

Original Research Article

Effect of Brine Concentration on Bacteria Isolated from Leafy Vegetables

Comment [MF1]: Effect of brine concentration on bacteria isolated from three types of leafy vegetables (*Talinum triangulare*, *Telfairia occidentalis*, *Vernonia amygdalina*).

Abstract

The effect of brine on bacteria isolated from leafy vegetables was evaluated. Fresh waterleaf (*Talinum triangulare*), Pumpkin leaves (*Telfairia occidentalis*) and Bitter leaves (*Vernonia amygdalina*) including cooking salt were bought from vendors in Mile 3 market, Port Harcourt, Rivers State. Brine concentrations: 1, 2, 3, 4 and 5% was prepared by dissolving appropriate grams of salt in distilled water. Nine millilitres of the respective concentrations were transferred into clean test tubes, labelled, stoppered with foil and autoclaved at 121°C for 15 minutes at 15psi. Sterile distilled water served as a control. The test isolates were standardized based on the 0.5McFarland and 1mL each was introduced into different brine concentrations. The standard plate count was used to monitor brine effects on isolates and this was done hourly for six hours. Inoculated plates were incubated at 37°C for 24 hours. After incubation, enumerated colonies were used to deduce isolate mortality. The total heterotrophic bacterial (THB) load of bitter, Pumpkin and water leaves were $3.49 \pm 0.4 \times 10^6$, $3.25 \pm 0.4 \times 10^6$ and $1.99 \pm 0.2 \times 10^6$ CFU/g, respectively. The staphylococcal counts for bitter, Pumpkin, and water leaves were $1.65 \pm 0.3 \times 10^4$, $3.13 \pm 0.5 \times 10^4$ and $1.55 \pm 0.4 \times 10^4$ CFU/g, respectively. Total coliform counts for bitter, Pumpkin, and water leaves were $1.52 \pm 0.8 \times 10^5$, $2.85 \pm 0.1 \times 10^5$ and $1.75 \pm 0.6 \times 10^5$ CFU/g, respectively. Staphylococcal counts of pumpkin leaf were significantly ($P \leq 0.05$) higher than those obtained for bitter leaf and water leaf. There was no significant difference ($P > 0.05$) in the THB and Coliform counts of all samples. *E. coli* was predominant in Pumpkin and water leaf while *Staphylococcus* sp was predominant in bitter leaf. The LC_{50} values for *E. coli*, *Klebsiella*, *Staphylococcus*, and *Bacillus* sp were; 5.39, 3.88, 1.62, and -0.41mg/ml, respectively. The LC_{50} showed that the brine was very lethal on *Bacillus* sp and *Staphylococcus* sp. High brine concentration is recommended to achieve reduced bacterial load.

Keywords: Brine, leafy vegetables, Bacterial isolates

Introduction

Due to continuous demands, lack of jobs and life style changes over the past decades, vegetable farming activities have increased in the urban and peri-urban areas, however, urbanization and increasing population size has led to scarcity of land and water with most farmers having access to smaller land size for farming as observed in other West African countries (Drechsel *et al.*, 2014). In a bid to supply nutrients and water to farmlands, farmers site their farms close to various water sources such as pipe, wells, streams, and drains for irrigation (Drechsel *et al.*, 2014). More so, fertilizers of inorganic and organic origins (poultry and cow manure) are applied in the farms and the most used organic fertilizers in vegetable farms are those of poultry origin since they are very cheap and readily available (Amoah *et al.*, 2007). Harvesting of leafy green vegetables into containers or sacks are done by hand with or without knives before they are transported to the market centers and other retail outlets under non refrigeration conditions by market women or middle men. Before these vegetables are presented for sale, they are most times washed with water to rid them of dirt (Quansah *et al.*, 2018).

Despite the nutritional importance of fresh vegetables, the danger of microbiological contaminants in vegetables is of public concern since it can serve as a route of microbial

infection (Kuan *et al.*, 2017); this worry is compounded by the way these vegetables are for the most part eaten raw (not cooked) and washing may not ensure cleaning, so any occupant microorganism effectively enter the nutritious trench. Subsequently, these public concerns are justifiably informed based on detailed cases regarding various foodborne disease episodes brought about by utilization of vegetables contaminated by microorganisms like *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp (CDC, 2011 and 2012; Maffei *et al.*, 2013). Different evaluations of occurrences of foodborne diseases flare-ups have brought about diseases, hospitalizations, death, and even food reviews in certain nations, especially the US (Scallan *et al.*, 2011; WHO, 2015; CDC, 2016). In a previous study, it was reported that increased consumption of fresh leafy vegetables has been associated with increasing number of foodborne outbreaks in the U.S., Canada, and European countries (Quansah *et al.*, 2018) and majority of such outbreaks are caused by bacteria (*S. enterica* and *E. coli*) or viruses (Hepatitis A and Norwalk virus) which can be transmitted through the fecal-oral route (Callejon *et al.*, 2015; Kozak *et al.*, 2013). Microbiological contamination of vegetables can occur directly or indirectly through (i) contact with soil, dust, water, and (ii) punctures and open cuts of tissues of vegetables; thus, contaminations of vegetables may occur internally or externally during cultivation, harvest, packaging, storage, transporting and marketing (Eni *et al.*, 2010).

Brining or salting is the oldest and cheapest way of preserving vegetables, meat, fish and other foods whilst maintaining a fair amount of their nutritional value (Fraser, 2005). Salt absorbs much of the water in the vegetables and makes it difficult for microorganisms to survive. In brine preservation of vegetables, the preserving effect is obtained by the combined action of the salt and the acid produced by fermentation. Four different ways of salting or brining vegetables has been identified by Fraser (2005): They comprise i) weak brine (5-15% by weight); ii) strong brine (15-25% by weight); iii) a weak brine (5-15% by weight) plus vinegar and iv) a strong brine (15-25% by weight) plus vinegar. Salt concentration has a significant influence on controlling pathogen growth in foods and also plays a critical role to ensure the food safety (Henney *et al.*, 2010). A study demonstrated that high salt concentrations result in higher osmotic pressures that alters metabolism in microorganisms thereby restricts their growth in fermented foods (Bautista *et al.*, 2008). Therefore, low brine concentration has the potential to increase food spoilage rates and the presence of pathogens. Thus, this study was carried out to investigate the toxicity of brine concentrations on bacterial isolates of leafy vegetables.

Materials and Method

Collection of Samples

Fresh leafy vegetables which include waterleaf (*Talinum triangulare*), Pumpkin leaves (*Telfairia occidentalis*) and Bitter leaves (*Vernonia amygdalina*) were bought from different vendors in Mile III market, Port Harcourt City Local Government Area, Rivers State. The samples were wrapped in foil and transported to the Microbiology Laboratory, Rivers State University for immediate analysis. The salt used in this study was also bought from the Mile III market.

Processing of Samples

The samples were processed by transferring 10g of each leaf sample into separate 250ml conical flask containing 90ml sterile normal saline. The flasks were swirled gently to dislodge the microflora in the leaves (Amadi *et al.*, 2014). After which, ten-fold serial dilution was carried out by transferring 1ml from the original stock (10^{-1}) to test tubes containing 9ml sterile saline. This was repeated serially to obtain 10^{-6} dilution.

Isolation of Total Heterotrophic Bacteria

The total heterotrophic bacterial load of the leaf was determined by transferring aliquot (0.1ml) of 10^{-3} and 10^{-4} dilution using a sterile 1mL pipette into freshly prepared pre-dried nutrient agar (NA) plates in duplicates. The plates were evenly spread with a flame sterilized bent glass rod, inverted and incubated at 37 °C for 24 hours. After incubation, distinct colonies on the plates were subcultured on pre-dried NA plates by picking the colony with a flamed inoculating loop. Plates were later incubated at 37 °C for 24 hours.

Isolation of Total Coliform

The total coliform and faecal coliform load of the leaves were determined by transferring aliquot (0.1ml) of 10^{-1} and 10^{-2} dilution into freshly prepared pre-dried Eosin Methylene Blue (EMB) agar plates in duplicates with the aid of a sterile 1mL pipette. The plates were evenly spread with a bent glass rod, inverted and incubated at 37 °C for 24 hours for total coliform while the faecal coliform plates were incubated at 44°C for 24 hours (Prescott *et al.*, 2011). After incubation, distinct colonies on the plates were subcultured on pre-dried NA plates by picking the colony with a flamed inoculating loop. Plates were later incubated at 37 °C for 24 hours.

Isolation of Total *Staphylococcus*

The total staphylococci in the leaves were determined by transferring aliquot (0.1ml) of 10^{-2} dilution using a pipette into freshly prepared pre-dried Mannitol Salt Agar (MSA) plates in duplicates. The plates were evenly spread with a flame sterilized bent glass rod, inverted and incubated at 37 °C for 24 hours (Prescott *et al.*, 2011). After incubation, distinct colonies on the plates were subcultured on pre-dried NA plates by picking the colony with a flamed inoculating loop. Plates were later incubated at 37 °C for 24 hours.

Identification of Bacterial Isolates

The bacterial isolates were identified based on their morphology and biochemical characteristics. Morphological characteristics employed were colonial morphology (colour, shape, size and texture of the colonies) and microscopic appearance (which include the gram reaction, cell shape and arrangements of the cells) under an oil emersion light microscope. Biochemical tests adopted include; catalase test, citrate utilization, oxidase, Methyl-Red, Voges Proskauer, coagulase, indole and sugar fermentation tests.

Preparation of Brine Concentration

The brine concentrations used were 1, 2, 3, 4 and 5%. The brine concentration was prepared according to the methods (Hajmeer *et al.*, 2006) with slight modification. This was done by dissolving 1g, 2g, 3g, 4g and 5g of normal cooking salt into well labelled 100ml conical flasks containing 9, 8, 7, 6 and 5ml distilled water, respectively (Williams and Dilosi, 2018). After which, 9ml of the respective concentrations were transferred into clean test tubes. Test tubes were well labelled according to the concentration of brine it contained and were stoppered with

cotton wool. These were later sterilized by autoclaving at 121°C for 15 minutes at 15psi. After sterilization, test tubes were arranged in racks according to their concentrations and used to test the various isolates. The control which is the 0% had no salt but only sterile distilled water.

Preparation and Determination of inoculum size

A colony of twenty-four hours old cultures was transferred into 9ml sterile peptone broth. This was later incubated at 37°C for eighteen hours. After incubation, 10-fold serial dilution was carried out by transferring 1ml from the eighteen hours old broth culture into test tubes containing sterile 9ml peptone broth. The serial dilution was carried out until the dilution of 10⁻⁶ was obtained. This was done for all the isolates. The different dilutions were compared with 0.5McFarland standard and aliquots from dilutions that matched the 0.5McFarland standard was transferred into pre-dried nutrient agar plates. This was spread evenly using a sterile bent glass rod, incubation at 37°C for 24 hours. This was done to determine the size of the initial inoculum.

Effect of Brine Concentration on Isolates

The effect of different brine concentrations on the isolates were determined by plate count method (Williams and Dilosi, 2018). In this method, 1mL each of the standardized bacterial inoculum was transferred into the different brine concentrations and incubated. The effect of the brine concentration on the test isolates were monitored hourly for six hours by inoculating aliquots from the set-up into pre-dried nutrient agar plates in duplicates. Inoculated plates were evenly spread using sterile bent glass rod and were incubated for 24 hours at 37°C. Counts from plates that showed growth were recorded and used in determining the % survival, %mortality and lethal concentration of brine on the isolate.

The formula used for the calculation were those adopted by Williams and Dilosi (2018) which state as follows;

$$LC_{50} = LC_{100} - \frac{\sum \text{Concentration Difference} \times \% \text{Mean Mortality}}{\% \text{ Control}} \quad \text{Eqn..... 1}$$

$$\% \text{ Survival} = \frac{\text{Log Concentration of Toxicants} \times 100}{\text{Log concentration of control}} \quad \text{Eqn..... 2}$$

$$\% \text{ Mortality} = 100 - \% \log \text{ survival} \quad \text{Eqn..... 3}$$

Statistical Analysis

The bacterial counts obtained were spread on Excel work sheet (Microsoft Excel 2016). The mean and standard deviations, % survival and mortality were all calculated using SPSS (v25). The Analysis of variance was carried out on the means and were there was significant differences, the Duncan Multiple Range test was employed to separate the means.

Result

The results for the total heterotrophic bacterial counts (THB), staphylococcal counts (SC), and coliform counts (TCC) of the vegetables is presented in Table 1. Result showed that the THB of bitter leaves, Pumpkin leaves and water leaves were 3.49±0.4×10⁶, 3.25±0.4×10⁶ and 1.99±0.2×10⁶ CFU/g, respectively. Results for the staphylococcal counts for bitter, Pumpkin and water leaves were 1.65±0.3×10⁴, 3.13±0.5×10⁴ and 1.55±0.4×10⁴ CFU/g, respectively. The total

coliform counts for bitter leaves, Pumpkin leaves and water leaves were $1.52 \pm 0.8 \times 10^5$, $2.85 \pm 0.1 \times 10^5$ and $1.75 \pm 0.6 \times 10^5$ CFU/g.

The cultural characteristics of the bacterial isolates showed that twenty-eight bacterial isolates belonging to *Bacillus*, *Staphylococcus*, *E. coli*, *Klebsiella*, *Proteus* and *Enterobacter* sp were isolated from the vegetables. The distribution of the bacterial isolates showed that they were not evenly distributed across the vegetable samples. That is, not all the isolates were isolated from a particular vegetable. This is illustrated in Fig1. For instance, *Proteus* sp which was not isolated from Pumpkin and Bitter leaves was isolated from water leaves. Also, *Klebsiella* sp was isolated in both water leaf and Pumpkin leaves but were not isolated in bitter leaves. However, *Bacillus* sp, *Staphylococcus* sp and *E. coli* were all isolated from the vegetables. The percentage occurrence showed that *E. coli* was the most dominant bacterial isolates in water leaves and Pumpkin leaves while *Staphylococcus* sp was the most dominant bacterial isolates in bitter leaves. The percentage occurrence of bacterial isolates in Pumpkin leaves was: *Bacillus* sp (28%), *Staphylococcus* sp (8%), *E. coli* (40%), *Klebsiella* sp (12%), *Proteus* sp (4%) and *Enterobacter* sp (8%). The percentage frequency of occurrence of bacterial isolates in water leaves is: *Bacillus* sp (29.4%), *Staphylococcus* sp (23.5%), *E. coli* (35.3%), *Klebsiella* sp (5.9%), *Proteus* sp (0%) and *Enterobacter* sp (5.9%). While the percentage frequency of occurrence of bacterial isolates in bitter leaves is: *Bacillus* sp (30%), *Staphylococcus* sp (50%), *E. coli* (10%), *Klebsiella* sp (0%), *Proteus* sp (0%) and *Enterobacter* sp (10%).

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Table 1: Variation of Microbial Population in the various Leafy Vegetables Examined

Samples	THB ($\times 10^6$)	SC ($\times 10^4$)	TCC ($\times 10^5$)
Bitter leaf	3.49 ± 0.4^a	1.65 ± 0.3^a	1.52 ± 0.8^a
Pumpkin leaf	3.25 ± 0.4^a	3.13 ± 0.5^b	2.85 ± 0.1^a
Water leaf	1.99 ± 0.2^a	1.55 ± 0.4^a	1.75 ± 0.6^a

*Means with same superscript are not significantly different ($P > 0.05$)

Key: THB: total heterotrophic bacteria; SC: staphylococci count; TCC: total coliform counts

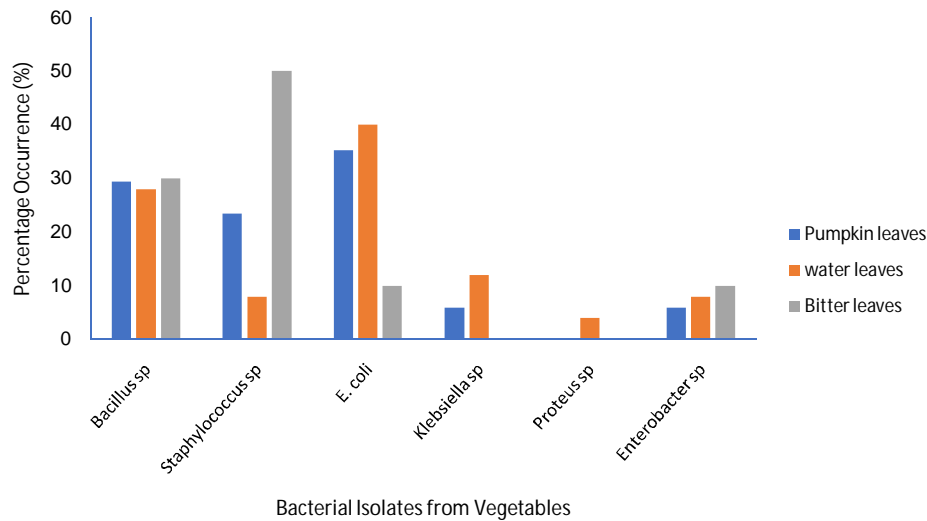


Fig. 1: Percentage occurrence of Bacterial Isolates from the Vegetables

The result of the percentage survival and mortality of brine concentrations at different hours for *E. coli*, *Klebsiella* sp, *Staphylococcus* sp and *Bacillus* sp is presented in Tables 2, 3, 4 and 5, respectively. In Table 2, result showed that there was no mortality of *E. coli* at 0 to 4% brine concentration in the first one hour. This was also seen in the 0 to 1% brine concentration after one hour interval while mortality was recorded in 2 to 5% brine concentrations after the one-hour interval. More so, it was observed that *E. coli* thrived in 1% salt concentrations in all the hours (i.e., from 1-6 hours) but declined in population as the concentration increased as well as extended time. The *Klebsiella* isolates, like the *E. coli* showed similar response to the brine concentrations in the first hour for the 0-3% concentrations in which there was no cell death but increased population (Table 3). The highest mortality of 31.1 was recorded in the 4% concentration at the 5th hour mark. The response of *Staphylococcus* sp to the different brine concentration showed that at 1% concentration of the first hour to the 5th hour, there was a decline in the population (Table 4). The highest mortality was observed in the 3% brine concentration in the 4th hour. The effect of brine on *Bacillus* sp as illustrated in Table 5 showed that the *Bacillus* isolates increased in population from what was recorded in the 0% concentration in the first hour for the 1-5% concentrations.

The effects of brine on the different bacterial isolates after six hours are presented in Table 6. Result showed that all the different brine concentrations had an impact on the growth of the bacterial isolates. The result showed that the response/ mortality of the bacterial isolates to the different concentrations varied with increased brine concentration. Statistically, there was no significant difference ($P \geq 0.05$) in the brine concentration on the isolates at 0, 1 and 2%. While at 3, 4 and 5%, there was significant difference ($P \leq 0.05$) in the response of the bacterial isolates. The mortality rate of *Bacillus* sp to the different brine concentration is given as 100.0 ± 0.00 ,

93.58±23.93, 81.80±26.24, 69.26±23.80, 75.40±25.41 and 77.37±20.92 CFU/ml for 0, 1, 2, 3, 4, and 5%, respectively. The mortality of *E. coli* to the brine concentration is given as 100.00±0.00, 105.92±2.23, 101.58±04.69, 100.97±7.75, 101.15±17.11 and 96.50±15.85CFU/ml for 0, 1, 2, 3, 4, and 5%, respectively. The response of *Klebsiellasp* to the brine concentration is given as 100.0±0.00, 102.28±8.18, 95.28±7.02, 92.37±18.41, 82.51±10.19 and 105.31±13.34 CFU/ml for 0, 1, 2, 3, 4, and 5%, respectively. The response of *Staphylococcus* sp to the brine concentration is given as 100.0±0.00, 95.97±12.68, 86.32±20.50, 80.12±13.86, 85.30±6.77 and 84.65±19.27 CFU/ml for 0, 1, 2, 3, 4, and 5%, respectively.

The result of the LC50 is presented in Table 7. Result showed that the LC50 values were not very high. The LC50 values for *E. coli*, *Klebsiella*, *Staphylococcus* and *Bacillus* sp were recorded as 5.39, 3.88, 1.62 and -0.41mg/ml, respectively.

Table 2: Percentage Survival and Mortality of Brine on *E. coli*

Time (Hour)		Concentrations (%)					
		0	1	2	3	4	5
1	%S	100	106.5	108.9	102.1	104.1	92.4
	%M	0	-6.5	-8.9	-2.1	-4.1	7.6
2	%S	100	104.1	97.5	94.1	97.1	87.3
	%M	0	-4.1	2.5	5.9	2.9	12.7
3	%S	100	108.4	105.1	94.1	94.3	97.5
	%M	0	-8.4	-5.1	5.9	5.7	2.5
4	%S	100	105.5	101.8	96.2	133.0	127.7
	%M	0	-5.1	-1.8	3.8	-33	-27.7
5	%S	100	102.8	96.9	105.7	82.4	88.1
	%M	0	-2.8	3.1	-5.7	17.6	11.9
6	%S	100	108.2	99.3	113.6	96.0	86.0
	%M	0	-8.2	0.7	-13.6	4	14.0

Keys: % S: percentage survival; % M: percentage mortality

Table 3: Percentage Survival and Mortality of Brine on *Klebsiellasp*

Time (Hour)		Concentrations (%)					
		0	1	2	3	4	5
1	%S	100	110.7	101.4	102.1	95.5	95.9
	%M	0	-10.7	-1.4	-2.1	4.5	4.1
2	%S	100	110.5	92.9	101.2	91.7	100.8
	%M	0	-10.5	7.1	-1.2	8.3	-0.8
3	%S	100	98.2	91.7	119.3	85.7	85.7
	%M	0	1.8	8.3	-19.3	14.3	14.3
4	%S	100	102.5	95.4	82.8	75.8	112.8
	%M	0	-2.5	4.6	17.2	24.2	-12.8
5	%S	100	102.9	104.9	68.9	68.9	117.8
	%M	0	-2.9	-4.9	31.1	31.1	-17.8
6	%S	100	88.9	85.4	79.9	77.5	118.9

%M	0	11.1	14.6	20.1	22.5	-18.9
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Keys: % S: percentage survival; % M: percentage mortality

Table 4: Percentage Survival and Mortality of Brine on *Staphylococcus* sp

Time (Hour)		Concentrations (%)					
		0	1	2	3	4	5
1	%S	100	98.4	124.3	92.8	83.6	67.5
	%M	0	1.6	-24	7.2	16.4	32.5
2	%S	100	85.0	86.7	87.5	77.7	67.5
	%M	0	15	13.3	12.5	22.3	32.5
3	%S	100	90.8	85.5	78.4	89.9	87.5
	%M	0	9.2	14.5	21.6	10.1	12.5
4	%S	100	91.7	68.9	54.1	81.5	87.5
	%M	0	8.3	31.1	45.9	18.5	12.5
5	%S	100	89.6	67.5	79.7	82.6	78.4
	%M	0	10.4	32.5	20.3	17.4	21.6
6	%S	100	120.3	85.0	88.2	96.5	119.5
	%M	0	-20.3	15	11.8	3.5	-19.5

Keys: % S: percentage survival; % M: percentage mortality

Table 5: Percentage Survival and Mortality of Brine on *Bacillus* sp

Time (Hour)		Concentrations (%)					
		0	1	2	3	4	5
1	%S	100	113.9	111.4	114.6	116.6	116.6
	%M	0	-13.9	-11.4	-14.6	-16.6	-16.6
2	%S	100	112.1	115.2	68.0	96.0	70.8
	%M	0	-12.1	-15.2	32	4	29.2
3	%S	100	71.4	71.4	45.0	64.5	68.8
	%M	0	28.6	28.6	55	35.5	31.2
4	%S	100	56.3	48.8	59.59	56.3	58.0
	%M	0	43.7	51.2	40.4	43.7	42
5	%S	100	101.5	77.6	59.59	51.8	83.6
	%M	0	-1.5	22.4	40.4	48.2	16.4
6	%S	100	106.3	66.4	68.8	67.2	66.4
	%M	0	-6.3	33.6	31.2	32.8	33.6

Keys: % S: percentage survival; % M: percentage mortality

Table 6: The Mean Percentage Survival (%) of Bacterial Isolates after Six hours

Isolates	Brine Concentrations					
	0%	1%	2%	3%	4%	5%
<i>Bacillus</i>	100.0±0.00 ^a	93.58±23.93 ^a	81.80±26.24 ^a	69.26±23.80 ^a	75.40±25.41 ^a	77.37±20.92 ^a
<i>E. coli</i>	100.00±0.00 ^a	105.92±2.23 ^a	101.58±04.69 ^a	100.97±7.75 ^b	101.15±17.11 ^b	96.50±15.85 ^{ab}
<i>Klebsiellasp</i>	100.0±0.00 ^a	102.28±8.18 ^a	95.28±7.02 ^a	92.37±18.41 ^b	82.51±10.19 ^{ab}	105.31±13.34 ^{ab}
<i>Staphylococcus</i> sp	100.0±0.00 ^a	95.97±12.68 ^a	86.32±20.50 ^a	80.12±13.86 ^{ab}	85.30±6.77 ^{ab}	84.65±19.27 ^b

Table 7: Lethal Concentration (LC₅₀) of Brine on the Isolates

Isolates	LC ₅₀ (%)
<i>E. coli</i>	5.39
<i>Klebsiellasp</i>	3.88
<i>Staphylococcus</i> spp	1.62
<i>Bacillus</i> spp	-0.41

Discussion

Vegetables play a remarkable role in human nutrition and health since they supply dietary fiber, phytochemicals, vitamins, and minerals (Dias and Imai, 2017). The effects of different concentration of brine on bacterial isolates from leafy vegetables was investigated. The microbial

result showed that bitter leaf had the highest total heterotrophic bacterial counts while Pumpkin leaf had the highest staphylococcal and coliform counts. Water leaf had the least total heterotrophic bacterial counts, staphylococcal counts. However, it had the second highest counts which was higher than those recorded for bitter leaf. Despite the variations in the counts, there was no significant differences ($P > 0.05$) in the total heterotrophic bacterial counts and total coliform counts of the various leaves. There was significant difference ($P < 0.05$) in the staphylococcal counts as the counts in the Pumpkin leaves were higher than those recorded for bitter leaf and water leaf which were not significantly different from each other. The total heterotrophic bacterial counts in all leafy vegetables were more than the counts recorded for *Staphylococcus* and coliforms. This observation could be attributed to the inhibitory effects in the special medium used in cultivating *Staphylococcus* sp, and coliforms while the medium used in enumerating the heterotrophic bacteria in the leafy vegetables is non selective. Thus, it allows for the growth of diverse microbial genera. This is well documented (Tom *et al.*, 2011; Prescott *et al.*, 2011). The high coliform counts recorded in the Pumpkin and water leaves could be attributed to the use of poultry droppings in treating the soils to enhance the fertility of the soil thereby increasing high yield. The total heterotrophic bacterial counts in this current study is lower than the range of 8.30 to 9.20 log CFU/g reported by Quansah *et al.* (2018) of leafy vegetables grown and sold in Accra. The microbial loads of leafy vegetables in this current study is generally high and agreed with previous work (OK *et al.*, 2010; Quansah *et al.*, 2018; Yafetto *et al.*, 2019).

The presence of these bacterial contaminants on leafy vegetables could be due to contamination from the environment or from the field and handling processes of sellers. More so, the presence of *E. coli* in leafy vegetables could as a result of contamination with faecal material from animals. *E. coli* are known as indicator organisms and their presence is an indication of faecal contaminations (Prescott *et al.*, 2011). Yafetto *et al.* (2019) in their study isolated *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Serratia marcescens* and *Staphylococcus* sp. Thus, with the exception of *Serratia marcescens* which was not isolated in this current study, all other isolates were similar to our findings. Furthermore, the bacterial isolates from this study have been reported by previous studies (Merlini *et al.*, 2018; Quansah *et al.*, 2018). Unsanitary postharvest practices, unhygienic postharvest handling practices and improper storage conditions have been reported to be the major cause of poor microbial quality (Quansah *et al.*, 2018). Vegetables transported to the markets are stored under non-refrigeration temperatures in sacks or boxes kept in rooms or in the open at the market centres. Some vegetables are washed to remove dirt before being displayed at the market for sale using water which is not changed regularly. This practice is known to promote cross contamination and microbial growth on vegetables (Quansah *et al.*, 2018).

The effect of the brine concentration on the bacterial isolates after six hours showed that the concentration changes influenced the growth of the isolates. The bacterial load decreased as the concentration increased but fluctuated with respect to concentration. This was observed in all isolates. For *E. coli* sp and *Staphylococcus* sp, the populations declined with increased brine concentration while the population of *Bacillus* sp and *Klebsiella* sp which declined gradually with respect to the increased concentration increased at 5% brine concentration. More so, none of the brine concentration completely inhibited the growth of the bacterial isolates. This agreed with Omotoyinbo (2016) who also reported that higher the NaCl concentration affected the optimal growth of *S. aureus* and *E. coli*. The growth of bacteria and increase in population in environment with sufficient nutrients and the decline in population due to limited nutrient or inhibitory substances in the environment is well understood (Hajmeer *et al.*, 2006). In this study,

Bacillus sp, *E. coli*, *Klebsiella* sp and *Staphylococcus* sp at 1 hour at 1% were multiplying and this could be that the growth environment was still suitable for microbial proliferation. As the concentration increased and the incubation period of cells was extended to 6 hours, shock related stress caused by the brine concentrations could have caused accumulation of waste metabolites and toxins in the cultures which could have resulted in morphological damage and plausible death of cells. The agreed with Hajmeer *et al.* (2006) who observed the effects of brine on *E. coli* and *S. aureus* for six and twelve hours. The decrease in the bacterial growth as a result of increasing salt concentration, was also reported by Abdulkarim *et al.* (2009), who further suggested that observed effect could be due to hyper osmotic effect on the bacteria which led to osmotic shock on the organisms thereby causing growth suppression. The LC₅₀ showed that the brine concentration has a bactericidal effect on *Bacillus* sp and *Staphylococcus* sp which had the least LC₅₀ values, and therefore, means that the brine was more effective on *Bacillus* and *Staphylococcus* sp. Thus, this finding contradicts previous reports which have shown that *Bacillus* and *Staphylococcus* sp could tolerate higher salt concentrations (Prescott *et al.*, 2011).

Conclusion

As the demand for vegetables increases so as to meet up the high demand, techniques in agricultural practices have improved. Many subsistence farmers use cheap substances to enhance soil fertility thereby increasing the yield of their crops. Such acts result in contamination of the crops with faecal matter which could cause spoilage and diseases

The different brine concentration on the bacterial isolates despite having intermediate inhibitory effects was not enough to completely inhibit the bacterial growth and multiplication.

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Comment [MF4]: It would be better to add some recent references(one or two).

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