Effect of attachment time followed by chlorine washing on the survival of inoculated *Listeria monocytogenes* on tomatoes and spinach

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ABSTRACT

The effect of attachment time (30 min, 24, 48 & 72 h) followed by chlorine washing (200 ppm) on the survival of inoculated *Listeria monocytogenes* on the surface and sub-surface of tomatoes and spinach were studied. The work was done to determine the efficacy of chlorine to decontaminate surface and sub-surface pathogens that may have come into contact with produce during pre-harvest. Tomatoes and spinach leaves were inoculated with a 6 log cfu/ml 18 h culture of *L. monocytogenes* ATCC 7644 (LM) on the surface and sub-surface and incubated at 20 ^{o}C for either, 30 min, 24, 48 or 72 h. LM attached and survived on the surface and sub-surface structures of both control and chlorine washed vegetables after each attachment time, up to 72 h. Higher levels of LM attachment and survival was however noticed on the sub-surface structures. Chlorine had a greater effect on the LM on the surface structures compared to those in the sub-surface structures, possibly because chlorine was not able to access the sub-surface structures where the pathogens were located. Chlorine was not effective in totally inactivating the surface LM on spinach and tomato. This research indicated that LM could attach to both surface and sub-surface structures of both tomatoes and spinach, within 30 min, and that even after 72 h, it still remained viable.

Keywords: *Listeria monocytogenes*, inoculation, surface, sub-surface, spinach, tomato, chlorine, survival, attachment time.

Practical Application: This study will inform the produce industry on the ability of *L. monocytogenes* ATCC 7644 to attach and grow on the surface and sub-surface structures of tomato and spinach during pre-harvest and post-harvest. More attention should be given to this phenomenon because the use of fresh or minimally processed fruits and vegetables are recommended as part of a healthy diet. It also indicates that minimal processors should avoid using vegetables with wounds since *L. monocytogenes* attached more to the sub-surfaces structures of the produce. Moreover, the use of sanitizers such as chlorine is less effective under these conditions. It has also brought to light the inability of chlorine to effectively decontaminate pathogens. This will make it mandatory for the industry to implement preventive measures i.e., Hazard Analysis Critical Control Point (HACCP), Good Agricultural Practice (GAP) and Good Hygiene Practice (GHP) instead of corrective measures.

INTRODUCTION

A major pre-harvest source of contamination of produce is irrigation water (Beuchat & Ryu, 1997; Beuchat, 2002). Ibenyassine *et al.,* (2006) reported that contaminated irrigation waters and surface run-off waters are the major sources of pathogenic microorganisms that contaminate fruits and vegetables. Steel *et al.,* (2005) carried out a survey on 500 irrigation water samples used for production of fruit and vegetables in Canada and found about 25 % of the samples to be contaminated with faecal *E. coli* and faecal *Streptococci*. Surface water when used to irrigate produce poses a health risk of contamination with *Salmonella* (Johnson *et al.,* 1997). Most surface waters were also found to be contaminated with *Listeria.* Combarro *et al.,* (1997) frequently isolated *Listeria* species from river water in Spain. Pathogens in irrigation water can attach to the surface of vegetables during pre-harvest (Ijabadeniyi *et al.,* 2008; Solomon *et al.,* 2006; Kenney & Beuchat, 2002). Different workers have shown that attachment of *Listeria monocytogenes* is possible through the release of an enzyme to surrounding host tissue or produce to facilitate bacterial attachment and infiltration (Hall-Stoodley & Stoodley, 2005; Jedrzejas, 2001). Production of extracellular fibrils and flagellin have also been reported to be used by *Listeria monocytogenes* to enhance attachment (Kalmokoff *et al*., 2008; Lemon *et al*., 2007) After attachment, they can gain access to the sub-surface structures through natural openings and wounds on

vegetable surfaces; a process called internalization (Warriner *et al.,* 2003; Bartz, 2006; Solomon *et al.,* 2006). Internalization is possible because of the natural openings such as stem scars, stomata, lenticels, root systems and broken trichomes (Quadt-Hallman *et al.,* 1997; Allen *et al.,* 1990), as well as due to damage of the waxy cuticles on the plant tissues (Solomon *et al.,* 2006).

Chlorine is routinely used as a sanitizer in wash, spray and flume waters in the fresh and minimal processed fruit and vegetable industries (Fonseca, 2006 & Bhagwat, 2006). Antimicrobial activity depends on the amount of free available chlorine or hypochlorous acid in water that comes into contact with microbial cells (Beuchat & Ryu, 1997; Beuchat, *et al.,* 1998). The concentration normally used is between 50-200 ppm and the contact time is 1- 2 min (Beuchat, 1998). In South Africa, sodium hypochlorite is commonly used to sanitize fresh vegetables (Clasen & Edmondson, 2006).

Antimicrobial agents, such as chlorine, hydrogen peroxide, ozone are not effective in completely eliminating all the bacteria on the surface of plants or vegetables (Solomon *et al.,* 2006; Doyle & Erickson, 2008). Internalization is a major problem in the fresh produce industry because pathogens present within the sub-surface structures of plant or vegetable are protected from the sanitizing effect of antimicrobial agents such as chlorine, hydrogen peroxide, ozone (Solomon *et al.,* 2006; Doyle & Erickson, 2008).

Although a lot of research work has been reported on the ability of pathogens like *E. coli* O157: H7 and *Salmonella* spp. to attach and gain access to the sub-surface structures of vegetables; not many reports have focused on *L. monocytogenes* (Beuchat, 1996). *L. monocytogenes* has the potential to cause human listeriosis after consumption of contaminated raw vegetables (Beuchat, 1996). *L. monocytogenes* has the ability to overcome food preservation and safety barriers such as refrigeration temperature, low pH and high salt concentration (Gandhi & Chikindas, 2007; Gorski *et al.,* 2005; Brandl, 2006). B*r*occoli, cabbage, salad greens and other vegetables pose even a higher risk of being associated with listeriosis because of enhanced *L. monocytogenes* attachment (Ijabadeniyi *et al.,* 2009; Ukuku *et al.,* 2005; US

FDA/CFSAN, 2008). Attachment and growth on some produce including spinach has been reported (Gorski *et al.,* 2004; Jablasone *et al.,* 2005)

The aim of this study was therefore to determine the effect of attachment time on the survival of *L. monocytogenes* on the surface and sub-surface structures of tomatoes and spinach. Subsequently, the effect of chlorine on the sub-surface and surface of *L. monocytogenes* on tomatoes and spinach after harvest was determined

MATERIALS AND METHODS

Reference strain

Listeria monocytogenes ATCC 7644 (LM) was obtained from the Agricultural Research Council, Irene, South Africa. The strain was cultured in Fraser Broth (FB) (Oxoid Ltd; Basingstoke, Hampshire, England) for 24 h at 37 °C and then stored at 4 °C. The working stock culture was sub-cultured into FB twice a month.

Tomatoes and spinach

Fresh tomatoes and spinach were purchased from a retail outlet on three separate occasions in Pretoria (South Africa). Tomatoes and spinach were examined and those with visual defects were not used. Tomatoes and spinach were washed with 70 % alcohol and tested for the presence of LM.

Inoculation of surface and subsurface structures of tomatoes with *L. monocytogenes* ATCC 7644

A 6 log cfu/ml, 18 h culture of LM, determined using McFarland standards (Andrews, 2005), was used as inoculum for all the experiments. This method uses optical density to determine titer. Eight tomatoes were inoculated on the surface and 8 within the sub-surface per experimental repetition. The experiment was repeated three times. To inoculate the tomatoes within the sub-surface structures, wounding was first simulated at 5 locations per tomato by using a sterile 1 ml plastic pipette tip, according to the method of Walderhaug *et al.* (1999). Five locations on the tomatoes were inoculated with 0.2 ml LM, to allow for even distribution of the inoculum into the tomato (Walderhaug *et al.,* 1999). To inoculate the surface of the tomatoes 1 ml of LM was

released over the surface of each tomato with a sterile pipette. Tomatoes were brought into contact with roll-off liquid on the sterile inoculating dish, using sterile tweezers, to assure that roll-off liquid was absorbed onto the tomato surface

Inoculation of surface and subsurface structures of spinach with *L. monocytogenes* ATCC 7644

Eight spinach leaves were inoculated on the surface and 8 within the sub-surface per experimental repetition. To inoculate the spinach on the sub-surface structures, a sterile needle was used to make a thin line in-between the leaf petiole (stem of a leaf) and 1 ml of the LM culture was introduced across the thin line (Walderhaug *et al.,* 1999). To inoculate the surface of spinach leaves, a sterile pipette was used to release 1 ml of the LM culture over its surface while the leaves were lying flat. After inoculation, they were allowed to attach and extent of attachment of LM was studied.

Chlorine washing of inoculated vegetables

After attachment of LM for 30 min, both surface inoculated and sub-surface inoculated tomatoes were washed by dipping into 200 ppm of chlorine for 1 min (Beuchat, 1998). The control was washed by dipping in distilled water. To disallow tomatoes from floating during washing, sterile tweezer was used to submerge the tomatoes in the chlorine water. The procedure was repeated for the treated and control samples after attachment of LM for 24, 48, and 72 h respectively.

After attachment of LM for 30 min, both surface inoculated and sub-surface inoculated spinach leaves were washed by dipping into 200 ppm of chlorine for 1 min (Beuchat, 1998). The control was washed by dipping in distilled water. The procedure was repeated for the treated sample and control after attachment of LM for 24, 48, and 72 h respectively.

Enumeration of *L. monocytogenes* ATCC 7644 on the surface and sub-surface structures of vegetables

To enumerate the number of LM on tomatoes, at each attachment time interval, on the surface and within the sub-surface, 100 g of tomato was added to 900 ml of distilled water after which maceration in stomacher lab-blender 400 (Fisher Scientific,

Mississauga, Canada) and plating on Palcam agar (Oxoid Ltd; Basingstoke, Hampshire, England) were done. Enumeration of LM was done with pour plate method.

To enumerate the number of LM on spinach leaves at each attachment time interval on the surface and within the sub-surface, 10 g of spinach leaf was added to 90 ml of distilled water after which maceration in stomacher lab-blender 400 (Fisher Scientific, Mississauga, Canada) and plated on Palcam agar (Oxoid Ltd; Basingstoke, Hampshire, England) were done. Enumeration of LM was done with pour plate method.

Preparation and observation of specimens for SEM

Pieces of tomato/spinach (about 2 by 2 mm area and 0.5 mm thickness) were gently cut off the inoculated surface of each tomato/spinach sample using a sterile blade. The cut pieces were fixed overnight in 4% glutaraldehyde, and rinsed twice with 0.1 M sodium phosphate buffer pH 7. 0. The samples were further fixed in 2% osmium tetroxide for 1 h and rinsed twice with 0. 1 M sodium phosphate buffer. Fixed samples were dehydrated in a graded ethanol series (30%, 50%, 70% and 100%). All procedures through dehydration were carried out at about 48C. The samples were dried in a LADD Critical-Point drier (LADD Research Industries, Inc., Burlington, Vermont, USA) with CO_2 as the transition gas. They were then mounted on specimen stubs and coated with approximate 30 nm layer of gold-palladium using a Hummer I sputter coater (ANATECH, LTD, Springfield, Virginia, USA). The samples were examined with a JEOL JSM-840 scanning electron microscope (JEOLUSA Inc., Peabody, Massachusetts, USA) at an accelerating voltage of 5 KV. Digital micrographs were collected at a resolution of 1280 x 960 and dwell time of 160 s. The digital images were adjusted using Adobe PhotoShop 5.0 and printed with a Codonics 1660 dye sublimation/thermal printer (Codonics, Inc., Middleburg Heights, Ohio, USA) using the thermal method.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether there was significant difference between the following factors inoculation site (surface vs. sub-surface),

chlorine and attachment time. The experiment was repeated three times (n=3). ANOVA was performed using Statistica software from windows version 7 (Tulsa, Oklohama, USA, 2003).

RESULTS

Effect of attachment time followed by chlorine washing on the survival of inoculated *Listeria monocytogenes* on tomatoes

Effect of attachment time

Attachment time, significantly ($p \leq 0.05$) affected the LM count on the surface and sub-surface structures of tomatoes (Table 1). LM attached and survived on the tomato after each attachment time. The level of LM that survived and attached on the surface of tomato was lowest after 24 h (3.81 log cfu/g) and highest after 72 h (4.78 log cfu/g) (Fig 1). The level of LM that survived and attached on the sub-surface of tomato was at similar levels after 30 min, 24 and 48 h, but increased significantly after 72 h of attachment time, to reach 5.39 log cfu/g (Fig 1). The greatest effect of attachment time was therefore observed after 72 h of attachment on both surface and sub-surfaces of tomatoes. The ability of LM to attach to the surface of tomato after 24 h of attachment was illustrated using scanning electron microscope (Fig 2).

Effect of chlorine

Overall, chlorine affected the LM counts significantly ($p \leq 0.05$) (Table 1). There was a significant difference ($p \leq 0.05$) between the LM counts for the control (washed with distilled water) and the inoculated tomatoes washed with chlorine in both surface and sub-surface inoculated samples and after each attachment time (Fig. 1, Table 1). The ability of LM after attachment on tomato for 24 h to survive sanitizing effect of chlorine was illustrated using scanning electron microscope (Fig 3).

After all attachment times, the LM levels for the control samples were higher than those of the chlorine washed samples. After 30 min of attachment time for the surface inoculated tomatoes, there was a 1.21 log cfu/g difference in LM levels between the control and the chlorine washed tomatoes. After 72 h attachment time, the difference

between the surface inoculated control and the chlorine washed tomatoes was significantly higher than for the other attachment times i.e., 2.26 log cfu/g (Fig. 1).

The LM levels for the sub-surface inoculated tomatoes followed the same trend, i.e. LM levels for the control higher than LM levels for the chlorine washed at different attachment times (Fig 1). The differences in LM on the sub-surface of control tomatoes and the treated ones followed the same trend like the surface inoculated samples. However, the effect after 72 h was not as pronounced between the two treatments.

Effect of inoculation site

There was a significant difference ($p \leq 0.05$) between the sub-surface inoculated LM and surface inoculated LM in tomatoes at different attachment times (Table 1). The LM levels for the sub-surface inoculated tomatoes were higher for both control and chlorine washed samples at each attachment time, than that of the surface inoculated tomatoes. The differences in LM between sub-surface inoculated and surface inoculated, control samples, decreased as the attachment time increased, i.e., log 1.3 log cfu/g after 30 min and 0.6 log cfu/g after 72 h of attachment (Fig 1). For the chlorine washed tomatoes the differences in LM, sub-surface inoculated and surface inoculated did not follow a similar trend, with the greatest difference in LM counts between the treatments after 30 min and 72 h of attachment, 1.26 and 1.04 log cfu/g respectively.

Effect of attachment time and chlorine washing on the survival of inoculated *Listeria monocytogenes* on spinach

Effect of attachment time

Attachment time did not significantly ($p \geq 0.05$) affect the LM count on the surface and sub-surface structures of spinach but there was a significant interaction ($p \leq 0.05$) effect between attachment time and chlorine washing on the inoculated LM (Table 1). LM attached and survived on the spinach after each attachment time as observed for tomato. The level of LM that survived and attached on the surface of spinach reduced as attachment time increased, 4.86 log cfu/g after 30 min and 3.41 log cfu/g after 72 h (Fig 4). The level of LM that survived and attached on the sub-

surface of spinach followed the same trend, reducing with increased attachment time, 5.17 log cfu/g after 30 min and 4.18 log cfu/g after 72 h (Fig 4). The ability of LM to attach to the surface of spinach after 24 h of attachment was shown with scanning electron microscope (Fig 2).

Effect of chlorine

As for tomato, overall, chlorine affected the LM counts significantly ($p \leq 0.05$) (Table 1). There was a significant difference ($p \leq 0.05$) between the LM counts for the control (washed with distilled water) and the inoculated spinach washed with chlorine in both surface and sub-surface inoculated samples and after each attachment time (Table 1). The ability of LM after attachment for 24 h on spinach to survive sanitizing effect of chlorine was illustrated using scanning electron microscope (Fig 3).

At all attachment times the LM levels for the control samples were higher than those of the chlorine washed samples. After 30 min of attachment time for the surface inoculated spinach, there was a 3.01 log cfu/g difference in LM levels between the control and the chlorine washed spinach. After 24, 48 and 72 h attachment time intervals, the differences between the surface inoculated control and the chlorine washed spinach reduced with increasing attachment time, i.e., 2.55, 1.38 and 1.54 log cfu/g respectively (Fig. 4).

The LM levels for the sub-surface inoculated spinach followed the same trend, i.e. LM levels for the control were higher than LM levels for the chlorine washed at different attachment times (Fig 4). The differences in LM on the sub-surface of control spinach followed a similar trend as noted for the surface inoculated samples. More than a two log difference was found after 30 min of attachment time with only a 0.91 log cfu/g reduction after 72 h of attachment time.

Effect of inoculation site

There was a significant difference ($p \leq 0.05$) between the sub-surface inoculated LM and surface inoculated LM in spinach at different attachment times (Table 1). The LM levels for the sub-surface inoculated spinach were higher for both control and chlorine washed samples at each attachment time than that of the surface inoculated tomatoes.

The differences in LM between sub-surface inoculated and surface inoculated, control samples increased with increase in attachment time, i.e. 0.3, 0.88, 1.31 and 0.77 log cfu/g after 30 min, 24, 48 and 72 h of attachment, respectively (Fig 4). For the chlorine washed spinach the differences in LM, sub-surface inoculated and surface inoculated, were comparable between attachment times. Differences in LM ranged between 0.86 and 1.4 log cfu/g (Fig 2).

DISCUSSION

It was evident from the results that LM was able to attach to both the surface and sub-surface structures of both spinach and tomatoes. This observation signifies that LM will attach to vegetables within 30 min of coming in contact with it in irrigation water or other sources. Although shorter attachment time was not determined in this work, Ells & Hansen (2006) reported that LM could attach to intact and cut cabbage within 5 min of exposure to intact and cut cabbage. Other workers reported the same time range of attachment of LM to lettuce, cantaloupe and *Arabidopsis thuliana* (Li *et al.,* 2002; Ukuku & Fett, 2002; Milillo *et al.,* 2008; Solomon *et al.,* 2006). It is evident that attachment of pathogenic bacteria to produce occurs in a rapid manner (Fonseca, 2006; Liao & Cooke, 2001)

LM survived on the sub-surface and surface of spinach and tomato up to 72 h. It has been found that pathogen could survive on tomatoes for a longer time. Elif *et al.,* (2006) showed that *Salmonella* Enteritidis can survive and grow during storage of tomatoes for 220 h.

The significant difference between sub-surface inoculated LM and surface inoculated LM in both vegetables at each time interval indicates that LM attaches in higher numbers to wounds or sub-surface structures than to undamaged surfaces (Takeuchi *et al.,* 2000). Timothy *et al.* (2006) showed that LM has a preference to attach to cut or wounded tissues compared to the intact leaf surfaces. This may be because surface structures of vegetables constitute a harsh environment with fluctuations in temperature unlike sub-surface structures (Solomon *et al.,* 2006). The sub-surface structures or cut surfaces also have significant amount of liquid containing nutrients that is utilized by the attached microorganisms (Bhagwat, 2006). Moreso, pathogens

are able to create a more hospitable microenvironment in the sub-surface structures unlike on the surface structures (Sapers, 2001).

In this study chlorine was relatively ineffective to decontaminate the surface inoculated LM on tomatoes and spinach. This observation was not different from several reports that emphasized that vigorous washing and treatment with chlorine does not remove all bacterial pathogens on fruit and vegetables (Solomon *et al.,* 2006; Doyle and Erickson, 2008). Ineffectiveness of chlorine may be due to the concentration (200 ppm) used. According to Kim *et al.* (1998) low levels (less than 200 ppm) of chlorine may not be effective against certain bacteria. Higher concentration (more than 200 ppm) is not used in the produce industry because it can generate residual by-products such as trihalomethanes in the waste water (Simpson *et al.,* 2000; Moriyama *et al.,* 2004). Higher concentration may also react with organic residues resulting in the formation of potentially mutagenic or carcinogenic reaction products (Moriyama *et al.,* 2004; Nakano *et al.,* 2000; Nukaya *et al.,* 2001; Rodgers *et al.,* 2004 ; Velazquez *et al.,* 2009)

Chlorine was more effective on the surface LM than on the sub- surface LM, probably because it was not able to access the sub-surface structures effectively, where the pathogens were located (Doyle and Erickson, 2008; Fonseca, 2006; Sapers *et al.,* 1990). This is in line with the observation of Liao and Cooke, (2001) who found that *Salmonella* Chester survived chlorine washing to a much greater extent when attached on the sub-surface structures of green pepper disks than on surface structures. According to Seymour *et al.* (2002) entrapped or internalized pathogens are not readily accessible to chlorine because of the components i.e., liquids leaking from sub-surface structures or wounds. The liquid is able to neutralize some of the chlorine before it reached the microbial cells (Bhagwat, 2006)

Chlorine was more effective on surface inoculated LM after 30 min attachment time compared to 72 h attachment time in spinach. This is in agreement with the work of Ukuku & Sapers (2001) who confirmed that *Salmonella* serovar Stanley populations in cantaloupes were reduced by 3 log cfu/ml after a sanitizer was applied immediately after inoculation but there was reduction by less than 1 log when sanitizer was applied

72 h post inoculation. The effectiveness of chlorine at an earlier attachment time was expected because sanitizer will easily remove pathogen that has just attached to the surface of produce compare to the one that has attached over a longer period of time (Sapers *et al.,* 1990). However, this was not the case in tomatoes in which chlorine was more effective on the surface inoculated LM after 72 h attachment time compare to after 30 min attachment time. This is because effectiveness of sanitizer on microbial reduction is dependent on the type of vegetable at any given attachment time (Abadias, *et al.,* 2008; Ukuku *et al.,* 2005). The difference may also be as a result of pathogen attachment, infiltration, internalization and biofilm formation which affect sanitizer effectiveness vary from one produce to another (Ukuku *et al.,* 2005). Also according to Fonseca (2006), differences in surface characteristics of the produce, physiological state of pathogen, and environmental stress conditions interact to influence the activity and efficiency of sanitizer. It may therefore be necessary to customise sanitizing treatments for different types of produce because of this complexity (Bhagwat, 2006)

CONCLUSION

These work shows that *Listeria monocytogenes* will attach within 30 min to spinach and tomato and it will remain viable after attachment even up to 72 h. Other workers have reported shorter attachment time of LM on other vegetables. Also, there is a difference in the attachment and survival of LM in both vegetables, showing that attachment and survival of LM vary from one vegetable to another. The present study also confirms that chlorine is more effective on the pathogens on the surface of vegetables than on the sub-surface, it could only reduce $\leq$ 3 logs inoculated and attached LM both on the surface and sub-surface structures.

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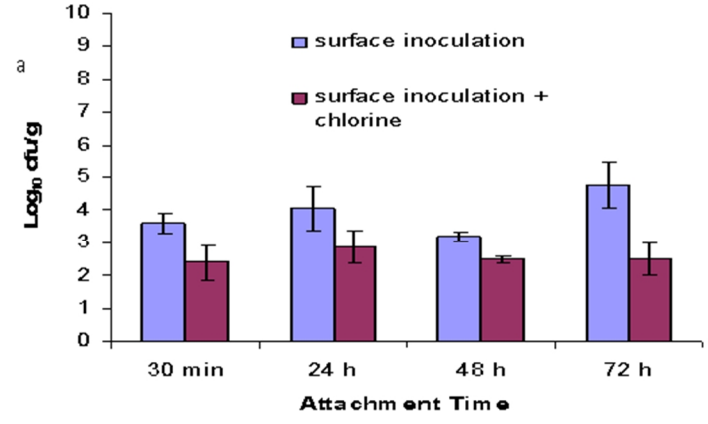
Table 1: P values of effect of chlorine, site, and attachment time on survival of inoculated *Listeria monocytogenes* on tomatoes and spinach

TREATMENT	**P value for Tomato**	**P value for Spinach**
Chlorine	0.001*	0.001*
Site	0.000*	0.000*
Attachment time	0.001*	0.246
Chlorine x Site	0.722	0.528
Chlorine x Attachment time	0.031*	0.021*
Site x Time	0.542	0.821
Chlorine x Site x Time	0.496	0.649

* $p \leq 0.05$



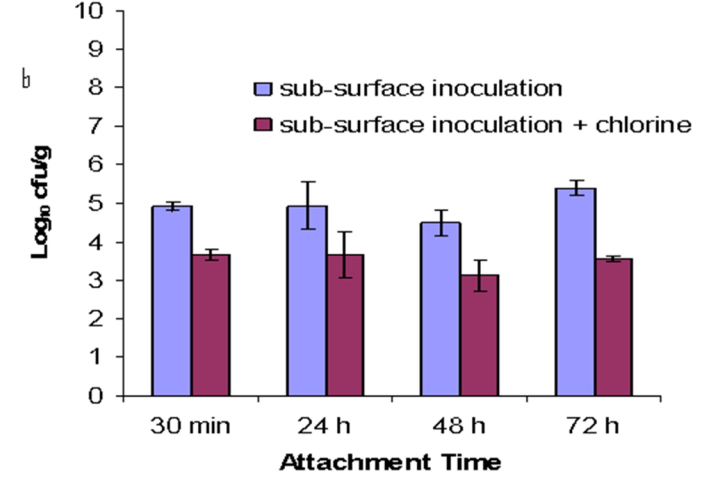


fig 1: Attachment and survival of L. monocytogenes on the surface (a) and subsurface (b) of tomatoes with or without chlorine washing

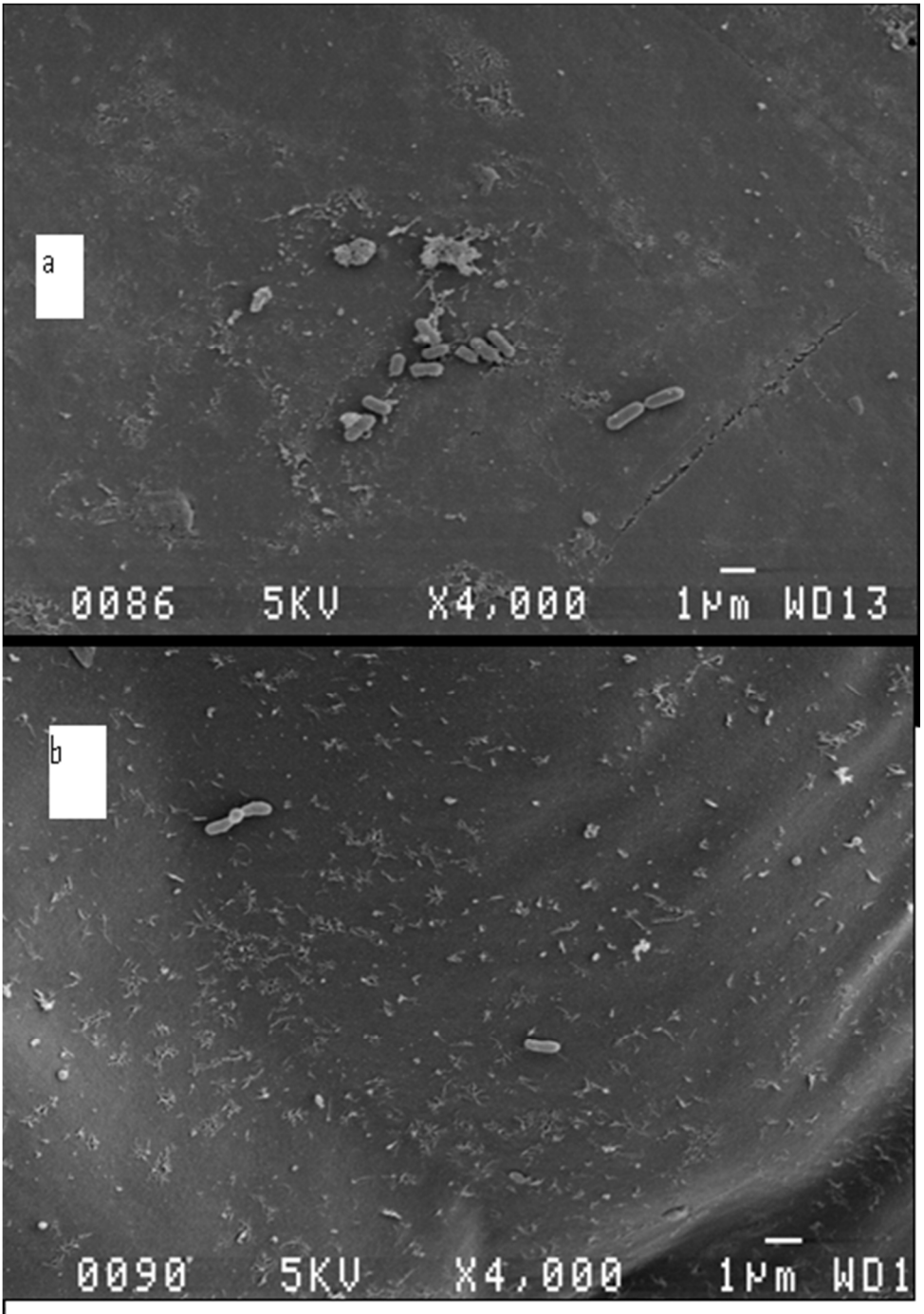


Fig 2: Scanning Electron Microscopy (SEM) of attachment of LM to the surface of tomato (a) and spinach (b)

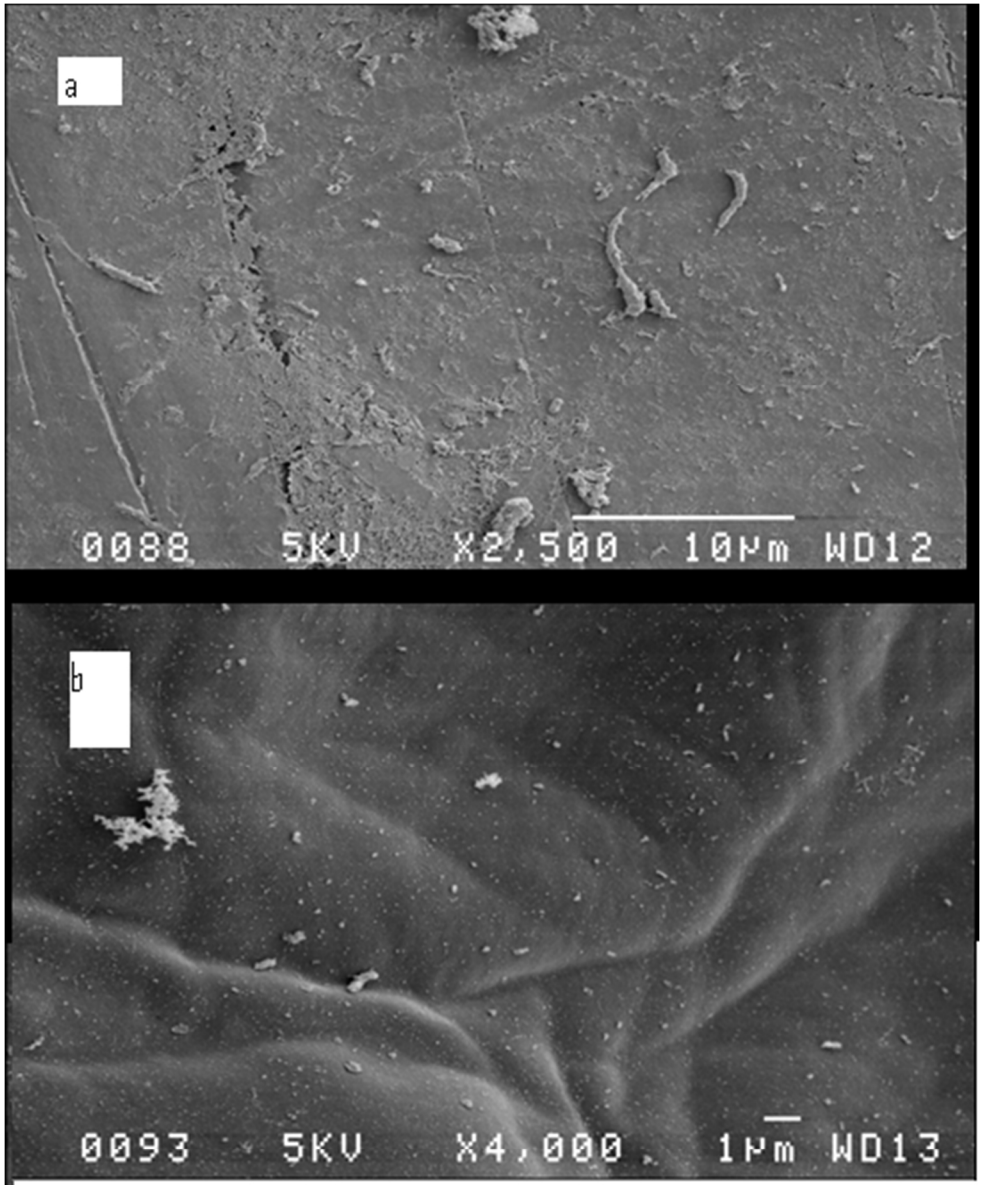
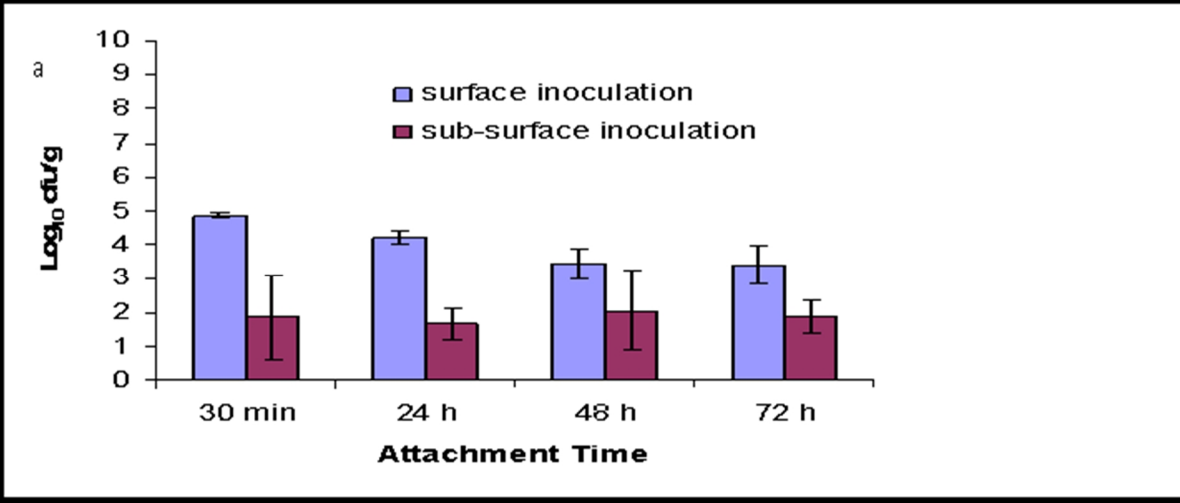


Fig 3: Scanning Electron Microscopy (SEM) of attachment of LM to the surface of tomato (a) and spinach (b) after 24h followed by chlorine washing



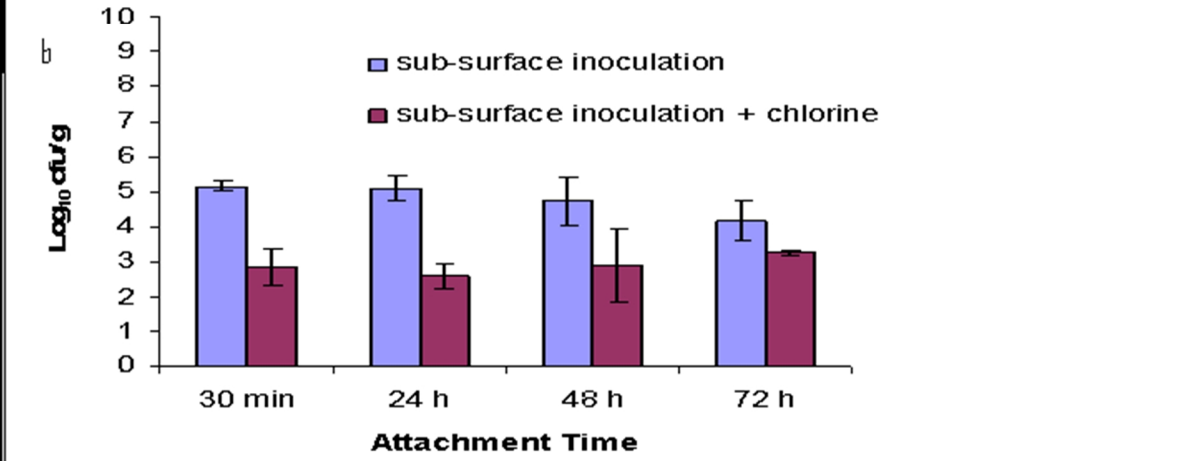


Fig 4: Attachment and survival of L. monocytogenes on the surface (a) and subsurface (b) of spinach leaves with or without chlorine washing