CHAPTER 3: RESEARCH

3.1 IRRIGATION WATER AS A POTENTIAL PRE-HARVEST SOURCE OF BACTERIAL CONTAMINATION OF VEGETABLES

ABSTRACT

The bacteriological quality of the irrigation canal from Loskop Dam, the two rivers that feed it and vegetables (broccoli and cauliflower) in Mpumalanga, SA, were investigated with respect to aerobic colony counts, aerobic sporeformers, anaerobic sporeformers and the presence of coliforms, faecal coliforms, *Escherichia coli*, *Salmonella* spp, *Listeria monocytogenes*, intestinal *Enterococci* and *Staphylococcus aureus*. Physico-chemical parameters determined for the surface water were pH, turbidity and chemical oxygen demand (COD). There were significant differences in the levels of COD and turbidity in the two rivers and the canal and the results of the three water samples were higher than WHO and SA water guidelines. Aerobic bacteria, aerobic spore bacteria and anaerobic spore bacteria in the two rivers, the canal and the vegetables followed the same trend. However, the level of aerobic bacteria (3–4 $\log_{10}$ cfu/g/ml), aerobic spore bacteria (1.6 $\log_{10}$ cfu/g/ml) and anaerobic spore bacteria (1.5 $\log_{10}$ cfu/g/ml) in both water and on vegetables during the period of sampling was low. Levels of faecal coliforms and *E. coli* were higher than the WHO standard. *S. aureus,* intestinal *Enterococci*, *Salmonella, L. monocytogenes* were recovered from the two rivers and the canal. Apart from *L. monocytogenes* that was not recovered from cauliflower, all bacterial pathogens recovered from the surface water were recovered from the vegetables. These results show that the rivers may contribute to the contamination in the irrigation canal and that may be a possible pre-harvest source of contamination of broccoli and cauliflower, which may in turn constitute a health risk to consumers.

3.1.1 Introduction

Commercial and small-scale farmers generally irrigate their produce with water from nearby rivers, streams, ponds, wells and dams most of which do not meet the required standard for irrigation (Westcot, 1997). Furthermore, the water is not treated before it is used for irrigation. According to the South African Water Quality Guidelines (SAWQG, 1996), irrigation water used in agriculture is mostly untreated water while home gardeners have access to treated water of high quality. South African's irrigation water sources are perceived to be at risk of contamination with human bacterial pathogens as a result of pollution caused by informal settlements and mines. According to Sigge & Fitchet (2009), 98% of South African water resources are fully utilized while 80% of its municipal sewerage systems are overburdened. South African surface water may be a source of contamination of fresh vegetables with bacterial pathogens due to the reasons given by Sigge and Fitchet (2009). The Berg River used for irrigation of vegetables in the Western Cape Province, SA, has also been reported to fall below the European Union (EU) microbiological standard allowed for vegetable production according to the *Cape Times* newspaper (2005). Similarly the *Landbouweekblad* magazine, of 24th August 2007, reported that water in Loskop Dam contained poisonous heavy metals and *E. coli* as a result of mines and municipalities dumping wastes in the rivers that feed the dam.

Tshivhandekano (2006) reported that irrigation water in the Tshwane metropolitan area of SA was highly contaminated with faecal coliform and *E. coli*. Hepatitis A Virus and rotavirus were also recovered from the Apies River in the same area (Tshivhandekano, 2006). There is also a concern over the safety of pickers, handlers, packers and farmers that participate in the production of vegetables during pre-harvest and post-harvest. It has been reported that young children from families of farming communities are the most vulnerable to *Salmonella* infection as a result of sewage irrigation (Ait & Hassani, 1999; FDA/CFSAN, 2001)

Although the nutritional and other benefits of a regular intake of fruits and vegetables are well documented **(**Fujiki, 1999; Potter, 1999; Lerici, Nicoli & Anese, 2000), internationally, health risk has been associated with the consumption of fresh fruit and vegetables (Beuchat, 1996; Beuchat & Ryu, 1997; De Roever, 1998; Beuchat, 2002). In September 2006, pre-packaged fresh spinach was recalled by the Food and Drug Administration (FDA) in the US as a result of an *E coli* outbreak in California, USA (IFT, 2007). Also, in the same year, fresh tomatoes consumed at restaurants in the USA were responsible for an outbreak of *Salmonella typhimurium*. There was also an *E. coli* 0157:H7 outbreak linked to lettuce from Taco Bell restaurants in the northern USA (IFT, 2007).

The microbial quality of irrigation water is critical because water contaminated with animal or human wastes can introduce pathogens into produce during pre-harvest and post-harvest (FDA/CFSAN, 2001). Indirect or direct contamination of produce from water or water aerosols of persistent pathogens on harvested vegetables has been long recognized as a potential hazard (FDA/CFSAN, 2001; WHO, 2003). The microbiological quality of the fresh vegetables is a significant concern for all stakeholders in the produce industry both local and international (Chang & Fang, 2007). According to Henneberry, Piewthongngam and Qiang (1999), the ten most common fresh vegetables consumed in the USA and other countries are broccoli, cauliflower, carrots, celery, lettuce, onions, tomatoes, cabbage, cucumbers and green peppers (Henneberry *et al*., 1999). The microbiological quality of irrigation water is therefore paramount to the safety of fresh and minimally processed vegetables (Bihn & Gravani, 2006; Solomon *et al.,* 2002).

Ibenyassine *et al.* (2006) reported that contaminated irrigation water and surface run-off water might be major sources of pathogenic microorganisms that contaminate fruits and vegetables in fields. Steele *et al.* (2005) surveyed 500 irrigation water samples used for the production of fruit and vegetables in Canada and found that 25% of the water samples were contaminated with

faecal *E. coli* and faecal *Streptococci*. River water used for both human and animal waste disposal poses a health risk due to contamination with *Salmonella* and *Listeria* when used for the irrigation of produce (Combarro *et al*., 1997; Johnson *et al*., 1997). Combarro *et al*. (1997) isolated different *Listeria* species from river water in Spain. The specie most isolated was *L. monocytogenes,* followed by *L. seeligeri*, *L. velshimeri* and *L. ivanovii*. Similarly, Geuenich *et al.* (1985) and Bernagozzi *et al.* (1994) also recovered mostly *L. monocytogenes*, 73% and 93% respectively, from river water.

The aim of this study was therefore to determine the effect of irrigation water on the bacterial quality of water in the canal it feeds and also the subsequent contribution to the bacterial contamination of fresh vegetables.

3.1.2 Materials and methods

Selection of rivers and vegetables

Due to various reports of contamination (Britz *et al.,* 2007, Tshivhandekano, 2006), the Loskop Dam irrigation scheme in the Mpumaplanga Province of SA was selected as the sampling area for this study.

Surface water samples were collected from three points: Loskop Canal from which the farmers irrigate and two rivers that feed the Loskop Dam, the Olifants River and the Wilge River. Water from the dam is subsequently released to the Loskop Canal system that is used to irrigate the vegetables. Surface water from the three points was aseptically collected at 12 intervals over a period of 12 months (November 2007 to October 2008) i.e., one interval per month. At each interval, 2 litres each of surface water was collected at the three points.

Three farms cultivating vegetables irrigated with water from the Loskop Dam irrigation scheme were also visited three times over a period of three months

for the collection of vegetables, namely, cauliflower and broccoli. Vegetables were picked randomly from the three farms and 25 g each was used for analyses. Farms were visited only three times because the vegetables are not grown all the year round.

Bacterial and physicochemical analyses of samples

Water and vegetable samples were examined for the presence of total coliforms, faecal coliforms, *E. coli, L. monocytogenes, Salmonella* sp., *Enterococcus, S. aureus*, aerobic sporeformers, anaerobic sporeformers, and aerobic colony counts were done. Apart from bacterial analysis, the following physico-chemical tests: temperature, pH, turbidity and COD, were determined in water samples.

Aerobic colony counts

Dilution series of water samples were prepared using buffered peptone water (BPW) (Oxoid Ltd; Basingstoke, Hampshire, England) and 0.1 ml each of the dilutions were pour-plated with Nutrient Agar (Oxoid) and incubated at 30 ℃ for 72 h (ISO, 1991).

Aerobic and anaerobic sporeformers

Water samples, 20 ml, were heated in a sterile test tube in a water bath (75 ℃) for 20 min (Austin, 1998). Serial dilutions were pour-plated. A set of plates were incubated aerobically at 37 ℃ for 48 h while the other set of plates were incubated an-aerobically in an anaerobic jar with anaerocult (Merck Ltd; Wadeville, Gauteng, South Africa) at 37 ℃ for 48 h.

Coliforms and faecal coliforms

Coliforms and faecal coliforms in the water samples were determined using the Most Probable Number (MPN) method (Christensen, Crawford & Szabo, 2002).

Escherichia coli

Positive *E. coli* Broth (MPN) samples were inoculated onto the surface of L-EMB (Oxoid) Agar plates with inoculating loop and incubated at 37 ℃ for 24 h (Christensen, *et al.,* 2002). Typical colonies from L-EMB were streaked onto *E. coli* Chromogenic Agar (Oxoid). Thereafter colonies were confirmed with API 20E (Oxoid Ltd; Basingstoke, Hampshire, England).

Listeria monocytogenes

Listeria monocytogenes was determined according ISO (2004). A 1 ml water sample was added to 9 ml of ½ Fraser Broth (Oxoid) and incubated at 37 ℃ for 48 h. 0.1 ml of the ½ Fraser Broth culture was then transferred into a test tube containing 10 ml of full Fraser Broth (Oxoid) and also incubated at 37^{o} C for 48 h. Oxford Agar (Oxoid) plates and Palcam (Oxoid) Agar plates were inoculated from culture from Fraser Broth. The plates were placed in an anaerobic jar and incubated microaerobically at 37 ℃ for 24 h. Typical colonies were streaked onto *Listeria* Chromogenic Agar (Oxoid). Thereafter colonies were confirmed with API *Listeria* (Oxoid).

Salmonella

Salmonella sp was determined according to ISO (1993). A 25 ml water sample was added to 225 ml sterile buffered peptone water and incubated at 37 ℃ for 24 h. The pre-enriched sample suspension, 10 ml, was transferred into 100 ml of Selenite cystine medium (Oxoid) and incubated at 37 ℃ for 24

h. About 0.1 ml of the same pre-enriched sample suspension was transferred into 10 ml of RVS (Merck Ltd; Wadeville, Gauteng, South Africa) and incubated at 37 ℃ for 24 h. Phenol Red/Brilliant Green Agar (Oxoid) and XLD (Oxoid) Agar plates were inoculated with cultures from Selenite cystine and RVS medium. The plates were incubated at 37 ℃ for 24 h. Typical colonies were streaked onto *Salmonella* Chromogenic Agar (Oxoid) and thereafter colonies were confirmed with API 20E (Oxoid Ltd; Basingstoke, Hampshire, England).

Staphylococcus aureus

S. aureus was determined according to ISO (1999). About 0.1 ml each of the dilutions were released on Baird Parker (Oxoid) Agar plates containing egg-yolk tellurite solution (Oxoid). Plates were incubated at 37 ℃ for 24 h. Catalase test was performed on positive colonies and confirmed with Staphylase test (Oxoid Ltd; Basingstoke, Hampshire, England).

Intestinal Enterococcus

About 100 ml of water samples was filtered through 0.45 µm membrane filter and placed on Slanetz and Bartley medium (Oxoid) mixed with 2, 3,5-triphenyltetrazolium chloride (Oxoid) after which plates were incubated at 37 ℃ for 44 h (ISO, 2000). Incubated 0.45 µm membrane filter that gave presumptive positive colonies was transferred to the surface of Bile Aesculin Azide Agar (Oxoid) and incubated at 44 ℃ for 2 h. Typical intestinal *Enterococci* colonies gave a tan to black colour.

Determination of physico-chemical parameters in surface water

The pH, temperature, turbidity, chemical oxygen demand (COD) of the irrigation water was determined concurrently with the microbiological analysis. The temperature of the surface water was measured with a Checktemp1

Portable digital thermometer (Hanna Instruments Inc. Woonsocket, R1, USA). The pH was measured with 211 Microprocessor pH meter (Hanna Instruments Inc. Woonsocket, R1, USA) while turbidity was determined with an H1 93703 Microprocessor turbidity meter (Hanna Instruments Inc. Woonsocket, R1, USA). Chemical Oxygen Demand (COD) was measured using the closed reflux colorimetric method, as described in standard methods (APHA, 2001). To a Teflon-coated tube, 2.5 ml of the sample was added, after which 1.5 ml of the digestion solution (10.2g/l K2Cr2O7, 170 ml/l concentrated H_2SO_4 and 33.3 g/l $HgSO_{4)}$ and 3.5 ml of concentrated H_2SO_4 were added. The tubes were placed in a COD reactor (HACD COD reactor) and refluxed for 2 h at 150 ℃. The tubes were allowed to cool and absorbance was read using a spectrophotometer (DR Lange Spectrophotometer, model CADAS 50S, Germany) at a wavelength of 600 nm. The absorbance of the samples was read along with potassium hydrogen phthalate standards that ranged from 0 to 1000mg-COD/l. The following formula was used to calculate the COD level of samples:

$$\text{COD (mg/l)} = \frac{\text{mg in final volume x 1000}}{\text{Sample volume}}$$

Statistical analysis

Analysis of variance (ANOVA), $p \leq 0.05$, (Tulsa, Oklohama, USA, 2003) was used to determine whether there were significant differences between the levels of turbidity, COD, aerobic plate count, aerobic sporeformer counts and anaerobic sporeformer counts in water samples from the Olifants River, Wilge River and Loskop Canal (n=12) as well as between the bacterial counts determined on the cauliflower and broccoli from three farms and the Loskop Canal (n=3).

3.1.3 Results

Physico-chemical properties of water from Loskop Canal, Olifants River and Wilge River

The turbidity of water samples differed significantly ($p \leq 0.05$) during the 12 sampling intervals (Table 8). During the sampling period, the Wilge River had the highest mean turbidity of 19.1 NTU followed by the Olifants River with 14.7 NTU and Loskop Canal with the lowest mean turbidity of 5.4 NTU (Figure 2). The mean turbidity level at all three sampling locations was higher than the international turbidity (1 NTU) standard for water (DWAF, 1996a). At some sampling intervals, there was a high variation between the NTU in both rivers and the canal. For example, the NTU for both rivers was very high at intervals 2, 5, 6, 7 and 12. However, no such trend was observed for the canal.

The COD of water samples also differed significantly ($p \leq 0.05$) during the 12 sampling intervals (Table 8). The Wilge River had the highest mean COD of 54.2 mg/l followed by the Olifants River with 53.5 mg/l and the Loskop Canal with the lowest COD of 50.4 mg/l. A similar trend to NTU was observed with COD higher at intervals 1 and 2, 4–7, for all 3 sites (Figure 3).

Table 8: Analysis of variance for turbidity, chemical oxygen demand (COD), aerobic colony count (ACC), aerobic sporeformers (ASF) and anaerobic sporeformers (AnSF) of water from Loskop Canal, Olifants River and Wilge River at 12 intervals for a period of twelve months

Effect	Degrees of freedom	Turbidity	COD	ACC	ASF	AnSF
Sampling interval	11	0.001	0.001	0.001	0.001	0.001
Location	2	0.001	0.010	0.001	0.001	0.001
Sampling interval x location	22	0.001	0.001	0.001	0.001	0.433

Statistical significance of main factor and interaction: $p \leq 0.05$

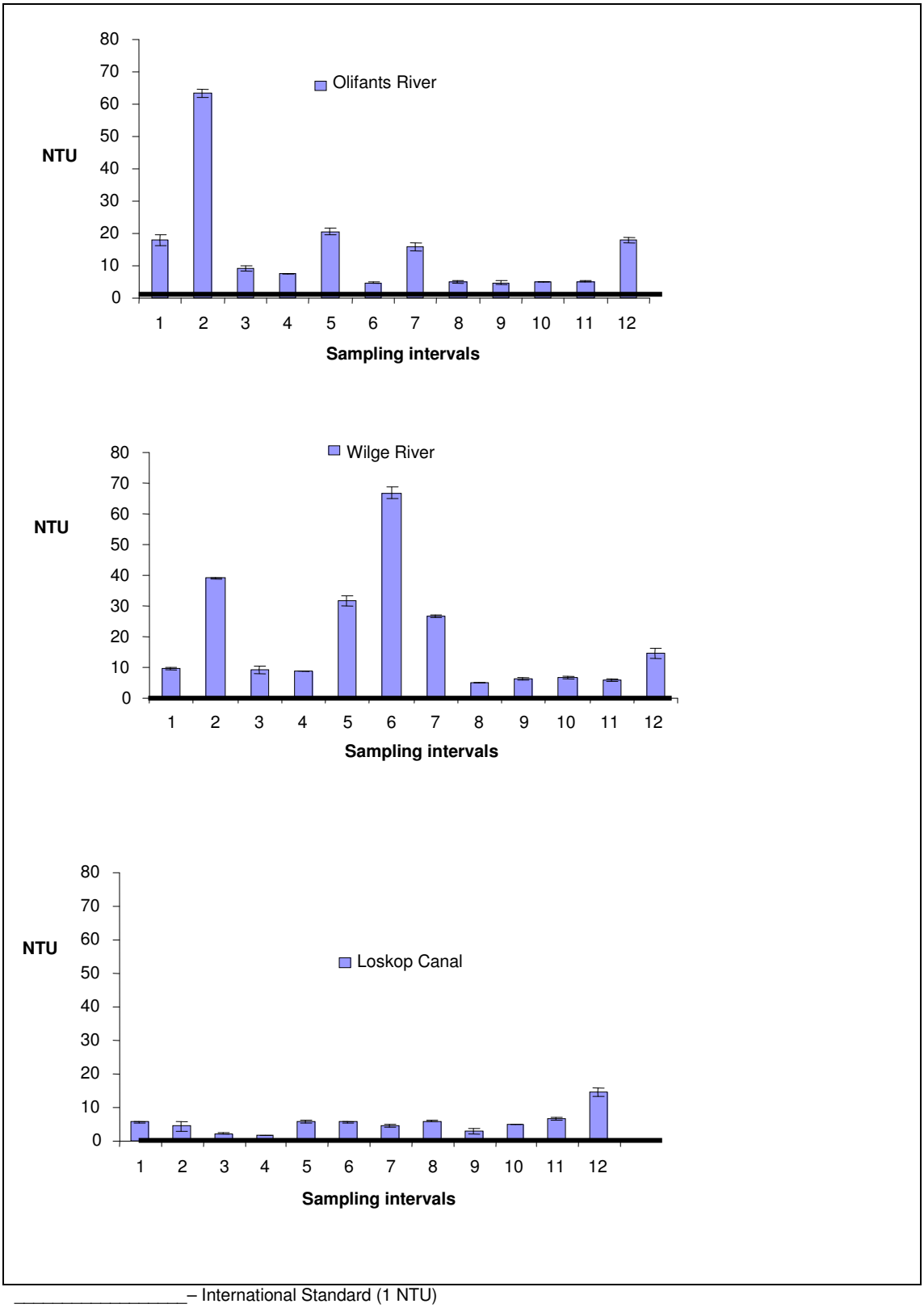
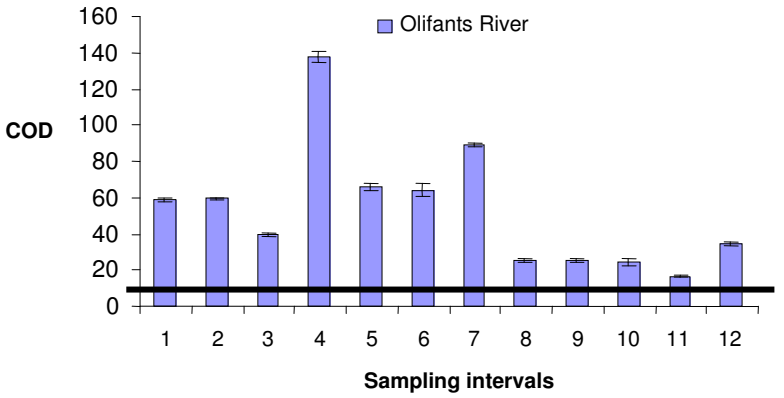
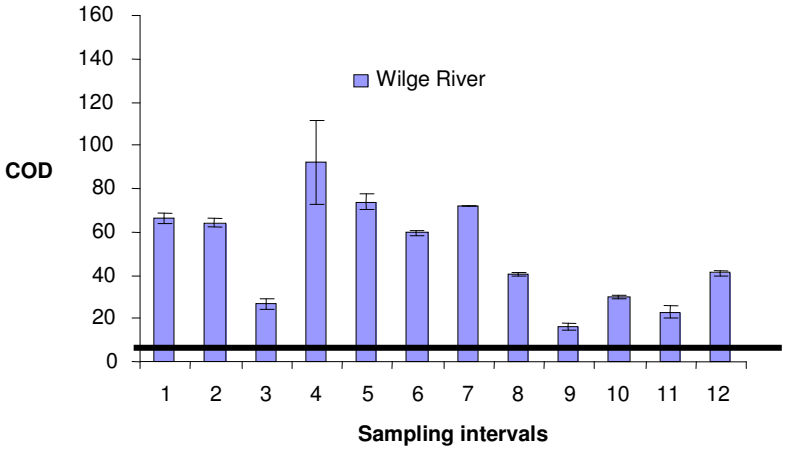
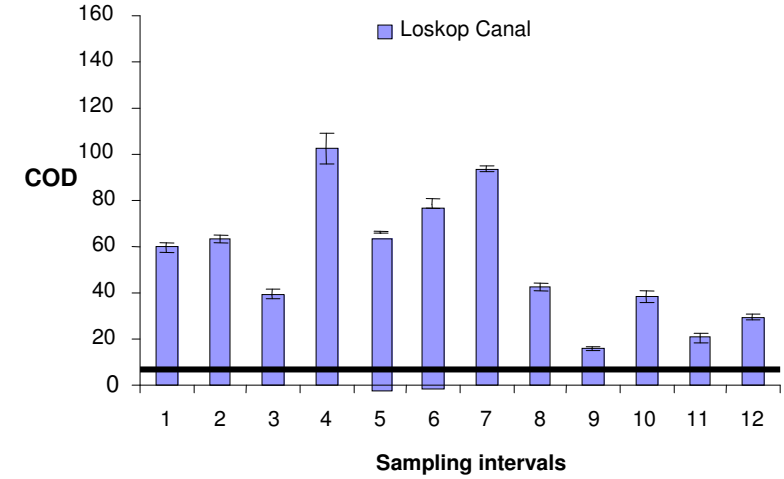


Figure 2: Turbidity of water from Loskop Canal, Olifants River and Wilge River during twelve sampling intervals







_____– International Standard (10 mg/l)

Figure 3: COD (mg/l) of water from Loskop Canal, Olifants River and Wilge River during twelve sampling intervals

The pH of the water samples from the Olifants River ranged between 7.02–7.88 (data not shown) for the 12 sampling intervals. The pH of water samples from the Wilge River and the Loskop Canal ranged between 7.00–7.62 and 7.03–9.71 respectively. In the canal, it was however unusually high during sampling intervals 1 and 2, 9.71 and 9.45 respectively. The average water temperature of the Loskop Canal ranged between 16–19 ℃ while it ranged between 17–23 ℃ for the Olifants River and 16–22 ℃ for the Wilge River during 12 sampling intervals (data not shown).

Incidence of aerobic bacteria (APC), aerobic sporeformer bacteria (ASF) and anaerobic sporeformer bacteria (AnSF) in the Loskop Canal, Olifants River and Wilge River

The mean APC count of water samples ranged between 2.9–3.2 log $_{10}$ cfu/ml and differed significantly ($P \leq 0.05$) over time (Table 8). Similar to turbidity and COD, the Wilge River had the highest mean APC counts of 3.2 $\log_{10}$ cfu/ml followed by Olifants River with 3 $\log_{10}$ cfu/ml and Loskop Canal with the lowest APC counts of 2.9 $\log_{10}$ cfu/ml during the 12 sampling intervals (Figure 4). The APC counts of the two rivers and the canal during the sampling period followed the same trend with higher and lower counts noted at the same time at the three locations. Also, the lowest APC levels at interval 9 correspond with low COD and turbidity levels determined at interval 9.

ASF at the three locations differed significantly ($p \leq 0.05$) during the 12 sampling intervals (Table 8). The Wilge River had the highest mean ASF count of 2 $\log_{10}$ cfu/ml followed by the Olifants River with 1.66 $\log_{10}$ cfu/ml and the Loskop Canal's mean ASF was 1.23 log $_{10}$ cfu/ml (Figure 5). While ASF was detected in the water samples from the Wilge River during all the sampling intervals, it was not detected at sampling interval 8 in the Olifants River and intervals 8 and 11 in the Loskop Canal.

The mean AnSF count for both the Loskop Canal and the Olifants River was 1.23 log_{10} cfu/ml while the mean AnSF count for the Wilge River was 1.93 log_{10} cfu/ml.

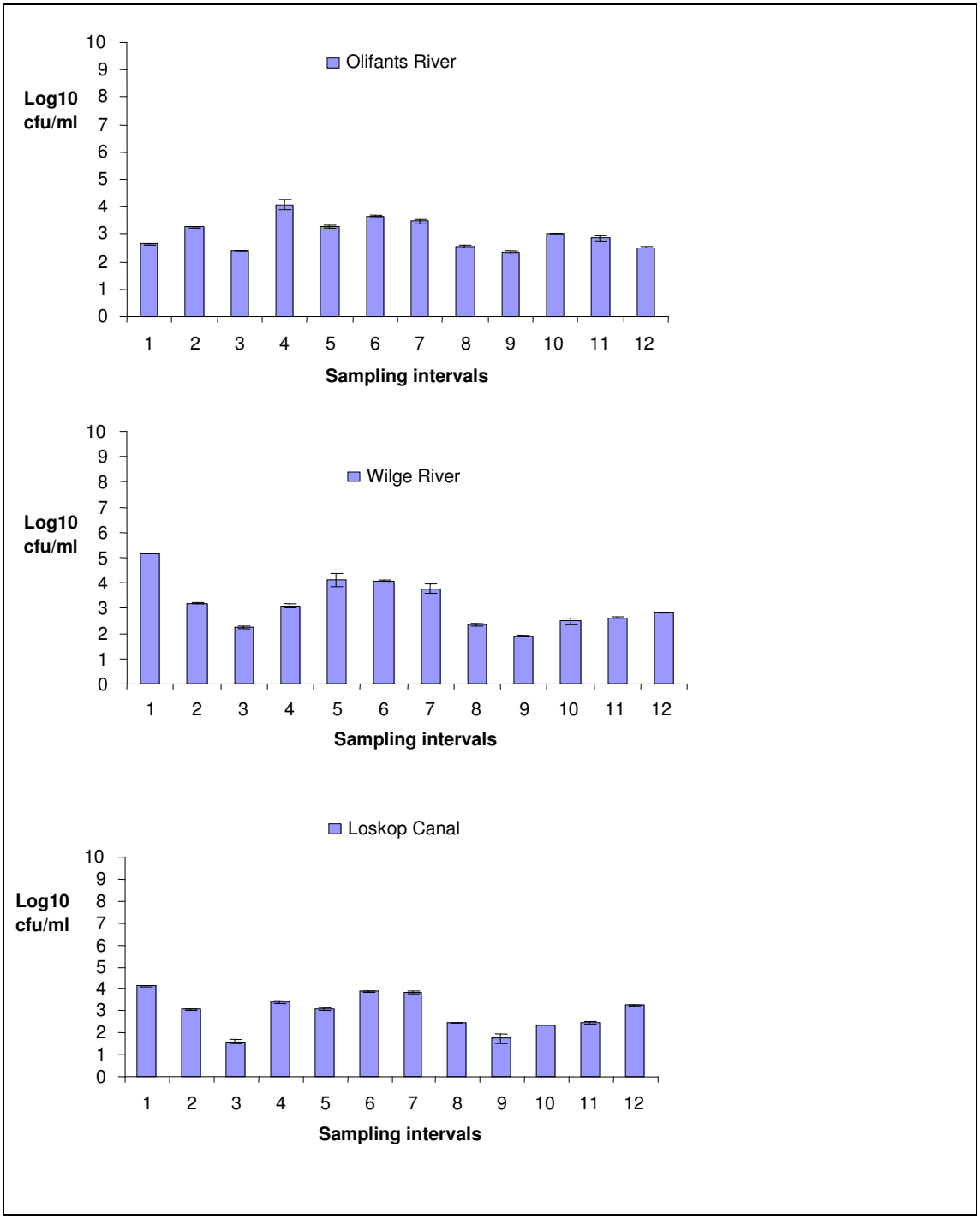


Figure 4: Aerobic colony counts (log 10cfu/ml) of water from Loskop Canal, Olifants River and Wilge River during twelve sampling intervals

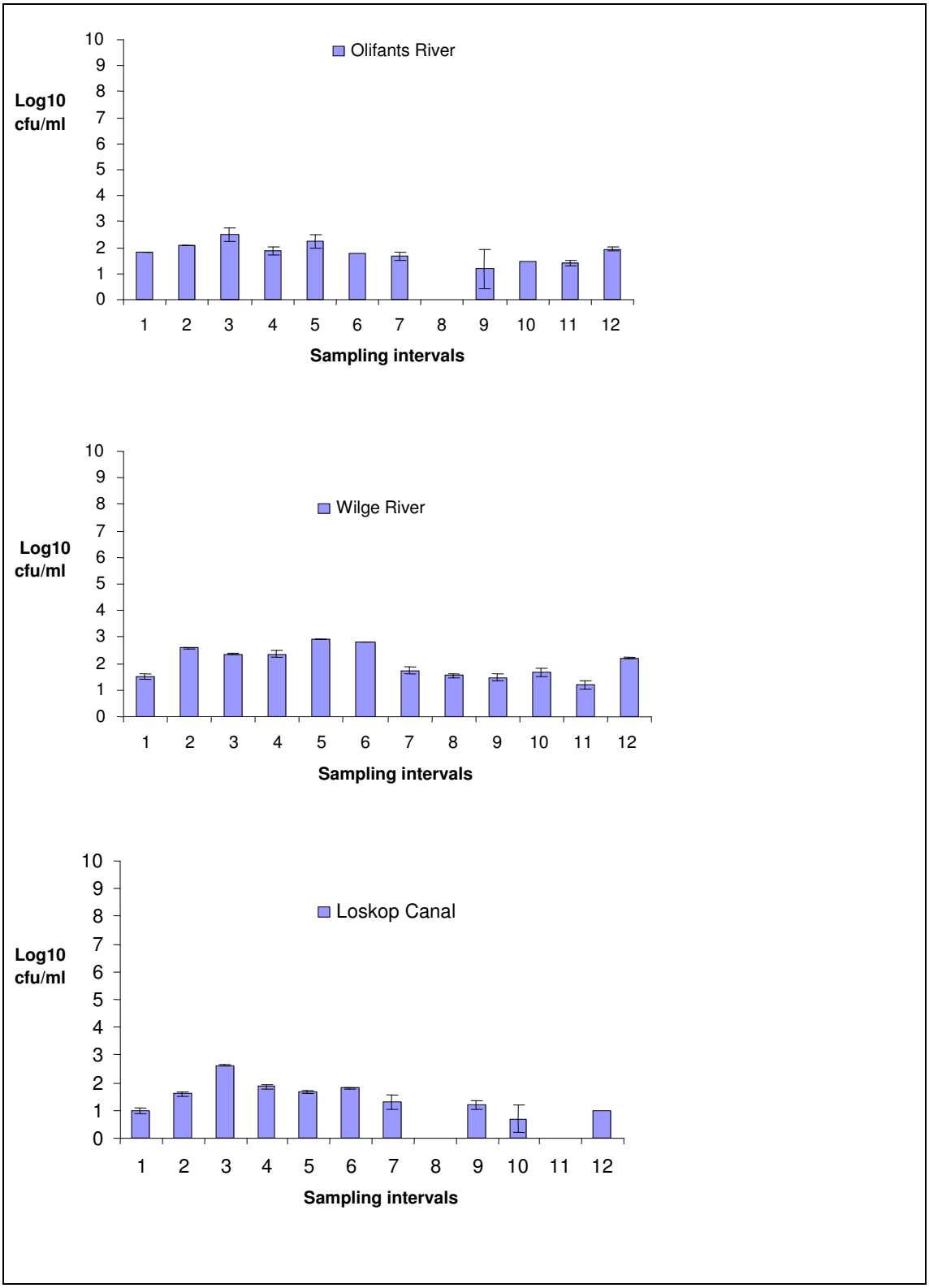
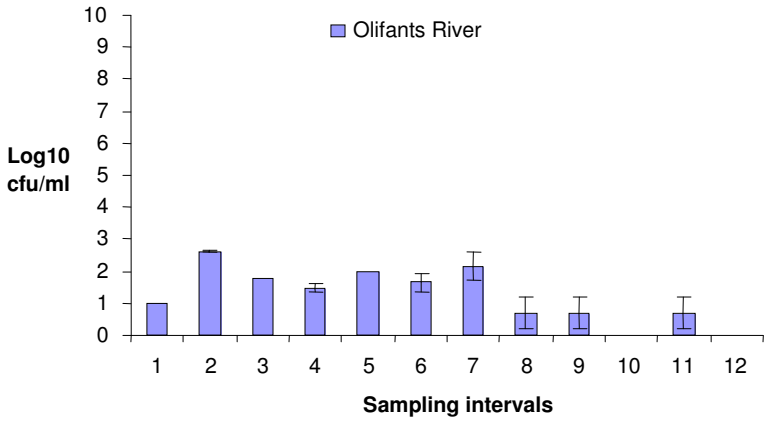
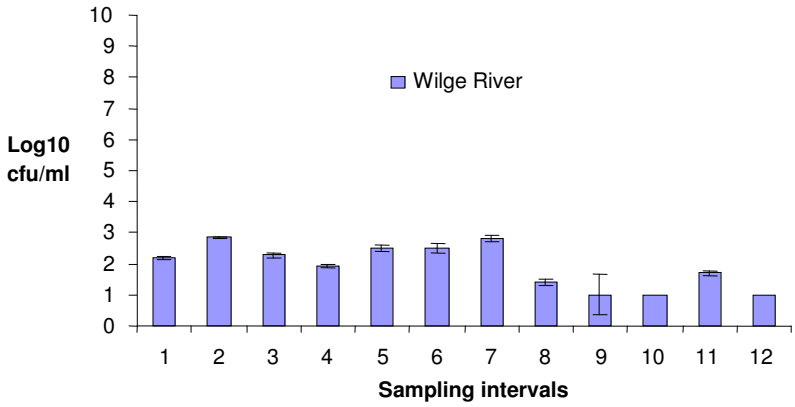


Figure 5: Aerobic sporeformer (log 10cfu/ml) counts of water from Loskop Canal, Olifants River and Wilge River during twelve sampling intervals

Similar to the ASF, AnSF was detected during all the sampling intervals in the Wilge River but it was not detected at sampling intervals 9, 11 and 12 in the Olifants River and at 10 and 12 in the Loskop Canal (Figure 6).





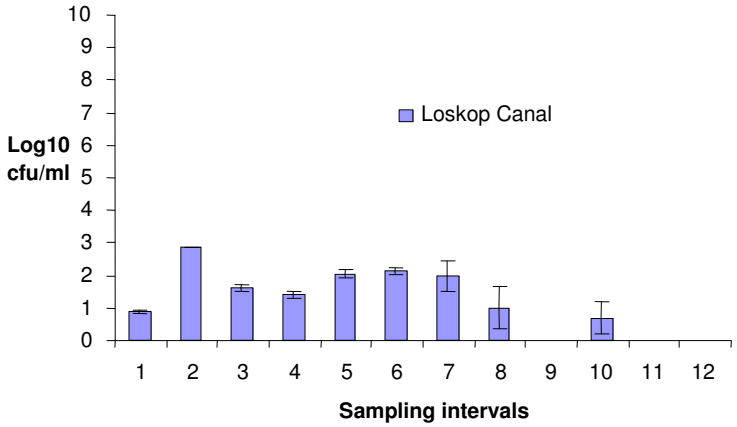


Figure 6: Anaerobic sporeformer (log10 cfu/ml) counts of water from Loskop Canal, Olifants River and Wilge River during twelve sampling intervals

Prevalence of S. aureus, E. coli, intestinal Enterococcus (IE), *Salmonella and L. monocytogenes in water from three surface water sites during the 12 sampling intervals*

Of the water samples collected during the 12 sampling intervals, 25% of the samples from the Olifants River, 33% from the Wilge River and 58% of the samples from the Loskop Canal were positive for *S. aureus* (Figure 7). However, the average *S. aureus* counts of water from the three surface water sampling sites were very low < 1 $\log_{10}$ cfu/ml. Incidence of *S. aureus* did not correspond between the sampling locations and only at interval 6 was *S. aureus* detected at all three locations (data not shown).

E. coli was recovered from the two rivers and the Loskop Canal during every sampling interval (Figure 7). Furthermore coliform and faecal coliform levels for the surface water met the international standard (1000 MPN/100ml) only once during the 12 sampling intervals in Loskop Canal water while at the Wilge River and Olifants River, the water samples met the standard during 25% and 30% of the 12 sampling intervals respectively.

IE was present in all the water samples collected from the Wilge River while incidence was lower in the Olifants River (67%) and the Loskop Canal (75%) (Figure 7). Incidence of *Salmonella* (50%) was higher in the Loskop Canal than in the Wilge River and the Olifants River (33% and 42% respectively). However, the incidence of *L. monocytogenes* (58%) in the Wilge River was higher than the 50% incidence observed in both the Loskop Canal and the Olifants River during the 12 sampling intervals (Figure 7).

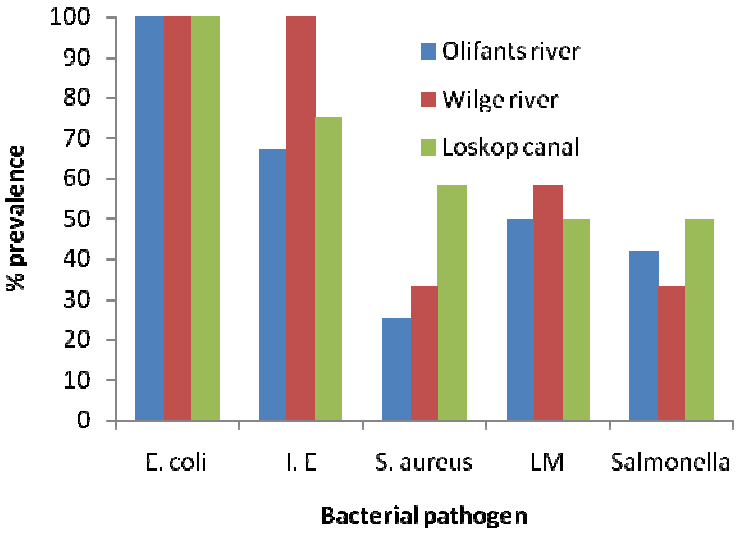


Figure 7: Prevalence of bacterial pathogens in the three water sources during twelve sampling intervals

Incidence of aerobic bacteria, aerobic spore bacteria and anaerobic spore bacteria on broccoli and cauliflower

The average APC, ASF and AnSF on the vegetables followed a similar trend. Although the numbers of the different groups of indigenous bacteria on broccoli were higher than on cauliflower during the three sampling intervals, the difference was less than 1 log (Figure 8).

The average APC on cauliflower was 3.8 $\log_{10}$ cfu/g while it was 4.1 $\log_{10}$ cfu/g on broccoli. Similarly, the average ASF and AnSF were also higher on broccoli. ASF on broccoli and cauliflower were 2 $\log_{10}$ cfu/g and 1.5 $\log_{10}$ cfu/g respectively while AnSF on broccoli and cauliflower were 1.6 $\log_{10}$ cfu/g and 1.4 $\log_{10}$ cfu/g respectively. There was no significant difference between the mean aerobic bacteria count of broccoli and cauliflower from the three farms whereas the mean anaerobic spore counts and aerobic spore counts differed significantly ($P \leq 0.05$) (Table 9). However, there was significant difference in aerobic colony count, aerobic spore counts and anaerobic spore counts in the two vegetables from the individual farms (Table 9).

The average APC in the three water samples from the Loskop Canal, Wilge River and Olifants River was lower than that on the two vegetables. However, the average ASF and AnSF were similar. Average APC, ASF and AnSF were 3.0, 1.6, 1.5 log_{10} cfu/ml while they were 3.9, 1.8, and 1.5 log_{10} cfu/g respectively on vegetables.

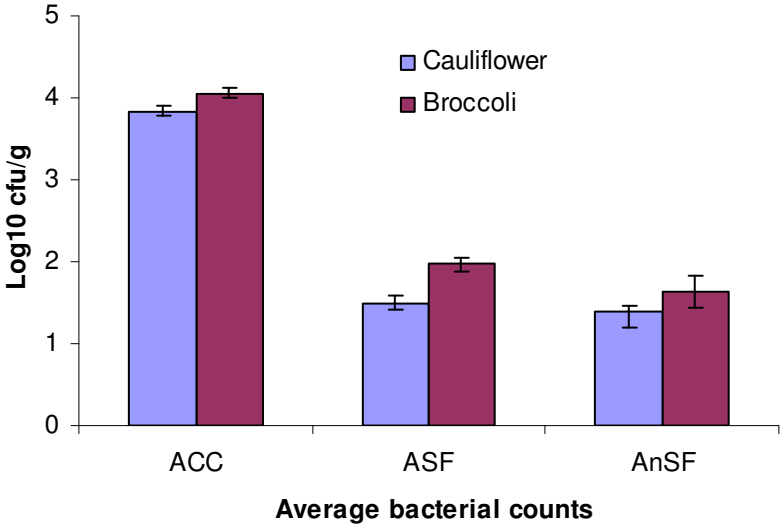


Figure 8: The average ACC, ASF, and AnSF on broccoli and cauliflower during three sampling intervals

Table 9: Analysis of variance for ACC, ASF, and AnSF of broccoli, cauliflower and irrigation water from the Loskop Canal during 3 sampling intervals

Effect	Degrees of freedom	ACC	ASF	AnSF
Sampling interval	2	0.266	0.001	0.002
Source	2	0.001	0.003	0.024
Sampling interval and source	4	0.001	0.001	0.101

Statistical significance of main factor and interaction: $p \leq 0.05$

Incidence of S. aureus, E. coli, intestinal Enteroccoci (I. E), Salmonella and L. monocytogenes (LM) on cauliflower, and broccoli

Incidence of *S. aureus* on broccoli (67%) was higher than on the cauliflower (33%). However, the average *S. aureus* counts on the vegetables during the three-month sampling period was very low < 1 $\log_{10}$ cfu/ml (Figure 9).

E. coli was recovered from the Loskop Canal, in cauliflower and broccoli during the three sampling intervals. Incidence of intestinal *Enterococcus* on broccoli was higher than that on cauliflower. The incidence was 44% and 33% respectively. However, it was 67% in the Loskop Canal. Also, the incidence of *Salmonella* (33%) in the Loskop Canal was higher than the 11% incidence observed on broccoli and cauliflower (Figure 9). Only broccoli was positive for *L. monocytogenes* during the three sampling intervals. However, *L. monocytogenes* were recovered from the Loskop Canal at other sampling intervals when vegetables were not examined. Also, with the exception of *L. monocytogenes* that was not recovered from cauliflower, all the bacterial pathogens isolated from the three water sources were also isolated from the two vegetables.

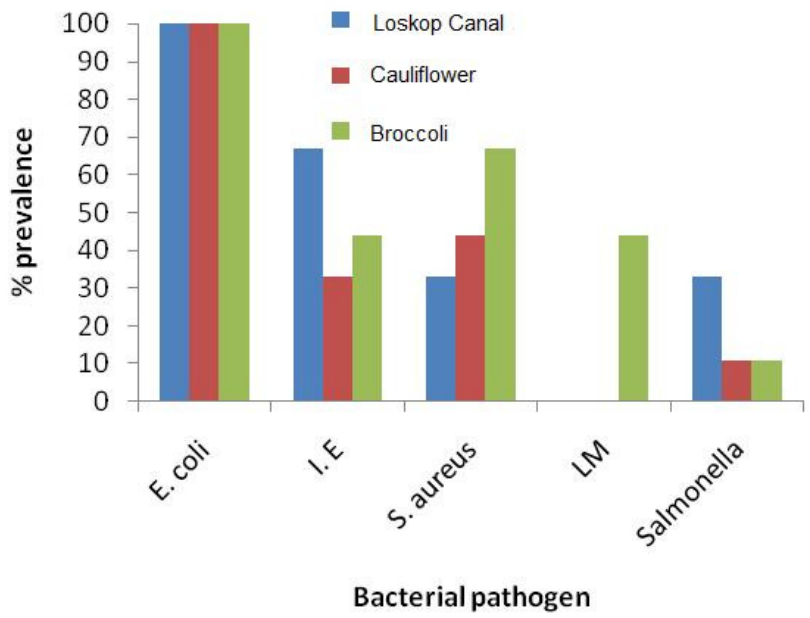


Figure 9: Prevalence of bacterial pathogens in the Loskop Canal and the two vegetables during three sampling intervals

3.1.4 Discussion

The temperature and pH values of the Loskop Canal and the two rivers that were conducive for bacterial growth may have influenced the survival of aerobic bacteria and bacterial pathogens in the water sources. According to Pautshwa *et al.* (2009), these two parameters could influence the level of faecal coliforms and intestinal *Enterococci*. The turbidity of the three water samples did not meet the SA water quality range for domestic water supply, 0 to 1 NTU (DWAF, 1996a–d). The turbidity range for water of good quality should be between 0 to 1NTU. The high turbidity level of surface water in this work corresponds with the river turbidity results of Fatoki *et al.* (2003). Fatoki *et al.* (2003) also found high turbidity levels in surface water indicated that soil erosion and run-off could be a source of high turbidity in the water system. The soil erosion and run-off could have been caused by the informal settlements around the two rivers. The COD results for all three water samples from Loskop Dam, Olifants River and Wilge River also did not meet

the WHO standard of 10mg/litre. This shows that the surface water contains organic pollutants that may have originated from the informal settlements and mines around the region where rivers are located.

Although the level of aerobic bacteria in both water and vegetable samples was low, a high prevalence of bacterial pathogens was observed in this study. This shows that aerobic bacteria levels are not a good determinant of the microbiological quality of irrigation water and produce.

The recovery of aerobic sporeformers from the three water samples is similar to the work of Fournnelle (1967) who recovered them from Alaska water at the same low level. However, the level of anaerobic sporeformers observed in our water samples was lower than has been reported by Molongoski and Klug (1976). Molongoski and Klug (1976) recovered up to 6 log of anaerobic sporeformers from freshwater lakes. Although low level of aerobic sporeformers were observed in the water samples, it may be unsuitable for the irrigation of fresh produce because of the possibility of microbial growth and cell division after attachment and infiltration on the vegetables.

The reason for a higher level of aerobic bacteria, aerobic sporeformers and anaerobic sporeformers in the Wilge River and the Olifants River, compared with those in Loskop Canal may be because the floor of the canal is cemented. It was noticed from the result that the higher difference was lower than 1 log and fell within the same level. This indicates that the Loskop Canal could have been contaminated by the two rivers namely, Wilge and Olifants Rivers. The average aerobic bacteria, aerobic sporeformers and anaerobic sporeformers in the water samples and on the vegetables were also within the same level, indicating that Loskop Canal could have contributed to the microbiota and contamination of the vegetables.

Although recovery of *S. aureus* from water samples is low, it may still pose a problem if such irrigation water is used for the production of produce that are

eaten raw (Khetarpaul, 2006). *S. aureus* was not expected to be recovered from the Loskop Canal, Wilge River or the Olifants River because its primary reservoir is the nasal cavity of humans (Jay, 2000). The presence of *S. aureus* in the two rivers and Loskop Canal also shows that the rivers may have contributed to the contamination level in the canal.

The result of heavy contamination of the three water sources, with *E. coli* and faecal coliforms corresponds to the work of Tshivhandekano (2006) on the Apies River, South Africa. This shows that the concern regarding contamination of surface water sources in SA may be valid and widespread. The two rivers may have been polluted with human faeces since *E. coli* and faecal coliforms are indicators of faecal pollution (Garcia & Servais, 2007). Human faeces contain higher faecal coliform counts, while animal faeces contain higher levels of faecal *Enterococci* (Gildreich & Kenner, 1969; Pautshwa *et al.,* 2009). The high incidence of *E. coli*, faecal coliforms and intestinal *Enterococcus* in the two rivers and the Loskop Canal indicate that the rivers are potential sources of contamination of the canal. In addition, the source of this contamination may be the informal settlements along the two rivers.

Contamination of water sources with other bacterial pathogens, namely, *L. monocytogenes* and *Salmonella* show that the two rivers and canal are of poor microbiological quality possibly as a result of faecal pollution. It also indicates that the two rivers are potential sources of contamination of the Loskop Canal. Other workers have reported the widespread contamination of faecal polluted surface water with these pathogens and this is a public health concern especially when water is used for agricultural purposes (Tymczyna *et al.,* 2000; Lyautey *et al.,* 2007; Garcia & Servais, 2007). According to Bhagwat (2006), the greatest concerns with human pathogens on fresh and minimally processed vegetables are *E. coli* 0157:H7, *Salmonella and L. monocytogenes.* The first two have low infective doses while *L. monocytogenes* grow very well under refrigeration storage conditions

(Bhagwat, 2006). Another safety concern with these pathogens is that they can form biofilms on the produce thereby making sanitizers ineffective (Somers *et al.,* 1994; Fonseca, 2006).

L. monocytogenes was not recovered from the Loskop Canal during the sampling intervals when incidence in the irrigation water source and vegetables were compared. However, it was recovered at previous sampling intervals. This signifies that *L. monocytogenes* may survive on the surface of broccoli for a long time after contact with irrigation water.

A lower incidence of *S. aureus*, *Salmonella*, intestinal *Enterococcus* and the absence of *L. monocytogenes* on cauliflower compared with broccoli show the possibility of differences in surface characteristics of the two produce affecting pathogen attachment and survival (Ukuku *et al.,* 2005; Fonseca, 2006). Broccoli among some other vegetables has been reported to pose a higher risk of being associated with listeriosis because of enhanced *L. monocytogenes* attachment (FDA/CFSAN, 2008).

The study clearly indicates the potential effect of raw sewage spillage, informal settlements and wastewater from mines and industries on irrigation water sources and pre-harvest vegetables.

3.1.5 Conclusion

The water used for irrigation in this study is a likely source of contamination of broccoli and cauliflower with bacterial pathogens and constitutes a food safety risk. The water should be properly treated when used for produce that may be eaten raw. This safety measure should be combined with Good Agricultural Practices (GAPs) and HACCP during the production of fresh vegetables.

3.2 EFFECT OF ATTACHMENT TIME FOLLOWED BY CHLORINE WASHING ON THE SURVIVAL OF INOCULATED *LISTERIA MONOCYTOGENES* ON TOMATOES AND SPINACH

Abstract

The effect of attachment time (30 min, 24, 48 and 72 h) followed by chlorine washing (200 ppm) on the survival of inoculated *Listeria monocytogenes* on the surface and subsurface of tomatoes and spinach was studied. The work was done to determine the efficacy of chlorine to decontaminate surface and subsurface 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 subsurface and incubated at 20 ℃ for either 30 min, 24, 48 or 72 h. LM attached and survived on the surface and subsurface structures of both control and chlorine-washed vegetables after each attachment time, up to 72 h. Higher levels of LM attachment and survival were however noticed on the subsurface structures. Chlorine had a greater effect on the LM on the surface structures compared with those in the subsurface structures, possibly because chlorine was not able to access the subsurface 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 subsurface structures of both tomatoes and spinach within 30 min, and that even after 72 h it still remained viable. It also indicated that chlorine treatment is more effective against surface LM compared to subsurface inoculated LM.

3.2.1 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 fruit and vegetables. Steele *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, Minnaar & Buys 2008; Solomon *et al.,* 2006; Kenney & Beuchat, 2002; Ruiz Vargas & Garcia-Villanova, 1987).

Different researchers have shown that attachment of *Listeria monocytogenes* is possible through the release of an enzyme to the surrounding host tissue or produce to facilitate bacterial attachment and infiltration (Hall-Stoodley & Stoodley, 2005; Jedrzejas, 2001). It has been reported that extracellular fibrils and flagellin have also been used by *Listeria monocytogenes* to enhance attachment (Kalmokoff *et al*., 2008; Lemon *et al*., 2007). After attachment, they can gain access to the subsurface 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 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; Ukuku *et al.,* 2005).

Chlorine is routinely used as a sanitizer in wash, spray and flume waters in the fresh and minimally-processed fruit and vegetable industries (Fonseca, 2006; Bhagwat, 2006; Beuchat, 1999). Antimicrobial activity depends on the amount of sodium hypochlorite 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 and 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 that are present within the subsurface structures of plants or vegetables are protected from the sanitizing effect of antimicrobial agents such as chlorine, hydrogen peroxide and ozone (Solomon *et al.,* 2006; Doyle & Erickson, 2008).

Although much research has reported on the ability of pathogens such as *E. coli* O157:H7 and *Salmonella* spp. to attach and gain access to the subsurface 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, Palumbo & Nguyen, 2004; Brandl, 2006). Broccoli, 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; FDA/CFSAN, 2008). Attachment to and growth on some produce including spinach have been reported (Gorski *et al.,* 2004; Jablasone, Warriner & Griffiths, 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 subsurface structures of tomatoes and spinach. Subsequently, the effect of chlorine on the subsurface and surface of *L. monocytogenes* on tomatoes and spinach after harvest was determined

3.2.2 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 subcultured 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 eight within the subsurface per experimental repetition. The experiment was repeated three times. To inoculate the tomatoes within the subsurface structures, wounding was first simulated at five 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 side of 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 ensure 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 eight within the subsurface per experimental repetition. To inoculate the spinach on the subsurface 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 the extent of attachment of LM was studied.

Chlorine washing of inoculated vegetables

After attachment of LM for 30 min, both surface-inoculated and subsurface inoculated tomatoes were washed by dipping into 200 ppm of chlorine for 1 min (Beuchat, 1998). The control was washed by dipping into distilled water. To disallow tomatoes from floating during washing, sterile tweezers were 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 subsurface inoculated spinach leaves were washed by dipping into 200 ppm of chlorine for 1 min (Beuchat, 1998). The control was washed by dipping into 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 subsurface structures of vegetables

To enumerate the number of LM on tomatoes, at each attachment time interval, on the surface and within the subsurface, about 100 g (one whole tomato) of tomato was added to 900 ml of distilled water after which maceration in a 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 the pour-plate method.

To enumerate the number of LM on spinach leaves at each attachment time interval on the surface and within the subsurface, about 10 g of spinach leaf was added to 90 ml of distilled water after which maceration in a 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 the 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 to dehydration were carried out at about 48 ℃. 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 a significant difference between the following factors: inoculation site (surface vs. subsurface), chlorine and attachment time. The experiment was repeated three times (n=3). ANOVA was performed using Statistica from Windows, version 7 (Tulsa, Oklohama, USA, 2003).

3.2.3 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 subsurface structures of tomatoes (Table 10). LM attached and survived on the tomato after each attachment time. The level of LM that survived and attached to the surface of tomato was lowest after 24 h (3.81 log cfu per tomato) and highest after 72 h (4.78 log cfu per tomato) (Fig 10). The level of LM that survived and attached to the subsurface 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 per tomato (Fig 10). The greatest effect of attachment time was therefore observed after 72 h of attachment to

both surface and subsurfaces of tomatoes. The ability of LM to attach to the surface of tomato after 24 h of attachment was illustrated using a scanning electron microscope (Figure 11).

Table 10: P values of effect of chlorine, site and attachment time on survival of inoculated *Listeria monocytogenes* on tomatoes and spinach

Treatment effect	P value for tomato	P value for spinach
Chlorine	0.001*	0.001*
Site	0.001*	0.001*
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

* Denotes statistical significant of treatment at $p \leq 0.05$

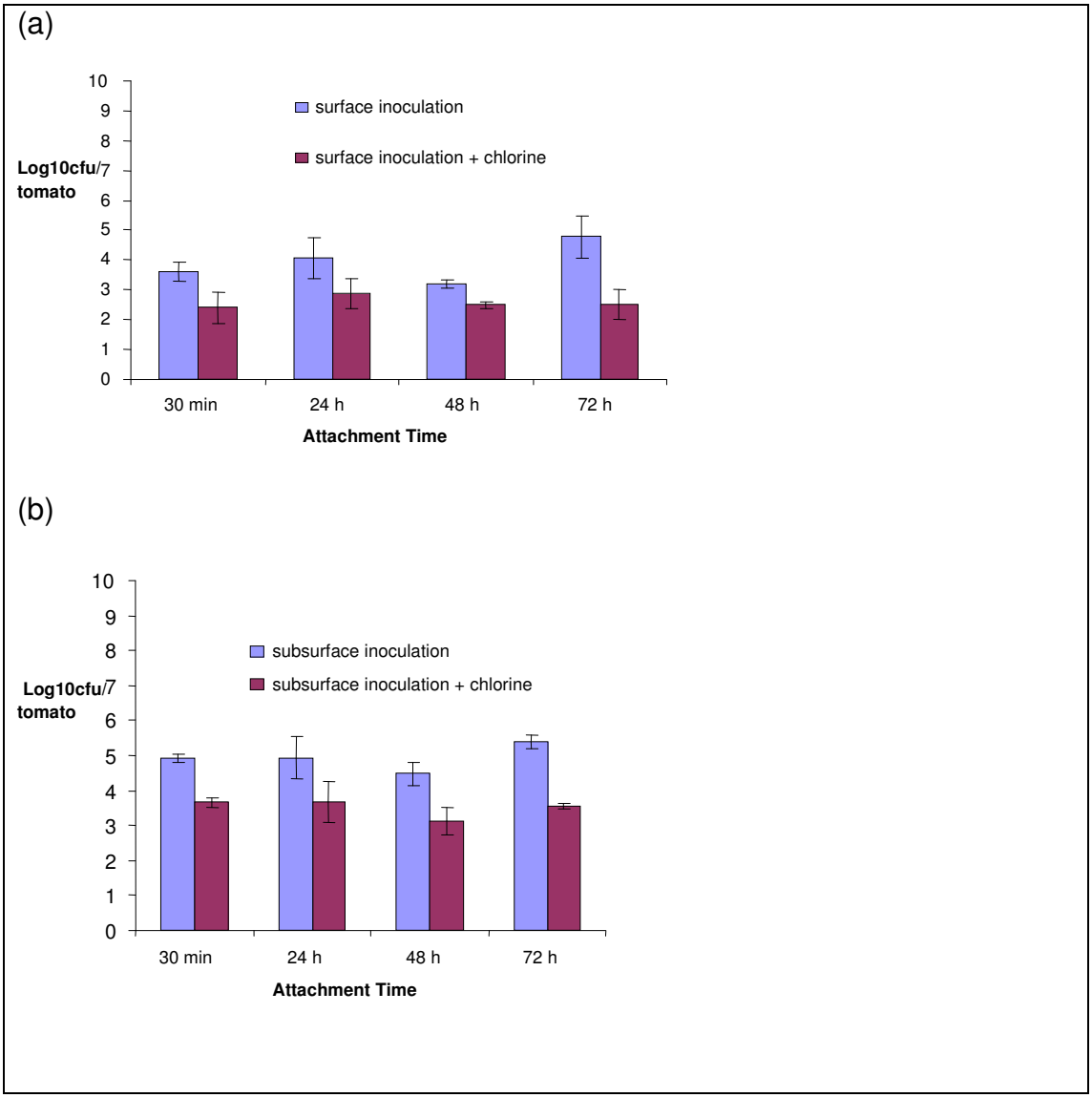


Figure 10: Attachment and survival of *L. monocytogenes* on the surface (a) and subsurface (b) of tomatoes with or without chlorine washing

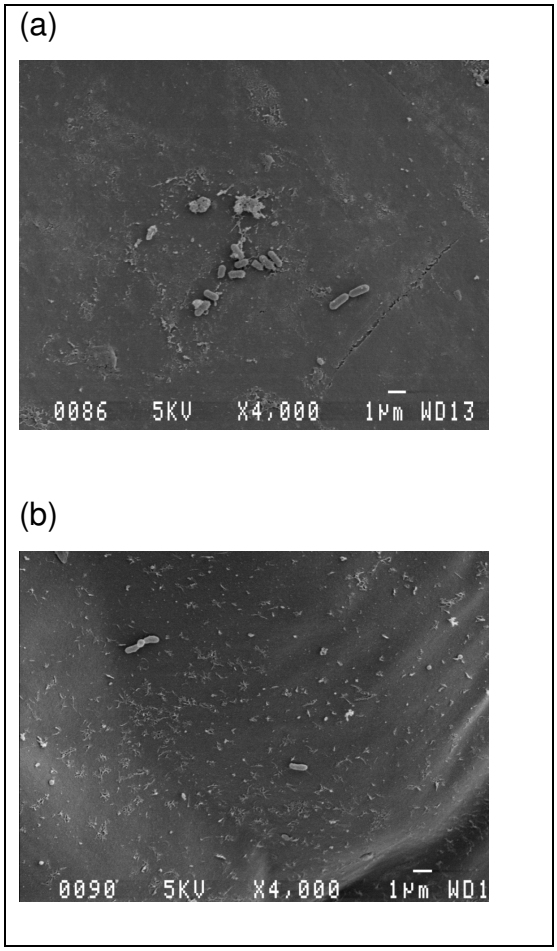


Figure 11: Scanning Electron Microscopy (SEM) of attachment of LM to the surface of tomato (a) and spinach (b) after 24 h

Effect of chlorine

Overall, chlorine affected the LM counts significantly ($p \leq 0.05$) (Table 10). 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 subsurface inoculated samples and after each attachment time (Figure 10, Table 10). The ability of LM to survive the sanitizing effect of chlorine after attachment to tomatoes for 24 h was illustrated using a scanning electron microscope (Figure 12).

After all attachment times, the LM levels for the control samples were higher than those for the chlorine-washed samples. After 30 min of attachment time

for the surface-inoculated tomatoes, there was a 1.21 log cfu per tomato 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 per tomato (Figure 10).

The LM levels for the subsurface-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 10). The differences in LM on the subsurface of control tomatoes and the treated ones followed the same trend as the surface-inoculated samples. However, the effect after 72 h was not as pronounced as that between the two treatments.

Effect of inoculation site

There was a significant difference ($p \leq 0.05$) between the subsurface-inoculated LM and surface-inoculated LM in tomatoes at different attachment times (Table 10). The LM levels for the subsurface-inoculated tomatoes were higher for both control and chlorine-washed samples at each attachment time, than those of the surface-inoculated tomatoes. The differences in LM between subsurface-inoculated and surface-inoculated control samples, decreased as the attachment time increased, i.e., 1.3 log cfu per tomato after 30 min and 0.6 log cfu per tomato after 72 h of attachment (Fig 10). For the chlorine-washed tomatoes the differences in LM, subsurface-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, namely, 1.26 and 1.04 log cfu per tomato respectively.

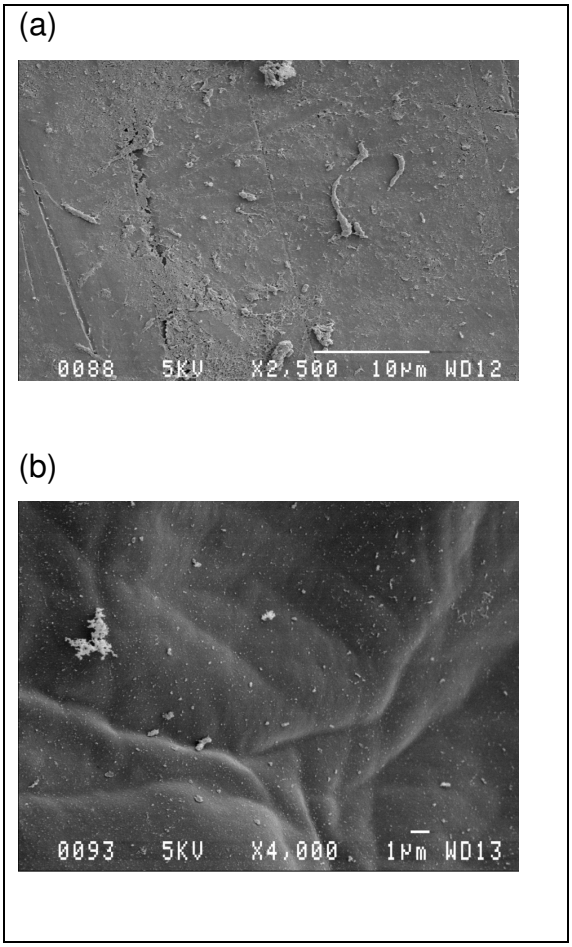


Figure 12: Scanning Electron Microscopy (SEM) of attachment of LM to the surface of tomato (a) and spinach (b) after 24 h followed by chlorine washing

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 subsurface structures of (Table 10). LM attached and survived on the spinach after each attachment time as observed for tomato. The level of LM that survived and attached to the surface of spinach reduced as attachment time increased, 4.86 log cfu per leaf after 30 min and 3.41 log cfu per leaf after 72 h (Figure 13). The level of LM that survived and attached to the subsurface of spinach followed the same trend, reducing with increased

attachment time, 5.17 log cfu per leaf after 30 min and 4.18 log cfu per leaf after 72 h (Figure 13). The ability of LM to attach to the surface of spinach after 24 h of attachment was shown with a scanning electron microscope (Figure 11).

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-inoculated and subsurface-inoculated samples and after each attachment time (Table 10). The ability of LM after attachment to spinach for 24 h to survive the sanitizing effect of chlorine was illustrated using a scanning electron microscope (Figure 12).

At all attachment times the LM levels for the control samples were higher than for those of the chlorine-washed samples. After 30 min of attachment time for the surface-inoculated spinach, there was a 3.01 log cfu per leaf 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 per leaf respectively (Figure 13).

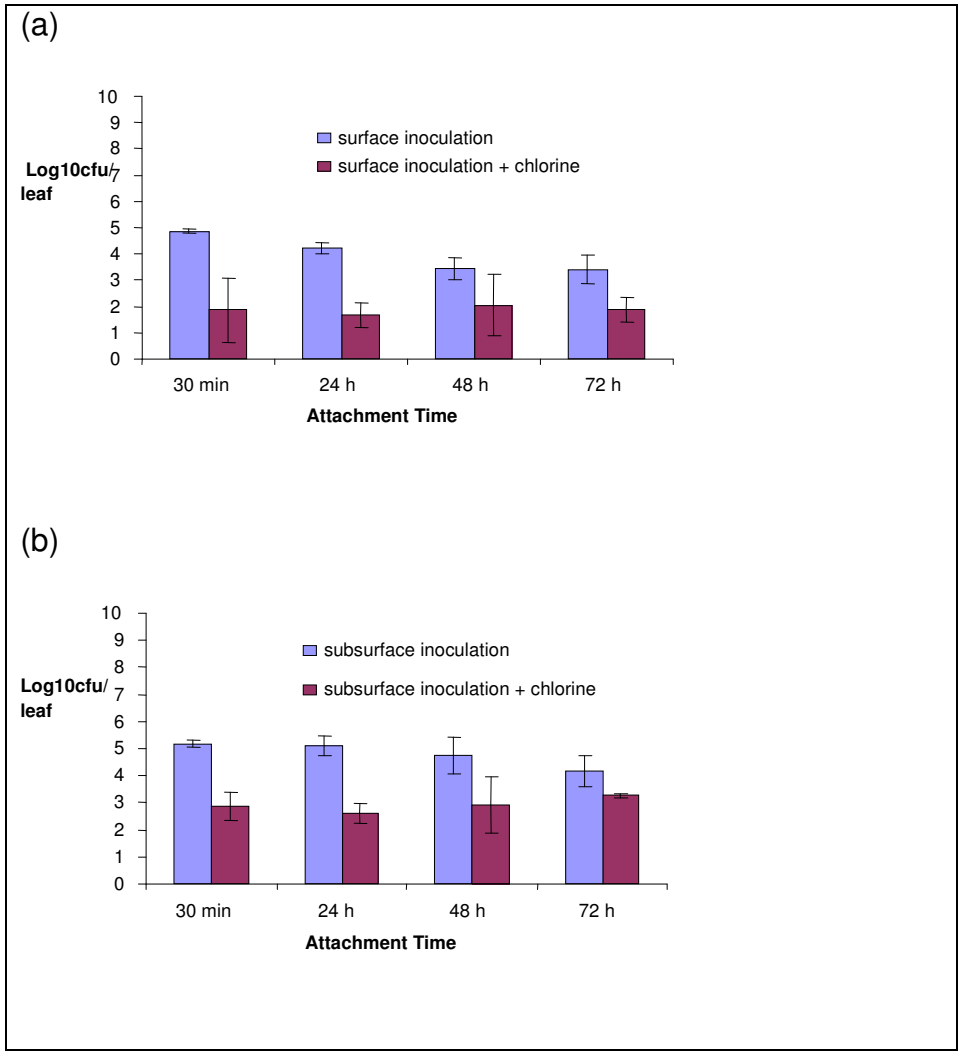


Figure 13: Attachment and survival of *L. monocytogenes* on the surface (a) and subsurface (b) of spinach leaves with or without chlorine washing

The LM levels for the subsurface-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 (Figure 13). The differences in LM on the subsurface 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 per leaf reduction after 72 h of attachment time.

Effect of inoculation site

There was a significant difference ($p \leq 0.05$) between the subsurface-inoculated LM and surface-inoculated LM in spinach at different attachment times (Table 10). The LM levels for the subsurface-inoculated spinach were higher for both control and chlorine-washed samples at each attachment time than for those of the surface-inoculated tomatoes. The differences in LM between subsurface-inoculated and surface-inoculated control samples increased with an 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 (Figure 13). For the chlorine-washed spinach the differences in LM, subsurface-inoculated and surface-inoculated, were comparable between attachment times. Differences in LM ranged between 0.86 and 1.4 log cfu/g (Figure 13).

3.2.4 Discussion

It was evident from the results that LM was able to attach to both the surface and subsurface structures of both spinach and tomatoes. This observation signifies that LM will attach to vegetables within 30 min of coming into contact with it in irrigation water or other sources. Although a shorter attachment time was not determined in this work, Ells and 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, Brackett & Beuchat, 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 subsurface and surface of spinach and tomato up to 72 h. It has been found that pathogens could survive on tomatoes for a longer time. Elif, Gurakan and Bayindirli (2006) showed that *Salmonella enteritidis* could survive and grow during storage of tomatoes for 220 h.

The significant difference between subsurface-inoculated LM and surface-inoculated LM in both vegetables at each time interval indicates that LM attaches in higher numbers to wounds or subsurface structures than to undamaged surfaces (Takeuchi *et al.,* 2000). Timothy and Hansen (2006) showed that LM has a preference to attach to cut or wounded tissues compared to intact leaf surfaces. This may be because surface structures of vegetables constitute a harsh environment with fluctuations in temperature unlike subsurface structures (Solomon *et al.,* 2006). The subsurface structures or cut surfaces also have a significant amount of liquid containing nutrients that is utilized by the attached microorganisms (Bhagwat, 2006). Furthermore, pathogens are able to create a more hospitable microenvironment in the subsurface structures than 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 emphasizing that vigorous washing and treatment with chlorine does not remove all bacterial pathogens on fruit and vegetables (Solomon *et al.,* 2006; Doyle & Erickson, 2008). Ineffectiveness of chlorine may be due to the low concentration (200 ppm) used and the ability of LM to form biofilms (Ukuku *et al.,* 2005). According to Kim, Yousef and Chism (1998), low levels of chlorine may not be effective against certain bacteria. A 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 wastewater (Simpson *et al.,* 2000; Moriyama *et al.,* 2004). It may also lead to a reaction 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 subsurface LM, probably because it was not able to access the subsurface structures

effectively, where the pathogens were located (Doyle & Erickson, 2008; Fonseca, 2006; Sapers *et al.,* 1990). This is in line with the observation of Liao & Cooke (2001) who found that *Salmonella* Chester survived chlorine washing to a much greater extent when attached to the subsurface 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., organic matter coming from the tissue exudates. The organic matter is able to neutralize some of the chlorine before it reaches 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 and Sapers (2001) who confirmed that *Salmonella* serovar Stanley populations in cantaloupes was 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 a pathogen that has just attached to the surface of produce compared 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 compared to an attachment time after 30 min. This is because the 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 and vary from one produce to another (Ukuku *et al.,* 2005). Also according to Fonseca (2006), differences in surface characteristics of the produce, the physiological state of a pathogen, and environmental stress conditions interact to influence the activity and efficiency of the sanitizer. It may therefore be necessary to customise sanitizing

treatments for different types of produce because of this complexity (Bhagwat, 2006).

3.2.5 Conclusion

This work shows that *Listeria monocytogenes* will attach to spinach and tomato within 30 min and it will remain viable after attachment even up to 72 h. Other authors have reported a 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 subsurface, as it could reduce only ≤ 3 logs inoculated and attached LM both on the surface and subsurface structures.

3.3 BACTERIAL PATHOGENS IN IRRIGATION WATER AND ON PRODUCE ARE AFFECTED BY CERTAIN PREDICTOR VARIABLES

Abstract

The possibility of predicting the presence of pathogens in irrigation water and on vegetables was determined. Logistic regression analysis was used to determine whether various predictor variables could be used to predict the occurrence of *L. monocytogenes*, *Salmonella* spp and intestinal *Enterococcus* in irrigation water and vegetables (cauliflower and broccoli). It was evident that COD was statistically reliable to predict *L monocytogenes*, turbidity, reliable to predict intestinal *Enterococcus* and faecal coliforms and coliforms, and reliable to predict *Salmonella* in irrigation water. Also, while the regression analysis showed that the aerobic colony count (ACC) and aerobic sporeformer count (AnSF) could be used to predict *Salmonella* and intestinal *Enteroccus* in vegetables, *S. aureus* and ACC were indicated to be significant parameters in predicting *L monocytogenes* on vegetables. This work showed

that in addition to the common indicators, i.e., *E. coli,* faecal coliforms, and faecal *Streptococci*, the microbiological quality of irrigation water and vegetables might be indicated after physico-chemical properties and ACC.

3.3.1 Introduction

The rate of foodborne disease outbreaks caused by produce contamination increased from 0.7% in the 1970s to 13% between 1990 and 2005 (Ailes *et al.,* 2008). There are ample avenues for produce to become contaminated during production and afterwards (Beuchat & Ryu, 1997; Beuchat, 2002; Beuchat, 2006). According to Johnston *et al.* (2006), contamination takes place at different stages of the growth, harvest, packing and distribution of produce. Contaminated irrigation water sources have been reported as a major way by which fruits and vegetables become contaminated with bacteria pathogens (Ibenyassine *et al.*, 2006).

According to Ailes *et al.* (2008), improved diagnostic methods and enhancements to foodborne disease surveillance systems have helped in produce safety and vegetable recall. Another thing that may lead to improved produce safety is the use of other indicator organisms different from the common ones, i.e., faecal coliforms, faecal *Streptoccoci* and *E.coli.* Physico-chemical properties may also be used for monitoring the microbiological safety of water (Horman *et al.* 2004). Horman *et al.* (2004) found that together with *E. coli* and faecal coliform, *C. perfrigens* could be used as an indicator of water safety. Furthermore, a combination of suitable indicators such as coliform and acid-fast bacteria, coliphages, the standard plate count, and fecal *Streptococci* has been recommended for adequate monitoring (Grabow *et al.,* 1983). In fact, Harwood *et al.* (2005) believed that public health cannot be adequately protected through simple monitoring schemes based on the use of *E. coli* alone but suggested that additional parameters should be used as indicators. Scott *et al.* (2002) also confirmed that the use

of other pathogens, chemical methods, genotypic and phenotypic methods are fundamental to microbial source tracking.

Our goal was to use logistic regression analysis and some predictor variables to predict the presence of selected bacterial pathogens, i.e., *Salmonella* spp, *L. monocytogenes* and intestinal *Enterococcus* in irrigation water and vegetables. Determination of the presence of all these pathogens in irrigation water and vegetables could be costly and also time consuming. Although the use of logistic regression analysis for prediction in irrigation water and fresh produce is uncommon, Ailes *et al.* (2008) used this model to confirm that microbial concentrations on fresh produce are predicted by post-harvest processing, importation and the season. Also, the absence of some indicators in water was significant to predict its safety through the logistic regression model (Horman *et al.,* 2004).

3.3.2 Materials and methods

For the selection of rivers and vegetables, bacterial and physical analyses of samples, refer to Ssection 3.1.2.

Statistical analyses

All statistical analyses were completed by using SAS version 9.2 (SAS Institute, Inc., Cary, NC). ACC, ASF, AnSF, and S. aureus were log-transformed to satisfy the assumption of normality. The associations of the occurrence of *L. monocytogenes, Salmonella* spp and intestinal *Enterococcus* in irrigation water and vegetables were explored using binary logistic regression analysis. For this analysis, we dichotomised the dependent variables, *L. monocytogenes, Salmonella* spp and intestinal *Enterococcus* where values for absence were coded as ‘0’ while values for presence were coded as ‘1’. For prediction of the three bacterial pathogens in irrigation water, four predictor variables (coliforms, faecal coliforms, COD and turbidiy)

were taken into the model. On the other hand, ACC, *S. aureus*, location, ASF, AnSF, coliforms and faecal coliforms were used as predictor variables in the model for prediction of the bacterial pathogens in vegetables. The resulting regression coefficients quantified the type of association between the predictor variable and the respective dependent variable. A p-value of ≤ 0.05 was considered statistically significant and all reported p-values were two-tailed.

3.3.3 Results and discussion

Predictive relationships between predictors

A pooled data set from the Loskop Canal, Olifants River and Wilge River were analysed to determine if the concentrations of any of the indicators, total coliforms, faecal coliforms, *S. aureus*, aerobic sporeformers, anaerobic sporeformers and aerobic colony counts, were correlated with each other and with physico-chemical parameters (turbidity and chemical oxygen demand). High significant correlations were observed between faecal coliforms and total coliforms ($r = 0.999$, p-value < 0.0001), aerobic sporeformers and anaerobic sporeformers ($r = 0.535$, p-value < 0.0001), *S. aureus*, aerobic sporeformers ($r = 0.498$, p-value < 0.0001), aerobic colony counts and anaerobic sporeformers ($r = 0.354$, p-value $= 0.0002$), aerobic colony counts and *S. aureus* ($r = 0.345$, p-value $= 0.0003$); and a significant correlation was observed between anaerobic sporeformers and *S. aureus* ($r = 0.203$, p-value $= 0.0354$). Except between turbidity and *S. aureus*, chemical oxygen demand and total coliforms, chemical oxygen demand and faecal coliforms, significant correlations were observed between the concentrations of any of the indicators with physico-chemical parameters.

Binary logistic regression was used to test the hypothesis that faecal coliform, location, COD and turbidity were predictive of the presence of *L. monocytogenes, Salmonella* sp and intestinal *Enterococcus* in irrigation water.

Binary logistic regression was also used to test the hypothesis that ACC, ASF, AnSF, *S. aureus*, faecal coliform and coliform were predictive of the presence of *L. monocytogenes, Salmonella* sp and intestinal *Enterococcus* on vegetables.

Prediction of L. monocytogenes, Salmonella and intestinal *Enterococcus in water samples from Loskop Canal, Wilge River and Olifants River*

Results of the logistic regression indicated that only one predictor, COD, was statistically reliable ($p \leq 0.05$) to predict the presence *L. monocytogenes.* The estimates of regression coefficients of the predictors $\hat{\beta}$, Wald statistic and p-values are presented in Table 11.

Table 11: Prediction of *L. monocytogenes* in irrigation water

Predictors	$\hat{\beta}$	**Wald**	**p-value**
Faecal coliforms	-0.0014	0.5785	0.4469
Coliforms	0.0001	0.5194	0.4711
Turbidity	-0.0199	0.6958	0.4042
COD	-0.0399	9.4825	0.0021

A p-value of ≤ 0.05 was considered statistically significant

Like the result of the prediction of *L. monocytogenes* in irrigation water samples in which only one predictor was associated with it, only one predictor, turbidity was found to be statistically significant ($p \leq 0.05$) to predict the presence of intestinal *Enterococcus* in the water samples from three sources (Table 12).

Table 12: Prediction of intestinal *Enterococcus* in irrigation water

Predictors	$\hat{\beta}$	Wald	p-value
Faecal coliforms	0.0013	0.4224	0.5157
Coliforms	-0.0001	0.3564	0.5505
Turbidity	-0.0544	5.7643	0.0164
COD	0.0264	2.4581	0.1169

A p-value of ≤ 0.05 was considered statistically significant

Faecal coliforms and coliforms however were found to be significant ($p \leq 0.05$) to predict the presence of *Salmonella* sp (Table 13).

Table 13: Prediction of *Salmonella* sp in irrigation water

Predictors	$\hat{\beta}$	Wald	p-value
Faecal coliforms	0.0048	3.8008	0.0500
Coliforms	-0.0005	3.8038	0.0500
Turbidity	0.0105	0.3399	0.5599
COD	0.0123	1.3747	0.2410

A p-value of ≤ 0.05 was considered statistically significant

Prediction of L. monocytogenes, Salmonella sp *and* intestinal *Enterococcus on vegetables*

The result of logistic regression analysis showed that two predictors, ACC and *S. aureus*, were statistically significant (both p-values are ≤ 0.05) to predict the presence of *L. monocytogenes* on vegetables. The estimates of regression coefficients of the predictors $\hat{\beta}$, Wald statistic and p-values are shown in Table 14.

Table 14: Prediction of *L. monocytogenes* on vegetables

Predictors	$\hat{\beta}$	Wald	p-value
ACC	-1.8486	17.9433	0.0001
ASF	-0.2353	0.3620	0.5474
AnSF	-0.0767	0.0586	0.8088
S. aureus	0.9414	6.9747	0.0083
Faecal coliforms	-0.0004	0.0855	0.7700
Coliforms	0.0001	0.0830	0.7733

A p-value of ≤ 0.05 was considered statistically significant

Also, from the result of the logistic regression analysis, ACC and AnSF were observed to be significant ($p \leq 0.05$) to predict the presence of intestinal *Enteroccocus* and *Salmonella* sp respectively (Table 15 and Table 16).

Table 15: Prediction of intestinal *Enterococcus* on vegetables

Predictors	$\hat{\beta}$	Wald	p-value
ACC	-0.7971	6.2123	0.0127
ASF	0.0152	0.0016	0.9682
AnSF	0.7324	5.2992	0.0213
S. aureus	-0.1662	0.2770	0.5986
Faecal coliforms	-0.0020	3.1176	0.0775
Coliforms	0.0002	3.3093	0.0689

A p-value of ≤ 0.05 was considered statistically significant

Table 16: Prediction of *Salmonella* sp on vegetables

Predictors	$\hat{\beta}$	Wald	p-value
ACC	-1.2487	9.7924	0.0018
ASF	0.1181	0.0932	0.7602
AnSF	0.6926	4.2584	0.0391
S. aureus	0.5469	2.4546	0.1172
Faecal coliforms	0.0007	0.3020	0.5827
Coliforms	-0.0001	0.2633	0.6079

A p-value of ≤ 0.05 was considered statistically significant

The result of the prediction of *L. monocytogenes* in irrigation water signifies that there may be a direct relationship between *L. monocytogenes* and COD in irrigation water. Higher COD results in water may result in a high concentration of *L. monocytogenes* in irrigation water. The reason why other predictors, i.e., faecal coliform, coliform and turbidity were not associated with *L. monocytogenes* in irrigation water is not clear. The result also signifies that there is a direct relationship between intestinal *Enteroccocus* and turbidity. Faecal coliforms and coliforms have long been known as indicators of enteric bacteria in water (Jay, 2000). The logistic regression result proved that faecal coliforms and coliforms can be used to predict the presence of *Salmonella* sp in water and that there is relationship between faecal coliform and *Salmonella* sp. This is similar to the observation of Polo *et al.* (1998) who showed that there is a direct relationship between the presence of *Salmonella* sp and indicators of faecal pollution, i.e., coliforms and faecal coliforms in rivers, freshwater reservoirs and seawater. Ferguson *et al.* (1996) also observed that the higher the concentration of faecal coliform, the higher the recovery of *Salmonella* sp in an aquatic habitat.

The reason why faecal coliforms and coliforms were not significantly associated with *L. monocytogenes* and intestinal *Enterococcus* may be because they are not usually found in human faeces, unlike *Salmonella* sp. According to Gildreich and Kenner (1969) and Pautshwa *et al.* (2009), human faeces contain higher faecal coliform counts, while animal faeces contain higher levels of faecal *Enterococci*. Wild birds and animals have also been shown to be the main source of contamination with *L monocytogenes* (Weiss & Seeliger, 1975).

The prediction of *L. monocytogenes*, intestinal *Enterococcus* and *Salmonella* in irrigation with the aerobic colony count (ACC) shows that it may be an important parameter to indicate the presence or absence of these pathogens. The relationship between the three bacterial pathogens and ACC may be an

indirect one, i.e., low aerobic colony count was associated with the prevalence of *Salmonella*, intestinal *Enterococcus* and *L. monocytogenes* on vegetables.

Several workers have reported that there is an indirect relationship between indigenous bacteria and foodborne pathogens (Johnston *et al.,* 2006; Ruiz *et al.,* 1987; Ukuku *et al.,* 2005). It was also observed from our study that there was a prevalence of these bacterial pathogens in irrigation water and vegetable samples while low aerobic colony counts were observed.

The logistic regression analysis may therefore be used as a tool for a predictive microbiology model which has an immediate practical application to predict microbial produce safety and quality, and provide quantitative understanding of the microbial ecology of irrigation water and produce (Ross, Dalgaard & Tienungoon, 2000).

3.3.4 Conclusion

Faecal coliforms and coliforms indicate a high probability of *Salmonella* presence in water and they may be used as risk parameters. There is a relationship between the physiochemical properties of water i.e., COD and turbidity and certain bacterial pathogens i.e., *L. monocytogenes* and intestinal *Enterococcus*.