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Scanning electron microscopy of *Salmonella* biofilms on various food-contact surfaces in catfish mucus

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ABSTRACT

The objective of this study was to determine the growth and survival of *Salmonella enterica* in the presence of high and low concentrations (375 μg/ml and 15 μg/ml) of catfish mucus extract at 10 °C and 22 °C for 63 days. The second objective of this study was to investigate the biofilm formation of *Salmonella enterica* serovar Blockley (7175) in catfish mucus extract for 48 h at 22 °C on four food-contact surfaces and to observe the biofilm populations using Scanning Electron Microscopy (SEM). The surface properties, surface roughness and surface energies were determined using contact angle measurement and atomic force microscopy. In 375 μg/ml of catfish mucus extract that was inoculated with 3 log CFU/ml, the growth of *Salmonella* counts were increased to a maximum of 6–7 log CFU/ml at 10 °C and 7–8 log CFU/ml at 22 °C in 7–14 d and decreased by 1–2 log CFU/ml from these peak levels at both 10 °C and 22 °C from 21 to 63 d. In 15 μg/ml of catfish mucus extract, *Salmonella* counts were in the range of 4–5 log CFU/ml at 10 °C and 5–6 log CFU/ml at 22 °C over 7–63 d of storage. By contrast, *Salmonella* counts were non-detectable in the absence of catfish mucus by 21–28 d of storage at 10 °C or 22 °C. The biofilm counts of *S.* Blockley (7175) on a stainless steel surface were 4 log CFU/cm^2 and 5.5 log CFU/cm^2 in 15 μg/ml and 375 μg/ml of catfish mucus extract respectively after 48 h incubation at 22 °C. SEM revelead that biofilm formation by *S.* Blockley (7175) was less in 15 μg/ml than 375 μg/ml of catfish mucus extract on stainless steel. In addition, SEM indicated that the visible biofilms were least on buna-N rubber as compared to stainless steel, polyethylene and polyurethane surfaces. Contact angle and atomic force microscopy confirmed that buna-N rubber was highly hydrophobic with low surface energy and low roughness when compared to other three surfaces. These findings indicate that *Salmonella* can utilize catfish mucus as a nutrient source to survive for longer periods and promote biofilm formation for its persistence on different food-contact surfaces.

1. Introduction

During commercial processing, channel catfish (*Ictalurus punctatus*) are stunned, deheaded, eviscerated, skinned, filleted, chilled, washed, sorted and packed (Silva et al., 2001). During these processing steps, bacteria from the skin surface and gut microflora from processing equipment may lead to cross-contamination of retail products such as steak, nuggets, skinless and boneless fillets (Bal'a et al., 1999; Rajkowski et al., 2009; Tucker and Hargreaves, 2004). In 2015, the United States Department of Agriculture (USDA)-Food Safety Inspection Service (FSIS) catfish risk assessment report prioritized *Listeria monocytogenes* and *Salmonella* as important foodborne pathogens associated with catfish due to their prevalence on the product (USDA-FSIS, 2015).

Salmonella spp. are facultative anaerobic, nonsporulating, Gram-negative bacterium with rod-shaped bacilli. Salmonellosis outbreaks have been linked to the consumption of seafood in the United States such as molluscs, crustaceans and finfish products between 1973 and 2006 and other seafood products such as shrimp, oysters, salmon and crab between 1998 and 2015 (Iwamoto et al., 2010; Elbashir et al., 2018). A *Salmonella enterica* ssp. *enterica* serovar Hadar outbreak with 10 cases that was reported from a New Jersey restaurant was linked to catfish. Due to this outbreak, Centers for Disease Control and Prevention (CDC) has listed catfish as a vehicle for the spread of *Salmonella* (USDA-FSIS, 2010). *Salmonella*

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can survive for long periods of time over months or years in aquatic environments (Amagliani et al., 2012; FAO, 2010; Lunestad et al., 2007; Winfield and Groisman, 2003). This pathogen has been isolated from freshwater catfish, catfish ponds and catfish skin, gills, feces, guts and intestines (Andrews et al., 1977; McCoy et al., 2011; Wyatt et al., 1979). *Salmonella* spp. was isolated from 30% of 77 tilapia mucus samples from different ponds in the Guangdong province of China (Li et al., 2017). LaiHao et al. (2009) also isolated *Salmonella* species from tilapia mucus.

Adhesion of various pathogenic and non-pathogenic bacteria to mucosal surfaces has been evaluated previously (Collado et al., 2007; Grześkowiak et al., 2011). For example, *Flavobacterium columnare* was able to grow and adhere to the polystyrene surface for up to 24 h in the presence of salmon (*Salmo salar*) mucus (Staroscik and Nelson, 2008). Shoemaker and LaFrentz (2015) demonstrated that *F. columnare* grew and survived in water for greater than 100 d when mixed with sterilized mucus from tilapia (*Oreochromis niloticus X O. aureus*). Our recent findings show that *Salmonella* uses catfish mucus as a nutrient source to grow and form biofilms on various food processing surfaces. Of the four food-contact surfaces, buna-N rubber formed least as compared to stainless steel, polyethylene and polyurethane surfaces (Dhowlaghar et al., 2018). The first objective of the present study is to determine the survival of *Salmonella* isolates in the presence of catfish mucus extract for up to 63 d at two temperatures (10 °C and 22 °C). The second objective of this study is to observe the biofilm formation by *Salmonella* on four food-contact surfaces by scanning electron microscopy in the presence of catfish mucus. In addition, the surface properties, surface roughness and surface energies were also determined using contact angle measurement and atomic force microscopy.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two strains of *S.* Typhimurium (49 and 55) isolated from chicken and *S.* Heidelberg isolated from turkey were provided by Dr. Sharma C.S (Poultry Science Department, Mississippi State University, MS). *S.* Hadar (7101), *S.* Virchow (7207) and *S.* Blockley (7175) isolated from imported frozen catfish fillets from thailand were provided by Dr. Shaohua Zhao (USDA, Laurel, MD). Strains were stored at −80 °C in tryptic soy broth that contained 0.6% yeast extract (TSBYE, pH 7.2; BD Bio sciences, San Jose, CA) and was supplemented with 16% glycerol. The working stock cultures for these strains were prepared by inoculating a colony on xylose lysine deoxycholate agar (XLD, BD Difco^{Tm}, San Jose, CA) and then incoulated in tryptic soy agar with 0.6% yeast extract (TSAYE). Slants were then stored at 4 °C for up to 4–5 weeks. The overnight culture of each strain was prepared by inoculating 10 ml of the TSBYE culture from the working stock and incubation in a shaker at 150 rpm (C24 Classic series incubator shaker, New Brunswick Scientific, Inc., Edison, NJ, USA) at 37 °C for 18–24 h to reach cell concentrations of approximately 9 log CFU/ml.

2.2. Preparation of catfish mucus extract

Mucus extract was prepared as described by Shoemaker and LaFrentz (2015) with modifications. Mucus was collected from a local catfish processing plant in Mississippi. A lot size of 25 catfish were sampled. Each catfish (~250 g) was placed into a stomaching bag containing 10 mL of ice-cold sterile physiological saline, and the skin surface was massaged for 30s. Samples were then stored on ice for 2 min prior to collecting the mucus solution into sterile 50 mL tubes. Mucus samples were then transferred to the lab within 30 min. The tubes were centrifuged at 800 × *g* (Beckman, Model TJ-6 centrifuge, IL, USA) for 5 min at 4 °C to remove particulate fecal matter, autoclaved for 15 min at 121 °C and stored at −60 °C until used. The concentration of mucus protein was determined using the Bradford protein assay kit (Bio-Rad, U.S.A).

2.3. Evaluation of growth and survival of Salmonella in catfish mucus at different temperatures

One milliliter of each *Salmonella* culture that was grown overnight was centrifuged seperately at 9000 × *g* (Marathon 21000R, Fisher Scientific) for 5 min at 4 °C. The resulting cell pellet was resuspended in 1 mL of physiological saline to obtain 9 log CFU/ml of stationary phase cells. Serial dilutions were performed in sterile physiological saline to yield 3 log CFU/ml in pre-adjusted catfish mucus (375 and 15 μg/ml) concentrations and incubated at both room temperature (22 °C) and 10 °C (VWR, Scientific Products, Sheldon manufacturing, INC., OR, 97113). Cell numbers were enumerated every 7 d for 63 d. Each sample was serially diluted in sterile physiological saline and then plated on TSA (Tryptic soy agar) for *Salmonella* and incubated at 37 °C (Imperial III Incubator, labline instrument Inc., IL, USA) for 48 h to obtain colony forming units (CFU).

2.4. Biofilm formation and scanning electron microscopy (SEM)

The four different processing surfaces that were used in this study include stainless steel, buna-N rubber, ultra-high molecular weight polyethylene and thermoplastic polyurethane coupons. The description of these surfaces are provided in Dhowlaghar et al. (2018). Two different assays for biofilm formation were performed using scanning electron microscopy. The first assay evaluated the effect of mucus concentration on biofilm formation on stainless steel surface and at 22 °C for 48 h. The second assay evaluated biofilm formation using 375 μg/ml of catfish mucus on all four surfaces listed above at 22 °C for 48 h. The surfaces were cut into 1 × 1.5 cm coupons. Stainless steel coupons were autoclaved at 121 °C for 15 min to sterilize the coupons. Ultra-high molecular weight polyethylene, thermoplastic polyurethane and buna-N rubber coupons were surface sterilized with 70% alcohol for 15 min and subsequent rinsing with sterile water to eliminate the alcohol. Sterile coupons were dried for 20 min inside a biosafety cabinet prior to use. The coupons were placed in 24-well plates with one coupon per well. One ml of stationary phase grown *Salmonella* Blockley (7175) was centrifuged at 9000 × *g* for 5 min at 4 °C. The pellet was resuspended in 1 ml of sterile physiological saline. After resuspension, the culture was serially diluted in sterile physiological saline to 3 log CFU/ml that contained either pre-adjusted concentrations of 375 or 15 μg/ml of catfish mucus extract. Two ml was added into each well that contained a stainless steel coupon in a 24-well plate and incubated at 22 °C for 48 h. After incubation, non-adhered cells were removed by washing the coupons three times with a 2.75 ml aliquot of sterile physiological saline at 48 h of incubation. The cells that adhered to the coupon were quantified by transferring the coupon into a plastic tube (15 ml-Tornado™) that contained 5 ml of 0.1% peptone water with 0.02% tween-80 and 5 sterilized glass beads, vortexed for 1 min and then enumerated.

2.5. Scanning electron microscopic (SEM) analysis of biofilm on various food-contact surfaces

After 48 h of incubation, the coupons were removed from the well and washed with 3 mL of sterile saline to remove the medium and non-adhered cells. The coupons were then fixed overnight with Karnofskys solution (2.5% glutaraldehyde in 0.1M sodium