CENTER for PRODUCE SAFETY

CPS 2018 RFP
FINAL PROJECT REPORT

Project Title
Preventative sanitation measures for the elimination of *Listeria monocytogenes* biofilms in critical postharvest sites

Project Period
January 1, 2019 – December 31, 2019 (extended to January 31, 2020)

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Objectives

1. *Construct testing beds from fabricated surfaces in the laboratory to replicate real-life conditions found in the packinghouse by identifying areas, materials, specific environmental conditions, and microflora in the packinghouse that can harbor Listeria monocytogenes. These test beds will be validated with conditions found in the packinghouse.*
2. *Determine cleaning and cleaning and sanitizing procedures for biofilms (Listeria monocytogenes single culture and in co-culture with the resident microflora) grown in conditions simulating the packinghouse. Compare and validate efficacy of the tested sanitizers against L. monocytogenes biofilms with the EPA requirements for hard surface (porous and non-porous) sanitizers.*

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CDFA SCBGP grant# 18-0001-080-SC

FINAL REPORT

Abstract
Surfaces in the stone fruit packinghouse can be fabricated from materials suitable for fruit packing and sorting but difficult to clean. In this project we investigated the formation of single species biofilms (*Listeria monocytogenes* Scott A) and mixed species biofilms of *Listeria monocytogenes* Scott A with two different background microflora found in fruit packinghouses, *Pseudomonas* spp. and *Burkholderia* spp. Biofilms were grown for 48 hours in laminar flow conditions and then exposed to different concentrations of chlorine, benzalkonium chloride, or peracetic acid for 5 to 20 minutes. To simulate conditions found in the packinghouse, biofilms were grown on modified surfaces with increased roughness and on control (smooth) surfaces. Results showed that the mixed species biofilms were, in general, more resistant to sanitizer treatment than were *L. monocytogenes* single-strain biofilms. For example, the mixed species biofilms showed less than 3.2 log reduction after 10-minute exposure to 200 ppm chlorine, while single species *L. monocytogenes* biofilms showed approximately 5.1 log reduction. In addition, surfaces with increased roughness protected biofilms against the antimicrobials. For example, exposure to 200 ppm chlorine for 15 minutes reduced *L. monocytogenes* Scott A populations in biofilms by 5.9 log on control (smooth) coupons with 0.2-µm surface roughness, or by 4.5 log on a surface with approximately 7-µm roughness. These results indicate that mixed species biofilms and surface imperfections require optimal sanitizer concentration and longer exposure time to avoid survival of residual microorganisms.

Background
In the past decade, growing consumer demand for fresh fruits and vegetables has led to an increase in the production of minimally processed produce. Produce can carry natural, generally non-pathogenic microflora but may potentially be contaminated with foodborne human pathogens from different sources. *Listeria monocytogenes* is a microorganism of particular concern because it is prevalent in wet environments and it grows in refrigeration conditions. In these conditions the pathogen is difficult to eradicate.

In order to survive in the food processing environment, microorganisms, including *Listeria monocytogenes*, can attach on surfaces and form embedded colonies surrounded by extracellular polymeric substances called biofilms. In the microbial world, biofilms have the advantage to protect bacteria against adverse conditions, such as shear forces associated with cleaning and sanitation, osmotic stress or desiccation, and survival with limited nutrients available. These microbial communities or biofilms can grow over time and detach to colonize other surfaces or food products. The significance of biofilms cannot be stressed enough from a sanitation point of view: while planktonic cells usually can be effectively removed by physical methods and/or killed by chemical sanitizers, microorganisms become much more difficult to remove when they form biofilms. The residual cells that are not eliminated by the sanitation process can regrow and contaminate food products.

Among the many cellular and environmental factors affecting bacterial attachment and biofilm formation, surface roughness plays a very important role. It is generally accepted that bacteria are more able to attach to crevices and pits where they are protected from unfavorable environmental disturbances. Surfaces with a mean surface roughness (Ra) value of ≤0.8 micrometers (µm) are typically considered “hygienic,” whereas those with Ra >0.8 µm are more susceptible to bacterial deposition. Bacteria may attach to rougher surfaces in greater quantities to avoid osmotic stress; on the other hand, rougher surfaces offer higher surface area for attachment, while protecting the cells from shear forces. Additionally, surface irregularities, such

as pits and crevices, make cleaning more difficult, leading to the presence of residual cells or "persister" cells that survive the sanitation process. The persister cells are important since over time they can develop resistance to the sanitizers used in the cleaning process.

Typically, food contact surfaces in the food industry are designed from materials easy to sanitize and resistant to scratching (such as stainless steel). However, stainless steel may not be the top choice material in the fruit industry, especially for equipment parts involved in fruit handling and sorting, since those surfaces must be more flexible in order to prevent fruit bruising and deterioration.

In laboratory conditions, colonization of surfaces by microorganisms is dependent on experimental conditions such as growth conditions, type of media, temperature. However, conditions may be different in the packinghouse. There are multiple factors for responsible bacterial fate in packinghouses and it seems reasonable to hypothesize that the combination of risk factors—microenvironments created by the imperfect material design, topographical features of the material, and the physiological state of the bacteria in response to the environmental factors—can induce the formation of persisters or resident cells resistant to sanitizers. The overall aim of the project was to characterize the conditions favorable for *L. monocytogenes* persistence on food contact and non-food contact surfaces, and design measures to reduce pathogen persistence and avoid further contamination. The study evaluated the impact of surface characteristics (hydrophobicity, roughness) on the *de novo* biofilm formation, especially in areas difficult to clean and to determine sanitation practices.

Research Methods

1. Bacterial strains and growth conditions:
All strains used in this study are listed in Table 1. In biofilm inactivation experiments, *L. monocytogenes* Scott A was used and the strain was transformed with a plasmid (pNF8) encoding for the green fluorescent protein (GFP) and erythromycin resistance. Strains were maintained as freezer stocks at -80°C in tryptic soy broth (TSB) with 25% (wt/vol) glycerol. Prior to each experiment, the strains were streaked onto tryptic soy agar (TSA) and incubated at 37°C for 24 hours. A single colony was used to inoculate 20 ml of TSB, followed by overnight incubation at 25°C with shaking (200 rpm). Overnight cultures were transferred twice in 2x Listeria synthetic medium (LSM), prepared as described below, and incubated at 25°C with shaking until late exponential phase (optical density at 600 nm [OD_{600}] = 0.7–0.8). When experiments involved fluorescence, the growth media was supplemented with 10 µg ml^{-1} of erythromycin for plasmid maintenance. Biofilms were grown in a chemically defined media (Listeria synthetic medium, LSM). Briefly, the media was prepared by mixing the eight concentrated stock solutions prepared in advance with glutamine and cysteine, which were mixed and added fresh. Cultures for filament inoculation were grown in 2x LSM, and biofilms were subsequently developed in 0.5x LSM.

2. Test surfaces:
New thermoplastic materials were obtained from DuPont USA, and new and used packing line materials were obtained from packinghouses in California and South Carolina. The following surfaces were tested: Hytrel® (thermoplastic polyester elastomer, used to fabricate rollers for fruit sorting lines); neoprene (or polychloroprene, used to fabricate soft surfaces such as dividers and coating surfaces for sorting tables); Hycar® (synthetic thermoplastic rubber, used for table coatings and belts); thermoplastic polyurethane, polyurethane, and polyvinyl chloride (PVC), all used for conveyer belts. The surface roughness and topography of the samples were determined with a surface profilometer (Mitutoyo SJ410). The instrument was used in the

packinghouse only for limited determinations (due to vibrations interfering with the instrument), but the used surface materials collected in the packinghouse were later measured in the laboratory. Contact angle (for hydrophobicity profile) was measured by estimating the contact angle of a single water droplet spotted on the surface.

To fabricate modified surfaces (to resemble used packinghouse surfaces), the surface of the new material was altered with a rotary platform, double-head abrader to yield the desired roughness. Based on the preliminary data collected in the field, and from used surfaces collected and analyzed in the laboratory, we aimed to obtain surfaces between approximately 2.0 ± 0.5 µm and 7.5 ± 0.5 µm surface roughness for fruit rollers (Hytrel) and 5.0 ± 0.5 µm and 10 ± 0.5 µm for conveyor belts and table protectors (Hycar). Individual coupons for test bed surfaces were cut from Hytrel and Hycar sheets to obtain 3 x1 inch coupons. The surfaces were cleaned by soaking the coupons in 1% alkaline cleaning solution for 15 minutes. Coupons were then rinsed copiously with distilled water, and then sterilized by placing in 70% ethanol for 10 minutes. Coupons were rinsed in sterile distilled water, removed with sterile tweezers, and allowed to dry in a safety cabinet and then mounted in the sterilized biofilm apparatus.

3. Biofilm apparatus:
A biofilm apparatus was developed for 3 x 1 inch coupons cut from surface materials. The apparatus consists of a multichannel peristaltic pump that flows media in from the media reservoir at a rate of 1 ml min^{-1}, connected to the flow cells and waste container through tubing and connectors (Figure A). For biofilm formation, individual coupons were mounted in the flow cells and sandwiched between a precut sterile silicone gasket and a sterile glass slide, within a biosafety cabinet. Flow cells were exposed overnight to ultraviolet light in the biosafety cabinet. Each experiment could run 8 individual flow cells (surfaces) simultaneously.

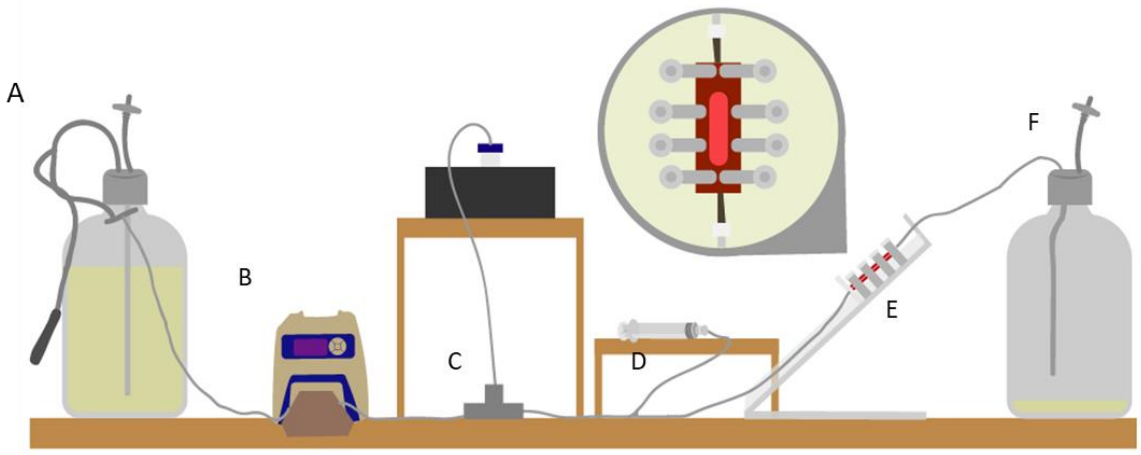

Figure A. Reactor for biofilm growth and sanitizer testing of modified substrata.
L. monocytogenes and background microflora were inoculated in each fluid cell using a sterile syringe, and bacteria were allowed to attach for 1 hour. Media flowed in from the reservoir while the tubing from the sanitizer container was clamped; the flow was constant (1 ml min^{-1}) from a calibrated peristaltic pump. The inset shows the typical elements required to assemble the fluid cells: a silicone gasket with inlet and outlet needles was placed on the top along with a plain microscope glass slide, and the whole assembly was sealed with metallic clamps.

4. Inoculation, biofilm formation, and treatment of biofilms with sanitizers:
Early exponential growth phase cultures of *L. monocytogenes* Scott A (optical density 0.15) grown in 2X LSM were inoculated in the flow cells (10 ml each, through a separate inlet tubing

with a sterile syringe) and the cells were allowed to attach for 1 hour. After the attachment phase, the media was pumped at a flow rate of 1 ml min^{-1}, and biofilms were grown for 48 h. When performing mixed-species biofilm experiments with cultures isolated from packinghouses, the isolates were grown in Luria Bertani broth, then washed twice in LSM to eliminate residual media, and mixed 1:1 with the *Listeria* culture used for inoculation. Lm biofilms, single species (*L. monocytogenes* Scott A) and mixed species culture, were developed on coupons and then each test surface was removed from the apparatus and placed in 50-ml conical tubes containing 20 ml of sanitizer solution (i.e., chlorine, benzalkonium chloride, or peracetic acid).

Chlorine solutions were prepared from a 6% stock solution of sodium hypochlorite (vol/vol, Ricca Chemical Company) by adding different volumes to phosphate buffer (pH 7.5) to obtain concentrations of 100 and 200 µg ml^{-1} (ppm). The concentration of chlorine was determined with a Hach kit with the DPH method. Benzalkonium chloride (BAC) solutions of 100 and 400 µg ml^{-1} (ppm) were prepared from a 50% stock solution (w/w, Alfa Aesar) in sterile water. Peracetic acid (PAA) solutions of 50 and 100 µg ml^{-1} were prepared from a stock solution (Sigma Aldrich).

Surfaces were exposed to sanitizer for selected time intervals and then aseptically removed and placed in sterile conical tubes containing 20 ml of TSB supplemented with 1% sodium thiosulphate (to neutralize the reaction) and 0.3 g of sterile acid-washed glass beads added to promote bacterial detachment. Tubes were vortexed and subjected to serial dilutions and standard bacterial enumeration. Samples were plated on TSA supplemented with 0.6% yeast extract and 10 µg ml^{-1} of erythromycin. In experiments where the surviving bacteria population was below the detection limit of the plate count assay (2.3 log CFU), an enrichment method was used. Briefly, a sterile silicone gasket (1/2 inch thick) was placed on the tested coupon and then filled with 3 mL of sterile TSB, assembled into a flow cell and incubated for 48 hours at 37°C. Coupons that presented *L. monocytogenes* Scott A growth were considered positive whereas flow cells with no growth indicated complete disinfection and were considered negative. For mixed biofilms, enumeration was performed on TSA-erythromycin plates and TSA without antibiotic (for mixed biofilms) and incubated at 37°C to determine the population of the surviving cells. The surviving population of the background microflora was calculated by subtracting the *L. monocytogenes* population (from TSA-erythromycin plates) from the total plate count on TSA.

5. Sampling of the stone fruit packinghouses:
To collect topographical data and recreate microenvironment conditions similar to the packinghouse, a total of 11 packing units were visited in California and South Carolina. Visits involved (i) collection of environmental swabs from fruit contact surfaces and non-food contact surfaces, (ii) measurements of temperature and relative humidity (RH) in different parts of the packinghouse to help simulate conditions in the laboratory, and (iii) measurements of surface roughness and hydrophobicity on select surfaces. The packinghouses visited ranged in size and production capacity, from a small outdoor packing shed with a limited daily schedule to automated large facilities packing millions of fruits. The visits were scheduled while the facilities were operational. Environmental swabs from areas difficult to clean were collected, shipped, and then processed in our laboratory at Clemson University. Temperature and RH in the packinghouses were monitored with wireless dataloggers (Fisher Scientific). Surface roughness measurements were collected, where possible, with a portable surface profilometer, and contact angle (for hydrophobicity profile) was estimated by the droplet method.

In the laboratory, swab samples were diluted with phosphate saline buffer (PBS, pH 7.5) and plated on non-selective agar (plate count agar) by two methods: spread plating and pour-plating. Plates were incubated in mesophilic conditions for 48 hours. Colonies were purified by streaking at least three times on fresh plates and then tested for biofilm formation with a 48-well plate assay format. Briefly, biofilms were grown in diluted TSB (1:10) for 8 hours and the total

biofilm biomass was quantified with a crystal violet assay. Experiments for biofilm development were repeated independently three times, and the data shown are means and standard deviations.

6. Identification of the select microbial isolates from the packinghouse:
Briefly, genomic DNA from select colonies was used as templates for amplification with universal 16S rRNA bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were purified and visualized under UV light on 1% agarose gels stained with ethidium bromide to confirm the presence of a 1,350 base pair band, and then bands were cut, purified, and the PCR products were cloned in pcr2.1 TOPO vector (Invitrogen). The plasmids with the cloned PCR product were used for bi-directional DNA sequencing with primers 27F and 1392R; sequencing was performed at Clemson University. Sequences were aligned and taxonomic information was obtained with the Silva RNA database (https://www.arb-silva.de/).

7. Statistical analysis:
All experiments were replicated 3 times or more. In testing of the sanitizers, 2 test surfaces were used in each replicate. Data sets were analyzed using the general linear model of the Statistical Analysis System (SAS). Duncan's multiple range test and proc *t* test were used to determine which conditions were significantly different. Significant differences in mean values are presented at a 95% confidence level ($P \leq 0.05$).

Outcomes and Accomplishments

Objective 1: Results demonstrated that most surfaces in the packinghouses were hydrophobic (based on the contact angle of a single water droplet test) except for the polyurethane surfaces, which were hydrophilic (with a contact angle around 30°). Materials used to manufacture these surfaces can have intrinsic hydrophobic properties; most new materials were measured in the laboratory were hydrophobic. Also, the coatings used for fruits may contain mineral or vegetable oil in composition and can also coat surfaces and increase the degree of hydrophobicity. Overall, the collection of environmental swabs from fruit contact surfaces and non-food contact surfaces revealed that brushes presented a higher microbial load than the rest of the sampled surfaces (**Table 2**). The range in microbial loads on the surfaces sampled can be explained by the sampling time (e.g., higher loads at the end of packing operations) and the state of the equipment (e.g., in some instances, heavily used and showing cracks). The source of the microorganisms was not significant for our study, but we identified strong biofilm forming microorganisms from the surfaces in the packinghouses, such as *Pseudomonas* spp., *Burkholderia* spp., and *Klebsiella* spp. (**Table 3**). The frequent isolation of some microorganisms from surfaces also suggests that some of these are established "residents" in the packinghouse. These biofilm formers can condition packinghouse surfaces for increased microbial adherence, or they can trap other microorganisms in the biofilm matrix.

Objective 2: The type of antimicrobial, concentration, and exposure time were important factors in biofilm inactivation. Chlorine efficacy against *L. monocytogenes* biofilms on the test bed surfaces made of Hytrel or Hycar was dependent on the concentration and exposure time. A 1-min treatment with 100 or 200 ppm chlorine showed similar efficacy against Lm biofilms; however, after longer exposure time (10 min) the log reduction values were significantly different for 100 ppm and 200 ppm treated samples of Hytrel (**Figure 1**) and Hycar (**Figure 4**). Mixed species biofilms were more resistant to chlorine than the Lm monoculture biofilms. On Hytrel, after 10-min treatment with 100 or 200 ppm chlorine, the reductions in Lm for single species

biofilms were 3.5 or 5.1 log, respectively, whereas the reductions for mixed biofilms were 2.7 or 3.2 log, respectively (Fig.1). A similar trend was observed for the single species and mixed biofilms on Hycar treated with chlorine (Fig. 4). Results also indicate that surface roughness has an important role in *L. monocytogenes* biofilm formation and resistance to sanitizers. Specifically, significantly more biomass ($P \leq 0.05$) formed on the uneven surfaces (roughness [Ra] of 7 and 10 µm) than on the control (smooth) surface. Chlorine efficacy was greatly reduced in the presence of mixed biofilms and increased surface roughness. For mixed biofilms grown on Hytrel, a 20-min treatment with 100 ppm chlorine resulted in 3.2 log reductions on the control surface compared with 2.7 log reductions on the Ra 7-µm surface. Chlorine was effective, especially at 200 ppm, but for better efficacy, especially when treating mixed biofilms, the sanitizer requires longer exposure time. On the control surface, surviving populations of *L. monocytogenes* exposed to the different concentrations of chlorine were significantly different after a 5-min treatment. Treatment with 200 ppm chlorine resulted in significant differences in biofilm inactivation on Hytrel after 5 min and on Hycar after 10 min.

Benzalkonium chloride (BAC) was more effective against mature *L. monocytogenes* biofilms, especially on the modified surfaces. More importantly, mixed species biofilms appeared to be more resistant to BAC than single species biofilms on Hytrel (**Figure 2**) and Hycar (**Figure 5**). A 10-min exposure to 100 ppm BAC resulted in lower reductions ($P < 0.05$) for both the single and mixed species biofilms on the modified Hytrel surfaces than on the control (smooth) surface. After 15-min or 20-min exposure to 100 ppm BAC, both single and mixed species biofilms grown on Hytrel or Hycar coupons with surface roughness of 7 µm or 10 µm, respectively, showed higher surviving Lm populations ($P < 0.05$) than the single and mixed biofilms grown on the control surfaces and those with moderate roughness (Ra 2 or 5 µm, respectively). In contrast, after treatment with 200 ppm BAC for 15 min, the mixed species biofilms on the modified surfaces showed a higher surviving Lm population ($P < 0.05$) and a lower maximum specific inactivation rate ($P < 0.05$) than did the single species biofilms; whereas no differences between single and mixed species biofilms were observed on the control surfaces.

Single and mixed species biofilms were more resistant against peracetic acid (PAA) treatments on Hytrel (**Figure 3**) and Hycar (**Figure 6**) compared with other sanitizers. The mixed species biofilms showed very high resistance against PAA treatments compared with single species biofilms. The log reduction curves after exposure for 15 min to 100 ppm PAA showed a higher surviving Lm population ($P < 0.05$) for Lm grown in mixed species biofilms compared with the single species biofilms.

Summary of Findings and Recommendations

Project results indicate that growth in mixed species biofilms can provide a protective effect on *L. monocytogenes* during exposure to sanitizers. Protective cultures can be found in the fruit packinghouse, and their repeat isolation from multiple units and locations suggests that these background non-pathogenic strains are well-adapted in the stone fruit packinghouse environment. Sanitizers were most efficient on smooth surfaces and sanitizer efficacy decreased with increasing surface roughness. In ideal conditions in terms of concentration and time of exposure (at least 10 minutes), sanitizer treatment can reduce biofilm biomass by more than 5 log. This project demonstrated that benzalkonium chloride and chlorine were the most efficient in reducing *L. monocytogenes* biofilms. The sanitizers tested have different properties (e.g., mechanism of action, diffusion coefficient, and reactivity) and thus the physico-chemical differences were reflected in the project results. A logical conclusion would be that future testing should include mixtures of antimicrobials for a cumulative effect against biofilms in the packinghouse.

Appendices

Publications

None to date

Presentations

The Principal Investigator presented preliminary results of the project at the annual CPS Research Symposium in June 2019 in Austin, TX (377 attendees).

Budget Summary

The total funds awarded for this project were $115,774. As of 31 March 2020, expenditures totaled $114,048; the team had expected to use the remaining funds for travel to the CPS Research Symposium in June 2020.

Tables and Figures (see below)

Tables 1– 4 and Figures 1–6

Table 1. Microbial strains used in this study

Strain	Relevant characteristics	Source
L. monocytogenes Scott A	Has pNF8 plasmid for green fluorescent protein; 4b serotype	This study
L. monocytogenes Petite Scott A (ATCC 49494)	4b serotype	ATCC
Pseudomonas spp.	Isolated from multiple locations in the packinghouse, and strong biofilm former	This study
Burkholderia spp.	Isolated from multiple locations in the packinghouse, and strong biofilm former	This study

Table 2. Summary of swabbing results collected from stone fruit packinghouses

Swabbed area of interest	Number of swabs collected	Range (min-max) of total microbial load detected through plate count (log CFU/ml)
Fruit defuzzing brush - green PSE (triangular-shaped polyethylene)	32	5.77-7.03
Fruit dewatering brush - red PSE	35	5.86-8.04
Dewatering sponge - polyester (only two facilities had sponges)	8	6.93-8.09
Wax brushes (Waxlon, Mix Waxlon and horsehair, and one facility had a red PSE brush as a wax brush)	34	6.82-7.88
Other brushes (blue PSE) at one facility present in the dry area - sorting equipment	6	5.24-6.84
Fruit conveyer - polyvinyl chloride (PVC)	12	5.42-5.51
Fruit belt - polyurethane	22	3.52-7.53
Fruit sizer carriers (Hytrel®)	25	3.50-7.83
Acrylic resin fruit dividers (Hycar®) - along packing line	16	4.88-5.62
Conveyor rod - galvanized iron	10	3.92-4.04
Stainless steel sorting table	15	2.64-3.48
Coating table fabric - PVC coated polyester	14	3.57-5.98
Floors, drains, cold room surfaces	38	5.06-7.32
Utensils, buckets	12	3.91-5.85
Unwashed fruit	24	5.42-6.01
TOTAL	**303**	

Table 3. Identification of microbial isolates from environmental swabs and their biofilm potential.

Microbial isolate	**Surface material**	**Biofilm formation capacity in 48-well plate** [a]
Acinetobacter spp.	Brushes, PVC	++
Bacillus spp.	Sizers, stainless steel, belts, cold room	+
Brevibacterium spp.	Brushes, conveyor belt	+
Burkholderia spp.	Brushes, sizers, drains, wet floor surfaces	++++
Enterobacter spp.	Drains, floors	++++
Flavobacterium spp.	Brushes, floor, drains, wet conveyor belts	++
Klebsiella spp.	Brushes, PVC	++++
Microbacterium spp.	Brushes, floor	ND
Pseudomonas spp.	Brushes, PVC, stainless steel	++++
Pectobacterium spp.	Brushes, iron rods	++
Rahnella spp.	Brushes, PVC conveyor belts	++
Rhisobium spp.	PVC, floors	+
Staphylococcus spp.	Floors, PVC, sizers	+++
Streptococcus spp.	Brushes, floors, PVC, sizers, drains	++
Sphingomonas spp.	Floors, drains	++
Stenotrophomonas spp.	PVC	++

[a] Biofilm-formation capacity (crystal violet assay) of isolates: ++++, very strong; +++, strong; ++, moderate; +, weak; and ND, not detected.

Table 4. Repeatability and reproducibility of *L. monocytogenes* inactivation on stainless steel coupons

Sanitizer	**Concentration**	**Mean log reduction**	**Repeatability standard deviation**	**Reproducibility standard deviation**
Chlorine	100 200	2.45 4.79	1.201 0.855	1.402 0.901
Benzalkonium chloride (BAC)	100 400	3.58 5.46	0.766 1.108	0.948 1.062
Peracetic acid (PAA)	50 100	3.93 4.58	0.732 1.663	0.993 1.281

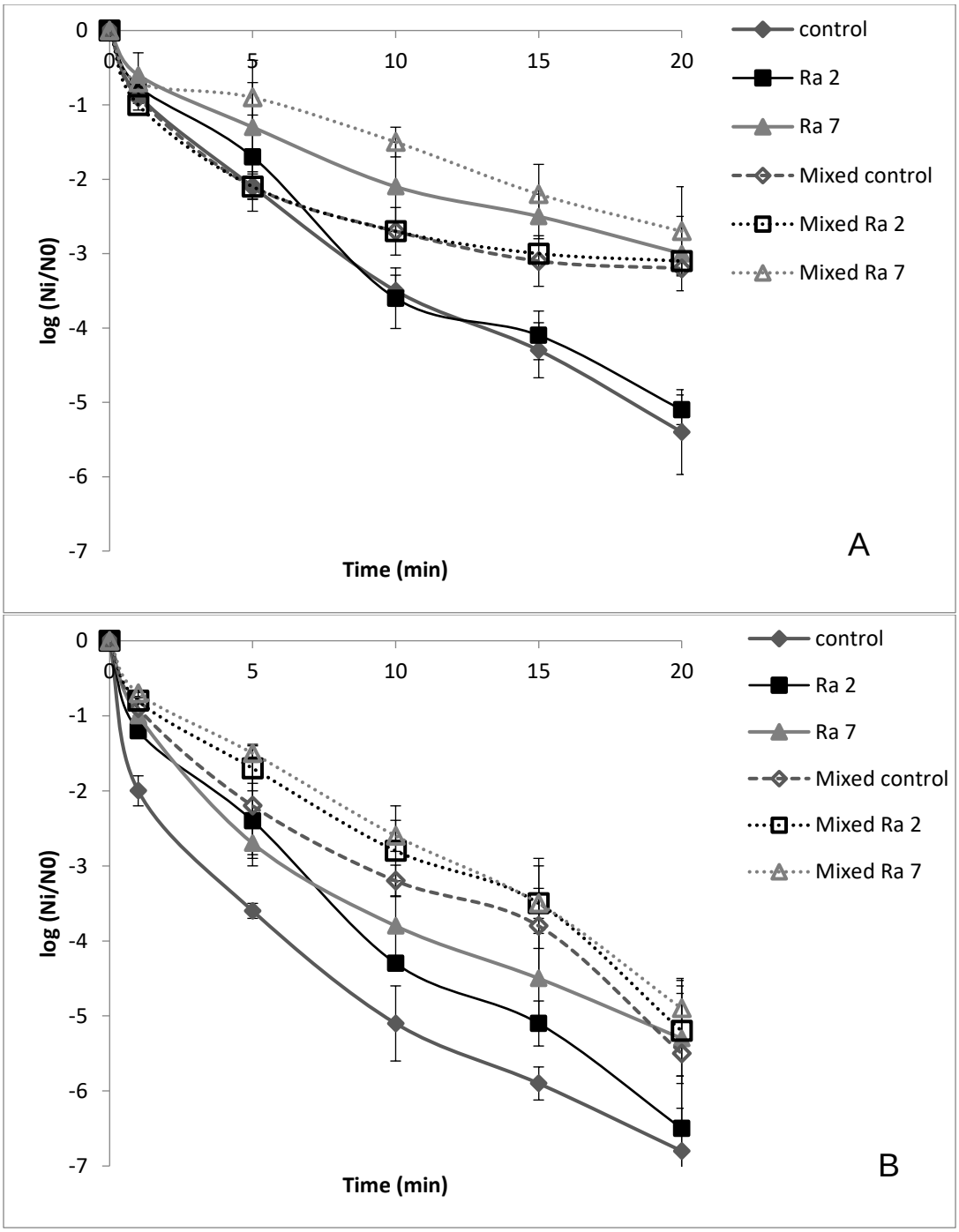

Figure 1. Log reductions of *L. monocytogenes* during chlorine treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hytrel® coupons (smooth [control] and with roughness of 2 µm [Ra 2] or 7 µm [Ra 7]: (A) treatment with 100 ppm active chlorine; (B) treatment with 200 ppm active chlorine. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.

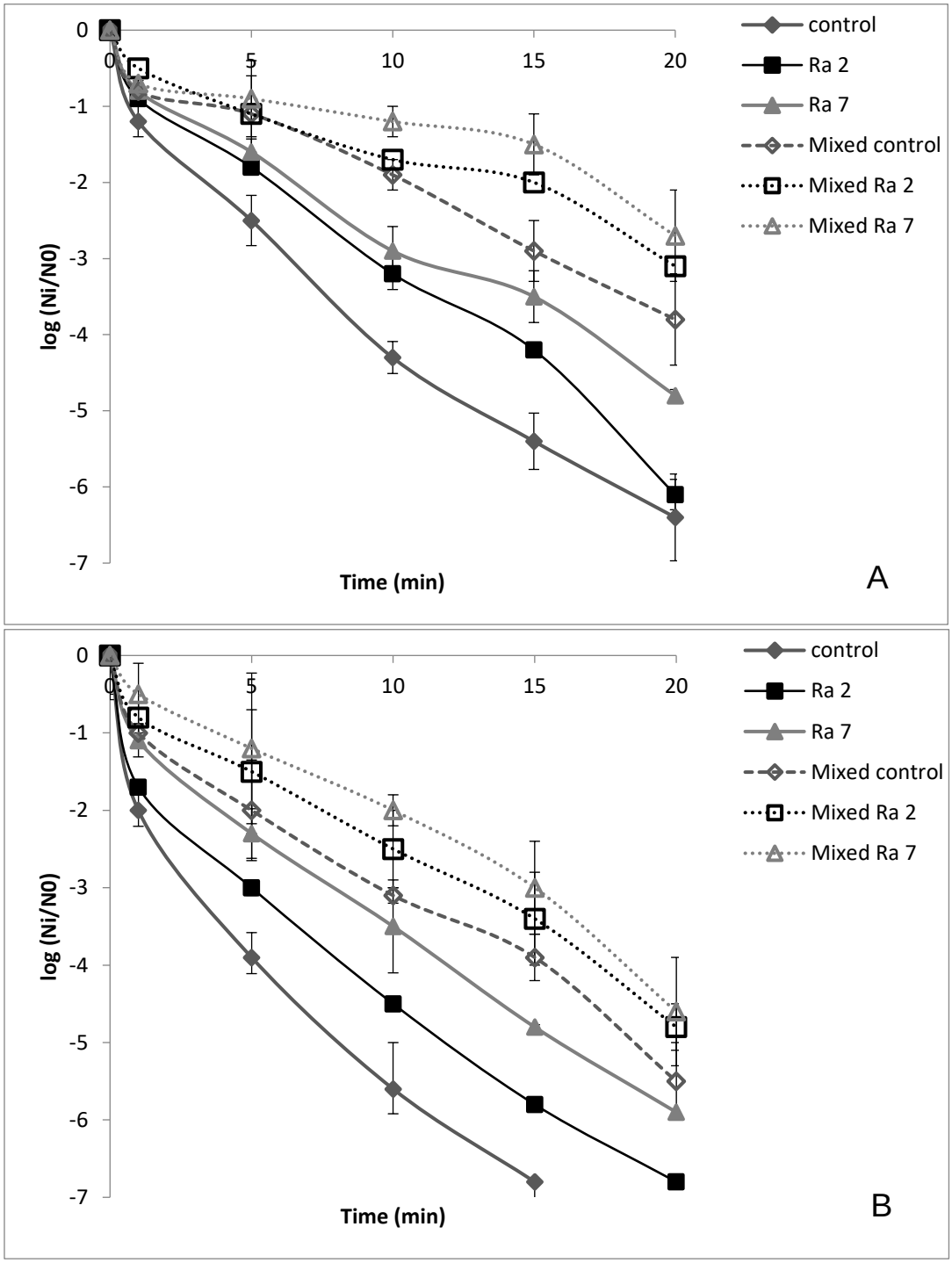

Figure 2. Log reductions of *L. monocytogenes* during benzalkonium chloride (BAC) treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hytrel® coupons (smooth [control] and with roughness of 2 µm [Ra 2] or 7 µm [Ra 7]: (A) treatment with 100 ppm BAC; (B) treatment with 400 ppm BAC. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.

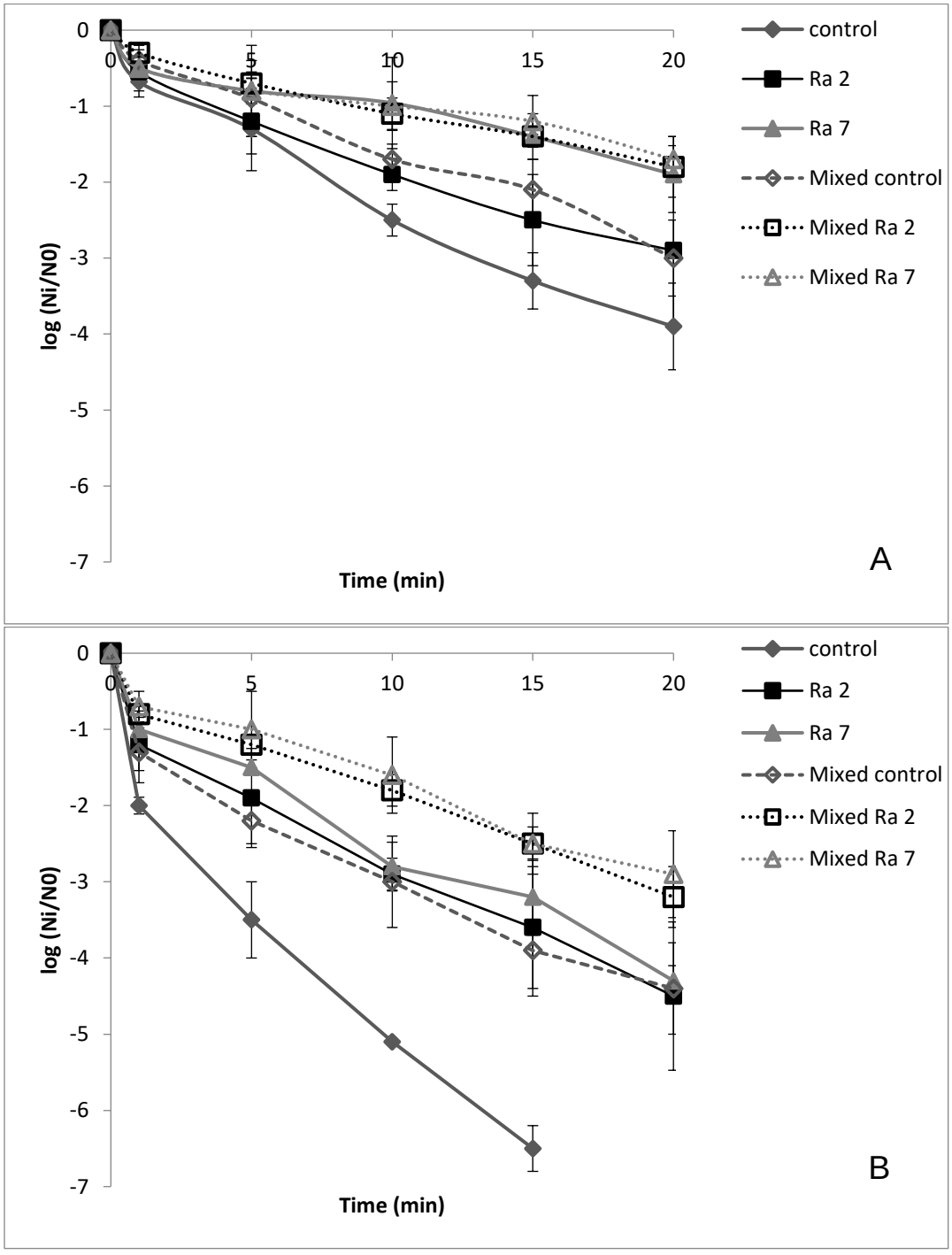

Figure 3. Log reductions of *L. monocytogenes* during peracetic acid (PAA) treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hytrel® coupons (smooth [control] and with roughness of 2 µm [Ra 2] or 7 µm [Ra 7]: (A) treatment with 50 ppm PAA; (B) treatment with 100 ppm PAA. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.

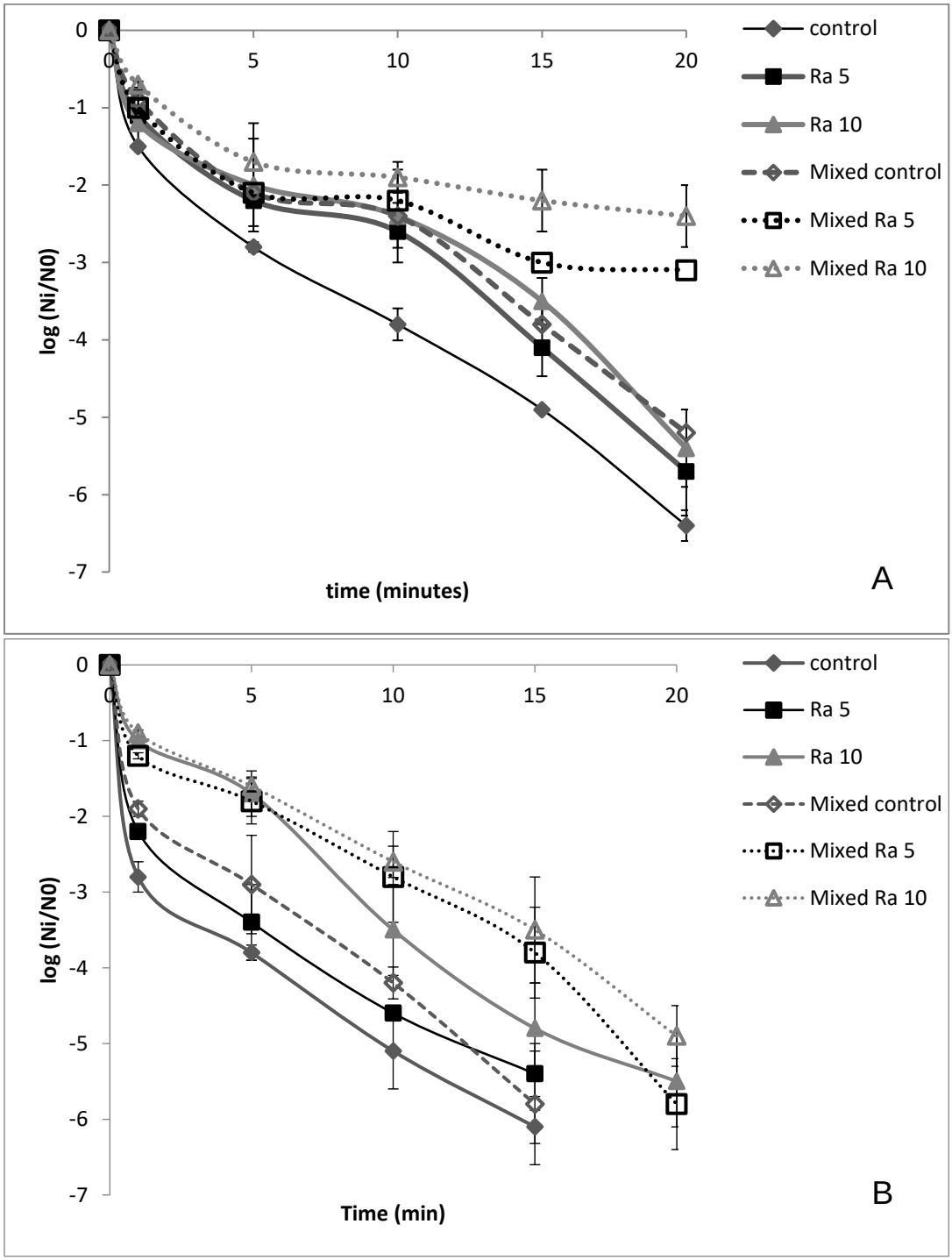

Figure 4. Log reductions of *L. monocytogenes* during chlorine treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hycar® coupons (smooth [control] and with roughness of 5 µm [Ra 5] or 10 µm [Ra 10]: (A) treatment with 100 ppm active chlorine; (B) treatment with 200 ppm active chlorine. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.

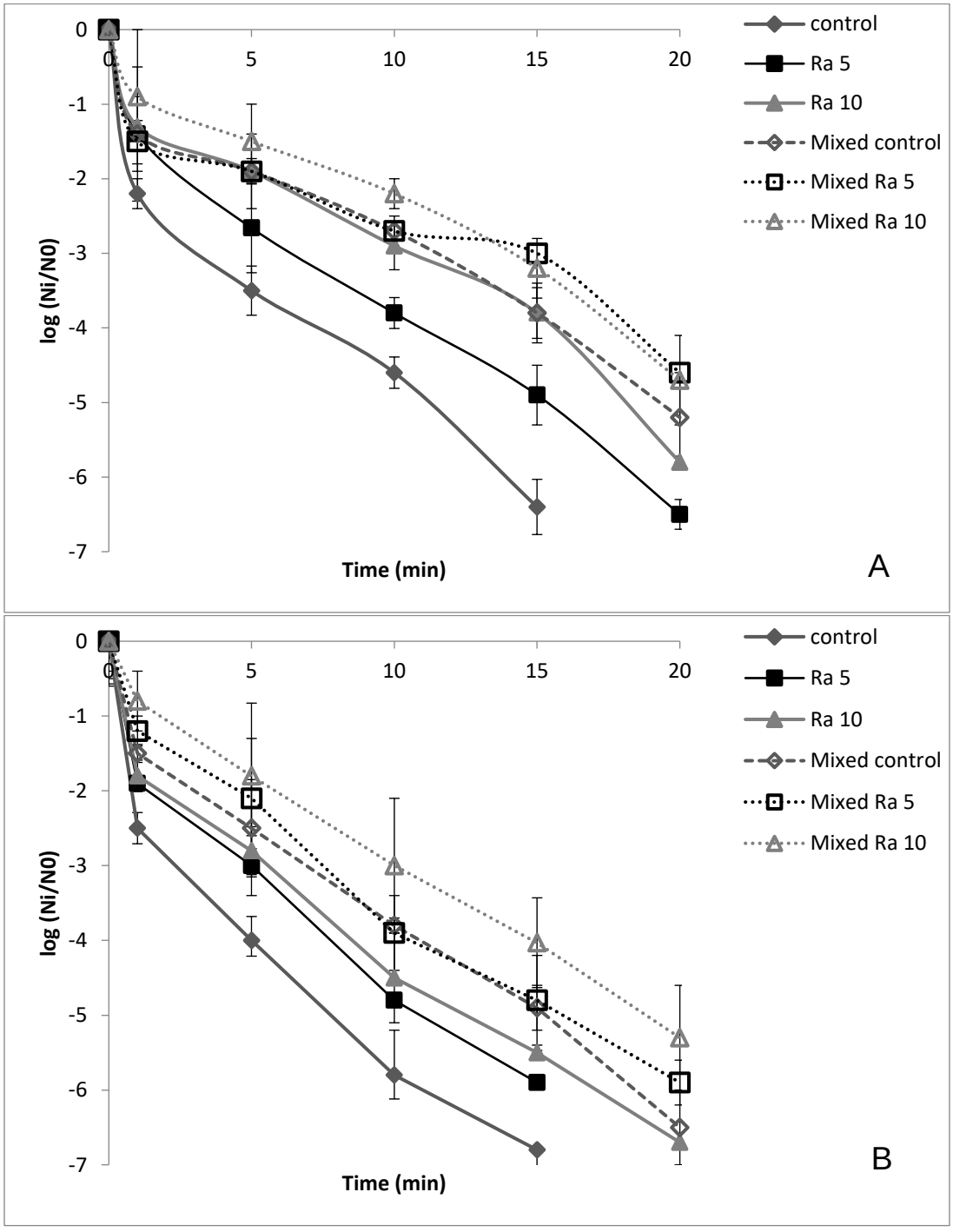

Figure 5. Log reductions of *L. monocytogenes* during benzalkonium chloride (BAC) treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hycar® coupons (smooth [control] and with roughness of 5 µm [Ra 5] or 10 µm [Ra 10]: (A) treatment with 100 ppm BAC; (B) treatment with 400 ppm BAC. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.

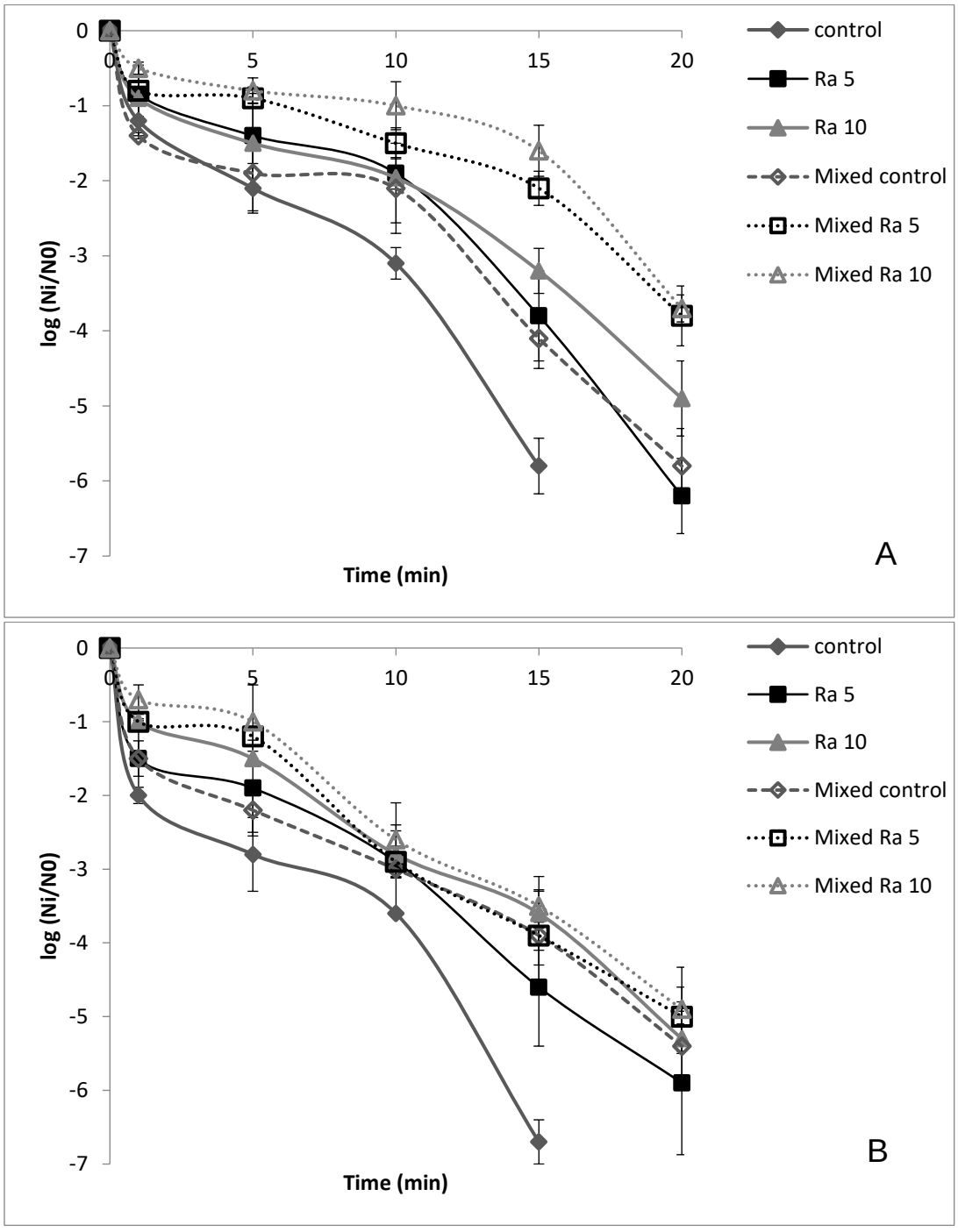

Figure 6. Log reductions of *L. monocytogenes* during peracetic acid (PAA) treatment on biofilms of single species (solid lines) and mixed species (dashed lines), grown on Hycar® coupons (smooth [control] and with roughness of 5 µm [Ra 5] or 10 µm [Ra 10]: (A) treatment with 50 ppm PAA; (B) treatment with 100 ppm PAA. The graphs show the average inactivation of three independent experiments of biofilms (48 h) grown and treated at 25°C. Data points below the detection limit (log10 (Nt/N0) ≈ -6.8) are not shown in the graphs.