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Characterization of attachment and biofilm formation by meat-borne *Enterobacteriaceae* strains associated with spoilage

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ABSTRACT

Meat wastes and losses were associated with spoilage microorganisms, usually sourcing from the biofilm cross-contamination. In this study, the attachment and biofilm formation of five meat-borne *Enterobacteriaceae* strains (*Enterobacter cloacae, Hafnia alvei, Proteus mirabilis, Citrobacter freundii and Klebsiella oxytoca*) were characterized by two simulated contamination scenarios. There were great variations in surface hydrophobicity, motility and adhesion ability on a polystyrene surface of 96-well microplates among these tested strains. *P. mirabilis* and *C. freundii* showed higher adhesion than other strains. More than 4.5 log CFU/cm^2 cells were transferred to stainless steel surface under a short-term (5 h) attachment scenario. More than 8 log CFU/cm^2 of biofilm formation on stainless steel surface under a long-term (7 d) growth scenario were found for all tested strains, and nearly 10.5 log CFU/cm^2 cells were observed for *E. cloacae* after 5-day incubation. A mature biofilm architecture was observed by confocal laser scanning microscopy (CLSM), consisting of multilayer aggregates of more than 30 μm thicknesses. This was further confirmed by attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR), and the dominant peaks in biofilm spectra were mainly associated with amide, polysaccharides and glycosidic linkage. This finding could provide the basic data for developing novel disinfectant strategies.

1. Introduction

The meat wastes derived from spoilage during distribution has been a worldwide concern, the Food and Agriculture Organization declared that approximately 1.3 billion tons of food are annually wasted or lost, including more than 20% of the 263 million tons of meat products (FAO, 2011). Although meat spoilage is a complex event associated with a combination of biological and chemical activities, the spoilage mainly depends on the counts and species of microorganisms on the meat. It is well known that the microorganisms on meat products mainly originate from animal intestines during slaughter and cross-contamination occurs during processing, transportation and storage (Yang, He, Badoni, Tran, & Wang, 2017a). Many effective approaches have been applied to reduce microorganisms and inhibit meat spoilage in the meat industry (Sohaib, Anjum, Arshad, & Rahman, 2016). For example, numerous automated devices have been used for animal evisceration to avoid damage to the intestine, and the disinfection of processing environments is carried out to decrease cross-contamination. Even so, many cross-contamination cases are still associated with processing contacted surfaces, mainly attributed to the presence of bacteria biofilm that forms on solid surfaces (Carrasco, Morales-Rueda, & Garcia-Gimeno, 2012), which is the dominant growth status of bacteria in food industrial settings rather than in planktonic scenario. Biofilm was broadly defined as a surface-attached bacterial communities encased in a matrix of extracellular polymeric substances secreted by the cells (Jahid & Ha, 2012). The cells in the biofilm could possess more than a 10-fold greater resistance to disinfectants compared to planktonic bacteria, making established biofilm particularly difficult to remove (Mah & O'Toole, 2001). The residue cells from biofilm can lead to further cross-contamination (Cappitelli, Polo, & Villa, 2014). Currently, much attention is focused on the elimination of food-borne pathogens whereas the attachment and biofilm status of microorganisms associated food spoilage has been largely overlooked (Teh, Lee, & Dykes, 2017; Yang, Hoe, Zheng, Chung, & Yuk, 2017b). Biofilm formation of spoilage bacteria should be paid more attention, since mechanical equipment highly increases the surfaces available and

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opportunities for cross-contamination from biofilm formation in the modern food industry.

The microbial species of meat spoilage highly depend on packaging conditions, demonstrating that *Enterobacteriaceae* and *Pseudomonas* are commonly present in aerobic packaging, and *Enterococcus* and lactic acid bacteria are associated with vacuum and air-modified packaging (Holl, Behr, & Vogel, 2016; Wang et al., 2016c). Extensive surveys have previously focused on biofilm formation of *Pseudomonas* in food microbiology investigations, and the effect of various growth conditions, including nutrient sources and temperatures, on biofilm formation of *Pseudomonas* isolates from food and processing equipment has been explored (Aswathanarayan & Vittal, 2014). However, less information is known about the attachment and biofilm formation by spoilage-related *Enterobacteriaceae* (Burgos, Perez-Pulido, Galvez, & Lucas, 2017).

It has been widely believed that biofilm formation of bacteria on surfaces is a complicated dynamic process determined by several factors, including mainly surface characteristics, external environment factors and intracellular processes, meaning "what is happening at the interface, on the outside and on the inside of the cells", respectively. Innovative research perspective has been focused on surface characteristics, including solid surfaces and cells surfaces. However, inconsistent findings between biofilm formation and cell surface properties have been reported in *Enterobacteriaceae* isolates, in particular of food-borne pathogens, and the potential positive link between biofilm formation and surface hydrophobicity and the motilities of *Salmonella* and *Campylobacter* have been confirmed (Nguyen, Turner, & Dykes, 2011; Wang et al., 2013). Conversely, no correlation was observed between hydrophobicity or swimming and biofilm production in *Escherichia coli* O157 and *Listeria monocytogenes*, respectively (Di Bonaventura et al., 2008; Goulter, Gentle, & Dykes, 2010). Thus, it is important to explore the link between attachment ability and the surface properties of *Enterobacteriaceae* isolates associated with meat spoilage.

Considering the above-mentioned, five *Enterobacteriaceae* isolates commonly encountered in meat processing plants were tested in this study to (i) obtain the relationship between cells surface characterization and attachment ability, (ii) determine the process of biofilm formation under short- and long-term growth periods, and (iii) explore the morphology and extracellular polymeric substances of formed biofilm. This work could provide new insights into the biofilm development process of spoilage-related isolates, which would provide basic data for developing novel disinfectant strategies.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Five strains of *Enterobacteriaceae,* including *Enterobacter cloacae*, *Proteus mirabilis*, *Hafnia alvei*, *Citrobacter freundii* and *Klebsiella oxytoca*, previously isolated from spoilage chicken carcasses and confirmed by 16sDNA sequences and typical biochemical reaction, were tested in this study. These strains have showed great potential ability to spoil meat. Stock cultures were frozen at - 60 °C in tryptone soy broth (TSB, Hopebio, Qingdao, China) containing 50% glycerol. Prior to experiments, each strain was activated on tryptone soy agar (TSA, Hopebio, Qingdao, China) plates incubated overnight at 30 °C. Each strain was cultured in 10 mL TSB at 30 °C for 20 h. The cells were harvested by centrifugation at 12,000 × g for 10 min at 4 °C and then washed three times with 0.85% NaCl solution.

2.2. Cell motility surface hydrophobicity and assays

The motility of tested strains was evaluated using soft-agar plate assays (Cong et al., 2011; Hidalgo, Chan, & Tufenkji, 2011) with swimming agar (10 g/L tryptone, 5 g/L NaCl, 2.5 g/L glucose and 0.3% agar) and swarming agar (25 g/L LB, 0.5 g/L glucose and 0.5% agar). Three μL of each cell suspension was spotted onto the surface of soft-agar plates, and the motilities were recorded after incubation at 30 °C for 8 h (swimming) or 20 h (swarming). Five independent assays were performed for each strain.

Cell surface hydrophobicity was evaluated as previously described with minor modifications (Darilmaz, Beyatli, & Yuksekdag, 2012). Briefly, 2 mL of each cell suspension, prepared by centrifuging cells culture at 12,000 × g for 10 min at 4 °C and washed three times and then vertexing with 0.85% NaCl solution ($OD_{600\ nm} = 0.6$, A_0), was added to 2 mL of xylene. The two-phase system was mixed using a vortex for 2 min. The mixture was stored for 15 min at room temperature to allow the separation of the two phases. The $OD_{600\ nm}$ of the aqueous phase was measured (A_1) by automatic microplate reader (Molecular Devices, USA). The percentage of cells dispersing into xylene was calculated as $[(A_0-A_1)/A_0] \times 100$ (Kim & Wei, 2007). Five independent assays of each strain were performed for each solvent.

2.3. Cell attachment and biofilm formation

To measure adhesion ability, prior to inoculation, the suspension of each strain was diluted with a 0.85% NaCl solution to a concentration of 10^6 CFU/mL, and an aliquot (20 μL) of each strain culture was transferred to 180 μL of fresh TSB in a 96-well polystyrene microplate. The microplates were then incubated for 1 and 3 days respectively used for 20 °C and 30 °C. The TSB, devoid of bacterial inocula, served as a negative control. Following incubation, wells were rinsed three times with sterile de-ionized water, the plates were air-dried for 30 min, and then each well was stained with 200 μL of 0.25% (wt/vol) crystal violet for 30 min (Kim & Wei, 2009). The staining solution was then removed, and the wells were rinsed three times with sterile de-ionized water. The crystal violet bound to the biofilm was then solubilized with 200 μL of 95% ethanol for 30 min. The absorbance was measured at 570 nm (Silagyi, Kim, Lo, & Wei, 2009).

Stainless steel plates (50 × 20 × 1 mm, grade 304, 2B finish) were used for short-term attachment and long-term biofilm formation, since stainless steel is a material commonly used in the manufacture of meat-processing equipment. Then, 100 μL of each cell suspension was transferred into the prepared TSB tubes containing stainless steel plates (initial cell concentrations were 6 log CFU/mL for short-term attachment and 2 log CFU/mL for long-term biofilm formation). All plates were incubated at 20 °C for 168 h without agitation. Cell counts were determined at 10, 20, 30, and 60 min and 2, 3, 4 and 5 h incubation times for short-term attachment and 1, 2, 3, 4, 5, 6 and 7 days for long-term biofilm formation, respectively. A single plate was removed for sampling and was rinsed three times with 0.85% NaCl solution to remove non-attached cells, whereas the attached biofilm cells were removed by the violent water-flapping method (Wang et al., 2016b). Cell counts were determined in five replications with a TSA plate incubated at 30 °C for 24 h. The results are expressed as log CFU/cm^2.

2.4. Confocal laser scanning microscopy (CLSM)

Stainless steel plates incubated for 1 day or 5 days were aseptically rinsed three times with 0.85% NaCl solution to remove planktonic cells. CLSM was used for identification of biofilm