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Effectiveness of chlorine wash on *Listeria monocytogenes* biofilm on onions

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ABSTRACT

An investigation into the effectiveness of chlorine wash on *Listeria monocytogenes* biofilm on onions was carried out. Whole white onion bulbs were purchased from a retail shop at Nottingham, UK, and a strain of *L. monocytogenes* (NCTC 11944) was obtained from Food Microbiology Laboratory, University of Nottingham, UK. Chlorine treatment on *L. monocytogenes* biofilm was done according to standard method. Using different chlorine concentrations (20, 50, 100, 150 and 200 mg/l) for different time durations (0.5, 1, 2, 5, 10, 15 and 20 minutes) at pH 6.5, inoculated onion pieces were treated. Bacterial enumeration was done using serial dilution and spread plate method on chromogenic *Listeria* selective plates. Reduction of *L. monocytogenes* biofilm ranged from 1.5 – 2.4 $\log_{10}$ with the maximum reduction observed with 200 mg/l chlorine at 20 minutes treatment time. Similar treatment of *L. monocytogenes* in suspension (planktonic cells) gave up to 5 log reduction when 200 mg/l chlorine was applied for 20 minutes. The effectiveness of chlorine was more on *L. monocytogenes* in suspension than as a biofilm on onion.

Keywords: Chlorine, biofilm, *Listeria monocytogenes*, onion, inoculation.

INTRODUCTION

Listeria monocytogenes is a food-borne pathogen of public health significance and economic importance to the food industry with a death rate of about 20% (Rocourt et al., 2000). *L. monocytogenes* is an opportunistic pathogen and can cause listeriosis in the immuno-compromised, elderly, infants and pregnant women (Montville and Karl, 2008). It has been shown that vegetables are a major route of transmission of *L. monocytogenes* to man. The first documented incidence of listeriosis, due to contaminated coleslaw, occurred in 1981 at Canada where 18 out of the 41 cases led to death (Schlech et al., 1983). *L. monocytogenes* has extreme resistance to adverse environmental conditions and this makes it a major concern for the food industry as it is able to persist as biofilms on surfaces and contaminate finished products.

The prevalence of contamination of foods by *L. monocytogenes* in fresh vegetables is 11%, raw meat (13%), dairy product (3%) and seafood (3%) (Juneja and Sofos, 2010). A zero-tolerance in 25 g sample of food is the regulation in the United Kingdom and United States and Italy. However, most European countries like Germany have a regulation of <100 CFU/g for foods that would not support

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the growth of *L. monocytogenes* and is not meant for susceptible populations (Birgit, 2000; Montville and Karl, 2008).

L. monocytogenes is a major concern in minimally processed vegetables with extended shelf-life because it is able to survive at refrigeration temperatures and increase in numbers during storage. The presence of *L. monocytogenes* in minimally processed vegetables has been reported (Froder et al., 2007; De Curtis et al., 2002). Though there is limited information about *L. monocytogenes* in onions, it has been shown that *L. monocytogenes* will grow and/or survive in most fresh produce (Porto and Uboldi, 2001). Onion, *Allium cepa*, belongs to the family *Allium* together with other vegetables such as garlic and leek (James, 2008). Contamination of onions with *L. monocytogenes* can occur at any step of the food chain from field to harvesting to factory processing, transportation to point of sale (Inatsu et al., 2010).

Biofilms are resistant to disinfecting agents, act as a reservoir for recontamination and once established can be very difficult to get rid of. *L. monocytogenes* is able to form biofilms and this limits the effectiveness of chlorine wash because of their ability to form adherent microcolony cells on surfaces of produce (Olmez and Kretzchmar, 2009).

Fresh-cut industries usually rely on washing with chemical disinfectants to produce safe end products with extended shelf-life. Produce washing is aimed at removing dirt, microorganisms and cell exudates that encourage their growth as well as pesticide residues (Gill et al., 2009). Chlorine is the most commonly used in fresh-cut industries because it is relatively inexpensive, has a wide spectrum application and is effective when correctly applied. The lack of understanding of key areas of chlorine chemistry, for example the control of pH, has resulted in less than maximum effectiveness (Gill et al., 2009). Chlorine effectiveness is also affected by organic matter and temperature. An industry standard of 50-200 ppm free chlorine is the established standard in the washing of commercial fruits and vegetables (Haute et al., 2013). Kim (2007) has noted however that though surface decontamination of fresh vegetables can improve shelf-life and product quality, it cannot be depended upon to totally remove pathogenic microorganisms present. Strict hygienic measures and HACCP must be in place at all times to ensure safety of vegetables. The aim of this project was to investigate the effectiveness of chlorine wash on *L. monocytogenes* as a biofilm on onion and as free-living in culture.

MATERIALS AND METHODS

A strain of *Listeria monocytogenes* (NCTC 11944) was obtained from the Food Microbiology Research Laboratory, Food Science Division, University of Nottingham, UK. Chlorine (NaOCl) granules were obtained from a UK-based company specializing in the supply of fresh fruits and vegetables. Onion samples were purchased from an open market at Nottingham, UK. The experiments were carried out at the Food Microbiology Laboratory, University of Nottingham, UK.

Chlorine treatment of L. monocytogenes-inoculated onion

This was done according to the method of Park et al. (2008) and it is as follows:

Preparation of inoculum

An overnight Brain Heart Infusion (BHI) broth culture of *L. monocytogenes* was centrifuged at 4000 x g for 30 minutes at 4 °C. Pelleted cells were re-washed trice in 5 ml buffered peptone water (Oxoid Ltd, UK) and re-suspended in 5 ml buffered peptone water to produce a culture of approximately 10^8 CFU/ml. Initial inoculum count was

enumerated by plating on chromogenic *Listeria* selective medium.

Preparation of inoculated onions

Onions were cut into roughly 5 x 5 cm pieces using a sterile scalpel. 10 g of onions were washed in sterile deionised water, placed on a sterile aluminium foil and allowed to dry in a laminar flow biological safety cabinet. Using a micropipettor, 0.1 ml of previously prepared and standardized (10^8 CFU/ml) *L. monocytogenes* culture was applied as 30 droplets on onion surface. Inoculated onion pieces were left to dry in the biological safety cabinet for 4 hours to allow the attachment of bacteria.

Treatment with chlorine

Onion pieces were dipped into various chlorine concentrations (20, 50, 100, 150 and 200 mg/l) for different treatment times (0.5. 1, 2, 5, 10, 15 and 20 minutes). Control was prepared by dipping into sterile deionized water. After each treatment time, each 10 g treated onion was immediately transferred to a stomacher bag and homogenized in 50 ml neutralizing broth (Difco) using a stomacher.

Bacterial enumeration

One **(**1) ml of stomached sample was serially diluted in 9 ml of buffered peptone water. Dilutions 10^{-4} and 10^{-3} (0.1 ml) were spread plated unto chromogenic *Listeria* selective plates and incubated at 37 °C for 24-48 hours after which colonies were counted.

Treatment of *L. monocytogenes* pure culture with chlorine was also carried out for effective comparison between *L. monocytogenes* in a biofilm and in free-living state.

An overnight broth culture of *L. monocytogenes* was prepared by incubating in a shaker at 37 °C (concentration of 10^8 CFU/ml). Chlorine solutions were prepared by dissolving Sodium hypochlorite (NaOCl) granules in sterile water to give different concentrations: 20, 50, 100, 150 and 200 mg/l and pH adjusted to 6.5 using 25% HCl. Broth culture was serial diluted (10-fold) with each chlorine solution. Using the Miles and Misra (1938) method, 0.01 ml of dilutions 10^{-5} and 10^{-6} were plated in triplicate on Brain Heart Infusion (BHI) plates, allowed to dry and incubated at 37 °C for 24±2 hours.

Tests were carried out in triplicates and Analysis of Variance (ANOVA) was performed using SPSS software, 15.0 version. Significant differences between results were determined at $P < 0.05$.

RESULTS

For the treatment of onions inoculated with *L. monocytogenes,* reduction of *L. monocytogenes* ranged from 1.5 – 2.4 $\log_{10}$ with the maximum reduction observed with 200 ppm chlorine at 20 minutes and minimum at 20 ppm at 0.5 minute treatment time. This is shown in Figures 1 and 2. Figure 1 shows an increasing gradient of log reduction as both time and concentration increase (significant at $P<0.05$).

Figure 2 represents survival curves for *L. monocytogenes* on inoculated onion surface. Beginning at log 8, different concentrations of chlorine wash water (ppm) and sterile water (control) and for varying treatment times (minutes) were used to treat *L. monocytogenes* on onions and least survival was observed at 200 ppm for 20 minutes (log 5.6). Primary reduction was observed within the first 30 seconds of exposure for all concentrations.

Results for the treatment of *L. monocytogenes* pure culture with varying concentrations of chlorine showed no growth in the 10^{-4} and 10^{-5} dilution plates for all concentrations depicting up to 5 log reduction.

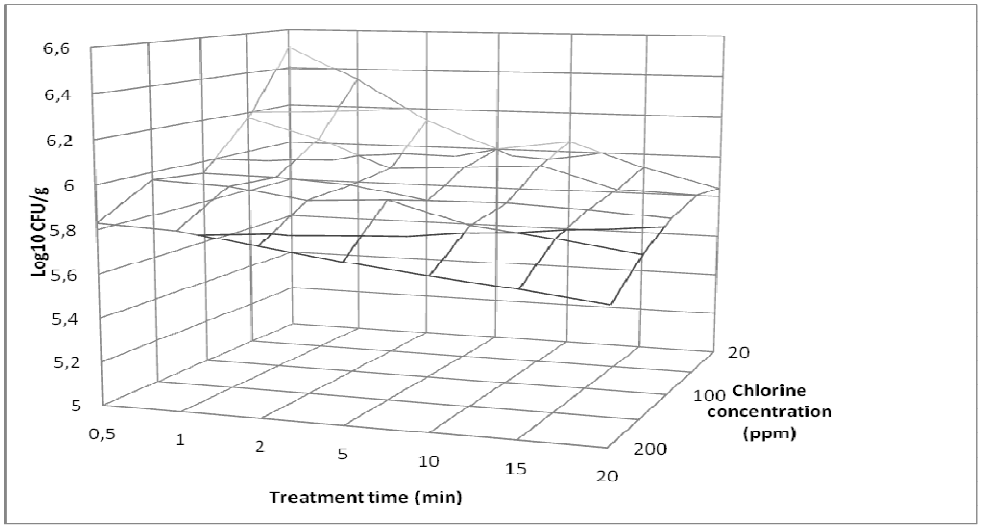

Figure 1: Reduction of *L. monocytogenes* on onions with chlorine treatment at various times.
A decreasing gradient of $\log_{10}$ CFU/g of L. monocytogenes is illustrated as both chlorine concentration and as time exposure increases. Highest $\log_{10}$ CFU/g (6.5) is seen at 20 ppm for 0.5 min and this decreases until the least log value (5.6) at 200 ppm for 20 min.

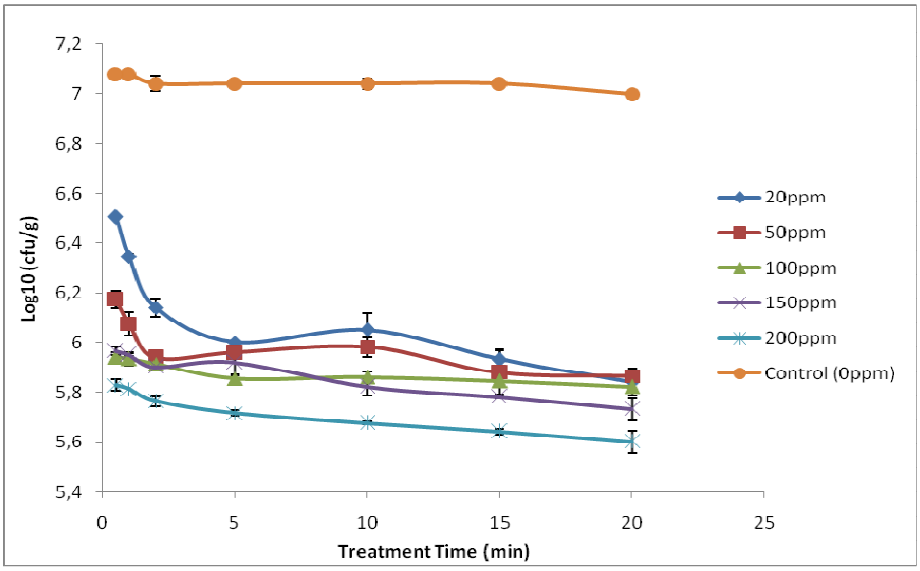

Figure 2: Survival curves for *L. monocytogenes* on inoculated onion surface.
Beginning at log 8, different concentrations of chlorine wash water (ppm) and sterile water (control) and for varying treatment times (min) were used to treat *L. monocytogenes* on onions. Least survival is observed at 200 ppm for 20 min (log 5.6). Log reduction occurred with increasing chlorine concentration and time (significant at $P<0.05$). Primary reduction is observed within the first 30 s of exposure for all concentrations.

DISCUSSION

From Figure 1, the effectiveness of the reduction of *Listeria monocytogenes* biofilm on onion surface increased with increasing chlorine concentration and time ranging from 1.5 – 2.4 $\log_{10}$. It has been reported that chlorine wash on fresh cut vegetables usually give 1 – 2 log reductions (Beuchat et al., 2004; Sapers, 2001). It is observed from Figure 2, that the greatest part of reduction occurred during the first 30 seconds of treatment for all concentrations. Added treatment time showed further reduction but minimal in comparison with the initial 30s. Kim et al. (2000) reported antimicrobial effect of chlorine on *L. monocytogenes* within the first 30 seconds of contact.

Treatment with water (control) at various treatment times gave a maximum of 1 log reduction. According to Beuchat et al. (2004), a vigorous wash in potable water can give a 1-2 log reduction of *L. monocytogenes*.

Results from the treatment of *L. monocytogenes* as a pure culture suggest the effectiveness of chlorine against *L. monocytogenes* in suspension (planktonic form) up to a 5 log reduction.

It is observed from the results of this research that *L. monocytogenes* was more resistant to the sanitizing effect of chlorine as a biofilm on cut onion surface than as a pure culture in suspension. Moreto and Langsrud (2004) noted that *L. monocytogenes* shows a significant resistance to disinfection as a biofilms than in the free-living state and that thick complex biofilms are more challenging to detach than adherent single cells. In biofilms, microorganisms are able to attach firmly to the vegetable surface because of embedding of cells into inaccessible parts (Olmez and Kretzchmar, 2009; Gill et al., 2009). It has been observed that the presence of biofilms on vegetables significantly affects the effectiveness of disinfectants while *L. monocytogenes*, in suspension, shows little or no resistance to mostly used disinfectants. This is especially relevant in the case of minimally processed vegetables requiring no further processing before consumption. The need for stricter hygiene measures and effective implementation of HACCP and GMP from farm to fork is emphasized to prevent contamination of onions by *L. monocytogenes*.

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