ISSN 2989-0705 (Online)|Vol 2|Issue 1|January 2024

Journal of Applied Learning | BMS CAMPUS make learning happen

Isolation and biochemical analysis of *Enterobacteriaceae* from *Cucumis sativus*

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Abstract

The aim of this experiment was to detect the presence of pathogenic *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella*, which was crucial to assess the safety of consuming raw cucumbers. Furthermore, to gain insight on the occurrence of *Enterobacteriaceae* in cucumbers. Ten cucumber samples were subjected to microbiological tests and biochemical tests. Streak plate method on MacConkey agar was used for differentiating lactose fermenters and non-lactose fermenters. The bacterial isolates were pure cultured and subjected to a series of biochemical tests according to the guidelines in the Bergey's Manual of Determinative Bacteriology. Based on microbiological test results, all cucumbers samples were tested positive for *Enterobacteriaceae*. 60% of the cucumber samples were found to contain lactose fermenters and 40% of the samples were found to contain non-lactose fermenters. A sequence of biochemical tests led to the identification of *Enterobacteriaceae* species such as *Klebsiella pneumoniae* and *Citrobacter diversus*. Out of the ten samples, *Klebsiella pneumoniae* was isolated from 5 cucumber samples whereas only 1 cucumber sample contained *Citrobacter diversus*. The other 4 cucumber samples were non-lactose fermenters which required ornithine decarboxylase test for confirmation of the *Enterobacteriaceae* species. The cucumber samples were tested negative for *Escherichia coli* and *Salmonella*, which indicated the cucumbers were safe for consumption. *Klebsiella pneumoniae* was found as the frequently occurring *Enterobacteriaceae* in *Cucumis sativus* and this result was supported by studies done in the past.

Keywords: *Enterobacteriaceae*, *Cucumis sativus*, food safety, biochemical analysis

1. Introduction

Fresh or less processed cucumbers that are whole or sliced can be suspects of microorganism contaminations, especially since they are not subjected to the steps necessary for sterilisation.[1] The probability of developing food-borne diseases can increase with the consumption of vegetables that are raw or lightly cooked.[2] Table 1 indicates the number of cases for food poisoning and dysentery in Sri Lanka from 2017 to 2020. Globally, an approximation of 550 million people fall prey to food-borne diseases annually, out of which, 230 individuals die.[3]

There was an increase in the number of food businesses in Sri Lanka and most of them were established without any confirmation on food safety. For instance, the number of venders in Sri Lanka increased after many individuals lost their jobs due to the coronavirus disease crisis in 2019.[8] In order to safeguard the health and hygiene of local consumers, it is crucial to evaluate the microbiological quality and safety of food. [9]

Table 1. Selected statistical data on food-borne illnesses documented in the Weekly Epidemiology Report (WER) Sri Lanka.[4-7]

Year	Number of cases	
	Food poisoning	Dysentery
2020	553[4]	875[4]
2019	918[5]	2229[5]
2018	1170[6]	2268[6]
2017	969[7]	2187[7]

Food-borne illnesses could arise due to contamination of food with agents such as bacteria, viruses, parasites, prions and chemicals.[3] Of the various contaminating agents, bacteria, for instance, *Escherichia coli*, *Salmonella* and *Campylobacter* have been responsible for many recurring incidents of food-borne diseases.[10]

1.1 Enterobacteriaceae associated contaminations and consequences. All bacteria that belong to the diverse *Enterobacteriaceae* family are gram negative, flagellated bacteria that are also facultative anaerobes with a size of approximately 0.3 – 1.0 * 1.0 – 6.0 µm. Figure 1 indicates some of the genera comprising the *Enterobacteriaceae* family.[11] Several bacterial species from this family have been the cause of numerous food-borne outbreaks by contaminating fresh vegetables and fruits.[12]

A serotype of *E. coli* known as *Escherichia coli* O157:H7 could trigger haemorrhagic diarrhoea and kidney failure because it produces a toxin called Shiga.[13] *Salmonella* is an organism similar to *E. coli*. *Salmonella bongori* and *Salmonella enterica* are two species of *Salmonella*.[14] *Salmonella* is known to cause salmonellosis which is also known as typhoid fever.[13]

Furthermore, a common nosocomial pathogen called *Klebsiella pneumoniae*, is known to cause Friedlander's pneumonia and rhinoscleroma. Apart from the two human specific infections, *Klebsiella pneumoniae* can also cause other infections associated with blood, urine and the respiratory system.[15]

Majority of the illnesses associated with fresh vegetables are known to be caused by *E. coli* and *Salmonella* species.[16] According to a study conducted in Sri Lanka to assess the microbiological quality of selected fresh vegetables, 6% of the leafy vegetable samples indicated presence of *Salmonella* spp. and 2% of the samples showed prevalence of *E. coli*. [17]

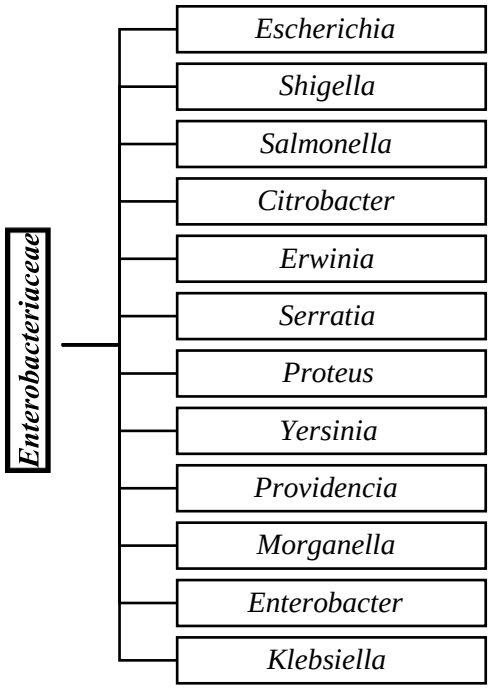

Figure 1. Classification of the *Enterobacteriaceae* family.[11]

Including Sri Lanka, a list of other countries: India, Vietnam, Philippines, USA, Mexico, Spain, Brazil, Canada and Thailand, have reported illness caused by consumption of fresh produce contaminated with *E. coli* and *Salmonella*.[13] Some of the pathogens associated with food-borne outbreaks in Sri Lanka include hepatitis A, *Salmonella*, *Listeria monocytogenes* and *Vibrio cholerae*.[18]

1.2 Contamination of C. sativus by Enterobacteriaceae. Fresh vegetables such as lettuce, cucumber, spinach, carrot and tomato are often eaten raw.[13] Figure 2 illustrates the varieties of cucumbers cultivated. It is a juicy fruit with a crisp texture, making it a highly preferred constituent of salads. Consumed in its unripe, green form, cucumber which is also known as *Cucumis sativus*, is the most cultivated member of the *Cucurbitaceae* family.[19] *Cucumis sativus* is one of the 825 species across the 118 genera comprising the *Cucurbitaceae* family.[20]

Fruits and vegetables are highly vulnerable to contamination by factors such as soil and irrigation water associated with the pre-harvesting process, and handling process after harvest.[16] Pathogenic microbes such as *E. coli* O157:H7 and *Salmonella* could contaminate the soil in cases where poultry litter is used as fertilizers for growing fruits and vegetables.[21] Fruits and vegetables could act as an intermediate for transmission of pathogens or opportunistic pathogens. Hence, over the years, there has been a positive correlation between consumption of vegetables and fruits, and human infections.[12]

Figure 2. Different cultivars of cucumber.[20]

1.3 Isolation and identification of Enterobacteriaceae. Selective media could be used to grow colonies of bacteria which in turn could be used to isolate *Enterobacteriaceae*, by subjecting them to phenotypic tests indicated in Figure 3.[22] The following research used a specific set of biochemical tests from the Bergey's Manual, as outlined in Figure 4 and Figure 5, to characterize *Enterobacteriaceae*.

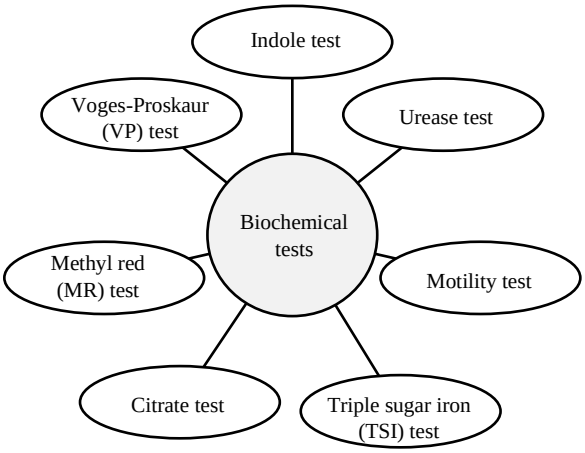

Figure 3. Selected biochemical tests used in the research for identification of *Enterobacteriaceae*.[22, 23]

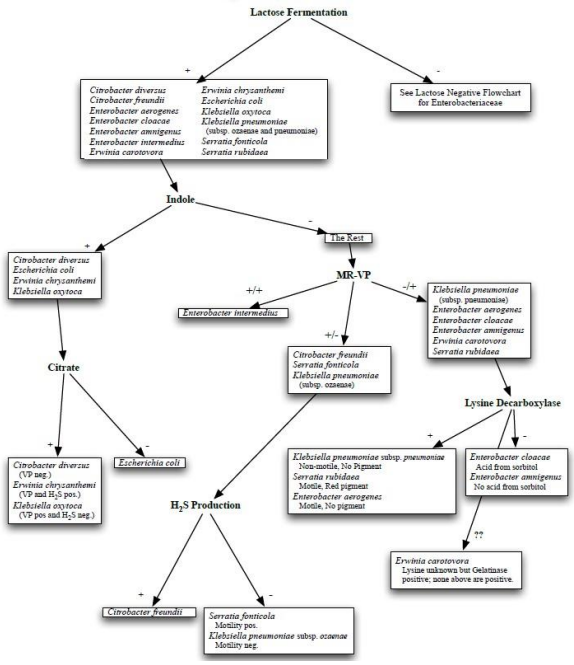

Figure 4. Biochemical tests to identify lactose fermenting *Enterobacteriaceae*.[24]

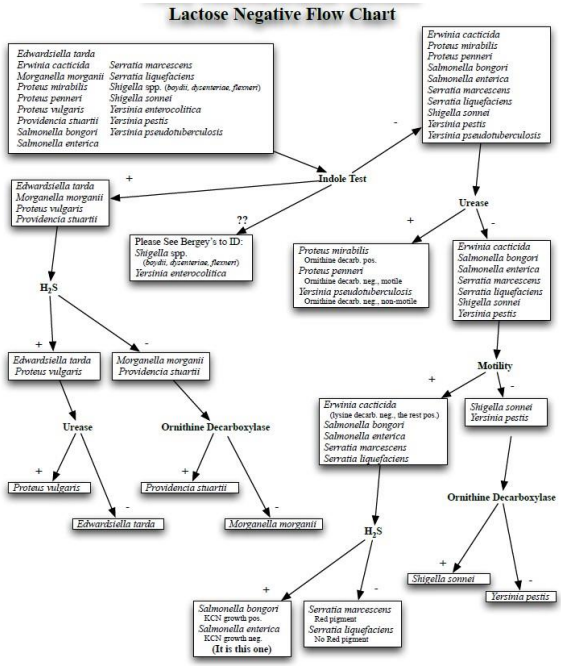

Figure 5. Biochemical tests to identify *Enterobacteriaceae* that do not ferment lactose.[24]

A tristate outbreak took place in 2016 due to the consumption of contaminated cucumbers. The FDA was able to confirm that cucumbers were the source of contamination due to the consistent investigations conducted on cucumbers to detect *Salmonella*.[25] Consistent investigations on food are essential to understand the hygienic conditions of food. This is important in Sri Lanka because there are few investigations on food hygiene.[17] Furthermore, food-borne diseases may not be detected as there are no standard diagnostic procedures to identify them in Sri Lanka.[17] Hence, scientific literatures may not cover most incidents of food-borne illnesses.[17] The occurrence of *Enterobacteriaceae* in *Cucumis sativus* in Sri Lanka can be understood from the subsequent research project. The study results can be used to understand the degree to which hygienic conditions must be refined for quality production of fresh cucumbers.[12]

2. Methodology

2.1 Sample collection. Ten fresh cucumbers were collected from different places (Table 2) within Wellawatta and Pettah, which are two zones in Colombo, Sri Lanka.

Table 2. Sample collection locations in Colombo.

Sample	Date of collection	Collection site	Location
1	18th February 2022	Vegetable market	Pettah
2		Roadside vegetable stand	Wellawatta
3		Fruit and vegetable market	Wellawatta
4		Grocery store	Wellawatta
5		Roadside vegetable market	Wellawatta
6	20th April 2022	Supermarket	Wellawatta
7		Grocery store	Pettah
8		Roadside vegetable stand	Pettah
9		Vegetable market	Pettah
10		Roadside vegetable stand	Pettah

2.2 Sample preparation. Five sterile beakers were labeled. Five fresh cucumbers were thoroughly washed using distilled water and then utilised for sample preparation. Slices of the mid region of the cucumber was cut and weighed. Approximately 100 g of the cucumber was blended to obtain homogenized cucumber sample. Sterile equipment were used for sampling.

Using a micropipette, 2 ml of the cucumber sample was transferred into sterile screw-capped containers. This was followed by the addition of 18 ml of freshly prepared peptone water. The preceding mixtures were mixed by swirling the closed container which was incubated at 37 °C for 48 hours. Thereafter, the samples were stored at -4 °C until analyzed further.

2.3 Streak plate method on MacConkey agar. The refrigerated samples were left out at room temperature. Near a Bunsen flame, five sterile petri plates were closely arranged and labeled. The surface of the biosafety cabinet was wiped with 70% ethanol and the five petri plates were appropriately aligned in a single file. Freshly prepared, MacConkey agar was poured into sterile petri plates and allowed to solidify.

Using sterile inoculating loops, quadrant streak was performed from all the samples. The preceding steps were replicated to culture another set of MacConkey agar plates to minimise errors. The streaked MacConkey agar plates were sealed with parafilm and incubated at 37 °C for 24 hours. Thereafter, the plates were stored at -4°C until analyzed further.

2.4 Preparation of pure culture. Freshly prepared sterile nutrient broth was poured into five sterile broth tubes in equal volumes and labeled. The nutrient broth tube was inoculated using a discrete colony picked from its corresponding MacConkey agar plate after incubation. The inoculated nutrient broth tubes were incubated at 37 °C for 24 hours, and then stored at -4 °C until analyzed further.

2.5 Biochemical tests. The *C. sativus* inoculums were subjected to a series of biochemical tests, as demonstrated in the results section. The test tubes involved were autoclaved at 100 °C for 45 minutes before being utilised in the biochemical tests. The test tubes were then labelled appropriately. Procedures were carried out under aseptic conditions to minimize cross contamination. For each test, two control tubes were prepared using *E. coli*.

2.5.1. Indole test. 5 ml of tryptophan broth was inoculated with pure culture. The inoculated tryptophan tubes along with the control tubes were incubated at 37 °C for 24 hours. Thereafter, 1-2 ml of Kovac's reagent was added and colour change was noted.

2.5.2. Citrate test. 5 ml of freshly prepared Simmons Citrate agar was poured into sterile test tubes and left in a slanted position for solidification. Pure culture was inoculated on the Simmons Citrate agar slants. The pure culture slants along with the control tubes were incubated at 37 °C for 24 hours.

2.5.3. Urease test. 80 ml of Christensen urea agar mixture and 4 ml of urea solution was used to produce urea slants. After solidification, 5 ml urea slants were inoculated with pure culture. The control tubes and streaked tubes were incubated at 37 °C for 24 hours.

2.5.4. Motility test. Sulphide Indole Motility (SIM) agar tubes were prepared and inoculated with pure culture by penetrating the inoculating loop half-way through the semi-solid agar. Along with the control tubes, all the test tubes were incubated at 37 °C for 24 hours.

2.5.5. Triple sugar iron test. The pure culture was streaked on its respective 5 ml triple sugar iron agar slant using a sterilised inoculating loop. The inoculated tubes with controls were incubated at 37 °C for 24 hours.

2.5.6. Methyl red test. Test tubes containing 5 ml of Methyl Red Voges-Proskauer (MRVP) broth were inoculated with their respective pure cultures using a sterile inoculating loop. The tubes were then incubated at 37°C. After 24 hours, 1 ml from the incubated MRVP tube was transferred to a clean tube and 2 to 3 drops of methyl red solution was added.

2.5.7. Voges-Proskauer test. Using a sterile inoculating loop, test tubes containing 5 ml of MRVP broth were inoculated with their respective pure cultures. After incubation at 37 °C for 24 hours, 6 drops of Barritt's reagent I and 2 drops of Barritt's reagent II was added.

3. Results

3.1 Analysis of microbiological test results. Out of the 10 samples involved in this experiment, 6 samples contained lactose fermenters (Figure 6) and 4 samples contained non-lactose fermenters (Figure 7), as indicated in table 3.

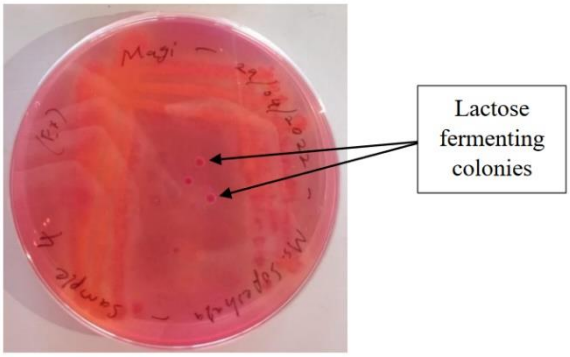

Figure 6. MacConkey agar plate with lactose fermenters.

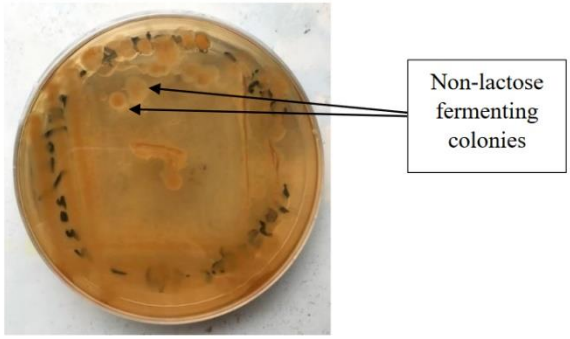

Figure 7. MacConkey agar plate with non-lactose fermenters.

Table 3. Cucumber samples assigned from 1 to 10 yielded lactose fermenters and non-lactose fermenters.

Samples						
Lactose fermenters	5	6	7	8	9	10
Non-lactose fermenters	1	2	3	4	-	

3.2 Analysis of biochemical test results. Table 4 contains the biochemical test results for all 10 cucumber samples. For making comparisons between the test results, reference microorganisms: positive control and a negative control, were involved in the experiment.[26] The bacterial species present were identified using the Bergey's Manual, shown in Figure 4 and Figure 5. Almost all tests had both a negative control (NC) and a positive control (PC).

In the MR test, the bacterial ability to ferment glucose and produce a strong acid is assessed. If the Embden-Meyerhof pathway used by bacteria led to the production of strong acid, positive results for MR test is seen, as demonstrated in Figure 8.[27] In the presence of a pH indicator such as methyl red, bacteria will induce a red colour change.[28] Negative result for MR test is indicated by a colour change to yellow.[23]

Table 4. Positive (P) and negative (N) biochemical test results for the ten cucumber samples.

Tests	**Samples**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Indole test	N	N	N	N	N	N	N	P	N	N
Citrate test	P	P	P	P	P	P	P	P	P	P
Methyl red test	-	-	-	-	P	P	P	-	P	P
Voges-Proskauer test	-	-	-	-	N	N	N	N	N	N
Triple sugar iron test	N	N	N	N	N	N	N	N	N	N
Motility test	N	N	N	N	N	N	N	N	N	N
Urease test	P	N	P	N	P	P	P	P	P	P

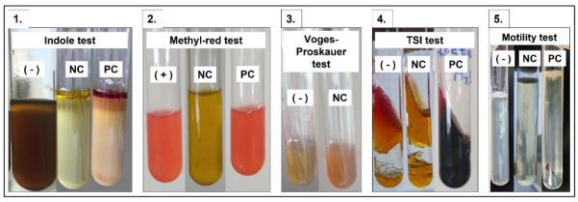

Figure 8. Biochemical tests confirming *Klebsiella pneumoniae*.

Red colour change of VP broth means a positive result.[28] The absence of colour change, as presented in Figure 8, is due to the inability of bacteria to ferment glucose and form pyruvic acid, a key compound necessary to form acetoin. Metabolic routes leading to acetoin formation is based on the bacterial type and their enzyme systems. Acetoin gives rise to diacetyl in the presence of 40% KOH and oxygen in air, and the red colouration is catalysed by α-naphthol.[29]

Constituents of Triple Sugar Iron (TSI) agar include 0.1% of glucose, sucrose and 1% of lactose.[22] The test assesses the bacterial ability to ferment these carbohydrates and produce hydrogen sulphide. Acidification is indicated by phenol red and H_2S production is indicated by the presence of ferrous sulphate.[29] The TSI positive control in Figure 8 evidences a black precipitate due to ferrous sulphide and H_2S gas generation.

The formation of carbon dioxide or hydrogen could be determined by the presence of bubbles and splits in the agar.[29]

The TSI negative result in Figure 8 is with an alkaline slant and an acid bottom, which indicates the bacteria is a glucose fermenter. Other situations that may occur is when both the slant and bottom are either alkaline or acidic, in which case the interpretations would go as, no sugars fermented or all three sugars fermented, respectively.[22]

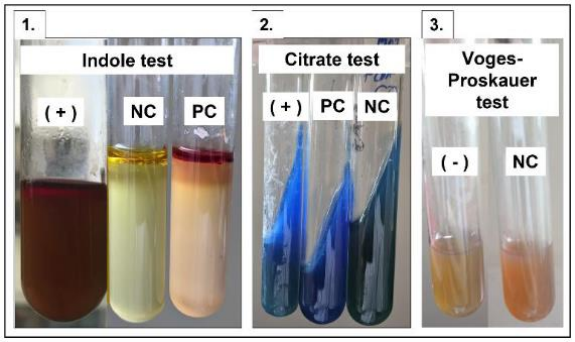

Figure 9. Biochemical tests confirming *Citrobacter diversus*.

Indole positive result witnessed in Figure 9 has a red ring which is a result of the reaction between indole and an aldehyde from the Kovac's reagent.[23] Indole test is used to understand if the bacteria is capable of using tryptophanase to generate indole, pyruvic acid and ammonia, by hydrolysing and deaminating tryptophan.[29] Indole negative result is identified by the presence of an orange-yellow ring, as evidenced in Figure 8.[22]

In the citrate test, bacteria obtain carbon and energy by degrading citrate. Bacteria produces the enzyme citritase which aids in breaking down the citrate in media into oxaloacetate and acetate. Pyruvate and carbon dioxide are produced by the further breakdown of oxaloacetate. The medium becomes alkaline due to the formation of sodium carbonate from sodium citrate, and this provokes a colour change from green to blue.[23] Bromothymol blue indicator in Simmon's citrate test will turn blue in case of a positive result, and stay green to in case of negative result, as presented in Figure 9.[15]

The positive control for motility test in Figure 8 shows signs of turbidity. This is due to the diffuse growth of bacteria, signifying its motile characteristic. The negative motility test result and the negative control show growth along the stab line. The agar was also clear, which together confirms that the bacteria is non-motile.[22]

Some of the above biochemical tests indicated the presence of *Klebsiella pneumoniae* in lactose fermenting samples: 5, 6, 7, 9 and 10. *Citrobacter diversus* was confirmed to be present in lactose fermenter sample 8.

Non-lactose fermenter samples: 1 and 3 could be either *Proteus mirabilis*, *Proteus penneri* or *Yersinia pseudotuberculosis*. The possibility of identifying one of these three species was confirmed by the negative indole results and positive urease results, as demonstrated in Figure 10. Non-lactose fermenter samples: 2 and 4 could either be *Shigella sonnei* or *Yersinia pestis*. This was concluded based on negative results for indole test, urease test and motility test, as indicated in Figure 10.

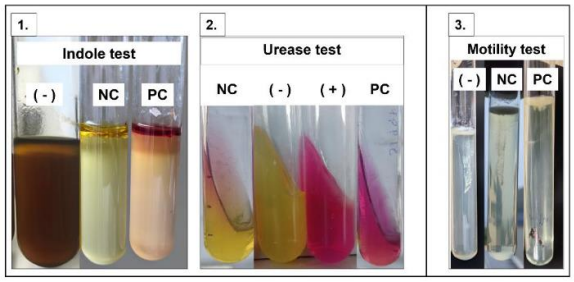

Figure 10. Indole test and urease test to specify *Enterobacteriaceae* in samples 1, 2, 3 and 4. Motility test was needed for further confirmation in samples 1 and 3.

Many bacterial species produce the enzyme urease.[29] Decomposition of urea into ammonia and carbon dioxide is catalysed by this

enzyme. Only urease-positive species are capable of triggering phenol red to turn pink by using the nutrients and pH buffers in the medium to synthesise sufficient ammonia.[28] Urease-negative species are distinguished by the absence of colour change, as visualized in Figure 10.[26]

4. Discussion

Microorganisms, for instance, *Enterobacteriaceae* could be distinguished from one another using a variety of biochemical tests.[28, 9] Biochemical testing is often restricted to essential substrates required to rule-in or rule-out the desired pathogens in resource-constrained situations. The *Enterobacteriaceae* species present in the sample could be identified by piecing together the results of the individual biochemical tests.[22]

Analysis of biochemical test results confirmed the presence of the *Enterobacteriaceae* in all ten *C. sativus*, specifically: *Klebsiella pneumoniae* (50%) and *Citrobacter diversus* (10%). In a similar study, 60% of fruits and 91% of vegetables out of the 105 fresh produces tested, were found to harbour *Enterobacteriaceae*. Along with other bacterial species such as *Enterococcus casseliflavus* and *Enterobacter cloacae*, the most abundant member after *E. coli* was *Klebsiella pneumoniae*.[12]

According to ALatawi *et al*[15], characterisation of bacteria in street stall vegetable samples using both biochemical tests and molecular tests revealed that 24 isolates were *Klebsiella* spp., and 6 isolates were *Enterobacter asburiae*. There were also isolates of other families including, three *Pseudomonas aeruginosa* isolates and two *Aeromonas caviae* isolates. Another study highlighted the prevalence of *Klebsiella pneumoniae* in which 100% occurrence was found in lettuce and 82.5% occurrence in cucumbers.[2]

As a whole, the abovementioned past studies and this research project evidence *Klebsiella* spp. as a frequently occurring *Enterobacteriaceae* member in vegetables. However, biochemical methods are time consuming and have poor specificity, therefore 16s rRNA gene sequencing; a DNA-based molecular method, can be used alternatively for highly specific identifications.[15]

One out of the ten cucumbers tested in this research project yielded a *Citrobacter* specie. Similarly, Adegun, Oluduro and Aregbesola[30] reported that only one out of the fifteen cucumber samples were contaminated with *Citrobacter* spp. However, their study findings on the percentage of Citrobacter spp. occurring in fruits and vegetables showed 68% of *Citrobacter freundii*, 24% of *Citrobacter braakii* and 8% of *Citrobacter youngae*. There is a possibility for low prevalence of *Citrobacter* species in cucumbers when compared to *Klebsiella pneumoniae*.[30]

When fresh vegetables are contaminated with microorganisms such as *Klebsiella pneumoniae*, food safety becomes a concern. People with weak immune systems are at a higher risk of developing diseases after consumption of contaminated fresh vegetables. One way to lower risk is to wash vegetables using disinfectants. This is because gentle washing will not dislodge *Klebsiella* so easily from the vegetable surface.[24] Colonisation and growth of microorganisms on vegetables, followed by metabolism of plant tissues for nutrients could be caused by the microorganisms present in the soil.[2]

The *Enterobacteriaceae* specifically inspected in this experiment were *E. coli* and *Salmonella*, but they were not found in any of the cucumber samples tested. This could be due to the small sample size. A study similar to this research project involved fifty fresh vegetable samples.[17] Along with *Listeria monocytogenes*, food-borne pathogens such as *E. coli* and *Salmonella* spp. were found to contaminate the fresh vegetables.[17, 31] In another study involving 30 ready-to-eat salad vegetables, *E. coli* was detected in 96.7% of

the samples.[32] Therefore, the chances of isolating *E. coli* are high in an experiment involving a large sample size.

On the contrary, results from Al-Kharousi *et al*[12] on cucumbers imported from Oman and UAE, showed the presence of *Enterobacteriaceae* but absence of *E. coli*. Results from Abakari, Cobbina and Yeleliere[32] highlighted *Salmonella* spp. as the least prevalent members in ready-to-eat salad vegetables. According to Erickson *et al*[33], there was no contamination found on 60% to 78% of the cucumbers, after the fruits were exposed to irrigation water infected with 3.8 log CFU/mL of *Salmonella typhimurium* and *E. coli* O157:H7.

Contamination of cucumbers may have been prevented by the cucumber plant canopy that could protect the fruits from exposure to irrigation water.[33] This way, contamination of cucumbers by pathogenic *Enterobacteriaceae* such as *E. coli* and *Salmonella* spp. may have been overcome. Poor handling by vendors and faecal contamination can lead to presence of *E. coli* in food.[32] Since *E. coli* was not detected in the ten cucumber samples tested, it could mean that the cucumbers were handled properly and were not exposed to any faecal contamination.

Each cucumber was brought from a different market or vegetable stall in order to increase the variation of *Enterobacteriaceae* members detected in this experiment. Based on results analysis, two species of *Enterobacteriaceae* members were confirmed and few other species are most likely to be present. This variation in the isolated species could be due to difference in hygiene conditions at the marketing areas and the agriculture from which the vegetables were initially sourced.[32]

Peer-reviewed literature has documented a variety of biochemical tests. Only a small fraction of substrates was found to be effective in identifying *Enterobacteriaceae*. Selective use of biochemical tests is done because, sometimes, certain substrates, though successful in identification of *Enterobacteriaceae*, are poisonous, unstable or expensive for regular use.[22] Furthermore, not all bacteria could be accurately identified using biochemical tests. For instance, since *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* share a large degree of similarity, the standard determination tests can lead to misidentification of these members.[34] Such misidentifications can be expected among the results of the experiment, especially since isolates include *Klebsiella pneumoniae* and *Citrobacter diversus*.

As biochemical reactions are simple detection methods, the medium quality can influence the precision of the tests results, and the *Enterobacteriaceae* specie identified.[27] Additional measures to prevent contamination should be taken into account.[27]

5. Conclusion

In conclusion, pathogenic microorganisms such as *E. coli* and *Salmonella* were not detected in the cucumbers tested. *Klebsiella pneumoniae* was the most prevalent *Enterobacteriaceae*. This pinpoints the risk of consuming raw cucumbers, especially by immunocompromised patients. Another lactose fermenter detected was *Citrobacter diversus*, apart from which, non-lactose fermenters were also detected. Specie confirmation of the non-lactose fermenters requires ornithine decarboxylase biochemical analysis. However, it can be concluded that *Enterobacteriaceae* were detected in all ten cucumber samples.

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