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Bacteriological Assessement of Vegetables Cultivated in Soils Treated with Poultry Manure and the Manure-Treated Soil Samples

OBI C. N.*

Department of Microbiology, College of Natural Sciences, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State, Nigeria
*Corresponding author: b4brocliff@gmail.com

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Abstract Bacteriological quality of four vegetables: waterleaf (W), pumpkin (P), cucumber (C) and tomatoes (T) cultivated in soils treated with poultry manure and the manure-treated soil samples (S1, S2, S3 and S4) were assessed using standard microbiological methods. *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Bacillus* spp, *Salmonella* spp. and *Shigella* spp were recovered from the soil and vegetable samples. The total bacterial count of the soil samples ranged from 1.75×10^9 (S3) to 5.9×10^4cfu/ml (S4) while the total bacterial count for the vegetable samples ranged from 2.65×10^9 (P) to 1.50×10^5cfu/ml (W). The total coliform count of the soil samples ranged from 1.65×10^7(S3) to 6.9×10^4cfu/ml (S4) while that of the vegetable samples ranged from 1.20×10^8(P) to 7.2×10^5cfu/ml (W). S1 was significantly different from S2 and S4 for the total bacteria count for the soil samples while P and W were significantly different for the vegetable samples ($P< 0.05$). For *Salmonella-Shigella* plate counts, S1 and S4 were significantly different and S2 and S3 were same ($P< 0.05$); while for the vegetable samples, P, W, C and T were significantly different ($P< 0.05$). S1, S2, S3, and S4 were significantly different for the total coliform count for the soil samples and same for P, W and C ($P<0.05$). Sensitivity screening for the isolates showed that *Bacillus* spp was most sensitive to Ofloxacin (25mm) while *Staphylococcus aureus* was most sensitive to Ofloxacin (22mm). *Shigella* was most sensitive to Ciprofloxacin (25mm) while *E. coli* showed highest sensitivity to Ciproflacin (25mm) and Ofloxacin (25mm). In addition, *Klebsiella* spp was most sensitive to Ciprofloxacin (23mm) and Ofloxacin (23mm) and *Salmonella* showed resistance to all the antibiotics. This study demonstrated that there is a high level of microbial contamination associated with the cultivation of vegetables in soils in which organic manure has been applied to which is of risk to the consumers.

Keywords: *bacterial pathogens, contamination, poultry manure, soil, vegetables*

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1. Introduction

Raw and minimally-processed vegetables are an essential part of people's diet all around the world because they serve as an extraordinary dietary source of nutrients, micronutrients, vitamins and fibre for humans and are thus vital for health and well being. Well balanced diets, rich in fruits and vegetables, are especially valuable for their ability to prevent vitamin C and A deficiencies and are also reported to reduce the risk of several diseases (Kalia and Gupta, 2006).

Vegetables are widely exposed to microbial contamination through contact with soil, dust and water and by handling at harvest or during postharvest processing. They therefore harbour a diverse range of microorganisms including plant and human pathogens (Carmo *et al.*, 2004). Vegetable consumption has been associated with a reduced risk for cardiovascular disease (Djoussé *et al.*, 2004; John and Ziebland, 2004), cancer (Riboli and Norat, 2003), stroke, and reduced mortality. Differences in microbial profiles of various vegetables result largely from unrelated factors such as resident microflora in the soil, application of nonresident microflora via animal manures, sewage or irrigation water, transportation and handling by individual retailers (Ray and Bhunia 2007; Ofor *et al.*, 2009).

In developing countries such as Nigeria, continued use of untreated waste water and manure as fertilizers for the production of fruits and vegetables is a major contributing factor to contamination (Amoah *et al.*, 2009). Despite their nutritional and health benefits, outbreaks of human infections associated with the consumption of fresh or minimally processed fruits and vegetables have increased in recent years (Beuchat, 2002). Enteric pathogens such as *Escherichia coli*, *Salmonella* and *Shigella* are among the greatest concerns during food-related outbreaks (Buck *et al.*, 2003). Several cases of typhoid fever outbreak have

been associated with eating contaminated vegetables grown in or fertilized with contaminated soil or sewage (Beuchat, 1998). These increases in fruits and vegetable borne infections may have resulted from increased consumption of contaminated fruits and vegetables both in the home and outside the home as most people spend long hours outside the home. In Nigeria for instance, street vending of handy ready-to-eat sliced fruit and vegetables has recently become very common and the market is thriving.

Even though vegetables have caught the eyes of many vegetable growers' attention in the world, the serious implication relating to the use of organic manure in the fertilization of vegetables has led to an increasing health problem as a result of contamination of vegetables by microorganisms (Maboko and Du Plooy, 2008).

2. Objectives of Research

Increased consumption of vegetables has increased their demand for these products which has led to an alarming increase associated with the negative health implication associated with consumption of contaminated batches of vegetables produced from soils treated with animal manure. This research work carried out in 2014 aimed at:

i. determining the microorganisms associated with selected vegetables cultivated in soil treated with poultry manure and the manure-treated soil samples;

ii. determining the antimicrobial susceptibility patterns of the isolates.

3. Soil Sampling and Analysis

Ten grams (10g) of soil surrounding a randomly selected vegetable plant at a depth of 2.5cm from the surface were aseptically collected from each treated plot of farmland for each crop using a sterile trowel previously sprayed with 70% ethanol. The samples were kept in a sterile plastic bag and transported to the laboratory for analysis. This collection was replicated four times four times for each vegetable sample.

One gram (1g) of each soil sample was serially diluted with 9ml of distilled water in eight test tubes. 0.1ml aliquot of the serially diluted samples (from 10^{-4} to 10^{-6}) was spread plated on MacConkey agar (Titan, Biotech limited), *Salmonella-Shigella* agar (Titan Biotech limited), and Nutrient agar (Titan Biotech limited). The plates were incubated at 25°C for 24hours for bacterial growth.

4. Vegetable Sampling and Analysis

Samples of four vegetables: Waterleaf (W), Pumpkin (P), Cucumber (C) and Tomatoes (T) samples were randomly selected from plants thinned or harvested and the edible portions (root for cucumber, fruit for tomatoes and leaves for pumpkin and water leaf) were separated using scissors previously sprayed with 70% ethanol, placed in sterile plastic bags and transported to the laboratory for analysis within one hour. The vegetables were sampled four times. MacConkey agar (Titan Biotech limited), *Salmonella-Shigella* agar (Titan Biotech limited) and Nutrient agar (Titan Biotech limited) were prepared according to manufacturer's instruction, and sterilized by autoclaving at 121°C for 15minutes except *Salmonella-Shigella* agar which does not require autoclaving and was sterilized by boiling for 15minutes.

Ten grams (10g) of each vegetable sample were weighed with a weighing balance and rinsed thoroughly in 90ml of distilled water. 1ml of the rinse water was then serially diluted (1:10) in six test tubes and 0.1ml aliquot from 10^{-3} and 10^{-4} dilutions were spread onto the various agar for determination of bacteria and incubated aerobically and anaerobically at 37°C for 24hours. The total number of colonies that developed were counted (30-300 colonies), and expressed as CFU/ml for microbial load. Colonies were differentiated on the basis of morphological and counts were made for the different colonies. Pure cultures of representative bacterial colonies were obtained by sub-culturing by streaking onto sterile Nutrient agar, MacConkey agar, and *Salmonella- Shigella* agar plates previously prepared. The plates were then incubated at 37°C for 24hours. Isolated colonies resulting from these cultures were examined with respect to colony features such as consistency, edge of colony, extent of growth, and pigmentation and was preserved on agar slants and subjected to Gram reaction and other biochemical tests (Baker and Breach, 1980; Cowan and Steel, 1985).

5. Biochemical Tests

The isolates were from the soil and vegetable samples analyzed were subjected to biochemical tests: catalase production, oxidase production, indole test, coagulase test and sugar fermentations.

5.1. Antibiotic Susceptibility Testing

5.1.1. Preparation of Turbidity Standard Equivalent to McFarland 0.5

One percent (1%) v/v solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water. 1% of barium chloride was also prepared by dissolving 0.5g of dehydrated barium chloride in 50ml of distilled water. 0.6ml of Barium chloride solution was added to 99.4ml of the sulphuric acid solution and mixed properly. The solution was preserved in the fridge.

5.2. Sensitivity Testing

The Kirby-Bauer disc diffusion technique was used (CLSI, 2009). Pure colonies of isolates were obtained from agar slants and sub-cultured on already prepared culture plates. After growth within 24hours, isolates from each of the plates were adjusted to 0.5 McFarland standards in 0.9% saline. Swab sticks were dipped into the solution of the isolate and normal saline and streaked uniformly on Muller-Hinton agar plates to obtain confluent growth. Multi antibiotics sensitivity discs were placed on the surface of the media, using a pair of forceps, little force was applied to ensure firm contact with agar plate. The plates were then inverted and incubated aerobically at 37°C for 18-24hours.

6. Measurement of Zone of Inhibition

The plates were examined for zone of inhibition. Using a ruler, the diameter of zone of inhibition was obtained in millimeters from the reverse of the plate. The zone sizes of each plate (both Gram positive and negative plates) were interpreted and zone sizes reported as susceptible, intermediate or resistant.

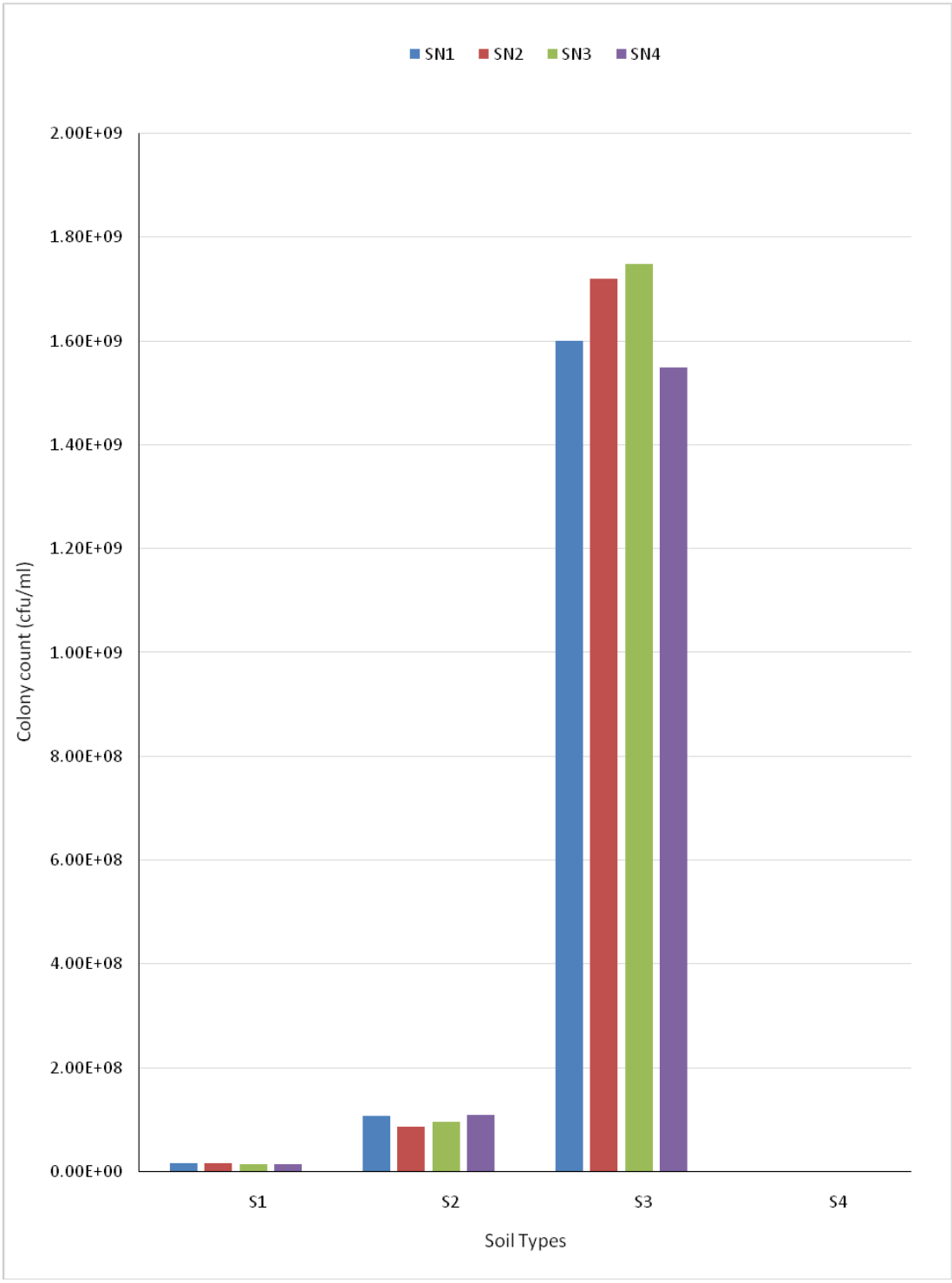

Figure 1. Viable plate count of Microorganisms for experimental soil samples

7. Results and Discussion

The analysis was conducted using four vegetable types and four soil samples for the respective vegetables namely: Pumpkin (coded P and S1 for the cultivated soil of pumpkin); Water leaf (coded W and S2 for the cultivated soil of water leaf), Cucumber (coded C and S3 for the cultivated soil) and Tomatoes (coded T and S4 for the cultivated soil). *Staphylococcus aureus*, *Escherichia coli*, *Klesbsiella* spp, *Shigella* spp., *Salmonella* spp, and *Bacillus* spp recovered from the vegetable and soil samples. The colony forming unit (cfu/ml) values are shown in Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7 and Figure 8 while Table 1 and Table 2 show the antibiotic sensitivity patterns for the Gram positive and Gram negative organisms respectively.

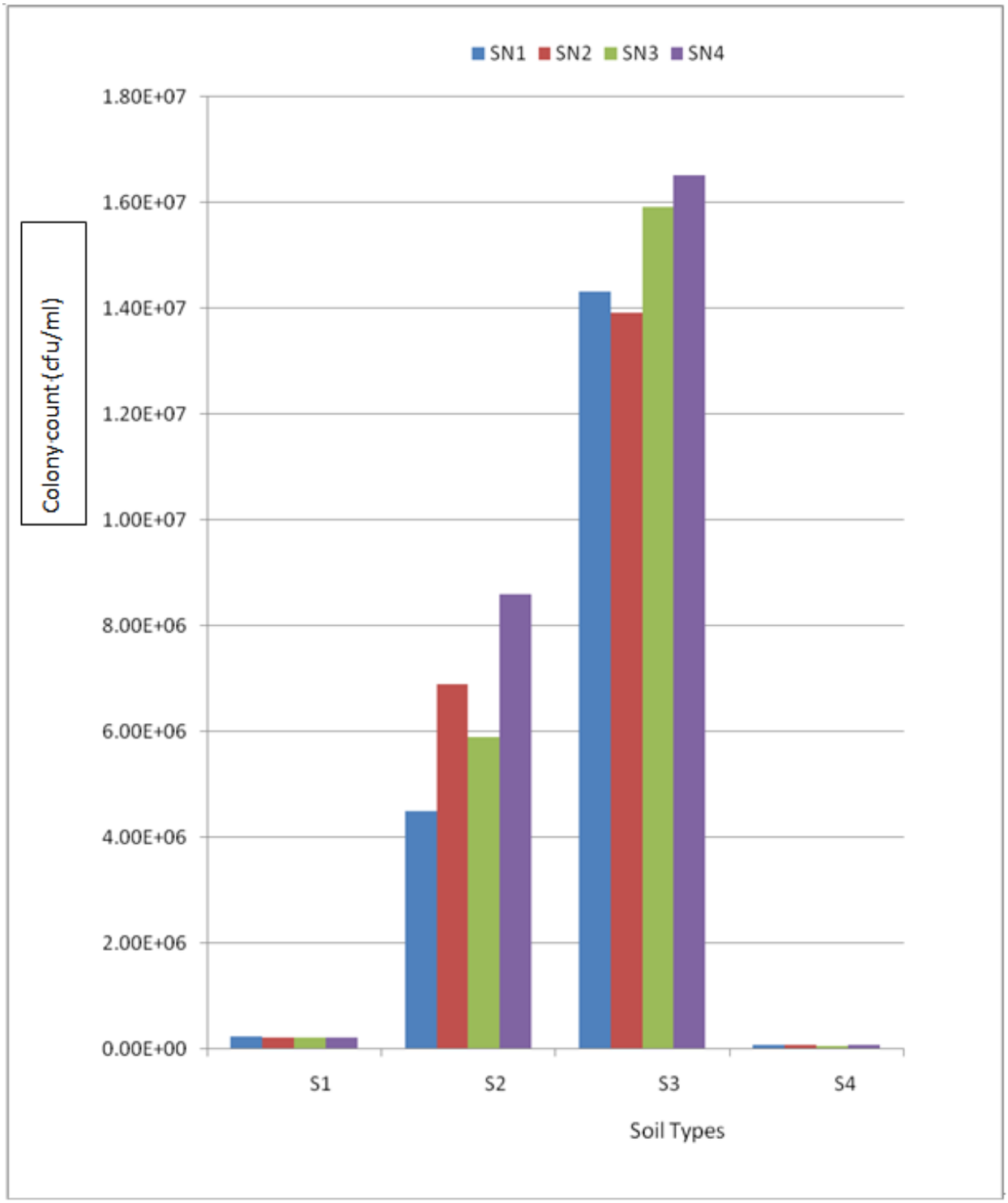

Figure 2. Total Coliform Count for the experimental soil samples

This study has shown that the application of poultry manure to soil cultivated with some ready-to-eat vegetables is of great microbial hazard. Therefore, vegetables may act as a reservoir for several pathogens and thereafter infect susceptible host (Beuchat, 1998). Almost any ready-to-eat vegetable that has been contaminated with pathogens either from the environment or from human or animal faeces or through storage, processing and handling could potentially cause disease (Beuchat, 2002).

The high Total bacterial and coliform counts, detected among the vegetable samples surveyed in this investigation revealed that the contamination of these vegetables by pathogenic microorganisms may present a potential health hazard to consumers. The Total bacteria count which ranged from 1.75×10^{9}cfu/ml to 5.9×10^{4}cfu/ml and 2.65×10^{9}cfu/ml to 1.50×10^{5}cfu/ml for the soil and vegetable samples respectively showed that a diverse population of bacteria can be isolated from vegetables and soil used in cultivating them. In comparison with the work carried out by Macklin *et al*., (2005), it is believed that the countable colonies fell in between 10^{4} to 10^{9} cfu/ml due to environmental factors affecting the soil and its constituent, competition between residual microorganisms which was not present in the fresh poultry manure.

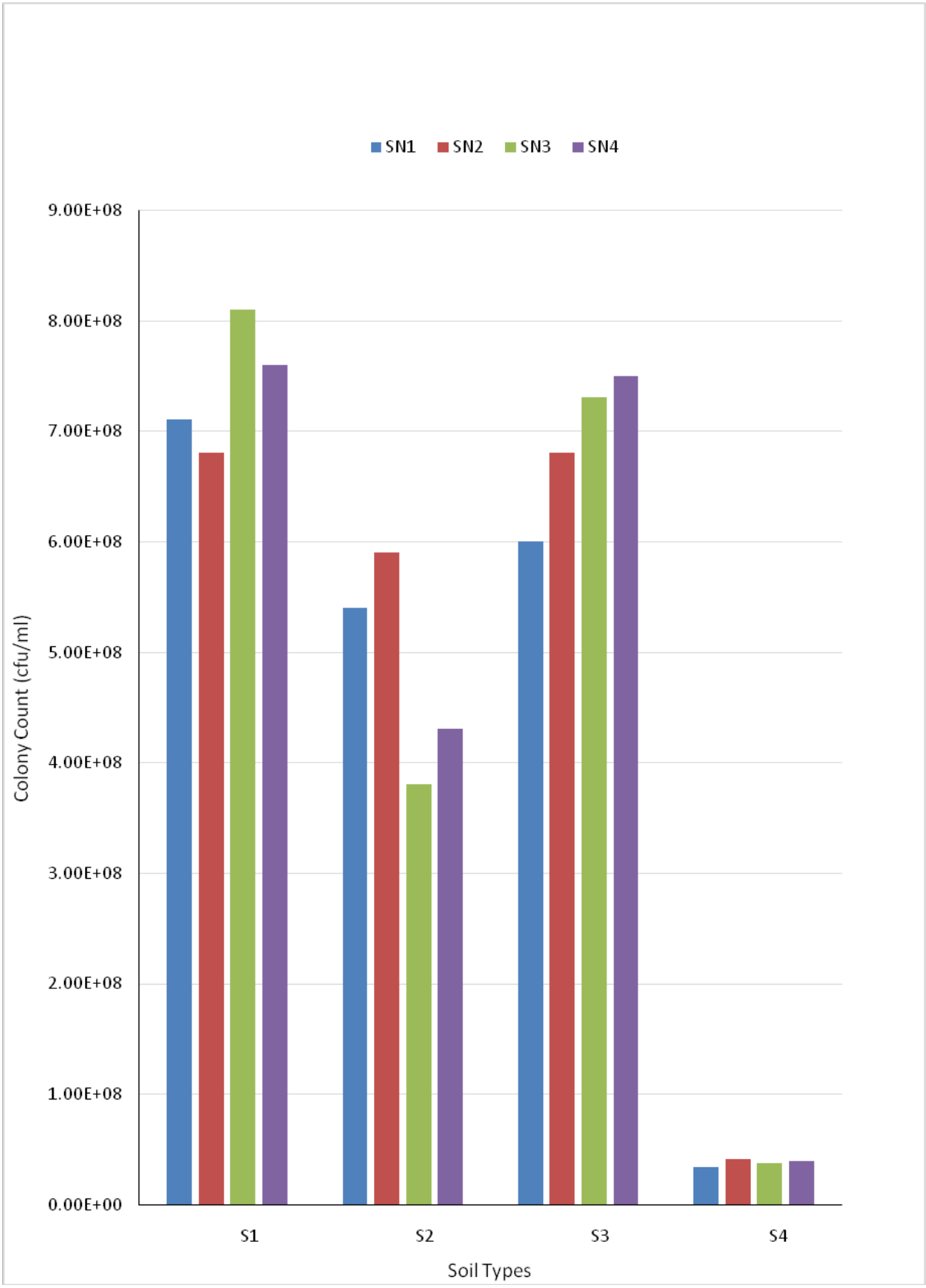

Figure 3. Total anaerobic bacteria count of the experimental soil samples

The Total coliform count which ranged from 1.65×10^{7} - 6.9×10^{4}cfu/ml and 1.20×10^{8} - 7.2×10^{5}cfu/ml showed that both the vegetable and soil harbored enteric organisms of faecal origin. Fruits and vegetables can become contaminated with pathogenic microorganisms during harvesting through faecal material, human handling, harvesting equipment, transport containers, wild and domestic animals, air, transport vehicles, ice or water (Beuchat, 1995).

The presence of high number of total coliform (*Klebsiella*) and faecal coliform (*Escherichia coli*) in the soil and vegetable samples revealed that the manure could

be a possible source of contamination from the soil to the vegetable. Kudva *et al.*, (1998) confirmed the occurrence of *E. coli* and other coliforms in birds used as poultry. *S. aureus* which is a chance contaminant was also detected. According to Houang *et al.*, (1991), there have been occurrences of *S. aureus* on ready-to-eat vegetables. Although *S. aureus* may have a diverse route in terms of contamination of vegetables viz the nasal passages of healthy food handlers and by irrigation of vegetable farm with contaminated water (Abdelnoor *et al.*, 1983).

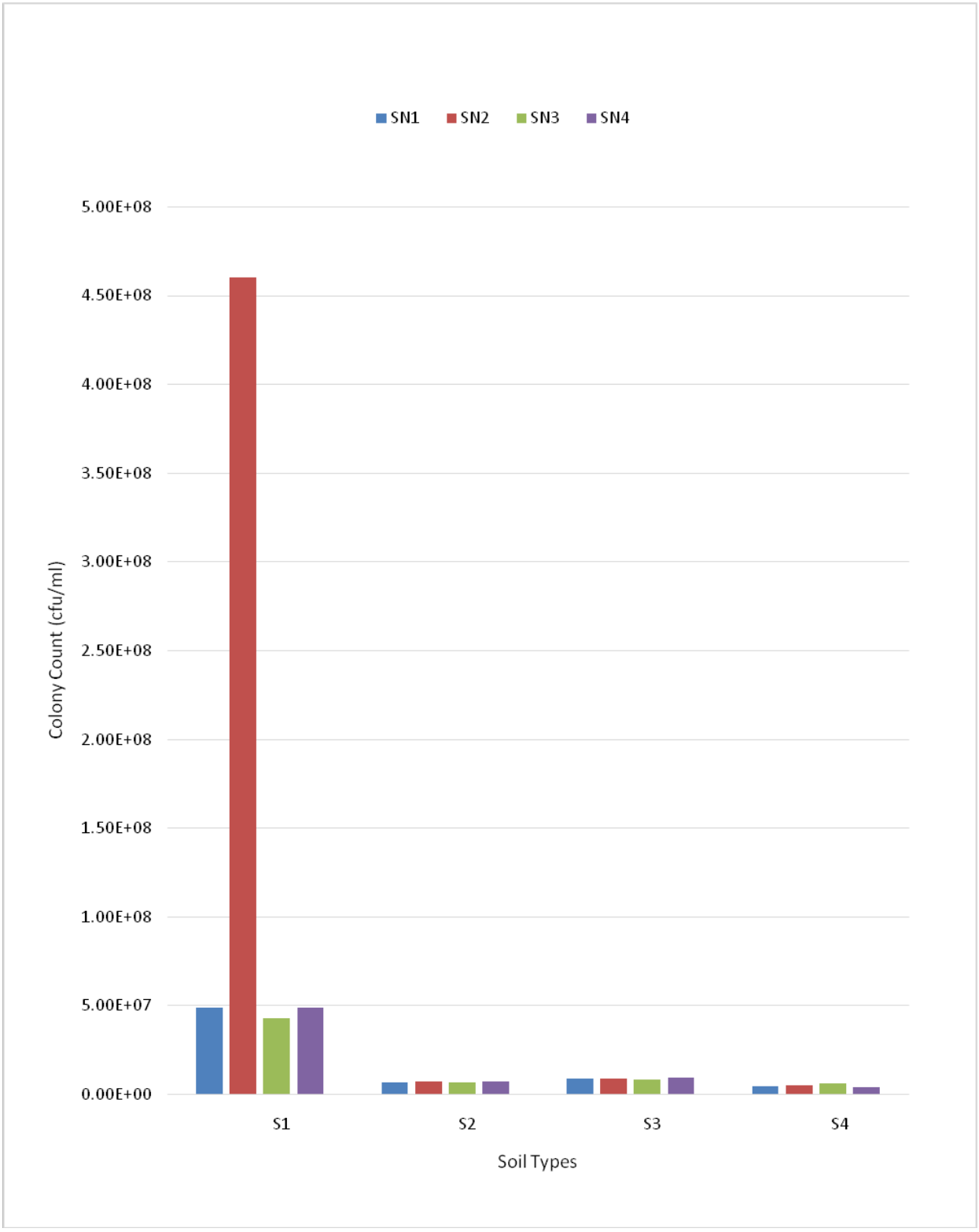

Figure 4. Total plate count for *Salmonella* and *Shigella* of the four experimental soil samples

Contamination of the different soil samples and vegetables like cucumber and watermelon by *Bacillus* spp was also noted. These medium (soil and rooted vegetables) provide an anaerobic environment that favours the growth of *Bacillus subtilis* (Solomon e*t al.*, 1990). *Salmonella* and *Shigella* contamination was also prominent in all the vegetable and soil samples analyzed. This posses a possible means of transfer from manure to soil and then to vegetable. The contamination by these species of organisms is believed to be through this route due to the

observation that the climate, soil conditions and growing practices failed to influence contamination. According to Frehund *et al.*, (1987) several large outbreaks of Shigellosis have been attributed to the consumption of contaminated raw fruits and vegetables. An outbreak of *Salmonella* in 1974 was attributed to watermelon that was contaminated with *Salmonella typhimurium* that had been collected from the ground, which had been fertilized with cow and poultry manure (Fisher and Golden, 1998).

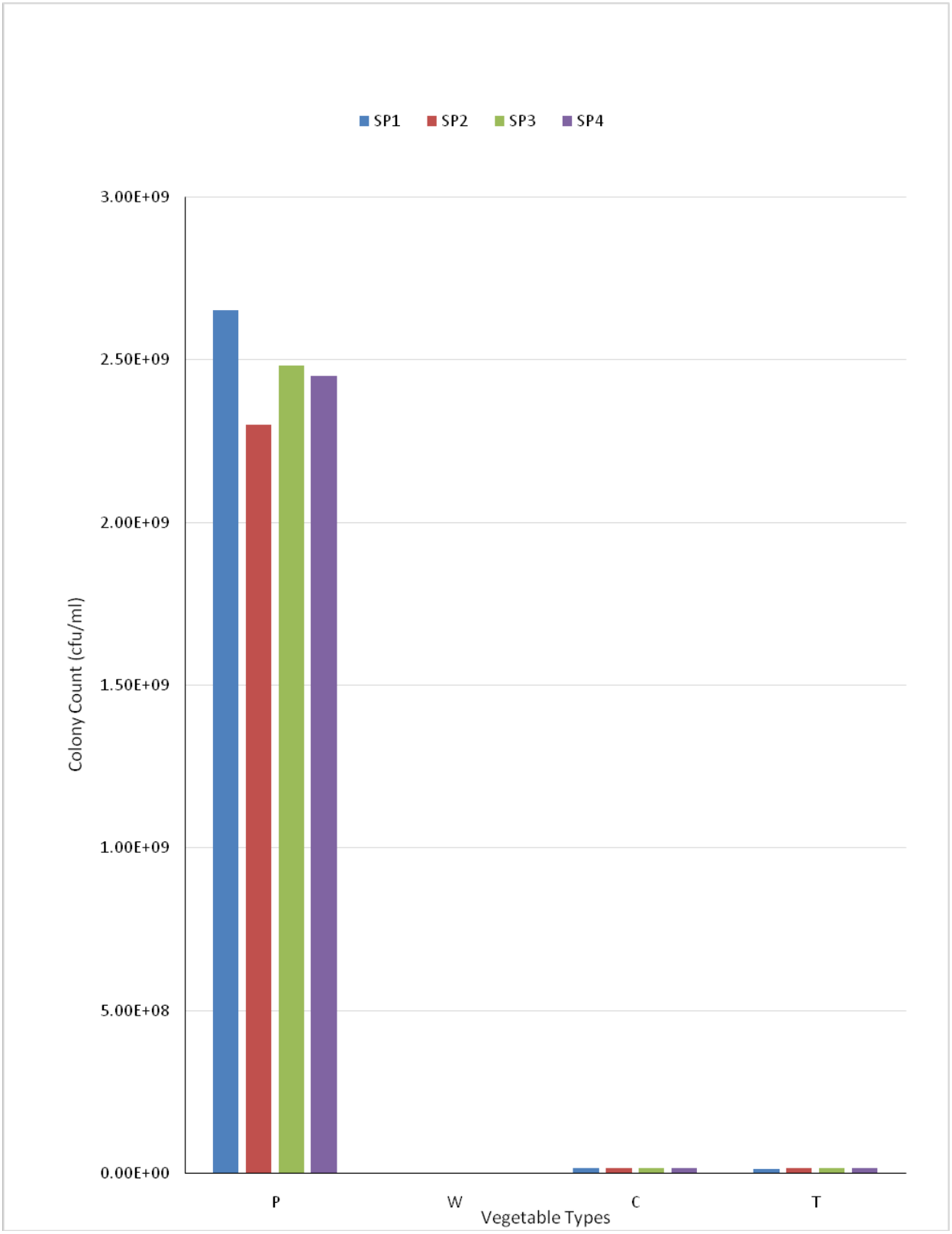

Figure 5. Viable plate count of Microorganisms for experimental vegetable samples

According to Schefferle, (1995) total litter bacteria concentration fall within the range of 10^{10} to 10^{11} colony forming units (cfu) per gram of litter. Total aerobic counts of poultry manure are lower at 10^{8} to 10^{10} (Macklin *et al.*, 2005). These earlier investigation matched this study based on the fact that most of the aerobic plate count produced high bacterial population at concentrations ranging 10^{7} to 10^{9} but the bacterial count in relation to

some of the soil and vegetable analyzed fell at concentrations between 10^4 to 10^6. This variation in bacterial count is believed to be as a result of the type of vegetable analyzed example cucumber is a root vegetable in which the edible portion is in close proximity with the soil in which manure has been applied to. This accounts for the high bacteria count gotten from the analysis of this vegetable.

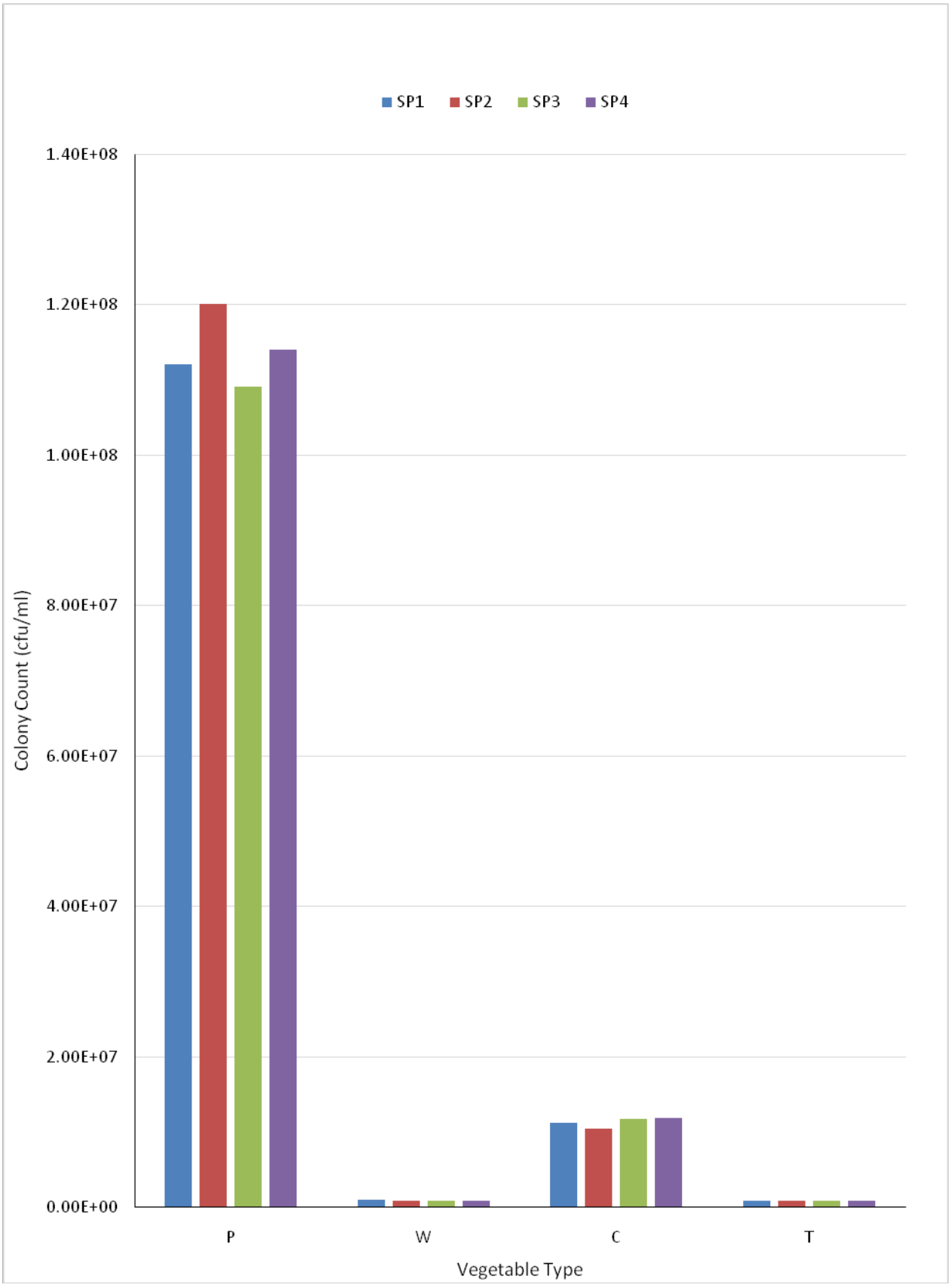

Figure 6. Total Coliform count of the experimental vegetable samples

Antibiotic sensitivity screening showed that *Bacillus subtillis* was most sensitive to Ofloxacin (25mm) and sensitive to Gentamycin (21mm) but had an intermediate susceptibility to Ceftriaxone (16mm). However, it was

resistant to other antibiotics tested. *S. aureus* was most sensitive to Ofloxacin (22mm), then to Gentamycin (16mm) and intermediately susceptible to Ceftriaxone (15mm) but resistant to other antibiotics tested.

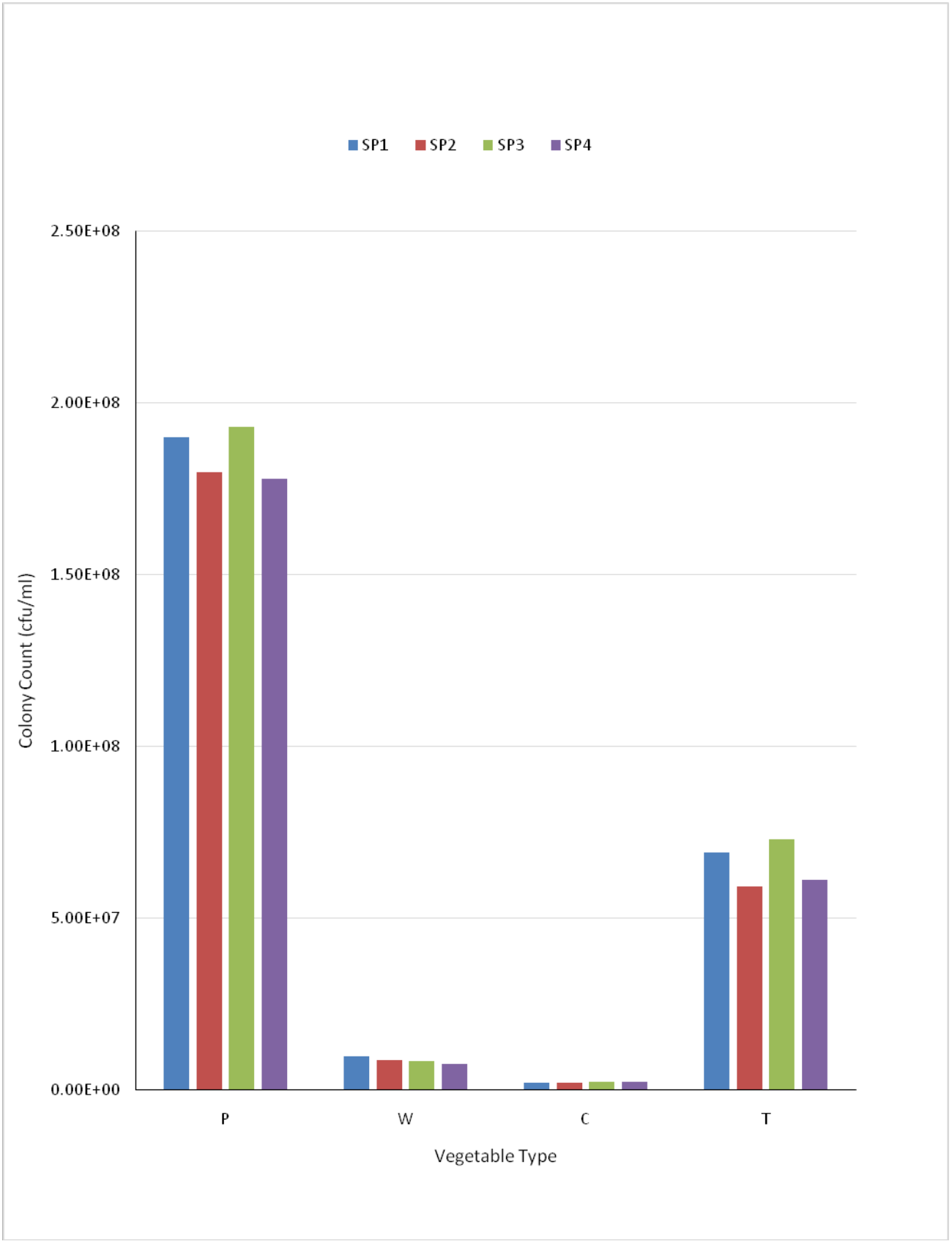

Figure 7. Total Anaerobic Bacteria Count of the experimental vegetable samples

Among all the Gram negative organisms isolated, *Shigella* spp was most sensitive to Ciprofloxacin (25mm), sensitive to Gentamycin (20mm), intermediately sensitive to Nitroxoline (15mm) but resistant to other antibiotics tested. *Salmonella* spp showed resistance to all the antibiotics. *E. coli* showed high sensitivity to Ciprofloxacin, Ofloxacin, Gentamycin and Nitroxoline antibiotics with inhibitory zones of 25mm, 25mm, 23mm,

and 21mm respectively and resistant to other antibiotics. *Klebsiella* spp was most sensitive to Ciprofloxacin and Ofloxacin with inhibitory zones of 23mm and 23mm and sensitive to Gentamycin at inhibitory zone of 22mm. However, it was resistant to Nitroxoline and other antibiotics contained in the disc tested.

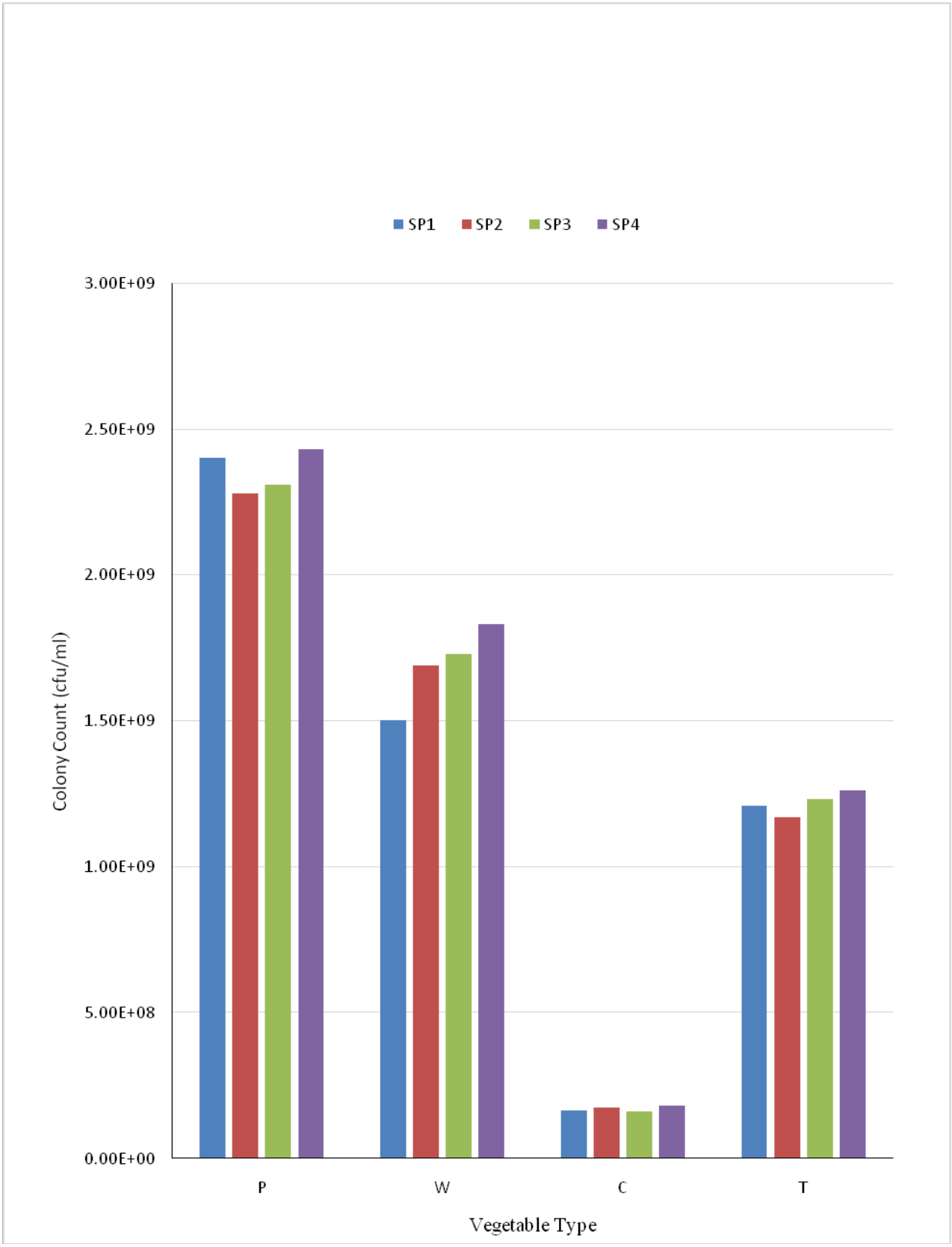

Figure 8. Total Plate Count for *Salmonella* and *Shigella* of the four experimental vegetable samples

From this study, it is deducted that diverse bacterial populations are associated with the soil and vegetable samples analyzed. In developing countries like Nigeria, the continued use of untreated or not-well-treated waste water and manure as fertilizers for the production of fruits and vegetables is a major contributing factor to microbial contamination (Amoah *et al*, 2009).

Table 1. Antibiotic Sensitivity pattern of Gram Positive Isolates (mm)

Grain positive	Ceftazidime (30µg)	Cefurizime(10µg)	Gentamycin(30µg)	Erythromycin (10µg)	Ciprofloxacin (30µg)	Cloxacillin (30µg)	Ofloxacin (30µg)	Augmentin (30µg)
Bacillus spp	R	R	21	R	16	R	25	R
Staphylococcus aureus	R	R	16	R	15	R	22	R

Key: Cfz- Ceftazidime, Crx- Cefurizime, Gen- Gentamycin, Ery- Erythromycin, Cxc-Cloxacillin, Ofl-Ofloxacin, Aug-Augmentin, Cpr- Ciprofloxacin. R = Resistant.

INTERPRETATIVE REFRENCE RANGE

CODE	SENSITIVE	INTERMEDIATE	RESISTANT
Cfz	≥18	11 – 15	≤10
Crx	≥17	12 – 16	≤11
Gen	≥15	13 – 14	≤12
Cpr	≥19	2 – 18	≤11
Ery	≥23	14 – 22	≤13
Cxc	≥14	10 – 13	≤9
Ofl	≥22	14 – 21	≤13
Aug	≥15	13 – 14	≤13

Table 2. Antibiotic Sensitivity pattern of Gram Negative isolates (mm)

Grain negative	Ceftazidime (30µg)	Cefurizime (30µg)	Gentamycin (30µg)	Cefuroxime (30µg)	Ofloxacin (30µg)	Augmentin (30µg)	Nitroxoline (30µg)	Ciprofloxacin (30µg)
Shigella spp	R	R	20	R	21	R	15	25
Salmonella spp	R	R	R	R	5	R	R	8
Escherichia coli	R	R	23	R	25	R	21	25
Klebsiella spp	R	R	22	R	23	R	11	23

Key: Cfz- Ceftazidime, Crx- Cefurizime, Gen- Gentamycin, Cxm- Cefuroxime, Ofl-Ofloxacin, Aug-Augmentin, Nit-Nitroxoline, Cpr- Ciprofloxacin. R= Resistant.

INTERPRETATIVE REFRENCE RANGE:

CODE	SENSITIVE	INTERMEDIATE	RESISTANT
Cfz	≥18	11 – 15	≤10
Crx	≥17	12 – 16	≤11
Gen	≥15	13 – 14	≤12
Cxm	≥23	15 – 22	≤14
Ofl	≥22	14 – 21	≤13
Aug	≥15	13 – 14	≤13
Nit	≥17	15 – 16	≤14
Cpr	≥21	16 – 20	≤15

8. Conclusion

The present study revealed that several pathogenic bacteria were involved in the contamination of vegetables and this is of high potential hazard to consumers especially the illiterate majority who are not aware of such risks and can go ahead to consume these vegetables without as much as washing them thoroughly. Due to the potential microbiological risks, the safety of these products can be improved by treatment of vegetables with certain surface disinfectants before consumption. Surface disinfectants like chlorine, hydrogen peroxide, ozone, chlorine dioxide can be used in combination with water for treatment of vegetables before consumption. Other methods like irradiation can be used to inactivate pathogens in ready-to-eat vegetables. Antibiotics ranging from; Gentamycin, Ofloxacin, Ciprofloxacin and Nitroxoline can also be used to treat the diseases in which these organisms cause. Organic fertilizers should be treated by composting to reduce the residual microorganisms. Water used in the production, irrigation and washing of vegetables should be of a good quality that does not introduce microorganisms at a level that might cause harm to the consumers.

Finally, harvesting at the appropriate time and keeping the harvested products under well-controlled conditions will help in restricting growth of pathogenic and post-harvest spoilage microorganisms.

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