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Removal of *Salmonella* biofilm formed under meat processing environment by surfactant in combination with bio-enzyme

Huhu Wang [a, b], Huawei Wang [a, b], Tong Xing [a, b], Na Wu [a, b], Xinglian Xu [a, b, *], Guanghong Zhou [a, b]

[a] National Center of Meat Quality and Safety Control, Nanjing Agricultural University, Nanjing 210095, PR China
[b] Synergetic Innovation Center of Food Safety and Nutrition, Nanjing Agricultural University, Nanjing 210095, PR China

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ABSTRACT

Salmonella outbreaks are commonly linked to consumption of contaminated food, its outbreaks have been associated with the biofilm formed on food processing surfaces, due to the acquired resistance that this bacterium possesses. In this study, four surfactants and five bio-enzymes were individually and conjunctively tested to remove the biofilm formed on stainless steel surfaces by a seven-strain cocktail of *Salmonella* grown in meat thawing-loss broth (MTLB). The results showed that cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) reduced greater numbers of biofilm cells than tween-80 and rhamnolipid. 1 mg/mL of CTAB and 10 mg/mL of SDS could remove 100% cells of biofilm at irreversible attachment phase (about 5.39 Log CFU/cm^2). Compared to proteinase K, dispaseII, glucoside amylase and subtilisin, cellulase reached greater reduction (85%, about 5.6 Log CFU/cm^2) of cells in mature biofilm, but still remaining a huge number of residual biofilm cells. The combination of cellulase following CTAB immersion was effective in removal mature biofilm (100%, about 6.2 Log CFU/cm^2 cells), which was supported by the observation of fluorescence microscopy. This study indicated that CTAB combined with cellulase can apply as an alternative strategy to drastically remove mature biofilm of *Salmonella* exposed to meat processing environments.

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1. Introduction

Salmonella spp. is recognized as a globally widespread food-borne pathogen and is considered to be the second most common cause (behind *Campylobacter* spp.) of food-borne bacterial illness all over the world (CDC, 2014; Vieira et al., 2009); In 2013, *Salmonella* was responsible for an estimated 82,694 human cases and 59 deaths in the EU, resulting in notification rate of 20.4 cases per 100,000 population (EFSA-ECDC, 2015). The two most commonly reported *Salmonella* serovars were still Enteritidis and Typhimurium, representing 39.5% and 20.2%, respectively, of all reported serovars in confirmed human cases (EFSA-ECDC, 2015). *Salmonella* was frequently detected in a variety of animal-food products such as meat, poultry and eggs. Consumption of these raw and undercooked corresponding food contaminated with *Salmonella* may lead to development of acute gastroenteritis characterized by emesis, diarrhea, etc. It is now commonly accepted that food-borne pathogens such as *Salmonella* may grow predominantly as biofilm on solid surfaces, in most of their growth habitats in natural and industrial settings, rather than in planktonic mode (Shi & Zhu, 2009; Thallinger, Prasetyo, Nyanhongo, & Guebitz, 2013). Biofilm of food-borne pathogens were wide present in a variety of food processing sites, including dairy, fish processing, poultry, and ready-to-eat foods (Srey, Jahid, & Ha, 2013), and many food-borne outbreaks have been associated with biofilm (Simoes, Simoes, & Vieira, 2010). An example of this is the *Salmonella* outbreak in Spanish, which resulted in 2138 cases due to consumption of pre-cooked chicken contaminated by the biofilm of *Salmonella* Hadar formed on a bend of a pipe transporting gravy in the processing site (Perez-Rodriguez, Valero, Carrasco, Garcıa, & Zurera, 2008).

The biofilm cells can be resistant to environmental stresses, antibiotics and disinfectants (Hoiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Mah & O'Toole, 2001), and as a consequence are extremely difficult to eradicate in food industry. Therefore, much effort has been devoted for developing strategies to interfere with

* Corresponding author. National Center of Meat Quality and Safety Control, Nanjing Agricultural University, Nanjing 210095, PR China.
E-mail address: xlxu@njau.edu.cn (X. Xu).

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biofilm formation. Srey et al. (2013) and Jahid and Ha (2012) have reviewed the conventional control strategies of biofilm used to gain more proximity to efficiently maintain good hygiene throughout food industries, including sodium hypochlorite, hydrogen peroxide, ozone, and peracetic acid. Giaouris et al. (2014) also have discussed several novel control methods of pathogens biofilm, such as essential oils and bacteriophages. Additionally, the metabolite molecules of microbial interactions such as N-acylhomoserine lactone (AHL), autoinducer-2 and c-di-GMP, have been successfully evaluated as an alternative means for preventing from biofilm formation (Park, Lim, & Choi, 2015; Wang, Ye, Zhang, Dong, Xu, & Zhou, 2013). However, recent concerns have been raised over the effectiveness, safety and convenience of these approaches, consequently many approaches studied in lab conditions were limited in actual food processing. Current measures of biofilm removal in food industry, in particular of chemical-based disinfectants, were commonly applied by directly spraying or/and immersing. This application can enable the pathogens to survive and proliferate under higher concentration of disinfectants. Bremer, Fillery, and McQuillan (2006) reported that a standard clean-in-place regime (water rinse, 1% sodium hydroxide at 65 °C for 10 min, water rinse, 1.0% nitric acid at 65 °C for 10 min, water rinse) did not ensure the removal of bacteria biofilm formed under dairy processing environments. In addition, similar type of control measures have some other well-known drawbacks, such as the limit permeability into EPS of biofilm, the possible toxicity of residues, the promotion of genetic exchange between different bacteria and the resistance to disinfectants. Therefore, new approaches to control biofilm in the food industry should be focused according to the dynamical processes or steps of biofilm formation, including initial irreversible attachment, rudimentary, maturation and dispersion (Mizan, Jahid, & Ha, 2015). The novel two-step approaches should be explored, the adhesion cells in biofilm are firstly detached and removed based on the reduction of hydrophobicity between attachment cells and solid surface, and the degradation of EPS of biofilm, then all the planktonic cells (detachment from surfaces) are disinfected by bactericides or other control approaches.

The environments involving in biofilm formation such as foodborne isolates, room or lower temperature, variety of contacted-surfaces and residues of food liquid, which could be commonly encountered during food processing, were critical for biofilm removal. The existing studies about controlling biofilm were focused on the biofilm of *Salmonella* grown in some standard lab growth conditions (Burgos, Lopez, Aguayo, Pulido, & Galvez, 2013; Islam et al., 2014), there is little information about the removal of *Salmonella* biofilm grown in meat-based substrate under meat processing environments. The residual of meat liquid on processing contact-surfaces may protect the cells in biofilm, and then weaken the cleaning efficiency of control strategies. Therefore, In this study, meat-borne isolates, stainless steel surfaces and a meat-based growth substrate were tested to simulate the conditions probably found in the meat processing plants, and the focus was highlighted on the effect of single tested surfactant, single tested bio-enzyme, and the combination of special surfactant and bio-enzyme on biofilm removal of *Salmonella*.

2. Materials and methods

2.1. Strains and incubation medium

Seven *Salmonella* strains (*S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *S.* Stanley, *S.* Agona, *S.* Derby and *S.* Indiana) isolated from meat processing surfaces and poultry meat, were used in this study. Each strain stored in 40% glycerol at −70 °C was twice separately cultured in Trypticase Soy Broth (TSB) at 37 °C for 20 h, resulting in early stationary phase culture, then a seven-strain cocktail of *Salmonella* strains was prepared as following: 5 mL of each strain was centrifuged at 10,000 g, 4 °C for 5 min, and then the cell pellets were washed with 0.85% of NaCl solution twice and were resuspended in NaCl solution. The concentrations of cell suspension were determined with OD_{600nm}. The ratio of each strain in the cocktail was 1:1:1:1:1:1:1, and the final concentration of cocktail was approximate 8 Log CFU/mL.

A meat-based growth medium (chicken meat thawing-loss broth, MTLB) was used for biofilm formation. The MTLB was prepared as described previously (Midelet & Carpentier, 2002). The final concentration of protein in MTLB was 1 mg/mL confirmed by the Biuret protein assay.

2.2. Biofilm formed on stainless steel

Stainless steel plates (50 × 20 × 1 mm, food grade 304, 2B finish), a material commonly used in the manufacture of meat-processing equipments, were used for biofilm formation. Prior to use, the plates were cleaned as previously described by Poimenidou et al. (2009). For biofilm formation, 100 μL of cocktail suspension prepared as described above was transferred into a centrifuge tube (40 mL) containing 10 mL of MTLB loading a stainless steel plate. The stainless steel plates were partly (10 cm^2) submerged into MTLB fluid, and part of each plate was exposed to the air-liquid interphase (Chorianopoulos, Giaouris, Kourkoutas, & Nychas, 2010), and then the tubes were incubated at 20 °C, and the biofilm were obtained at 36 h and 132 h.

2.3. Removal of biofilm by individual and combination measures

Four surfactants (the cetyltrimethyl ammonium bromide (CTAB, BioSharp), sodium dodecyl sulfate (SDS, BioSharp), rhamnolipid and tween-80 (SunShineBio)), three proteases (proteinase K, dispase and subtilisin, Sigma) and two glycosidases (cellulase (R-10), Yakult; glucoside amylase, Sigma), were used for biofilm removal. The detail of experiment design was shown in Table 1. A single stainless steel plate loading biofilm was rinsed three times with 0.85% NaCl solution to remove non-attached cells, and then the plates were immersed in appropriate surfactant or bio-enzyme solutions under special conditions (Table 1).

2.4. Cells numeration of the biofilm removal

The plates treated by variety treatments described in Table 1, were rinsed three times with 0.85% NaCl solution to remove residual surfactants, bio-enzymes and planktonic cells, then the residual cells in biofilm on stainless steel plates were determined by swabbing and plate counting method. The residual cells of biofilm were removed with sterile cotton swabs and the swabs were transferred to tubes containing 0.85% NaCl solution, vortexed with beads for about 5 min, and then serial dilutions were prepared (Winkelstroter, Gomes, Thomaz, Souza, & De Martinis, 2011). Results were expressed as the Log CFU/cm^2 (total of 10 cm^2), four replicates were tested for each treatment. The percentage of reduction biofilm cells (%) was calculated as following: (the cells numbers in control group − the cells numbers in treatment group)/the cells numbers in control group × 100.

2.5. Fluorescence microscopy analysis

Stainless steel plates incubated in MTLB at 20 °C for 36 h and 132 h were aseptically rinsed three times with 0.85% NaCl solution to remove planktonic cells. The procedure of fluorescence microscopy was followed as previously described by Wang, Ding, Dong,

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