5.88 ± 0.38	-5.48 ± 0.27	-5.94 ± 0.20	-0.56 ± 0.36^{a}	-1.88 ± 0.30^{a}	-2.42 ± 0.05^{a}	-0.68 ± 0.56^{a}	-1.02 ± 0.50^{a}	-1.20 ± 0.10^{a}	$-1.75 \pm 0.14^{a,b}$
		k	1.93 ± 0.28	1.93 ± 0.04	3.63 ± 0.10	0.03 ± 0.02^{a}	0.15 ± 0.03^{a}	0.13 ± 0.01^{a}	0.13 ± 0.03^{a}	0.07 ± 0.02^{a}	0.09 ± 0.01^{a}	0.13 ± 0.05^{a}
		t_s	1.27 ± 0.03	1.21 ± 0.07	0.78 ± 0.01	1.34 ± 3.85	0.26 ± 0.19^{a}	1.86 ± 0.62	9.21 ± 1.66^{a}	2.70 ± 2.35	$5.27 \pm 0.57^{a,b}$	6.27 ± 2.35
	100	A				-4.12 ± 0.08	-5.50 ± 0.32	-4.93 ± 0.42	-3.88 ± 0.08	-4.71 ± 0.15	-4.60 ± 0.18	-4.61 ± 0.11
		k				1.29 ± 0.21	0.73 ± 0.00	1.56 ± 0.07	1.43 ± 0.06	1.87 ± 0.38	1.37 ± 0.04^{b}	1.67 ± 0.02
		t_s				5.33 ± 0.25	1.20 ± 0.09	5.63 ± 0.15	8.68 ± 0.47^{b}	8.67 ± 0.28^{b}	5.39 ± 0.07^{b}	5.79 ± 0.03
BHI-Mn-G	20	A	-6.12 ± 0.10	-8.66 ± 1.24	-6.70 ± 0.36							
		k	2.06 ± 0.04	1.76 ± 0.06	1.80 ± 0.26							
		t_s	2.57 ± 0.23	2.83 ± 0.09	1.46 ± 0.03							
	50	A	-6.08 ± 0.24	-5.61 ± 0.32	-7.96 ± 0.02	-1.23 ± 0.56^{a}	-3.48 ± 0.46^{a}	-0.79 ± 0.18^{a}	-0.50 ± 0.24^{a}	-0.49 ± 0.22^{a}	$-0.28 \pm 0.15^{a,b}$	-0.59 ± 0.04^{a}
		k	2.75 ± 0.32	2.72 ± 0.38	2.78 ± 0.00	0.07 ± 0.01^{a}	0.24 ± 0.01^{a}	0.08 ± 0.07^{a}	$0.02 \pm 0.01^{a,b}$	0.05 ± 0.01^{a}	$0.02 \pm 0.01^{a,b}$	0.07 ± 0.02^{a}
		t_s	1.01 ± 0.12	0.95 ± 0.15	1.23 ± 0.15	6.32 ± 0.27^{a}	5.68 ± 2.44	0.65 ± 0.11^{a}	0.19 ± 1.76^{b}	0.79 ± 0.52	2.63 ± 2.59	0.44 ± 1.31
	100	A				-4.40 ± 0.35	-5.21 ± 0.26	-0.76 ± 0.04	-0.68 ± 0.28^{b}	-0.86 ± 0.08	-0.68 ± 0.29^{b}	-0.93 ± 0.57
		k				0.50 ± 0.09	0.86 ± 0.14	0.11 ± 0.06	0.07 ± 0.02^{b}	0.07 ± 0.00	0.09 ± 0.07^{b}	0.10 ± 0.04
		t_s				2.78 ± 0.41	2.28 ± 0.10	0.59 ± 1.05	1.49 ± 1.03	-0.35 ± 0.58	4.64 ± 5.89	1.21 ± 1.43

[a] Parameter estimate significantly different from planktonic cells ($p < 0.05$; t-test).
[b] Parameter estimate significantly different from single species biofilms ($p < 0.05$; t-test).
[c] Difference between the surviving population and the initial population ($\log_{10}$ cfu/well).
[d] Maximum specific inactivation rate ($\log_{10}$/min).
[e] Duration of the shoulder (min).

t-test) and a lower maximum specific inactivation rate (p<0.05, *t*-test) after exposure for 15 min to 100 µg/ml benzalkonium chloride compared with single species biofilms, while for *L. plantarum* no difference between single and mixed species biofilms was observed. These results indicate that growth in mixed species biofilms can provide a protective effect on *L. monocytogenes* and *L. plantarum* during exposure to benzalkonium chloride.

Single and mixed species biofilms are also more resistant against peracetic acid treatments than planktonic grown cells (p<0.05, *t*-test) (Fig. 4A and Table 3). However, the differences in peracetic acid resistance between single and mixed species biofilms are less pronounced (Fig. 4B and Table 3). In particular, *L. monocytogenes* grown in mixed species biofilms in BHI-Mn-G showed very high resistance against peracetic acid treatments compared with *L. monocytogenes* single species biofilms. The inactivation curves after exposure for 15 min to 100 µg/ml peracetic acid showed a higher surviving population (p<0.05, *t*-test) and a lower maximum specific inactivation rate (p<0.05, *t*-test) for *L. monocytogenes* grown in mixed species biofilms compared with the single species biofilms, while no difference for *L. plantarum* was observed. These results might indicate a protective effect of the presence of *L. plantarum* on *L. monocytogenes* in the mixed species biofilm grown in BHI-Mn-G during peracetic acid exposure.

4. Discussion

In this study, we investigated the formation of single and mixed species biofilms of *L. monocytogenes* and *L. plantarum* and the resistance of these biofilms to disinfectants. The contribution of each species to the mixed biofilm was dependent on the specific composition of the medium. In BHI, both *L. monocytogenes* strains dominated in the mixed species biofilm. However, the addition of manganese sulfate and/or glucose to BHI resulted in a decrease of the number of *L. monocytogenes* cells in the mixed species biofilm and an increase of the contribution of *L. plantarum*. *L. plantarum* accumulates high concentrations of intracellular manganese ions, which are used to combat reactive oxygen species thus providing resistance to oxidative stress (Archibald and Fridovich, 1981). Furthermore, *L. monocytogenes* and *L. plantarum* can metabolize glucose resulting in acidification of the medium. However, *L. monocytogenes* is not able to grow below pH 4.4 (van der Veen et al., 2008), while *L. plantarum* is able to grow down to pH 3.4 (Passos et al., 1993). Since the pH of the BHI-Mn-G medium during formation of mixed species biofilms of *L. monocytogenes* and *L. plantarum* reaches pH 3.4, the difference in acid tolerance between *L. monocytogenes* and *L. plantarum* provides the latter organism the opportunity to become the dominant organism in the mixed species biofilm.

So far, little work has been done on the resistance of mixed species biofilms to disinfectants. Previous work on hypochlorite resistance of a mixed biofilm of *L. monocytogenes*, *Pseudomonas fragi*, and *Staphylococcus xylosus* showed that it required 1000 ppm free chlorine to obtain 2 $\log_{10}$ reduction in *L. monocytogenes* viable counts after 20 min exposure, while planktonic cells were completely inactivated after 30 s exposure to 10 ppm free chlorine (Norwood and Gilmour, 2000). However, this study did not present results of single species biofilm resistance against hypochlorite, making it impossible to judge the protective or shielding effect of secondary species on *L. monocytogenes* in the mixed species biofilm. A study on the resistance of mixed species biofilms of *L. monocytogenes* and *Pseudomonas* sp. against hypochlorite and two peracid sanitizers with or without an organic challenge showed that peracid sanitizers were most effective, in particular with an organic challenge (Fatemi and Frank, 1999). Again this study did not include a comparison with resistance of single species biofilms or planktonic grown cells. In our study, we investigated the resistance of single and mixed species biofilms and planktonic cells of *L. monocytogenes* and *L. plantarum* against the two disinfectants benzalkonium chloride and peracetic acid. We showed that *L. monocytogenes* and *L. plantarum* grown in mixed species biofilms were in most conditions more resistant

to the disinfection treatments than single species biofilms. The mixed species biofilms grown in BHI, which contains the lowest number of *L. plantarum* cells, already showed higher resistance of both *L. monocytogenes* and *L. plantarum* against benzalkonium chloride treatments compared with single species biofilms. In BHI, no difference in final pH was observed between single and mixed species biofilms, suggesting that the increased resistance of the mixed species biofilms to benzalkonium chloride is dependent on the interaction between both species. In contrast, a large difference in peracetic acid resistance between single and mixed species biofilms was particularly observed in BHI-Mn-G, in which the mixed species biofilm contained the highest number of *L. plantarum* cells. This difference in peracetic acid resistance between single and mixed species biofilms in BHI-Mn-G was specific for *L. monocytogenes*, since *L. plantarum* grown in both single and mixed species biofilms showed very high resistance. Increased resistance of *L. monocytogenes* in the mixed species biofilms grown in BHI-Mn-G might be related with acid adaptation, since a lower final pH was reached in the culture medium. However, it has been shown for *L. monocytogenes* that acid adaptation does not result in increased peracetic acid resistance (Stopforth et al., 2002), suggesting that increased resistance of *L. monocytogenes* in the mixed species biofilm is related to other factors that remain to be elucidated.

The differences between benzalkonium chloride and peracetic acid resistance of the various single and mixed species biofilms might be related with the mode of action of both disinfectants. The mechanism of benzalkonium chloride disinfection is thought to be the disruption and dissociation of the lipid bilayer of the bacterial cell membrane leading to leakage of cytoplasmic material, while peracetic acid functions as an oxidizing agent (McDonnell and Russell, 1999). For *L. monocytogenes* it has been shown that adaptation and resistance to benzalkonium chloride is related with induction of non-specific efflux pumps and changes in the fatty acid composition of the cell membrane (Aase et al., 2000; To et al., 2002). Therefore, it will be interesting to investigate in future studies whether interactions between *L. monocytogenes* and *L. plantarum* in mixed species biofilms result in activation of similar defense systems or that resistance is conferred via other mechanisms. Peracetic acid is an oxidant that produces hydroxyl radicals that subsequently attack essential cell components such as DNA. Peracetic acid resistance mechanisms might therefore consist of systems involved in DNA repair, such as the SOS response. Recently, it was shown for *L. monocytogenes* that its SOS response was important for oxidative stress resistance (van der Veen et al., 2010), and that the SOS response was specifically activated during continuous flow biofilm formation and not during static biofilm formation (van der Veen and Abee, 2010). Whether the SOS response is activated during mixed species biofilm formation remains to be elucidated. In contrast to *L. monocytogenes* and other low GC Gram-positives, *L. plantarum* contains a specific oxidative stress resistance mechanism that includes accumulation of high concentrations of intracellular manganese ions, acting as radical scavengers (Archibald and Fridovich, 1981). Our results showed that peracetic acid resistance of *L. plantarum* grown in biofilms was increased in BHI-Mn-G but not in BHI-Mn pointing to a more prominent role of acid adaptation in peracetic acid resistance.

In conclusion, our approach highlighted the impact of mixed species biofilm formation on disinfection resistance. In future studies we will investigate the specific factors involved in mixed species biofilm formation, including intra- and interspecies communication, and the mechanisms that confer disinfection resistance.

Appendix 1. Sequences of optimized genes

EGFP

ATGGTTAGTAAAGGAGAGGAATTATTCACAGGTGTGGTGCCAATCTTA
GTAGAATTAGATGGAGATGTAAACGGCCATAAATTTTCTGTATCTGGCGAA

GGCGAAGGTGATGCTACTTACGGTAAATTAACGTTAAAATTCATTTGTACGA
CTGGTAAATTACCTGTACCTTGGCCGACATTAGTAACAACATTAACGTATGG
TGTTCAATGCTTCTCTCGTTATCCAGATCACATGAAACAGCACGATTTCTTT
AAATCAGCAATGCCAGAAGGATACGTGCAAGAACGTACAATTTTCTTTAA
AGACGACGGCAACTACAAAACGCGCGCTGAAGTTAAATTTGAAGGCGATA
CATTAGTGAATCGTATTGAGTTAAAAGGAATTGACTTTAAAGAAGATGGTA
ATATCTTAGGACACAAATTAGAGTATAATTATAATAGTCATAATGTTTATAT
TATGGCGGACAAACAGAAAAATGGAATCAAAGTTAATTTTAAAATTCGTCA
TAATATTGAAGATGGTAGCGTTCAATTAGCAGATCATTATCAGCAAAACAC
ACCTATTGGTGATGGTCCAGTGTTATTACCAGATAACCATTATTTATCAACA
CAATCTGCTTTAAGCAAAGACCCGAATGAAAAACGTGATCATATGGTTTTAT
TAGAGTTCGTAACGGCAGCGGGAATCACTTTAGGAATGGATGAATTATACA
AATAA

ECFP

ATGGTTAGTAAAGGAGAAGAATTATTTACTGGCGTTGTTCCAATTTTA
GTAGAATTAGACGGxTGATGTAAATGGTCATAAATTCAGCGTTTCTGGTG
AGGGTGAAGGTGATGCGACTTATGGCAAATTAACGTTAAAATTCATCTGC
ACAACGGGCAAATTACCTGTACCGTGGCCAACATTAGTTACAACGTTAACA
TGGGGCGTGCAATGTTTTAGCCGTTATCCGGATCACATGAAACAACATGAT
TTCTTCAAATCTGCAATGCCAGAAGGTTATGTTCAAGAACGCACAATTTTCT
TTAAAGATGACGGTAATTACAAAACACGTGCGGAGGTAAAATTTGAAGGC
GATACGTTAGTGAACCGTATTGAATTAAAAGGCATTGATTTCAAAGAAG
ACGGAAACATTTTAGGTCACAAATTAGAATATAATTATATTAGCCACAATGT
TTATATTACAGCAGATAAACAGAAAAACGGAATCAAAGCTAACTTCAAAATC
CGCCATAATATTGAAGATGGATCAGTACAATTAGCAGACCATTACCAACAAA
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ACACAATCAGCATTAAGTAAAGACCCTAATGAAAAACGTGATCATATGGTAT
TATTAGAGTTTGTTACTGCTGCTGGAATTACTTTAGGTATGGATGAATTATAC
AAATAA

EYFP

ATGGTTTCTAAAGGAGAAGAATTATTCACGGGCGTTGTTCCTATTTTAG
TAGAATTAGATGGAGACGTAAATGGCCATAAATTTAGTGTGAGTGGTGAGG
GAGAAGGTGATGCGACATATGGCAAATTAACTTTAAAATTTATCTGTACAAC
AGGTAAATTACCAGTACCGTGGCCTACTTTAGTAACTACATTGGCTACGGAT
TACAGTGCTTCGCGCGTTACCCAGACCACATGAAACAACATGACTTTTTCAAA
AGCGCTATGCCGGAAGGCTATGTTCAAGAACGCACTATTTTCTTTAAAGATG
ATGGAAACTACAAAACGCGTGCAGAAGTTAAATTCGAGGGCGATACGTTAG
TAAATCGTATCGAATTAAAAGGTATTGATTTTAAAGAGGATGGAAACATTTT
AGGTCATAAATTAGAATACAATTATAATTCTCATAATGTTTATATTATGGCTC
ACAAACAGAAAAATGGTATCAAAGTGAACTTTAAAATTCGTCATAACATTGA
AGATGGTAGCGTTCAATTAGCGGATCACTACCAACAAAATACACCAATCGG
AGATGGTCCAGTTTTATTACCAGACAATCATTATTTATCATATCAATCTGCTTT
ATCAAAAGATCCAAATGAAAAACGTGATCACATGGTATTATTAGAGTTCGTG
ACAGCAGCAGGAATCACTTTAGGCATGGACGAATTATACAAATAA

DsRed

ATGGACAATACTGAAGATGTGATCAAAGAGTTTATGCAATTCAAAGTAC
GTATGGAAGGCAGTGTAAATGGTCACTATTTCGAAATTGAAGGTGAGGGAG
AAGGTAAACCATATGAAGGAACGCAGACAGCGAAATTACAAGTTACAAAAG
GCGGTCCATTACCTTTCGCTTGGGATATCCTATCACCTCAATTTCAGTATGGA
AGTAAAGCATATGTTAAACATCCGGCTGATATTCCGGACTATATGAAATTAT
CATTTCCAGAAGGTTTTACTTGGGAGCGTAGTATGAATTTTGAAGATGGCGG
CGTGGTTGAAGTTCAACAAGACAGTTCTTTACAAGATGGCACTTTTATCTACA
AAGTAAAATTCAAAGGGAGTGAATTTCCCAGCTGATGGTCCTGTAATGCAAA
AAAAAACTGCGGGATGGGAGCCAAGCACAGAAAAACTATATCCACAAGAT
GGTGTACTAAAAGGCGAAATTAGCCATGCTTTAAAGTTAAAAGACGGTGGA
CATTACACGTGTGATTTTAAAACAGTATACAAAGCAAAAAAACCTGTTCAATT
ACCAGGAAATCATTACGTTGATTCAAAATTAGATATCACAAACCATAACGAA
GATTATACGGTTGTGGAGCAATACGAACACGCAGAAGCGCGCCATAGTGGA
TCTCAATAA

Appendix 2. Single species biofilms of *L. monocytogenes* EGD-e (A), LR-991 (B), and *L. plantarum* WCFS1 (C). See legend of Fig. 2 for a detailed description
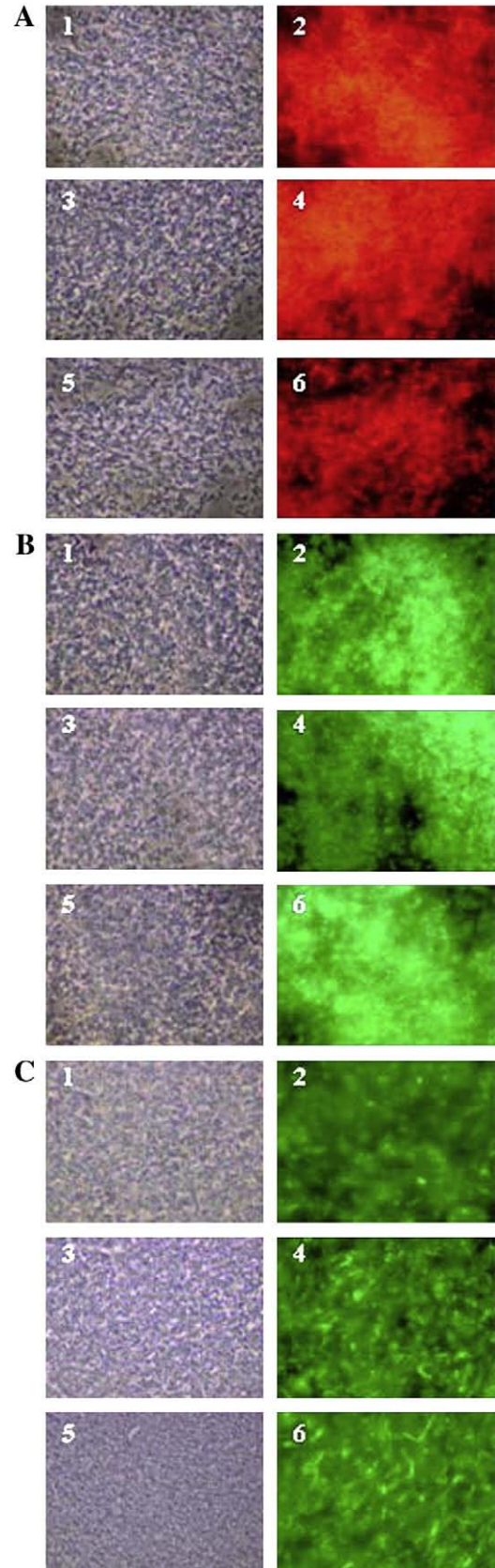
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