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Short communication

Biofilm formation of *Yersinia enterocolitica* and its persistence following treatment with different sanitation agents

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ABSTRACT

The biofilm formation by *Yersinia enterocolitica* was studied under conditions simulating a meat processing environment. *Y. enterocoltica*, especially biotype 4, readily formed biofilm in pork meat juice (MJ: meat juice). Biofilm was detectable after repeated treatment simulating the everyday cleaning routine which implies the potential to survive and persist in a pork processing environment. Sodium hypochlorite was more effective a sanitizer than quaternary ammonium compound (QAC: quaternary ammonium compound) in cleaning biofilm of *Y. enterocolitica*.

1. Introduction

Foodborne diseases are a growing public health problem worldwide (Schlundt, 2002). Yersiniosis due to infection with *Y. enterocolitica*, is the most frequently reported zoonotic gastrointestinal disease after campylobacteriosis and salmonellosis in many countries (Eurosurveillance Editorial, 2012). *Y. enterocolitica* species are heterogeneous, containing six biotypes - 1A, 1B, 2, 3, 4 and 5 (Wauters, Kandolo, & Janssens, 1987) with biotype 4 being the most common biotype associated with pig and human infection worldwide (Bottone, 1999). Some case-control studies identified the consumption of undercooked pork as one of the major risk factors in *Y. enterocolitica* infections (Boqvist, Pettersson, Svensson, & Andersson, 2009; Ostroff et al., 1994; Satterthwaite, Pritchard, Floyd, & Law, 1999). Pigs are asymptomatic carriers of human pathogenic *Y. enterocolitica*, frequently found in the tonsils and intestinal contents of clinically healthy pigs at slaughterhouses (Bhaduri, 2005; Fredriksson-Ahomaa, Hielm, & Korkeala, 1999). Evidence of the transmission of pathogenic *Y. enterocolitica* from the slaughterhouse environment to carcasses has been shown (Laukkanen et al., 2009).

Biofilms are a Food Safety concern as they increase the resistance of bacteria to many physical and chemical factors used in controlling hygiene in the food industry (Brown & Gilbert, 1993). The role of biofilms as a source of foodborne human pathogens is well established (Bridier et al., 2015). However, in case of *Y. enterocolitica* the role of biofilms and the factors leading to biofilm formation remain largely unknown.

In the food industry, chemicals are routinely used to sanitize and disinfect product contact surfaces. These chemicals provide a necessary and required step to ensure that the foods produced and consumed are as free as possible from microorganisms that can cause foodborne illness. According to AOAC Official Methods of Analysis, the sanitization standard for contamination reduction of food contact surfaces is generally accepted as 99.999% (a 5-log reduction) achieved in 30 s (Gaithersburg, 2012). Hypochlorites are the most widely used sanitizers due to their effectiveness and low cost. Hypochlorites kill microorganisms by damaging their outer membrane, losing permeability control and eventual cell lysis (Virto, Manas, Alvarez, Condon, & Raso, 2005). These compounds also destroy enzymes and DNA. However, hypochlorites cause concern, due to their effects on equipment and human health as they are corrosive to metals and may irritate skin and damage mucous membranes. Hypochlorites may cause environmental contamination because the chlorine compounds in hypochlorites can form toxic compounds after combining with organic materials

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(Cray, 2000). Therefore, hypochlorites are recommended for use at low concentrations (<200 ppm) (Pfunter, 2011). Quaternary ammonium compounds (QAC) have a relatively mild bactericidal effect compared to the hypochlorites but still function well as a sanitizer. They block the nutritional uptake of bacteria by binding with the acidic phospholipids in the bacterial cell wall (McBain, Ledder, Moore, Catrenich, & Gilbert, 2004; Pfunter, 2011). They are commonly used because they are usually noncorrosive and relatively nontoxic to users. Also, they are biodegradable hence pose less burden on the environment (Van Ginkel & Kolvenbach, 1991).

Bacteria may become resistant after repeated exposure to chemical treatment due to the inherent resistance of a particular bacterium or populations of bacteria. In addition, the development of a biofilm may provide additional resistance due to the physical protection from EPS that are associated with biofilm. This study aimed to investigate the potential of *Y. enterocolitica* biofilm, formed under conditions that are likely to happen in meat processing plant, to survive sanitizer treatments commonly used in the meat industry. Meat drip (juice) was used as the medium for biofilm development to represent the conditions in a meat processing plant. According to the Processed Meats Code of Practice in New Zealand (NZFSA, 2010), operators of meat processing plants are required to develop their own cleaning and sanitation programmes including detergents/sanitizers to be used, their concentrations, application methods and contact times. In this study, biofilm formation was monitored under an assumed cleaning regime in a pork processing plant over a period of five days. The effectiveness of sodium hypochlorite and QAC, were compared.

2. Methods and materials

2.1. Bacterial strains and culture preparation

A total of 16 *Y. enterocolitica* isolates recovered from food and provided by the Enteric Reference Laboratory in ESR (The Institute of Environmental Science and Research) New Zealand were used in this study (Table 1). Of the 16 *Y. enterocolitica* isolates, seven belonged to biotype 1A, one belonged to biotype 2, two strains were from biotype 3 and six from biotype 4.

The strains were pre-cultured in Tryptic soy broth (TSB) (Becton Dickinson, New Jersey) for 18 h at 22 °C. The cultures were harvested and washed twice by centrifugation (2400 rcf for 5 min). The pelleted bacteria was re-suspended in 0.85% NaCl to an optical density of 0.5 at 600 nm (equivalent to 10^8 CFU/ml) before use to inoculate either a 96-well microtitre plate (Falcon®, Corning Inc., USA) or stainless steel coupons for biofilm growth.

2.2. Preparation of meat juice (MJ)

Fresh pork meat was purchased from PAK'nSAVE (Palmerston North, New Zealand) in February 2015 and ground in a meat mincer. The pork mince was repeatedly put through a freeze/thaw cycle (−20 °C/4 °C) and MJ was collected during each thawing step until no more could be extracted. The MJ was centrifuged at 2400 rcf for 10 min to remove the large particles. The supernatant was adjusted to pH 6.8 using 0.1 mol NaOH and then was filter-sterilized using the Minsart® 0.45 μm micron syringe filter units before being stored at −80 °C.

2.3. Surface preparation for biofilm formation

Stainless steel coupons (grade 316, 2b, 1 cm dia, BioSurface Technologies Corporation, USA) were used in this study as it is the most common bench surface material used in the meat processing industry. The coupons were soaked in detergent (Trigene Advanced, 1%) for at least 30 min, washed with distilled water and dried before autoclaving for 15 min at 121 °C.

2.4. Microtitre plate biofilm assay

A microtitre plate assay was used to screen the biofilm formation by all 16 isolates in MJ and TSB at 22 °C as described by O'Toole (2011) with minor modification. A 20 μl volume of bacterial solution made to $A_{600} = 0.5$ was inoculated into 200 μl of media in each well and incubated for 24 h. The absorbance at 600 nm was measured to record bacterial growth using a spectrophotometer plate reader (SPECTROstar Nano, BMG LABTECH). To measure biofilm formation, the 200 μl of culture was drained and the plate was washed with distilled water three times and dried at 40 °C for 30 min. Then, the plate was stained with 0.5% crystal violet and incubated at room temperature for 15 min before being drained, rinsed and dried again as described above. Finally, the retained crystal violet in each well was solubilized using 95% ethanol and the microtiter plate was incubated at room temperature for 10–15 min before the absorbance at 595 nm was measured. The extent of biofilm formation was expressed as biofilm formation index (BFI) A_{595nm}/A_{600nm}.

The background from media was subtracted by including the result from un-inoculated MJ and TSB ($n = 4$). The average of A_{595nm} and twice the standard deviation was chosen as the cut-off value. Biofilm formation of each well was expressed as the difference between the tested A_{595nm} value and the cut-off value corresponding to its culture medium. Each stain in each culture medium was tested in quadruplicate.

2.5. Biofilm growth on stainless steel under a Simulated Meat Processing Regime

Stainless steel coupons were put into each well of a 24-well microtitre plate (Falcon®, Corning inc., USA) containing 2 ml MJ or TSB. Subsequently, 200 μl of designated bacterial suspension ($A_{600nm} = 0.5$) was inoculated into each well. The 24-well plate was incubated in a static culture at 22 °C for 24 h to allow biofilm formation. Then, the *Y. enterocolitica* biofilm formed on each stainless steel coupon was subjected to treatment with different sanitizers in a simulated daily cleaning regime for 5 days. Triplicate samples were taken on days 1, 3 and 5. The cell number on each coupon was enumerated by an impedance method established previously (Wang, Palmer, & Flint, 2016). All cell enumerations for this study were performed using method described by Wang et al. (2016) in which the time taken for bacteria to alter the media impedance by

Table 1
Y. enterocolitica isolates from food used in this study (Provided by ESR, Porirua, New Zealand).

Biotypes	Strain number
1A	ERL072345, ERL072346, ERL072347, ERL073947, ERL082059, ERL093846, ERL10460
2	ERL032126
3	ERL104253, ERL112277
4	ERL032122, ERL032123, ERL032124, ERL032125, ERL072344, ERL114165