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Growth, yield and microbial contamination of lettuce (*Lactuca sativa L.*) grown under two types of cultivation system as influenced by different organic fertilizers

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ABSTRACT

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Increasing popularity of lettuce in the tropics is becoming evident due to its widespread use in health and nutrition. Lettuce, a ready-to-eat vegetable is expected to be safe and of good quality. A study was conducted to assess the effect of different organic fertilizers on the growth, yield and microbial contamination of lettuce grown under two types of cultivation system. This was carried out in a combined analysis arranged in randomized complete block design with the following treatments: without organic fertilizer (control), cow manure, fresh chicken dung, dried chicken dung, vermicast and goat manure. Results showed that lettuce grown under structure performed better than in open field as manifested by early heading, bigger polar head size, longer and broader leaves and more compact head. Among the organic fertilizers used, dried chicken dung and vermicast enhanced the yield and yield components of lettuce. Microbial contaminants like *E. coli* were detected in soils applied with organic fertilizers. The highest microbial count was recorded in fresh chicken dung-treated soils in both open field and under structure. *E. coli* contamination in lettuce leaves was more evident in the open field than under structure. Regardless of soil and crop samples, treated or not, positive detection of *Salmonella* was observed.

Keywords: lettuce, protective structure, vermicast, dried chicken dung, *E. coli, Salmonella*

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INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a temperate annual or biennial plant that belongs to the Asteraceae family and is gaining popularity in the tropics. It is one of the most important vegetables in the human diet which is commonly eaten raw, used as a salad ingredient and in sandwiches (Richter 2014). Its crispy, green or crimson-red leaves are a low fat and low-calorie source of essential nutrients that benefit health. It is a good source of vitamin A and C and folic acid (USDA 2019). Lettuce requires considerable quantities of water during the growing period. Thus, the soil must be humus-rich to retain and supply the moisture needed by the growing plants.

In the tropics, the cultivation of most vegetables provides a significant source of income and fresh food for small and large-scale growers. However, conventional open field cultivation faces a number of problems, such as the plants being subjected to drought, wind breakage, pests and diseases. As a solution, the adoption of protected cultivation in low-cost protective structures is used. Protective structures have the potential for generating increased yield and better quality of vegetables specifically in tropical climates (Gonzaga et al 2013).

Nowadays with greater consumer awareness concerning health and environmental risk issues, farmers are engaging in organic farming as an alternative to conventional agriculture. Organic farming relies on the incorporation of organic materials derived from animals or plants, with or without undergoing certain processes like fermentation, that are used as fertilizers for growing crops (Lampkin 1990). These organic fertilizers are known to be good sources of macro and micronutrients. They also help in improving soil fertility and structure, thereby increasing the ability of the soil to retain water and nutrients, creating an appropriate medium for plant growth (Evanylo et al 2008, PCARRD 2008). Furthermore, the use of organic fertilizers diminishes the environmental pollution from manure disposal and contributes to the recycling of wastes.

However, organic fertilizers are a potential source of human pathogenic bacteria like *Salmonella* spp. and *Escherichia coli* (Oliveira et al 2010, Benjamin et al 2013). Trace-back investigations of occurrences linked to lettuce have concluded that contamination was in the field and the sources were the water used for irrigation, pesticides and fertilizers. Hence, high outbreak occurrences of foodborne diseases are associated with the agricultural sector. If not prevented, these pathogens may become incorporated into the supply chain leading to an epidemic and may infect the neighbouring environment.

The use of organic fertilizers is necessary to promote the growth and yield of crops. Few studies have been done in the Philippines on the analysis of microbial contaminants, specifically *Salmonella* spp. and *E. coli* in lettuce applied with organic fertilizers. This study aimed to compare the performance, determine the effects of different organic fertilizers and detect microbial contaminants of lettuce under conventional and protected cultivation. Results of this research provide information and awareness on the safety of lettuce consumption which is an important vegetable and salad ingredient.

MATERIALS AND METHODS

Land Preparation and the Protective Structure

Two experimental areas measuring 5mx15m ($75m^2$), one under protective structure and the other in an open field were prepared three weeks before the conduct of the study. The areas were cleaned, plowed and harrowed two times weekly to allow thorough decomposition of plant residues and pulverization of the soil. Small canals were constructed along the sides of the structure to serve as drainage for excess water. For the protective structure, a bamboo house type by the ACIAR-GAP project with an entirely curved roof and open sides was used. The structure measured 5mx40m and stood 4m high covered with UV treated plastic film with a thickness of 0.005in (Gonzaga et al 2013).

Experimental Design and Treatment

Two separate studies were conducted simultaneously in two single-factor experiments arranged in Randomized Complete Block Design (RCBD) with four (4) replications and six (6) treatments. Combined analysis of variance (ANOVA) arranged in factorial was done with homogenous variances. Each treatment plot measured 1mx1.5m with a distance of 50cm between treatment plots and a planting distance of 25cmx25cm. The different treatments applied to both cultivation systems were as follows: control (no organic fertilizer), cow manure, fresh chicken dung, dried chicken dung, vermicast and goat manure.

Cultural Management

Lettuce seeds of head type (var. Grande) were sown in a seedbox filled with sterilized mixture of garden soil and carbonized rice hull at a 1:1 ratio. The seedlings were pricked to seedling trays upon reaching the first true leaves. They were hardened one week before transplanting by gradual exposure to sunlight and withdrawal of water until they showed signs of temporary wilting. After hardening, seedlings were then transplanted, generally three to four weeks after sowing and having three to four true leaves. This was done late in the afternoon to minimize transplanting stress.

Regular watering was done using drip irrigation. Supplemental fertilization was administered two weeks after planting by drenching 150g of calcium nitrate dissolved in 16L of water. Hand weeding was done as necessary. Harvesting was done manually when the plants were big enough and before bolting, which was about 25 to 30 days after transplanting, segregated according to treatment and washed with tap water.

Acquisition and Application of Organic Fertilizers

The goat and cow manure were obtained at the Department of Animal Science, Visayas State University (VSU), Baybay City, Leyte while fresh and dried chicken dung were brought from a nearby poultry farm. Vermicast was acquired from EcoFarmi at VSU. The organic fertilizers were applied per hill before transplanting

by incorporating them into the soil to a depth of 10cm at a rate of 3.75kg per plot with 24 plants. Each plant receiving 156.25g of organic fertilizer.

Gathering of Samples for Microbial Analysis

Before sampling, all tools such as shovel and scoop were sterilized by autoclaving at 15psi for 15min. Tools were cleaned and sterilized by spraying with 70% alcohol when moving to another sampling plot. Gloves were worn to avoid cross-contamination and were regularly sanitized with 70% alcohol or replaced between samplings. Sampling was done before planting and during harvesting. Soil samples from seedling trays used in seedling production and lettuce seedlings before transplanting were also obtained and tested. All collected samples were placed in sterile plastic bags, sealed, labeled accordingly and placed in a closed plastic container for transport to the laboratory for microbial analyses.

Five hundred grams of soil subsamples at a depth of 10cm were collected around the periphery of three randomly selected crops per treatment plot using a sterile shovel. A composite sample was made from each treatment. Lettuce samples (three plants per treatment plot) were collected consistent with local harvesting practices using sterile scissors/knives and placed in sterilized plastic bags. Composite samples from each treatment were made.

Water used in irrigation was tested at each sampling, ie, during transplanting and harvesting by obtaining approximately 500mL from the water source that was placed in a sterile bottle. The water was allowed to run first for 1min before collection. Samples gathered were sealed, labeled accordingly and placed in a Styrofoam box for transport to the laboratory and analyzed within 24h.

Microbial Analysis

Lettuce and soil samples obtained from replicated treatment plots were mixed together to have a composite sample of each treatment. Two replicates were provided for microbial analysis.

Soil and lettuce samples were analyzed for *E. coli* using Compact Dry EC. Samples weighing 25g were placed in a stomacher bag added with 225mL of sterile buffered peptone water. Samples were placed in a stomacher for 3±1min. A 1.0mL (diluted if necessary) of the homogenized sample was placed in the middle of the Compact Dry plate and incubated for 24±2h at a temperature of 35±2°C. Observation, proper labeling and counting were done. *E. coli* positive plates formed blue to blue-purple colonies. In case of any difficulties counting colonies due to the high numbers of colonies grown, the bacteria count was obtained by multiplying by 20 the average number of colonies per grid counted from several grids.

Salmonella detection was performed using a rapid test Compact Dry SL. Samples weighing 25g were placed in stomacher bags with 225mL of sterile buffered peptone water for 3±1min and incubated for 20-24h at 35-37°C for pre-enrichment. After that, the bags were taken out and rubbed for homogenization. A pipette was used to transfer 0.1mL of each of the enriched specimens that was dropped approximately 1cm from the edge of the plate and a drop of 1mL sterile water was dropped at the opposite side of the plate. These were incubated for 20-24h at a temperature of 41-43°C. Observation and proper labeling were done. A positive test for *Salmonella* was recorded when black to green isolated or fused

colonies were observed and the sheet around the colonies changed to yellow. If a large quantity of *Salmonella* was present, no isolated colonies were formed (there may be several spots with fused black or green colonies), but the whole plate sheet became yellow. *Salmonella* negative is indicated by no color or a color change to red or reddish-purple of the medium, with no black or green colonies observed.

Irrigation water samples were tested for the presence or absence of *Escherichia coli* using the Colitag™ in a 16-48h testing window. Water samples were transferred to a 100mL container labeled accordingly. One sachet of Colitag™ was added to the water samples previously pre-warmed at 44 to 45°C for 7-10min in a water bath and shaken until no clumps formed. This was incubated for 16-48h at 35±0.5°C. Afterwards, it was visually checked for yellow color indicating positive for total coliforms. If the sample fluoresces when illuminated under longwave (365nm) UV lamp, it is positive for *E. coli* bacteria.

Data Gathered

Various horticultural characteristics were measured such as days from transplanting to heading; percent head formation; the number of wrapper leaves and leaf size, which were determined by counting the number of days from transplanting up to the time when the youngest leaf curled inward; the number of sample plants that produced heads per treatment plot at the time of harvest; the number of wrapper leaves per plant produced at harvest; and by measuring the length and width of the most significant or most prolonged leaf.

Yield parameters including head size was determined by measuring the polar (top to bottom) and equatorial (left to right) size of the lettuce head at harvest using a tape measure and the diameter was calculated by dividing the circumference by π. Compactness was assessed at the time of harvest by hand pressure method following the scale: 1-very loose, 2-loose, 3-compact and 4-very compact. Yield per plot and tons/ha were determined by combining the weights of all lettuce plants per treatment plot and then converting to tons/ha.

Data Analysis

All data gathered was statistically analyzed using the computer software Statistical Tool for Agricultural Research (STAR) version 2.0.1. The presence of significant differences among treatments was determined using combined analysis of variance (ANOVA) laid out in Randomized Complete Block Design (RCBD). A Bartlett's test was done for homogeneity of error variances between types of cultivation systems. Comparison of treatment means was done using Least Significant Difference (LSD) at a 5% level of significance.

RESULTS AND DISCUSSION

Horticultural Characteristics

The types of cultivation system significantly affected the number of days from transplanting to heading and leaf size (Table 1). Lettuce under protective structure formed heads four days earlier and had significantly longer (23.83cm) and broader

(18.53cm) leaves than in the open field (19.36cm and 16.80cm, respectively). This result was most likely attributed to the microclimate condition provided by the protective structure. Majumder (2010 and Nair and Nagouajio (2010) reported that elevated air temperature inside the structure and improved moisture status enhances root development, increasing the uptake of nutrients such as potassium and nitrogen, thereby favoring good crop growth. Lettuce in the open field are prone to environmental stresses such as heat and radiation stress that impose detrimental effects on the crop such as reduced plant photosynthetic and transpiration efficiency that negatively impact root development, resulting in delayed head formation. There was a higher light intensity recorded in the open field which ranged from 2,233-3,336µmol $m^{-2}s^{-1}$ compared to that under the structure that ranged from 1,146-1430µmol $m^{-2}s^{-1}$ (Figure 1). Lettuce was observed having symptoms of temporary wilting in the open field during its early growth stage. There was no significant observed effect of the types of cultivation system on the number of wrapper leaves and percentage head formation.

Table 1. Horticultural characteristics of lettuce (*L. sativa* var. Grande) as influenced by types of cultivation system and organic fertilizers

Treatment	Days from Transplanting to Heading	No. of Wrapper Leaves	Head Formation (%)		Leaf Size (cm)	
					Length	Width
Cultivation System						
Open field	17.41b	3.68	62.44		19.36b	16.80b
Protective structure	13.73a	3.56	65.02		23.83a	18.53a
Organic Fertilizers			*OF*	*PS*		
Control	16.21	3.66	57.67b	62.03	20.52b	15.66c
Cow manure	15.80	3.33	62.73ab	64.60	21.18b	17.45bc
Fresh chicken dung	15.01	3.66	62.60ab	65.93	21.43b	18.41ab
Dried chicken dung	15.03	3.98	66.33a	66.33	23.49a	19.94a
Vermicast	15.57	3.51	63.85ab	65.28	21.69b	17.35bc
Goat manure	15.80	3.59	61.45ab	65.95	21.29b	17.19bc
CV (a) %	4.44	14.56			2.37	8.33
CV (b) %	5.38	14.13	5.29	4.78	5.14	7.52
HV	ns	ns	*		ns	ns

Data is homogeneous (ns) or heterogeneous (*) between types of cultivation system
Mean separation within columns by Least Significant Difference (LSD) at 5% level
Open field (OF), Protective structure (PS) and Homogeneity of variances (HV)
Coefficient of variation (CV (a)) Types of cultivation system and (CV (b)) Types of organic fertilizers

Different organic fertilizers did not significantly influence the number of days from transplanting to heading and number of wrapper leaves. However, lettuce heads were promoted with the application of dried chicken dung in the open field but were not significantly different from other organic fertilizers applied. However, the application of dried chicken dung significantly increased lettuce leaf length (23.49cm) and width (19.94cm) compared to other treatments. The remaining organic fertilizer treatments used were notably comparable with each other giving leaf length ofless than 21.69cm but greater than 20.52cm and leaf width measuring less than 18.41cm but greater than 15.66cm. The micro and macro elements of dried chicken dung help increase soil porosity and soil microbial activity, enhancing

crop productivity through increased root systems (Sharply and Smith 1991, Omisore et al 2009). The improved morphological characteristic of lettuce along with increased root system could also be due to the readily available nutrients such as nitrogen and phosphorus in the soil. Nitrogen is a constituent of the chlorophyll molecule with phosphorus playing an important role in energy transfer, photosynthesis, the transformation of sugars and starches and nutrient movement within the plant (Ahemad et al 2011).

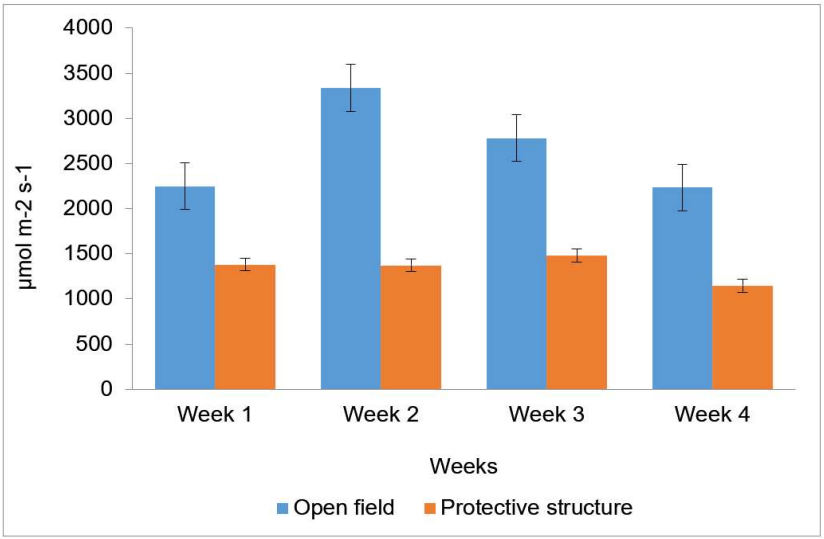

Figure 1. Weekly light intensity in open field and protective structure

Yield and Yield Components

The type of cultivation system significantly affected the head size and compactness of lettuce (Table 2). Lettuce grown under the protective structure had larger polar diameters but were more compact than those in the open field. The bigger head size, in terms of polar diameter, of lettuce grown under the structure than in the open field was attributed to some parameters such as time of heading initiation. The compactness of the lettuce heads in the study only fell into the categories described as very loose and loose with an equivalent rating of 1 and 2, respectively and no samples were ratedas 3 and 4 with a description of compact and very compact, respectively. Significant results obtained in the protective structure were correlated with the earlier head formation, which also contributed to more compact heads.

Regarding the different organic fertilizers applied, dried chicken dung promoted bigger head size (polar and equatorial diameter). The application of organic fertilizers enhanced compactness of the lettuce head. The application of organic fertilizers stimulates the natural cycles that ameliorate and enrich the soil, thus, nutrients are released over the years for crop growth and development (Snyder 2009). Likewise, organic fertilizers increase water holding capacity, improve aeration and water infiltration (Davis and Wilson 2012). Further, Stephens and Kostevicz (2009) stated that organic fertilizer preserves and enhances the fertility

of the soil because it encourages beneficial insects and microorganisms and minimizes the flow of toxic pesticides into waterways.

Table 2. Head size and compactness of lettuce (*L. sativa* var. Grande) as influenced by types of cultivation system and organic fertilizers

Treatment	Head size (cm)		Compactness
	Polar diameter	Equatorial diameter	
Cultivation System			
Open field	9.86b	9.21	1.75b
Protective structure	10.71a	9.87	2.20a
Organic Fertilizers			
Control	9.33c	8.43b	1.52b
Cow manure	10.12bc	9.07ab	1.90ab
Fresh chicken dung	10.67ab	9.94ab	2.02a
Dried chicken dung	11.03a	10.25a	2.23a
Vermicast	10.27ab	9.74ab	2.06a
Goat manure	10.31ab	9.79ab	2.11a
CV (a) %	3.80	8.90	12.55
CV (b) %	5.60	4.97	15.79
HV	ns	ns	ns

Data is homogeneous (^{ns}) or heterogeneous (*) between types of cultivation systems
Mean separation within columns by Least Significant Difference (LSD) at 5% level
Open field (OF), Protective structure (PS) and Homogeneity of variances (HV)
Coefficient of variation (CV (a))Types of cultivation system and (CV (b))Types of organic fertilizer

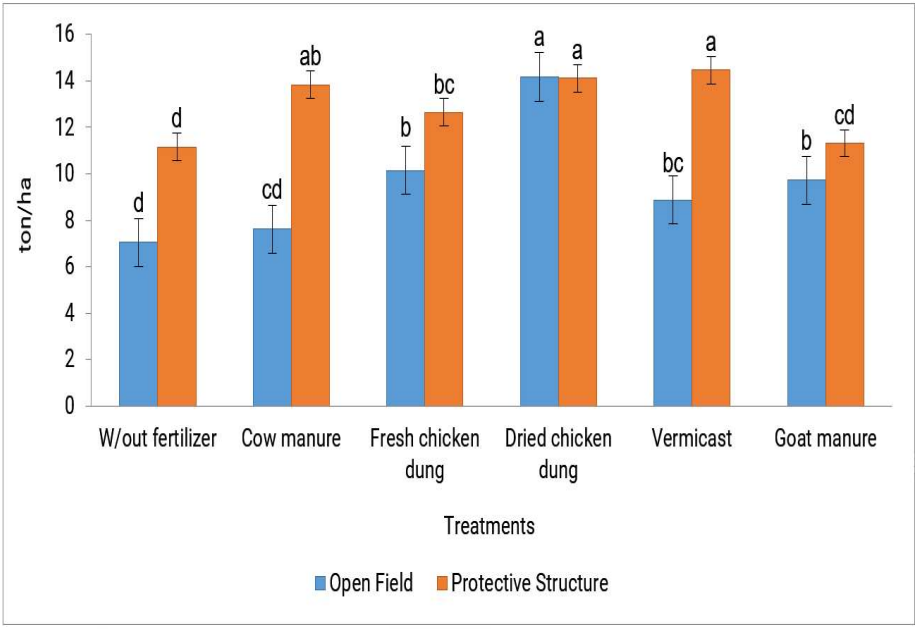

Figure 2. Yield (ton/ha) of lettuce (*L. sativa* var. Grande) as influenced by types of cultivation system and organic fertilizers

In terms of yield, the different organic fertilizers showed significant differences (Figure 2). It was observed that lettuce applied with dried chicken dung produced the highest yield per plot and tons per hectare in the open field (2.13kg and 14.17t ha^{-1}, respectively) while in the protective structure, the yield was enhanced by the application of both vermicast and dried chicken dung. These results were manifested by the high percentage of head formation, longer polar length and wider equatorial diameter. Similar results have been reported by Ghanbarian et al (2008) and Ouda and Mahadeen (2008) that plants fertilized with dried chicken manure displayed the highest growth parameter and marketable yield. Moreover, Arancon et al (2004) and Mishra et al (2005) showed that vermicast had beneficial effects on the growth and yield of both strawberries and rice due to plant hormones and humate.

Microbial Contamination

Microbial analysis of organic fertilizer treated soils before transplanting showed *E. coli* contamination in both the open field and the protective structure (Table 3). Plots applied with fresh chicken dung obtained the highest microbial count of 4.58Log g^{-1} in the protective structure and 4.48Log g^{-1} in the open field, which were above the lowest limit of detection of 2Log g^{-1} (10^2CFU g^{-1}) (Johannessen et al 2005). Moreover, a high *E. coli* in the soil sample from the seedling tray could be attributed to improper sterilization of the soil medium used during seedling production. No *E. coli* contamination of the irrigation water was observed.

Table 3. Microbial contamination of soil and lettuce as influenced by types of cultivation system and organic fertilizers

Treatment	Soil								Lettuce			
	Open Field				Protective Structure				Open Field		Protective Structure	
	Initial		Final		Initial		Final		Final		Final	
	EC	SL	EC	SL	EC	SL	EC	SL	EC	SL	EC	SL
Control	0.00	+	0.00	+	0.00	+	0.00	+	0.70	+	0.00	+
Cow manure	2.63	+	1.85	+	3.33	+	1.74	+	0.00	+	0.00	+
Fresh chicken dung	4.48	+	0.70	+	4.58	+	0.70	+	1.74	+	0.00	+
Dried chicken dung	2.06	+	1.00	+	2.23	+	1.93	+	0.70	+	0.00	+
Vermicast	0.70	+	1.40	+	3.15	+	2.75	+	0.00	+	0.00	+
Goat manure	2.30	+	2.23	+	1.65	+	1.48	+	0.00	+	0.70	+

EC (*Escherichia coli,* Log g^{-1}); SL (*Salmonella,* + positive); Seedling tray soil- 3.15 Log_{10} g^{-1}; Lettuce for transplanting- 0 Log_{10} g^{-1}; Limit of detection: for ready to eat foods (FSANZ 2018) - >2 Log or 10^2 CFU g^{-1} (Unsatisfactory); 0.5-<2 Log or 3-<10^2CFU g^{-1} (Marginal); <0.5 Log or <3 CFU g^{-1} (Satisfactory); for soil (Johannessen et al 2005) - 2 Log or 10^2 CFU g^{-1}; No *E. coli* contamination in irrigation water used throughout the study and turbidity is clear (9)

There was a decrease in the *E. coli* count for the positive samples obtained during the final soil sampling except for vermicast, where an increase of count from 0.70 to 1.40Log g^{-1} was observed in the open field.This can be attributed to the promotion of microbial activity by the vermicast providing a rich carbon source, an important food source for *E. coli* (Ndegwa and Thompson 2001) which is

significantly sustained under warm temperatures (Winfield and Groisman 2003). *E. coli* are considered copiotrophic strategists because they proliferate in nutrient-rich environments but are relatively low in number when nutrients are limited. Consistently, no microbial contamination was observed in untreated soils. The majority of *E. coli* contaminated soils in the protective structure and the open field showed a decline in the microbial count over time. The differences can be attributed to the fact that different organic fertilizers have different nutrients, structure, numbers and types of background microflora, presence of antibiotics and different pH levels affecting the growth of microorganisms, specifically *E. coli* (Rus and Yanko 1981, Sidhu et al 2001).

E. coli on the lettuce plants was not detected on the ready to transplant seedlings, however, an increase in the microbial count was noted at harvest of 0.70Log g^{-1} (5CFU g^{-1}) which was recorded on goat manure-treated plants grown under structure. In the open field, plants with the highest count were recorded for fresh chicken dung (1.74Log g^{-1}) followed by dried chicken dung and also untreated lettuce plots (0.70Log g^{-1}). This indicated that *E. coli* has the possibility of adhering to lettuce leaves since the soil samples analyzed turned positive. A high susceptibility of lettuce to microbial contamination could be due to its large surface area, allowing microbes to cling more easily. Moreover, an increase in the likelihood of rain drops splashing soil from the treated plots can lead to the transfer of microorganisms in the open field. Nevertheless, these values are far from unsatisfactory ratings (>2Log g^{-1} (FSANZ 2018)) for ready-to-eat (RTE) fresh produce.

The results of the *Salmonella* test in soil and lettuce samples at initial and final analysis were all positive in both protected and open field cultivation and samples from the seedling tray soil and lettuce seedlings ready for transplanting (Table 3). Once *Salmonella* is in the soil, a high possibility of contamination of crops is possible via the rhizosphere and root as well as via soil splashing onto the leaves, flowers or fruits during overhead irrigation or rainfall. All these might result in internalization of *Salmonella* into the edible plant parts, as reviewed by Hirneisen et al (2012). Moreover, an increase in the likelihood of *Salmonella* persistence for more extended periods has been reported, from a few days up to 332 days in manure-amended soils (Holley et al 2006, Islam et al 2004, You et al 2006). However, it is unclear how these positive detections in the samples relate to contamination risk specifically of the lettuce crop which is eaten raw, since the microbial *Salmonella* population cannot be enumerated. Certain measures such as withholding periods for manure application and harvest, and the proper washing of lettuce should be implemented.

CONCLUSION

Production of vegetables under protective structures and using manures are deemed proven and effective. Results revealed that lettuce under protective structure formed heads four days earlier and produced more compact heads than in the open field. Dried chicken dung produced the heaviest yield in lettuce grown in the open field while vermicast and dried chicken dung produced the heaviest yield under the protective structure. This study also proves that organic fertilizers can

potentially introduce human pathogens onto food. Plants applied with fresh chicken dung had the highest microbial count. There was a decrease of the soil *E. coli* microbial count for most of the samples from the initial to the final analysis, except for vermicast, where an increase in microbial count was recorded. Increased contamination potential of lettuce grown in open field cultivation than under the protective structure. For all organic fertilizers tested, there was a positive detection for *Salmonella* in the soil and lettuce samples at the initial and final analyses in both protected and open field cultivation.

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