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# Mixed culture biofilms of *Salmonella* Typhimurium and cultivable indigenous microorganisms on lettuce show enhanced resistance of their sessile cells to cold oxygen plasma

Iqbal Kabir Jahid [a, b], Noori Han [a], Cheng-Yi Zhang [a], Sang-Do Ha [a, *]

[a] School of Food Science and Technology, Chung-Ang University, 72–1 Nae-Ri, Daedeok-Myun, Anseong, Gyunggido 456–756, South Korea
[b] Department of Microbiology, Jessore University of Science and Technology, Jessore 7408, Bangladesh

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ABSTRACT

Control of foodborne pathogens in fresh produce is crucial for food safety, and numerous *Salmonella* Typhimurium (ST) outbreaks have been reported already. The present study was done to assess effectiveness of cold oxygen plasma (COP) against biofilms of ST mixed with cultivable indigenous microorganisms (CIM). ST and CIM were grown at 15 °C as monocultures and mixed cultures for planktonic state, biofilm on stainless steel, and lettuce leaves. Thereafter, the samples were treated with COP and surviving populations were counted using plate counting methods. Biofilms and stomatal colonization were examined using field emission scanning electron microscopy (FESEM) and food quality was assessed after treatment. Mixed cultures of ST and CIM showed an antagonistic interaction on lettuce but not on SS or in planktonic state. Mixed cultures showed significantly ($p < 0.05$) greater resistance to COP compared to monoculture biofilms on lettuce but not on SS or planktonic state. Shift from smooth to rugose colony type was found for planktonic and for biofilms on SS but not on lettuce for ST. Mixed culture biofilms colonized stomata on the inside as demonstrated by FESEM. Although, lettuce quality was not affected by COP, this technology has to be optimized for further development of the successful inactivation of complex multispecies biofilm structures presented by real food environment.


## 1. Introduction

Fresh produce is an important source of nutrients, vitamins, and fiber for human sustenance (Anonymous, 2013). The incidence of foodborne disease, including salmonellosis, linked to fresh produce has risen worldwide during the last few years because of high consumption, changing consumer habits, and broader worldwide distribution (FDA, 2013). In the USA alone, 1527 outbreaks were identified between 2009 and 2010, resulting in 29,444 cases of illness with 1118 hospitalizations and 23 deaths (MMWR, 2013). *Salmonella* spp. was the most common bacterial pathogen reported during this period, accounting for 30% of foodborne illnesses (MMWR, 2013). *Salmonella enterica* serovar Typhimurium (ST) is one of the most clinically important foodborne pathogens, with 6 outbreaks caused by ST in the USA in 2013 (CDC, 2013). The US Department of Agriculture Economic Research Service reported 1.4 million cases of salmonellosis in 2010, costing 2.65 billion dollars and resulting in 415 deaths (USDA-ERS, 2010). The European Food Safety Authority (EFSA, 2013) reported over 100,000 human cases of illness due to *Salmonella* sp., costing approximately €3 billion annually.

Biofilms are a 3-dimensional sessile community of microorganisms attached to a solid surface shielded inside an endogenously produced extracellular polymeric substance (EPS) secreted by the colony, representing a common mode of microorganism growth in natural and industrial settings (Agle, 2007). Biofilm production on the surface of produce is a common phenomenon under natural conditions (Rayner et al., 2004), and 10–80% of the total natural population of leaves contains biofilms (Morris et al., 1998; Lindow and Brandl, 2003). Rayner et al. (2004) also identified natural biofilms on fresh tomatoes, carrots, and mushrooms.

Artificially seeded biofilms of foodborne pathogens and methods for their reduction have been extensively studied and reviewed (Niemira and Cooke, 2010; Ölmez and Temur, 2010; Patel and Sharma, 2010; Jahid and Ha, 2012; Olaimat and Holley, 2012). The results of these studies indicate that foodborne pathogens in

* Corresponding author. School of Food Science and Technology, Chung-Ang University, 72–1 Nae-Ri, Daedeok-Myun, Anseong, Gyunggido 456-756, South Korea. Tel.: +82 031 670 4831; fax: +82 031 675 4853.
*E-mail address:* sangdoha@cau.ac.kr (S.-D. Ha).

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the form of biofilms are resistant to common disinfectants, and therefore represent a substantial food safety problem (Jahid and Ha, 2012).

Most of the clinical, environmental, and food sample studies on biofilms conducted thus far have examined pure culture biofilms; however, such biofilms are uncommon in regular food. Data obtained thus far on mixed culture biofilms in food have been reviewed previously (Manuzon and Wang, 2007), and the complex interactions between cell populations forming biofilms have been discussed elsewhere (Moons et al., 2009). In nature, certain microorganisms first colonize/attach to the surface, and other microbes then follow to form biofilms (Kolenblander, 2000). Compared to monocultures on food contact surfaces, mixed culture biofilms are more resistant to common disinfectants (Behnke et al., 2011; Behnke and Camper, 2012; Kostaki et al., 2012; van der Veen and Abee, 2011; Ibusquiza et al., 2012; Jahid et al., 2014b).

Currently, there are no disinfectants that can achieve a 5-log reduction of foodborne pathogen biofilms on fresh produce; this state of affairs poses a major food safety problem (Jahid and Ha, 2012). Biofilms formed by foodborne pathogens are resistant to many disinfectants, particularly biofilms consisting of mixed culture. Novel alternative methods are therefore needed to address this growing health concern. Cold oxygen plasma (COP) has been used successfully to disinfect various foods such as lettuce (Fernández and Thompson, 2012; Jahid et al., 2014a), mangoes (Perni et al., 2008), melons (Perni et al., 2008), apples (Critzer et al., 2007), and cantaloupe (Critzer et al., 2007). Few studies, however, have tested the ability of COP to combat biofilms (Vleugels et al., 2005).

Many bacteria, such as *Vibrio cholerae* (Yildiz and Schoolnik, 1999), *Vibrio parahaemolyticus* (Chen et al., 2010), *Escherichia coli*, and ST (de Rezende et al., 2005) alter their phenotypes and shift from the smooth to rugose colony type and *vice versa*. Shifts from smooth to rugose variants can be induced by exposure to adverse conditions such as nutrient deprivation (Ali et al., 2002), temperature (Anriany et al., 2001), and predation (Matz et al., 2005). In general, compared to the smooth type, rugose colonies form thicker biofilms and exhibit lower motility, higher resistance to chlorine, and greater exopolysaccharide formation (Yildiz and Schoolnik, 1999).

Although microbial inactivation on food and food contact surfaces can easily be described using linear regression analysis, most disinfectants have inactivation kinetics that are nonlinear; i.e., microbial survival curves exhibit upward or downward concavity and a sigmoid shape (Mafart et al., 2002; Buzrul and Alpas, 2007; van Boekel, 2002). Accordingly, many authors have used nonlinear modeling methods, such as logistic models (Cole et al., 1993), the Gompertz equation (Veen and Abee, 2011), the Weibull model (Buzrul and Alpas, 2007), and the Fermi equation (Peleg, 1996). Many researchers have employed the Weibull model for analyzing the inactivation kinetics of planktonic (Buzrul and Alpas, 2007; Chun et al., 2010; van Boekel, 2002) and biofilm populations (Vaid et al., 2010), whereas others have modified the Weibull model to fit the data (equation) on foods and on the food industry (Mafart et al., 2002; Albert and Mafart, 2005; Coroller et al., 2006). GInaFiT 1.6 software is available free of charge (http://cit.kuleuven.be/biotec/downloads.php) and is easy to use for analyses of various linear and nonlinear equations in Microsoft Excel, including the Weibull model (Geeraerd et al., 2005). This software has previously been applied to analysis of biofilms on stainless-steel (SS) surfaces (Posada-Izquierdo et al., 2013).

The present study focuses on the inactivation kinetics of mixed culture ST and cultivable indigenous microorganisms on lettuce in the state of planktonic growth and in biofilms. The experiments were conducted either on SS coupons or on lettuce treated with COP; the data were analyzed using the Weibull model in addition to assessment of a shift to the rugose phenotype.

## 2. Materials and methods

### 2.1. Experimental design

Indigenous microorganisms present on the surface of lettuce were isolated from cleaned lettuce leaves, and the isolated strains were representative of cultivable indigenous microorganisms (CIM). Lettuce juice broth (LJB) was then prepared by blending and filtering using a 0.22-μm filter, and used to grow either planktonic cells or biofilms on SS coupons. Lettuce leaves were cut to the same size and background flora was removed. Bacterial inocula of CIM and ST were then prepared and seeded as monocultures and mixed cultures for either planktonic growth or biofilm formation on SS coupons and lettuce leaves. After incubation and washing, the samples were treated with COP at different exposure times, and the inactivation kinetics were determined. Smooth and rugose colonies were counted and photographed. Field emission scanning electron microscopy (FESEM) was used to examine biofilm formation on SS coupons and on lettuce leaves and stomatal colonization on lettuce leaves. The color and texture were recorded to assess food quality of the lettuce after COP treatment. The entire experimental design is outlined in Fig. 1.

### 2.2. Isolation of cultivable indigenous microorganisms; media and growth conditions

Fresh iceberg lettuce (*Lactuca sativa*) was purchased from a local grocery store in Anseong, Republic of Korea on the day of harvest, and transported to the laboratory within 30 min under refrigerated conditions. The core and outer 2 layers were discarded by hand, and the internal parts were cut into 5 × 3 $cm^2$ sections using a sterile scalpel. The resulting lettuce coupons were washed with sterile distilled water (SDW) to remove unattached cells, and then immersed in Dulbecco's phosphate-buffered saline (DPBS; Sigma−Aldrich, Inc.; St. Louis, MO) and incubated at 15 °C or 20 °C for 24 h. Microorganisms were then plated on brilliant green agar (BGA; Difco; Becton Dickinson, Franklin Lakes, NJ) with 25 μg/mL of nalixic acid (NA) and 25 μg/mL of novobiocin (NO) to identify ST-like colonies present in the samples.

ST-negative samples were then mixed together and the solution was termed CIM. ST used in this study was obtained from Chemical Regulation and Food Safety Center, Exponent, Inc. (Bowie, MD, USA) .Ha et al., 1995) which is resistance to NO and NA. The strain was selected for the study because of its properties of resistance to both antibiotics which was used to differentiate from CIM. All the strains were preserved at −70 °C in $T_1N_1$ broth (1 g tryptose/100 mL and 1 g NaCl/100 mL) containing 15% glycerol, and one cryovial was thawed and resuspended in tryptic soy broth (TSB); incubation for 24 h for 20 °C at 220 rpm was used in each experiment.

### 2.3. Preparation of lettuce and reduction of background flora

Coupons were used immediately after preparation or stored at 4 °C until use. To reduce background flora, coupons were placed on sterile petri dishes and treated with COP for 5 min on both sides. Sterility of lettuce coupons was then confirmed according to procedures described in Section 2.10. The results of this procedure showed that background microflora was removed successfully by COP, with counts below the detection level of 1 log CFU/mL or $cm^2$.