Resistance of *Listeria monocytogenes* Biofilms to Disinfectants

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Abstract

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We studied the optimal conditions for the biofilm development by *Listeria monocytogenes* on a model system represented by microtiter plates, and also for determined some effective disinfectant agents. *Listeria monocytogenes* ATCC 13932 and an industrial isolate of *Listeria monocytogenes* Lm-24 were compared as to their abilities to form biofilms. The starting concentration of the cells leading to the most reproducible results was 0.5 McFarland. The temperatures tested ranged between 8°C to 37°C, the optimal values to form biofilm in buffered peptone water (BPW) with 0.05% glucose were 25°C and 30°C. Under comparable conditions the persistent strain *L. monocytogenes* Lm-24 constituted more massive biofilm than did the reference strain. The following disinfectants were applied: Savo, Merades Alco, benzalalkonium chloride. A persistent industry in isolate *Listeria monocytogenes* Lm-24 was used as the model organism for these tests. Benzalalkonium chloride treatment was found to be the most efficient way to damage the biofilm. One minute treatment with 500 mg/l was lethal for the biofilm cells, and that with 125 mg/l for planctonic cells. Savo suppresed the viability of the biofilm cells only by about 20% on average while being lethal for planctonic cells. Merades Alco exhibited only a weak effect on both the biofilm and planctonic cells.

Keywords: *Listeria monocytogenes*; biofilm; disinfectants

Listeria monocytogenes is a Gram-positive foodborn pathogenic bacterium which is ubiquitous in the outdoor environment. *Listeria monocytogenes* is responsible for listeriosis with an overall mortality rate around 20–30%, and it can persist in food-processing environments over many years, which is an important cause of the food contamination (Borucki *et al.* 2003).

L. monocytogenes attaches to and forms biofilms, that are protective layers of proteins and polysaccharides surrounding the bacteria, and sticks onto numerous surfaces and equipments (Kalmokoff *et al.* 2001). Many bacteria are able to attach to and colonise the environmental surfaces by producing a three-dimensional matrix of extracellular polymeric substances (EPS) called biofilm (Blaschek *et al.* 2007). Biofilms are a collection of microorganisms surrounded by the slime they secrete, attached to either inert or living surfaces (Poulsen 1999). Planctonic cells in contrast to biofilm are freely and individually living in liquids. The biofilm arrangment allows the microorgan-

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isms to persist in the environment and to resist desiccation, UV ligth, and the treatment with antimicrobial and sanitising agents. Biofilms can be difficult to control since they can form where water is plentiful and the cleaning is not performed properly. The common sites for *L. monocytogenes* isolation from dairy industry processing plants are the filling or packaging equipment, floor drains, walls, cooling pipes, conveyors, collators used for assembling the products for packaging, racks for transporting products, hand tools or gloves, and freezers (Tompkin *et al.*1999).

In order to enable reproducible studies of bacterial attachment and colonisation, a variety of direct and indirect experimental procedures have been developed (Lindsay & von Holy 1997; Poulsen 1999). The biofilm formation is a complex process regulated by diverse characteristics of the growth medium, substrate, and cell surface.

The microtiter plate procedure belongs to the indirect methods for the estimation of the amount of bacteria *in situ* and can be modified for various biofilm formation assays. In this study, the procedure using the microtiter plates according to Djordjevic *et al.* (2002) was employed. The objective of the experiments was to find a suitable medium for biofilm formating by a reference strain in comparison with an industrial isolate, and to test the efficiency of the commonly used disinfectants against biofilm.

MATERIALS AND METHODS

Culture preparation. *Listeria monocytogenes* ATCC 13932 and an industrial isolate (cheese factory) of *L. monocytogenes* labelled as Lm-24 were used throughout. The stock cultures were stored at –80°C in brain-heart infusion – BHI (Merck, Darmstadt, Germany) and 15% glycerol. The working cultures were maintained on plate count agar – PCA (Merck, Darmstadt, Germany) slants at 4 °C for 30 days. Prior to each experiment, a loopful of the culture was grown in10 ml of BHI at 37°C for 18 hours.

Media. The biofilm production assays were performed using the following media: buffered peptone water – BPW (Merck, Darmstadt, Germany), BPW with 5.0% NaCl, BPW with 0.5% NaCl, BPW with 0.5% glucose, BPW with 0.05% glucose, BPW with 5.0% NaCl and 0.5% glucose, BPW with 0.5% NaCl and 0.05% glucose, physiological saline – PS, brain heart infusion – BHI, 10 times diluted BHI.

Microtiter plate biofilm production assaay. Microtiter plates COSTAR 3797 (Corning Incorporated, Lowell, USA) were chosen as the standard tools in all experiments. Overnight cultures in BHI were diluted to above mentioned media and equilibrated at 0.5 McFarland value (app. 10^7 CFU/ml), which gave the best results in comparison to the less diluted starting concentrations tested before.

The microtiter plates were washed with 70% ethanol and air dried. To each well of the respective microtiter plate, 100 µl of the individual culture dilution of the tested media was transferred. For one type of medium and dilution, 16 parallels (2 rows with 8 wells) were measured. Before and after 22 h incubation the microtiter plates were measured on Tecan-Spectra 9440012 spectrophotometer (Tecan Austria GmbH, Grödig, Austria) at 620 nm. After 22 h of incubation the medium was removed from the wells and the wells were washed five times

Table 1. List of tested disinfectants

Name	Composition	Recommended concentration	Tested concentration	Time of treatment (min)
Savo S (commercial name)	NaClO (min. 5%) + NaOH (min. 2%)	1:10 in water = 1×	2×, 1×, 0.5×, 0.25×	5, 10, 30
Merades Alko MA (commercial name)	propan-2-ol (20–30%) + ethanol (30–50%)	without dilution = 1×	1×, 0.5×, 0.25×, 0.125×	5, 10, 30
Benzalalkonium Chloride BC (component in disinfectants)	quarternary ammonium salts	tested from 0.5 g/l (Desam Extra, Biochemie, Czech Republic) to 40 g/l (Microbac Forte, Hartmann-Rico, Germany; Hexaquart Forte, B. Braun, Germany)	1000, 500, 250, 125 mg/l	1, 5, 10, 30

with sterile distilled water to remove the loosely associated bacteria. The plates were air dried for 45 min and each well was stained with 150 µl of 1% crystal violet solution in water for 45 minutes. After staining, the plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. After 45 min of destaining, 100 µl from each well was transferred to a new microtiter plate and the level (OD) of crystal violet present in the destaining solution was measured at 620 nm at Tecan-Spectra 9440012. The biofilm formation was tested at following temperatures: 8, 25, 30, and 37°C.

Disinfectants assay. Three different disinfectants were tested: Savo S (mixture of NaClO and NaOH, Penta, Prague, Czech Republic), generally used in the food industry and household, Merades Alco – MA (mixture of ethanol and propanol, Merak, Prague, Czech Republic), plentifully used in dairy industry, and benzalalkonium chloride – BC (quartenary ammonium salt, QUAT group, Fluka Analytical, St. Louis, USA), approved for food industry. The microtiter plates were treated after 22 h of incubation by adding 100 µl of the agent tested directly in the well to simulate in the best way the real conditions. For the treatment times and concentrations used see Table 1. In the case of A – assessment of the disinfectant impact on the biofilm after the giving time, the microtiter plates were washed and treated using the same biofilm staining procedure as mentioned above. The destruction of the biofilm by agent tested was measured in this mode immediately after the treatment. To measure B – viability of the biofilm cells after the disinfectant treatment, the microtiter plates after disinfectants treatment were washed with sterile water and 100 µl of fresh BPW containing 0.05% glucose was put into the wells. The microtiter plates were incubated at 30°C for 22 h and the resulting density and biofilm formation were compared with those obtained without the disinfectant treatment. For the evaluation of C – disinfectant influence on planctonic cells, 10 µl of the treated suspension was transferred into a new well with 90 µl of BPW containing 0.05% glucose and was then incubated at 30°C for 22 hours. The resulting density and biofilm formation were compared with those obtained without the disinfectants treatment.

Statistics. All measured values were statistically evaluated using software Statistica 8 (StatSoft, Inc., Tulsa, USA). The insufficient washing out of unbound crystal violet was assumed to be a possible source of errors, so only the values lying in the 75% lower percentile were taken in account for the determination of the average and standard deviations, while those in 75% upper percentile were omitted as outline values. The interval determined by the average and the double standard deviation consists of 95% of the considered values.

RESULTS AND DISCUSSION

The bacteria involved in the biofilm formation undergo transitions from planctonic and motile forms to an aggregated mode that is essentially

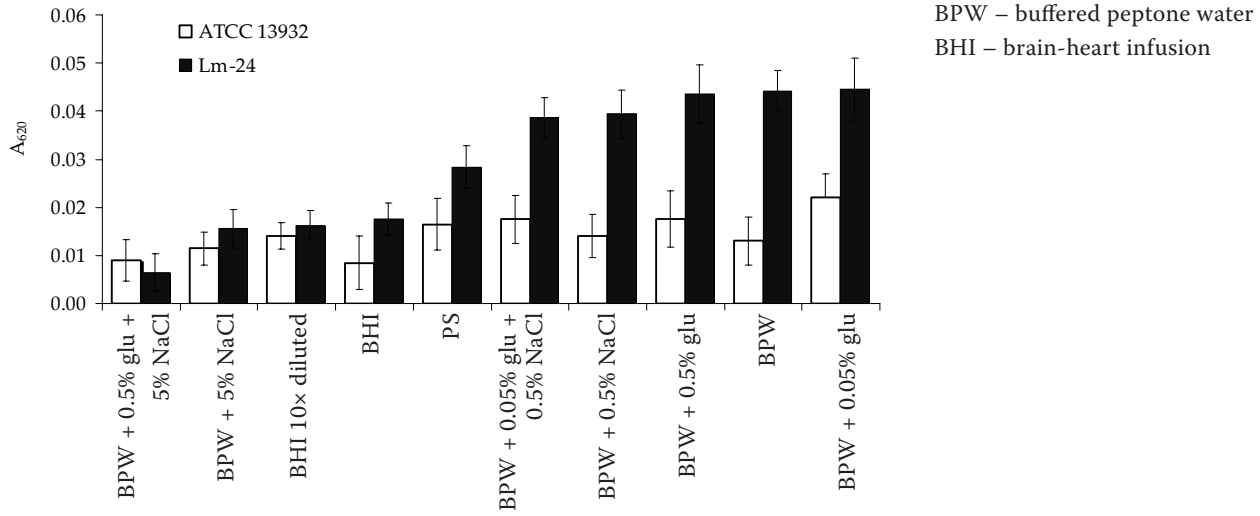

Figure 1. Effect of medium composition on biofilm formation by two strains *L. monocytogenes* in different media at 30°C

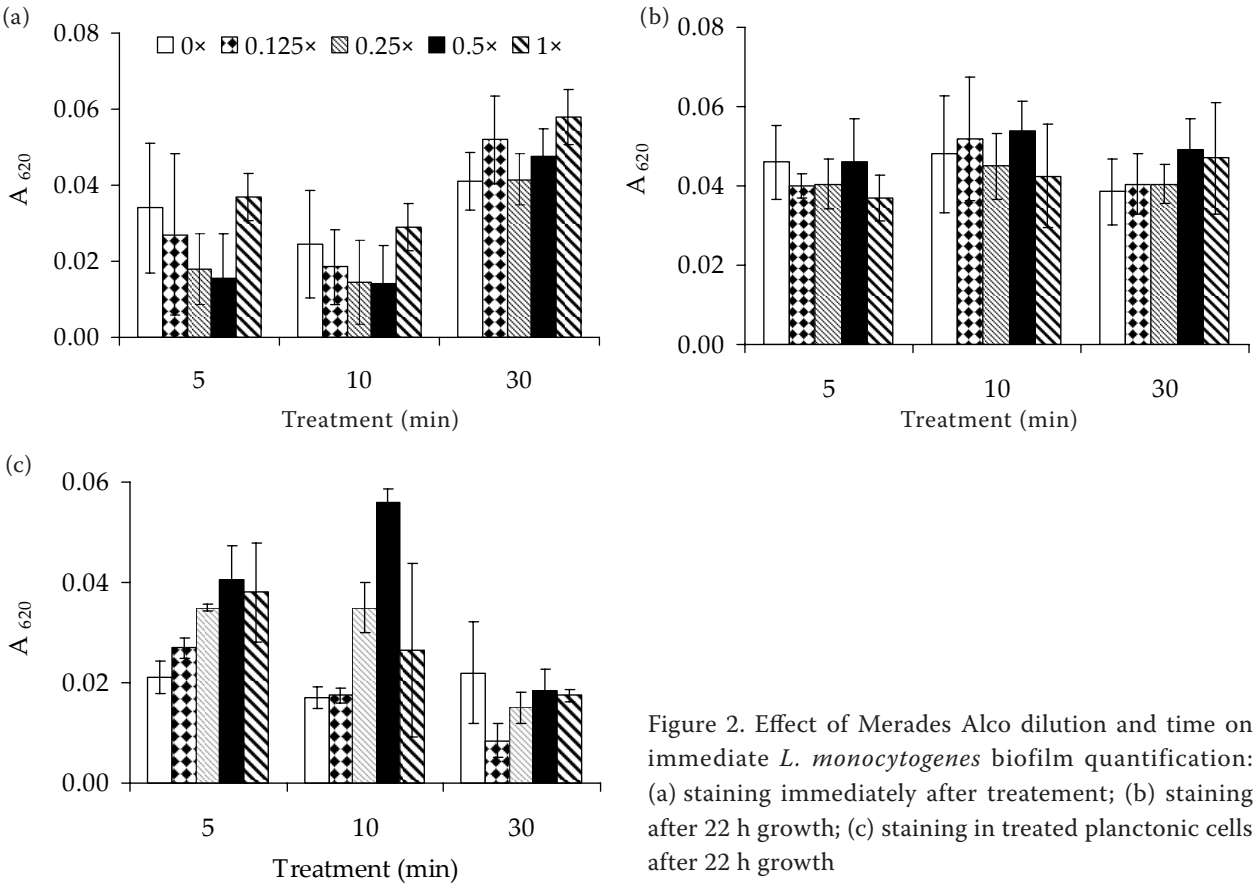

Figure 2. Effect of Merades Alco dilution and time on immediate *L. monocytogenes* biofilm quantification: (a) staining immediately after treatement; (b) staining after 22 h growth; (c) staining in treated planctonic cells after 22 h growth

sessile and embodied in an exopolymer matrix. The abilities of *L. monocytogenes* ATCC 13932 and *L. monocytogenes* Lm-24 to produce biofilms in the microtiter plates in ten different types of media at 30°C are shown in Figure 1. Similar results were obtained for 25°C, data not shown. The incubation time was 22 hours. The prolongation of this time did not enhance the biofilm formation by either strain at any temperatures tested (8, 25, 30, 37°C), data not shown. The type of medium exhibited a more determinative effect on the biofilm production than did the temperature. The media selected represented a wide range of nutrition demands, from a very complex medium like brain heart infusion (BHI) to a poor medium like buffered peptone water (BPW) ending by physiological saline (PS) only. The most intensive biofilm formation occurred in the media based on BPW with different

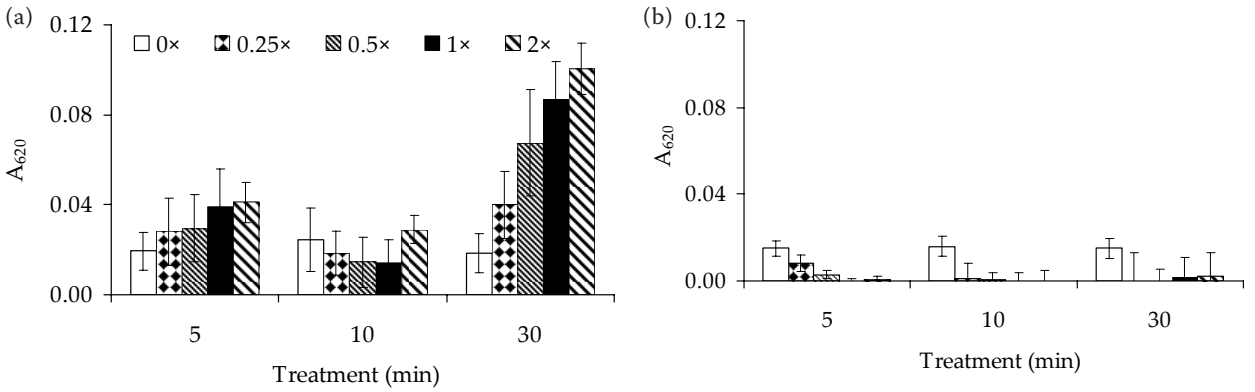

Figure 3. Effect of Savo dilution and time of treatment on on immediate *L. monocytogenes* biofilm quantification: (a) staining immediately after treatement; (b) staining after 22 h growth

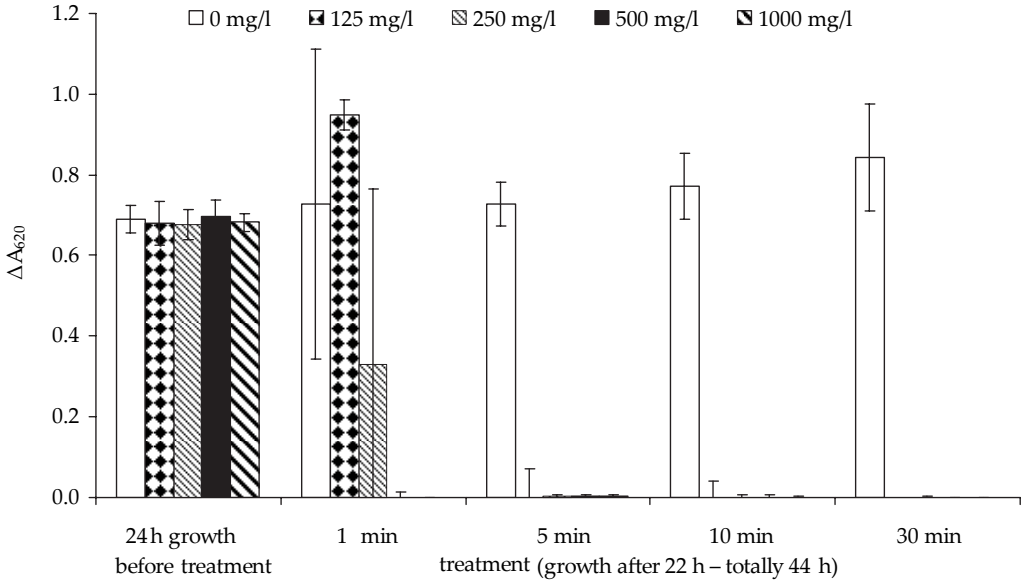

Figure 4. Effect of benzalalkonium chloride on *L. monocytogenes* biofilm cells and their subsequent growth from biofilm treated different media

additions of glucose and NaCl. The results of the absorbance measurement were comparable with the data obtained for *L. monocytogenes* biofilm by Pan *et al*. (2010).

For testing disinfectants efficiency, only the better biofilm forming industrial isolate Lm-24 was used. MA was tested as the first agent. Figure 2a shows the decrease in absorbance in 5 min and 10 min periods at lower concentrations, which can be explained as the effect of partial biofilm removal, while the higher concentration and longer time periods led to an increased number of dead cells that produce a matter absorbing crystal violet to a greater extent. When the microtitre plates were washed after MA treatment and filled with fresh medium, the effect obtained is shown in Figure 2b. It is evident that after 22 h the biofilm was either restored or its quantity slightly increased. The last test was done with planctonic cells. The results shown in Figure 2c give evidence that planctonic cells treated for shorter time periods can cause an increased biofilm formation after the transfer in fresh medium. In the case of 5 min treatment, the higher the concentration of disinfectants the more increased biofilm. When planctonic cells were treated with 0.5× concentration, the highest increase was measured. After 30 min treatment the biofilm formation was decreased by this ac-

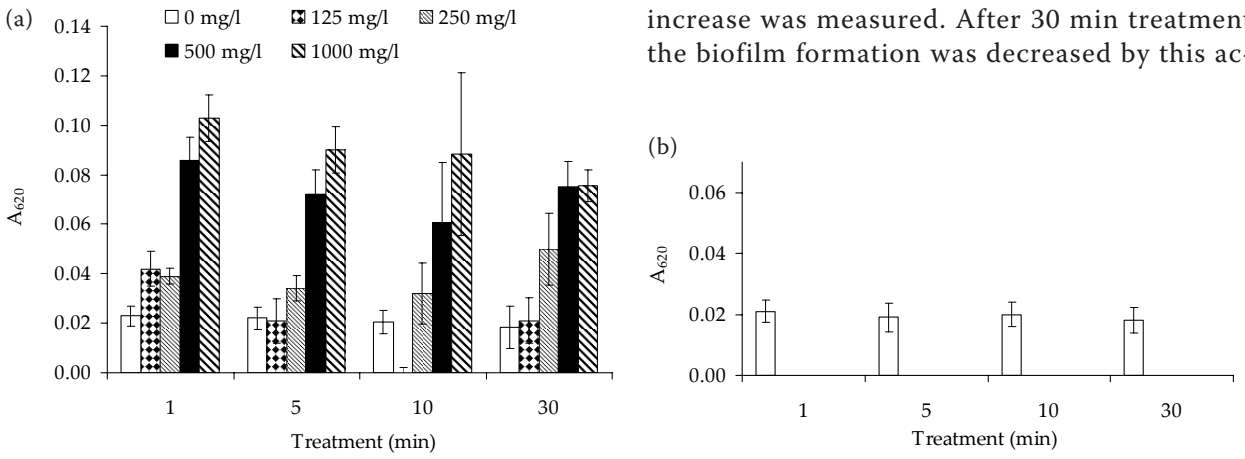

Figure 5. Effect of benzalalkonium chloride on *L. monocytogenes* biofilm cells: (a) staining done immediately after treatment; (b) expresed as biofilm formed after 22 hours

tion. MA had only a slight effect on the viability and structure of the biofilm with the longest time period (30 min) in the concentrations used. Its effect on planctonic cells was slightly bit supporting their growth and biofilm formation reliant on the treatment duration.

The effect of S dilutions and treatment times on biofilm is presented in Figure 3a. The growth of the biofilm cells treated with S for 5 min and 10 min after 22 h incubation led to a decrease of the subsequent growth by 20% in comparison with the untreated biofilm cells. Even 30 min treatment did not cause any significant decrease in the growth, data not shown. Planctonic cells after the treatment with S were deeply repressed to form biofilm (Figure 3b).

BC exhibited the strongest lethal effect on both planctonic cells and those growing in the biofilm. Figure 4 shows the results of BC treatment on the biofilm cells. After 22 h of subsequent cultivation, no biofilm was formed. The following Figure 5a presents the results received after the staining of the biofilm with crystal violet immediately after BC treatment. It is obvious that the non removed dead cells increased the resulting absorbance. The length of the treatment was not determinative; the effect of the agent concentration was more significant. In Figure 5 is it visible that the cells in the biofilm were devitalised by 500 mg of BC/l, while for planctonic cells was 125 mg/l the critical lethal concentration. These results clearly proved the generally accepted fact of the decreased sensitivity of biofilm cells to disinfectants in the comparison to the planctonic cells. Saginur *et al*. (2006) proved it for pathogenic strains of staphylococci treated by antibitiocs, Houari and Martino (2007) for strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* growing in the presence of chlorhexidine and benzalkonium chloride, Moretro *et al*. (2009) for *Salmonella* in biofilm on stainless steel. For *Listeria monocytogenes* it was observed for the wide range of disinfectants: sodium hypochlorite, quaternary ammonium compound and peroxyacetic acid (Stopforth *et al*. 2002), ozone, chlorine, and hydrogen peroxide (Robbins *et al*. 2005). The tested strain *L. monocytogenes* cells in biofilms were at least 1000 times more resistant to BC than in the planktonic form and to kill almost all the live cells in the biofilms concentrations of BC higher then 10 g/l should be applied for at least 30 min (Romanova *et al*. 2007). This resistance can be caused not only by the difficult difusion of the disinfectants to the biofilm cells (Xu *et al*. 1996) or the protective effect of the matrix (Pan *et al*. 2006), but also by the different physiological state of biofilm cells (Evans *et al*. 1994) caused by their specific gene expression, dissimilar from the planctonic cells (Ren *et al*. 2004). Biofilms are generally removed physically, for example, by scraping them off with a brush, etc. However, because biofilms adhere tightly to the carrier surface, this is not particularly effective.

Considering complex signaling and multifactorial relay-like processes in a biofilm, advancements in analytical techniques will be crucial for the studies on the complex interactions between the environments of food plants and biofilm bacteria to defend food against contamination. Numerous genes have been found to influence the biofilms physiology (Jefferson 2004), but the key principles of the biofilm performance will deserve continuous effort. The choice of disinfectants or cleaning agents along with their optimum concentrations and times of action is very important for destroying microbes. It is also important that the resistance of microbes to different disinfectants and cleaning agents be taken into account when planning the cleaning process.

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