Acta Univ. Sapientiae, Alimentaria, **5** (2012) 5–17

Formation and removal of *Listeria monocytogenes* and *Lactococcus lactis* biofilms

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Abstract. Since the presence of biofilms causes significant problems in food industry and human pathogenic bacteria can take part in biofilm formation, within the frame of this study, we wanted to determine the biofilm-forming ability of *Listeria monocytogenes* in monoculture and mixed culture under industrial circumstances. As lactic acid bacteria used in different technological processes can inhibit the growth of pathogens, the effect of *Lactococcus lactis* on the biofilm formation of *L. monocytogenes* was tested. The multiplication ability of two *L. monocytogenes* was investigated in BH broth and UHT milk. As differences in growth of the tested strains were found at 10 °C, it is supposed that their biofilm-forming capacity differs at low temperature as well. During biofilm formation in monoculture, *L. monocytogenes* cells attached to the surface of metal coupon in higher number than those of *Lactococcus lactis*, but in the case of mixed culture lactic acid bacteria inhibited the biofilm formation of *L. monocytogenes*. The destructive effect of Florasept (a chlorinated disinfectant) was higher in biofilms of *L. monocytogenes*, since resistance to the disinfectant was significant in *Lactococcus*

Key words and phrases: biofilm, cleaning, *Listeria monocytogenes*, *Lactococcus lactis*.

lactis biofilms. Based on our results, it can be concluded that – though *L. monocytogenes* can attach to metal surfaces under industrial circumstances – its cell number is low in biofilms. Nevertheless, lactic acid bacteria can reduce or inhibit the formation of biofilms of pathogenic bacteria.

1 Introduction

Bacteria in aqueous environment are rarely planktonic; instead they tend to colonize solid surfaces, forming biofilms. A biofilm is a complex and heterogeneous structure of cells that is surrounded by an extracellular matrix of exopolysaccharide (EPS) (called glycocalyx) secreted by those cells. Both spoilage organisms and foodborne pathogens, e.g., *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are able to form biofilms on abiotic surfaces and have enhanced tolerance to different antimicrobial agents, e.g., disinfectants when their cells form biofilms (*Costerton et al.*, 1987, *Stewart et al.*, 2000).

L. monocytogenes is a foodborne pathogen of particular concern in the food industry, ubiquitous in the environment, on plant materials and in the soil. As a consequence, the occurrence of *Listeria monocytogenes* biofilms can cause post-processing contamination and a considerable risk to food safety.

Several studies have demonstrated that *L. monocytogene*s can be present in food-processing environments (*Chmielewski & Frank*, 2003; *Martin & Fisher*, 1999; *Tompkin*, 2002) and it is able to persist in processing factories, sometimes for many years with consequent contamination of food products (*Senczek et al.*, 2000; *Bagge-Ravn et al.*, 2003). This leads to lowered shelf-life of products and transmission of diseases (*Carpentier & Cerf*, 1993; *Mittelman*, 1998). The vast majority of organisms in the natural environment and in the food-processing environment occur in multispecies biofilms (*Costerton et al.*, 1987). *Listeria monocytogenes* will also most likely grow with other microorganisms in mixed species biofilms in food-processing environments (*Carpentier & Chassaing*, 2004; *Habimana et al.*, 2009). The presence and the diversity of microbial groups can facilitate or limit the persistence of *L. monocytogenes* on surfaces (*Carpentier* and *Chassaing*, 2004; *Zhao et al.*, 2004). When growing in co-culture biofilm with strains of the genus *Pseudomonas*, *Staphylococcus* or *Flavobacterium*, the population of *L. monocytogenes* could be increased or decreased depending on the strain (*Carpentier* and *Chassaing*, 2004; *Norwood & Gilmour*, 2000, 2001).

Several methods have been evaluated to kill and remove biofilm organisms from food-processing facilities. These methods include various physical cleaning methods such as high pressure cleaning (*Meyer*, 2003) or ultrasound (*Mott et al.*, 1998). Chemical methods are widely used for the inactivation of biofilm organisms. These include alkali/acid wash (*Parkar et al.*, 2004), chlorine (*Meyer*, 2003), peracid sanitizer (*Fatemi & Frank*, 1999) and acidic electrolysed water (*Ayebah et al.*, 2006). However, these methods are limited to small areas and have not yet found their way to practical application. The antimicrobial activity of nisin on planktonic cells of *Listeria monocytogenes* has been well-documented (*Budde & Jacobsen*, 2000). However, studies addressing the effect of nisin-producing strains on *L. monocytogenes* biofilms are scarce (*Leriche et al.*, 1999).

In our study, we investigated the formation of mixed species biofilms of *L. monocytogenes* in combination with *Lactococcus lactis* and their resistance to disinfection treatment.

2 Materials and methods

Cultures

Two strains of *Listeria monocytogenes* (H10 and H24) isolated from meat industrial plant were used in the experiments together with a nisin-producing culture collection strain of *Lactococcus lactis* (ATCC 11454). All bacterial strains were stored at refrigerated temperatures (4 °C). *L. monocytogenes* strains were maintained on BH agar slants (brain extract, heart extract and peptons 17.5 g; glucose 2.0 g; yeast extract 2.5 g; sodium chloride 5.0 g; di-sodium hydrogen phosphate 2.5 g; agar agar 15.0 g; distilled water 1.0 l) (Merck 1.10493)), while *L. lactis* was stored on MRS agar slants.

Overnight cultures of *Listeria strains* (incubated at 37 °C) were used in the experiments. *L. lactis* was cultivated in BH broth and incubated at 30 °C for 24 hours. A stock suspension of 10^9 cells ml^{-1} was prepared from each strain and used after appropriate dilutions.

Growth measurements

The growth of *Listeria monocytogenes* strains and *Lactococcus lactis* was examined individually and in mixed cultures at 10 °C to simulate the cold manufacturing temperatures of a milk (fat content 2.8%) factory. Growth curves were recorded in BH broth and in milk. Ten ml of the growth medium was

inoculated with the bacteria at the level of 10^6 cells ml^{-1}. When biofilm formation was followed in mixed cultures, the initial proportion of *L. monocytogenes* and *L. lactis* was 1:1000. Samples were taken at 0, 24, 120 and 144 hours. Cell numbers were determined by pour-plating with TGE agar (5.0 g Tripton, 1.0 g Glucose, 2.5 g yeast extract, 1.0 l distilled water) in the case of *Listeria* strains, while in the case of *Lactococcus* strain MRS agar was used in double layer.

Biofilm formation

Biofilm formation was carried out in BH broth. Petri dishes containing 20 ml BH broth were inoculated with *Listeria monocytogenes* strains and *Lactococcus lactis* individually or in mixed cultures with an initial number of 10^7 cells ml^{-1}. Two sizes of stainless steel coupons were used in the experiments for the detection of viable cell counts (30×9 mm and 75×9 mm). Metal coupons were immersed and left into the inoculated growth medium for an hour to attach to the test microorganisms. Then coupons were removed from the Petri dishes and rinsed with sterile distilled water to remove the unattached cells. Coupons were then placed into other Petri dishes containing sterile uninoculated BH broth. Attached cells served as "inocula" for biofilm initiation. The number of biofilm-producing cells was determined by pour-plating and fluorescent microscopy after 0, 24 and 144 hours.

Detection of viable cell counts in biofilms

At sampling times, metal coupons were removed from the Petri dishes containing BH broth, and rinsed with sterile water to remove unattached cells. Small coupons were placed into test tubes containing sterile glass beads (diameter $0.4 - 0.6$ mm) and 10 ml sterile diluents (1 g pepton, 8.5 g NaCl, 1 l distilled water). Tubes were vortexed for 2 minutes to remove attached cells from the surface of the coupons. Appropriate dilutions of these suspensions were pour-plated and incubated at 30 °C for $24 - 48$ hours. All measurements were carried out in duplicates.

Microscopic investigations

Microscopic slide size coupons were investigated with epifluorescent microscope (Olympus BH-2, Olympus Optical Gmbh, Germany) after acridine orange (AO, 0.02 g/100 ml water, Merck 14281) staining for 2 minutes.

Biofilm removal

After sampling, coupons were rinsed with sterile water to remove unattached cells and placed into disinfectant solution (8 ml Florasept in 250 ml distilled water). Coupons, after rinsing with sterile distilled water, were placed into Petri dishes containing 15 ml Florasept suspension. They were left there for 15 minutes' contact time and then rinsed with sterile neutralizer (3 g lecitin, 30 ml Tween 80.5 g Na-tiosulphate, 1 g α-hisztidin, 10 ml phosphate buffer (34 g KH_2PO_4 in 500 ml destilled water), 1000 ml destilled water)) to stop the effect of the disinfectant. Control coupons without disinfection and disinfected coupons were examined under fluorescent microscope (see Section 2.5) and the number of surviving cells was determined as described in Section 2.4 in the case of individual cells. When mixed cultures were examined, samples were also spread-plated on *Listeria*-selective Palcam agar (MERCK, 1.11755.0500).

Fluorescent *In situ* hybridization (FISH)

Fluorescent in situ hybridization technique was used to separate *Listeria* cells from *L. lactis* in biofilms of mixed cultures under fluorescent microscope. The VIT-Listeria kit (Vermicon, Munich) was used for the analysis by the instructions of the manufacturer.

3 Results and discussion

Comparison of the multiplication ability of *Listeria monocytogenes* and *Lactococcus lactis* strains in BH broth and UHT milk

To be able to determine the biofilm-forming ability of *L. monocytogenes* strains in BH broth and UHT milk, their growing capacities were tested in different culture media. Changes of the colony-forming units of *L. monocytogenes* H10 and H24, depending on the sampling time, are summarized in Table 1.

L. monocytogenes H10 could propagate well in both media. Its initial cell number grew two orders of magnitude during the 144 hours (6-day-long) incubation time and, as it can be seen from Table 1, it could multiply better in UHT milk since the cell concentration was ten times higher in milk than in BH broth after 24 hours of incubation. In contrast with these observations, *L. monocytogenes* H24 could not multiply efficiently either in milk or in BH broth as its cell number remained 10^6 ml^{-1} during the incubation time.

Table 1: Changes of colony-forming units of *L. monocytogenes* H10 and H24 depending on the sampling time

Sampling time (hours)	*L. monocytogenes* H10 (log_{10} CFU ml^{-1})		*L. monocytogenes* H24 (log_{10} CFU ml^{-1})		*Lactococcus lactis* ATCC 11454 (log_{10} CFU ml^{-1})	
	BH broth	UHT milk	BH broth	UHT milk	BH broth	UHT milk
0	6.2	6.2	6.1	6.1	6.6	6.4
24	6.3	7.2	6.1	6.3	6.4	6.5
120	7.7	7.6	6.5	6.7	8.2	8.2
144	8.4	8.3	6.7	6.9	8.6	8.7

Although both *L. monocytogenes* strains originate from meat samples and were isolated from similar conditions (data are not shown), differences in their growing capacity could be observed. Since *L. monocytogenes* H10 showed better multiplication ability at 10 °C, it was chosen for further investigations.

Lactococcus lactis ATCC 11454 could grow well in both BH broth and UHT milk. The initial cell concentration (10^6 CFU ml^{-1}) reached 10^8 CFU ml^{-1} during 120 hours of incubation and did not change significantly until the 6^{th} day of the experiment.

Culture media used for the propagation of the tested bacteria did not have significant effect on the multiplication ability of the strains; thus, BH broth was used as culture medium in studying biofilm formation.

Biofilm formation of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 strains in monoculture

Determination of biofilm-forming ability for *Listeria monocytogenes* H10 and Lactococcus lactis ATCC 11454 was performed in separate experiments from monocultures. As it can be seen in Figure 1, until the first sampling time (after 1 hour of incubation), more *L. monocytogenes* cells were adhered to the surfaces of the metal coupons than that of *Lactococcus lactis*. Furthermore, in the course of the analysis, higher numbers of *L. monocytogenes* H10 were characteristic of biofilm formation (104-105 cells ml^{-1}), while it was only 101-103 cells ml^{-1} in the case of *Lactococcus lactis* ATCC11454. This observation can correlate with the elevated adherence capacity of *L. monocytogenes*, the significant resistance to different stress factors and the ability to propagate even at lower temperature.

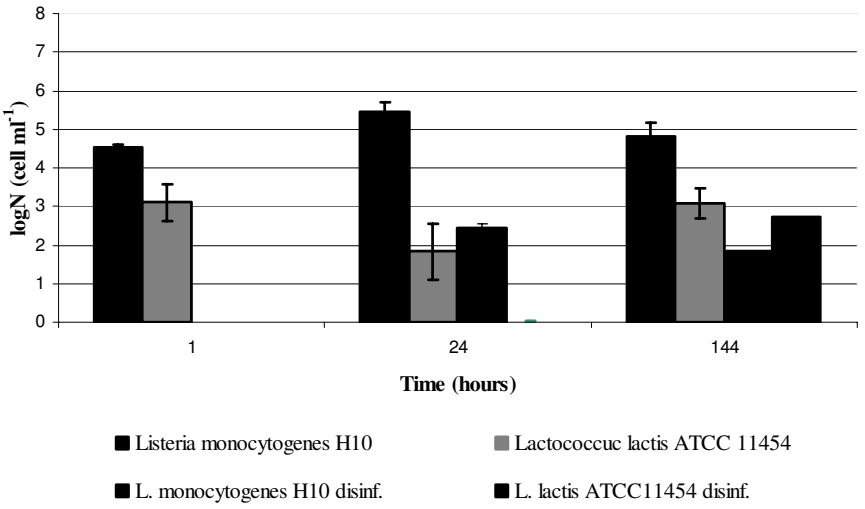

Figure 1: The concentrations of adhered *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 cells took part in the biofilm formation separately. Changes in cell numbers are indicated in the case of control and disinfected samples

Disinfection proved to be adequate in the case of biofilms of the tested bacteria since significant changes could be observed in the cell numbers of treated samples after 24 hours of incubation. Cell concentration decreased three orders of magnitude in the case of *Listeria monocytogenes*, while the presence of Lactococcus lactis living cells was not detected. Since efficiency of chlorinated disinfectants against L. monocytogenes biofilm had been previously observed (Alasri et al., 1992; Green, 1993), the effectiveness of Florasept was confirmed. After six days, a similar decline could be seen in *L. monocytogenes* cell concentration; however, biofilm formed by Lactococcus lactis could not be removed as intensively as before (Figure 1). Presumably, the resistance of the cells that constituted the biofilm increased significantly, which feature is well-known in the process of biofilm formation.

Results of modelling for biofilm formation by fluorescent microscopy

Biofilm formation of the cells was followed by fluorescent microscopy, parallel with viable cell counting. After staining the metal coupons with acridine orange, the results were evaluated by microscopic observation of the cells at-

taching together. As it can be seen in Figure 2 (A/1-A/3), the number of the attached cells in the case of the control sample increased continuously during incubation time. Nevertheless, a relatively low number of *L. monocytogenes* cells took part in biofilm formation. This observation confirms the tendency obtained by the culturing of viable cell counts in 3.2.

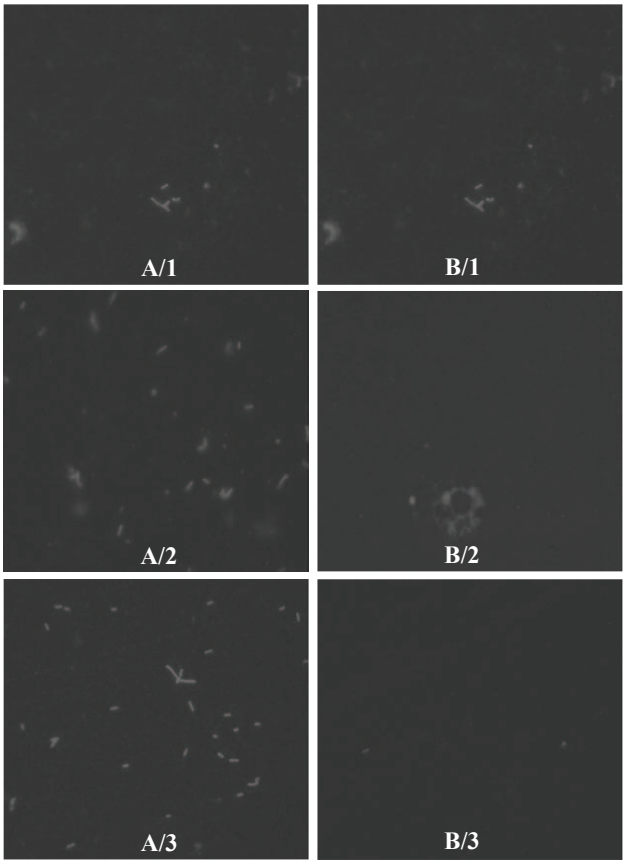

Figure 2: Results of fluorescent microscopic investigation for control and disinfected samples of *Listeria monocytogenes* biofilm at different sampling times.

A/1: control sample after 1 hour, A/2: control sample after 24 hours, A/3: control sample after 144 hours; B/1: disinfected sample after 1 hour, B/2: disinfected sample after 24 hours, B/3: disinfected sample after 144 hours

Based on pictures of metal coupons treated with Florasept (Figure 2. B/1-B/3), it can be observed that the applied disinfectant was effective in the removal of *L. monocytogenes* biofilm. On the surfaces of the sanitized coupons, the number of biofilm-forming cells decreased significantly at both sampling times.

Development of biofilm from the mixed culture of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 strains

After investigating the biofilm-forming capacity of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 in monocultures, the development of the biofilm from mixed culture was analysed. The inoculation ratio of the two bacteria (*L. monocytogenes* and *Lactococcus lactis*) was 1:1000.

As it is shown in Figure 3, the cell concentration of *Listeria monocytogenes* was relatively low during the experiment ($10^1 - 10^2$ CFU ml^{-1}), but the total cell number was fairly high (10^7 CFU ml^{-1} after 1 hour of incubation), which indicates the dominance of *Lactococcus lactis* cells in biofilms.

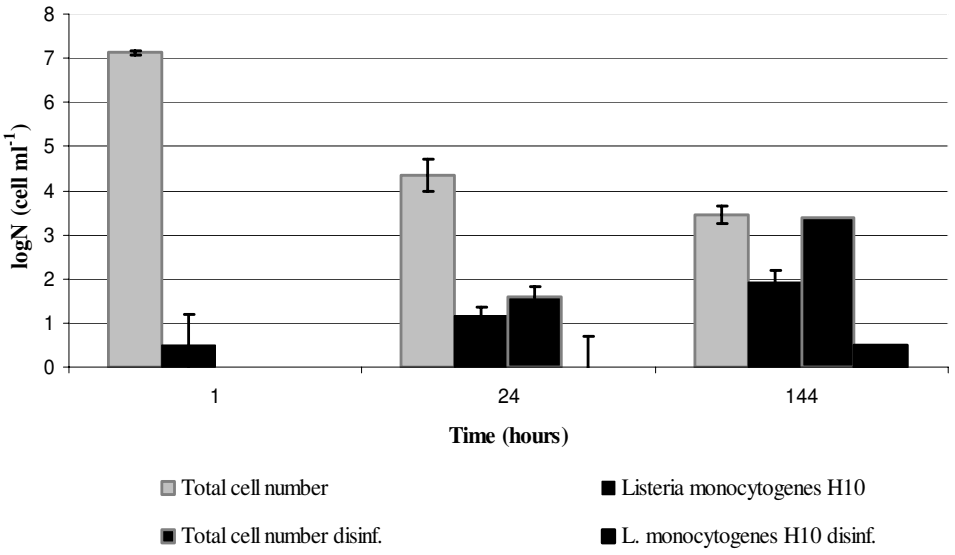

Figure 3: Changes of cell numbers for mixed cultures of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC11454 in control and disinfected samples

The concentration of *Listeria monocytogenes* increased through the incubation; however, the total bacterium count decreased (from 10^7 to 10^3 CFU ml^{-1}), which highlights the reduction of *Lactococcus lactis* cells in the biofilm and the higher resistance of *L. monocytogenes* to lower temperature.

Treatment with disinfectant proved to be efficient in the case of *Listeria monocytogenes*, but lactic acid bacteria were resistant to sanitation, since after six days of incubation their number was approximately 10^3 cells ml^{-1}.

Results of fluorescent in situ hybridization

Biofilm formation of mixed culture for *L. monocytogenes* and *Lactococcus lactis* was to be detected by FISH method that can differentiate the cells of *Listeria monocytogenes* from non-*monocytogenes Listeria* and other bacteria during in situ analysis.

As it was observed in 3.4, only a low number of *L. monocytogenes* cells ($10^1 - 10^2$ CFU ml^{-1}) took part in biofilm formation when mixed cultures were investigated. Since the detection level of FISH is 4×10^5 CFU ml^{-1} (*Hogardt et al.*, 2000), *L. monocytogenes* cells were not detected on metal coupons; however, they were present in form of biofilm.

4 Conclusions

In the last 20 years, biofilms have had a particular concern with food industry as bacteria can colonize different surfaces of food-processing plants, thus contributing not only to economic losses but also to the development of food-borne illnesses. Resistance of biofilms to different cleaning and disinfection processes increases; therefore, the removing of biofilms is a big challenge for food industry.

In our study, the biofilm-forming capacity of *Listeria monocytogenes* and *Lactococcus lactis* was investigated in monoculture and mixed culture. *L. monocytogenes* was able to form biofilm at 10 °C on metal surfaces in relatively high number, while *Lactococcus lactis* biofilm formation was partially inhibited. In mixed culture, lactic acid bacteria inhibited the biofilm formation of *L. monocytogenes* when a comparatively high number of *Lactococcus lactis* was used. The effect of a chlorinated disinfectant (Florasept) was higher in biofilms of *L. monocytogenes*, since resistance to the disinfectant was noteworthy in *Lactococcus lactis* biofilms. Lactic acid bacteria applied in technological processes of food industry can help us inhibit the attachment and colonization of harmful microorganisms, amongst them: human pathogenic bacteria.

Removal of biofilms is a complicated task for food industry; thus, cleaning and disinfection of tools and equipments used for food processing are essential. Application of surfaces without scratches is also important in confining hidden bacteria which can take part in biofilm formation.

5 Acknowledgements

The authors are grateful to Vermicon for providing the FISH test.

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