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Determination of Enterobacteriaceae (*Escherichia coli, Salmonella*) and *Vibrio cholerae* in *Centella asiatica* (Gotukola) samples

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Abstract

Healthy diets should include leafy green veggies. The usage of leafy greens, such as *Centella asiatica* (Gotukola), has increased in recent years as a result of suggestions for a healthier life. Nevertheless, as it is typically eaten raw or barely cooked, gotukola is prone to spreading foodborne illnesses. Controlling bacterial contamination during the pre-harvest and post-harvest stages is needed to avoid foodborne disease outbreaks. The purpose of this study was to detect the presence of *Escherichia coli*, *Salmonella*, and *Vibrio cholerae* in Sri Lankan gotukola leaves. Ten fresh gotukola samples were collected for this study from supermarkets and open air markets in Western, Southern, Central and North - Western provinces of Sri Lanka. All samples were evaluated using MacConkey agar culture, TCBS agar culture and biochemical assays for further bacterial confirmation of *Escherichia coli*, *Salmonella*, and *Vibrio cholerae*.

Keywords: *Escherichia coli*, *Centella asiatica*, Gotukola, antimicrobial activity

1. Introduction

The recent worldwide trend of switching from synthetic to herbal medication is known as "return to nature." As a result, both developing and developed countries have boosted their demand for plant-based medications. Non-toxicity, great effectiveness, and affordability are few reasons for this current shift to herbal treatments.[1] *Centella asiatica* is a medicinal plant that has therapeutic properties. It is a flowering plant in the Apiaceae family that grows as a perennial herbaceous plant.[2] *Centella asiatica*, often known as gotukola in Sri Lanka, can be found in household gardens. India, Sri Lanka, Thailand, China, Indonesia, and Madagascar, as well as other tropical and subtropical Asian countries, are home to this species.[3] It is generally used as a culinary vegetable as well as a medicinal herb and can be used in a variety of ways. Essential nutrients, carbohydrates, proteins, vitamins, and minerals, as well as potent antioxidants and plant-based bio chemicals, are abundant in *Centella asiatica*.[4] Gotukola is a blood purifier that helps the circulatory system's veins and capillaries stay healthy. It's also widely used to aid children with hyperactivity and memory problems.[5] Additionally, Ayurvedic treatment makes use of gotukola juice, powder, herbal oil, tea and tablets.[6]

Foodborne pathogens are bacteria that can infect humans due to the consumption of contaminated foods.[7] This is seen as a major public health threat that affects both developing and industrialized countries. The onset of foodborne pathogen-associated illnesses is influenced by host sensitivity, pathogen evolution and adaptation, changes in lifestyle, and pre and post-harvest stages of food production and manufacturing process.[8] Fresh vegetable consumption is becoming increasingly linked to human foodborne illnesses.[9] Because these are taken uncooked, there is a chance that germs could be present, posing a health risk.[10] During harvesting, handling, processing, and packing, fresh vegetables are at risk of contamination from chemical fertilizers, polluted water, unsanitary conditions and other sources.[11]

The purpose of this study was to use biochemical and microbiological techniques to determine the presence of *Escherichia coli*, *Salmonella* and *Vibrio cholerae* in Sri Lankan gotukola samples. Isolation and identification of bacterial colonies using MacConkey and TCBS agar medium, followed by a series of

biochemical assays depending on the bacteria's ability for lactose and non-lactose fermentation are the objectives of this study. In addition, the microbiological quality of the *Centella asiatica* samples were compared with different locations based on their geographical and storage conditions. *Enterobacteriaceae* is a vast bacterial family that includes numerous well-known members such as *E. coli*, *Salmonella*, *Shigella*, *Citrobacter* and *Klebsiella*.[12] They are Gram-negative, rod-shaped, facultatively anaerobic, motile bacteria that are commonly utilized for research due to their ease of handling and adaptability. They can be classified based on their ability to ferment lactose.[13]

Vibrio cholerae is a Gram-negative, facultative anaerobe bacterium with a comma-shaped morphology.[14] A flagellum is located at one of the bacterium's poles, and pili can be found all over its cell surface.[15] MacConkey agar culture media is used to isolate and distinguish gram negative lactose fermenting and lactose non-fermenting bacteria, specifically those from the *Enterobacteriaceae* family.[16] The MacConkey agar contains peptones, which are necessary nutrients for microbial growth.[5] Bile salts and crystal red hinder the growth of gram-positive bacteria, resulting in bacterial selection.[17] Neutral red acts as a pH indicator, turning pink when the pH falls below 6.8.[18] To isolate *Enterobacteriaceae* colonies, samples are grown on MacConkey agar media in the current study. To isolate and cultivate *Vibrio cholerae* and other *Vibrio* species from samples, the selective differential medium TCBS Agar is utilized.[19] Depending on whether *Vibrio* spp. can ferment sucrose, they either create yellow or green colonies on TCBS agar culture.[20] When sucrose is fermented, *Vibrio cholerae* produces yellow colonies, but *Vibrio parahaemolyticus* produces green colonies in TCBS agar medium.[21]

Biochemical assays are also carried out to establish the presence of *Enterobacteriaceae* in the cultured sample. Biochemical analysis of each sample is carried out in accordance with Bergey's manual. Because the study's main focus was on *E. coli*, *Salmonella*, and *Vibrio cholera* the lactose fermentation indole positive pathway and lactose non-fermentation indole negative pathway are also investigated. The capability of an organism to use citrate as its main source of energy is measured using the citrate test.[22] The capability of an organism to synthesize and maintain acid end products from glucose fermentation is assessed by the methyl red test.[23] Hydrogen sulfide test is mostly used to detect members of the *Enterobacteriaceae* family. This test aids in the recognition and classification of *Enterobacteriaceae* members.[24] The indole test determines whether or not an organism can digest tryptophan and create indole. The motility test is used to evaluate whether or not an organism is motile. The urease test identifies the formation of microorganisms that can hydrolyze urea to create ammonia and carbon dioxide.[25] The VP test is used to detect acetone in bacterial broth cultures. Alpha-naphthol and potassium hydroxide are added to bacteria-inoculated VP broth to perform this test.[26]

2. Methodology

2.1. Sample Collection. The *Centella asiatica* plant's fresh, undamaged, infection free leaves were collected from ten different geographical locations in Sri Lanka (Table 1). All samples were collected in ziplock bags and delivered to the lab within 24 hours, kept at room temperature before being analysed.

2.2 Sample Preparation. Collected samples were gently grinded using mortar and pestle and transferred to beakers. Peptone water was prepared, and was added to small beakers and mixed well (5 g of each gotukola sample was mixed with 20 ml of peptone buffer). Samples were filtered into labeled sterile plastic containers and kept in the incubator for 24 hours at 37°C and finally, refrigerated.

Table 1: Sample collection data sheet

Sample code	Date	Province	Location	Type of store
A_1	18/02/2022	Western	Aluthgama	Open air market
A_2	23/02/2022	Central	Dambulla	Open air market
B_1	19/02/2022	North-Western	Ganewatta	Supermarket
B_2	23/02/2022	Western	Kalutara	Open air market
C_1	18/02/2022	North-Western	Galgamuwa	Open air market
C_2	23/04/2022	North-Western	Kurunagala	Supermarket
D_1	18/04/2022	Western	Beruwala	Open air market
D_2	23/04/2022	Western	Panadura	Open air market
E_1	19/04/2022	Western	Matugama	Open air market
E_2	22/04/2022	Southern	Ambalangoda	Supermarket

2.3 MacConkey Agar Culturing. MacConkey agar was prepared. Inside the biosafety cabinet, MacConkey agar was poured into petri plates and kept until the culture medium solidified. Under aseptic conditions, a loop of prepared sample was streaked on MacConkey agar using the quadrant streaking technique. Incubated for 48 hours at 37°C and refrigerated[27].

2.4 TCBS agar culturing. Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) agar was prepared. Inside the biosafety cabinet, TCBS agar was poured into petri plates and kept until the culture medium is solidified. Using the quadrant streaking technique, a loop of each prepared sample was streaked on TCBS agar under aseptic conditions. Incubated for 48 hours in 37°C and refrigerated[28].

2.5 Nutrient broth preparation. Nutrient broth was prepared. 35ml of nutrient broth was transferred into the falcon tubes. A distinct colony was isolated from a MacConkey agar plate and dissolved into its nutrient broth. This procedure was done for all the remaining samples near the bunsen flame and incubated for 48 hours at 37°C[29].

2.6 Biochemical tests. Bergey's manual was followed for performing biochemical tests on each sample. The lactose fermentation indole positive pathway and lactose non-fermentation Indole negative pathway were investigated because the study was focused on bacteria. Biochemical tests were performed under aseptic conditions near the bunsen flame and work benches were cleaned previously with 70% ethanol. A set of biochemical tests such as Indole test, Citrate test, Hydrogen sulfide test, Urease test, Motility test, Voges - Proskauer test and Methyl - Red test were performed to detect bacteria in each sample.

2.7 Indole test. Indole test was performed on both fermented and non-fermented samples. Tryptophan broth was prepared. In Tryptophan broth, isolated pink colonies from sub cultured agar plates of each gotukola sample were incubated at 37°C for 48 hours[30]. After the incubation period, 1-2 ml of Kovac's indole reagent was added, and the color changes were noted and recorded.

2.8 Citrate test. For all Indole positive results, Simmons citrate agar was prepared. Colonies from each sub cultivated gotukola sample were stab cultured on citrate agar slants. After, the agar slants were incubated for 48 hours at 37°C, and the color variations were noted and recorded.

2.9 Hydrogen sulfide test. Triple iron sugar agar was prepared for the Indole positive samples. 5mL of solution was kept at a slant form. Loop full of colony was taken and inoculated into the medium and incubated for 48 hours at 37°C[31]. The color changes were noted and recorded after 24 hours.

2.10 Urease test. For Indole positive results, Christensen's urea was prepared and mixed with 40% urea solution. 5mL of agar medium was added to test tubes in slanting position. Loop of isolated colony was taken and inoculated on the urea surface. Finally, test tubes were incubated at 37°C for 48 hours[32]. The color change was noted and recorded after 24 hours.

2.11 Motility test. Sulphide Indole Motility (SIM) agar was prepared for the Indole negative samples. On motility semi-solid agar, precise colonies from the subcultured samples were stab inoculated to the depth of ½ inch and

incubated at 37°C for 24 hours[33]. Results were noticed and recorded after 48 hours.

2.12 Voges-Proskauer test. The samples that tested negative for Indole performed Voges-Proskauer test. MRVP broth, Alpha-Napthol solution and KOH solution was prepared. 1mL of each MRVP broth with the bacterial growth was transferred into labeled sterile test tubes. Then 6 drops of Alpha-Napthol solution was added and 2 drops of KOH solution was added[34]. Color changes were observed and recorded after 20 minutes.

2.13 Methyl Red test. Methyl Red test was done for the samples negative for Indole test. MRVP broth and Methyl Red indicator were prepared. 5mL of the broth was introduced to the labeled sterile test tubes. Each sample of nutritional broth contained a bacterial colony that was transferred to sterile test tubes and incubated for 48 hours at 35°C to 37°C.[35] Then, 4 - 6 drops of Methyl Red indicator were added and color changes were observed and recorded.

3. Results

3.1 Microbiology analysis results. Lactose-fermenting bacterial colonies are pink, while non-lactose-fermenting bacterial colonies are colorless (Figure 1). Dark pink colorless dome-shaped colonies were prioritized when choosing colonies for biochemical tests.[1]

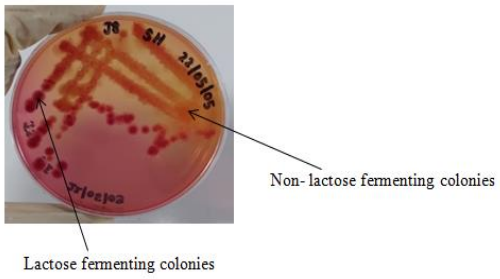


Figure 1. MacConkey agar colonies

Six of the ten samples used, formed pink colonies, accounting for 60% of the total, and lactose-fermenting bacteria were discovered in all six samples. And five samples (50% of the total) produced colorless colonies. D_2 sample was contained both pink and colorless colonies. After isolation of gram negative bacteria, they were tested for the presence of lactose and non-lactose fermenting bacteria.

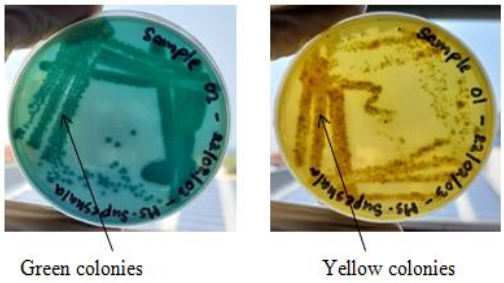


Figure 2. TCBS agar colonies

As shown in Figure 2, green colonies contained *Vibrio parahemolyticus* while yellow colonies contained *Vibrio cholera.*[36] Only 4 (Sample A_1, B_1, E_1, D_1) out of the 5 samples or 80% of the entire sample produced the yellow colonies that represented *Vibrio cholerae*.

3.2 Biochemical analysis results

Table 2. Biochemical test results interpretation

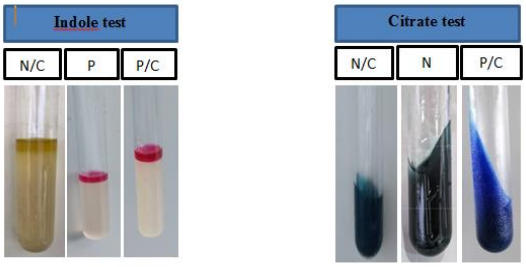
Biochemical test	Positive test result	Negative test result
Indole test	Cherry red ring	Yellow ring
Citrate test	Blue slant	Green slant
Urease test	Pink	Yellow
MR test	Red color	Remained yellow
VP test	Cherry red	Yellow brown
Motility test	Diffuse, hazy growths that cover the medium	Sharply confined to the stab line
H_2S test	Blacking precipitate	No black precipitate

3.3 Summary of the results obtained from microbiological and biochemical analysis. Based on the results from biochemical analysis for each sample organisms in each sample was identified as follows.

Among the 11 samples, *E. coli* was observed in 4 samples (36.37%) (Samples A_1, B_2, C_1, E_1). Lactose fermentation ability of the bacteria in these samples were confirmed using MacConkey agar growth results and

biochemical data for Indole positive and Citrate negative bacteria (Figure 3).

Figure 3. *E.coli* confirmatory biochemical test



result

There was evidence of *Klebsiella oxytoca* in one of the 11 samples (9.09%) (Sample D_1). Gram negative lactose fermenting which tested positive for Indole, Citrate, and VP test but negative for H_2S test (Figure 4) showed for the presence of *Klebsiella oxytoca*.

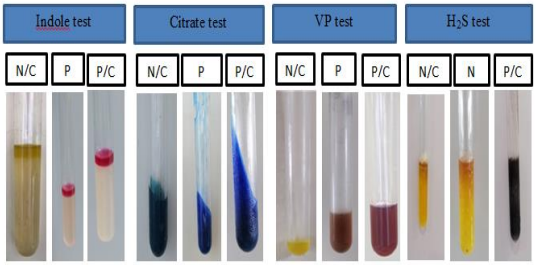


Figure 4. *Klebsiella oxytoca* confirmatory biochemical results

Out of the 11 samples only D_2 sample was confirmed the presence of *Citrobacter freundii* (9.09%). Gram negative lactose fermenting Indole and VP negative but MR and H_2S positive bacterial colonies indicated the presence of bacteria (Figure 5).

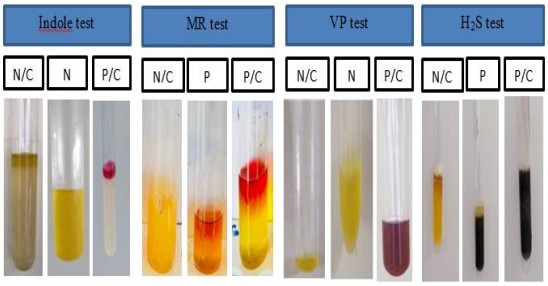
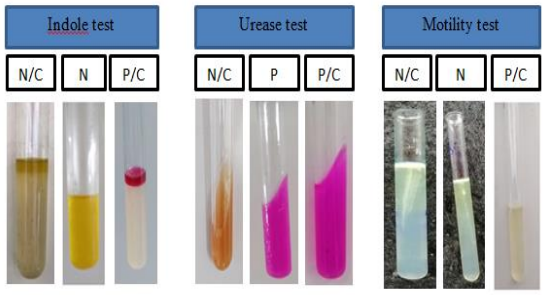


Figure 5. *Citrobacter freundii* confirmatory biochemical test results

Yersinia pseudotuberculosis was found 2 samples out of 11 samples (Samples E2 and D2) in 18.18%. These samples had colonies which were gram-negative and non-lactose fermenting and they tested negative for Indole and Motility test but positive for Urease test (Figure 6).

Figure 6. *Yersinia pseudotuberculosis*



confirmatory biochemical test results

Gram negative, non-lactose fermenting colonies, which tested negative for Indole and Urease and positive for Motility, and H_2S test (Figure 07) found in 2 out of the 11 samples, verified the presence of salmonella (18.18%) (Samples B1, C2).

Out of the 11 samples only A_2 sample was confirmed the presence of *Proteus vulgarius* (9.09%). This sample showed gram negative, non-lactose fermenting colonies which positive for Indole, Urease and H_2S tests (Figure 8).

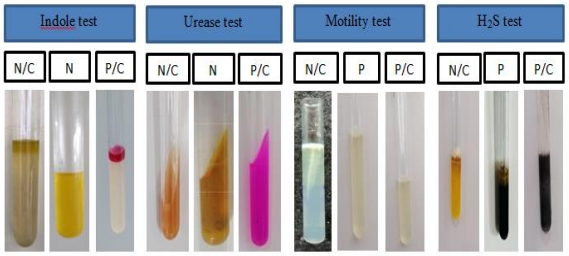


Figure 7. *Salmonella* confirmatory biochemical test results

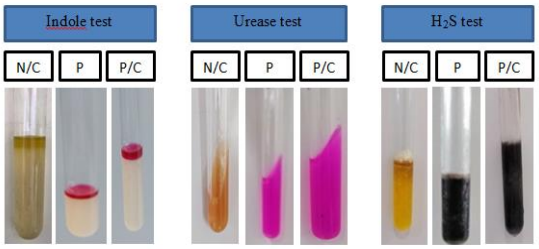


Figure 8. *Proteus vulgarius* confirmatory biochemical test results

Table 3. Summary of the results

Sample	MacConkey agar	Indole test	Citrate test	Urease test	VP test	MR test	Motility test	H_2S test	Confirmed Organism
D_2	Pink colonies	(-)			(-)	(+)		(+)	*Citrobacter freundii*
A_1		(+)	(-)						*E.coli*
B_2		(+)	(-)						*E.coli*
C_1		(+)	(-)						*E.coli*
E_1		(+)	(-)						*E.coli*
D_1		(+)	(+)		(+)			(-)	*Klebsiella oxytoca*
E_2	Color less colonies	(-)		(+)			(-)		*Yersinia pseudotuberculosis*
D_2		(-)		(+)			(-)		*Yersinia pseudotuberculosis*
B_1		(-)		(-)			(+)	(+)	*Salmonella*
C_2		(-)		(-)			(+)	(+)	*Salmonella*
A_2		(+)		(+)				(+)	*Proteus vulgarius*

Summary of the results obtained for all 10 samples were as follows (Table 3). D2 sample was obtained both pink and colorless colonies. Therefore, D2 was taken as two samples.

As shown in figure 9, the gotukola samples from the four provinces contained *Escherichia coli* (36.37%), *Citrobacter freundii* (9.09%), *Klebsiella oxytoca* (9.09%), *Yersinia pseudotuberculosis* (18.18%), *Salmonella* (18.18%), and *Proteus vulgarius* (9.09%).

Bacteria had a substantial distribution in the Western and North - Western provinces. Also, Western province demonstrated a wide distribution of bacteria due to the heavily polluted, largely urbanized, and unsanitary conditions (Figure 10).

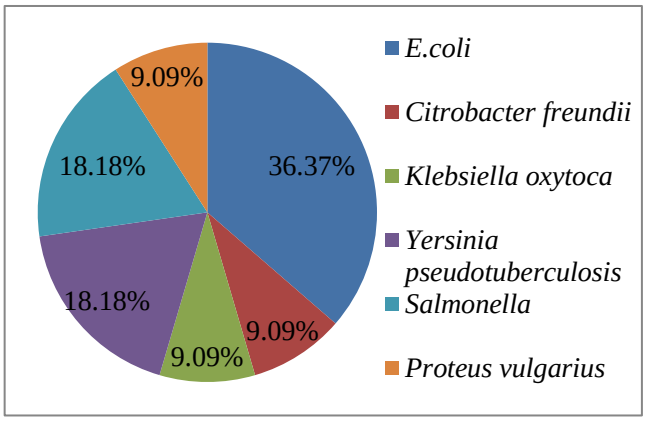


Figure 9. Microbiological and biochemical analysis results of gotukola

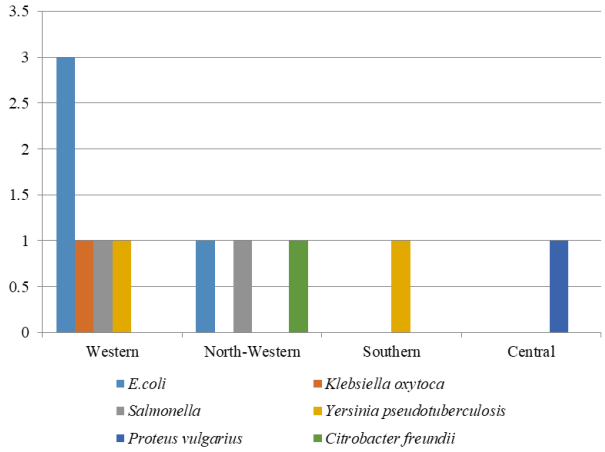


Figure 10. Regional distribution of bacterial contamination in *Centella asiatica*

4. Discussion

Centella asiatica (gotukola) leaves were collected from various regions of Sri Lanka to detect the presence of *Escherichia coli, Salmonella, and Vibrio cholerae* in gotukola samples representing different conditions and geographical locations. Young, undamaged, and infection-free leaves were chosen for the sample collection, as shown in the methodology. The goal of the current study was to determine the presence of bacteria *E. coli, Salmonella, and Vibrio cholera* in gotukola leaf samples from Western, Southern, Central, and Northern-Western Provinces of Sri Lanka. Samples were stored under different conditions upon buying from open air markets and supermarkets. Considering that they were collected from several regions, there is a high

probability for many samples to be contaminated with faeces[40].

In the current investigation, only 4 of the 5 samples (Samples A_1, B_1, E_1, and D_1), or 80% of the overall sample, formed the yellow colonies that represented *Vibrio cholera* during the TCBS agar culture examination (Figure 02). The green colonies represented *Vibrio parahemolyticus*, were only grown in one sample (Sample C_1), which accounted for 20% of the whole sample. Samples A_1, B_1, C_1, D_1, and E_1 were cultured on TCBS agar. After analysis, it was proved that *E. coli* contamination rate of gotukola samples from open air markets (36.37%) was greater than those from supermarkets. Many local markets are open air markets where food is exposed and handled room temperature. The dispersion of bacteria was significantly high in all four provinces. Hence, this study also showed that highly populated, largely industrialized areas in developing countries were more vulnerable to bacterial contamination of food due to the polluted water and unhygienic conditions.

Despite the fact that consuming green leafy vegetables like gotukola is crucial for maintaining good health, this study indicates that these foods can harbor a variety of food-borne pathogens such as *Escherichia coli, Salmonella, and Vibrio cholera*. This is due to the fact that several bacteria types were identified in all the samples. *Escherichia coli* showed that greatest dispersion in samples from the Western and North-western Provinces (36.37 %), whereas the bacteria had the least distribution in samples from the Southern and Central provinces.

When compared to the Western and North-Western provinces, Southern and Central provinces are less urbanized with low overall pollution. This may have caused the lower spread of bacteria.

Salmonella species was present (18.18%) in the samples from Western and North-Western Provinces, but was absent from the samples from southern and Central provinces. This might be due to the fact that, the respective samples were taken from areas with a lower level of microbial population and less population.

Green leafy products are prone to microbial contamination during both pre and post harvesting periods[39]. The risk of direct contamination rises during post- harvesting, when pathogenic bacteria get established on growing crops, prior to harvest[41]. Due to irrigation water pollution, contaminated containers and fertilizers containing animal manure, leafy crops are susceptible to contamination during pre-harvesting[13]. Especially, the widespread use of untreated irrigation water and sewage fertilizer in Sri Lankan agricultural practices is a major contributor to the pre-harvest microbial contamination of leafy vegetables[14].

According to the studies, *Klebsiella oxytoca* is seldom isolated from organic materials like vegetables[41]. Infected water is often where *Klebsiella oxytoca* is found. Also, it is possible that contaminated water used to wash the green leaves led to the presence of *Klebsiella oxytoca* in sample D_1 from the western province's seaside Beruwala region from open-air market. Certain investigation, imply that the contamination was directly caused by water infected with pathogenic *E. coli* that was sprayed on gotukola leaves[25]. The water sprinkled on green leaves may have been polluted and might be even contaminated with bacteria[36]. Bundling and storing gotukola with other vegetables may also increase the exposure to contamination of pathogens[25,35].

Moreover, it has been found that contaminated soil also can be infected the gotukola plant[17]. This indicates that post-harvest washing greatly affects the microbial contamination of leafy greens. This study was able to detect a noticeable dissemination of bacteria despite the samples being from local open air and super markets with diverse storage conditions. A study conducted in Thailand, discovered that samples of leafy green vegetables from open air markets and supermarkets respectively had 44% and 15%, bacterial contribution[28]. This is a notable difference in percentages. Therefore, it may be assumed that varied environmental and sanitary

conditions would have a substantial impact bacterial establishment. The findings demonstrate that *Proteus vulgarius* was detected in sample A_2 from the open air market in Dambulla, Central province. It is highly likely that gotukola samples were either directly or indirectly exposed to faecal contamination, as *Proteus vulgarius* was found in water and leafy vegetables. The poor hygiene of the merchants may also be responsible for this contamination[42].

In the samples taken from Aluthgama, Kalutara, Beruwala, Panadura and Mathugam, *Escherichia coli, Klebsiella oxytoca, Yersinia pseudotuberculosis*, and *Salmonella* were discovered. This could be due to the western province being one of the most populated and industrialized regions in the country, which shows a greater susceptibility for contamination of food products. The Western province has a broader bacterial distribution than the other provinces. The vegetable cages were surrounded by a lot of flies and market places were close to drainage lines carrying polluted water in the Western province. Additionally, insects are yet another potential cause of bacterial contamination. Certain studies show that bacteria can be directly transferred from contaminated files to crops.[7]

According to the current study, in order to prevent food-borne illnesses, gotukola should be properly cleansed with fresh water and disinfectants before consumption. During cooking, people frequently use turmeric water, vinegar, and salt solutions to wash green leafy vegetables.[12] But past studies have demonstrated that such disinfectants are poor at eliminating faecal coliform bacteria36. Given that gotukola is frequently consumed raw, it is important to consider washing procedures before consumption, as all the studied gotukola samples confirmed the presence of pathogenic bacteria.[43]

New advanced technologies can be suggested for future perspectives in order to detect the pathogens within a short time period accurately. Therefore, advanced methods are introduced in the molecular detection of microorganisms for further detection in this area of study. The most recent next-generation technology being utilized for the quick identification and categorization of microorganisms is Matrix-Assisted Laser Desorption/Ionization (MALDI). Its foundation is the use of brief laser pulses to ionize microbial cells, followed by the use of an electric field to accelerate the particles in a vacuum system[11]. Standard molecular techniques for detecting bacteria are 16S ribosomal RNA gene sequencing, restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) [27]. These techniques are used in both clinical and laboratory circumstances. The 16S rRNA gene is a prime target for identification since it is unique to each bacterial species[44].

Ribotyping, which is an rRNA, based phylogenetic analysis, flow cytometry, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) can also be used to identify the morphological characteristics of any pathogen of interest[45]. Nucleic acid-based detection techniques like Real-time PCR and microarray technologies like DNA micro array can be used to detect pathogens accurately[46]. The creation of microbe detection limits will continue to be an important step in microbiology and combination of these approaches and technology will develop the capacity for pathogen identification.

Conclusion

According to results of the microbiological and biochemical analysis, *Escherichia coli* (36.37%), *Citrobacter freundii* (9.09%), *Klebsiella oxytoca* (9.09%), *Yersinia pseudotuberculosis* (18.18%), *Salmonella* (18.18%), and *Proteus vulgarius* (9.09%) were substantially contaminated in the gotukola samples from the all four provinces.

Further research can be done to determine whether other bacterial species are present in gotukola. It is still possible to find out whether other bacterial species are present in gotukola by conducting further research. Hence in conclusion, gotukola leaves should not be consumed in their raw state due to the presence of pathogens in all of the samples

analyzed. Furthermore, follow washing treatments prior to the consumption and cooking it on a low flame will lessen the danger of infected with food borne diseases.

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