Comparative Analysis of the Presence of Microorganisms in Lettuce in Three Markets in Metro Manila

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Abstract

The development of multi-drug resistant (MDR) pathogens caused by the overuse and misuse of antimicrobials is going to be the next pandemic, according to The World Health Organization (WHO). Thus, conducting studies on how the MDR pathogens spread should also be prioritized. For instance, microbes, a number of which are potentially antimicrobial resistant, spread among vegetables and other food products due to improper sanitation and handling. The lack of clean water and sanitation as well as inadequate infection prevention and control promotes their spread, some of which can be resistant to antimicrobial treatment. In this study, lettuce grown in the Philippines has been subjected to investigation to determine the presence of pathogenic microorganisms. Lettuce has become popular through the introduction of foreign delicacies such as salads, wraps, and samgyeupsal. Three samples were procured in different markets in Metro Manila: Marikina wet market, Farmers' market Cubao, and Supermarket (Cubao). Bacteria from the lettuce were cultured in Eosin Methylene Blue Agar (EMB) for 24 h at 37^{0}C, counted using the colony counter and analyzed using Independent Samples Kruskal-Wallis H Test at .05 Level of Significance. Gram staining was then performed for three colonies exhibiting different colors and were isolated and subcultured in nutrient broth to obtain pure culture. All samples were done in triplicates. The genomic DNA of the microorganisms were extracted using the xanthogenate method, and the specific DNA regions were amplified using 16s rRNA. DNA sequencing to identify the microbes at the species level. Results indicate that there is no significant difference between the number of colonies from the samples in the three markets. Moreover, three microbes namely *Raoultella ornithinolytica (Marikina wet* market), *Acinetobacter pittii (Farmer's market), and Acinetobacter soli (supermarket) match the results in BLAST (Basic Local Alignment Search Tool); hence, they were classified as biosafety level 2. Further investigation on the source of contamination is highly recommended.*

Keywords: microorganisms, lettuce, genomic DNA, PCR, DNA sequence, *Acinetobacter soli, Acinetobacter pittii, Raoultella ornithinolytica*

Introduction

Microorganisms can be classified as either pathogenic or nonpathogenic. Pathogenic organisms such as bacteria, fungi, protozoa, or viruses may cause diseases. For more than half a century, the treatment of infectious diseases has greatly relied upon microbial agents, resulting in a significant decrease in morbidity and mortality rates related to these (Republic of Ghana, 2017; Dache, Dona, & Ejeso, 2021). In addition, health systems have developed and improved over the years, thus paving the way for the public to gain easy access to antimicrobials.

While the use of microbial agents is a welcome progress, their extensive use caused some of these pathogens to adapt to antibiotics, thus rendering them less effective (Centers for Disease Control and Prevention [CDC], 2019). This phenomenon is known as multi-drug resistance (MDR), which is defined as the nonsusceptibility of pathogens to known antibiotics through mechanisms such as accumulation and modification of the antimicrobial target and enzymatic modification of the drug (Munita & Arias, 2016; Kapoor, Saigal, & Elongavan, 2017). This now has become a global concern in antimicrobial therapy since without effective antibiotics, the ability to treat common and life-threatening infections will be undermined (WHO 2023). It is important to note that in the recent pandemic, antibiotics (i.e., macrolides, cephalosporins, fluoroquinolones, and penicillin) have been prescribed to many COVID–19 patients to remedy secondary bacterial infections. Hence, there is a significant possibility that MDR and associated fatalities cannot be ruled out soon (Gagliotti *et al. 2020; Chedid et al. 2021).*

The pathogenic microorganisms have been nicknamed “super bugs” because they have developed a high degree of resistance against antibiotics (Tanwar, Das, Fatima, & Hameed, 2014; Dache, Dona, & Ejeso, 2021). Some of the most common MDR as per WHO are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa, and Enterobacter species (ESKAPE* pathogens*)* (Levin *et al.* 1999; Miller *et al.* 2005). Others include *Escherichia coli* (Liu *et al.* 2016; Rapoport *et al.* 2016; Yamamoto *et al.* 2019; Dache, Dona, & Ejeso, 2021), *Streptococcus pneumonia* (Chang *et al. 2003;* Falagas, Kasiakou, & Saravolatz, 2005; Arias & Murray, 2012; Gurung *et al.* 2013; Dotel, O’Sullivan, & Gilbert, 2017; Oatman *et al.* 2019) *and Plasmodia spp., Leishmania* spp.*, Trichomonasvaginalis,* etc., which are resistant to cephalosporin, macrolides, rifampicin, and others (Berkow & Lockhart, 2017). Resistance caused by these microorganisms against microbial agents may result in blood infection, urinary tract infection, pneumonia, meningitis, otitis, AIDS, influenza, and hepatitis (Tanwar, Das, Fatima, & Hameed, 2014).

For this reason, the development of MDR strains which are primarily caused by the overuse and the improper use of antibiotics is going to be the next pandemic, according to the World Health Organization. As case in point, approximately 700,000 patients from across the globe die due to being infected by multi-drug resistant bacteria (World Health Organization [WHO], 2019).

Thus, awareness on how the MDR pathogens spread should also be investigated. One way microbes enter our system is through the food we consume. Reports on outbreaks on vegetables and other food products which contained potentially MDR pathogens may be caused by improper sanitation and handling of the produce (Janeway, Travers, Walport, & Shlomchik*,* 2001).

Among the vegetables available in the market today, lettuce (*Lactuca sativa*) has become a trend due to the introduction of foreign delicacies such as salads, wraps, and *samgyeupsal*. It has also become an ideal vegetable for those who are dieting because of its high fiber and low nutrient content. Lettuce is an annual plant which belongs to the aster family (*Asteraceae*). It has five distinct types: Cos or Romaine, Crisphead, Butterhead, Loose-leaf, and Stem or Asparagus lettuce (DA BPI 2010). However, despite lettuce’s vibrant reputation, news reports and other studies on lettuce outbreaks in other countries linked to pathogenic microorganisms pose a potential risk for lettuce consumers. In 2005, 121 total outbreaks and 11,404 illnesses and deaths were associated with *E coli O157*. 120 cases of which were in Sweden wherein consumption of contaminated lettuce resulted in illnesses (Ackers *et al*. 1998; Poimenidou, Chatzithoma, Nychas, & Skandamis, 2016). Just recently in 2018, multistate outbreak was also reported to be associated with *E. coli*-contaminated lettuce (Infectious Diseases Society of America [IDSA], 2019; U.S. Food and Drug Administration, 2019). At least 32 individuals from 11 states have been documented to be infected with *E. coli* O157:H7 from October 8 to October 31, 2018. 13 of them were hospitalized and one was reported to have acquired hemolytic uremic syndrome. Based on epidemiologic evidence, the same bacteria

isolated from Cos lettuce was the culprit of the outbreak. This outbreak is still being investigated as it is difficult to identify its source since lettuce has no common grower, brand, or distributor (IDSA, 2019).

To make matters worse, the occurrence of resistant phenotypes/resistant genes of these pathogenic bacteria post a threat to public safety since lettuce is a ready-to-eat vegetable (RTE). Most pathogenic bacteria can only be eliminated by thorough cooking with at least 70^{0} C to higher temperature (WHO, 2018). This means that even a healthy person can be at risk. Previous studies have identified methods to eliminate pathogenic microorganisms in lettuce and other vegetables. However, despite efforts in exploring ways to eliminate these pathogens, researchers have yet to identify the best method. To date, the use of sanitizing agents is recommended to at least reduce the bacterial load. Thus, in handling lettuce, water quality, sanitizer, and contact time, among other factors, must be taken into consideration (Bencardino, Vitali, & Petrelli, 2018).

At present, there is a dearth of studies that discuss the extent of the microbial presence on lettuce in local markets in the Philippines, specifically in Metro Manila. The Brazilian standard and United Kingdom guidelines have set a fecal coliform limit of < 20 CFU/g- 100 CFU/g which means that higher than the prescribed limit is regarded as unsatisfactory (Valentin-Bon *et al.* 2008). As for microbial loads, Hazard Analysis and Critical Control Points'-Total Quality Management (HACCPTQM) have set a standard for microbial loads where in the quality for raw foods containing greater than >2 $_{log}$CFUg^{-1} is considered average to poor or spoiled category. Moreover, plate count of aerobic mesophilic bacteria that exceeds the prescribe limit for microbial count is regarded harmful even if the organisms are not known to be pathogens (Aycicek *et al.* 2006).Thus, this paper aimed to investigate locally grown and sold lettuce to determine total number of microorganisms (microbial loads) found in it if will pass the prescribed permissible level of microbes by (HACCPTQM) and to isolate and identify the presence of pathogenic bacteria in the sample lettuce from different markets in Metro Manila to determine if the microorganisms are pathogenic and belong to some of the most common MDR identified by WHO.

The study may be deemed as significant in understanding the extent of microbial presence in lettuce available in local markets in the Philippines as it may serve as the basis for implementing measures that can ascertain the safety of consumers. Moreover, it will also shed light as to the status of MDR in our country.

Methodology

Microorganisms can be classified as either pathogenic or nonpathogenic. Pathogenic organisms such as bacteria, fungi, protozoa, or viruses may cause diseases. For more than half a century, the treatment of infectious diseases has greatly relied upon microbial agents, resulting in a significant decrease in morbidity and mortality rates related to these (Republic of Ghana, 2017; Dache, Dona, & Ejeso, 2021). In addition, health systems have developed and improved over the years, thus paving the way for the public to gain easy access to antimicrobials.

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Discussion

Cultures and Culture Conditions

Eosin-methylene blue (EMB) and nutrient broth (NB) were sterilized as the media for bacterial growth. Sterile water blanks as well as other materials were also autoclave-sterilized at 121°C, 15 psi for 15 minutes (Cappuccino & Welsh, 2018).

Sampling

A total of three (3) vegetable samples (lettuce) were collected from retail vegetable stores in the three (3) selected markets in Metro, Manila (Marikina wet market, Farmer's Cubao, and Supermarket (Cubao). Samples were randomly collected in designated (labeled) sterile plastic bags and transported to the Microbiology Laboratory of the Ateneo de Manila University for analysis.

Isolation and Identification of bacteria

Each sample was homogenized in a sterile electrical blender for five minutes (each containing 20g of sample and 180 ml of sterile water. Serial dilutions up to 10−3 were made. 1ml of 10−1 dilution (sample in the blender) was transferred into its labeled 99-ml sterile water blank. Using another pipette, 0.1 ml from 10−3 (sample in 99 ml sterile water blank + 1ml of sample dilution 10−1 dilution), was transferred to the plate labeled 10−3 EMB agar for plating (for colony counting and gram-staining). The plates were incubated at 37 0C in an inverted position for 24 hours. Colonies for each plate were counted using the colony counter. Colonies that exhibited different colors on the EMB agar were isolated and subcultured in nutrient broth to obtain pure culture. All samples were done in triplicates (Cappuccino & Welsh, 2018).

Gram Staining (Differential staining)

Bacterial smears of 24-hour isolates (1-Markina; 2-Farmer's; and 3-Supermarket) were prepared. Each smear was covered with crystal violet (primary stain) for one minute. The excess stain was poured off and rinsed with distilled water. Iodine (stain affinity enhancer) was added for one minute and was rinsed gently with distilled water. This step was repeated once more. The slide was then washed with 95% ethanol (decolorizer) for 10-15 seconds to remove excess stain. Lastly, the smear was covered with safranin (counterstain) for one minute. The excess stain was poured off and the slide was rinsed gently with distilled water before blotting dry with tissue paper. The specimen was then examined under the microscope in LPO, HPO, and then OIO (Gulpeo n.d.)

DNA Extraction

Xanthogenate method was used to extract the DNA from the vegetable samples. 1 mL of the incubated bacterial culture (pure culture) in nutrient broth was centrifuged for 3 mins at 4,000 rpm. The supernatant was decanted from the media and then resuspended with 600 µL of XS buffer pipetted into the microcentrifuge tube. The tubes were incubated at 70 °C using dry bath for 15 minutes and then vortexed for 10 s. The tubes were incubated on the freezer for 10 mins and centrifuged for another 5 mins at maximum speed. The pellet was discarded, and the supernatant was transferred into a new microcentrifuge tube and 750 µL of isopropanol was added. The tubes were mixed by gentle inversions and then incubated for 5 mins at room temperature. After which, it was centrifuged for 5 mins at maximum speed. The supernatant was discarded, and the pellet was added with 70% ethanol (ETOH), vortexed to mixed and then centrifuged for another 5 minutes at maximum speed. Supernatant was discarded and the pellet was air-dried. Finally, the pellet was resuspended in 50 µL TE buffer (Constantino 2017).

Agarose Gel Electrophoresis (AGE)

DNA from the xanthogenate extraction was visualized through agarose gel

electrophoresis (AGE). 1% agarose gel was prepared by using agarose powder and 1x TAE buffer (0.3g agarose in 30 ml 1x TAE). The mixture was stained using 3 µL of ethidium bromide (EtBr). 5 µL of DNA extracted from cultures were loaded into the wells of the agarose gel after it was mixed with the 3 µL of gel loading dye (GLB) (mixed in the clean side of parafilm). The gel was run in an electrophoresis tank at 100 V for 30 mins. The molecular weight markers used were the Vivantis VC1kb ladder (for the genomic DNA) and the Vivantis 100 bp plus ladder (for Polymerase Chain Reaction (PCR)). The gel was visualized under UV light (Constantino 2017).

Polymerase Chain Reaction (PCR)

The DNA extracted from the lettuce was amplified through PCR. In a sterile microcentrifuge tube, the mastermix was prepared: 12.5 µL 2X Vivantis Taq Master mix, 0.25 µL of 50 mM MgCl2, and 7.75 µL nuclease-free water. Reaction tubes contained 10 µM of 1.25 µL forward primer, 1.25 µL reverse primer, and 2 µL of the DNA template.

Table 1. Primer sequences used in PCR.

Primer	Sequence (5'-3')	Direction
27F	AGAGTTT-	Forward
1492R	CGATAATAACAACAG	Reverse

Table 1 shows the primer sequences used in PCR. The amplification for primer pair F and R was conducted as follows: initial denaturation at 94 °C at 5 mins, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and 1 cycle of a final extension of 72 °C for 5 mins. 5µL PCR product was then viewed in 1% agarose gel electrophoresis (Suardana, 2014).

DNA sequencing and Identification

The PCR products were sent to Asiagel for DNA sequencing. The sequences were then edited using Chromas version 2.6.6 developed by Technelysium Pty. Ltd. and assembled using Prabi-Doua:CAP3 Sequence Assemby (Huang and Madan 1999). It was then identified using NCBI BLAST.

Statistical Data Analysis

Non-parametric correlation Independent Samples Kruskal-Wallis Test was performed to determine the relationship between target variables of concern (IBM SPSS Statistics 2015). A 95% confidence interval was used in presenting the ranges of data.

RESULTS

The total number of microorganisms in the lettuce samples in the EMB agar were counted using the colony counter. An average of 160 colonies were counted from Farmer's wet market sample followed by Marikina wet market with 150 colonies, and then the Supermarket with 132 colonies. In terms of logCFUg−1 (ranged from 6.12 to 6.20) it is higher than the prescribed permissible level of microbes by Hazard Analysis and Critical Control Points'-Total Quality Management (HACCPTQM).

Table 2. Average Number of bacteria isolated from raw vegetables in EMB agar.

Source of Lettuce Sample	Dilution	Average No. of Colonies per Plate	CFU/ml	$_{log}$CFUg^{-1}
Marikina Wet Market	10^{-3}	150	1.5×10^{6}	6.17
Farmer's Market	10^{-3}	160	1.6×10^{6}	6.20
Cubao Supermarket	10^{-3}	132	1.32×10^{6}	6.12

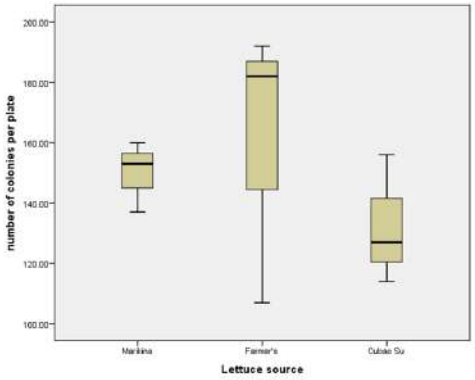


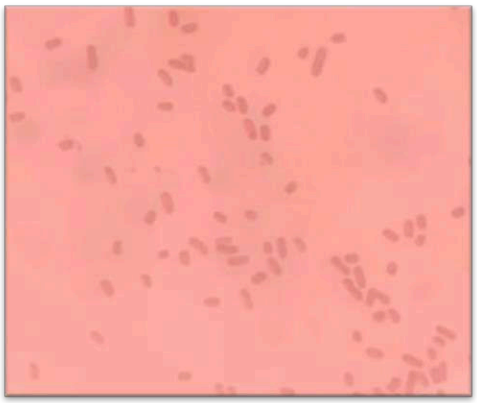
Figure 1. Number of colonies with their standard deviation. Marikina colonies 150 ± 11.78983, Farmer's market 160 ±46.45787, and Supermarket 132 ±21.50194.

The bacterial load per source of lettuce varies as seen in Figure 1. In Marikina market the minimum bacterial load is 137 and the maximum is 160 (as indicated by whiskers of the boxplot). For Farmer's market the minimum is 107 and the maximum is 192 160 (as indicated by whiskers of the boxplot). And for Supermarket, the minimum is 114 and the maximum is 156 160 (as indicated by whiskers of the boxplot). Overall, the minimum count is around 110 colonies and the maximum count is 190 plus.

Morphological/Phenotypic Identification

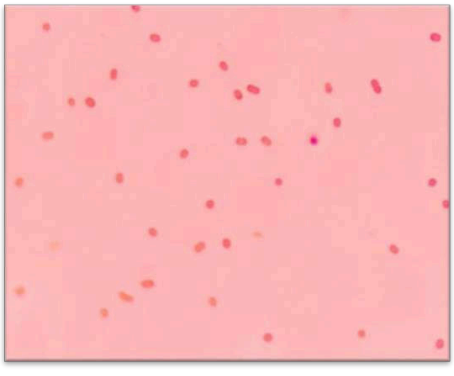
Preliminary Identification

Figure 2. Gram-stain result for Marikina market sample.



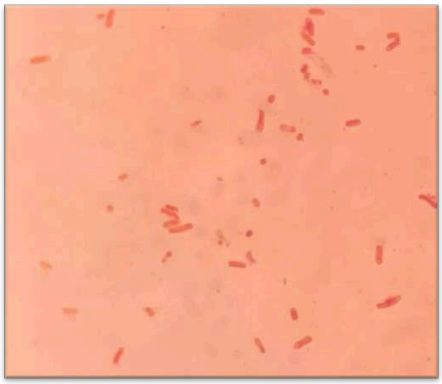
Microscopic view under (OIO | 1000x)

Figure 3. Gram-stain result for Farmer's market sample.



Microscopic view under (OIO | 1000x)

Figure 4. Gram-stain result for Supermarket sample.

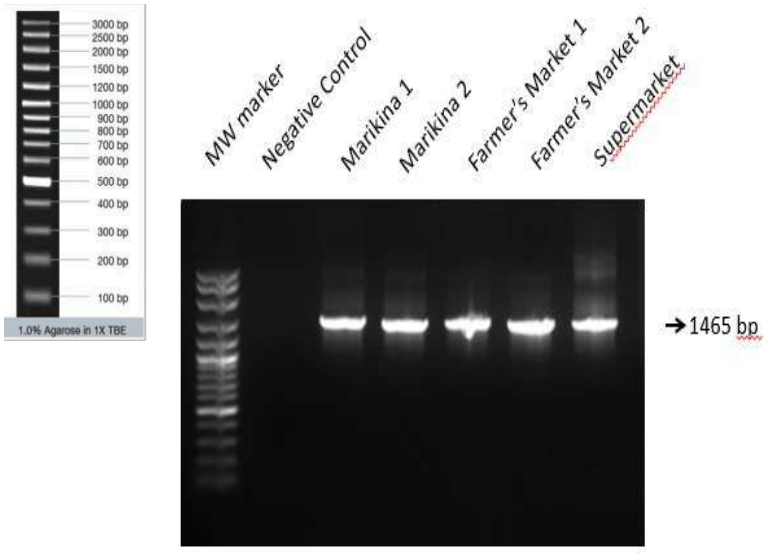


Microscopic view under (OIO | 1000x)

Molecular Identification

DNA Extraction and PCR.

Figure 5. PCR genomic DNA



MW marker: 100 bp plus ladder (Vivantis)

The presence of bands in Figure 5 contained the estimated molecular weight MW ~1465 bp corresponded to a fragment of 16srRNA gene. This indicates the proper identification of the target gene. DNA sequencing was performed via Asiagel to further identify the PCR products. The sequences were then edited using Chromas version 2.6.6 and assembled using Prabi-Doua:CAP3 Sequence Assemby which are all available online. They were then identified using ncbi BLAST.

Figure 6. Sanger Sequencing and BLAST

Isolate code	Closest Species Match (Accession Number)	Seq/per Identity (%)	Query Cover-age (%)
1	NR_114502.1	99.85%	100%
2	NR_117621.1	100%	100%
3	NR_044454.1	99.45%	100%

Isolates Identification

Isolate code 1: Marikina Market

Raoultella ornithinolytica was the closest bacterial identity for isolate 1 with 99.85% sequence/percentage identity. Below is the scientific classification of this bacterium:

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacterales
Family	*Enterobacteriaceae*
Genus	*Raoultella*

Isolate code 2: Farmer's Market

Acinetobacter pittii was the candidate for isolate 2 with 100% sequence/percentage identity. Below is the scientific classification of this bacterium:

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonales
Family	Moraxellaceae
Genus	*Acinetobacter*

Isolate code 3: Supermarket

Acinetobacter soli was the closest bacterial identity for isolate 3 with 99.45% sequence/percentage identity. Its scientific classification is the following:

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonales
Family	Moraxellaceae
Genus	*Acinetobacter*

DISCUSSION

Data analysis using Kruskal-Wallis H test in Figure 1 shows that the number of bacteria isolated from lettuce collected in three markets in terms of the distribution of number of colonies per plate across categories of lettuce source is not statistically significant ($p>.05$). This suggests that the lettuce collected from the open markets (Marikina and Farmer's) are similar in terms of bacterial load with their seemingly cleaner counterparts found in a Supermarket. The store where lettuce was bought is insignificant because sources are exposed to bacterial contamination. This result reflects the poor practices either during harvest and handling of lettuce in the Philippines. It is also noticeable that the bacterial load per source of lettuce varies as seen in Figure 1. This variation in bacterial load is probably due to some bacteria hindering or slowing down the growth of other bacteria (Valentin-Bon, Jacobson, Monday, & Feng 2008). As to microbial loads, Hazard Analysis and Critical Control Points'-Total Quality Management (HACCPTQM) have set a standard where in the quality of raw foods registering less than $<2\ _{log}CFUg^{-1}$ is considered good quality and greater than $>2\ _{log}CFUg^{-1}$ is considered average to poor or spoiled category. The plate count of aerobic mesophilic microorganisms is regarded as harmful if it exceeds the prescribed limit for microbial count even if the organisms are not known to be pathogens (Allen, Edberg, & Reasoner, 2004). In Brazil, a fecal coliform limit of 100 CFU/g has been set; on the other hand, the United Kingdom has set the E. coli count limit at < 20 CFU/g- 100 CFU/g. Higher than the prescribed limit is regarded as unsatisfactory (Valentin-Bon, Jacobson, Monday, & Feng 2008). The result of this study in terms of $_{log}CFUg^{-1}$ (aerobic plate count) is higher than the prescribed permissible level of microbes by (HACCPTQM). This study is comparable to the other studies such as study of vegetables including lettuce in Ethiopia where in the results are also higher (ranged from 5.24 to 6.54 log $CFUg^{-1}$). Similar study in the US on 100 bagged lettuce and spinach mixes have found a mean of 7.2 to 7.3 log10 CFU/g (Valentin-Bon, Jacobson, Monday, & Feng 2008). Another study from Saudi Arabia showed 105 lettuce samples (ranged from 4.97-5.99 log10 CFU/g) (Hamad, Al-Amer, & Al -Otaibi, 2013). Also, in Brazil, 50% of 133 RTE leafy salads were found to have a mean of >6.0 log10 CFU/g (Fröder et al. 2007). All these studies have reported that the bacterial contamination may have come from either pre-harvesting or post harvesting including the distribution and handling of the vegetable.

To further identify and analyze the bacteria grown from EMB agar, the colonies were examined, and gram staining was then performed. Three (3) colonies exhibiting different colors (green-Marikina, blueish-Farmer's, and pink-Supermarket) were selected. The colored colonies indicate their ability to ferment lactose and produce of acids. Non-fermenters of lactose are normally colorless colonies (Lal and Cheeptham 2007). Based on the preliminary identification, the three bacteria were all gram negative as they had grown in Eosin-methylene blue agar (EMB) which is a selective medium for gram-negative bacteria. The ability of the bacteria to vigorously ferment lactose and produce acids made them appear as dark colored colonies which can be classified in general as enteric microorganisms. Figure 2 (Marikina) shows a dark colony (green) (producing metallic sheen). This metallic green sheen is also an indicator of fecal coliforms. Based on the said characteristics, the bacterium isolated from Marikina is an enteric bacterium and due to its bacterial shape (rod-shaped) and its green metallic sheen appearance, it is most probably E. coli. Most members of this family form the gut microbiota of animals and humans. Most of them are harmless symbionts but some strains could be pathogenic (Shearer et al. 2012; Derrien, Alvarez, & de Vos, 2019). The Supermarket (Fig. 4) isolate most likely belongs to another family even though its bacterial structure is the same as the ones found in Marikina (rod-shaped) due to its ability to ferment lactose. Both bluish (Farmer's) and Supermarket (pink) colonies are classified to other families which weakly or slowly ferment lactose. Moreover, Farmer's isolate (Fig. 3) has a bacterial shape that is intermediate between cocci and bacilli (very short rods) which makes it a gram-negative coccobacillus. Among the other gram-negative coccobacillus, Acinetobacter spp are ubiquitous in soil and water; hence, this can possibly infect the lettuce during pre-harvest. Some studies have also linked this bacterium with fruit and vegetable contamination (Mesbah Zekar et al. 2017. Based on the above-mentioned characteristics, the isolated bacteria from Fig.3 could belong to this group.

Molecular identification of the three isolates identified Raoultella ornithinolytica from the Marikina market, Acinetobacter pittii from Farmer's market, and Acinetobacter soli from the Supermarket. R. ornithinolytica is a gram negative, aerobic, oxidase-negative,

facultatively anaerobe and nonmotile bacillus that belongs to Enterobacteriaceae family. It was initially classified to belong in Klebsiella genus but reclassified as Raoultella genus based on 16srDNA and gyrA, gyB, and rpo B genes. Raoultella spp. can be isolated in natural environment such as soil and aquatic environments. They ferment lactose and produce catalase with 2,3-butanediol as their major fermentation product. They have low nutritional requirement and can grow between 4^{0}C - 40 ^{0}C with histidine as the only carbon source (Drancourt, Bollet, Carta, & Rousselier 2001; Park et al. 2011). R. ornithinolytica has gained attention in the clinical field in the recent years due to its isolation frequency from patients with various forms of infection. It was considered as an impurity and non-pathogenic at first, but recent studies have proved its pathogenicity; it can have adverse effects to humans' health, particularly in organs. It can also cause systemic infections in patients who have undergone invasive surgeries such as surgery in the abdomen. The presence of this bacteria under favorable conditions may cause colonization of the gut leading to infections (Tayo & Kyame, 2022). Other Infections associated with R. ornithinolytica include: urinary tract infection (UTI), gastrointestinal infections, wound and skin infections as well as bacteraemia, respiratory infections (pneumonia and pleural effusion), mediastinitis, osteomyelitis, cerebral abscess, pericarditis, conjunctivitis, otitis, and meningitis. The mortality rate related to R. ornithinolytica infection is 5% (Seng et al., 2016; Seng et al., 2016). The multidrug resistance ability of R. ornithinolytica classifies this species as biosafety level 2 (BSL2) (Trapotsis, 2019). Strains of R. ornithinolytica have been reported to be resistant to ampicillin, clavulanic acid, amoxicillin doxycycline, co-trimoxazole, rifampicin, tetracycline, trimethoprim, oxytetracycline, colistin, kanamycin, and pefloxacin. Moreover, patient's renal monitoring and therapeutic drug monitoring are required to avoid toxicity (Germovsek et al. 2017). According to USFDA, food poisoning in this bacterium in healthy individual will require a dose of at least 50 mg histamine in fish or fishery products or consuming a 250 g single serving size of fish (200 mg/kg -limit). It is also noteworthy to think that this threshold may vary according to individual exposure and health status. Lower hazard level may be considered to some individuals that have increased sensitivity in relation to metabolic differences, age or drug therapies or physiological conditions.

Acinetobacter pittii is under a large group of Acinetobacter spp. Acinetobacter baumanii (the most common cause of infections in humans). A. pittii (formerly known as Acinetobacter genomic species 3) belongs within the Acinetobacter calcoaceticus-baumanii complex. They are ubiquitous in nature particularly in water and soil, but they can be occasionally present in food stocks. They are also found in human skin (part of normal microbiota of skin). It can colonize the skin and upper respiratory tract without causing an infection but can result in infection if the immune defense is compromised. This can be dangerous to human health especially those with immunosuppression or weakened immune system (Nemec et al. 2011). This bacterium can also be associated with diarrhea, bacteraemia, meningitis, pulmonary infections, and nosocomial infections. Acinetobacter ranked 9th after S. aureus, E. coli, P. aerugenosa, Klebsiella spp. Enterococci, Serratia, Enterobacter and C. albicans as agents of nosocomial infections. Acinetobacter spp. are the second most isolated bacteria in humans and their incidence is on the increase as well as mortality rates. The mortality rates related to this genus was 20 to 60% (Doughari, Ndakidemi, Human, & Benade, 2011; Nemec et al. 2011; Wang, Chen, Yu, Lü, & Zong, 2013). The emergence of these types of bacteria has also become a major concern recently in the medical field due to their multidrug resistance capability. It also has the capability to survive in a very dry environment for a long time due to their rapid profundity in transformation. Colonization of this bacterium increases as one stays longer in the hospital. A. pittii was normally susceptible to aminoglycosides, carbapenems, and fluoroquinolones. However, there is an emerging carbapenem-resistant A. pitti which can increase its pathogenicity due to its acquired resistance genes. Carbapenem-resistant Acinetobacter was reported to have caused an estimated of 8,500 infections among hospitalized patients (healthcare settings) and around 700 deaths in the US in 2017 (CDC, 2019). Further investigation on the virulence mechanism of this pathogen should be therefore undertaken to discover more effective control measures (Nemec et al. 2011).

Acinetobacter soli is one of the newly pathogens described under Acinetobacter spp. A. soli was previously isolated from forest soils. However, this bacterium is now

present in clinical specimens. The presence of this bacterium can cause bloodstream infections (BSI) in newborns. This bacterium may have the potential to cause outbreaks in newborns (Pellegrino et al. 2011). Just like the two previous isolates, A. soli is also a multidrug resistant bacterium. A. soli strain B1T, has resistance to amikacin and gentamicin.

CONCLUSION and RECOMMENDATIONS

Results suggest that lettuce grown in the Philippines (from the 3 markets) in terms of $_{log}$CFUg–1 (ranged from 6.12 to 6.20) is higher than the prescribed permissible level of microbes by (HACCPTQM). This reflects the poor practices either during harvest or handling of lettuce in the country. The lettuce also contains pathogenic bacteria that can cause diseases. Isolating pathogenic bacteria such as *R. ornithinolytica* in Marikina market, *Acinetobacter pittii* in Farmer's market, and *Acinetobacter soli* in Supermarket in Cubao which are all multi-drug resistant are indications that we cannot not simply ignore the possibility of contamination or an outbreak in our country given that the lettuce bacterial load can only be reduced using disinfectant. It is therefore recommended to further investigate the source of contamination in lettuce. People should also be aware of the increasing virulence and rapid development of these kinds of pathogens to observe good hygiene practices.

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