Biocontrol Science, 2018, Vol. 23, No. 3, 107－119

Original

Characteristics of Biofilms Formed by Co-Culture of *Listeria monocytogenes* with *Pseudomonas aeruginosa* at Low Temperatures and Their Sensitivity to Antibacterial Substances

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Received 27 May, 2017/Accepted 15 February, 2018

We assessed the properties of biofilms (BFs) formed by mono- and co-cultures of *Listeria monocytogenes* and *Pseudomonas aeruginosa* (L+P-BF) at low temperatures and examined their sensitivity to several antibacterial substances. *L. monocytogenes* viable counts comprised only 1-10% of total L+P-BF viable counts at 10℃ and 15℃, indicating the significant prevalence of *P. aeruginosa* in co-cultures. L+P-BF formed at 10℃ and 15℃ showed very high resistance to antibiotics and NaClO. Examination of the effects of nattokinase and nisin, natural food additives with antibacterial properties, showed that their application alone failed to inhibit L+P-BF development at 10℃ and 15℃. However, a combined treatment with nisin and ethylenediaminetetraacetic acid, a food additive that can be used as a permeabilizing agent, suppressed the formation of L+P-BF at 10℃ and 15℃. Microscopy observations of L+P-BF did not reveal pronounced morphological changes in bacterial cell morphology. We also noted that *P. aeruginosa* resistance to the action of nisin during BF formation was higher when it was maintained in co-culture with *L. monocytogenes*. The results of the present study are an important step toward developing a safe formulation of acceptable food additives that could be used for suppression of BFs formed by pathogenic bacteria during food storage.

Key words：Biofilm / *Listeria monocytogenes* / *Pseudomonas aeruginosa* / Nisin / Ethylenediaminetetraacetic acid (EDTA).

INTRODUCTION

Bacteria commonly adhere to surfaces and form structured communities called biofilms (BFs). BFs are enclosed in a self-produced polymeric matrix that adheres to inert or living surfaces (Costerton et al., 1999). BF presence on food contact surfaces is considered as a health hazard (Di Cicco et al., 2015). This risk is especially serious because bacteria within BFs have decreased susceptibility to antimicrobial agents compared with those existing in planktonic form (Giaouris et al., 2005).

Listeria monocytogenes is a gram-positive foodborne pathogen implicated in severe human and animal diseases. Listeriosis occurs primarily in pregnant women, newborn infants, elderly persons, and immunocompromised individuals. It incurs a high mortality rate (16-38 %) (Mitjà et al., 2009). *L. monocytogenes* can grow under a wide range of environmental stresses, such as extreme pH, high salinity, or low temperature. Consequently, this bacterium is difficult to eradicate from food processing facilities (Lourenço et al., 2011). The ability of *L. monocytogenes* to grow at low temperatures is a serious problem that has caused numerous foodborne outbreaks (Buchanan et al., 2017). In addition, *L. monocytogenes* forms BFs in which two or more bacterial species can coexist (Ibusquiza et al., 2012). Generally, co-culture BFs are less susceptible to biocides than the respective pure culture of their respective

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constituents (Lourenço et al., 2011). Recently, a limited number of studies examined BFs formed by *L. monocytogenes* in combination with other bacteria, such as *Staphylococcus* spp. (de Carvalho et al., 2015; Norwood and Gilmour, 2001; Reiu et al., 2008), *Salmonella* sp. (Kostaki et al., 2012), *Pseudomonas putida* (Giaouris et al., 2013; Ibusquiza et al., 2012), and *P. fluorescens* (Alavi and Hansen, 2013; Puga et al., 2015; 2016;).

To our knowledge, the report by Lourenço et al. (2011) has been the only study of BFs formed by co-cultures of *L. monocytogenes* with *P. aeruginosa* (L+P-BF). The authors reported that stress tolerance to biocides of L+P-BF formed at 12℃ was higher than that at 37℃. *P. aeruginosa* is a common gram-negative bacterium widely distributed in the environment, e.g., in the soil, fresh water, and seawater. *P. aeruginosa* can cause serious diseases as an opportunistic pathogen, and it shows high resistance to many antimicrobials (Poole, 2002). *P. aeruginosa* also is a BF-forming bacterium that can grow at a wide range of temperatures, from 4℃ to 42℃ (LaBauve and Wargo, 2015).

Expansion of food cold chain logistics has greatly contributed to the prevention of food poisoning by microorganisms. However, there is a concern that food may be poisoned in cold storage by some bacteria that are widely distributed in the environment and capable of forming BFs at low temperature conditions. Therefore, in this study, we investigated the characteristics of L+P-BF formed at low temperatures and effects of food additives on BF formation. We found that L+P-BF formed at low temperatures had extremely high resistance to sanitizers, but the addition of nisin in combination with ethylenediaminetetraacetic acid (EDTA) inhibited L+P-BF formation.

MATERIALS AND METHODS

Test bacteria

L. monocytogenes ATCC 19114, ATCC 19115, and *P. aeruginosa* ATCC 7700 were used as test strains. The bacteria were stored in 10% glycerol solution at −85℃. Prior to the experiment, they were thawed and pre-incubated in the nutrient broth (Eiken Chemicals Co. Ltd., Tokyo, Japan) at 37℃ for 24 h. Pre-incubated bacterial cells were washed twice (3,000 rpm, 5 min × 2), and resuspended with sterile pure water to a cell density of 10^5 CFU/ml.

Evaluation of the ability of test strains to form BFs and determination of BF viable cell counts

Two hundred microliters of Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, UK) were poured into a polystyrene 96-well flat bottom microplate (Iwaki, Asahi Glass Co. Ltd., Tokyo, Japan). Resuspended bacterial suspensions of *L. monocytogenes* and *P. aeruginosa* were mixed in the same volume. The mixed bacterial suspension (10 µl) was inoculated into the wells. The initial cell density of bacterial cells was approximately 10^3 CFU/well. Microplates were incubated at 10℃ and 15℃ for 5-14 days. After incubation, the supernatant was discarded, and the well was washed with sterilized pure water twice and then dried on a clean bench for 1 h. BFs formed on the surface of the microplate well walls were stained with 1/100 diluted crystal violet stain (Wako Pure Chemicals Co. Ltd., Osaka, Japan) for 30 min. Then, after the staining solution was removed, the wells were washed with pure water twice and allowed to dry on a clean bench for 1 h again. After that, 250 µl of 99.5% ethanol was dispensed and left in each well for 10 min to extract the stain. The supernatant (200 µl) was transferred to another microplate, and absorbance at 590 nm ($Abs._{590}$) was measured with a microplate reader (TECAN Infinite® 200 PRO, Tecan Trading AG, Hombrechtikon, Switzerland). BF-forming abilities were evaluated based on $Abs._{590}$ values.

To enumerate viable counts in the BF, the supernatants were discarded after incubation and washed with sterile pure water to remove unattached cells, and the BFs were removed by swabbing with sterile cotton swabs (Joseph et al., 1998, Holah et al., 2002). The swabs were transferred to 1 ml of sterile pure water in a sterilized 15 ml centrifuge tube. The tube was shaken vigorously and exposed to ultrasonic treatment for 10 min to remove the bacteria attached to the swab. We preliminarily confirmed that viable counts were not negatively affected by ultrasonic treatment. Total viable counts of bacteria and counts of *L. monocytogenes* in L+P-BF were determined by standard spread plate technique by using nutrient agar (Eiken Chemicals) and PALCAM Listeria-Selective agar (Merck KGaA, Darmstadt, Germany), respectively. The counts of *P. aeruginosa* in L+P-BF were determined by subtracting the counts of *L. monocytogenes* from total bacterial counts.

Suspensions containing single bacterial species (10 µl), *L. monocytogenes* or *P. aeruginosa*, were also inoculated into the wells at an initial cell density of approximately 10^3 CFU/well, and the BF amount and BF viable counts in monocultures of *L. monocytogenes* (L-BF) or *P. aeruginosa* (P-BF) were evaluated using the same procedure as described above.

The growth behavior of the bacteria in the cultures during incubation at 10℃ was monitored. The monoculture and co-culture were prepared as described above using the 96- well plate and incubated at 10℃. The culture solution (180 µl) sampled from the wells of the 96-plate during incubation were poured into another plate and the $Abs._{590}$ was measured by the microplate

reader up to 48 h. When sampling, the contents of the 96-well plate were mixed by vibration for 15 s in the microplate reader.

Microscopy observation

Mixed bacterial suspensions (50 µl) of *L. monocytogenes* ATCC 19114 with *P. aeruginosa* ATCC 7700 prepared in Todd-Hewitt broth were put on slide glass plates (Matsunami Glass Ind., Ltd., Osaka, Japan) in sterile plastic petri dishes. The dishes were covered with the lid to prevent evaporation of the liquid and incubated at 10℃ or at 15℃. We previously confirmed that capacities to form BFs in 96-well microplates and on the glass plates for up to five days were similar for L+P-BF, L-BF, and P-BF ($P > 0.05$). BFs formed on the plates were observed by phase-contrast microscopy with the EVOS® cell imaging system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Furthermore, *L. monocytogenes* and *P. aeruginosa* cells were detected by fluorescent double staining using the RNA *in situ* hybridization method to visualize the typical localization of the cells. We used fluorescent RNAscope® Target probes (Advanced Cell Diagnostic Inc., Hayward, CA, 94545, USA) that were designed based on 16S rRNA sequences of *L. monocytogenes* ATCC 19114 (red fluorescence) or *P. aeruginosa* ATCC 7700 (green fluorescence). A mixed bacterial suspension of *L. monocytogenes* ATCC 19114 and *P. aeruginosa* ATCC 7700 was prepared in Todd-Hewitt broth, and a portion of that suspension (100 µl) was inoculated on the slide glass plate. After five days of incubation, the BFs formed on the glass plate were stained with RNAscope® and observed by fluorescent microscopy with the EVOS® cell imaging system.

The staining procedure was in accordance with the instructions given in the RNAscope® 2-Plex Detection Kit (Chromogenic) user manual (Wang et al., 2012).

Resistance of BFs to antibacterial substances

The sensitivity of BFs to antibiotics and sodium hypochlorite was examined as follows. After BFs were formed in microplate wells after five days of incubation at 10℃ or 15℃, the supernatant was discarded and the well was washed with sterilized pure water twice and dried on a clean bench for 1 h. Two hundred microliters of an antibiotic- or sanitizer-containing solution (penicillin G, chloramphenicol, or sodium hypochlorite) was then added to each well and the microplate was incubated at 37℃ for 24 h. Each supernatant solution containing the drug was then discarded, and the well was washed twice with sterilized pure water. Thereafter, 200 µl of nutrient broth was poured into each well and the wells were incubated at 37℃ for 24 h. Then, the change in turbidity was inspected visually. The antibiotics and sodium hypochlorite were purchased from Wako Pure Chemicals.

Effects of antibacterial food additives on BF formation

Antibacterial properties of the food additives nattokinase (Wako Pure Chemicals) and nisin from *Lactobacillus lactis* (Sigma-Aldrich, St. Louis, MO, USA) were examined. The nisin preparation contained nisin at a concentration of 2.5%. Ethylenediaminetetraacetic acid (EDTA: Wako Pure Chemicals) was also used as a permeabilizing agent. Todd-Hewitt broth (100 µl) was dispensed into microplate wells at a concentration twice higher than usual. Each food additive was diluted with sterile pure water. EDTA was mixed with nattokinase or nisin. Then, 50 µl of food additive-containing solution was dispensed into microplate wells. Single or mixed bacterial suspensions (50 µl), which were diluted to a cell density of 1/5, were also inoculated into microplate wells. The initial number of bacteria was about 10^3 CFU/well. After five days of incubation at 10℃, 15℃, or 37℃, the BF amount and BF viable counts were measured using the same procedures as described earlier.

Statistical analysis

All experiments were performed in triplicate. Data are presented as the mean ± standard error of the mean. To determine the significance of differences in mean values, data were analyzed using one- or two-way analysis of variance followed by post hoc Tukey's test, if appropriate. BellCurve for Excel® version 2.0.3 (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for calculations. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

BF formation

Table 1 shows changes in $Abs._{590}$ values that were proportional to the amount of BF in mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* at 10℃. The amount of L+P-BF at 10℃ gradually increased until the 5th day in culture and did not change significantly ($P > 0.05$) until the 14th day of culture. The L-BF amount in *L. monocytogenes* monocultures increased until days 3-5 of culture and did not change significantly until day 14 ($P > 0.05$). Notably, L-BFs were significantly smaller than L+P-BF or P-BF ($P < 0.05$). The P-BF amount increased until the 5th day in culture and did not change significantly until day14 ($P > 0.05$). In addition, there were no significant differences in the BF amount between L+P-BF and P-BF cultures across all days in culture ($P < 0.05$), suggesting that the L+P-BF amount at 10℃ was dependent primarily on *P. aeruginosa*. In addition,

TABLE 1. Biofilm formation in the wells of polystyrene microplates by mono- and co-cultures of *L. monocytogenes* and *P. aeruginosa* at 10℃.

Culture	Absorbance (590 nm)					
	0 day	1 day	3 day	5 day	8 day	14 day
L.m. ATCC 19114 + P.a.^{A}	0.14 ± 0.03 a,A	0.19 ± 0.01 a,A	0.49 ± 0.07 b,A	0.53 ± 0.10 b,A	0.46 ± 0.08 b,A	0.42 ± 0.01 b,A
L.m. ATCC 19115 + P.a.^{A}	0.14 ± 0.03 a,A	0.20 ± 0.05 ab,A	0.43 ± 0.02 c,A	0.58 ± 0.17 c,A	0.38 ± 0.06 bc,AC	0.46 ± 0.10 c,A
L.m. ATCC 19114^{B}	0.14 ± 0.01 a,A	0.17 ± 0.02 a,A	0.32 ± 0.14 ab,A	0.36 ± 0.05 ab,B	0.28 ± 0.11 ab,C	0.36 ± 0.19 b,A
L.m. ATCC 19115^{B}	0.14 ± 0.01 a,A	0.16 ± 0.01 a,A	0.35 ± 0.18 b,A	0.28 ± 0.05 ab,B	0.25 ± 0.08 ab,C	0.31 ± 0.13 ab,A
P.a. ATCC 7700^{A}	0.14 ± 0.03 a,A	0.17 ± 0.02 b,A	0.41 ± 0.12 c,A	0.63 ± 0.08 cd,A	0.60 ± 0.03 d,D	0.43 ± 0.04 cd,A

Different lowercase or capital letters indicate significantly different mean values within the row or column, respectively ($P < 0.05$).
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

TABLE 2. Biofilm formation in the wells of polystyrene microplates by mono- and co-cultures of *L. monocytogenes* and *P. aeruginosa* at 15℃.

Culture	Absorbance (590 nm)					
	0 day	1 day	3 day	5 day	8 day	14 day
L.m. ATCC 19114 + P.a.^{A}	0.14 ± 0.03 a,A	0.20 ± 0.01 a,A	0.46 ± 0.07 b,A	0.33 ± 0.06 c,A	0.43 ± 0.06 d,A	0.31 ± 0.06 c,A
L.m. ATCC 19115 + P.a.^{A}	0.14 ± 0.03 a,A	0.19 ± 0.03 a,A	0.41 ± 0.08 ab,A	0.31 ± 0.06 bc,A	0.37 ± 0.08 c,A	0.30 ± 0.02 bc,A
L.m. ATCC 19114^{AB}	0.14 ± 0.01 a,A	0.18 ± 0.02 a,A	0.22 ± 0.01 a,B	0.38 ± 0.09 b,AB	0.61 ± 0.08 c,B	0.42 ± 0.12 b,A
L.m. ATCC 19115^{AB}	0.14 ± 0.01 a,A	0.17 ± 0.00 a,A	0.22 ± 0.02 ab,B	0.34 ± 0.16 bc,AB	0.42 ± 0.16 c,AC	0.34 ± 0.03 c,A
P.a. ATCC 7700^{B}	0.14 ± 0.03 a,A	0.19 ± 0.05 a,A	0.44 ± 0.14 b,A	0.63 ± 0.10 c,C	0.52 ± 0.09 c,D	0.37 ± 0.06 bd,A

Different lowercase or capital letters indicate significantly different mean values within the row or column, respectively ($P < 0.05$).
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

TABLE 3. Total viable counts or viable counts of *L. monocytogenes* in biofilms formed by mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* at 10℃ (unit: $\log_{10}$ CFU/well).

Culture		Incubation time (day)				
		1	3	5	8	14
L.m. ATCC 19114 + P.a.	Total counts^{A}	4.0 ± 0.3 a, ABC	6.2 ± 0.4 b,A	7.4 ± 0.5 c,A	7.7 ± 0.4 c,A	7.4 ± 0.3 c,AC
	Listeria counts^{B}	3.1 ± 0.3 a,BC	4.8 ± 0.3 b,BC	5.6 ± 0.4 bc,B	6.2 ± 0.1 c,B	4.9 ± 0.4 b,B
L.m. ATCC 19115 + P.a.	Total counts^{A}	4.4 ± 0.2 a,A	6.2 ± 0.3 b,A	7.6 ± 0.1 c,A	7.7 ± 0.2 c,A	7.4 ± 0.1 c,AC
	Listeria counts^{B}	3.1 ± 0.4 a,C	4.5 ± 0.5 bd,C	5.6 ± 0.3 c,B	6.3 ± 0.3 d,B	4.9 ± 0.5 bc,B
L.m. ATCC 19114	Counts^{C}	3.9 ± 0.6 a,AC	5.4 ± 0.6 b,A	5.8 ± 0.6 bc,B	6.4 ± 0.3 c,B	6.5 ± 0.6 c,CD
L.m. ATCC 19115	Counts^{C}	3.8 ± 0.4 a,AC	5.4 ± 0.5 b,A	5.9 ± 0.2 b,C	6.2 ± 0.2 b,B	6.0 ± 0.6 b,D
P.a.	Counts^{A}	4.5 ± 0.1 a,A	5.7 ± 0.8 b,AB	7.0 ± 1.0 c,A	7.7 ± 0.3 c,A	7.4 ± 0.0 c,AC

Different lowercase or capital letters indicate significantly different mean values within the row or column, respectively ($P < 0.05$).
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

no significant differences were observed between BF amounts of the two strains of *L. monocytogenes* examined (Table 1, $P > 0.05$).

At 15℃, the L+P-BF amount increased until day 3 in culture and showed little change until day 14 (Table 2). In addition, L-BF and P-BF amounts were maximal on day 8 and day 5 in culture, respectively. No significant differences in BF amounts were observed between the two strains of *L. monocytogenes* ($P > 0.05$) cultured at 15℃. BF amounts of both *L. monocytogenes* ATCC 19114 and *L. monocytogenes* ATCC 19115 cultures at 15℃ were significantly higher than those at 10℃. At the same time, there were no significant differences in the amounts of L+P-BF and P-BF cultured at 10℃ and 15℃ ($P > 0.05$).

BF viable counts

Table 3 shows viable counts in L+P-BF, L-BF, and

TABLE 4. Total viable counts or viable counts of *L. monocytogenes* in biofilms formed by mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* at 15℃ (unit: $\log_{10}$ CFU/well).

Culture		Incubation time (day)				
		1	3	5	8	14
L.m. ATCC 19114 + P.a.	Total counts[A]	4.8 ± 0.3 a,A	4.8 ± 2.0 a,ACDE	7.6 ± 0.4 b,AC	7.1 ± 0.8 b,AB	7.0 ± 0.2 b,A
	Listeria counts[B]	2.8 ± 0.4 a,B	4.0 ± 0.5 ab,B	5.6 ± 0.6 c,B	6.1 ± 0.7 c,B	5.2 ± 0.1 b,B
L.m. ATCC 19115 + P.a.	Total counts[A]	4.7 ± 0.2 a,A	6.1 ± 0.9 b,C	7.9 ± 0.2 b,A	7.0 ± 1.0 b,A	7.1 ± 0.2 b,A
	Listeria counts[B]	2.7 ± 0.3 a,B	4.3 ± 0.7 b,BD	5.6 ± 0.2 b,B	5.6 ± 0.4 b,C	5.5 ± 0.3 b,B
L.m. ATCC 19114	Counts[A]	4.8 ± 0.2 a,A	4.3 ± 0.6 a,ABE	7.2 ± 0.4 b,AC	6.5 ± 1.5 b,AB	7.3 ± 0.2 b,A
L.m. ATCC 19115	Counts[A]	4.6 ± 0.1 a,A	5.1 ± 0.4 ab,ABC	6.4 ± 1.0 b,BC	7.3 ± 0.9 c,A	7.2 ± 0.1 c,A
P.a.	Counts[A]	4.6 ± 0.3 a,A	6.1 ± 1.2 b,CE	7.3 ± 0.6 b,AC	7.1 ± 0.5 b,AB	7.0 ± 0.5 b,A

Different lowercase or capital letters indicate significantly different mean values within the row or column, respectively ($P < 0.05$). Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

TABLE 5. Ratio of viable counts of *Listeria monocytogenes* to total counts in biofilms formed in co-culture of *L. monocytogenes* with *P. aeruginosa* at 10℃ and 15℃.

Temperature (℃)	Culture	Listeria ratio (%)				
		1 day	3 day	5 day	8 day	14 day
10	L.m. ATCC 19114 + P.a.[A]	13.7 ± 9.6 a,A	5.9 ± 4.1 a,A	2.0 ± 0.7 b,A	3.4 ± 2.7 b,A	0.6 ± 0.6 b,A
	L.m. ATCC 19115 + P.a.[A]	6.4 ± 6.0 a,AB	3.2 ± 2.4 a,A	1.2 ± 0.6 a,A	5.5 ± 5.6 a,A	0.4 ± 0.4 a,A
15	L.m. ATCC 19114 + P.a.[A]	1.2 ± 0.7 a,B	4.1 ± 3.1 a,A	2.6 ± 3.5 a,A	10.0 ± 10.9 a,A	1.6 ± 0.5 a,A
	L.m. ATCC 19115 + P.a.[A]	1.3 ± 0.9 a,B	3.3 ± 4.5 a,A	0.7 ± 0.6 a,A	1.8 ± 0.9 a,A	4.1 ± 4.1 a,A

Different lowercase or capital letters indicate significantly different mean values within the row or column, respectively ($P < 0.05$). Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

P-BF formed at 10℃. Total bacterial counts of L+P-BF in the period between day 5 and day 14 in culture were within 7.4-7.7 $\log_{10}$ CFU. *P. aeruginosa* viable counts in L+P-BF were determined by subtracting viable counts of *L. monocytogenes* from total bacterial counts. L+P-BF predominantly contained *P. aeruginosa*, whereas *L. monocytogenes* viable counts generally comprised 10% or less of total counts (less than 1% on day 14; Table 3). There was no significant difference between total L+P-BF counts and *P. aeruginosa* counts in P-BF ($P > 0.05$). Furthermore, it was found that the variation in *L. monocytogenes* counts in L+P-BF were significantly different from those in L-BF at 10℃, as slight inhibition of growth was apparent in L+P-BF ($P < 0.05$).

Table 4 shows bacterial counts in mono- and co-cultures of *L. monocytogenes* and *P. aeruginosa* cultured at 15℃. In the period between day 5 and day 14 in culture, total bacterial counts of L+P-BF were 7.0-7.9 $\log_{10}$ CFU. In *L. monocytogenes* 19114 + *P. aeruginosa* co-culture, total counts and *Listeria* counts of cultures grown at 10℃ and 15℃ were significantly different ($P < 0.05$). In addition, *L. monocytogenes* growth was suppressed in L+P-BF formed at 15℃. As in the case of cultures grown at the incubation temperature of 10℃, L+P-BF mostly contained *P. aeruginosa*, whereas *L. monocytogenes* comprised less than 10% of total counts (Table 5). There was no significant difference between L+P-BF and P-BF total counts ($P > 0.05$). The L-BF amount at 15℃ was significantly higher than at 10℃. Examination of the relationship between BF amounts (Tables 1 and 2) and BF viable bacterial counts (Tables 3 and 4) showed fairly high correlation between these parameters: $R^2 = 0.78$ at 10℃, $R^2 = 0.56$ at 15℃, and $R^2 = 0.67$ when data at both two temperatures were combined.

On the other hand, the changes in $Abs._{590}$ values of the cultures are shown in Fig.1. The growth rate in descending order after 2 days in mono-culture was L.m. ATCC19115 > P.a. ATCC7700 > L.m. ATCC 19114 (Fig.1). *P. aeruginosa*, which was the dominant strain in BF, did not have the highest growth rate. In the case of co-culture of L.m. ATCC19115+P.a. ATCC 7700, the value of $Abs._{590}$ was almost the sum of that of the mono-culture of each bacterium. On the other hand, the growth of P.a. aTCC 7700 was depressed by co-culturing with L.m. ATCC 19114.

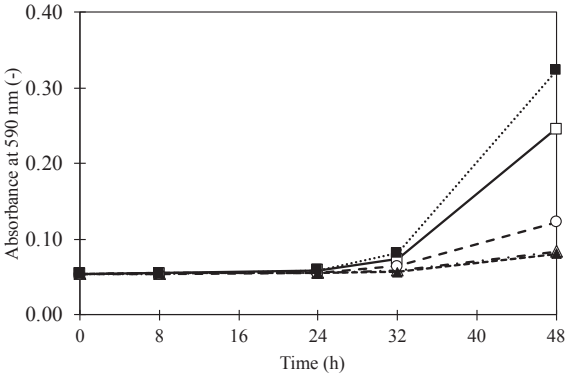

FIG. 1. Changes in absorbance at 590 nm of cultures at 10℃ in monoculture and co-culture. △: *Listeria monocytogenes* ATCC19114, □: *Listeria monocytogenes* ATCC19115, ○: *Pseudomonas aeruginosa* ATCC7700, ▲: *Listeria monocytogenes* ATCC19114 + *Pseudomonas aeruginosa* ATCC7700, ■: *Listeria monocytogenes* ATCC19115 + *Pseudomonas aeruginosa* ATCC7700.

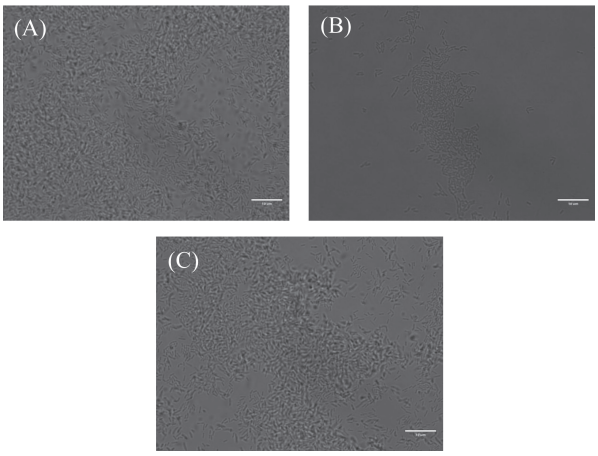

FIG. 2. Phase-contrast microscopy images of biofilms formed at 10℃ on day 5 in culture. (A) Biofilm formed by a monoculture of *Pseudomonas aeruginosa* ATCC7700. (B) Biofilm formed by a monoculture of *Listeria monocytogenes* ATCC19114. (C) Biofilm formed by a co-culture of *L. monocytogenes* ATCC19114 and *P. aeruginosa* ATCC7700.

Microscopy observation of L+P-BFs

Fig.2(A) shows a phase contrast micrograph of P-BF after five days in culture at 10℃. P-BF contained rods of approximately 0.5×2 μm that overlapped on the glass plate. In contrast, L-BF cells spread in monolayers after five days in culture [Fig.2(B)]. The L+P-BF appearance after five days was apparently the same as that of P-BF, indicating that *P. aeruginosa* was the predominant species of L+P-BF [Fig.2(C)].

In order to reveal the localizations of the acterial cells

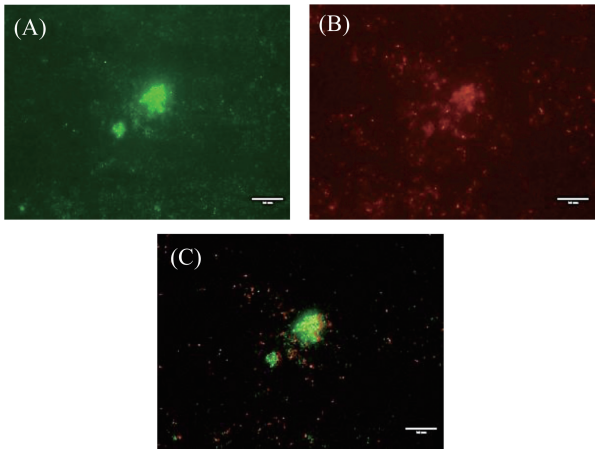

FIG. 3. Fluorescence microscopy images of biofilms formed at 10℃ on day 5 in culture. Biofilms formed by a co-culture of *Listeria monocytogenes* ATCC19114 with *Pseudomonas aeruginosa* ATCC7700 were double-stained with RNAscope® target probes to red and green fluorescence, respectively. (A) *P. aeruginosa* ATCC7700. (B) *L. monocytogenes* ATCC19114. (C) Merged image.

of the two species in L+P-BF, *L. monocytogenes* and *P. aeruginosa* were stained green [Fig.3(A)] and red [Fig.3(B)], respectively, by using RNAscope® and observed under a fluorescence microscope. As illustrated by the merged image in Fig.3(C), *P. aeruginosa* and *L. monocytogenes* cells in P+L-BFs were intermixed. Similar results were also obtained in co-cultures grown at 15℃.

Resistance of BFs to antibiotics and sodium hypochlorite

Resistance of L+P-BFs formed at 10℃ and 15℃ to antibiotics and sodium hypochlorite was examined after five days in culture. At 10℃, the growth of BFs could not be suppressed at all even when the concentration of penicillin G or chloramphenicol was 5000 μg/ml. Only NaClO inhibited the growth of L+P-BFs and P-BF, and L-BF at 1000 μg/ml and 500 μg/ml, respectively. Then, the antibiotics and NaClO were applied at 37℃ to the BFs to enhance the action (Table 6).

Penicillin G failed to inhibit BF growth even at a relatively high concentration of 5,000 μg/ml even at 37℃. Chloramphenicol treatment attenuated the growth of L-BFs at 5,000 μg/ml. NaClO treatment negatively affected L-BF at 125 μg/ml, whereas suppressive action on L+P-BFs was evident only at concentrations higher than 500 μg/ml. The results obtained from cultures grown at 15℃ were similar to those obtained at 10℃.

The MBC after contact between the antibiotics or NaClO and the planktonic cells of the single or mixed

TABLE 6. Resistance to antibiotics and sodium hypochlorite of biofilms formed by mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* at 10℃ after five days in culture.

Reagnets	Concentration (μg/ml)	L.m. ATCC 19114 + P.a.	L.m. ATCC 19115 + P.a.	L.m. ATCC 19114	L.m. ATCC 19115	P.a.
Penicillin G	1,250	+	+	+	+	+
	2,500	+	+	+	+	+
	5,000	+	+	+	+	+
Chloramphenicol	1,250	+	+	+	+	+
	2,500	+	+	+	+	+
	5,000	+	+	−	−	+
Sodium hypochlorite	62.5	+	+	+	+	+
	125	+	+	−	−	+
	250	+	+	−	−	+
	500	−	−	−	−	−
	1,000	−	−	−	−	−

+: growth, −: no gorwth
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

TABLE 7. Effect of nattokinase or nisin on biofilm formation in mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* after five days in culture.

Reagents, Concentration	Temperature (℃)	L.m ATCC 19114 + P.a.	L.m ATCC 19115 + P.a.	L.m ATCC 19114	L.m ATCC 19115	P.a.
Nattokinase 1000 μg/ml	10	+	+	+	+	+
	15	+	+	+	+	+
	37	−	−	−	−	−
Nisin 50 μg/ml	10	+	+	−	−	+
	15	+	+	−	−	+
	37	+	+	−	−	+

+: growth, −: no gorwth
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC7700.

bacteria cultures for 24 h was <625 μg/ml, <625 μg/ml or <1 μg/ml for PCG, CP or NaClO, respectively. The resistance of the BFs was remarkably higher than that of the planktonic cells.

Effects of nattokinase and nisin on BF formation

Table 7 shows the effects of nattokinase and nisin on BF formation to be dependent on the incubation temperature. Although in Table 7 the results for 1,000 μg/ml nattokinase are shown, in fact the formation of L+P-BF, L-BF, and P-BF at 37℃ was suppressed by nattokinase at concentrations as low as 15.6 μg/ml. In contrast, at 10℃ and 15℃, BF formation could not be suppressed in any type of culture even by 1,000 μg/ml nattokinase. These results showed that nattokinase did not inhibit BF formation at low temperatures.

Nisin inhibited L-BF formation at 10℃, 15℃ and 37℃ at a concentration of 0.78 μg/ml and higher. The growth of neither L+P-BF or P-BF was suppressed by 50 μg/ml nisin.

Effect of nisin + EDTA on BF formation

Both nattokinase and nisin when applied alone could not suppress L+P-BF formation at low temperatures (Table 7). Therefore, EDTA was added to the culture medium as an agent that would facilitate the penetration of nattokinase and nisin into the bacteria. EDTA is a metal chelating compound that itself possesses antibacterial activity (Leive et al., 1965). EDTA alone could suppress L-BF formation at a concentration of 0.4% or higher, whereas the growth of P-BF and L+P-BF could be inhibited by 2.0% EDTA or higher after five days of incubation (data not shown). In the present study, 0.1 and 0.2% EDTA solutions were used for combined treatment with nattokinase or nisin, because EDTA alone did not affect BF formation at these concentrations.

TABLE 8. Effects of nisin and EDTA on the amount of biofilms formed in the wells of polystyrene microplates by mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* after five days in culture (unit: absorbance at 590 nm).

Culture	EDTA (%)	Temperature (℃)	Control*	Nisin concentration (μg/ml)		
				0	6.25	12.5
L.m. ATCC 19114 + P.a. ATCC 7700	0.1	10	0.19 ± 0.01 a	0.73 ± 0.08 b	0.73 ± 0.16 b	0.76 ± 0.16 b
		15	0.19 ± 0.01 a	0.56 ± 0.12 b	0.65 ± 0.16 b	0.63 ± 0.24 b
		37	0.17 ± 0.00 a	0.41 ± 0.01 ab	0.38 ± 0.15 ab	0.49 ± 0.24 b
	0.2	10	0.18 ± 0.01 a	0.58 ± 0.11 b	0.36 ± 0.16 ab	0.19 ± 0.03 ac
		15	0.19 ± 0.01 a	0.54 ± 0.23 b	0.54 ± 0.04 b	0.40 ± 0.17 ab
		37	0.17 ± 0.01 a	0.36 ± 0.05 a	0.20 ± 0.04 a	0.25 ± 0.04 a
L.m. ATCC 19115 + P.a. ATCC 7700	0.1	10	0.19 ± 0.01 a	0.51 ± 0.09 b	0.61 ± 0.00 b	0.57 ± 0.01 b
		15	0.19 ± 0.02 a	0.63 ± 0.08 b	0.71 ± 0.18 b	0.66 ± 0.20 b
		37	0.17 ± 0.01 a	0.33 ± 0.05 ab	0.48 ± 0.09 b	0.57 ± 0.09 b
	0.2	10	0.18 ± 0.01 a	0.56 ± 0.20 b	0.28 ± 0.14 a	0.15 ± 0.01 a
		15	0.19 ± 0.02 a	0.53 ± 0.12 b	0.46 ± 0.28 b	0.41 ± 0.25 ab
		37	0.17 ± 0.01 a	0.38 ± 0.11 a	0.28 ± 0.04 a	0.27 ± 0.04 a
L.m. ATCC 19114	0.1	10	0.19 ± 0.01 ab	0.29 ± 0.09 b	0.16 ± 0.01 a	0.16 ± 0.02 a
		15	0.19 ± 0.01 a	0.29 ± 0.05 a	0.24 ± 0.06 a	0.22 ± 0.05 a
		37	0.17 ± 0.01 a	0.42 ± 0.13 b	0.20 ± 0.11 a	0.18 ± 0.09 a
	0.2	10	0.18 ± 0.03 ab	0.27 ± 0.08 b	0.16 ± 0.02 ab	0.15 ± 0.01 a
		15	0.17 ± 0.03 a	0.24 ± 0.106 a	0.15 ± 0.03 a	0.14 ± 0.02 a
		37	0.19 ± 0.02 a	0.24 ± 0.03 a	0.14 ± 0.01 a	0.13 ± 0.00 a
L.m. ATCC 19115	0.1	10	0.19 ± 0.02 a	0.24 ± 0.08 a	0.16 ± 0.01 a	0.15 ± 0.02 a
		15	0.18 ± 0.01 a	0.30 ± 0.04 b	0.18 ± 0.03 a	0.18 ± 0.05 a
		37	0.17 ± 0.01 a	0.34 ± 0.14 b	0.21 ± 0.13 a	0.19 ± 0.11 a
	0.2	10	0.17 ± 0.02 a	0.25 ± 0.03 a	0.18 ± 0.04 a	0.16 ± 0.01 a
		15	0.18 ± 0.01 a	0.16 ± 0.01 a	0.17 ± 0.01 a	0.16 ± 0.01 a
		37	0.19 ± 0.01 ab	0.25 ± 0.03 b	0.15 ± 0.00 ab	0.13 ± 0.01 a
P.a. ATCC 7700	0.1	10	0.19 ± 0.01 a	0.61 ± 0.11 b	0.61 ± 0.04 b	0.57 ± 0.08 b
		15	0.18 ± 0.01 a	0.56 ± 0.11 b	0.70 ± 0.17 b	0.61 ± 0.08 b
		37	0.19 ± 0.01 a	0.37 ± 0.06 ab	0.46 ± 0.12 ab	0.51 ± 0.15 b
	0.2	10	0.18 ± 0.01 a	0.62 ± 0.22 b	0.18 ± 0.01 a	0.15 ± 0.01 a
		15	0.19 ± 0.01 a	0.64 ± 0.17 b	0.62 ± 0.25 b	0.48 ± 0.29 b
		37	0.17 ± 0.00 a	0.41 ± 0.14 a	0.24 ± 0.07 a	0.35 ± 0.11 a

*The value of absorbance at 590 nm when bacterial suspension was not added.
Different lowercase letters within a column indicate significantly different mean values ($P < 0.05$).
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa*.

L-BF formation could be suppressed by a combination of EDTA at a concentration of 0.1% or higher and nattokinase at a concentration of 15.6 μg/ml or higher. However, the formation of P-BF and L+P-BF was not affected at any temperature, even by a combination of 0.2% EDTA and 1000 μg/ml nattokinase (data not shown).

Table 8 shows the results of the combined treatment with nisin and EDTA. L-BF formation was suppressed at all temperatures tested by a combination of nisin and EDTA at a concentration of 0.1% or higher. In fact, L-BF formation could be suppressed by a combination of 0.1% EDTA and nisin at a concentration of 0.75 μg/ml or higher. In contrast, the formation of P-BFs and L+P-BFs was not inhibited even after the treatment with 0.1% EDTA and 12.5 μg/ml nisin. Partial attenuation of P-BFs and L+P-BFs was achieved by a combined treatment with 0.2% EDTA and 6.25 μg/ml nisin. In the latter

TABLE 9. Effect of nisin + 0.2% EDTA on total viable counts or viable counts of *L. monocytogenes* in the biofilms formed by mono- or co-cultures of *L. monocytogenes* and *P. aeruginosa* at 10℃ after five days in culture (unit: absorbance at 590 nm).

Culture	Nisin Conc. (μg/ml)	Total counts (log_{10} CFU/well)	*Listeria* counts (log_{10} CFU/well)
L.m. ATCC 19114 + P.a.		7.4 ± 0.5 a	5.6 ± 0.4 a
L.m. ATCC 19115 + P.a.		7.6 ± 0.1 ab	5.6 ± 0.3 a
L.m. ATCC 19114	0	−	5.8 ± 0.5 a
L.m. ATCC 19115		−	5.9 ± 0.2 a
P.a.		7.0 ± 0.9 a	−
L.m. ATCC 19114 + P.a.		6.1 ± 0.3 a	N.D.
L.m. ATCC 19115 + P.a.		5.9 ± 0.5 ac	N.D.
L.m. ATCC 19114	6.25	−	N.D.
L.m. ATCC 19115		−	N.D.
P.a.		3.9 ± 1.1 b	−
L.m. ATCC 19114 + P.a.		3.2 ± 0.6 b	N.D.
L.m. ATCC 19115 + P.a.		2.5 ± 0.4 b	N.D.
L.m. ATCC 19114	12.5	−	N.D.
L.m. ATCC 19115		−	N.D.
P.a.		3.0 ± 0.4 b	−

Different lowercase letters within a column indicate significantly different mean values ($P < 0.05$)
N.D. : below detection limit (< 2 log_{10} CFU/well)
Abbreviations: L.m., *Listeria monocytogenes*. P.a., *Pseudomonas aeruginosa* ATCC 7700.

case, viable counts were 5.9 to 6.1 log_{10} CFU in L+P-BF (Table 9), which was lower than viable counts obtained in control cultures at day 5 (Table 4). *L. monocytogenes* viable counts in L+P-BFs were below the detection limit. In addition, *L. monocytogenes* was not detected even after culturing the immersion solution of the swab scraped off in the well with nutrient broth, which indicated that *L. monocytogenes* was completely eliminated, and only *P. aeruginosa* survived in the presence of 0.2% EDTA and 6.25 μg/ml nisin. It should be noted that *P. aeruginosa* survival counts in L+P-BF were significantly larger than those in P-BF after the treatment with 0.2% EDTA and 6.25 μg/ml nisin ($P < 0.05$). This result suggested that *P. aeruginosa* may have higher resistance toward antibacterial agents in co-culture with *L. monocytogenes*.

Following 5-day incubation with 0.2% EDTA and 12.5 μg/ml nisin, the absorbance values of L+P-BF and P-BF decreased until there were no significant differences from the values in the control cultures ($P > 0.05$). Nonetheless, viable counts of approximately 3 log_{10} CFU/well were detected from the well wall surface after five days in culture. As in the case of the combination of 0.2% EDTA and 6.25 μg/ml nisin, *L. monocytogenes* could not be detected after incubation with 0.2% EDTA and 12.5 μg/ml nisin because only *P. aeruginosa* survived.

DISCUSSION

The capacity of various microorganisms to withstand treatment with antibacterial substances is greatly increased if they can form BFs. Although instances of food poisoning by microorganisms have been kept low by widespread use of cold chain logistics, some bacteria are known to be able to form BFs even at low temperatures. In the present study, we assessed the properties of BFs formed by mono- and co-cultures of *L. monocytogenes* and *P. aeruginosa* grown at low temperatures and examined their sensitivity to several antibacterial substances. We found that the L+P-BF formed at 10℃ and 15℃ showed very high resistance to antibiotics and NaClO. However, a combined treatment with nisin and EDTA suppressed the formation of L+P-BF.

Characteristics of biofilms formed by co-cultures of *L. monocytogenes* with *P. aeruginosa* at low temperatures

The fraction of *L. monocytogenes* viable counts in L+P-BF total viable counts was within 1-10% at 10℃ and 15℃, indicating the predominance of *P. aeruginosa* in L+P-BFs. The finding of a lower proportion of *L. monocytogenes* in such co-cultures was consistent with the results of previous studies of co-cultures of *L. monocytogenes* with *Pseudomonas* spp. (Fatemi and Frank, 1999; Lourenço et al., 2011). In most cases,

Pseudomonas spp. tends to be the predominant species in co-culture BFs, for example, in a co-culture with *E. coli* on silicone surfaces (Cerqueira et al., 2013) and with *E. coli* O157:H7 on glass slides (Uhlich et al., 2010). As shown in Fig.1, up to 2 days the growth rate of *L. monocytogenes* ATCC 19115 was the fastest, followed by that of *P. aeruginosa* and *L. monocytogenes* ATCC 19114. Therefore, the dominance of *P. aeruginosa* in the co-cultured BF originated not from its growth rate but because *P. aeruginosa* could easily to attach to surfaces and form BF.

Reiu et al. (2008) revealed a close relationship between *L. monocytogenes* and *Staphylococcus aureus* in dual species BFs by scanning electron microscopy. The spatial organization of *L. monocytogenes* cells within monoculture BFs was flat and homogeneous. In contrast, BFs formed by *S. aureus* in pure culture were heterogeneous. Stainless steel surfaces were partly colonized, and cells were aggregated in distinct clusters. In dual species BFs, spatial organization features of *L. monocytogenes* with *S. aureus* cells were different from those in monocultures. For example, larger *L. monocytogenes* rods were observed (10-20 μm) that were mainly found within microcolonies of *S. aureus*. Phase-contrast microscopy observations in the present study did not allow clear conclusions about the extent of the association between *L. monocytogenes* and *P. aeruginosa*. However, no macroscopic differences were observed between L+P-BFs and P-BFs.

It has been reported that the number of *L. monocytogenes* cells in BFs could be increased in the presence of the *S. aureus* cell-free supernatant. That effect persisted after ultrafiltration (< 3 kDa), and was heat stable, but became lost after proteinase K treatment (Rieu et al., 2008). In addition, *L. monocytogenes* attached in significantly greater numbers (>3-log difference) to surfaces with preexisting *P. putida* BFs than to *Pseudomonas*-free surfaces (Hassan et al., 2004). Autoinducers produced by *L. monocytogenes* appear to contribute to the BF-forming capacity. It was demonstrated that S-ribosyl homocysteine, a precursor of autoinducer-2, stimulated dense BF formation by *L. monocytogenes* (Belval et al. 2006). Similarly, the accessory gene regulator quorum sensing system, described for *S. aureus*, also appears to be involved in the modulation of adhesion and early stages of BF formation by *L. monocytogenes* (Rieu et al., 2007). It is not possible at the present stage to explain comprehensively the influence of the counterpart bacteria on *L. monocytogenes* in co-culture. Future studies examining quorum sensing in bacterial co-cultures will be necessary in future.

Resistance of L+P-BF to antibacterial substances

In this study, L+P-BFs formed at 10℃ and 15℃ showed very high resistance to antibiotics and NaClO, which suggested the necessity to repress BF formation, especially in food. Norwood and Gilmour (2000) investigated the resistance of multispecies BF comprising *L. monocytogenes*, *P. fragi*, and *S. xylosus*. In that study, BFs were exposed to NaClO concentrations of 200, 500, and 1000 mg/l for 20 min, but a substantial two-log cycle drop in bacterial numbers was only achieved at 1000 mg/l. In contrast, in planktonic culture, all three microorganisms were completely eliminated when exposed to 10 mg/L NaClO for a 30-s period.

Lourenço et al. (2011) examined the resistance of L+P-BFs formed at 37℃ and 12℃ to four commercial dairy sanitizers (one alkyl amine acetate based, two chlorine based, and one phosphoric acid based). Their study revealed that L+P-BFs were generally less susceptible than pure *L. monocytogenes* cultures. Notably, BFs formed at 12℃ were usually less susceptible to 20-min exposures to sanitizers than BFs formed at 37℃. In that study, minimum BF-eradicating concentrations of those four commercial dairy sanitizers ranged from 200 to 32,000 mg/l, and L+P-BF formed at a low temperature also showed very high resistance to sanitizers. Thus, the results of the report by Lourenço et al. (2011) and data from the present study reinforced the notion that BFs formed by co-cultures of *L. monocytogenes* and *Pseudomonas* spp. exhibit extreme resistance to sanitizers. Therefore, the emergence of L+B-BFs should be prevented, especially in foods.

Effect of natural food additives on the formation of L+P-BF at low temperatures

The use of proteolytic enzymes specific for the molecules involved in the adhesion and BF formation as anti-BF compounds has been reported previously (Sato et al., 1983). Narisawa et al. (2014) found that the proteolytic enzyme nattokinase, which is present in the traditional fermented food "Natto" and belongs to the alkaline serine protease family, inhibited sucrose-dependent formation of BFs by cariogenic streptococci. The presence of nattokinase is known to reduce the amount of water-insoluble glucan that promotes adhesion to the tooth surface and aggregation of bacterial cells within a BF. Furthermore, nattokinase and plasmin were demonstrated to disperse *S. aureus* BFs that exhibited high antimicrobial resistance to rifampicin and vancomycin over time (> 48 h) (Zapotoczna et al., 2015). In the present study, nattokinase at 100 μg/ml inhibited BF formed by all mono- and co-cultures only at 37℃. Purified nattokinase has high thermostability at 30℃ to 50℃, and the optimal temperature for nattokinase activity is 40℃ (Wang, et al., 2009). In our experiments, we

revealed that nattokinase, however, could not inhibit BF formation at low temperatures (10℃ and 15℃).

Nisin is a natural polypeptide bacteriocin produced by some strains of *L. lactis* (Leriche et al., 1999). It has a "generally recognized as safe" designation and is used in over 50 countries as a food preservative and antimicrobial substance. Inhibition of the gram-positive foodborne pathogens *S. aureus*, *L. monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens* by nisin has been demonstrated (Pimentel-Filho et al., 2014). Nostro et al. (2010) reported that nisin incorporated in polyethylene-co-vinyl acetate films reduced formation of BFs by gram-positive bacteria, such as *L. monocytogenes*. Minei at al. (2008) showed that growth of *L. monocytogenes* on stainless steel coupons is reduced in the presence of 1,000 IU/ml nisin. However, the treatment with nisin did not fully eradicate bacteria and after 24 h of incubation, renewed BF formation was detected in *L. monocytogenes* cultures.

In the present study, we also showed that nisin suppressed only L-BF formation, but not the formation of L+P-BF or P-BF. To augment the efficacy of nattokinase and nisin, EDTA, which is also a food additive, was used as a permeabilizing agent. Ayres et al. (1998) revealed that treatment with 0.3% EDTA enhanced the antibacterial activity of chlorhexidine diacetate, triclosan, and benzalkonium chloride against *P. aeruginosa* BFs. Lebeaux et al. (2015) reported that 0.3% EDTA increased the inhibitory effects of gentamicin, amikacin, and vancomycin on BF formation by gram-positive and gram-negative microorganisms, including *P. aeruginosa*.

Treatment of gram-negative bacteria, such as *E. coli*, with EDTA released 50% of the outer membrane polysaccharides (Wooley and Jones 1983). Nisin disrupts the structure of the cytoplasmic membrane of susceptible species by binding to anionic phospholipids, especially lipid II, which is an essential element of the cell wall. Subsequently, nisin permeates and incorporates into the membrane, forming an ion channel. This causes the efflux of intracellular components and, consequently, cell damage and death (Müller-Auffermann et al., 2015). Gram-negative bacteria are resistant to nisin because their cell envelopes, especially the outer membrane, are far less permeable than those of gram-positive bacteria. EDTA and other chelating agents remove Mg^{2+} and Ca^{2+} ions from the cell wall of gram-negative bacteria, causing the release of phospholipids and, as a result, increase cell wall permeability (Suganthi et al., 2012). These properties of nisin and EDTA likely accounted for the efficient suppression of L+P-BFs and P-BFs documented in this study.

The maximum doses of nisin and EDTA according to the Food Sanitation Act of Japan are 12.5 mg/kg for nisin and 0.025% for EDTA, respectively. In this study, nisin was used within the recommended concentration range, whereas EDTA was employed at concentrations that exceeded the recommended level. Today, nisin is widely used in a variety of food products around the world. In many countries, there are no concentration limits for its application, whereas some countries specify nisin levels for specific product categories. For example, the maximum acceptable level of nisin in cheese in EU is also 12.5 mg/kg (Müller-Auffermann et al., 2015).

In conclusion, we obtained evidence that BFs formed by mono- and co-cultures of *L. monocytogenes* and *P. aeruginosa* can be controlled by the combined application of nisin and EDTA. However, further studies of the combination variations will be needed in order to find the formulation that would be compatible with the acceptable levels of food additives currently stipulated by food safety regulations.

REFERENCES

Ayres, H. M., Payne, D. N., Furr, J. R., and Russell, A. D. (1998) Effect of permeabilizing agents on antibacterial activity against a simple *Pseudomonas aeruginosa* biofilm. *Lett. Appl. Microbiol.*, **27**, 79-82.

Belval, S. C., Gal, L., Margiewes, S., Garmyn, D., Piveteau, P., and Guzzo, J. (2006) Assessment of the roles of luxS, S-ribosyl homocysteine, and auto- inducer 2 in cell attachment during biofilm formation by *Listeria monocytogenes* EDG-e. *Appl. Environ. Microbiol.*, **72**, 2644-2650.

Buchanan, R. L., Gorris, L. G., Hayman, M. M., Jackson, T. C., and Whiting, R. C. (2017) A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Cont.*, **75**, 1-13.

Cerqueira, L., Oliveira, J. A., Nicolau, A., Azevedo, N. F., and Vieira, M. J. (2013) Biofilm formation with mixed cultures of *Pseudomonas aeruginosa*/*Escherichia coli* on silicone using artificial urine to mimic urinary catheters. *Biofouling*, **29**, 829-840.

Costerton, J. W. (1999) Introduction to biofilm. *Int. J. Antimicrob. Agents*, **11**, 217-221.

Daneshvar Alavi, H. E., and Truelstrup Hansen, L. (2013) Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surfaces. *Biofouling*, **29**, 1253-1268.

de Carvalho, R. J., de Souza, G. T., Honório, V. G., de Sousa, J. P., da Conceição, M. L., Maganani, M., and de Souza, E. L. (2015) Comparative inhibitory effects of *Thymus vulgaris* L. essential oil against *Staphylococcus aureus*, *Listeria monocytogenes* and mesophilic starter co-culture in cheese-mimicking models. *Food Microbiol.*, **52**, 59-65.

Di Ciccio, P., Vergara, A., Festino, A. R., Paludi, D., Zanardi, E., Ghidini, S., and Ianieri, A. (2015) Biofilm formation by *Staphylococcus aureus* on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. *Food Cont.*, **50**, 930-936.

Fatemi, P., and Frank, J. F. (1999). Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracid sanitizers. *J. Food Prot.*, **62**, 761-765.

Giaouris, E., Chorianopoulos, N., and Nychas, G. J. (2005)

Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* Enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *J. Food Prot*., **68**, 2149-2154.

Giaouris, E., Chorianopoulos, N., Doulgeraki, A., and Nychas, G. J. (2013) Co-culture with *Listeria monocytogenes* within a dual-species biofilm community strongly increases resistance of *Pseudomonas putida* to benzalkonium chloride. *PLoS One*, **8**, e77276.

Hassan, A. N., Birt, D. M., and Frank, J. F. (2004) Behavior of *Listeria monocytogenes* in a *Pseudomonas putida* biofilm on a condensate-forming surface. *J. Food Prot*., **67**, 322-327.

Holah, J. T., Taylor, J. H., Dawson, D. J., and Hall, K. E. (2002) Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *J. Applied Microbiol., Symp. Suppl*., **92**, 111S-120S.

Ibusquiza, P. S., Herrera, J. J., Vázquez-Sánchez, D., and Cabo, M. L. (2012) Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*. *Food Cont*., **25**, 202-210.

Joseph, B., Otta, S. K., and Karunasagar, I. (2001) Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int. J. Food Microbiol*., **64**, 367-372.

Kostaki, M., Chorianopoulos, N., Braxou, E., Nychas, G. J., and Giaouris, E. (2012) Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. *Appl. Environ. Microbiol*., **78**, 2586-2595.

LaBauve, A. E., and Wargo, M. J. (2015) Growth and laboratory maintenance of *Pseudomonas aeruginosa*. In: *Current Protocols in Microbiology*. (Cohen, L. E et al. ed.) Unit.-6E.1., Online ISBN: 9780471729259, Wiley Online Library.

Lebeaux, D., Leflon-Guibout, V., Ghigo, J. M., and Beloin, C. (2015) *In vitro* activity of gentamicin, vancomycin or amikacin combined with EDTA or l-arginine as lock therapy against a wide spectrum of biofilm-forming clinical strains isolated from catheter-related infections. *J. Antimicrob. Chemother*., **70**, 1704-1712.

Leive, L. (1965) Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochem. Biophys. Res. Commun*., **21**, 290-296.

Leriche, V., D. Chassaing, and B. Carpentier. (1999) Behaviour of *L. monocytogenes* in an artificially made biofilm of a nisin-producing strain of *Lactococcus lactis*. *Int. J. Food Microbiol*., **51**, 169-182.

Lourenço, A., Machado, H., and Brito, L. (2011) Biofilms of *Listeria monocytogenes* produced at 12℃ either in pure culture or in co-culture with *Pseudomonas aeruginosa* showed reduced susceptibility to sanitizers. *J. Food Sci*., **76**, M143-M148.

Minei, C. C., Gomes, B. C., Ratti, R. P., D'angelis, C. E., and De Martinis, E. C. (2008) Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *J. Food Prot*., **71**, 634-638.

Mitjà, O., Pigrau, C., Ruiz, I., Vidal, X., Almirante, B., Planes, A. M., Molina, I., Rodríguez, D., and Pahissa, A. (2009) Predictors of mortality and impact of aminoglycosides on outcome in listeriosis in a retrospective cohort study. *J. Antimicrob. Chemother*., **64**, 416-423.

Müller-Auffermann, K., Grijalva, F., Jacob, F., and Hutzler, M. (2015) Nisin and its usage in breweries: a review and discussion. *J. Inst. Brew*., **121**, 309-319.

Narisawa, N., Kawasaki, Y., Nakashima, K., Abe, S., Torii, Y., and Takenaga, F. (2014) Interference effects of proteolytic nattokinase on biofilm formation of cariogenic streptococci. *Food Preserv. Sci*., **40**, 273-278.

Norwood, D. E., and Gilmour, A. (2000). The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *J. Appl. Microbiol*., **88**, 512-520.

Norwood, D. E., and Gilmour, A. (2001) The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Lett. Appl. Microbiol*., **33**, 320-324.

Nostro, A., Scaffaro, R., Ginestra, G., D' Arrigo, M., Botta, L., Marino, A., and Bisignano, G. (2010) Control of biofilm formation by poly-ethylene-co-vinyl acetate films incorporating nisin. *Appl. Microbiol. Biotechnol*., **87**, 729-737.

Pimentel-Filho, N. J, Martins, M. C., Nogueira, G. B., Mantovani, H. C., and Vanetti, M. C. D. (2014) Bovicin HC5 and nisin reduce *Staphylococcus aureus* adhesion to polystyrene and change the hydrophobicity profile and Gibbs free energy of adhesion. *Int. J. Food Microbiol*., **190**, 1-8.

Poole, K. (2002) Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. *Curr. Pharm. Biotechnol*., **3**, 77-98.

Puga, C. H., Orgaz, B., and SanJose, C. (2016). *Listeria monocytogenes* impact on mature or old pseudomonas fluorescens biofilms during growth at 4 and 20℃. *Front. Microbiol*., **7**, 134.

Puga, C. H., Orgaz, B., Muñoz, S., and SanJose, C. (2015) Cold stress and presence of *Pseudomonas fluorescens* Affect *Listeria monocytogenes* biofilm structure and response to chitosan. *J. Mol. Genet. Med*., **9**, 4.

Rieu, A., Weidmann, S., Garmyn, D., Piveteau, P., and Guzzo, J. (2007) Agr system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. *Appl. Environ. Microbiol*., **73**, 6125-6133.

Rieu, A., Lemaître, J. P., Guzzo, J., and Piveteau, P. (2008) Interactions in dual species biofilms between *Listeria monocytogenes* EGD-e and several strains of *Staphylococcus aureus*. *Int. J. Food Microbiol*., **126**, 76-82.

Sato, S., Koga, T., and Inoue, M. (1983) Degradation of the microbial and salivary components participating in human dental plaque formation by proteases elaborated by plaque bacteria. *Arch. Oral Biol*. **28**, 211-216.

Suganthi, V., Selvarajan, E., Subathradevi, C., and Mohanasrinivasan, V. (2012) Lantibiotic nisin: Natural preservative from *Lactococcus lactis*. *Int. J. Pharm. Pharm. Sci*., **3**, 13-19.

Uhlich, G. A., Rogers, D. P., and Mosier, D. A. (2010) *Escherichia coli* serotype O157: H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathogens and Disease*, **7**, 935-943.

Wang, C., Du, M., Zheng, D., Kong, F., Zu, G., and Feng, Y. (2009) Purification and characterization of nattokinase from *Bacillus subtilis* Natto B-12. *J. Agri. Food Chem*., **57**, 9722-9729.

Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo., H. T., Ma, X. J., and Luo, Y. (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn*., **14**, 22-29.

Wooley, R. E., and Jones, M. S., (1983) Action of EDTA-Tris and antimicrobial agent combinations on selected patho-

genic bacteria. *Vet. Microbiol.*, **8**, 271-280.
Zapotoczna, M., McCarthy, H., Rudkin, J. K., O' Gara, J. P., and O'Neill, E. (2015) An essential role for coagulase in *Staphylococcus aureus* biofilm development reveals new therapeutic possibilities for device-related infections. *J. Infect. Dis.*, **212**, 1883-1893.