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DETERMINATION OF THE PRESENCE OF ESCHERICHIA COLI IN LACTUCA SATIVA OF WESTERN, SOUTHERN, CENTRAL AND NORTH-WESTERN PROVINCES OF SRI LANKA

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ABSTRACT

Escherichia coli (E. coli) is one of the most frequent foodborne pathogens associated with many of the worldwide outbreaks. Out of which, E. coli contaminating lettuce leaves has shown the highest incidence. Furthermore, E. coli has also been studied extensively as an important indicator organism of faecal contamination. Hence it serves a prime role in determining the microbiological quality of food and water sources. The present study was carried out in order to detect the presence of E. coli in lettuce leaves (Lactuca sativa) of Sri Lanka via culture, biochemical and molecular techniques. Lettuce leaves were collected from 21 different open air markets and supermarkets located in North-Western, Southern, Western and Central provinces of Sri Lanka.

Out of the 21 samples tested, 14.3% of the samples were contaminated with E. coli. In which, highest incidence was observed in lettuce leaves obtained from North-Western, Southern and Central provinces of Sri Lanka. Besides, E. coli contamination in lettuce leaves obtained from supermarkets was noted to be higher compared to open air markets. In addition, coliforms such as Citrobacter diversus (42.9%), Klebsiella oxytoca (28.6%), Klebsiella pneumonia subsp. ozaenae (4.8%) too were detected. Furthermore plant pathogens such as Erwinia chrysanthemi which promotes the growth of E. coli was detected in two of the samples. Hence the total of 21 samples were considered to be not suitable for consumption in its' raw state, due to the detection of these pathogens. Whereas, 14.3% of the samples which harboured E. coli, were considered to be faecal contaminated.

Keywords: E. coli, Lactuca sativa, Open air markets, Supermarket

INTRODUCTION

Foodborne pathogens are organisms that are capable of infecting humans through contaminated food. This has been considered as a major health hazard affecting both developing and developed countries (Prieto et al., 2015). A series of emerging foodborne pathogen associated diseases are driven by factors such as pathogen evolution and adaptation, lifestyle changes, host susceptibility, pre and postharvest stages of food production and manufacturing. Fresh produce consumption has been increasingly associated with food borne pathogens of humans. A report from centres for disease control and prevention (CDC) indicated that among produce linked to outbreaks, leafy greens such as lettuce and spinach were of the highest frequency (Berry et al., 2015). This could be linked to the growing

interest towards ready to eat salads, promoting healthy living. As these are consumed raw, it carries a potential risk of containing pathogens that would cause a health problem (Cerna-Cortes et al., 2015). The contamination risk of fresh produce is associated with fertilizers, untreated water and other sources during harvesting, handling, processing and packaging (Taban and Halkman, 2011). Among the foodborne pathogens, Escherichia coli (E. coli) are one of the most frequent foodborne pathogens, which cause diseases through various sources. Out of which, food sources are the most prominent (Clark, 2018).

E. coli is a gram negative, rod shaped, facultative anaerobic coliform bacterium which is usually present in the intestines of vertebrates (Tenaillon et al., 2010). It belongs to the Enterobacteriaceae family. Most E. coli strains exist as normal micro biota of the gut and remain harmless. Whereas few strains such as E. coli 0157:H7, when contaminated mostly via food, causes serious illnesses. Therefore considered as pathogenic. Since most E. coli resides in the gut, it passes out along with faeces. Thus E. coli is considered as an indicator organism of faecal contamination, giving consideration to its long survival period in faeces as well. However, proliferation of E. coli takes place only with the re-entry into the gut via contaminated food or water, thereby causing diseases such as diarrhoea, meningitis, pneumonia and urinary tract infections (Tenaillon et al., 2011; Russell and Jarvis, 2001). A semi quantitative risk ranking tool was developed to analyse the pathogen-produce pair attribution risk in USA. Where, this was able to indicate leafy greens and E. coli pair as the highest ranking pathogen-produce (Anderson et al., 2011). Cattle are considered as a significant source of E. coli 0157:H7 strain. Hence outbreaks of E. coli 0157:H7 via lettuce consumption have gained the focus on cattle as the source of contamination. Animal slurry is often utilized as a fertilizer for cultivation, thus pathogens such as E. coli from animal slurry could migrate through the soil, contaminating the crop (Russell and Jarvis, 2001). A study that was conducted in lettuce grown in soils amended with animal slurry indicated the presence of E. coli in 47% of the crops (Jensen et al., 2013). Furthermore, E. coli residing in cattle can also be transmitted through air, contaminating the crops. This was depicted in a study that was conducted in a lettuce cultivation field which was in close proximity to a beef cattle feedlot. Where, an increased risk of crop being contaminated with E. coli was demonstrated when the distance between beef cattle feedlot and cultivation field was 180m or less (Berry et al., 2015).

In addition, untreated water usage during harvesting process too carries a potential risk in contaminating the crops. A study governing lettuce plants which were treated with artificially contaminated E. coli water was able to detect the presence of E. coli in the crops which were repeatedly exposed to the contaminated water (Solomon, Pang and Matthews, 2003). Nevertheless, in another study, lettuce samples were inoculated with E. coli and then chilled on uncontaminated ice. This was compared with uncontaminated lettuce samples which were treated with E. coli contaminated ice. Thus it was able to depict that the ice used for transportation of lettuce when melted, could transfer the contaminant from the contaminated lettuce leaves for the rest of the transporting leaves. Similarly if the ice used for transportation is contaminated, it also could contaminate the leaves (Kim and Harrison, 2007). Therefore it is certain that the contamination of lettuce with E. coli can occur during any stage from pre-harvesting to packaging. Hence, determination of E. coli in fresh produce is critical prior to its distribution. Especially, as it is being consumed raw with minimal

effort of decontamination. Therefore during this study, the presence of E. coli was tested in lettuce leaves (Letuca sativa) via microbiological, biochemical and molecular techniques.

Traditional approaches in analysing E. coli has been relying on cultural techniques. Hence many differential media have been developed. E. coli is a lactose fermenting bacteria which can be cultured easily in a laboratory setting. As E. coli is a chemoheterotroph, the culture medium should include a source of carbon and energy (Yaratha, Perloff and Changala, 2017). MacConkey agar is the first solid differential media formulated to isolate and differentiate gram negative lactose fermenting and lactose non fermenting bacteria, particularly of Enterobacteriaceae family (MacConkey, 1905). Peptones composed in the MacConkey agar provide the essential nutrients for the growth of microorganisms. Whereas, lactose monohydrate is a fermentable carbohydrate source which in the context of E. coli is fermented to produce hydrogen sulphide. The bile salts and crystal red inhibit the growth of gram positive bacteria, thereby causing selective growth of bacteria. Neutral red behaves as a pH indicator which turns pink when pH is below 6.8. Therefore, colonies of lactose fermenting bacteria such as E. coli turns into pink, as the resulting acid produced by the lactose fermentation causes the indicator to be turned into pink. Thus in the present study, samples were cultured in MacConkey agar media to isolate E. coli colonies.

In addition, biochemical tests such as indole test and citrate test were performed to further confirm the presence of E. coli in cultured sample. Furthermore, molecular techniques have shown higher sensitivity compared to microbiological and biochemical techniques. Hence molecular detection techniques were carried out in this study as a confirmatory test for the determination of E. coli. Comparison of the genomic sequences of bacterial species has shown that the 16S ribosomal RNA (rRNA) gene is a highly conserved gene within a species and also among the same genus. Therefore, molecular techniques focussing on 16S rRNA gene have been utilized as a gold standard for the identification of specific bacteria (Suardana, 2014; Woo et al., 2000). Thus during this study, a hypervariable region of the 16S rRNA gene of E. coli was amplified in order to confirm its' presence.

The main scope of the present study was to determine the presence E. coli in lettuce leaves of Sri Lanka via microbiological, biochemical and molecular techniques. Alongside, the microbiological quality of the lettuce samples were analysed as well.

METHODOLOGY

Sample collection

Total of 21 lettuce samples (Lactuca sativa) of 100g each were collected from North-Western, Central, Southern and Western provinces of Sri Lanka. These samples were collected from 21 different stores which were either supermarkets or open air markets located within these provinces (Table 1). All the samples were collected in sealed zip bags, and were transported to the laboratory within 24 hours. These samples were then stored at 4°C until use.

Sample code	Province	Location	Type of store
L01	North-Western	Nattandiya	Open air market
L02	North-Western	Dankotuwa	Supermarket
L03	North-Western	Kochchikade	Supermarket
L04	North-Western	Kochchikade	Supermarket
L05	North-Western	Kochchikade	Open air market
L06	Central	Gelioya	Open air market
L07	Central	Amunugama	Open air market
L08	Central	Madawala	Open air market
L09	Central	Katugasthota	Open air market
L10	Central	Kandy	Supermarket
L11	Central	Katugasthota	Supermarket
L12	Southern	Hikkaduwa	Supermarket
L13	Southern	Hikkaduwa	Open air market
L14	Southern	Baddegama	Supermarket
L15	Southern	Galle	Supermarket
L16	Southern	Galle	Supermarket
L17	Southern	Karapitiya	Supermarket
L18	Western	Wellampitiya	Supermarket
L19	Western	Wellawatte	Supermarket
L20	Western	Thalahena	Supermarket
L21	Western	Wellawatte	Open air market

Sample preparation

Lettuce leaves were homogenized in peptone water buffer (5g from each lettuce sample was homogenized in 45mL of peptone water buffer). Afterwards the content in each falcon tube was allowed to mix in the roller mixer at high speed for 2 hours. Later incubated overnight at 37°C and was stored at 4°C, until use (Niguma, Pelayo and Oliveira, 2017). Subsequently, homogenized samples were streaked on MacConkey agar plates and resulting pink colonies were isolated to confirm the presence of lactose fermenting bacteria.

ANALYSIS

Microbiological analysis

Gram staining technique was carried out for the lettuce samples. Next, the stained slides were observed through 100x objective lens of the light microscope. Visualization of pink stained bacteria were considered to be gram negative bacteria, while purple colour stained bacteria were distinguished as gram positive bacteria (Nagoba and Pichare, 2007).

Biochemical analysis

Biochemical tests were carried out for each sample according to the identification flow chart for lactose positive Enterobacteriaceae of Bergey's manual for systematic bacteriology (Kreig et al., 2011).

Indole test: Isolated pink colonies from sub-cultured agar plates of each lettuce sample were cultured in Tryptophan broth

for 24 hours at 37°C. At the end of the incubation, 1-2mL of Kovac's indole reagent was added drop wise to each test tube and the colour changes were noted.

Citrate test: Initially Simmons citrate agar was produced for all the indole positive samples. Afterwards, colonies of each sub-cultured lettuce samples were stab cultured on citrate agar slants. The agar slants were then incubated at 37°C for 24 hours and the colour changes in each test tube were noted.

Voges-Proskauer test: All samples were cultured in MR-VP broth. Into each MR-VP broth cultured samples, 0.6mL of 5% α-naphthol and 0.2mL of 40% KOH was added. Tubes were gently shaken to react with atmospheric oxygen and were left undisturbed for 10-15 minutes. The colour changes were then noted for each sample.

Methyl red test: For the MR-VP broth cultured indole negative sample, Five drops of methyl red reagent was added and the colour changes were noted.

TSI test: TSI agar was prepared for the samples which are either indole negative or citrate positive. Next, sub-cultured colonies of each sample were stab cultured on TSI agar. The test tubes were then covered with aluminium foil and were incubated for 24 hours at 37°C. At the end of 24 hours, the colour changes were noted for each sample.

Motility test: Motility semi-solid agar was prepared for the indole negative, VP negative, MR positive, TSI negative sample. Precise colonies from the sub-cultured samples were stab cultured on motility semi-solid agar. The test tubes were then incubated for 24 hours at 37°C. At the end of 24 hours, the colour change was noted (Vasanthakumari, 2009).

Molecular analysis

E. coli positive samples were cultured in MacConkey broth in order to carry out DNA extraction for these samples.

DNA extraction: DNA extraction was performed for the E. coli positive samples in order to further confirm the E. coli contamination. DNA extraction was carried out using kit extraction method according to the manufacturer's instructions (Promega, 2017). The extracted DNA were then stored at 2°C until use.

Polymerase chain reaction: A hypervariable region of 16S rRNA gene in E. coli was amplified using 51-GTT GTA AGG CAC TTT GAG TGG TGA GGA-31 forward primer and 51¬¬¬¬¬-GCC TCA AGG GCA CAA CTT CCA AG-31 reverse primer. 6X PCR mixture was prepared by adding 82.5 µL PCR water, 9 µL MgCl_2, 30 µL 5X green go Taq buffer, 3 µL dNTP and 1.5 µL Taq polymerase. Prior to addition of Taq polymerase, all the reagents were tapped and spun. PCR program was carried out in 26µL reaction volumes containing 3µL of extracted DNA, 1 µL forward primer, 1 µL reverse primer and 21µL of the PCR mix (1X) (Sabat et al., 2000). The PCR cycle was programmed with initial DNA denaturation at 94°C for 2 minutes followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 45 seconds, extension at 72°C for 1 minute and 30 seconds and this was followed by final extension at 72°C for 10 minutes.

Agarose gel electrophoresis: 5 µL of PCR product of each sample was analysed by agarose gel electrophoresis in 1.5% agarose gel. The gel was allowed to run at 75V, 250mA for 1 hour. Later, the gel was visualized using the UV illuminator. Visualization of 544bp was utilized to confirm E. coli (Sabat et al., 2000).

RESULTS

Microbiological analysis results

Lactose fermenting bacteria such as E. coli forms pink colonies when cultured in MacConkey media due to the production

of acid along with lactose fermentation. Therefore the pH indicator in the media turns into pink colour, making lactose fermenting bacteria easily distinguishable. Meanwhile non-lactose fermenting bacteria form yellow or colourless colonies. Hence through the isolation of pink colonies, 100% positive results for the presence of lactose fermenting bacteria were obtained for all 21 samples. Meanwhile, gram staining carried out for pink colonies isolated by MacConkey agar of all 21 samples, determined that all the samples (100%) contained gram negative rod shaped, lactose fermenting bacteria.

Biochemical analysis results

Indole test results: Indole test determines the ability of a microorganism to hydrolyse tryptophan into indole, pyuruvate and ammonium. Where, the addition of Kovac's reagent, reacts with indole to produce a cherry red colour compound (Acharya, 2012). Out of the 21 samples, 20 samples were identified as indole positive.

Citrate test results: Citrate test determines the ability of a bacterium to use citrate as a source of energy. Bacteria that use carbon as the source of energy possess citrate-permease enzyme. This converts citrate to pyruvate along with ammonium salt hydrolyzation into ammonia, which makes the medium alkaline. Hence the bromothymol blue pH indicator in the media turns from green to blue, indicating the presence of autotrophic organisms (Citrobacter diversus, Erwinia chrysanthemi, Klebsiella oxytoca). Likewise, in the presence of heterotrophic organisms such as E. coli, the media remains in green colour.

According to the Bergey's manual, citrate test was done for indole positive samples. In which, out of the 20 indole positive samples, 17 samples were distinguished as citrate positive while 3 samples were citrate negative. Hence, three samples that were positive for lactose fermentation, gram negative rod shaped bacteria which is indole positive and citrate negative, were confirmed as E. coli.

Voges-Proskauer test results: Voges-Proskauer (VP) test determines the ability of an organism to produce acetylemethyl carbinol via glucose fermentation. Acetylemethyl is then oxidized to diacetyl in the presence of α-naphthol and atmospheric oxygen in alkaline medium. Diacetyl along with guanidine containing compounds in the VP broth thereby condenses to form a pinkish red polymer. Out of the 18 samples which were either indole negative or citrate positive, 8 samples were indicated to be VP positive, while 10 samples were VP negative. Out of the 17 citrate positive samples, 9 samples were VP negative. Hence these samples were confirmed as Citrobacter diversus.

Methyl red test results: Methyl red (MR) test detects the ability of a microorganism to perform mixed acid fermentation from glucose. These large quantities of acid, causes the pH of the medium to be low (below 4.4) which is detected by the methyl red indicator by turning into red. VP test detects only the 2,3 butanediol pathway of glucose fermentation. Hence, MR test along with the VP test results provide an accurate confirmation of the glucose fermentation pathway the organism performs (Karki, 2018). Methyl red test was performed for the indole and VP negative sample. This sample was detected to be MR positive. As this result is common for three types of bacteria according to Bergey's manual, TSI test results were taken into account to confirm the type of bacteria.

Triple sugar ion (TSI) test results: Triple sugar iron agar is a differential media that is composed of lactose, sucrose and glucose in a ratio of 10:10:1 along with ferrous sulphate and phenol red pH indicator. Hence it detects the ability of an organism to reduce sulphur and ferment carbohydrates. If an organism could

reduce sulphur, it forms hydrogen sulphide gas which reacts with ferrous to form ferrous sulphide. Thus appears as a black precipitate. Out of the citrate and VP positive samples, 6 samples indicated to be TSI negative and 2 samples TSI positive. Therefore the 6 TSI negative samples were confirmed as Klebsiella oxytoca, while the 2 TSI positive samples were regarded as Erwinia chrysanthemi. Meanwhile, the indole and VP negative sample was detected to be TSI negative, hence the results of motility test was regarded to confirm the type of organism.

Motility test results: Motility test media allows motile bacteria to readily migrate through the media which would result in diffused cloudiness of the media. Whereas, the non-motile bacteria would distinctly grow only in the stab region of the inoculated area. Thereby makes motile bacteria easily distinguishable from non-motile bacteria (Acharya, 2015).

Motility test performed for the indole, VP, TSI negative and MR positive sample. This was indicated to be motility negative. Hence it was confirmed as Klebsiella pneumonia subsp. Ozaenae.

Summary of the results obtained from microbiological and biochemical analysis

According to the results obtained from biochemical results collectively for each sample, the organism of each sample was determined as follows.

E. coli was confirmed in 3 out of the 21 samples (14.3%). This was confirmed by lactose fermentation positivity by culturing in MacConkey agar, followed by gram negative staining for rod shaped bacteria, along with biochemical results of indole positive and citrate negative (Figure 1). Furthermore, due to the comparative higher sensitivity of molecular techniques, molecular results were too taken into consideration for the confirmation of *E. coli*. These three samples were thereby confirmed for faecal contamination due to the presence of faecal indicator.

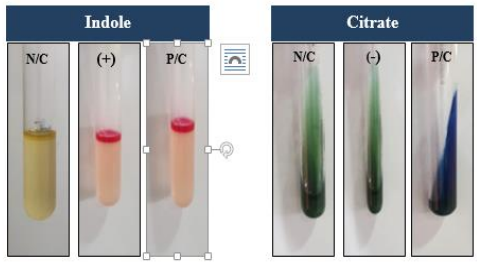

Figure 1- E. coli confirmatory biochemical test results

Citrobacter diversus was confirmed for 9 out of the 21 samples (42.9%). It was confirmed with the aid of lactose fermentation positive nature indicated by MaConkey agar, gram negative staining for rod shaped bacteria followed by positive for indole and citrate and negative for VP test (Figure 2).

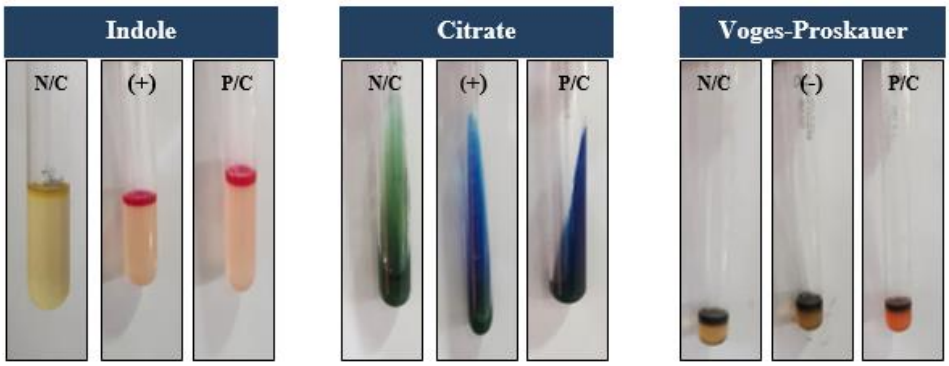

Figure 2- Citrobacter diversus confirmatory biochemical test results

Klebsiella oxytoca was confirmed for 6 out of the 21 samples (28.6%). Samples that were lactose fermentation positive, gram negative rod shaped bacteria, positive for indole, citrate and VP and negative for TSI, were confirmed as positive for Klebsiella oxytoca (Figure 3).

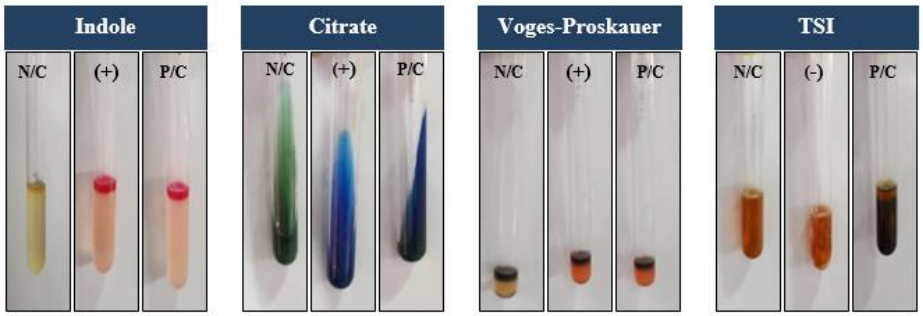

Figure 3- Klebsiella oxytoca confirmatory biochemical test results

Erwinia chrysanthemi was confirmed for 2 out of 21 samples (9.5%). It was confirmed based on lactose fermentation positive, gram negative rod shaped bacteria, positive for all indole, citrate, VP and TSI tests (Figure 4).

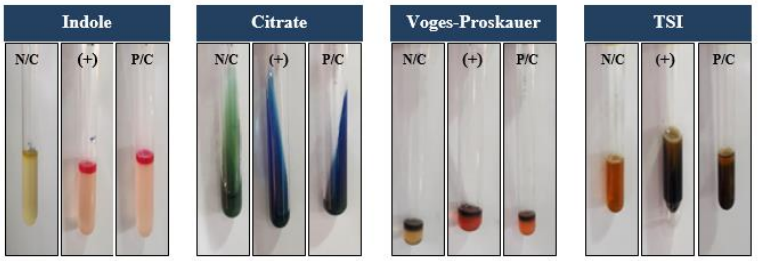

Figure 4- Erwinia chrysanthemi confirmatory biochemical test results

Klebsiella pneumonia subsp. *ozaenae* was confirmed for 1 sample out of the 21 samples (4.8%). It was confirmed according to the positive results obtained for lactose fermentation, gram negative rod shaped bacteria along with negative results for indole, positive for methyl red, negative for TSI and motility tests (Figure 5).

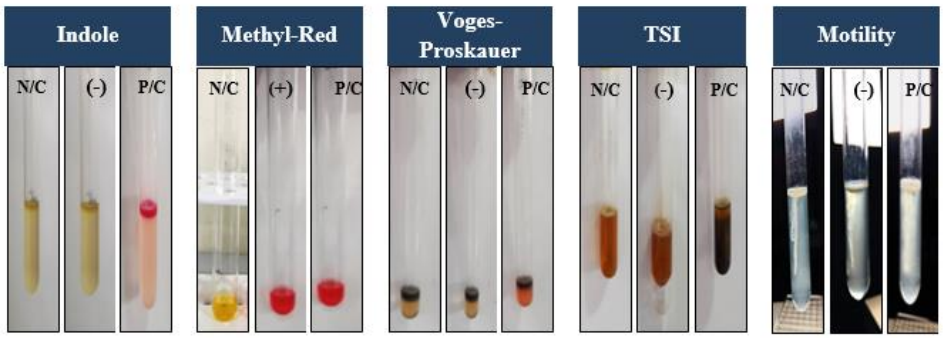

Figure 5- Klebsiella pneumonia subsp. ozaenae confirmatory biochemical test results

Summary of the results obtained for all 21 samples were as follows (Table 2 and Figure 6).

Sample code	MacConkey media results (presence of pink colonies)	Indole test results	Citrate test results	VP test results	Methyl red test results	TSI test results	Motility test results	Confirmed organism
L01	Positive	Negative	-	Negative	Positive	Negative	Negative	*Klebsiella ozaenae*
L02	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L03	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L04	Positive	Positive	Negative	-	-	-	-	*E. coli*
L05	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L06	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L07	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L08	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L09	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L10	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L11	Positive	Positive	Negative	-	-	-	-	*E. coli*
L12	Positive	Positive	Positive	Positive	-	Positive	-	*Erwinia chrysanthemi*
L13	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L14	Positive	Positive	Positive	Positive	-	Positive	-	*Erwinia chrysanthemi*

L15	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L16	Positive	Positive	Negative	-	-	-	-	*E. coli*
L17	Positive	Positive	Positive	Positive	-	Negative	-	*Klebsiella oxytoca*
L18	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L19	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L20	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*
L21	Positive	Positive	Positive	Negative	-	-	-	*Citrobacter diversus*

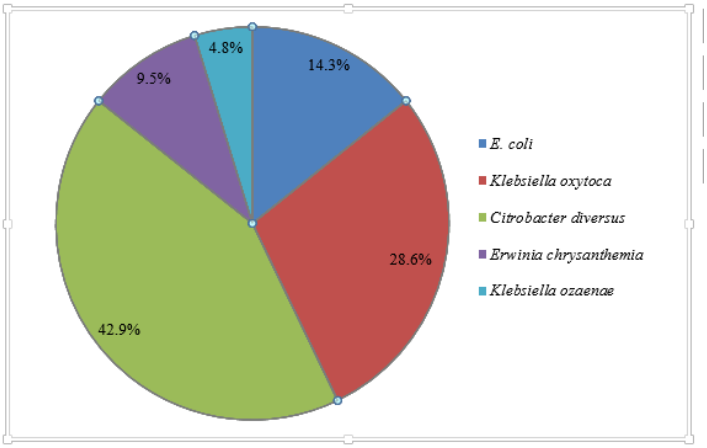

Figure 6- Microbiological and biochemical analysis results of the 21 samples

Molecular test results

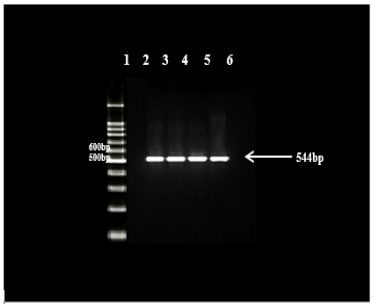

Figure 7- Agarose gel image for the LO4, L11 and L16 samples. Lane 1= 100bp ladder, Lane 2- negative control, Lane 3- positive control, Lane 4- PCR products of LO4, Lane 5- PCR products of L11, Lane 6- PCR products of L16.

The 3 samples (L04, L11 and L16) which were detected to be positive for E. coli via microbiological and biochemical analysis, were further confirmed by molecular testing. DNA extracted from these samples were subjected for PCR in order to amplify a hyper variable region of the 16S rRNA gene of E. coli. Hence when agarose gel electrophoresis was performed

for these PCR products, all three samples indicated 544bp DNA fragments when visualized under UV illuminator (Figure 7). Thereby further confirming for the presence of E. coli in these three samples (Sabat et al., 2000).

Data analysis of faecal contaminated samples according to the location and type of store.

According to the locations of the three lettuce samples that were confirmed for E. coli contamination, it could be confirmed that, out of the 4 provinces analysed, 3 provinces of Sri Lanka acquired the highest faecal contamination of lettuce leaves. These provinces would be Southern, North-Western and Central provinces (Figure 8). Whereas lettuce leaves obtained from Western province was not observed to be E. coli contaminated.

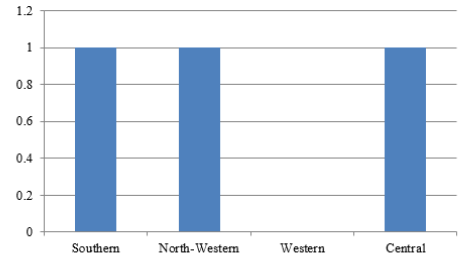

Figure 8- Data analysis for the E. coli positive samples based on their locations

Furthermore according to the type of store these samples were obtained from, confirms that lettuce leaves obtained from supermarkets are more prone to be contaminated with E. coli (23.1%) than the lettuce leaves obtained from open air markets.

DISCUSSION

In the present study, it was intended to determine the presence of *E. coli* in the lettuce leaves obtained from Western, Southern, Central and Northern-Western provinces of Sri Lanka. Following the microbiological, biochemical analysis, and further confirmation through the molecular techniques, 14.3% (3 samples out of the 21 samples) were positive for *E. coli*. As these samples were obtained from North-Western, Central and Southern provinces, it can be confirmed that the lettuce leaves obtained from these areas are more prone for faecal contamination. In addition, it was also identified that lettuce samples of supermarkets were more contaminated with *E. coli* (23.1%) in comparison to open air markets.

However, alongside microbiological analysis of the lettuce samples indicated 4.8% (1 sample) positivity for *Klebsiella pneumonia* subsp. *ozaenae*, 9.5% (2 samples) for *Erwinia chrysanthemi*, 28.6% (6 samples) for *Klebsiella oxytoca* and 42.9% (9 samples) for *Citrobacter diversus* following the biochemical tests. Hence due to the detection of all these pathogens, it was confirmed that all 21 lettuce samples are not suitable for consumption in its' raw state. These indicated coliform bacteria are rod shaped gram negative, non-spore forming bacteria that are capable of fermenting lactose with the production of gas and acid. They have been extensively used as indicators of food and water sanitary quality. Even though coliforms themselves are not considered to cause serious illness, their presence has been utilized to indicate the presence of other pathogenic organisms of faecal origin. However it should be given significance that only *E. coli* serves as the main indicator of faecal contamination, due to their long survival periods in faeces.

Similar to the present study, several studies have been carried out globally with regards to microbiological analysis of lettuce leaves. A study conducted in Spain, detected *E. coli* in 22.2% of samples which was collected from 9 different lettuce fields (Oliveira *et al*, 2010). While in Saudi Arabia, 5 out of the 45 lettuce

samples tested determined the presence of *E. coli* (Hamad, Al-Amer and Al-Otaibi, 2013). Similarly in USA, 16% of the ready to eat bagged lettuce samples were identified to be positive for faecal contamination due to the presence of *E. coli* (Valentine-Bon *et al*., 2008). Furthermore, similar to the present study, a study was conducted in Philippines where, the detection of *E. coli* in lettuce leaves from supermarkets and open air markets were compared. In which, 1.20-3.92 $\log_{10}$ CFU/g of *E. coli* was detected in lettuce leaves obtained from open air markets and 3.09-3.15 $\log_{10}$ CFU/g of *E. coli* in lettuce leaves obtained from supermarkets. Even though, the detected amount of *E. coli* were higher in supermarkets compared to open air markets, according to the statistical analysis the difference of microbial counts were considered to be not significant (Vital *et al*., 2014). A similar study conducted in Philippines itself, determined the presence of antimicrobial resistant *E. coli* in similar amounts in both supermarket and open air market lettuce leaves (Vital, Caballes and Rivera, 2017). However, a study conducted in Italy, involved comparison of *E. coli* detection in ready to eat lettuce leaves and fresh produce. This was able to indicate that, *E. coli* count was higher in ready to eat lettuce leaves obtained from supermarket chains in comparison to fresh produce. Hence indicating similar results compared to the present study. Thereby it provides evidence that industrial processing and storage of lettuce leaves in vegetable coolers in supermarkets, results in an additional source for *E. coli* contamination (Bencardino, Vitali and Petrelli, 2018).

Furthermore, a study conducted in Spain in fresh vegetables and leaves indicated the highest incidence of *E. coli* (10%) in lettuce leaves compared to other vegetables. Meanwhile, *Klebsiella ozaenae* (5%), *Klebsiella oxytoca* (5%) were also isolated from the lettuce leaves (Soriano *et al*., 2001). Whereas in Malaysia, 200 samples tested were identified to be harbouring *Klebsiella pneumonia* in all the samples (Puspanandan *et al*., 2012).

In the present study, apart from *Klebsiella* and *E. coli*, 9.5% of *Erwinia chrysanthemi* too were indicated. *Erwinia chrysanthemi* is considered to be a plant pathogen which is responsible for soft-rot disease in plant species (Lee, Chen and Hsu, 2006). A study that was conducted in USA, showed that during postharvest, lettuce leaves affected by soft rot disease due to *Erwinia chrysanthemi* infection, enhanced the colonization by *E. coli* O157:H7 by 27 times. Comparatively to healthy middle aged lettuce leaves on postharvest (Brandl, 2008). In addition another study conducted in USA, confirmed that the causative factors for this enhanced growth of *E. coli* O157:H7, are the virulence factors of *Erwinia chrysanthemi* (Yamazaki *et al*., 2011). Hence, confirming that even though *Erwinia chrysanthemi* is a plant pathogen, it also promotes the growth of human foodborne pathogens such as *E. coli* O157:H7.

Furthermore, it was noted that 42.9% of the lettuce samples were indicative of *Citrobacter diversus* during this study. However, *Citrobacter diversus* has not been noted as a prominent bacterium in previous studies of microbiological analyses carried out in lettuce leaves. Nevertheless, a study conducted in Netherlands, was able to indicate the presence of *Citrobacter diversus* in one sample out of thee 75 samples analysed (Blaak *et al*., 2014). *Citrobacter diversus* is considered as an important neonatal pathogen responsible for causing meningitis (Baylis *et al*., 2011). Thus its' presence in lettuce leaves indicates the unsuitability for consumption.

When comparing these previous studies conducted in lettuce leaves with the present study, it is notable that the

diversity of microbial community in lettuce leaves differs from study to study. The main causative factor of this is the environmental factors that promote the bacterial phyllosphere colonization. Pyllosphere is known as the aerial parts of the plant and it has been considered to promote the growth of diverse microbial communities. This bacterial phyllosphere has been known to be affected by a range of environmental factors such as rainfall, wind, temperature and solar radiation. These factors thereby have shown to play a predominant role in determining the patterns of bacterial colonization in the phyllosphere. In addition to this, plant morphology such as the position, height of the leaves and age of the leaves have also been shown to affect the diversity of bacterial colonization (Hunter *et al*., 2010).

Considering that all 21 lettuce leaf samples obtained for this study, were confirmed for its unsuitability for consumption in its' raw state, it is important to consider treatments that would reduce the colonization of these coliforms in lettuce leaves. A study conducted in Saudi Arabia involving lettuce leaves demonstrated, washing with tap water and vinegar (5% acetic acid) significantly reduced the level of microbial contamination to a level that is suitable for human consumption (Hamad, Al-Amer and Al-Otaibi, 2013). Meanwhile a study was conducted in Ethiopia to identify an effective treatment method to drastically reduce faecal coliform content in lettuce leaves. This was able to indicate, rinsing with portable tap water for 2 minutes followed by dipping in vinegar solution of 15 000 ppm for a minute was responsible for the highest faecal coliform reduction, in comparison to other methods (Woldetsadik *et al*., 2017).

CONCLUSION

It was observed that all lettuce leaves tested were positive for the presence of coliforms. Where highest incidence accounts for Citrobacter diversus with 42.9% of leaves positive. Even though, presence of E. coli was not prominent as much as Citrobacter diversus, 14.3% of leaves were positive for E. coli confirming their faecal contamination. Out of the samples contaminated with E. coli, highest contamination was observed in supermarkets than the open air markets with most accounting for North-Western, Southern and Central provinces of Sri Lanka. Hence in conclusion, considering the confirmation of coliform presence in all the lettuce leaves tested, it is important to consider washing treatments prior to the consumption as lettuce leaves are mostly being consumed raw.

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REFERENCE LIST

Acharya, T. (2012) Indole test: principle procedure and results, at https://microbeonline.com/indole-test-

principle-procedure-results/, Microbe Online, visited 22 August 2018.

Acharya, T. (2015) Tests for bacterial motility: procedure and results, at https://microbeonline.com/tests-bacterial-motility-procedure-results/, Microbe Online, visited 22 August 2018.

Acharya, T. (2016) Streak plate method principle purpose procedure and results, at https://microbeonline.com/streak-plate-method-principle-purpose-procedure-results/, visited 22 August 2018.

Anderson, M., Jaykus, L., Beaulieu, S. & Dennis, S. (2011) Pathogen-produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation, Food Control, Vol. 22, pp. 1865-1872.

Anon (2017) Wizard genomic DNA purification kit, at https://worldwide.promega.com/-/media/files/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol.pdf?la=en, Promega, visited 22 August 2018.

Aryal, S. (2018) Citrate utilization test principle media procedure and result, at https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result, Microbioogy Info, visited 22 August 2018.

Aryal, S. (2018) MacConkey agar composition principle uses preparation and colony morphology, at https://microbiologyinfo.com/macconkey-agar-composition-principle-uses-preparation-and-colony-morphology/, Microbioogy Info, visited 22 August 2018.

Aryal, S. (2018) Voges-Proskauer test principle reagents procedure and result, at https://microbiologyinfo.com/voges-proskauer-vp-test-principle-reagents-procedure-and-result/, Microbioogy Info, visited 22 August 2018.

Baylis, C., Uyttendaele, M., Joosten, H. & Davies, A. (2011) The Enterobacteriaceae and their significance to the food industry, International life sciences institute.

Bencardino, D., Vitali, L. A. & Petrelli, D. (2018) Microbiological evaluation of ready-to-eat iceberg lettuce during shelf-life and effectiveness of household washing methods, Italian Journal of Food Safety, Vol. 7, pp. 50-54.

Berry, E. D., Wells, J. E., Bono, J. L., Woodbury, B. L., Kalchayanand, N., Norman, K. N., Suslow, T. V., Lopez-Velasco, G. & Millner, P. D. (2015) Effect of proximity to a cattle feedlot on Escherichia coli 0157:H7 contamination of leafy greens and evaluation of the potential for airborne transmission, Applied and Environmental Microbiology, Vol. 81, pp. 1101-1110.

Blaak, H., Hoek, A. H. A. M. V., Veenman, C., Leeuwen, A. E. D. V., Lynch, G., Overbeek, W. M. V. & Husman, A. M. D. R. (2014) Extended spectrum β-lactamase and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment, International Journal of Food Microbiology, Vol. 2014, pp. 168-169.

Brandl, M. T. (2008) Plant lesions promote the rapid multiplication of Escherichia coli 0157:H7 on postharvest lettuce, Applied and Environmental Microbiology, Vol. 74, pp. 5285-5289.

Cerna-Cortes, J. F., Leon-Montes, N., Cortes-Cueto, A. L., Salas-Rangel, L. P., Helguera-Repetto, A. C., Lopez-Hernandez, D., Rivera-Gutierrez, S., Fernandez-Rendon, E. & Gonzalez-y-Merchand, J. A. (2015) Microbiological quality of ready-to-eat vegetables collected in Mexico city: occurrence of aerobic-mesophilic bacteria, fecal coliforms, and potentially pathogenic nontuberculous Mycobacteria, Biomed Research International, Vol. 2015, pp. 1-9.

Clark, M. (2018) Foodborne illnesses, at https://foodborneillness.com/ecoli_food_poisoning/, Marler Clark, visited 22 August 2018.

Hamad, S. H., Al-Amer, J. J. & Al-Otaibi, M. M. (2013) Assessment of the microbiological quality and wash treatments of lettuce produced in Hofuf city, Saudi Arabia, Asian Journal of Plant Pathology, Vol.7, pp. 84-91.

Hunter, P. J., Hand, P., Pink, D., Whipps, J. M. & Bending, G. D. (2010) Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (Lactuca species) phyllosphere, Applied and Environmental Microbiology, Vol. 76, pp. 8117-8125.

Jensen, A. N., Storm, C., Forslund, A., Baggesen, D. L. & Dalsgaard, A. (2013) Escherichia coli contamination of lettuce grown in soils amended with animal slurry,

Journal of Food Protection, Vol. 76, pp. 1137-1144.

Karki, G. (2018) Voges-Proskauer test objective principle procedure and result, at http://www.biologypractical.com/voges-proskauer-test-vp-test-objective-principle-procedure-and-result/, Biology Practical, visited 22 August 2018.

Kim, J. K. & Harrison, M. A. (2008) Transfer of Escherichia coli 0157:H7 to Romaine lettuce due to contact water from melting ice, Journal of Food Protection, Vol. 71, pp. 252-256.

Krieg, N. R., Ludwig, W., Whitman, W. B., Hedlund, B. P., Paster, B. J., Staley, J. T., Ward, N. & Brown, D. (2011) Bergey's manual of systematic bacteriology (2nd Edition) Springer.

Lee, Y. A., Chen, K. P. & Hsu, Y. W. (2006) Characterization of Erwinia chrysanthemi, the soft-rot pathogen of white-flowered calla lily, based on pathogenicity and PCR-RFLP and PFGE analyses, Plant Pathology, Vol. 55, pp. 530-536.

*MacConkey, A. (1905) Lactose-fermenting bacteria in faeces, Epidemiology and Infection, **5**, 333-379.*

Nagoba, B.S. & Pichare A. (2007) Medical microbiology (2nd Edition) Elsevier.

*Niguma, N. H., Pelayo, J. S. & Oliveira, T. C. R. M. D. (2017) Microbiological evaluation of lettuce produced by conventional and organic systems in farms of Londrina, PR, Semina Ciencias Agrarias**,** Vol. 38, pp. 175-184.*

Oliveira, M., Usall, J., Vinas, I., Anguera, M., Gatius, F. & Abadias, M. (2010) Microbiological quality of fresh lettuce from organic and conventional production, Food Microbiology, Vol. 27, pp. 679-684.

Prieto, M., Colin, P., Fernandez-Escamez, P. & Alvarez-Ordonez, A. (2015) Epidemiology, detection and control of foodborne microbial pathogens, Biomed Research International, Vol. 2015, pp. 1-2.

Puspanandan, S., Afsah-Hejri, L., Loo, Y. Y., Nillian, E., Kuan, C. H., Goh, S. G., Chang, W. S., Lye, Y. L., John, Y. H. T., Rukayadi, Y., Yoshitsugu, N., Nishibuchi, M. & Son, R. (2012) Detection of Klebsiella pneumonia in raw vegetables using most probable number-polymerase chain reaction (MPN-PCR), International Food Research Journal, Vol. 19, pp. 1757-1762.

Russell, J. B. & Jarvis, G. N. (2001) Practical mechanisms for interrupting the oral-fecal lifecycle of Escherichia coli, Journal of Molecular Biology and Biotechnology, Vol. 3, pp. 265-272.

Sabat, G., Rose, P., Hickey, W. J. & Harkin, J. M. (2000) Selective and sensitive method for PCR amplification of Escherichia coli 16S rRNA genes in soil, Applied and Environmental Microbiology, Vol. 66, pp. 844-849.

Solomon, E. B., Pang, H. & Matthews, K. R. (2003) Persistence of Escherichia coli 0157:H7 on lettuce plants following spray irrigation with contaminated water, Journal of Food Protection, Vol. 66, pp. 2198-2202.

Soriano, J. M., Rico, H., Molto, J. C. & Manes, J. (2001) Incidence of microbial flora in lettuce, meat and Spanish potato omelette from restaurants, Food Microbiology, Vol. 18, pp. 159-163.

Suardana, I. W. (2014) Analysis of nucleotide sequences of the 16S rRNA gene of novel Escherichia coli strains isolated from feces of human and Bali cattle, Journal of Nucleic Acids, Vol. 2014, pp. 1-7.

Taban, B. M. & Halkman, A. K. (2011) Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens?, Anaerobe, Vol. 17, pp. 286-287.

Tenaillon, O., Skurnik, D., Picard, B. & Denamur, E. (2010) The population genetics of commensal Escherichia coli, Nature Reviews Microbiology, Vol. 8, pp. 207-217.

Valentin-Bon, I., Jacobson, A., Monday, S. R. & Feng, P. C. H. (2008) Microbiological quality of bagged cut spinach and lettuce mixes, Applied and Environmental Microbiology, Vol. 74, pp. 1240-1242.

Vasanthakumari, R. (2009) Practical microbiology BI publications Pvt Ltd.

Vital, P. G., Caballes, M. B. D. & Rivera, W. L. (2017) Antimicrobial resistance in Escherichia coli and Salmonella spp. Isolates from fresh produce and the impact to food safety, Journal of Environmental Science and Health, Vol. 52, pp. 683-689.

Vital, P. G., Dimasuay, K. G. B., Widmer, K. W. & Rivera, W. L. (2014) Microbiological quality of fresh produce from open air markets and supermarkets in the Philippines, The Scientific World Journal, Vol. 2014, pp. 1-7.

Woldetsadik, D., Drechsel, P., Keraita, B., Itanna, F., Erko, B. & Gebrekidan, H. (2017)

Microbiological quality of lettuce (Lactuca sativa) irrigated with wastewater in Addis Ababa, Ethiopia and effect of green salads washing methods, International Journal of Food Contamination, Vol. 4, pp. 1-9.

Woo, P. C. Y., Leung, P. K. L., Leung, K. W. & Yuen, K. Y. (2000) Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient, Molecular Pathology, Vol. 53, pp. 211-215.

Yamazaki, A., Li, J., Hutchins, W. C., Wang, L., Ma, J., Ibekwe, A. M. & Yang, C. (2011) Commensal effect of pectate lysases secreted from Dickeya dadantii on proliferation of Escherichia coli 0157:H7 EDL933 on lettuce leaves, Applied and Environmental Microbiology, Vol. 77, pp. 156-162.

Yaratha, G., Perloff, S. & Changala, K. (2017) Lactose vs non-lactose fermenting E. coli: epidemiology, clinical outcomes and resistance, Open Forum Infectious Diseases, Vol. 4, pp. 589-590.