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Response of long-term acid stress to biofilm formation of meat-related *Salmonella* Enteritidis

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ABSTRACT

Salmonella commonly preferred to survive in the pattern of biofilm rather than planktonic cells in food processing environment, where various stress was widely encountered. In the present study, the response of biofilm formation of four-isolate cocktail of *Salmonella* Enteritidis on stainless steel surface to long-term acid stress was determined within a pH range of 5.0–7.2. The results showed that a short-term weak acid stress (pH 6.0 and 5.5) could enhance the attachment of *S.* Enteritidis, whereas short-term harsh stress significantly reduced cells attachment; Long-term weak and harsh stress obviously inhibited the biofilm formation of *S.* Enteritidis as compared with control group (pH 7.2), and the inhibition was significant depend on pH values, however, no difference was observed in planktonic cells numbers. Many small cell clusters rather than large cells aggregates were observed by fluorescence microscopy in harsh stress group, however, a classical process of biofilm formation and the complex three-dimensional structure formed by dense aggregates were found in control group. Consistent finding was also revealed by the observation of ATR-FTIR, which suggested that the harsh acid stress decreased the amount of various components formed in biofilm matrix, in particular of polysaccharides and proteins. Our finding suggested that long-term harsh acid stress could exert a great impact on biofilm formation of *S.* Enteritidis, and the obtained information was benefit for developing novel disinfection procedures in food processing industry.

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1. Introduction

Salmonella sp. has been widely concerned due to its increasing contamination in the food industry and serious risks to human health. More than 2500 *Salmonella* serovars preferred a wide variety of habitats have been associated with animal or human infection, in particular of the Enteritidis serovar, which is the global predominant serovar confirmed in food outbreaks and infection (Vieira et al., 2009). According to the annual report of EFSA-ECDC in 2015, *Salmonella* Enteritidis has resulted in a total of 29,090 (39.5%) confirmed cases of human salmonellosis in 2013 (EFSA-ECDC, 2015); meanwhile, more than 14% of laboratory-confirmed human *Salmonella* infections has been directly associated with Enteritidis in U.S, topping the serovars list (CDC, 2014).

Although many causes or sources have been associated with pathogens contamination in the food industry, bacteria persistence or biofilm formation on food processing-contact surfaces have been identified as a principal source (Bridier, et al., 2015). Most pathogens involved in food-borne outbreaks are able to form biofilm on various solid surfaces and under almost all the environmental conditions encountered in food processing industry, such as poultry, meat, fish processing, dairy and ready-to-eat (RTE) foods industry (Srey, Jahid, & Ha, 2013). Consequently, much effort has been devoted to removing the biofilm by regular cleaning and disinfecting, and preventing biofilm formation by reducing cells attachment to contact surfaces; many chemical products have been used in removing biofilm in food processing plants, including oxidative, acid-based, chlorine-based, surfactants and electrolyzed water. A few residues of disinfectants could, in many cases, remain on contact surfaces in the practice of food plants due to the unique design of the equipment and non-standard operational procedures. Thus, the bacteria cells are subjected to the stress of disinfection residues, inducing the variation of cell properties including adhesion ability and biofilm formation.

So far, many research concerns have been focused on the planktonic bacteria or corresponding biofilm in response to

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environmental stress encountered in food plants, such as heat, acid, sanitizers, starvation, and dehydration (Ban, Kang, & Yoon, 2015; Burin, Jr., Silva, & Nero, 2014; Galvao, Prudencio, & Vanetti, 2015; Gruzdev, Pinto, & Sela, 2012; Lagha et al., 2015). The attachment ability of *Salmonella* isolates in response to short-term (2 h) stress of pH and salt were evaluated by Zulfakar, White, Ross, & Tamplin, 2013 who observed that there was no significant effect on attachment to meat proteins within a pH range of 5–9, and the effect of salt type and concentration varied depending on strains; Yang et al. (2016) demonstrated that the environmental stress commonly encountered in food plants could alter *Salmonella* biofilm resistance to chlorine treatment possibly by acting on the cellulose production. From the general survey of existing documents about biofilm formation of pathogens subjected to environmental stress, it is easy to find that the research emphases were focused on the effect of short-term stress on cells attachment, the resistance of formed-biofilm of pathogens to various stress and the adhesion ability of stressed cells in a friendly environment (Chen, Zhao, & Doyle, 2015; Nguyen & Yuk, 2013); However, limit information about long-term acid stress on pathogens attachment and biofilm formation has been known. In addition, the biofilm formation of cells in response to stress were commonly assessed using polystyrene micro-wells, but the correlation between biofilm production *in vitro* using polystyrene micro-wells and biofilm formation on processing contacted-surfaces in food facilities (e.g. stainless steel surfaces) has not yet been fully determined (Lourenco, Rego, Brito, & Frank, 2012). Considering the above, the present study was therefore carried out to determine the effect of long-term acid stress on biofilm formation of meat-related *Salmonella* Enteritidis on stainless steel surface, stimulating the situation generally encountered in food processing plants. Revealing biofilm formation in response to acid stress would contribute to microbiological risk assessment during food processing in an actual facility, and would also be of benefit for developing novel disinfectants and disinfection measures.

2. Materials and methods

2.1. Strains and incubation medium

A four-isolate cocktail of *Salmonella* Enteritidis previously isolated from meat and processing surfaces was tested in this study. Each isolate stored in 40% glycerol at −70 °C was twice separately cultured in tryptic soy agar (TSA, Luqiao Technology Co. Ltd., Beijing, China) at 37 °C for 18 h, then each isolate was incubated in TSB (Luqiao Technology Co. Ltd., Beijing, China) for 18 h. The cells were harvested by centrifugation at 8000 g for 10 min at 4 °C and washed twice with 0.85% NaCl solution, then the pellets were re-suspended in equivalent amounts of NaCl solution. Individual suspension of isolates was mixed to prepare the cocktail, the ratio of each isolate in the cocktail was 1:1:1:1, and the final concentration of cocktail was approximate 10^8 CFU/mL.

2.2. Biofilm formation in response to acid stress and cells numeration

Food grade stainless steel plates (75 × 25 × 1 mm, 304 type, 2b finish, Zhongyi stainless steel material Co. Ltd., Dongguan, China) were used for biofilm formation. One milliliter (10^8 CFU/mL) of cell suspension prepared as described above was transferred into a tube containing 100 mL of normal TSB (control, pH 7.2) or acid TSB (treatment) together with a stainless steel plate, and then incubated at 20 °C for 3, 6, 9, 12, 24, 48, 72, 96, 120, 144 or 168 h without agitation. The acidity of treatment group was individually adjusted to a pH value of 6, 5.5 and 5 with 0.1 M of hydrochloric acid. The stainless steel plates were partly submerged into TSB fluid, and part of each plate was exposed to the air-liquid interphase. The numbers of biofilm cells and planktonic cells (no stainless steel plate in TSB fluid) were enumerated at appropriate incubation time. A single plate was removed for sampling and rinsed three times with 0.85% NaCl solution to remove non-attached cells, whereas the attached biofilm cells were removed with a violent water-flapping method, which has been proved for effectively detaching biofilm cells on solid surfaces (Wang, Zhang, Dong, Xu, & Zhou, 2015), the violent water-flapping was mainly operated as following: the plates attached biofilm cells were put into a stomacher blender bag and then shaken at a frequency of 200 oscillations/min for 2 min using a bag mixer (BagMixer 400VW, Interscience). No difference in detaching biofilm cells was found between violent water-flapping method and cotton-swabbing method, cotton-swabbing has been confirmed as the most suitable method for removal of attached cells (Poimenidou et al., 2009). The numbers of biofilm cells were determined in four trials with triplicate samples using tryptic soytone agar plate (TSA) and xylose lysine deoxycholate agar plates (XLD). Results were expressed as log CFU/cm^2. The survival percent of biofilm cells was estimated by the following formula: survival percent (%) = (cell counts on selective medium (XLD)/cell counts on nonselective medium (TSA)) × 100 (Al-Qadiri, Lu, Al-Alami, & Rasco, 2011).

2.3. Fluorescence microscopy analysis

Stainless steel plates grown in TSB with pH 7.2 (control group) and TSB with pH 5.0 (harsh stress group) for 3, 24, 72, 120 and 168 h were aseptically rinsed three times with 0.85% NaCl solution to remove unattached cells (planktonic cells), and then the plates attaching biofilm cells were stained with 4,6-diamidino-2-phenylindole (DAPA, 2 μg/mL, Beyotime, Ltd., Shanghai, China) in the dark at room temperature for 10 min. Finally, the tested plates were rinsed three times with sterile 0.85% NaCl solution to remove excess stain. Images of biofilm were obtained with fluorescence microscopy (AX10, Zeiss, German) using a 100× oil immersion objective.

2.4. ATR-FTIR analysis

Mature biofilm obtained in TSB with pH 7.2 and pH 5.0 for 120 h were aseptically rinsed three times with 0.85% NaCl solution to remove unattached cells, and then each stainless steel plate loading biofilm was air dried. The attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR) was used in this study, and the germanium crystal was used to determine the accuracy of analysis. The spectra from 3500 to 780 cm^{-1} were acquired with a ATR-FTIR spectrometer (NEXUS 670, Thermo Nicolet, USA) with 2 cm^{-1} spectral resolution. To improve the signal-to-noise ratio, 256 scans were measured for each sample (Wang, Ding, Wang, Xu, & Zhou, 2013). Each planktonic cells suspensions were washed and re-suspended in sterile 0.85% NaCl solution. 300 μL aliquot of each planktonic suspension was transferred to the ATR crystal, and then transmission spectra were recorded as mentioned before. The spectra obtained from biofilm and planktonic cells samples were used to remove the spectral background: the NaCl solution spectrum for planktonic cells and a stainless steel plate spectrum for biofilm.

2.5. Statistical analysis

Biofilm cells was determined in four trials with triplicate samples (n = 4), the results were expressed as mean ± standard deviation. Statistical significance was determined by a one-way

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