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Encapsulation of *Lactobacillus* sp. isolated from yoghurt and evaluation of their antibacterial activity

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Abstract

Probiotics are living microorganisms that when ingested in the suitable quantities, provide numerous health benefits to the host organism. This study focused on *Lactobacillus* strains isolated from five different brands of yoghurt found in local markets and the effect of encapsulation on its antibacterial activity was observed. The isolated strains were labelled A to E and gram staining, catalase test, PCR (polymerase chain reaction), agarose gel electrophoresis and sequencing were done to identify *Lactobacillus* species. The bacteria were observed to be rod-shaped, gram positive and catalase negative and upon species specific PCR and sequencing, samples A and B were identified to be *Lactobacillus delbrueckii* subsp. *jakobsenii* and Sample E was *Lactobacillus rhamnosus* but samples C and D were identified to be *Streptococcus thermophilus* and *Streptococcus macedonicus* respectively. The identified *Lactobacillus* were then encapsulated in sodium alginate beads coated with chitosan. The CFS (cell-free supernatant) of encapsulated as well as non-encapsulated bacteria were subjected to a well-diffusion assay to assess the antibacterial activity of *Lactobacillus* against pathogenic *Escherichia coli* and *Staphylococcus aureus*. The results showed no antibacterial activity by both encapsulated and non-encapsulated bacteria against *Escherichia coli and Staphylococcus aureus*. This study helps understand that all *Lactobacillus* spp. may not have antibacterial activity. Therefore, finding mechanisms to improve the antibacterial activity of *Lactobacillus* and the use of those improved methods in dairy products would be more beneficial to human health.

Keywords: Antibacterial activity, Encapsulation, *Lactobacillus* spp., Probiotics, Yoghurt

1. Introduction

Probiotics are living microorganisms that when ingested in the suitable quantities, provide numerous health benefits to the host organism.[1] They help maintain the gut microflora. Bacteria that are commonly used as probiotics belong to the genera *Bifidobacterium, Lactobacillus, Saccharomyces, Enterococcus, Streptococcus and Lactococcus*.[1] Most commonly used probiotics in the food industry are lactic acid bacteria (LAB) which include the genera *Bifidobacterium* and *Lactobacillus*.[2] The type of probiotic bacteria that was focused on this study was *Lactobacillus*. This genus consists of various rod-shaped, gram-positive, catalase negative and non-spore forming bacteria. *Lactobacillus* spp. are classified as generally regarded as safe (GRAS) and are considered safe for human consumption. These bacteria are also naturally found in the GI (gastrointestinal) tract as part of the human microbiota. *Lactobacillus* are commonly found in dairy products and also added as starter cultures in the production of certain food products to improve the flavour and to impart health benefits to the consumer. Probiotic genus, *Lactobacillus* is widespread for its numerous medical functions which include the decrease of enteric infections, cholesterol infections, intestinal tumours, lactose intolerance and to boost immunity.[3,4] *Lactobacillus* has many probiotic properties which include acid tolerance, bile tolerance, antibacterial activity, antibiotic susceptibility, hydrophobicity, co-aggregation and auto-aggregation.[2,5] This study focus antibacterial activity as the main probiotic characteristic.

Yoghurt is one of the main sources of probiotics. Many studies have observed

Lactobacillus spp. in yoghurt. A study observed *Lactobacillus* strains in commercially available yoghurt samples which exhibited probiotic properties.[6] Another study also observed and isolated bacteriocin producing *Lactobacillus* spp. which exhibited antibacterial activity.[7] Since yoghurt is considered healthy for consumption and due to the presence of *Lactobacillus* spp. in it, yoghurt was chosen as the sample in this study.

Lactobacillus species play a major role in maintaining a healthy gut microflora by inhibiting the growth and activity of pathogenic bacteria. This is achieved by various mechanisms such as production of antibacterial compounds like bacteriocins, organic acids and hydrogen peroxide, competition for nutrients, competition for colonizing sites, competitive exclusion and modulation of host immune system.[8,9] Among the various mechanisms bacteriocin and organic acid production is considered the most common mode of maintaining a healthy gut microflora.[8,10]

Bacteriocins are low molecular weight peptides or proteins that inhibit the growth of pathogenic bacteria, particularly those associated with GI infections. They can function as a colonizing peptide, killing peptide or signalling peptide. For example, Plantaricin A, a bacteriocin secreted by *Lactobacillus plantarum* can function as both a killing peptide as well as a signalling peptide.[10, 11] A study observed Lactobacillus *plantarum* was able to produce bacteriocins which inhibited common pathogenic bacteria.[12] Moreover, another study observed that the bacteriocin produced by *Lactobacillus acidophilus* had inhibitory activity against some pathogens.[13]

Organic acids are end products of the fermentative metabolism of *Lactobacillus*. They have an antagonistic effect on pathogenic bacteria by causing intracellular acidification of cells and permeabilizing the membrane by disrupting the lipopolysaccharide layer of the outer membrane.[14] The main metabolic product released by *Lactobacillus* strains as observed by Serrano-Nino *et al.,*[15] was lactic acid which causes the decrease in pH in the environment creating unfavourable conditions for the growth of pathogenic bacteria. As lactic acid has the potential to permeabilize the membrane, it could lead to the entry of hydrogen peroxide into the bacterial cells which produces reactive and cytotoxic oxygen species which can damage the nucleic acids, proteins and lipids.[16,17]

Encapsulation is a technique to immobilize the bacterial cells within a protective matrix. It increases cell density, improve resistance to contamination, protect the probiotics from external disturbances during storage and processing, enhance the production and secretion of secondary metabolites and to protect the chemical and physical stability of the cell. Encapsulation is an efficient method to improve the viability of probiotics in food and GI tract. The most common material used for microencapsulation is alginate.[18, 19] Alginate is considered as a GRAS material that can be safely incorporated into food. Microencapsulation with alginate alone has low effectiveness as alginate has low stability in acidic conditions and in the presence of chelating agents.[20] Therefore, coating alginate microbeads with a polycation such as chitosan can decrease the porosity of the bead and enhance its stability thereby increasing the viability of the microencapsulated bacterial cells.[20] Moreover, through encapsulation probiotics can be released in a controlled manner at the required target site. A study observed an increased viability in microencapsulated *Lactobacillus rhamnosus* cells compared to free cells. They also showed greater tolerance to bile salts and acid, higher survival rates in stimulated gastric juice conditions and higher inhibition zones were observed in the antibacterial assay by the microencapsulated cells.[21] Another study also observed microencapsulated *Lactobacillus casei* cells to show an increased resistance to GI conditions. The significance of this study is to investigate the effect of encapsulation in the

antibacterial activity of *Lactobacillus* spp. The findings will be useful for manufacturers as they can aid in enhancing the quality of probiotic products. This study can help manufacturers to understand how encapsulation improves the viability and enhances the antibacterial activity of probiotic bacteria.

2. Methodology

2.1 Culturing of samples. The samples collected were cultured on De Man, Rogosa and Sharpe (MRS) agar under aseptic conditions and were subjected to gram staining and catalase tests.[6]

2.2. Biochemical test and staining. Lactobacillus species were identified by observing the colony morphology (size, shape and elevation) and through tests such as gram staining and catalase tests.

2.2.1 Gram staining. A bacterial smear was prepared and was stained with Gram-staining. The bacteria were then observed under the microscope at 100x magnification.[6]

2.2.2 Catalase test. A bacterial smear was prepared and a few drops of 3% H_2O_2 were added to the smears. The smears were observed for the bubble formation to confirm the presence of catalase enzymes.[6]

2.3 DNA extraction (QIAGEN DNeasy Kit method). The DNA was extracted by following the manufacturer's instructions provided. The extracted DNA was then stored in -20^{o}C.

2.4 Identification of Lactobacillus by PCR. The extracted DNA was amplified using universal primers 27F and 1492R (IDT) (Table 1). Master mix was prepared for five samples, positive control (known *Lactobacillus* DNA), negative control (autoclaved distilled water) and an extra reaction and 12.25µL of it was aliquoted into each PCR tube. Autoclaved distilled water (11.75µL) and DNA were then added into the PCR tubes. The final volume of PCR mix was 25µL. The PCR thermal cyclic conditions followed are mentioned in Table 2.

2.5 Visualization of PCR products. In 2% agarose gel, 6µL of the PCR products, positive and negative control and 2µL of 100bp DNA ladder was loaded into the wells. The gel was first run for 20 mins at a voltage of 45V and then run for 30 mins at 50V. The DNA bands were then visualized under UV.

2.6 Sequencing. Samples confirmed from PCR were sent to Macrogen, Korea to be sequenced (16SrRNA sequencing) to identify the bacterial species. The results were interpreted using BioEdit and NCBI BLAST. The bacteria identified as *Lactobacillus* were carried forward to the assay.

2.7 Encapsulation

2.7.1 Sodium alginate and chitosan preparation. Sodium alginate with a concentration of 3% (w/v) was prepared and sterilized. Upon cooling, 5 mL of *Lactobacillus* bacteria suspension was added into it slowly and mixed well. Chitosan with a concentration of 0.2% (w/v) was also prepared and sterilized.

*2.7.2 Gel beads formation***.** The prepared 3% sodium alginate was transferred to a 50mL syringe. The sodium alginate solution was released from the syringe drop-wise slowly into 100mL of $CaCl_2$ solution (32g/L) while gently swirling the beaker. The beads formed were filtered, washed with distilled water and immersed in the 0.2% chitosan solution for 15 mins. The beads were filtered again, washed with distilled water and was stored in MRS broth in the refrigerator This method was modified from Djaenudin *et al.,* 2020.[19]

2.8 Antibacterial assay. To compare the antibacterial activity of encapsulated and non-encapsulated bacteria, cell-free supernatants (CFS) of both bacteria were initially prepared.

Table 1. Universal primers used for DNA amplification.

Primer	Sequence (5' to 3')	Expected PCR product	References
Forward primer (27F)	AGAGTTTGATCMTGGCTCAG	1500bp	Dharmasiri *et al.*[29]
Reverse primer (1492R)	GGTTACCTTGTTACGACTT	1500bp	Dharmasiri *et al.*[29]

Table 2. PCR thermal cycling conditions

Steps	Temperature (°C)	Duration (minutes)	Number of cycles
Initial denaturation	94	5	
Denaturation	94	1	35
Annealing	60	1	
Extension	72	2	
Final extension	72	12	
Final hold	4	∞	

2.8.1 Encapsulated bacteria CFS preparation. First, 2.00g of beads were transferred to a 50 mL falcon tube and were dissolved in 15mL of phosphate buffer saline (PBS) buffer by mixing by vortex gently. The solution was centrifuged at 4000 rpm for 5 mins and the supernatant was discarded. Then, 5mL of MRS broth was added to the pellet and the tubes were left to incubate at 37°C for 20 hours. The mixture was then heated at 100°C for 20 mins and immediately frozen at -20°C for 20 mins. Finally, it was centrifuged at 4000 rpm for 5 mins to get the CFS.[18]

2.8.2 Non-encapsulated bacteria CFS preparation. Initially, 5mL of subculture was transferred into 50mL falcon tube, centrifuged at 4000 rpm for 5 mins and the supernatant was discarded. Then, 5mL of MRS broth was added to the pellet and incubated at 37°C for 20 hours. The mixture was then heated at 100°C for 20 mins and immediately frozen at -20°C for 20 mins. Finally, it was centrifuged at 4000 rpm for 5 mins to get the CFS.[18]

2.8.3. Pathogenic bacterial suspension preparation. *Escherichia coli* and *Staphylococcus aureus* were inoculated in nutrient broth and left to incubate at 37°C for 20 hours. The broth cultures were then centrifuged at 4000 rpm for 5 mins to obtain the pellet. PBS buffer was added to the pellet and the absorbance of the suspension was adjusted to 0.2 at a wavelength of 600nm.[23]

2.8.4 Antibacterial Assay. Antibacterial activity was assessed through well-diffusion method.[24] The CFS of the samples were heated at 100°C for 20 mins. The Mueller-Hinton agar was prepared, poured into the petri plates and was left to solidify under UV for 15 mins. Pathogenic bacterial suspensions (100µL) were spread onto the plate and the wells were prepared. The heat-treated CFS (100µL) were added into the appropriate wells along with a negative (autoclaved distilled water) and positive (gentamycin) control. The plates were incubated at 37°C for 48 hours (modified from Trabelsi *et al.*[24], 2014). The diameter of the inhibition zones formed were measured.

3. Results

3.1 Culturing of samples

Yoghurt samples were cultured on MRS agar exhibited milky white colonies with circular form, entire margin and convex elevation (Figure 1).

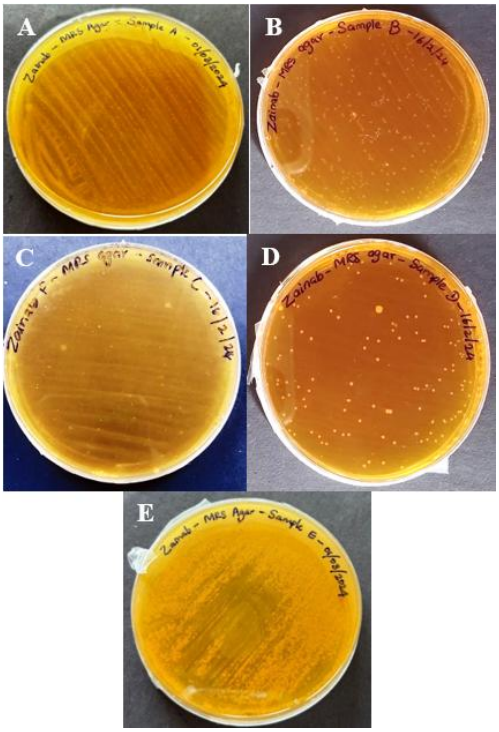

Figure 1. Yoghurt samples cultured on MRS agar (A to E – bacterial colonies from the yoghurt samples)

3.2 Gram staining

Microscopic observation of Gram-stained samples (Figure 2) revealed purple colour, rod-shaped bacteria in all the samples.

Table 3. Gram stain results

Bacterial isolates	Gram stain	Shape
A	+	rod-shaped
B	+	rod-shaped
C	+	rod-shaped
D	+	rod-shaped
E	+	rod-shaped

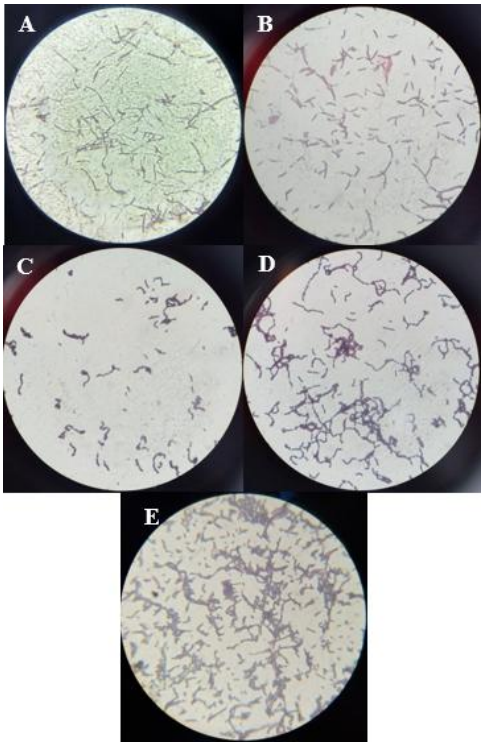

Figure 2. Microscopic images of bacterial isolates after gram staining (100x). (A to E – bacteria cultured on MRS agar)

3.3 Catalase test

Catalase test results revealed no bubble formation in any of the samples even under microscope.

3.4 Visualization of PCR products

PCR products visualized by agarose gel electrophoresis (Figure 3) showed bands of approximately 1500 bp in all samples and the positive control, but no band was observed in the negative control.

Figure 3. PCR products visualized on gel image. Lane 1: 100bp DNA ladder, Lanes 2-6: Samples A to E respectively, Lane 7: positive control, Lane 8: negative control.

3.5 Sequencing

Results of bacterial species identification through sequencing (Macrogen, Korea) is mentioned in Table 4. Sample A and B were identified to be *Lactobacillus delbrueckii* subsp. *jakobsenii* and Sample E was *Lactobacillus rhamnosus* but samples C and D were identified to be *Streptococcus thermophilus* and *Streptococcus macedonicus* respectively. Samples C and D were not carried forward to the assay.

Table 4. Sequencing results

Sample	Bacterial species	Accession number
A	*Lactobacillus delbrueckii* subsp. *jakobsenii* ZN7a-9 = DSM 26046	NZ_CP018218.1
B	*Lactobacillus delbrueckii* subsp. j*akobseni*i ZN7a-9	CP018218.1
C	*Streptococcus thermophilus* isolate STH_CIRM_65	NZ_LR822015.1
D	*Streptococcus macedonicus* strain NWAFU7001	MG550989.1
E	*Lactobacillus rhamnosus* strain 330	KJ939337.1

3.6 Encapsulation

Lactobacillus isolates encapsulated in sodium alginate coated with chitosan is shown below. Encapsulated beads of the isolates were observed to be small and spherical in shape (Figure 4).

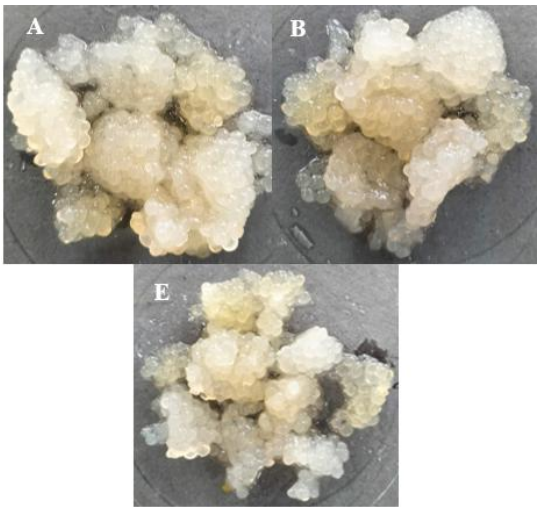

Figure 4. Bacterial isolates encapsulated in sodium alginate coated with chitosan.

3.7 Antibacterial Assay

Antibacterial activity of CFS of bacterial sample against pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* in well-diffusion assay after incubation for 48 hours is depicted in Table 5. Encapsulated and non-encapsulated bacterial samples showed no antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Only the positive control showed a ZOI.

4. Discussion

The bacteria *Lactobacillus* has various probiotic properties and play a major role in maintaining the gut microflora. Therefore, *Lactobacillus* is used in food industry to improve human health. This study isolated *Lactobacillus* from yoghurt and the antibacterial activity of encapsulated and non-encapsulated bacteria were compared.

The yoghurt samples were cultured on MRS agar as they are selective for *Lactobacillus*. MRS agar contains sodium acetate and ammonium citrate that prevent the growth of other microorganisms like *Escherichia coli* but allow the growth of *Lactobacillus*.[25] Amphotericin B was also added to MRS agar during MRS agar preparation. This prevents the growth of fungi and yeast thereby preventing contamination.[26]

Table 5. Inhibition zone sizes against *Escherichia coli* and *Staphylococcus aureus*.

Pathogenic Bacteria	Sample	Encapsulated bacteria	Non-encapsulated bacteria	Positive control (mm)	Negative control
Escherichia coli	A	-	-	20.00	-
	B	-	-	20.00	-
	E	-	-	20.00	-
Staphylococcus aureus	A	-	-	18.00	-
	B	-	-	18.00	-
	E	-	-	18.00	-

Bacteria were initially identified by observing colony morphology. Similar colony morphology was observed by Taye *et al*. [27] and Kaboosi [28] for *Lactobacillus* spp. Gram staining results as seen in figure 2 observed the bacteria to be rod-shaped gram-positive bacteria. The following results is supported by Prabhurajeshwar and Chandrakanth [7] who observed *Lactobacillus* as gram-positive rod-shaped bacteria. Catalase test results showed no bubbles formation even when observed under a microscope. This indicates that all the samples are catalase negative. Hoque *et al*. [29] and Abid *et al*., [30] also observed similar results for *Lactobacillus* spp. These results strongly correlated to the results observed in this study thereby suggesting the bacteria isolated could be a *Lactobacillus* sp.

Upon species specific PCR and sequencing, samples A and B were identified to be *Lactobacillus delbrueckii* subsp. *jakobsenii* and sample E was identified as *Lactobacillus rhamnosus*. The remaining samples C and D were identified to be *Streptococcus thermophilus* and *Streptococcus macedonicus* respectively which are also types of bacteria found in probiotic food (table 4). Since this study is focused on *Lactobacillus*, samples C and D were not carried forward to the assay.

Lactobacillus are widely studied and observed to exhibit many probiotic properties and this study focused on their antibacterial activity. Release of antibacterial compounds such as organic acids, bacteriocins and hydrogen peroxide is an important mechanism as mentioned previously.[8, 9] Some organic acids produced by *Lactobacillus* spp. are lactic acid, acetic acid, citric acid and butyric acid.[33] The most common organic acid involved is lactic acid, which is a main metabolic compound released by *Lactobacillus*.[34] The isolated *Lactobacillus* bacterial samples were encapsulated to compare the effect of encapsulation on the antibacterial effect of *Lactobacillus* with non-encapsulated bacteria. Encapsulation of bacteria has been observed to show improved viability and activity compared to non-encapsulated bacteria in various studies. Oberoi *et al.* [21] and Iznaga *et al*.,[22] observed better activity in microencapsulated cells compared to free cells. Larger zones of inhibition (ZOI) of encapsulated cells against pathogenic bacteria were observed by Oberoi *et al*. [21] and Phoem *et al*.[35] compared to free non-encapsulated cells.

In this study, none of the isolates *Lactobacillus delbrueckii* subsp. *jakobsenii* and *Lactobacillus rhamnosus* showed antibacterial activity. Both encapsulated and non-encapsulated bacteria were observed to show no ZOI against *Escherichia coli* and *Staphylococcus aureus*. These results are supported by Sharma *et al*.,[36] in which the CFS of *Lactobacillus* strains isolated from curd and breast milk showed no antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The *Lactobacillus* strains isolated

included *Lactobacillus delbrueckii* which is the same species isolated in this study as well. Sharma *et al*.,[36] also tested the antibacterial activity of the CFS of *Lactobacillus rhamnosus* as a reference strain which also showed no ZOI against *Escherichia coli* and *Staphylococcus aureus*. A study conducted by Hawas[32] observed that the CFS of some *Lactobacillus* species isolated from curd showed no ZOI. *Lactobacillus delbrueckii* at a low concentration showed no ZOI. CFS of *Lactobacillus rhamnosus* were also observed to show no ZOI against *Escherichia coli* and *Staphylococcus aureus* even when the CFS concentrations were increased. Another study by Jose, Bunt and Hussain[37], observed no inhibition of *Escherichia coli* by all *Lactobacillus* isolates which included *Lactobacillus rhamnosus*. They also observed that the *Lactobacillus* isolated from rumen of cows had better growth inhibition of pathogenic bacteria compared to the *Lactobacillus* isolated from dairy products. They thought it was probably because the *Lactobacillus* found in the gut may have to actively fight against these pathogens when encountered. Serrano-Niño *et al*.,[15] observed *Lactobacillus delbrueckii* to have antibacterial activity against *Escherichia coli* but not *Staphylococcus aureus* but Lopes *et al*.,[38] reported that *Lactobacillus delbrueckii* showed no ZOI against *Escherichia coli*.

In this study, the CFS was heat treated at 100ºC to check the antibacterial activity of antibacterial compounds on treating it with heat. The results showed no ZOI as mentioned before but, various studies have observed antibacterial compounds in CFS to be heat insensitive. Pompilio *et al*.,[39] heated the CFS at 70ºC and 100ºC to check if heat affects it and it was observed that there was no significant difference in the antibacterial potency between the heated and unheated CFS samples. Trabelsi *et al.,*[24] also heated the CFS of the samples up to 121ºC but observed no change on the antibacterial effect and clear ZOI were observed. Therefore, the reason for not observing an antibacterial activity in this study may not be due to the heat treatment of CFS of the samples.

One of the reasons for not observing ZOI could be due to the resistance of *Escherichia coli* and *Staphylococcus aureus* against the antibacterial compounds. Yang *et al*.[40], observed tolerance mechanisms of evolved acid-tolerant *Escherichia coli* against organic acids. The evolved strains of *Escherichia coli* showed an ability to maintain the intracellular ATP at high concentrations in increased acidic conditions, showed better cell membrane integrity compared to parental strains and showed increased accumulation of peptidoglycan in their cell wall by upregulating the respective genes resulting in a denser peptidoglycan layer.[40] This could block the entry of acid into the cells thus maintaining a better intracellular pH (pHi). *Escherichia coli* also can use acid resistance mechanisms that utilize protons and reduce proton influx by membrane modifications. Similar mechanisms are also present in *Staphylococcus aureus.*[41] In anaerobic conditions, protons in cytoplasm are converted to hydrogen gas by formate hydrogen lyase complex thereby preventing intracellular acidification.[42]

In *Staphylococcus aureus*, acetic acid dissociates in cytosol and the acetate ions interact with D-alanyl-D-alanine ligase (Ddl) and inhibit their activity. Panda *et al*.[43] observed that *Staphylococcus aureus* could resist acetate toxicity by alanine racemase which can increase the D-Ala-D-Ala pools. Some other studies have identified changes in gene expression in *Staphylococcus aureus* on exposure to acidic stress which help alleviate the intracellular acidification thereby ensuring survival.[44,45] A common response of *Staphylococcus aureus* to acid stress is increased urease activity, NADH mediated proton excretion and macromolecule repair mechanisms.[46]

Another reason could be because the potency of antibacterial activity is strain dependent. Qian *et al.,*[47] used *Lactobacillus*

delbrueckii as a reference strain and observed it to have a weaker activity compared to the other *Lactobacillus* spp. isolated from yoghurt. Jose, Bunt and Hussain[37] also observed some *Lactobacillus* spp. having antibacterial activity while some did not show any inhibition. Same was observed by Hawas[32] in which some *Lactobacillus* species exhibited ZOI against pathogenic bacteria while others did not. This could be due to the differences in gene expression. Some *Lactobacillus* spp. may contain a gene which has better antibacterial activity compared to others. Qian *et al.,*[47] also identified certain genes responsible for bacteriocin production. The *Lactobacillus* spp. that showed inhibitory activity had expressed these genes. Maybe the *Lactobacillus* isolated in this study lacks the genes involved in bacteriocin production and may also have reduced gene expression due to a mutation leading to poor antibacterial activity. An interesting observation reported by Osset *et al*.,[48] was that the inhibitory activity of the *Lactobacillus* spp. in solid and liquid medium were greatly different. *Lactobacillus* showed much better inhibition in liquid medium compared to solid medium. The species which showed good inhibitory activity in liquid medium failed to inhibit pathogen growth in solid medium. A similar observation was observed by Jamalifar *et al.,*[49]. The reason for this was thought to be due to better diffusion of antibacterial compounds in liquid medium. In this study, the antibacterial activity was assessed in solid medium thereby poor diffusion of antibacterial compounds in agar could be a potential reason for not observing any ZOI.

Above research evidences provide the possible reasons for no antibacterial activity. Identifying *Lactobacillus* species with good antibacterial activity and incorporating them in food products would be much more beneficial. Since antibacterial activity is strain dependent, use of different species together would give out a better result. The antibacterial activity of *Lactobacillus* could also be enhanced by incorporating genes responsible for bacteriocin and organic acid production. Ma *et al*.,[50] observed enhanced antibacterial activity of *Lactobacillus reuteri* after genetically modifying it through random mutagenesis. Bartkiene *et al*.,[51] utilized the byproducts of berries and fruits to improve the antimicrobial activity of some lactic acid bacteria. Similar mechanisms can be followed to enhance the antibacterial activity of *Lactobacillus* and incorporate them in the manufacture of probiotic food products thereby providing better health benefits to the consumer.

5. Conclusion

The *Lactobacillus* spp isolated from yoghurt were identified to be *Lactobacillus delbreuckii* and *Lactobacillus rhamnosus*. Both encapsulated and non-encapsulated *Lactobacillus delbreuckii* and *Lactobacillus rhamnosus* showed no antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Further research with a larger sample size is required to confirm the results. The antibacterial activity of *Lactobacillus* can be further improved by genetic modification and combining different strains together to confer better probiotic properties to humans.

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A verdant approach to the synthesis of silver nanoparticles using the leaf extracts of Jackfruits; estimation of the antioxidant and photocatalytic activity, antibacterial properties, melamine adulteration in milk, Para nitrophenol catalysis and cytotoxicity studies.

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Abstract

Nanotechnology is a vast area which attracts researchers due to molecules being within the nanoscale. *Artocarpus heterophyllus* is a medicinal fruit with many health benefits. In this study, five different variants of Jackfruit leaves; Red, Durian, Maharagama, Mandoor and Nirosha were used to synthesize silver nanoparticles (AgNP) via green approach. The shape and size of the AgNP was analyzed using a scanning electron microscope, which showed spherical AgNP in the range of 20-60 nm. The water extracts (WE) and their AgNP were evaluated on their total flavonoid content, total phenolic content and total antioxidant content, which showed higher values for AgNP. The antioxidant activity was determined via the 2,2-diphenyl-1 picrylhydrazyl assay and their IC_{50} value was determined, showing that the Durian and Mandoor WE had an IC_{50} higher than their AgNP. The cytotoxicity of AgNP was determined using *Artemia salina* and all of them had a 100% viability. The degradation of para-nitrophenol was also observed in the presence of AgNP and Durian_AgNP showed the highest degradation rate. Furthermore, the photocatalytic degradation of methylene blue was performed with 4000 ppm and 266.67 ppm AgNP. 4000 ppm AgNP showed a faster degradation in the presence of $NaBH_4$ and no visible degradation was observed under sunlight. Detection of melamine in milk was performed using Mandoor_AgNP, melamine was detected in water but not in milk. Lastly, the antibacterial activity was tested against *E. coli* and *S. aureus* using the well diffusion method. A zone of inhibition was observed with all the AgNP with *E. coli* and *S. aureus*. These research findings can be utilized in drug delivery, as alternatives for antibiotics and in the degradation of dye before entering the environment.

Keywords: *Artocarpus heterophyllus*, Green Synthesis, Scanning Electron Microscope (SEM), Silver Nanoparticles (AgNP), Melamine, Antioxidants

1. Introduction

Nanotechnology is a vast area that attracts many researchers due to its contribution in fields like science, engineering and computer science. "Nanoscience is the study of structures and molecules on the scales of nanometers ranging between 1 and 100 nm, and the technology that utilizes it in practical applications is called nanotechnology."[10]

Nanoparticles are materials that have a dimension within the nanoscale.[45] There are many types of nanoparticles out of which AgNP have drawn great attention in recent years due to its chemical stability, increased surface area to volume ratio, anti-microbial properties, catalytic activity,[71] along with optical, thermal and electrical conductivity.[79] AgNP has a variety of applications in anti-cancer therapy, wound healing and drug delivery.[15] It is also used in cosmetics, textile, electronics, sensors and water treatments.[32]

Metal nanoparticles can be synthesized using 2 approaches; the top-down approach and the bottom-up approach, illustrated in Figure 01. In the top-down approach, bulk materials are used as the starting material for nanoparticle synthesis. Bottom-up approach starts with atoms and builds on to produce nanoparticles.[76] Under these two approaches, there are three ways to synthesize nanoparticles, which are physical, chemical and biological methods.

Physical method uses techniques like laser ablation and evaporation-condensation.[36] This uses mechanical processes to reduce the size of the bulk material.[76] In chemical methods, the silver precursors are reduced by organic/ inorganic solvents which leads to AgNP synthesis.[46] Biological methods or the green approach involves the use of plants and microbes, causing it to be an eco-friendly, cost-effective, simple approach yet high yield of AgNP can be produced.[77] In this research plants were used as the use of microbes requires time and aseptic conditions.[2]

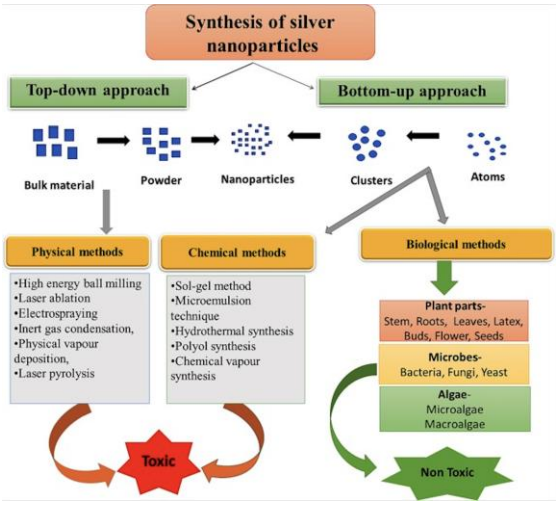


Figure 01: Different methods of AgNP synthesis.[20]

Artocarpus heterophyllus or Jackfruits are known for its taste, appearance as well as its health benefits. However, the pulp and seeds of the fruit is usually consumed and the other parts like the rind and leaves are often wasted despite them having many health benefits such as high antioxidant activity, the ability to control blood glucose levels, possessing anticarcinogenic, antimicrobial, anti-inflammatory and for wound healing.[55] Therefore, this research focuses on the synthesis of AgNP using Jackfruit leaves and performing various other tests from the synthesized AgNP and evaluating their results. In this research, five different variants of Jackfruit leaves were used: Red, Durian, Maharagama (MG), Mandoor and Nirosha.

Substances (natural or synthetic) that prevent or delay cellular damage caused by oxidants are known as antioxidants.[11] These oxidative stresses are associated with diseases like cancer, diabetes, atherosclerosis, Alzheimer's disease.[25] The AgNP can act as an antioxidant either by the single electron transfer or the hydrogen transfer and thus prevents or delay the damages caused to the cell by the oxidative stresses.[12]

Since nanoparticles can be used in the health field, it is important to test for its possible toxicity before its final use in the industry. *Artemia salina* (brine shrimps) are used to test for the lethality of AgNP. These are common model organisms, as it is cost-effective, has a short life cycle, increased offspring production and easy availability of the cysts.[8] The nauplii has a higher sensitivity to the toxic components compared to the adult brine shrimp. Other techniques to detect toxicity in cells include dye exclusion, colorimetric and fluorometric assays. However, these have their disadvantages such as insensitivity, time-consuming and labor intensive.[7]

Para-nitrophenol (PNP) is a toxic substance released from the dye, explosives and pesticides industries that causes harm to the environment, especially to the aquatic system and humans.[63] Since PNP degradation is too slow, the AgNP acts as a catalyst and allows the reaction to proceed. Advantages of AgNP involve them being biodegradable, cost-effective and the lack of secondary pollution production.[63] Despite there being other techniques like electro or photocatalyst and adsorption, these use high energy and expensive instruments.[75]

Synthetic dyes are widely used in the textile industries, where the waste is discarded into the water system. These dyes are either non-biodegradable[35] or can be transformed into carcinogens upon degradation of microbes present inside humans or animals. Even though, there are many wastewater treatment processes like reverse osmosis, incineration, filtration, electrochemical oxidation, they have their disadvantages such as the production of volatile toxic gases, increased reaction time, smelly odour, sludge formation and more.[60] Since AgNP have a large surface area, high adsorption properties and increased equilibrium rates, they can be used in the removal of these harmful dyes before entering the water system.[42]

Melamine is a chemical compound rich in nitrogen that is used as an adulterant in dairy

products, to increase their protein content. AgNP can be used in the detection of melamine (in milk) using colorimetric methods.[34] Melamine can also be detected by gas chromatography and high-performance liquid chromatography. In contrast, these techniques are time consuming, involve the use of expensive equipment, extensive sample preparation and require skilled personnel.[57]

Recently, antibiotics were becoming less effective against bacteria due to the increased usage and mutations in the bacterial cell. It is difficult to develop new antibiotics, as it is time consuming to study the efficacy and safety of the new drug and a large number of resources are needed. Meanwhile, the infections from the resistant microbes continue to spread.[14] Therefore, AgNP can be an excellent alternative to antibiotics, due to their antibacterial properties of releasing Ag^+ ions, inducing oxidative stress and the surface-binding of the bacteria to the AgNP, making it difficult for the bacteria to develop resistance against AgNP,[65] thus, allowing AgNP to be incorporated into medical products like catheters and other surgical tools.

This study aimed to investigate the use of leaf extracts from five different variants of Jackfruits to synthesize AgNP. The AgNP produced was used in assessing their antioxidant and photocatalytic activity, antibacterial properties, melamine adulteration in milk, para-nitrophenol catalysis and cytotoxicity studies. Scanning electron microscope (SEM) was used to analyze the shape and size of the AgNP. 2,2-diphenyl-1 picrylhydrazyl (DPPH) assay was used in the estimation of the antioxidant properties, whereas methylene blue (MB) and $NaBH_4$ was used in the estimation of the photocatalytic activity. As for the PNP catalysis, the synthesized AgNP was used to catalyze the reaction between PNP and $NaBH_4$. *E. coli* and *S. aureus* were used in the evaluation of the antibacterial properties via well diffusion and the cytotoxicity was tested on *Artemia salina*. Hence, utilizing this research results in wastewater treatment, industrial and medical fields.

2. Methodology

Throughout the research, a good laboratory practice was followed along with the use of personal protective equipment.

2.1 Sample Collection. In this research, five different variants of Jackfruit leaves, collected from Diyatha Uyana, Battaramulla were used. The variants used are Red, Durian, Maharagama (MG), Mandoor and Nirosha, illustrated in Figure 02.

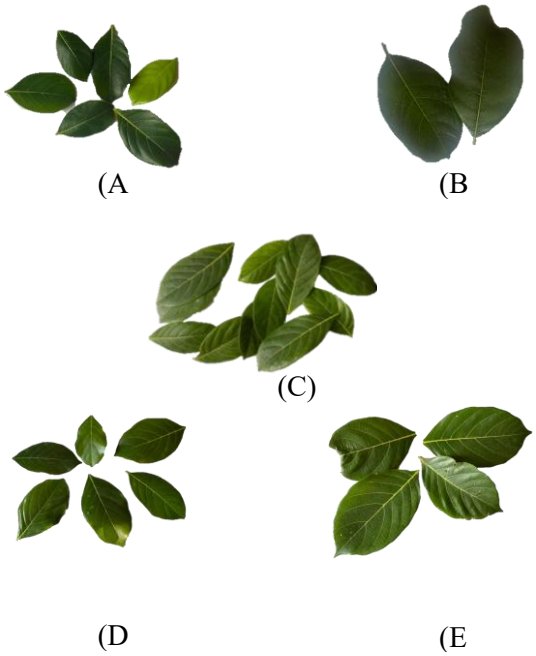


Figure 02: Different variants of Jackfruit leaves; (A) Red (B) Durian (C) MG (D) Mandoor (E) Nirosha

2.2 Preparation of water extraction of Jackfruit leaves. Dried Jackfruit leaves were cut into smaller pieces and 2 g of each variant was soaked in 50 mL distilled water ($d.H_2O$) . This was placed in the hot oven for 1 hour at 100 ºC and was then allowed to cool. Afterwards, all the five different water extracts (WE) were filtered using Whatman Filter paper and were stored at – 4 ºC until further use.[39]

2.3 Qualitative analysis of phytochemicals. This qualitative analysis was performed with WE.

Table 01: Shows the Phytochemicals test performed and their expected results.

Phytochemical Tests	Methodology	Expected Results
Alkaloids	A volume of 0.5 mL from each WE were placed into an oven to obtain residues. Afterwards, 1.5mL of the 2% (v/v) HCl was added into the WE residues and was dissolved. To this 1 drop of Mayer's reagent was added.	A white precipitate.[66]
Amino acids	Ninhydrin reagent (2 drops) was added into 0.5 mL of WE and was heated in a water bath	Colour change to blue.[13]
Anthocyanins	A volume of 0.5 mL of concentrated HCl was added into 0.5 mL of WE	Anthocyanins appear red in acidic conditions.[31]
Carbohydrates	To 0.5 mL WE, 2 drops of Iodine solution was added	Blue-black colour observed.[23]
Flavonoids	A volume of 125 µL of 1% $AlCl_3$ was added into 0.5 mL of WE.	Colour changes to yellow.[40]
Glycosides	WE (0.5 mL) were placed in an oven to reduce their volume in half. To this 0.5 mL of NH_4OH was added and was shaken.	Cherish red colour.[66]
Phenols	A total of 2 drops of 10% ferric chloride was added into 0.5mL of WE	Colour changes to black.[40]
Reducing sugars	To 0.5 mL of each WE, 1mL of Benedict's reagent was added and was heated in a water bath	Brick red indicates a positive result for reducing sugar.[13]
Saponin	A volume of 0.5 mL of the WE were added in their respective test tubes and were shaken vigorously using a Vortex meter.	Observation of foams on the surface of the test tube.[66]
Tannin	A total of 3 drops of 1% ferric chloride was added into 0.5 mL of WE	Colour changes to black.[13]

2.4 Synthesis of AgNP. A volume of 1 mL of the prepared WE was mixed with 9 mL of 0.02M $AgNO_3$.[41] This was then placed in the hot air oven at 90 ºC and 60 ºC for 15, 30, 45 and 60 minutes as well as at room temperature for 24 hours. A spectrophotometer reading was recorded in the range 320-520 nm with distilled water as the blank.

2.5 Evaluation of the Bandgap energy of AgNP. Bandgap energy is the energy required for an electron to move from the valence band (VB) to the conduction band (CB).[24]
Equation 01:

$$E = h\,x\,\frac{c}{\lambda}$$

E = Bandgap energy
h = Plank's constant (6.626×10^{-34} Js)
c = Speed of light (3×10^{8} ms^{-1})
λ = Wavelength of AgNP

2.6 SEM Analysis. An aliquot of Red_AgNP was centrifuged at 13,000 rpm for 30 seconds and the supernatant was removed, the sample was centrifuged each time after adding an aliquot of Red_AgNP and the supernatant was removed until a prominent pellet was obtained. Afterwards, this was allowed to dry in a hot air oven at 40ºC and was sent to Sri Lanka Institute of Nanotechnology (SLINTEC).

2.7 Quantitative analysis of TFC, TPC and TAC of WE and AgNP. The WE and AgNP were diluted before these studies were performed. All these studies were done in triplicates and d.H_2O was used as the blank.

2.7.1 Total Flavonoid Content (TFC)
A volume of 1 mL of 20% $AlCl_3$ was added into 1 mL of the samples and a drop of acetic acid was added. The absorbance was measured at 415 nm.[47] Then TFC was calculated using the quercetin as the standard curve and was expressed as µg/QE/100 g.

2.7.2 Total Phenolic Content (TPC)
A volume of 0.25 mL of 10% Folin-ciocalteu phenol reagent (FCR) was added into 0.25 mL of the sample and was mixed well. After 5 minutes, 2.5 mL of 7% Na_2CO_3 was added and was then incubated at room temperature for 90 minutes. The absorbance was then recorded at 765 nm.[47] Afterwards, TPC was calculated using the standard curve (gallic acid) and was expressed as g/GEA/100 g.

2.7.3 Total Antioxidant Capacity (TAC)

A volume of 5 mL of reagent solution was added into 0.5 mL of the sample. The reagent solution was prepared by mixing 0.60 $molL^{-1}$ H_2SO_4, 28 $mmolL^{-1}$ NaH_2PO_4 and 4 $mmolL^{-1}$ $(NH_4)_2MoO_4$ in a 1:1:1 ratio. The samples were then incubated at 95 ºC for 90 minutes. Afterwards, the samples were allowed to reach room temperature before the absorbance was measured at 695 nm.[72] TAC was then calculated using ascorbic acid as the standard curve and was expressed as g/AA/100 g.

2.8 Antioxidant Scavenging Activity of WE and AgNP via DPPH assay. DPPH solution (0.1 mM) was used as the control and methanol was used as the blank. Afterwards, 20, 40, 60, 80 and 100% of the diluted WE/AgNP were prepared and to this 1 mL of the DPPH solution was added. The absorbance was then recorded at 517 nm.[44]

Equation 02:

$$\%\ \text{Scavenging Activity} = \frac{Absorbance(DPPH) - Absorbance(Sample)}{Absorbance(DPPH)\ x\ 100} \times 100$$

Then, the IC_{50} values were calculated using the % scavenging activity (Equation 02).

*2.9 Cytotoxicity studies of AgNP on Brine shrimps***.** Brine shrimps were hatched in filtered sea water and were placed under a light source for 24 hours. In a 96 well plate, 800 ppm and 240 ppm of AgNP were added into separate wells and were filled to a total of 250µl using seawater. This was done in triplicates. A control was also prepared using only (250µl of) seawater. To this, 2 nauplii brine shrimps were added and were incubated for 24 hours next to a light source and the % viability was calculated[61] using Equation 03.

Equation 03:

$$\%\ \text{Viability} = \frac{Total No. of viable shrimps - Total No. of non viable shrimps \times 100}{Total\ No. of\ shrimps}$$

2.10 Degradation PNP using AgNP as a catalyst. Initially, 10^{-4} M of PNP and 10^{-1} M of $NaBH_4$ was prepared. The absorbance of PNP from 280-520 nm was used as the control. Then the readings were taken for a mixture of 2 mL of PNP and 1 mL of $NaBH_4$. Lastly, 20 µL of 4000 ppm AgNP was added into 2 mL of PNP and 1 mL of $NaBH_4$ and the absorbances were recorded until the complete degradation of PNP was observed.[29] d.H_2O was used as the blank and this procedure was repeated with all the AgNP. The rate constants were then calculated using equation 04.

Equation 04:

$$ln\ ln\left(\frac{Ct}{C0}\right) = -Kt$$

$$y = mx + c$$

$$mx = -kt$$

$$y = -kt + c$$

C_t = [PNP] at each interval
C_0 = Initial [PNP]
t = Time interval
k = Rate constant

2.11 Photocatalytic degradation of Methylene blue (MB) using AgNP. To 100 mL of 0.03 mM of MB, 1 mL of each 4000 ppm and 266.67 ppm of AgNP was added and the absorbance was recorded from 400-720 nm. This was done for 2 hours with a 30-minute interval. This was then repeated with the addition of 1 mL of $NaBH_4$.[33] A control was also recorded using only MB and d.H_2O was used as the blank.

2.12 Melamine Detection in AgNP. Concentrations of 2 ppm and 8 ppm melamine were spiked by adding 600 µL of melamine to 800 µL of 4000 ppm Mandoor_AgNP. The absorbance was measured from 320-720 nm and d.H_2O was used as the blank.

2.12.1 Melamine Adulteration in milk. Melamine spiked in milk involved the heating of 100mL of fresh milk, till it reached 90ºC and was allowed to cool down to 60ºC. To this, 25 drops of 0.25M of citric acid were added and kept at room temperature for 30 minutes.. Then it was centrifuged for 20 minutes at 4000 rpm. Afterwards, the supernatant was filtered twice using a Whatman filter paper. Then the absorbance of 600µl of milk, 600µl of melamine and 800µl of Mandoor_AgNP was measured from 320-720nm along with the absorbance of 600µl of milk and 800µl of AgNP.[54]

2.13 Antibacterial Activity. Initially, 15.2 g of Mueller-Hinton agar was dissolved in 400 mL of d.H2O and was heated on a Bunsen burner

until the agar was fully dissolved and the media turned clear. This was then autoclaved at 121 ºC for 15 minutes and was poured into labelled petri dishes and was allowed to solidify. The petri dish was divided into 4 and was labelled as Positive (+), Negative (-), Sample 1 (S_1) and Sample 2 (S_2) (Figure 03). The *E. coli* and *S. aureus* inoculum was prepared by using sterile cotton swabs to collect each species and this was then suspended in individual test tubes containing saline. Afterwards, the plates were inoculated with their respective inocula. Following this, two drops of WE/AgNP were added into the S_1 and S_2 wells producing duplicates of the sample. The samples were prepared by adding 1 mL of WE and AgNP to individually labelled watch glasses and this was allowed to dry in a hot air oven until residues were formed. This was then dissolved in 200µl of d.H2O and was then added into the wells. Saline (two drops) was used as the negative control and Gentamycin was the positive control. This was then incubated at 37 ºC for 24 hours and the zone of inhibition (ZOI) was measured.[16]

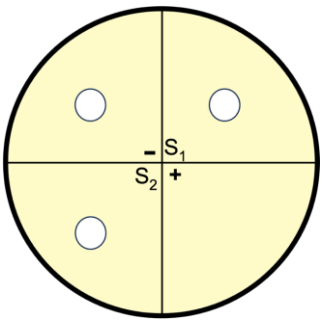


Figure 03: A labelled petri dish with wells only on -, S_1 and S_2

2.14 Statistical Analysis. The results obtained from each of these tests were then tabulated in Microsoft Excel Version 16.75.2 and were subjected to statistical analysis using SPSS. Comparison between species were performed via the one-way Anova test for TFC, TPC, TAC and antibacterial activity. Pearson's correlation was done to observe the correlation between TFC, TPC and TAC.

3. Results

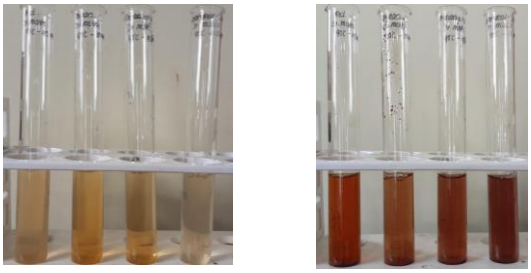
3.1 Qualitative Analysis of Phytochemicals present in the WE

Table 02*:* Qualitative studies of Phytochemicals present in WE (✓ indicates present and ✗ indicates not present)

Phytochemicals	Red	Durian	MG	Mandoor	Nirosha
Saponins	✓	✓	✓	✓	✓
Glycoside	✓	✓	✓	✓	✓
Alkaloid	✓	✓	✓	✓	✓
Reducing Sugar	✓	✓	✓	✓	✓
Phenols	✓	✓	✓	✓	✓
Flavonoid	✓	✓	✓	✓	✓
Tanins	✓	✓	✓	✓	✓
Carbohydrate	✓	✓	✓	✓	✓
Amino acids	✗	✗	✗	✗	✗
Anthocyanin	✗	✗	✗	✗	✗

All the WE showed a positive result for the phytochemicals except for amino acids and anthocyanins.

3.2 Synthesis of AgNP using Jackfruit WE



(A) (B)

Figure 04: Synthesis of AgNP before (A) and after (B) incubation

Table 03: Optimization of AgNP (✓ indicates synthesized and ✗ indicates not synthesized)

Samples	Incubation at 90 ºC				Incubation at 60 ºC			
	Time (mins)				Time (mins)			
	60	45	30	15	60	45	30	15

Red	X	✓	X	X	X	X	X	X
Durian	X	X	✓	X	X	X	X	X
MG	X	✓	X	X	X	X	X	X
Mandoor	X	✓	✓	X	X	X	X	X
Nirosha	X	X	X	X	X	X	✓	X

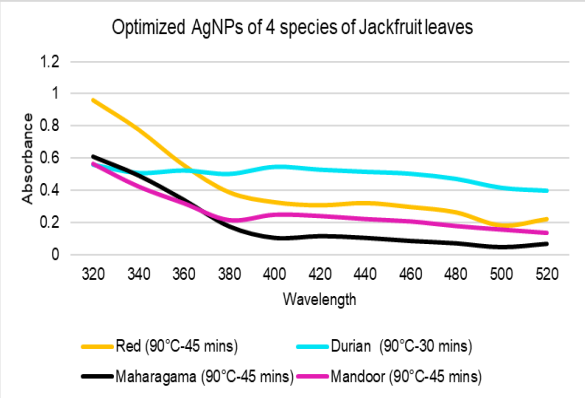


Figure 05: Absorbances of optimized AgNPs of 4 different species of Jackfruit leaves at 90 ºC - 45 minutes and 90 ºC - 30 minutes.

Red, MG and Mandoor showed the presence of AgNP at 90ºC- 45 minutes and Durian and Mandoor showed AgNP presence at 90ºC- 30 minutes and AgNP was detected in Nirosha at 60 ºC- 30 minutes. A colour change to dark brown was first observed (Figure 04) and the variant 'Durian' had the highest absorbance (Figure 05).

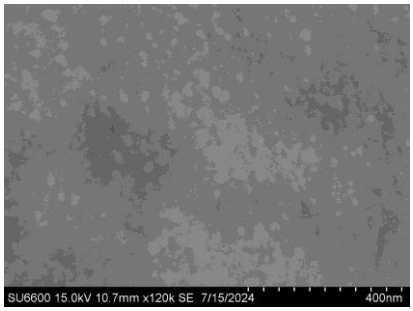
3.3 Bandgap energy of AgNP

Table 04: The band gap energy of AgNP.

Sample	Bandgap energy (eV)	Classifications
Red_AgNP	2.82	Semi-conductors
Durian_AgNP	3.10	Semi-conductors
MG_AgNP	2.95	Semi-conductors
Mandoor_AgNP	3.10	Semi-conductors

3.4 SEM Analysis

(A)



(B)

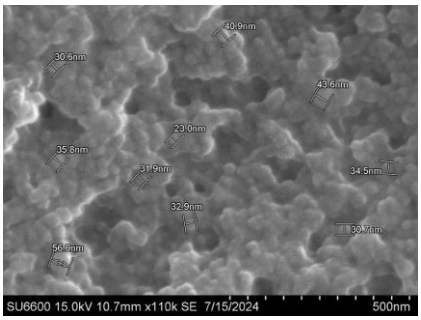


Figure 06: SEM analysis of Red_AgNP at 15.0 kV 10.7 mm x 120 k, 400 nm (A) 15.0 kV 10.7 mm x 110 k, 500 nm with diameters (B)

SEM analysis showed spherical shapes of Red_AgNP with diameters in the range 20-60 nm.

3.5 Quantitative Analysis of TFC, TPC and TAC of WE and AgNP

3.5.1 TFC

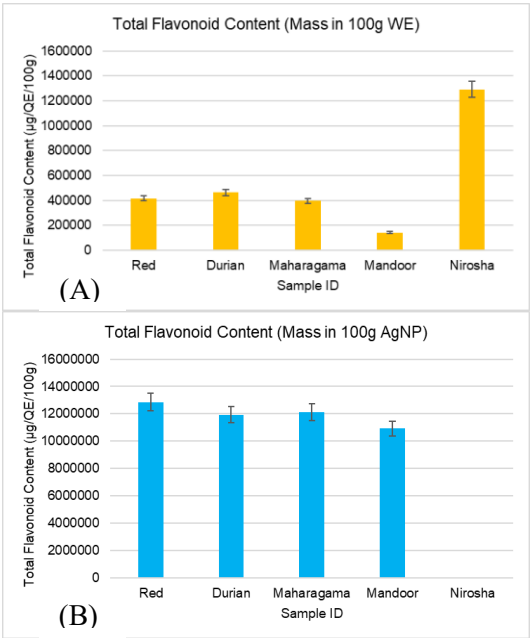
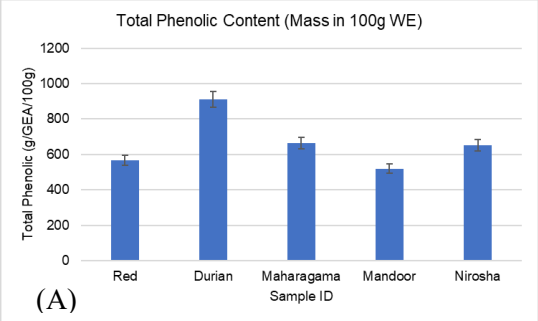


Figure 07: Average TFC of (A) WE and (B) AgNP measured in triplicates

3.5.2 TPC



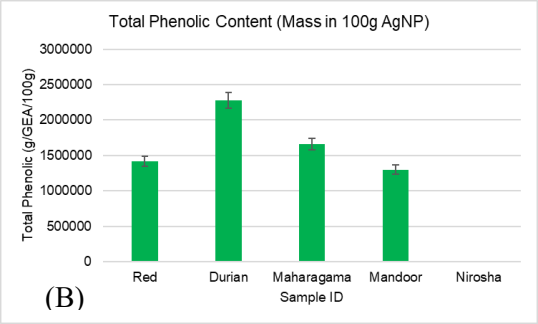


Figure 08: Average TPC of (A) WE and (B) AgNP measured in triplicates

3.5.3 TAC

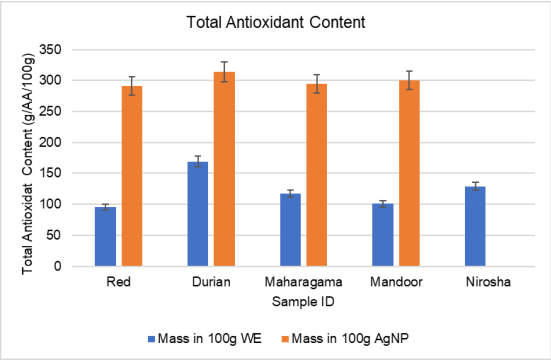


Figure 09: Average TAC of WE and AgNP measured in triplicates

The TFC, TPC and TAC of AgNP were higher than WE and there was a significant difference ($p < 0.05$) between the AgNP and WE.

3.6 Antioxidant Scavenging Activity of WE and AgNP via DPPH assay.

Table 05: IC_{50} values of WE and AgNP

Sample ID	WE	AgNP
Red	0.36	0.39
Durian	2.43	0.04
MG	0.081	0.39
Mandoor	0.30	0.19
Nirosha	0.13	-

The IC_{50} of Durian and Mandoor WE were higher compared to their AgNP.

3.7 Cytotoxicity studies of AgNP on Brine shrimps

Table 06: Viability of Brine shrimps in 800 ppm AgNP

Sample	Well 01	Well 02	Well 03	Viability	Results
Red	02/02	02/02	02/02	100%	
Durian	02/02	02/02	02/02	100%	
MG	02/02	02/02	02/02	100%	
Mandoor	02/02	02/02	02/02	100%	
Control	02/02	02/02	02/02	100%	

Table 07: Viability of Brine shrimps in 240 ppm AgNP

Sample	Well 01	Well 02	Well 03	Viability	Results
Red	02/02	02/02	02/02	100%	
Durian	02/02	02/02	02/02	100%	
MG	02/02	02/02	02/02	100%	
Mandoor	02/02	02/02	02/02	100%	
Control	02/02	02/02	02/02	100%	

The results showed that the brine shrimps had a 100% viability in the presence of both 240 ppm and 800 ppm AgNP.

3.8 Degradation PNP using AgNP as a catalyst.

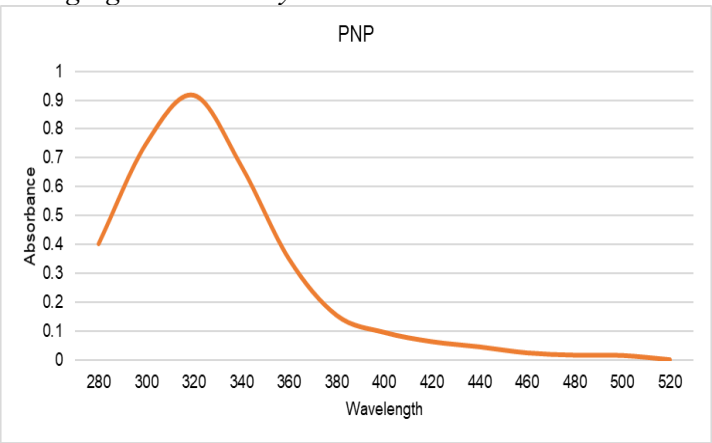


Figure 10: Absorbance of PNP

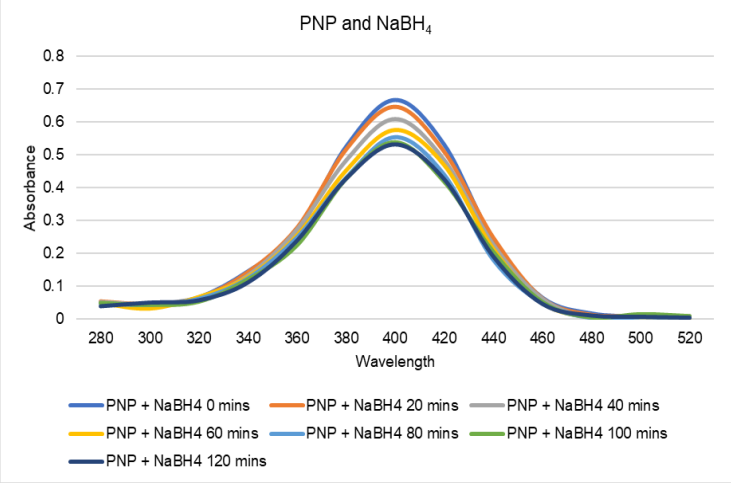


Figure 11: Absorbance during PNP degradation by $NaBH_4$ over 2 hours (without AgNP)

Degradation was not observed by the addition of $NaBH_4$ to PNP.

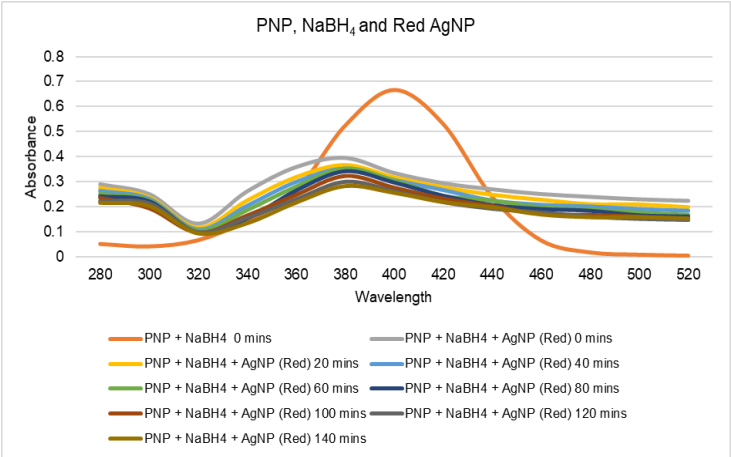


Figure 12: Absorbance of PNP and $NaBH_4$ in the presence of Red_AgNP

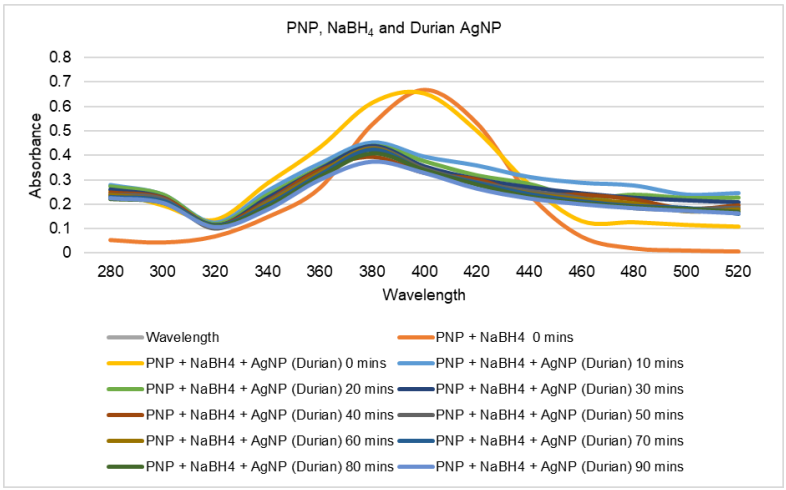


Figure 13: Absorbance of PNP, $NaBH_4$ in the presence of Durian_AgNP

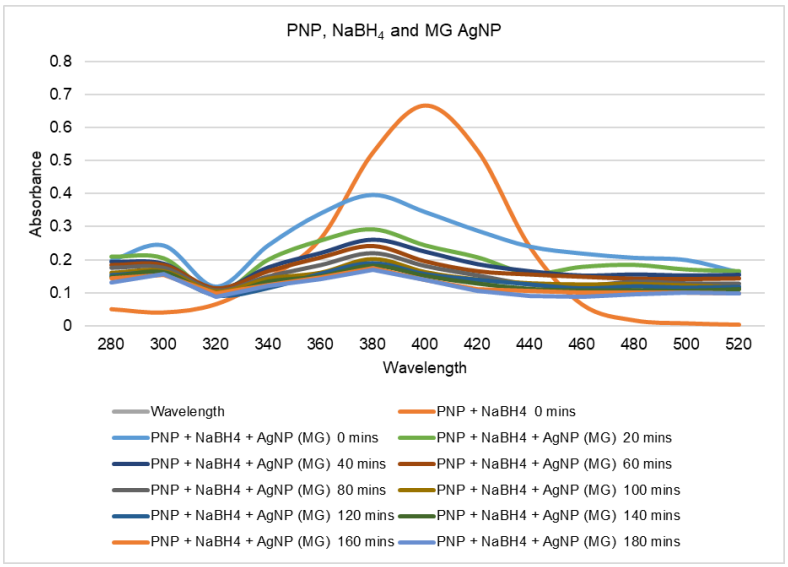


Figure 14: Absorbance of PNP, $NaBH_4$ in the presence of MG_AgNP

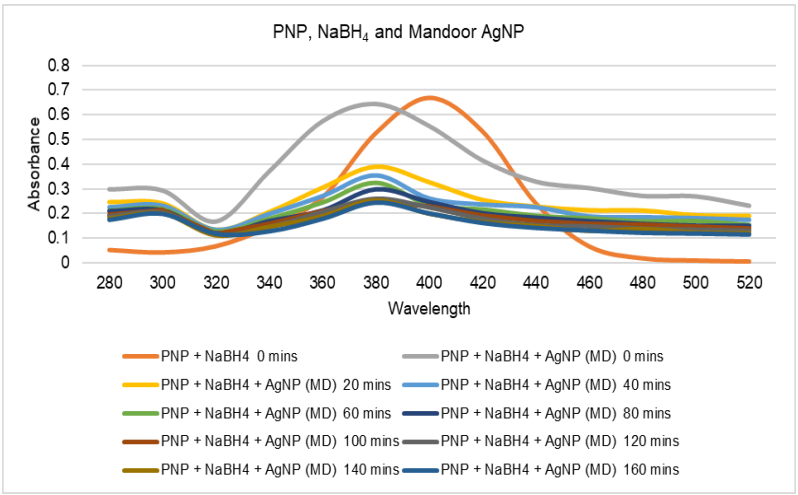


Figure 15: Absorbance of PNP, $NaBH_4$ in the presence of Mandoor_AgNP

PNP degradation was observed with all the AgNP.

3.9 Photocatalytic degradation of MB using AgNP

3.8.1 Degradation of MB under sunlight using AgNP

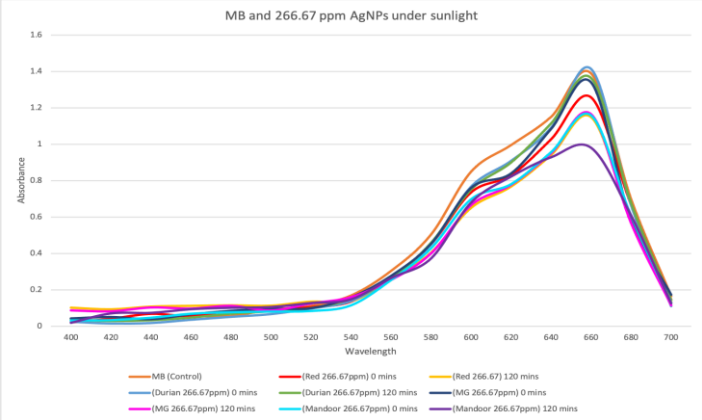


Figure 16: Absorbance of MB and all four 266.67 ppm AgNPs at 0 and 120 minutes

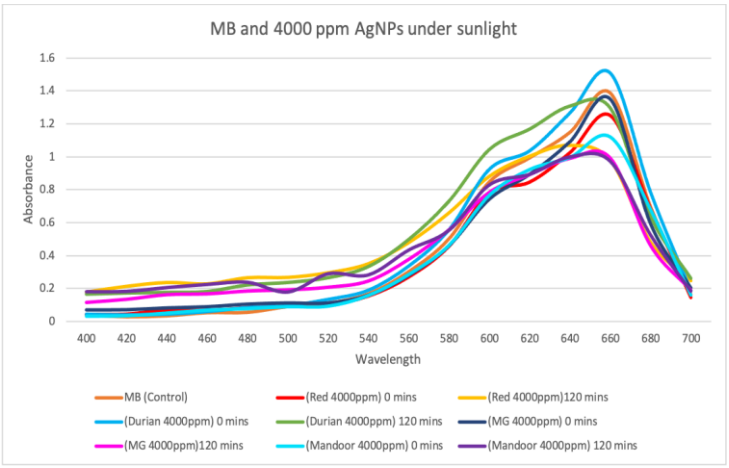


Figure 17: Absorbance of MB and all four 4000 ppm AgNPs at 0 and 120 minutes

MB did not show any degradation with AgNP, under sunlight.

3.8.2 Degradation of MB under sunlight using AgNP and $NaBH_4$

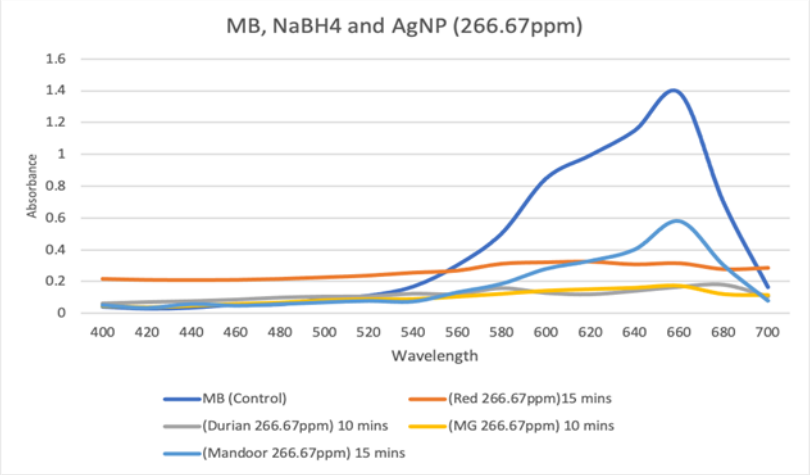


Figure 18: Absorbance of MB, $NaBH_4$ and all four 266.67 ppm AgNP

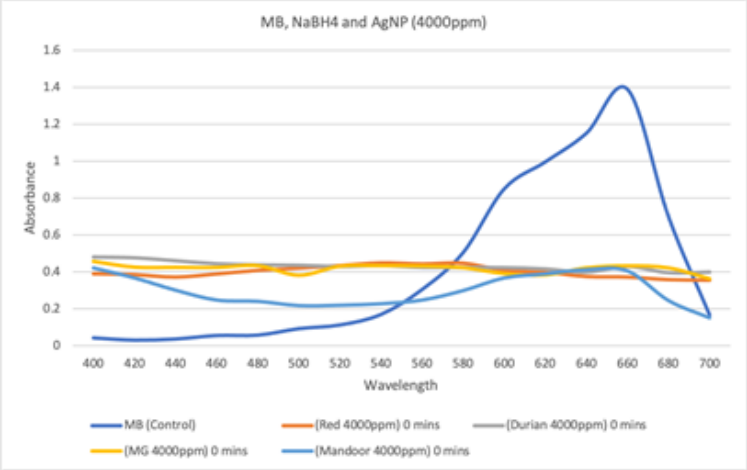


Figure 19: Absorbance of MB, $NaBH_4$ and all four 4000 ppm AgNP

An instant degradation was observed with 4000 ppm AgNP and $NaBH_4$ compared to 266.67 ppm and $NaBH_4$, which took 10-15 mins.

3.10 Melamine adulteration in milk

3.9.1 Detection of melamine in water

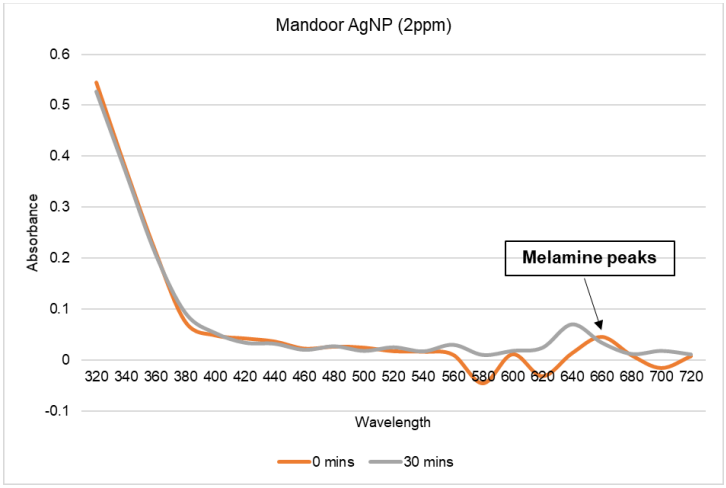


Figure 20: Melamine detection in water (2ppm).

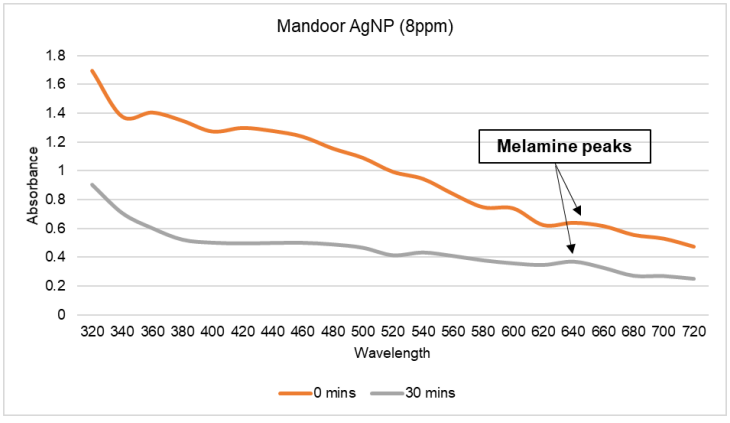


Figure 21: Melamine detection in water (8ppm).

Peaks were observed with 2 ppm and 8 ppm melamine, when spiked in water (Figure 20 and 21).

3.9.2 Melamine adulteration in milk using Mandoor_AgNP

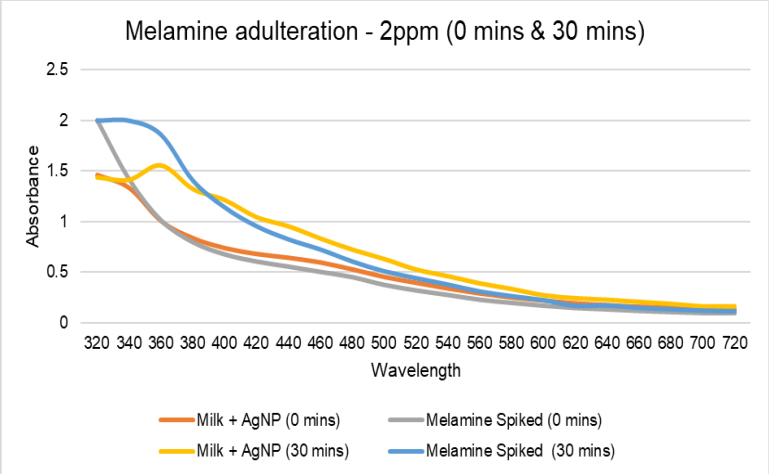


Figure 22: Melamine adulteration in milk (2 ppm)

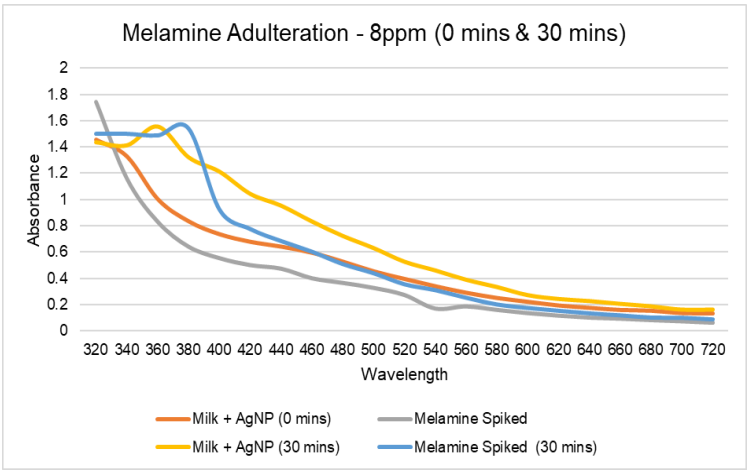


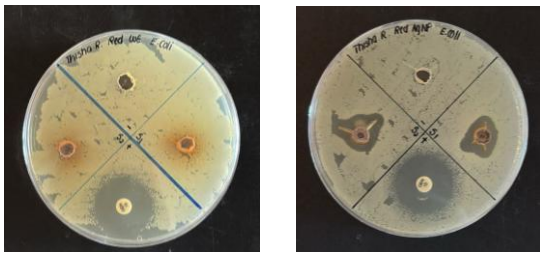
Figure 23: Melamine adulteration in milk (8 ppm).

No peaks were observed in melamine adulteration using milk (Figure 22 and 23).

3.11 Antibacterial Activity of WE and AgNP

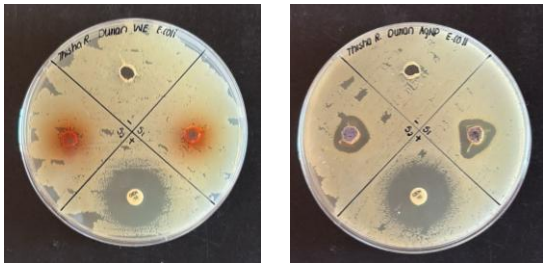
Table 08: Antibacterial activity of WE and AgNP using *E. coli*

Sample ID	S_1 (cm)	S_2 (cm)	Average (cm)	(+) (cm)
Red_WE	-	-	-	2.2
Durian_WE	-	-	-	2.2
MG_WE	-	-	-	2.2
Mandoor_WE	-	-	-	2.4
Nirosha_WE	-	-	-	2.4
Red_AgNP	1.2	1.5	1.35	2.2
Durian_AgNP	1.3	1.3	1.3	2.2
MG_AgNP	1.4	1.3	1.35	2.3
Mandoor_AgNP	1.3	1.2	1.25	2.2



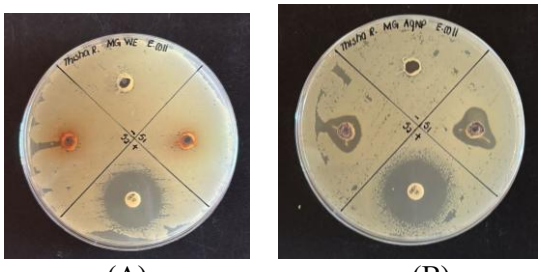
(A) (B)

Figure 24: Antibacterial activity of Red WE (A) and AgNP (B) using *E. coli*



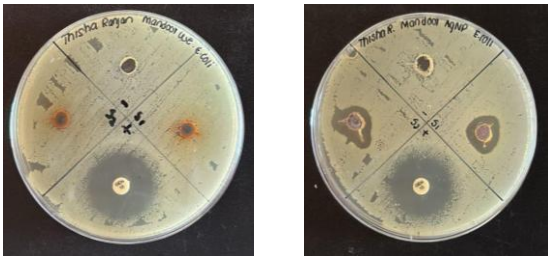
(A) (B)

Figure 25: Antibacterial activity of Durian WE (A) and AgNP (B) using *E. coli*



(A) (B)

Figure 26: Antibacterial activity of MG WE (A) and AgNP (B) using *E. coli*



(A) (B)

Figure 27 : Antibacterial activity of Mandoor WE (A) and AgNP (B) using *E. coli*

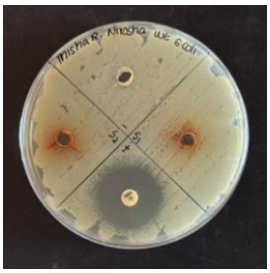
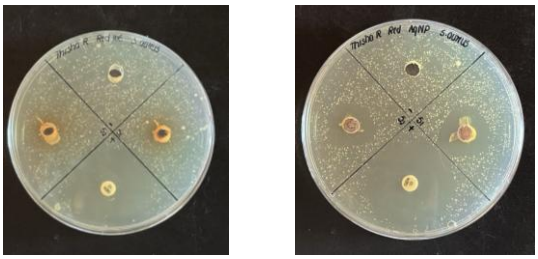


Figure 28 : Antibacterial activity of Nirosha WE using *E. coli*

Antibacterial activity was observed in all the AgNP whereas no activity was seen in WE with *E. coli* (Figure 24 - 28).

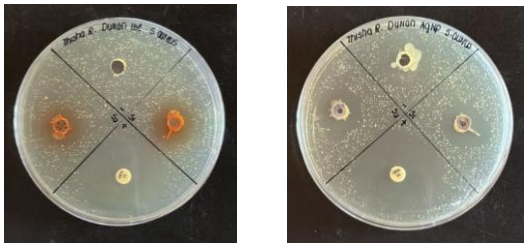
Table 09: Antibacterial activity of WE and AgNP using *S. aureus*

Sample ID	S_1 /(cm)	S_2 /cm)	Average /(cm)	(+) /(cm)
Red_WE	-	-	-	3.5
Durian_WE	1.5	1.2	1.35	3.5
MG_WE	1.1	1.1	1.1	3.5
Mandoor_WE	-	-	-	3.4
Nirosha_WE	1.2	1.1	1.45	3.4
Red_AgNP	1.4	1.5	1.5	3.3
Durian_AgNP	1.5	1.5	1.55	3.4
MG_AgNP	1.5	1.6	1.6	3.4
Mandoor_AgNP	1.6	1.6	1.45	3.4



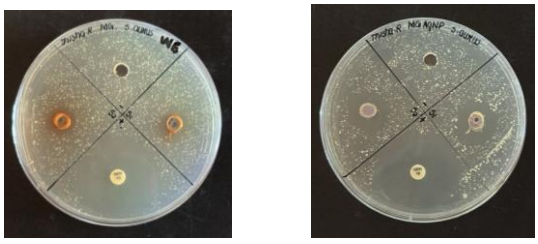
(A) (B)

Figure 29 : Antibacterial activity of Red WE (A) and AgNP (B) using *S. aureus*



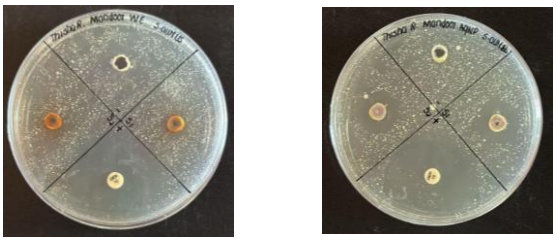
(A) (B)

Figure 30 : Antibacterial activity of Durian WE (A) and AgNP (B) using *S. aureus*



(A) (B)

Figure 31 : Antibacterial activity of MG WE (A) and AgNP (B) using *S. aureus*



(A) (B)

Figure 32: Antibacterial activity of Mandoor WE (A) and AgNP (B) using *S. aureus*

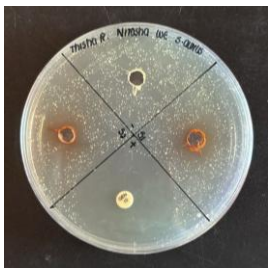


Figure 33: Antibacterial activity of Nirosha WE using *S. aureus*

Antibacterial activities were observed only with Durian, MG and Nirosha WE and all the AgNP showed a ZOI with *S. aureus* (Figure 29-33).

The Anova table showed a p value less than 0.05 ($p = 0.00185$) indicating a significant difference between the ZOI of *E. coli* and *S. aureus*.

4. Discussion

Jackfruit leaves are usually wasted despite them having many medicinal properties. In addition, research on jackfruit and its parts are limited, altogether, leading us to conducting this research. Since, this research followed the green approach, water was used as the extraction solvent as it is environmentally friendly. Phytochemical analysis showed the presence of saponins, glycosides, alkaloids, reducing sugar, phenols, flavonoids, tannins and carbohydrates in WE.[19] However, negative results were obtained for both amino acids and anthocyanins.

Ag^+ is reduced to Ag by the phytochemicals present in the plants as in Figure 34. Phytochemicals act as stabilizing, reducing and capping agents, resulting in AgNP formation.[52] Nanoparticle synthesis is affected by the incubation time, temperature, components/concentrations of WE, pH and metal ion concentrations.[18]

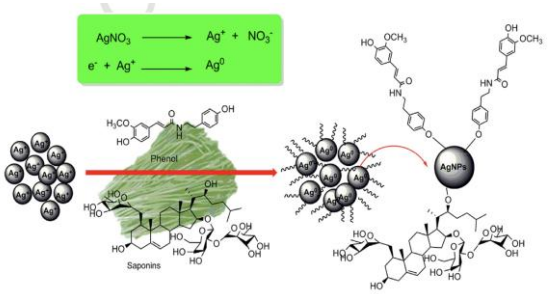


Figure 34: Green synthesis of AgNP.[28]

The first observation was a colour change from pale yellow to dark brown (Figure 04) due to the excitation of the Surface Plasmon Resonance (SPR). The oscillations of the conductive electrons on the AgNP surface due to excitation by light is known as the SPR.[28] Red, MG and Mandoor showed AgNP at 90 ºC - 45 minutes whereas at 90 ºC - 30 minutes, AgNP was detected in Durian and Mandoor and at 60 ºC - 30 minutes for Nirosha. These temperatures were also observed with Anu and her team.[4] The detection of AgNP in the range 400-500 nm is also due to the SPR.[5]

Semi-conductors have a bandgap energy < 3eV and insulators have a bandgap energy >4eV. Since some AgNP had a bandgap energy >3eV

but <4eV (Table 04), they were also classified as semi-conductors.

The shape and size of Red_AgNP was determined using SEM.[6] SEM analysis showed spherical AgNP of sizes in between 20-60nm (Figure 06), similar findings were also shown by Manjare.[41] However, AgNP clustering was observed[27] and this could be due to the increased Ag^+ concentration or increased pH of the extracts.[9]

The principles of TFC, shown in Figure 35, involves $AlCl_3$ forming a stable acid complex with the ketone groups along with the orthodihydroxyl group of the flavonoids, giving a yellow colour.[38] The TFC of AgNP were higher than their WE. TFC was high in Nirosha_WE and Red_AgNP (Figure 07). A p value of $2.14x10^{-8}$, shows a significant difference between AgNP and WE.

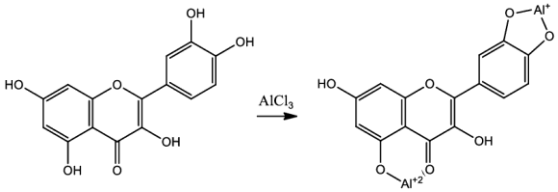


Figure 35: Principles of quantitative TFC.[37]

In TPC, the oxidation of the phenol group by the FCR enables the reduction of the phosphomolybdate-phosphotungstate in the FCR into molybdenum-tungsten complex (Figure 36), giving rise to a blue complex. Na_2CO_3 provides an alkaline atmosphere which facilitates the dissociation of protons from the phenolic compound.[49]

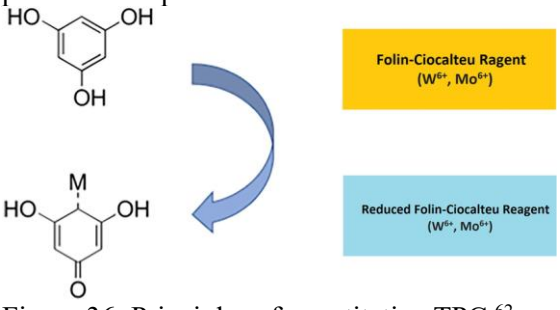


Figure 36: Principles of quantitative TPC.[62]

The TPC of AgNP was higher than the WE. Durian_WE and AgNP showed the highest TPC value (Figure 08). A p value of $5.37x10^{-5}$ ($p < 0.05$) suggests a significant difference between the TPC of the WE and AgNP.

TAC assay involves the reduction of molybdenum (VI) into molybdenum (V) in the presence of an antioxidant, giving a greenish-blue colour (Figure 37). The TAC of AgNP was higher than the WE and Durian_WE and AgNP had the highest TAC value (Figure 09). A p value of $8.67x10^{-6}$ ($p < 0.05$) shows a significant difference between the WE and AgNP. However, studies were not conducted on Jackfruits to support this.

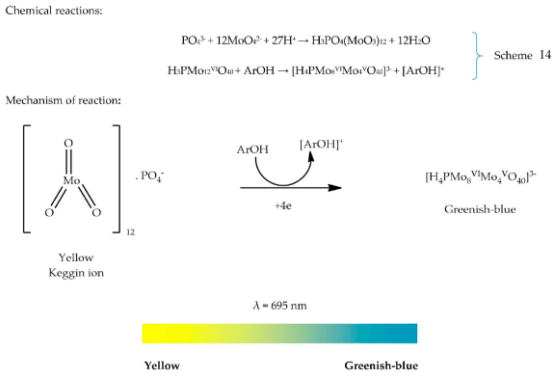


Figure 37: Principles of Quantitative TAC.[59]

The TFC, TPC and TAC of the AgNP were higher than their respective WE. However, the increase in TFC and TPC in WE compared to AgNP[17] could be due to different locations, variants and the involvement of the phytochemicals during AgNP synthesis.[67]

A strong correlation was observed among TFC, TPC and TAC in the Pearson correlation analysis (Figure 38). The TFC-TAC was higher compared to the others, indicating that flavonoids were the predominant phenolic compounds in jackfruit leaves that attributed to their antioxidant activities.[78]

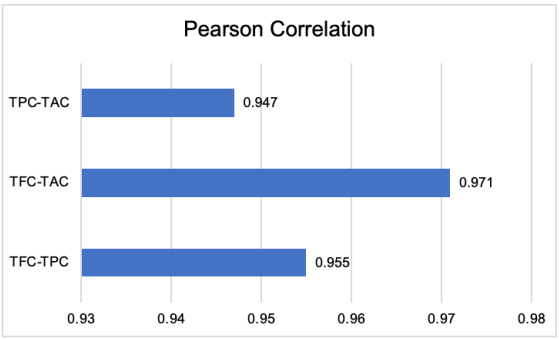


Figure 38: Pearson correlation between TFC, TPC and TAC

DPPH is a stable free radical that contains free electrons in its nitrogen atom which can be reduced by the H atom of the antioxidant.

DPPH has a deep purple colour which changes to pale yellow colour when it is reduced. There is a strong absorbance at 517 nm due to the presence of an odd electron. The AgNP's antioxidant activity, in Figure 39, is due to the bioactive molecules and secondary metabolites on the AgNP surface.[30]

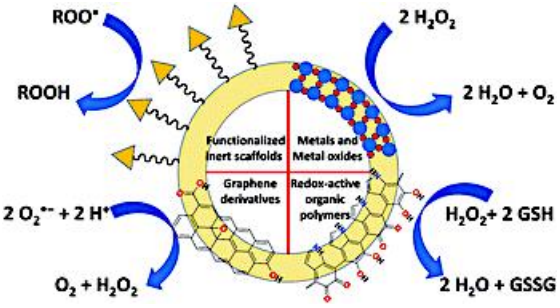


Figure 39: Antioxidant activity of Nanoparticles.[69]

The IC_{50} is the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals.[48] A high antioxidant activity is indicated by a low IC_{50} value. The results showed that Durian and Mandoor WE along with Red and MG AgNP had higher antioxidant activities (low IC_{50}) than their respective AgNP/WE.[26] Antioxidant activity of WE could be due to their increased phenolic and flavonoid content.[17]

AgNP's cytotoxic effect involves the interaction between the chitin of the brine shrimp and the Ag^+ ions, leading to the deformation of the chitin structure and eventually death.[51] AgNP can also enter the cell through diffusion or endocytosis. AgNP or Ag^+ generates ROS and elevated ROS levels produces oxidative stresses which eventually results in damage to the DNA and proteins in the cell leading to cell apoptosis,[3] as shown in Figure 40.

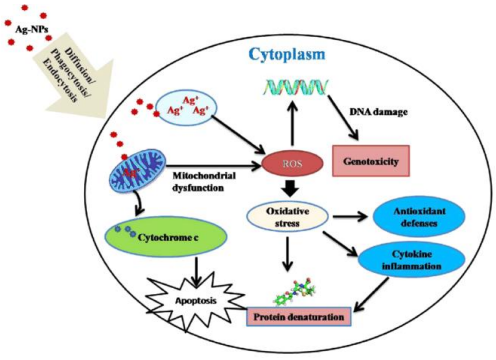


Figure 40: Cytotoxicity of AgNP.[3]

However, the brine shrimps had a 100% viability with 240 ppm and 800 ppm AgNP, due the production of AgNP via green synthesis. Phytochemicals involved in nanoparticle formation are usually compatible with the biological systems, resulting in a decreased cytotoxicity.[70]

PNP has 2 different absorbances based on its pH, 317 and 400 nm. In acidic conditions, a peak at 317nm is observed and upon the addition of $NaBH_4$, the peak shifts to the right and forms at 400nm (Figure 10).[73] PNP degradation to p-aminophenolate (PAP) by $NaBH_4$ is kinetically too slow due to the presence of a potential difference and a kinetic barrier between the donor (borohydride) and the acceptor (p-nitrophenolate ions), therefore, a slow degradation was observed with PNP and $NaBH_4$ (Figure 11).

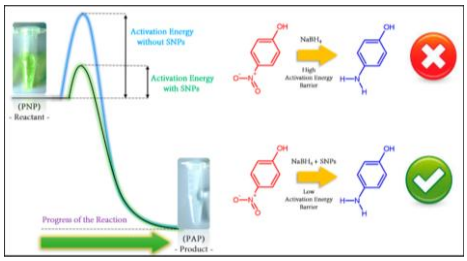


Figure 41: Catalytic degradation of PNP by AgNP.[63]

With AgNP, the addition of $NaBH_4$, forms hydrogen gas, which is adsorbed onto the AgNP. Thereafter, the adsorption of p-nitrophenolate ions onto the AgNP allows the reduction process, eventually causing the desorption of PAP from the AgNP. Hence, acting as a catalyst by facilitating the transfer of electrons from the donor to the acceptor and thus overcoming the kinetic barrier,[63] demonstrated in Figure 41. A colour change

from yellow to colourless also shows the degradation of PNP to PAP.[53]

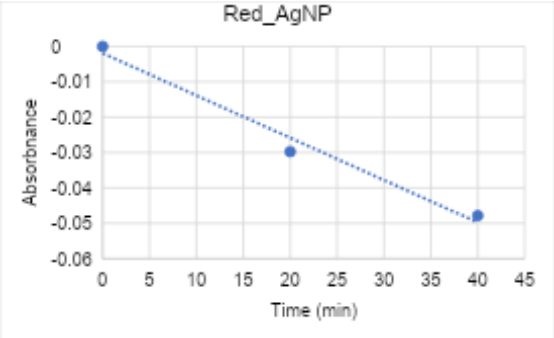


Figure 42: The rate of reaction for Red_AgNP

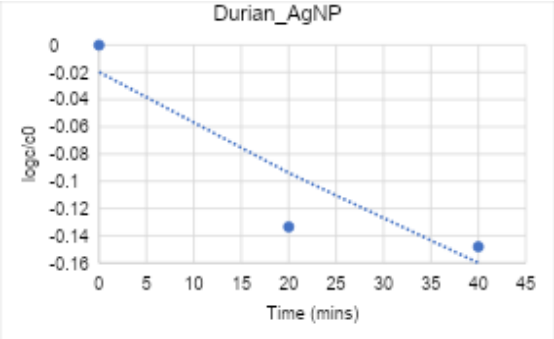


Figure 43: The rate of reaction for Durian_AgNP

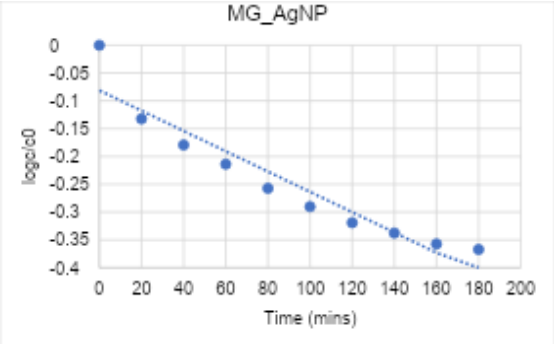


Figure 44: The rate of reaction for MG_AgNP

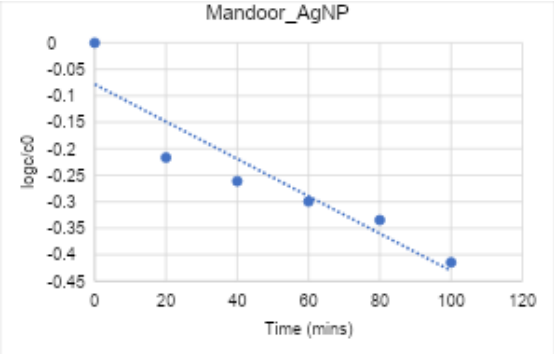


Figure 45: The rate of reaction for Mandoor_AgNP

Table 10: Rate constants of AgNP

Sample	Rate constant K
Red_AgNP	0.0012
Durian_AgNP	0.0037
MG_AgNP	0.0018
Mandoor_AgNP	0.0035

The reaction rate increased in the order Red < MG < Mandoor < Durian AgNP, shown in Figures 42-45 and table 10.

MB degradation by AgNP involves the excitation of the electrons in the VB into the CB upon irradiation. This generates an electron-hole which results in the production of hydroxyl radicals, which acts as an oxidizing agent, leading to the degradation of MB,[42] shown in Figure 46. In the presence of $NaBH_4$, adsorption of the BH_4^- onto the AgNP facilitates the transfer of electrons to MB eventually leading to a faster degradation,[22] explaining why there was no visible degradation of MB under sunlight, in the absence of $NaBH_4$.

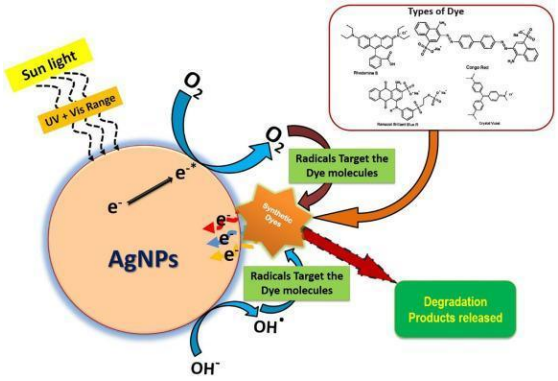


Figure 46: Photocatalytic degradation of MB using AgNP.[42]

With high concentrations of AgNP, there is an increased dispersion and an increased availability of active sites, explaining why an instant degradation of MB was observed with 4000 ppm AgNP and $NaBH_4$, whereas 10-15 minutes was needed for the complete degradation of MB using 266.67 ppm AgNP. The increase in absorbance observed with 4000 ppm AgNP and $NaBH_4$ after 0 minutes could be due to the agglomeration of excess AgNP.[68]

Since melamine has a high nitrogen content, it is used in milk adulteration.[56] AgNP plays a role in melamine detection via the electrostatic interaction between the negatively charged nanoparticle surface and the positively charged

amino group of melamine. The aggregation of the AgNP results in a colour change from yellow to red,[54] shown in Figure 47. A peak in the range 600 nm corresponds to the conversion of the dispersed AgNP into its aggregated form.[64]

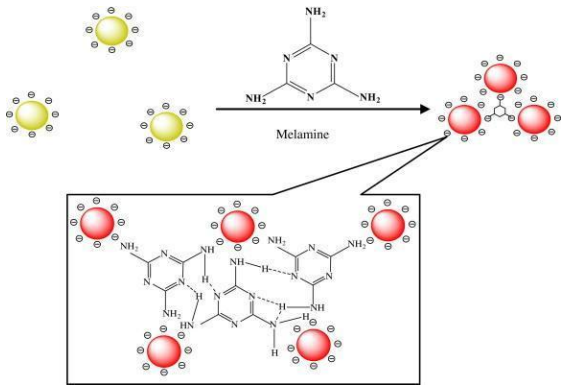


Figure 47: The MOA of Melamine detection by AgNP.[50]

Even though Mandoor_AgNP detected melamine (2ppm and 8ppm) in water, in milk, no peaks were observed for both AgNP and melamine, showing the absence of melamine in milk. The absence of AgNP peaks (Figure 22, 23), could be due to the interaction between existing whey proteins and AgNP, which inhibits the binding of melamine to the AgNP. However, this point was not proven with AgNP but this phenomenon was observed with gold nanoparticles,[43] assuming this could be the same with AgNP.

The AgNP exhibits its antibacterial activity by accumulating in the pits formed on the cell wall when they attach themselves, leading to the disruption of the cell wall and membrane permeability. The main MOA involves the release of Ag^+ from AgNP, which attaches to the bacterial cell wall and membrane via electrostatic interactions between the sulfur proteins, increasing the permeability of the cell membrane. Upon the penetration of the cell membrane, the Ag^+ deactivates the respiratory enzymes leading to the production of reactive oxidative species (ROS) which causes damage to the DNA. Since the DNA mainly consists of phosphate and sulphur, the Ag^+ interacts with the DNA affecting DNA replication. Furthermore, protein synthesis is also inhibited due to denaturation of the ribosome components,[1] shown in Figure 48.

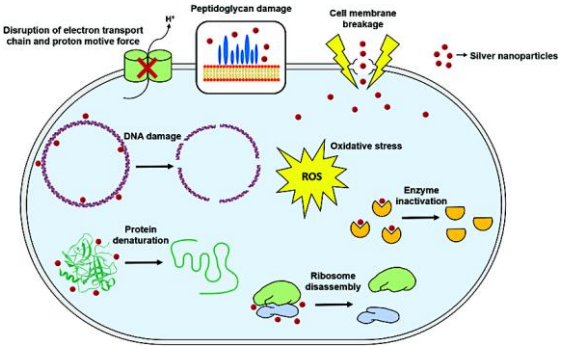


Figure 48: Antibacterial activity of AgNP.[58]

A ZOI was observed with all the AgNP in both *E. coli* and *S. aureus* but no ZOI was seen with WE in *E. coli*. However, Durian, MG and Nirosha WE showed a ZOI with *S. aureus*. This is due to the presence of phytochemicals, which gives WE their antibacterial properties, supported by the studies from Dhierllate.[21] The AgNP was more potent against *S. aureus* compared to *E. coli*, similar results were observed by Manjare.[41] The reason behind *S. aureus* being more sensitive to AgNP could be due to the lack of an outer film surrounding the peptidoglycan layers of the cell wall, allowing the straightforward interaction of AgNP and the bacterial outer membrane.[74] A p value of 0.002 shows a significant difference between the ZOI of *S. aureus* and *E. coli*.

5. Conclusion

In conclusion, AgNP was synthesized from 5 different variants of Jackfruit leaves. Optimization of AgNP occurred at 90 ºC -30 and 45 minutes and at 60 ºC - 30 minutes. All the AgNP were classified as semi-conductors. SEM analysis showed spherical AgNP of diameters between 20-60 nm. TFC, TPC and TAC were high in AgNP compared to their WE. The IC_{50} of Durian and Mandoor WE were found to be higher than their AgNP. Cytotoxicity study using *Artemia salina* showed 100% viability. Faster degradation of PNP was observed in Durian_AgNP. The photocatalytic degradation of MB was also rapid with all AgNP and $NaBH_4$. Mandoor_AgNP detected melamine in water but not in milk. All the AgNP showed antibacterial activity against *S. aureus* and *E. coli* and was more potent against *S. aureus*.

Therefore, AgNP synthesized from Jackfruit leaves can be used in many industries.

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Comprehensive analysis of effects of abiotic stress on the *Spondias dulcis* plant and evaluating the antibacterial properties

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Abstract

Plant stress is the term used to describe environmental factors that cause abrupt changes in respiration, photosynthesis, senescence, flowering, seed germination and development, cellular metabolism, gene expression, and growth in plants, all of which reduce crop yields and productivity. The detrimental effects that abiotic influences have on plant tissues are known as abiotic stress. In contrast to biotic stress, which is triggered by living organisms, abiotic stress is caused by non-living elements. This study investigates the morphological changes, and quantitative and qualitative evaluations of the phytochemical composition of *Spondias dulcis* under abiotic stress conditions such as drought, high salinity, flood, and nutrient deficiency, in addition to its antibacterial properties. The morphological changes of the stress-induced plants exhibited symptoms of abiotic stresses such as leaf yellowing, wilting, and sparser foliage. The qualitative assays indicated the presence of phytochemicals such as alkaloids, tannins, coumarins, phenols, cardiac glycosides, terpenoids, steroids, even under different stress conditions and indicated the absence of flavonoids and saponins. Drought had a considerable impact on total phenolic content, total antioxidant capacity and total protein content of the quantitative analysis. The total antioxidant capacity increased in response to high salinity. High salinity, nutrient deficiency, and flooding contributed to an increase in total flavonoid content. Methanolic extract of *S. dulcis* exhibited antibacterial activity, suppressing the growth of *Escherichia coli* and *Staphylococcus aureus*. The results of the investigation showed that Spondias dulcis has potential in medicine due to its antioxidant and antibacterial properties, as well as its ability to survive abiotic stressors in various habitats.

Keywords: *Spondias dulcis* leaves, Abiotic stress, Phenolic compounds, Antibacterial activity, Antioxidant activity

1. Introduction

External stresses that impact the plant's growth, yield and its life cycle are considered as plant stress. Plant stress can be divided into two major groups as biotics and abiotic stress (Figure 1). Biotic stress is a biological damage a plant undergoes such as disease or insects. Abiotic stress is a result of chemical or physical factors such as (light, water, or salt) which harm the plant and its surrounding environment. Stress triggers plants to exhibit abnormalities in their development and metabolic processes. When stress is minimal in plants they recover quickly. However, plants experiencing prolonged or excessive stress exhibit inhibition of their developmental processes, which leads to plant mortality.[1]

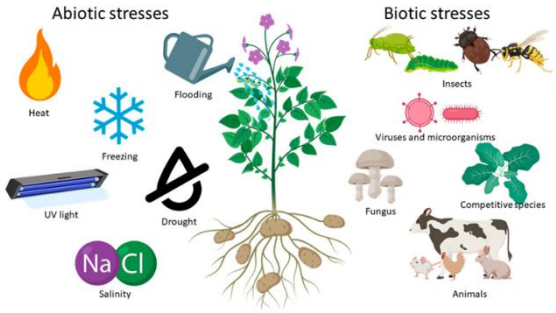


Figure 1. Several types of biotic and abiotic stresses that can affect plants.[2]

1.1 Abiotic stresses. The antagonistic effects of abiotic factors on a plant in a particular environment is referred to as abiotic stress. The stress influences biological processes like gene expression and cell metabolism that impacts growth and development.[3] Abiotic stressors include extreme temperature, changes in water

supply, extreme salt conditions, heavy metal contaminations, and nutritional stress. Different stressors elicit different effects like increase in reactive oxygen species and decrease in photosynthetic activity, plant growth and yield as shown in the Figure 2 and 3.[4]

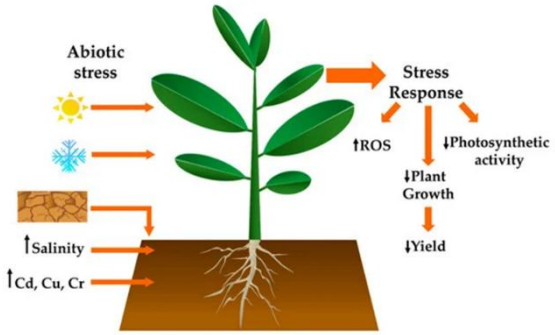


Figure 2. Abiotic stresses in plants and their stress responses.[5]

Water is crucial for plant survival and nutrient delivery. Since environmental conditions create a decrease in water in the soil, plants experience drought stress, resulting in an ongoing loss of water through transpiration or evaporation. This stress causes cellular water loss, plasmolysis, and cell death.[6]

Insufficient drainage infrastructure, increased soil deprivation and climate change has contributed to an increase in floods over the past few years.[7] Therefore, the flooding stress causes hypoxia and anoxia in plant tissues leading to cell death. Waterlogging stress in which the plants leaves and stems are partially submerged and submergence stress in which the plant is fully submerged, are the two types of flooding stress.[8]

Macronutrients such as Nitrogen (N), Phosphorous (P) and Potassium (K) and micronutrients such as Boron (B), Zinc (Zn) and Manganese (Mn) affects the plants functions in agriculture and natural ecosystems. The shortage of macronutrients and micronutrients negatively influences the growth of the plant leading to a nutrient deficient condition. Excess macronutrients have an adverse effect on the soil. Moreover, nutrients are responsible for antioxidant production. Absence of antioxidants can disrupt the plants system.[9]

Increased salt content in the soil causes salinity stress. This stress is a global and a life-threatening issue to the agricultural biosphere. Plant growth is hindered by high salt concentrations. The greater osmotic potential and specific ion toxicity damages the plant's development stages and inhibits seed germination, which adversely affect the quality and quantity of plant production.[10]

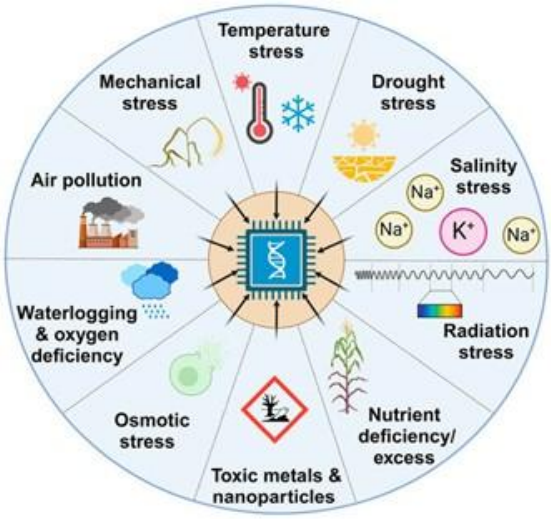


Figure 3. Types of abiotic stresses in plant tissues.[11]

1.2 Spondias dulcis. The genus *Spondias* (*Anacardiaceae*) consists of seventeen species; ten of which are indigenous to tropical Asia. This plant consists of edible fruits, tiny white flowers, and glossy leaves. *Spondias dulcis* is a fast-growing equatorial tree popular in Sri Lanka known as "Ambarella" while its commonly called as golden apple or hog plum.[12] The fruit can be eaten raw or used to make jams and juice drinks. Mature leaves are used in salads while young leaves and the fruit are cooked as a vegetable. Tribal members in Cambodia uses the bark of the plant to treat diarrhoea. The fruits and the leaves of *Spondias dulcis* have been utilized to treat eye infections, improve vision, reduce itching, internal ulcers, sore throats, and skin inflammation. The plant *Spondias dulcis* is more commonly used as a fruit in all parts of the world, which has numerous benefits due to the nutrient dense composition (Figure 4). The leaves also exhibit important properties such as antimicrobial, antioxidant, enzymatic inhibition and thrombolytic. High incident diseases like Alzheimer's, cancer, diabetes, and obesity have been studied as a novel treatment with the use of *Spondias dulcis* leaves.[13]

Figure 4. Ambarella leaves**.**

From sea level to 700m, the plant thrives in the subhumid, frost-free topics. *Spondias dulcis* can withstand 12-35℃, it thrives in regions with yearly daytime temperatures between 22-27℃ in response to abiotic stress conditions the leaves show signs of curling/wilting with yellowing and decreased lateral branching. While the plant can withstand temperatures as low as -3℃ when dormant, 0℃ can harm young plants. The plant can withstand 600-2200mm of rainfall per year but prefers 900-1800mm of rain and the leaves show slight wilting/ curling in response to the stress.[14] *Spondias dulcis* thrives on acid sand and soils formed from limestone, but the soil needs to be well drained. It can tolerate a pH between 4.5 and 8, preferring a pH between 5.5-6.5 for the plant growth. Although the plant may momentarily lose their leaves when in stress conditions, mature plants can withstand drought conditions.[15]

In recent years, scientists have used plants as sources of medications and bioactive substances which includes well known medicinal species but also plants are used as traditional medicines and food in several countries. The public interest in natural products and the significance of medicinal plants in the healthcare sector have increased due to the low toxicity, strong pharmacological action, and commercial availability. Moreover, plants contain bioactive constituents such as polyphenols, carotenoids, antioxidants, and proteins each of which exposes important biological activities.[16] To increase agricultural productivity and sustainability, it is crucial to investigate the effect of abiotic factors including heat, salinity, and drought on plants. Food security is at risk due to these pressures, which restrict crop yields, particularly considering climate change. Understanding how plants react to these circumstances can help us create crops that are more resilient, use water more efficiently, and manage resources effectively by promoting ecosystem protection.

Therefore, this study aimed to analyse the effects of abiotic stress on *Spondias dulcis* plant and evaluate the phytochemicals, antioxidant concentration and, antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus.*

2. Methodology

2.1 Inducing of abiotic stress for Spondias dulcis. Healthy *Spondias dulcis* plants were collected from Seed & Plant Retail Shop, a plant nursery located in Colombo 07. A total number of healthy forty-two plants were randomly selected and transported to the BMS campus premises for further processing to ensure consistency in the analysis. Seven *Spondias dulcis* plants were assigned to each of the drought, flood, nutrient deficiency, and high salinity conditions and the stress was induced according to Table 1. A batch of 7 plants were also maintained as control plants without inducing stress and 100 mL of distilled water were added to the plants every morning and evening.

Table 1. Abiotic stress inducing process.

Stress	Methodology
High salinity	For fourteen days, the plant was treated with 100 mL of 200 mM sodium chloride (NaCl) solution every morning and evening.[17]
Flood	After adding 5600 mL of water to a container, the plant roots and a part of the stem were immersed completely in water for 14 days.[18]
Nutrient deficiency	The plants original soil was replaced with autoclaved sand. 100 mL of distilled water was added to the plant

	each morning and evening for 14 days.[19]
Drought	For 14 days, 100 mL of 10% Polyethylene glycol (PEG 4000) were added each morning and evening.[20]

2.2 Spondias dulcis aqueous extract preparation. The leaves were collected, weighed, and cleansed with tap water, followed by distilled water to get rid of impurities. Leaves were patted and air-dried for 30 minutes and were cut into small pieces and evenly spread on an aluminium foil. Leaves were then placed in dry oven (Meditry instrument, China) at 50℃ for 48 hours. Once dried, they were crushed and weighed. Distilled water was added in a 1:10 ratio. The aqueous extract was then placed in dry oven at 90℃ for 15 minutes. After cooling to room temperature, the extract was transferred to a 50 mL falcon tube and centrifuged (Gemmy industrial corporation, Taiwan) at 4000 rpm for 10 minutes. The extract was filtered using Whatman No.1 filter papers. The filtrate was collected in a 50 mL falcon tube and stored in a refrigerator at 4℃.[21]

2.3 Spondias dulcis methanolic plant extract preparation. The leaves were dried as explained in 2.2. After drying, leaves were weighed to obtain 5 g using an analytical balance. The measured powder was transferred to a beaker, and 35 mL of methanol was added. The mixture was left to macerate in a watch glass at RT for three days. After maceration, mixture was filtered using Whatman No.1 filter papers to remove plant debris, and the filtrate was transferred to a beaker. The beaker was then placed in a dry oven set at 40°C. Once the solvent had evaporated, the concentrated residue was dissolved in 2 mL of methanol. The dissolved extract was transferred into a clean, sterile falcon tube and stored in refrigerator at 4℃.[22]

2.4 Determination of moisture content. The moisture content of plant leaves was found by following the below equation.[23]

$$\text{Moisture Content} = (w-d)/w \times 100$$

w= Fresh weight

d= Dry weight

2.5 Qualitative assays for phytochemicals. Qualitative tests were conducted to determine the presence of phytochemicals as shown in Table 2.

Table 2. Methodology for Qualitative assays

Test	**Methodology**
Alkaloids - Wagner Test	A drop of Wagner's reagent (Iodo-potassium iodide) was added to 1 mL of plant extract.[24]
Flavonoid - NaOH test	A mixture containing 0.5 mL of plant extract, few drops of 2% NaOH, and 2–3 drops of 1% HCl were mixed.[25]
Tannins	1 mL of 5% ferric chloride was added to 0.5 mL of plant extract.[26]
Saponins	A combination of 0.5 mL of plant extract and 5 mL of distilled water was shaken thoroughly.[27]
Coumarins	0.5 mL of 10% NaOH was added to 0.5 mL of plant extract.[28]
Phenols	A mixture consisting of 1 mL of plant extract, 1 mL of distilled water, and few drops of 10% $FeCl_3$ was added.[29]
Cardiac glycosides - Keller Kiliani test	A mixture containing 1 mL of plant extract, 0.5 mL of glacial acetic acid, 0.5 mL of $FeCl_3$, and 0.5 mL of concentrated H_2SO_4 was added.[30]
Terpenoids	A combination of 2.5 mL of plant extract, 1 mL of chloroform, and 1.5 mL of concentrated H_2SO_4 was added.[31]
Steroid	A mixture of 0.5 mL of plant extract, 0.5 mL of chloroform, and few drops of concentrated H_2SO_4 was added.[32]

2.6 Determination of Total Phenolic Content (TPC) using Folin-Ciocalteu method. Gallic acid (1 mg/mL in distilled water) was used as the standard solution. A standard series of 0.01, 0.05, 0.1, 0.25, and 0.5 mg/mL was prepared for the standard curve. Plant extract dilutions were

made by adding 100 µL of each sample with 500 µL of distilled water in test tubes. Reaction was initiated by mixing 100 µL of Folin-Ciocalteu reagent, followed by incubation in dark for 6 minutes. 1 mL of 7% sodium carbonate was added, with 500 µL of distilled water. The mixture was incubated at room RT for 90 minutes. Absorbance was measured using a UV-Visible spectrophotometer at 760 nm, and a standard curve was plotted. TPC was expressed as Gallic acid equivalents (mg GAE/g).[33] Distilled water was used as blank.

2.7 Determination of Total Antioxidant Capacity (TAC) using phosphomolybdate method. Ascorbic acid (1 mg/mL in distilled water) was used as the standard solution. A standard series of 0.01, 0.05, 0.1, 0.25, and 0.5 mg/mL was prepared for the standard curve. Plant extract dilutions were formulated by adding 0.1 mL of each sample into 0.5 mL of distilled water to a test tube. The reaction was initiated by mixing 1 mL of reagent solution, consisting of 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, which had been thoroughly mixed and set aside. The test tubes were capped with aluminium foil and incubated in a water bath at 95°C for 90 minutes. After incubation, the samples were allowed to cool to RT. Absorbance was measured at 765 nm using a UV-Visible spectrophotometer, and a standard curve was plotted. TAC was expressed as Ascorbic acid equivalents (mg AAE/g).[34] Distilled water was used as blank.

2.8 Determination of Total Flavonoid Content (TFC) using aluminium chloride assay. Quercetin (1 mg/mL in distilled water) was used as the standard solution. A standard series of 0.01, 0.05, 0.1, 0.25, and 0.5 mg/mL was prepared for the standard curve. Plant extract dilutions were formulated by adding 100 µL of each sample (15x) with 500 µL of distilled water in test tubes. To each mixture, 100 µL of 5% sodium nitrate was added, and the solution was allowed to stand for 6 minutes. 150 µL of 10% aluminium chloride solution was added and left to stand for 5 minutes. Afterwards, 200 µL of 1 M sodium hydroxide was added, and the mixture was gently stirred to ensure complete reaction. Absorbance was measured using a UV-Visible spectrophotometer at 510 nm, and a standard curve was plotted. TFC was expressed as Quercetin equivalents (mg QE/g).[33] Distilled water was used as blank.

2.9 Determination of Total Protein Content (TPrC) using lowry assay. Bovine Serum Albumin (BSA) (1 mg/mL in distilled water) was used as the standard solution and a standard series of 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, and 1 mg/mL was prepared for the standard curve. Plant extract dilutions were formulated by mixing 5 mL of reagent solution to 20X diluted sample. The reagent solution was prepared by mixing 48 mL of 2% sodium carbonate in 0.1N sodium hydroxide with 1 mL of 0.5% copper sulphate and 1 mL of 1% sodium potassium tartrate. The reaction mixture was allowed to stand at room temperature for 15 minutes. 0.5 mL of freshly prepared Folin-Ciocalteu reagent (1:1 dilution with water) was added and mixed well to ensure thorough reaction with the protein in the sample. The test tubes were incubated in the dark for 30 minutes. Absorbance was measured using a UV-Visible spectrophotometer at 660 nm, and a BSA standard curve was plotted. TPrC was expressed as BSA equivalents (mg BSA/g).[35] Distilled water was used as blank.

2.10 Determination of antimicrobial effect of Spondias dulcis using well diffusion-Antibiotic susceptibility test. Mueller-Hinton agar (MHA) was prepared by boiling the agar for few minutes until completely dissolved, followed by autoclaving at 121°C for 15 minutes. The agar was then cooled before being poured into petri dishes. A volume of 20 mL of agar was poured into each petri dish and allowed to solidify. Bacterial suspensions of *Escherichia coli* and *Staphylococcus aureus* was spread onto the MHA plates using a sterile cotton swab. Four wells were created in each MHA plate using a sterile micropipette tip, ensuring that the wells were evenly spaced and did not overlap. Wells 1 and 2 were filled with methanolic plant extract, while well 3 (negative control) was filled with methanol, and well 4 (positive control) was filled with Ciprofloxacin (100 µg/mL) solution (Figure 5). The plates were incubated at 37°C for 24 hours. Following incubation, the zones of inhibition around the wells were observed and measured to evaluate antibacterial activity.[36]

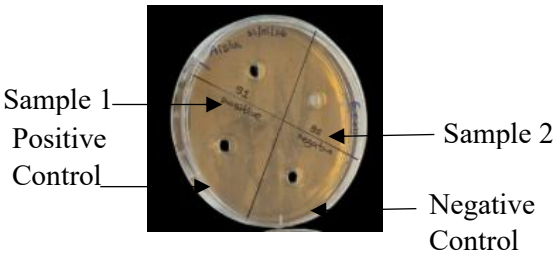


Figure 5. ABST plates before streaking before incubation

2.11 Statistical analysis. All values are expressed as mean ± Standard error. Microsoft Excel 2023 was used to calculate the standard error of the mean value.

3. Results and Discussion/Analysis and Findings

3.1 Morphological changes of control and abiotic stress. Observed morphological changes of the control plant and abiotic stress induced plants during the 14 days is shown in Table 3.

Table 3. Morphological changes of the plant control plant and in stress-induced plants

Condition	Morphological changes
Control	• More branching • Broader and healthier leaves • Long and bushier growth • Thicker stems • Even distribution of leaves
High salinity	• Sparser foliage • Thinner stems • Leaf wilting and damage • Less bushy appearance
Flood	• More elongated growth • Less compact foliage • Leaning structure • Slightly wilting/ curling leaves
Nutrient deficiency	• Elongated stems • Reduced lateral branching. • Smaller leaf size and number • Leaf yellowing
Drought	• Elongated stems • Reduced lateral branching. • Smaller leaf size and number • Leaf yellowing • Leaf wilting and damage

3.2 Qualitative phytochemical assays. Qualitative phytochemicals assay results conducted for control and stress-induced plants are shown in Table 4 as Presence (P) and Absence (Ab) of phytochemicals.

Table 4. Phytochemicals for qualitative assays

Tests	Control	Drought	High salinity	Heavy metal	Nutrient Deficiency	Flood
Alkaloid	P	P	P	P	P	P
Tannins	P	P	P	P	P	P
Phenolic Compound	P	P	P	P	P	P
Saponins	Ab	Ab	Ab	Ab	Ab	Ab
Flavonoid	Ab	Ab	Ab	Ab	Ab	Ab
Coumarins	P	P	P	P	P	P
Terpenoids	P	P	P	P	Ab	Ab
Cardiac Glycoside	P	P	P	P	P	P
Steroids	P	P	P	P	P	P

3.3 Total Moisture Content (TMC)

The plant aqueous extract obtained from the control had the highest TMC and the lowest was obtained in the drought condition. At the 10% error margins, the error bars of high salinity, nutrient deficiency, flood, and drought overlapped with control plants, whereas the drought condition did not overlap (Figure 6).

Therefore, the decreased moisture content in drought was statistically significant compared to control plants.

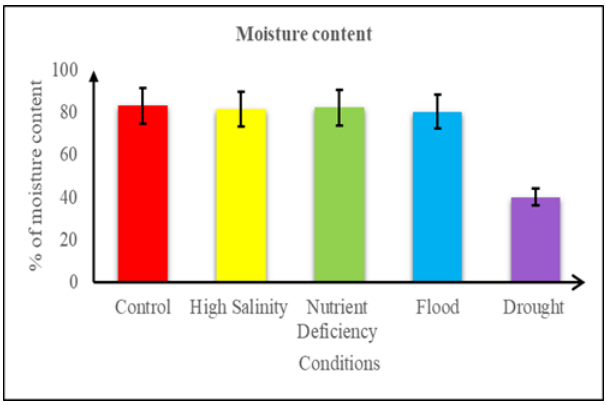


Figure 6. Bar chart of moisture content in each test group

3.4 Total Phenolic Content (TPC)

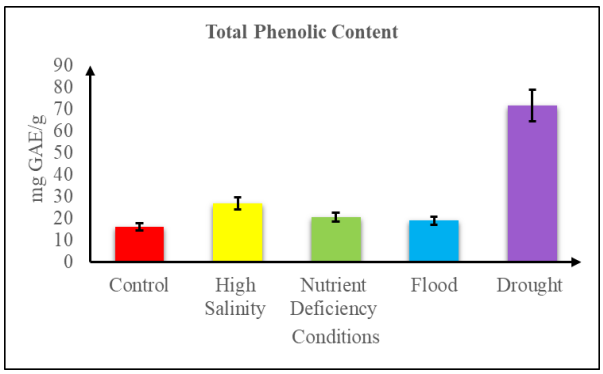


Figure 7. Total phenolic content in each test group

The plant aqueous extract obtained from the drought condition had the highest TPC and the lowest was obtained in the control plant. At the 10% error margins, the error bars of flood and nutrient deficiency overlapped with the control plants whereas high salinity and drought did not overlap (Figure 7) Therefore, the increased total phenolic content in high salinity, and drought were statistically significant compared to control plants.

3.5 Total Antioxidant Capacity (TAC). The plant aqueous extract obtained from the drought condition had the highest TAC and the lowest was obtained in the flood condition.

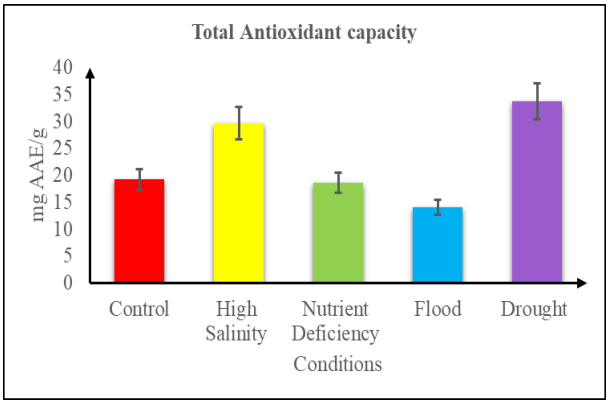


Figure 8. Total antioxidant capacity in each test group.

At the 10% error margin, the error bars of nutrient deficiency overlapped with control plants whereas high salinity, flood, and drought did not overlap (Figure 8). Therefore, the increased total antioxidant capacity in high salinity, drought and decreased TAC in flood conditions were statistically significant compared to control plants.

3.5 Total Flavonoid Content (TFC)

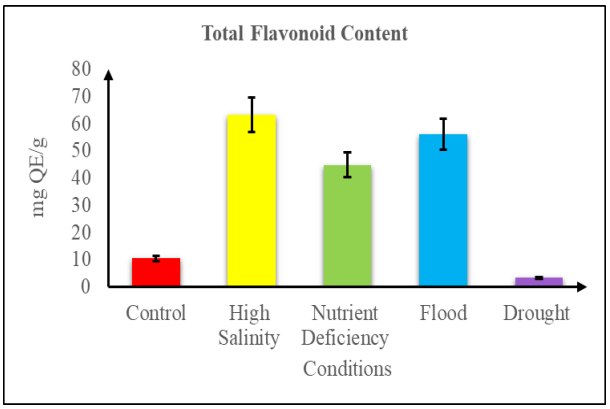


Figure 9. Total flavonoid content in each test group.

The plant aqueous extract obtained from the high salinity condition had the highest TFC and the lowest was obtained in the drought condition. At the 10% error margins, the error bars of high salinity, flood, nutrient deficiency, and drought did not overlap (Figure 9). Therefore, the increased total flavonoid content in high salinity, flood, nutrient deficiency, and decreased TFC in drought conditions were statistically significant compared to control plants.

3.6 Total Protein Content (TPrC)

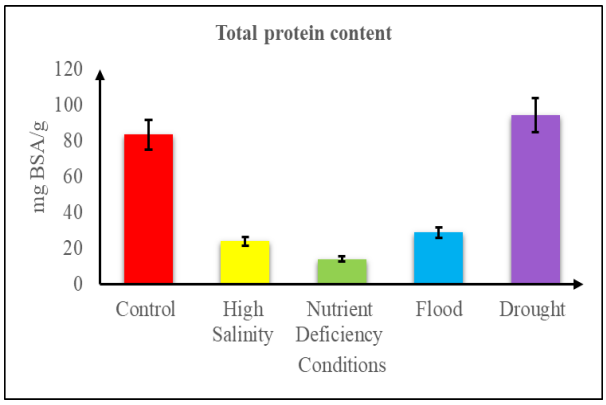
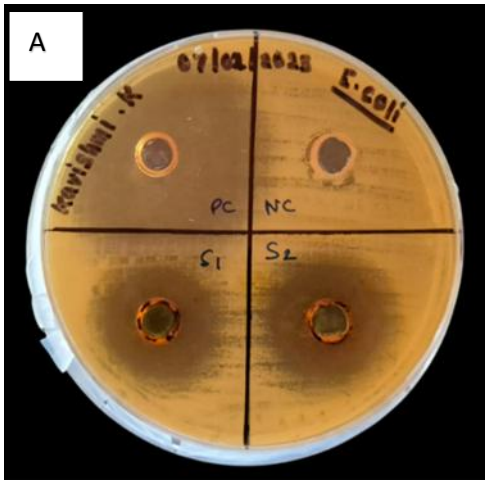


Figure 10. Total protein content in each test group.

The plant aqueous extract obtained from the drought condition had the highest TPrC and the lowest was obtained in the nutrient deficiency condition. At the 10% error margins, the error bars of drought overlapped with control. Whereas the error bars of high salinity, nutrient deficiency, and flood conditions did not overlap (Figure 10). Therefore, the decreased total protein content in high salinity, flood, nutrient deficiency, and increased TPrC drought conditions were statistically significant compared to control plants.

3.7 Antibacterial activity



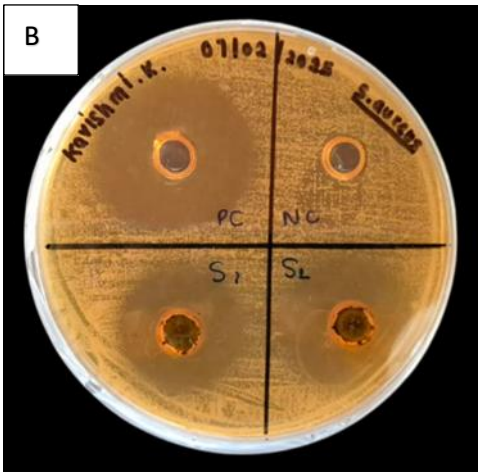


Figure 11. Antibacterial results of *Spondias dulcis* activity against *Escherichia coli* (A) and *Staphylococcus aureus* (B)

The antibacterial activity of *Spondias dulcis* activity against *Escherichia coli* (Figure 11A) and *Staphylococcus aureus* (Figure 11B). The methanolic extract of *Spondias dulcis* showed strong inhibitory activity against both *Escherichia coli* and *Staphylococcus aureus* as indicated by the clear zones of inhibition (Table 5).

Table 5. Zones of inhibition against bacterial species

Bacteria	**Zone of inhibition**	
	Sample 1	Sample 2
Escherichia coli	10 mm	9 mm
Staphylococcus aureus	10 mm	11 mm

4. Discussion

To maintain agricultural sustainability in different environment conditions triggered by climate change, this research focused on abiotic stresses like high salinity, flood, nutrient deficiency, and drought on *Spondias dulcis* plants due to the growing use of medicinal plants and evaluating the adaptation mechanism exhibited by the plant. Qualitative and quantitative analyses of the aqueous plant extract were done following antimicrobial potential of the methanolic plant extract of the *Spondias dulcis* leaves.

Due to various environmental circumstances, plants exhibit substantial variations in growth and morphology. Under optimal conditions control plants show greater branching, broader and healthier leaves, lengthy and bushier growth, thicker stems, and even leaf distribution, all of which represent the ideal water balance and nutrient availability (Table 4).[37]

In the high salinity condition, 200 mM of NaCl solution was chosen to add to the soil, due to the concentration having a depressive effect on the plant while giving a suitable condition for the salt stress.[38] Plants grown in high salinity environments resulted in thinner stems, withering leaves, and less bushy appearance due to osmotic stress brought on by salt accumulation, which interferes with water absorption and lowers leaf turgor while preventing lateral growth.[39] Moreover, a greater uptake of Na^+ and Cl^- ions from the soil lowers the photosynthetic efficiency, which has a detrimental effect on the leaves causing sparser foliage.[40]

In flood conditions, when stems and roots are submerged, plant experience temporary hypoxia until water subdues and normoxia returns. This gives a suitable condition for waterlogging stress in *Spondias dulcis* leaves and the plant showed curled and withering leaves.[41] In reaction to hypoxia in the root zone, flooded plants exhibit extended growth, as a tolerance mechanism,[42] less compact foliage, a leaning structure due to the stems elongation to access better aerated areas.

Elongated stems, less lateral branching, smaller leaves, and leaf yellowing (chlorosis) are all symptoms of nutrient deficiency condition which was obtained from autoclaving sand, free from nutrients and microorganisms.[43] Observations in the plant leaves were due to the lack of vital nutrients like Potassium and Nitrogen which are necessary for cell division and the synthesis of chlorophyll.[44] The plant development and yield causing less lateral branching and smaller leaves are caused due to magnesium deficiency.[45]

Drought stress was facilitated by 10% PEG 4000 as an osmotic agent to stimulate water deficit conditions.[46] Plants under drought stress resulted in longer stems, yellowing and wilting of the leaves due to a lack of water, promoting stomatal closure, limiting photosynthesis, and decreased cell growth to save water.[47] As the stress increases the plants showed reduced stature which affects the plants' yield. Cellular free radical metabolism is disturbed resulting in the buildup of free radicals and ROS in plants.[48]

Moisture content (MC) of the *Spondias dulcis* plant extract in the control condition was high compared to the abiotic stress induced plants. In the abiotic stresses, nutrient deficiency was the highest (82.2%) due to stunted growth, which retains more water within its tissues, and the lowest was obtained from the drought (40%) causing low absorption of water in the soil for roots. The determination of MC in nutrient deficient environments align with previous research articles.[15] confirming that due to the Nutrient Deficiency stress plants like *Spondias dulcis* (drought tolerant) maintain higher moisture content. In general drought stress reduces the MC of the plant due to loss of turgor and reduced uptake of water.[49] However, in *Spondias dulcis* plant, strategies such as leaf shedding and wilting are observations to control moisture loss during drought, the scientific literature currently lacks precise quantitative information on the leaf's MC. To measure the alterations in specific to the plant, more empirical research is required.

According to the results from the qualitative phytochemical analysis, the control plant parameters are consistent with other studies that have found alkaloids, tannins, coumarins, phenols, cardiac glycosides, terpenoids and steroids presence in *Spondias dulcis*.[12,13] These phytochemicals contain antibacterial, antidiarrheal, antiviral properties in addition to the antioxidant properties.[50] However, the presence of flavonoids and saponins were reported to be present in *Spondias dulcis* in previous research articles.[51] In comparison, in the present study both the phytochemicals were absent from *S. dulcis* leaf

extract. Different environmental factors, the plant parts used, the development stage variation, different extraction techniques employed and concentration below a detectable limit could be the reasons for the negative results obtained.[51]

The abiotic stresses and their phytochemical composition produced consistent results for the presence of alkaloids, tannins, coumarins, phenols, cardiac glycosides under all stress conditions. However, the absence of saponins and flavonoids were observed to be consistent throughout the abiotic stresses. Terpenoids were present in high salinity and drought conditions and were absent in flood and nutrient deficient conditions. Steroids were present only in drought conditions and were absent in high salinity, flood, and nutrient deficiency conditions. The variation could be a result of adaptive mechanism displayed by the plant on different stress conditions. During extreme conditions, the phytochemical composition changes due to metabolic pathways that help plants survive, which can affect synthesis of secondary metabolites.[52] Qualitative data on analysis of abiotic stress conditions phytochemical composition are currently lacking in the scientific literature therefore further investigations is required.

According to the TPC results, the highest TPC was caused drought stress (71.69 GAE g^{-1}). Since phenolics function as antioxidants to lessen the harm promoted by ROS, the rise is the result of oxidative stress.[53] A moderate increase in salinity (26.69 GAE g^{-1}) causes oxidative stress which in turn increases the phenols.[54] Conversely nutrient deficiency (20.59 GAE g^{-1}) and flood stress (18.83 GAE g^{-1}) resulted in slight increase indicating no significant difference compared to the control which was the baseline for the comparison of TPC.

According to the TAC results, the highest was caused by drought stress (33.765 AAE g^{-1}) indicating a protective mechanism for the oxidative stress by ROS .[55] Moderate increase in high salinity (29.76 AAE g^{-1}) is reflected by the plant's response to prevent oxidative damage and preserve cellular homeostasis.[56] The low levels of TAC in flood (14.145 AAE g^{-1}) are due to the hypoxia condition leading to root damage thus lowering the ascorbic acid levels.[57] Conversely nutrient deficiency (18.705 AAE g^{-1}) resulted in slight increase indicating no significant difference compared to the control which was the baseline for the comparison of TAC.

According to the TFC results, the highest was caused by high salinity stress (63.405 QE g^{-1}), indicating a protective mechanism for the oxidative stress by ROS, reducing the ion toxicity and altering the osmotic pressure.[58] Moderate increase in flood (56.19 QE g^{-1}) and nutrient deficiency (44.895 QE g^{-1}) is reflected by the plant's tolerance and the stress signalling pathway.[59] The low levels of TFC in drought (3.39 QE g^{-1}) are due to the hypoxia condition leading to root damage thus lowering the quercetin levels.

According to the TPrC results, the highest was reported by drought (94.36 BSA g^{-1}), indicating a protective mechanism for stress by producing proteins and proteases which help in increasing the protein content.[60] In high salinity (29.76 BSA g^{-1}) denaturation of proteins take place causing the protein levels to decrease. In flood (14.145 BSA g^{-1}) and nutrient deficit (18.705 BSA g^{-1}) conditions, oxygen deprivation and low levels of nutrients hinders protein synthesis thus lowering the protein content.[61]

Methanolic extract of *S. dulcis* indicated antibacterial activity in the antibiotic sensitivity testing using well diffusion against a gram-negative bacteria *E. coli* and a gram-positive bacteria *S. aureus*. The positive control (ciprofloxacin) inhibited the bacterial growth with inhibition zone while the negative control (methanol) no inhibition zone suggesting the methanol did not affect the growth. Inhibition of *E. coli* and *S. aureus* in the *Spondias dulcis* plant confirms the presence of antimicrobial activity, also in previously mentioned in research articles.[12] Investigating plants under abiotic stress is essential for enhancing

agricultural resilience, securing food supply, and responding to climate changes. It assists scientists in understanding plant responses, developing stress-tolerant plants, promoting ecological restoration and driving biotechnological advancements for sustainable agriculture.

5. Conclusion

In conclusion, this research examined abiotic stresses like high salinity, flood, nutrient deficiency and drought responds to S. dulcis plant. The phytochemical composition of the plant suggests presence of alkaloids, tannins, coumarins, phenols, cardiac glycosides, terpenoids, steroids, under different stress conditions and indicated the absence of flavonoids and saponins. The quantitative biochemical analysis showed, drought induced plants had a significant impact on TPC, TAC and TPrC. High salinity increased TAC and TFC. Nutrient deficiency and flooding also increased TFC. Methanolic extract of *S. dulcis* displayed antibacterial activity against *E. coli* and *S. aureus.* The study's findings have shown Spondias dulcis to have potential health benefits as antioxidant, and antibacterial properties, while withstanding abiotic stresses in different environments. Plant research under abiotic stress conditions contributes to the development of resilient plants, improved food security, and sustainable agriculture during environmental changes.

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Detection of *Leptospira* DNA in environmental water samples collected from the Gampaha District by qPCR.

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Abstract

Leptospirosis is a widely spread zoonotic disease in the world, caused by 11 pathogenic *Leptospira* belonging to the family Leptospiraceae. Sri Lanka, particularly the Gampaha District has been identified as an area with a high prevalence of leptospirosis. Wild and feral animals, livestock, and domestic pets are the main reservoirs. Sampling environmental water samples for the detection of Leptospira is used as a predictor of disease transmission. Therefore, the study aimed to detect the presence of Leptospira as evidence of environmental contamination.

In this study, we tested Leptospira DNA in water samples collected from the disease prevalent Gampaha district, using real-time polymerase chain reaction (PCR). The water samples were collected from 10 various locations in the Gampaha District. The processing was done using a pre-validated protocol for DNA extraction and the extracted DNA was then subjected to real-time PCR targeting specific genetic markers of Leptospira. No Leptospira DNA was detected from the tested samples, most probably due to the period that the study was conducted. The samples were collected in the month of April, which is a month the country reports a minimum number of cases.

Leptospirosis prevalence varies with geographical location, climate, and seasonality. Temperature, pH, and the presence of other microorganisms can all influence the survival and identification of leptospirosis bacteria in water samples. Therefore, further studies need to be done in a periodic manner to establish transmission dynamics in the Gampaha district.

Keywords: Leptospirosis, *Leptospira* DNA, water samples, DNA extraction real-time PCR

1. Introduction

Leptospirosis is a widely distributed zoonotic disease that affects humans and animals living in regions with humid, tropical, and subtropical climates.[1] It is a well-established infectious disease in Sri Lanka and is associated with high morbidity and mortality. The disease is caused by 11 pathogenic species of Leptospira belonging to the family Leptospiraceae. Leptospira have a helix-shaped body and an internal locomotor apparatus, the endo flagellum, which allows them to move freely even in the most viscous conditions (Figure 1). Wild and feral animals, livestock, and domestic pets are the main reservoirs that maintain disease in the environment. In Sri Lanka, minimum data is available on reservoir animals of leptospirosis.[2]

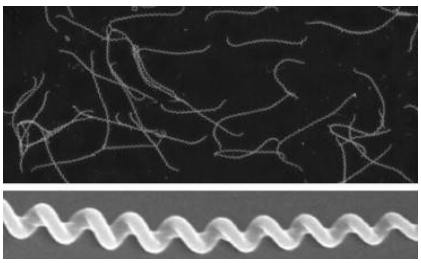

Figure 1: Electron micrographs of Leptospires.[3]

The disease leptospirosis is distributed throughout the country. In the previous two decades, the highest number of leptospirosis cases have been reported from the districts of Colombo, Kurunegala, Gampaha, Matale, and Kegalle.[4] The disease is endemic in some parts of the country and, in 2008, the country experienced its greatest outbreak of leptospirosis, with 7423 suspected cases and 204 deaths.[5] Leptospirosis is a well- known contributor to infectious disease epidemics caused by meteorological disasters in Sri Lanka.[6] Leptospirosis was first documented in Sri Lanka in 1953, among caught rats in Colombo. The country has experienced three major outbreaks in years 2003, 2008, and 2011 with significant death tolls. The 2008 epidemic was the worst ever reported in the country and the second- highest leptospirosis outbreak in the globe. Leptospirosis primarily affects agricultural populations such as paddy farmers and Chena cultivators in Sri Lanka and is linked to certain environmental conditions, mainly to monsoons.[7]

The infection manifests as mild to severe life-threatening diseases. The initial clinical presentation of leptospirosis is non-specific, and mimics other common infective and non-infective diseases in the country. The non-specific presentation challenges clinicians to diagnose the infection clinically. Therefore, knowing the transmission dynamics of the local geographical regions is essential to obtain exposure. The usual practice is to manage patients clinically and to obtain laboratory confirmation by serology retrospectively. Obtaining laboratory confirmation is a major challenge in tropical and subtropical regions due to limited laboratory facilities. However, the classic Weil's disease, which manifests as jaundice, renal failure, and bleeding, can be identified clinically but is associated with high morbidity and mortality.

There are two stages of leptospirosis. There are two phases: leptospiremic and immune. During the leptospiremic phase, you will have symptoms similar to the flu. They will begin a few days to weeks after you are first exposed to leptospira bacteria. Symptoms can persist for up to ten days. Once you enter the immunological phase, leptospira bacteria are present in your organs, particularly your kidneys. Urine tests will reveal the bacteria, and your body will develop immunity to them. During this phase, you may become ill with Weil's syndrome

The study of infectious diseases encompasses both the course of the disease in its host and how it spreads between hosts. Understanding illness transmission is critical for establishing effective interventions, protecting healthcare workers, and guiding a successful public health response.[8] Identifying the elements that contribute to disease spread is crucial for effective control and prevention actions.[9] Sampling the environment water samples for the detection of pathogens transmitted via contaminated water including leptospirosis is an important public health measure that allows to understand the transmission dynamics.

Travelers are more likely to contract leptospirosis if they visit flooded or recently flooded areas, swim, wade, kayak, or raft in possibly contaminated fresh water, such as lakes and rivers. Infection can spread by contact with infected animals, such as mice, cows, sheep, goats, pigs, horses, dogs, and wildlife, especially in metropolitan settings with poor sanitation. There is no vaccine licensed in the United States to prevent leptospirosis in humans.

Travelers should avoid touching fresh water or soil that may have been contaminated with animal urine. Avoid direct contact with materials potentially contaminated with animal urine, such as animal bedding. Refrain from wading, swimming, or submerging the head in floodwater or natural bodies of water including lakes, rivers, and swamps, particularly following periods of flooding or heavy rainfall. Minimize exposure to freshwater sources during and after such events. When contact with floodwater or freshwater is unavoidable, wear appropriate protective clothing, ensure all cuts and abrasions are securely covered with waterproof dressings, and consume only water that has been properly treated or boiled to

ensure safety.[10] The study aimed to detect the presence of *Leptospira* as evidence of environmental contamination.

2. Methodology

2.1 Sample collection. The water collections close to paddy fields, and human dwellings are well known to be contaminated with pathogenic and intermediate Leptospira. Ten replicates of water samples (50 ml) possibly contaminated with Leptospira were collected into sterile polypropylene centrifuge tubes. The collection sites were decided based on the published literature as source of transmission of Leptospirosis such as water retain in the paddy field, natural water collections in rat dwellings areas and marshy lands. The samples were transported in room temperature to the Molecular Medicine Unit, Faculty of Medicine, Ragama for detection of Leptospira DNA by a pretested and validated qPCR protocol.

2.2 DNA extraction from water samples. DNA from the collected water (200 µl) samples were extracted using the QIAamp viral RNA mini kit according to the manufactured instructions. All buffers (AVL, AW1, and AW2) were prepared according to the manufacturer's instructions. If precipitate had formed in the AVL buffer, it was dissolved by incubating at 56°C. Preprocessed water in 1XPBS (200 µl) was added to a 1.5 ml microcentrifuge tube. Then, 560 µl of prepared buffer AVL containing carrier RNA added to the sample and mixed by pulse vortexing for 15 seconds. Then the homogenate was incubated at room temperature (25°C) for 10 minutes. Thereafter, the sample was briefly centrifuged to remove drops from the inside of the lid. Following that 560 µl of absolute ethanol was added to the sample and mixed by pulse vortexing for 15 seconds. After mixing, the sample was briefly centrifuged to remove drops from the inside of the lid, and 630 µl of the homogenate was added to the QIAamp mini spin column (in a 2 ml collection tube) without wetting the rim. Subsequently, it was centrifuged at 8000 rpm for 1 minute, and the tube containing the filtrate was discarded. Then 500 µl of buffer AW1 was added to the column without wetting the rim and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded, and buffer AW2 (500 µl) was added to the column and centrifuged at 13400 rpm for 4 minutes. The column was placed again in a new collection tube and centrifuged at the same speed for 1 minute to remove any residual binding buffers from the DNA. Then the column was placed in a new 1.5 ml microcentrifuge tube, 60 µl of buffer AVE was added to the column, and it was incubated at room temperature for 1 minute. Finally, the column was centrifuged at 8,000 rpm for 1 minute, and the filtrate (DNA) was stored at -20°C until it was analyzed by PCR. The same procedure followed for all ten samples to extract DNA.

2.3 Primer design. Pathogenic Leptospira specific primers were selected from the fragment of the *secY* gene homologous to the L. interrogans S10-spc-a locus (Genbank accession number AF115283). The primers amplify 202 bp fragment between the locus positions 15 744 and 15 946 of *secY* gene that is located within the S10-spc-α locus containing genes for ribosomal proteins and it encodes preproteintranslocase for Leptospira.[11]

2.4 Reagents and reaction conditions for real time PCR. To detect leptospirosis, real-time PCR was performed. Before preparing the master mix, calculations were made to determine the exact volumes of the components required to prepare 10 samples, The amplification process was carried out after initial denaturation at 950°C for 10 minutes. The thermal process of amplification for 40 cycles was carried out including steps of denaturation at 950°C for 15 seconds, annealing at 540°C for 30 seconds, extension at 720°C for 30 seconds and with an extended final incubation at 720°C for 8 minutes. After cooling 300°C for 1 minute, melting curve (Tm) analysis from 65- 940°C with readings every 0.50°C was performed according to the manufacturer's instructions. The cut-off for the analysis was set at Threshold Cycle (Ct) value 35 and the approximate total time taken to run the entire program was about 2 hours. All samples were repeatedly tested at least twice for maximum reproducibility. All the data was

analyzed using the software provided by the Esco e Swift Spectrum 48 fluorescence qPCR detection system.[12,13]

Table 2. Optimal reaction conditions for real time PCR

Reagents	Optimized concentration range	Final Concentration
Double distilled water		
Forward primer SecY IV F	80-250 nM	2.5 mM
Reverse primer Sec Y IV R	80-250 nM	2.5 mM
SYBR Green qPCR Master mix	1X-5X	1X
Template DNA	<100 ng	Variable

Table 3. Optimal thermal conditions for real time PCR

Program	Cycles	Target Temperature	Incubation time
Initial Denaturation		950°C	15 minutes
Denaturation	40 Cycles	950°C	15 seconds
Annealing		540°C	30 seconds
Extension		720°C	30 seconds
Final Extension		720°C	8 minutes
Melting Temperature		650°C-950°C	

3. Results and Discussion/Analysis and Findings

Only the two positive controls show the amplification curves. All 10 samples did not get any amplification curves. All the 10 samples were negative.

The detection of Leptospira DNA from suspected environmental water samples is widely used as a predictor in surveillance to confirm the spreading of pathogens beyond the geographical locations and importantly as a predictor of an outbreak. However, such a procedure has its limitations. The sample testing is only a representation based on the existing knowledge. Low pathogen levels and inadequate volumes used may lead to false negative results.

Figure 2: Fluorescence Curves of the amplified product of the secY gene

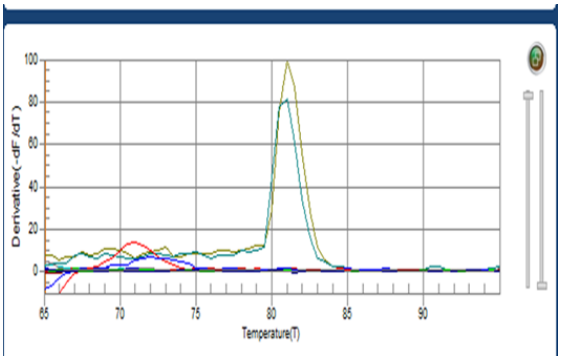

Figure 3: Melting Curves of the amplified product of the secY gene

In this study, all 10 water samples tested were negative for Leptospira DNA. The most possible reasons for a such result are the absence of Leptospira in water during the study period. The study was done in the month of April which was dry enough to have no reservoir water collections. The timing was just before the main monsoon of the country and were experiencing very high temperatures. All these climatic conditions probably impacted negatively on the study. If the area where the water samples were collected has a low prevalence of leptospirosis during the study period, it's possible that none of the samples contained the bacteria although the area is a highly prevalent region.[13] Environmental factors such as temperature, pH, and the presence of other microorganisms can affect the survival and detection of leptospirosis bacteria in water samples. The study indicates that

health authorities need not continue with prescribing prophylaxis to risk people during such time of the year.

The presence of carrier RNA may have contributed to improved DNA recovery from low-concentration samples. This is in agreement with Levesque-Sergerie *et al*., who reported that carrier RNA enhances the yield and stability of nucleic acids during extraction, especially when working with small sample volumes.[14]

The PCR results further validated the quality of the extracted DNA. Positive control samples produced clear and specific amplification bands, while the negative controls showed no amplification, confirming the absence of contamination.

In the DNA extraction process buffer AVL was a viral lysis buffer designed to isolate viral nucleic acids. It can be given with or without carrier RNA. When purifying small amounts of DNA, adding carrier RNA was improved DNA recovery.10 Carrier keeps the minimal amount of target nucleic acid in the sample from being irretrievably bound. The centrifuge process was used to harvest the bacterial sample, and this separates the sample into solids and liquids. After adding the sample to the carrier RNA and AVL mixture and incubating it at room temperature for ten minutes, carrier RNA is added to the sample to aid in the effective binding of DNA to the silica membrane of the spin column in future phases. Incubating at room temperature allows carrier RNA to bind to the DNA molecules, increasing their stability and avoiding destruction. Ethanol is frequently used in DNA extraction processes because of its ability to precipitate DNA from solution. Ethanol is added to the DNA-containing solution to cause precipitation. When ethanol is added to a solution, the solubility of DNA molecules decreases, leading them to cluster together and become insoluble in the aqueous solution. This precipitation separates the DNA from the other dissolved cellular components, such as proteins and lipids.[15] Buffers AW1 and AW2 function as a washing agent for DNA, ensuring that it is free of other bimolecular components.[16] Buffer AW1 washes away pollutants and impurities from the spin column's DNA-bound silica membrane. After centrifugation, the filtrate with these contaminants is discarded. Using a new spin column keeps the DNA bonded to a clean membrane, free of impurities that could interfere with downstream uses. Buffer AE elutes DNA from the spin column membrane into the microcentrifuge collection tube, allowing for stable DNA storage.

Positive controls are reviewed to ensure that the method is capable of amplifying the target nucleic acid, and negative controls are examined to ensure that no contamination occurs. Taq DNA Polymerase is important in Polymerase Chain Reaction (PCR) because of its role in synthesizing and amplifying new strands of DNA.[17] It is important to optimize PCR to avoid a non- specific amplification.

4. Conclusion

The study indicates the absence of Leptospira in environmental water samples during January, February, March and April of the year. The risk groups need not provide with antibiotic prophylaxis during such times of the year. Thus, this indicates a need for an island-wide study, to decide on appropriate control measures.

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Evaluation of the presence and the pathogenicity of a novel HNF1A p.Lys120Gln mutation identified in a patient suspected of Maturity Onset Diabetes of the Young.

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Abstract

Maturity Onset Diabetes of the Young (MODY) is a form of monogenic diabetes characterized by autosomal dominant inheritance and early onset. At least 14 different subtypes of MODY have been characterized based on the gene involved, and the specific subtype determines the most effective treatment. Hepatocyte nuclear factor-1 alpha (HNF1A)-MODY, also called MODY3 is one of the most common MODY subtypes seen among 20-60% of MODY patients. Patients with HNF1A-MODY are highly sensitive to sulfonylureas, and do not require insulin treatment in early stages. Therefore, proper genetic characterization is important in the diagnosis of MODY. In prior research aimed at developing a Next Generation Sequencing (NGS) based test for the diagnosis of MODY, a novel HNF1A c.358 A>C (p.Lys120Gln) mutation has been identified, which has not previously been reported, and the pathogenicity unknown. The objective of the current research was to confirm the presence of this mutation by an alternative method, and to carry out *in silico* structural and functional characterization, as well as population studies in order to determine its pathogenicity. We have confirmed the presence of this mutation in the tested sample by Sanger sequencing, thereby verifying the accuracy of NGS results. *In silico* structural analysis predicts possible deleterious effects of this mutation. Population databases show extremely low incidence of this mutation in the general population. Together with interpretations from other relevant literature and databases, we have deduced the HNF1A c.358 A>C (p.Lys120Gln) mutation to be a likely pathogenic variant causative of MODY, which may be utilized for the diagnosis of HNF1A-MODY.

Keywords: MODY3, HNF1A, p.Lys120Gln, Sanger sequencing, Pathogenicity

1. Introduction

MODY is a subset of monogenic diabetes characterized by an autosomal dominant inheritance that can be transmitted by either parent or occur as a de-novo mutation.[1] It is classically characterized by a non-acute and non-ketotic presentation in lean subjects, typically before 25 years of age.[2] About 1-6% of patients with diabetes suffer from MODY, and distinguishing it from type 1 or type 2 diabetes is a diagnostic challenge.[2,3,4] MODY often faces misclassification as type 1 diabetes (T1D) due to its onset at a young age, or as early onset type 2 diabetes (T2D) due to relatively low risk of ketosis, and the low-dependence on insulin. MODY stands apart from T1D and T2D genetically, as it is primarily caused by a single gene mutation.

There are at least 14 different subtypes of MODY, categorized based on the specific gene that is mutated. The most common among these, MODY1, MODY2 and MODY3 are associated with HNF4α, GCK and HNF1α respectively. Together these account for up to 80% of MODY cases, while the rest have mutations in one of the other MODY genes, namely, PDX, HNF1β, NEUROD1, KLF11, CEL, PAX4, INS, BLK, ABCC8, KCNJ11, and

APPL1. The different genes varied in terms of the age at which symptoms appear, how well they respond to treatments, and if extra-pancreatic symptoms are present.[5] Epidemiologically, variants in MODY genes have been reported from every region of the world.[5] Very limited studies have been carried out thus far with Sri Lankan patients suspected of MODY, which have identified mutations in some of these genes including HNF1A.[6]

The most frequently mutated gene in MODY overall is GCK followed by HNF1A.[5] However, in some populations, mutations in HNF1A gene are the most common cause of MODY.[7] HNF1A is a transcription factor that is expressed in the gut, kidney, liver, and pancreas, among other organs, and the gene is located on chromosome 12 (NC_000012.12) in the region 12q24.2.[8]

HNF1A is a regulatory protein that controls the expression of many genes in pancreatic beta cells, liver, kidneys and intestines. HNF1A-MODY occurs due to inhibition of the key steps of glucose transport and metabolism as well as mitochondrial metabolism in pancreatic-β cells.[6] In a study involving human islets from a person with a missense variation in the HNF1A gene, researchers revealed that, even though having normal β cell mass and key β cell characteristics, the individual experienced difficulty releasing insulin in response to glucose. This issue was linked to changes in genes related to glucose metabolism and ATP production. Additionally, significant alterations were observed in various metabolic functions, including gene transcription, protein synthesis and degradation, and cellular communication. The study suggests that a mutation in HNF1A may contribute to diabetes not by reducing β cell mass, but by disrupting the normal function of β cells required for insulin release in response to glucose, impacting β cell transcriptional regulatory networks.[9]

Individuals with HNF1A-MODY are usually non-insulin dependent at diagnosis, but their beta-cell function declines over time, leading to worsening hyperglycaemia. They show increased sensitivity to sulfonylureas, which can restore insulin secretion by bypassing the defective pathways, often making them more effective than insulin in the early stages.

In a previous study aimed at developing a Next Generation Sequencing (NGS) based test for the diagnosis of MODY, we have identified a patient with a novel HNF1A c.358 A>C (p.Lys120Gln) variant (unpublished research). This variant has not been previously reported in any MODY patient but given the role of HNF1A gene in MODY and the general attributes of the specific mutation, we carried out further verification of this variant and its pathogenicity analysis. Our studies confirmed the presence of this variant by the alternative method of Sanger sequencing[10], and established the variant as likely pathogenic in MODY.

2. Methodology

This study was approved by the Ethics Committee of the National Hospital of Sri Lanka (approval AAJ/ETH/COM/2024/OCT).

2.1. Samples. The c.358A>C (p.Lys120Gln) variant in the HNF1A gene was initially identified in the SN10 sample by NGS method. To confirm the presence of the detected variant, PCR & Sanger sequencing was subsequently performed with SN10 sample, along with SN05 (sample negative by NGS) and no template (nuclease free water) negative control.

Specific primers targeting the HNF1A gene were designed using Primer 3 Plus tool as below: Forward primer 5'-TACCTCACCGTCCCTGAGTC-3' and reverse primer 5'-CTGGTTGAGGCCAGTGGTAT–3'. The specificity of the primers for the target region was confirmed using Primer BLAST.

2.2. Sanger sequencing process. Genomic DNA were extracted from peripheral blood samples using the QIAmp DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocols. PCR amplification of the target region was performed in a 15µL reaction using 5X FIREPol® Master Mix (Solis Biodyne, Estonia), primers, nuclease-free water, and genomic DNA. PCR Thermocycling was conducted with an initial 10 minute denaturation at 95°C, followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes using a SimpliAmp™ Thermal Cycler.

Following amplification, PCR products were loaded on a 1% agarose gel and resolved at 70V for 15 minutes, with a 100bp DNA ladder used as a molecular size marker. Gel was visualized through the UV-transilluminator. PCR products were then purified using magnetic bead purification methods to eliminate unincorporated primers, nucleotides, and other impurities before downstream Sanger sequencing.

Chain termination PCR was performed using the bead purified PCR products as templates. The 10 µL reaction mixture contained 0.5µL of 2.5X Ready Reaction Mix, 1.75 µL of 5X Dilution Buffer, 2µL of 0.8 µM HNF1A forward primer, and 5.75µL of purified template. PCR thermal cycling was conducted with an initial denaturation at 96°C for 1 minute, followed by 25 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes, with a final hold at 4°C.

Post-PCR cleanup was performed using EDTA (0.125µM) and ethanol purification. DNA pellets were dissolved in 10µL of Hi-Di Formamide (Thermo Fisher Scientific). Purified products were then subjected to capillary electrophoresis using the SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Sequence data were analyzed using the BioEdit sequence alignment editor.

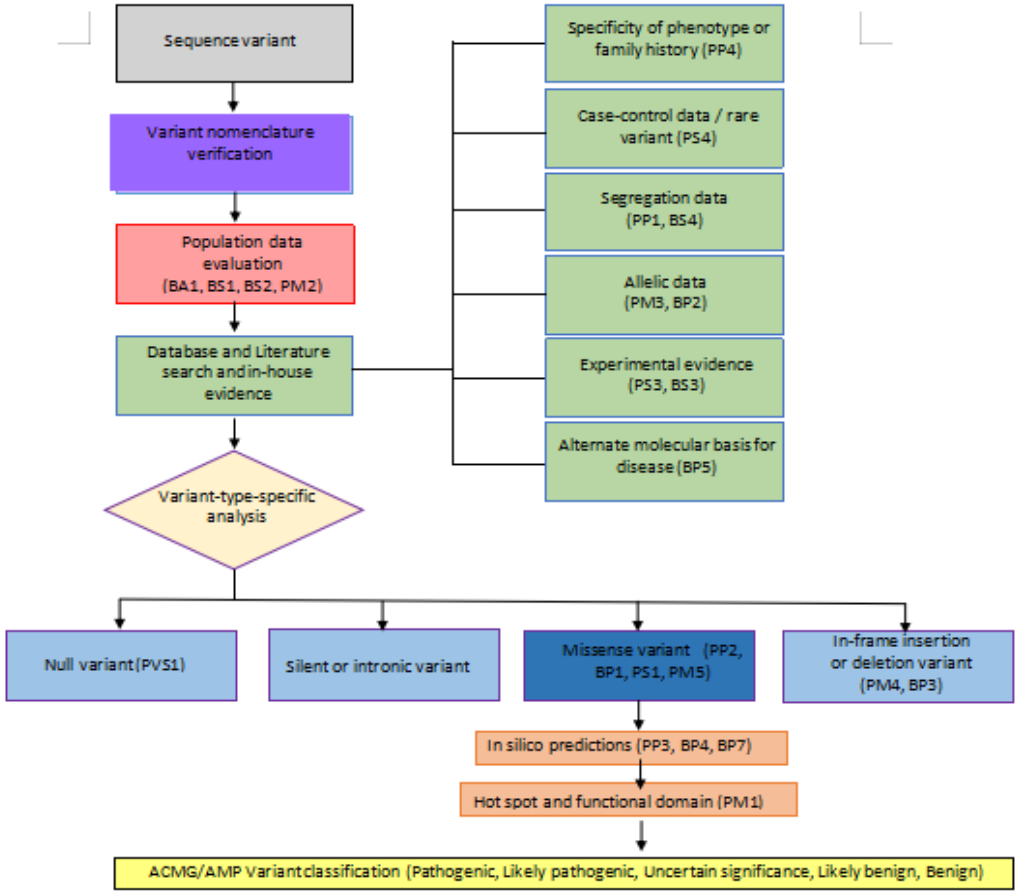

Figure 01: The variant classification and interpretation workflow as per ACMG/AMP guidelines (2015). [11]

2.3. Variant data pathogenicity analysis

2.3.1. Preliminary NGS analysis. Post NGS analysis was conducted using the Ion Reporter platform (ThermoFisher Scientific) with the in-built annotation workflow to identify and annotate potential variants.

The HNF1A c.358A>C (p.Lys120Gln) variant identified in the SN10 sample was further confirmed by aligning and evaluating the sequencing reads using the Integrative Genomics Viewer (IGV).

2.3.2. Assessment of the clinical significance. To assess the clinical significance of the identified HNF1A gene variant of SN10 patient, first followed the standardized variant interpretation workflow recommended by the American College of Medical Genetics and Genomics (ACMG). The analysis began with the identification and nomenclature verification of the variant according to the Human Genome Variation Society (HGVS) standards.[12] Population frequency was evaluated using public databases such as Genome Aggregation Database (gnomAD), Exome Aggregation Consortium (ExAC), 1000 Genomes and Bravo to determine the rarity or absence of the variant.[13] Next, a literature and clinical databases search was conducted, including resources like NCBI, Google scholar, Clinvar, ClinGen, Franklin and OMIM as well as internal data maintained by Genelabs Medical Pvt Ltd for any prior classifications of the variant, to check for any previously reported clinical significance. Variant interpretation also included evaluations based on its type among missense, silent, in-frame insertion/ deletion, intronic or null variants. [12]

In silico predictions using tools like SIFT, Phylop, Polyphen, Grantham, FATHMM, Mutation Taster and CADD scores were used to evaluate the potential impact on protein function/ amino acid substitution.[14] Additionally, the variant was examined based on whether it's occurred within a known mutational hotspot or functional domain. Supporting data such as segregation analysis, phenotype specificity, functional studies and de novo occurrence were analyzed. Based on the combination of these criteria, the variant was classified into one of five ACMG categories: pathogenic, likely pathogenic, uncertain significance, likely benign or benign.[12] The framework method outlined in Figure 01 ensures a comprehensive and evidence-based interpretation of the genetic variant's clinical relevance according to ACMG/ AMP standards and guidelines.

3. Results and Data Analysis and Findings

3.1. Patient Characteristics

The patient (SN10), a 33 years old female of Sinhala ethnicity, clinically diagnosed with diabetes at 19 years of age. At the time of diagnosis, her height was 154cm and weight 47kg, consistent with a normal body mass index (BMI). The clinical presentation was consistent with non-ketotic diabetes. Patient has a strong family history of diabetes mellitus, with the mother diagnosed with type 2 diabetes and her sister also developing diabetes in early adulthood (20s). Treatment data specific to sulfonylurea response in this patient were not available at the time of this analysis and familial segregation analysis was not performed as relatives were not available for genetic testing.

3.2. Variant Detection and confirmation

A heterozygous missense variant described as c.358A>C, leading to an amino acid substitution p.Lys120Gln was identified by NGS in SN10 patient sample. This variant is located on chromosome 12 at position Chr12:120988864 (hg38). This is a missense variant, resulting in a change from a positively charged lysine (Lys) to a polar, uncharged glutamine (Gln) at codon 120 within the highly conserved DNA binding domain of the HNF1A protein.

3.3. Sanger sequencing analysis

In gel electrophoresis of the PCR products of the region of interest, both SN05 and SN10 samples showed distinct bands at

approximately 389 base pairs, indicating the successful amplification of the targeted HNF1A gene region in both samples. Conversely, no band was present in the negative control (No template), indicating that there was no contamination in the PCR process. (Figure 02)

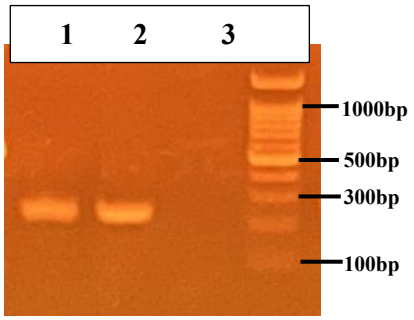

Figure 02: Gel electrophoresis of the PCR product. Lane 1-SN05 (negative sample), Lane 2-SN10 (positive sample), Lane 3: No template control, Lane 4: 100bp Ladder.

The PCR products of both SN05 and SN10 were sequenced by Sanger sequencing, and the electropherogram demonstrated a double peak at nucleotide position c.358 for sample SN10, confirming the presence of a heterozygous A>C (M: degenerative nucleotide) substitution (Figure 03). This mutation was not present in the SN05 sample, which was also negative by NGS.

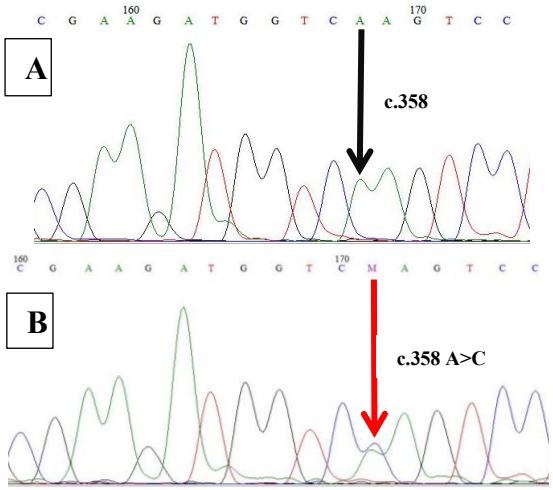

Figure 03: Electropherograms of the SN05 (A) and SN10 (B) samples. The arrows indicate the HNF1A c.358 position where an Adenine nucleotide is present in the wild type sequence of SN05 samples, and the A>C mutation is detected the SN10 mutated sample.

3.4. Population Frequency Analysis

Assessment of population frequency data was conducted using gnomAd, ExAC, 1000 Genomes and Bravo. The variant was not identified in any of these databases.[13] The absence of the variant in a large population database indicates that it is extremely rare or potentially novel in the general population and it supports the ACMG criterion PM2 (moderate evidence of pathogenic).[13]

3.5. Database and Literature Search

A search for clinical interpretations for this variant did not show any entries in available variant databases, including Clinvar, ClinGen and OMIM. Comprehensive literature searches using databases such as NCBI/PubMed, Google scholar, Google and Mastermind showed no prior studies or cases of the c.358A>C (p.Lys120Gln) variant on HNF1A gene. [12]

However, the variant analysis using Franklin by Genoox platform, showed the potential pathogenic significance by providing a computational prediction based on bioinformatics and other relevant data and giving a Likely Pathogenic outcome.

3.6. In silico (computational) Prediction Results

In silico analysis predictions collectively implied a probable damaging effect of the p.Lys120Gln mutation on protein (Table 01)

Table 01: *In silico* prediction scores

***In silico* tool**	**Result**	**Interpretation**
SIFT	0	Damaging
PolyPhen-2	0.927	Probably Damaging
Grantham	53	Moderately Radical Change
Phylop	8.87	Highly conserved
Other tools	Not available	No data

Several other tools such as FATHMM, Mutation Taster and CADD scores were used, but did not show interpretable scores for this specific variant.[14]

Although strong support was provided by *in silico* predictions and structural analysis, there was no available additional data for familial segregation analysis, phenotype specificity, de novo occurrence, or functional studies. Furthermore, there were no previous internal case records from our laboratory identifying this variant.[12]

Table 2. Variant classification and interpretation for HNF1A c.358A>C according to ACMG/AMP (2015) guidelines. [12]

ACMG Criteria	**Description**	**Results (Strength)**	**Evidence**
PM1	Located in a critical functional domain (DNA binding domain) with no benign variation	Moderate	DNA binding domain of HNF1A
PM2	Absent from population databases	Moderate	Not found in population databases (gnomAD v4.1.0, n=1614242)
PM5	Novel missense change at an amino acid residue where different pathogenic missense changes have been seen previously	Moderate	P.Lys120Glu previously reported as likely pathogenic
PP2	Missense variant in a gene where missense is a common mechanism of disease	Supporting	HNF1A intolerant to benign missense variation

PP3	Multiple *in silico* tools predict a deleterious effect. Aggregated score predicts a deleterious effect.	Supporting Aggregated prediction score ranges: Benign supporting 0-0.15, Pathogenic Supporting 0.7-0.8, Pathogenic Moderate 0.8-0.9, Pathogenic Strong 0.9-1.0 (Franklin Genoox)	SIFT, Polyphen, Phylop and Grantham

3.7. ACMG/AMP Classification

Based on three moderate (PM1, PM2 &PM5) and two supporting (PP2, PP3) ACMG criteria, this HNF1A c.358A>C (p.Lys120Gln) variant was classified as Likely Pathogenic.[12]

Table 2 summarizes the available interpretation of this variant classification according to the ACMG/ AMP guidelines.

4. Discussion and Conclusion

HNF1A-MODY (MODY3) is a monogenic disease with autosomal-dominant inheritance due to HNF1A haplo-insufficiency, where the loss of one functional copy of the HNF1A gene results in insufficient protein levels to sustain normal pancreatic beta cell function and glucose regulation, or dominant-negative effects.[15] The HNF1A gene has ten exons and a promoter that are prone to mutations, with exons 1–6 accounting for more than 80% of the total.[16] Specifically, most of the mutations have been found in HNF1A exons 2 and 4, while the lowest number have been found in exons 5 and 10.[15] Missense, frame shift, nonsense, splicing mutations, in-frame deletions, insertions and duplications of amino acids, and partial or whole-gene deletions are among the variations that have been reported to far. The dimerization domain, DNA-binding domain, and transactivation domain are among the 631 amino acids that make up the HNF1A protein.[17]

We have previously identified c.358A>C (p.Lys120Gln) mutation in Exon 2 of HNF1A in a suspected MODY patient (unpublished data), using an NGS-based method. In the current study we have verified this result by an alternative method of Sanger sequencing, to rule out sequencing artifacts in NGS. While the heterozygous substitution was clearly observed in SN10 through Sanger sequencing, its absence in SN05 confirms the variant is real, and is unlikely to be a sequencing artifact. Such use of multiple complementary approaches minimized the likelihood of false positive findings, which is particularly important in interpretation of rare variants.[11]

It was important to understand the significance of this variant in MODY, since it was not available in commonly used variant databases. Thus a systematic analysis was carried out to determine its pathogenicity in MODY, following the ACMG/AMP standards and guidelines.

The HNF1A gene encodes a transcription factor essential for pancreatic beta-cell development and glucose homeostasis, with pathogenic variants well established in association with Maturity-Onset Diabetes of the Young type 3 (MODY3).[18] The variant c.358A>C (p.Lys120Gln) identified in this study is located within the DNA-binding domain, a highly conserved and functionally critical region of the protein. Therefore, the variant supports the PM1 ACMG criteria.[12]

Pathogenicity in HNF1A is predominantly driven by missense mutations, with low rate of benign missense variation at the amino acid level. The p.Lys120Gln variant is consistent with this known disease mechanism, supporting the classification of PP2.[12]

Although the specific variant p.Lys120Gln has not been previously reported in major clinical variant databases, another substitution at the same codon p.Lys120Glu has been classified as likely pathogenic. This highlights the functional importance of lysine at position 120. The substitution to glutamine represents a potentially disruptive change in physicochemical properties and may impair protein-DNA interactions required for transcriptional regulation. The identification of another pathogenic variant at this residue supports the application of the PM5 criterion.[12]

The absence of this variant in large-scale population databases such as gnomAD, ExAc, 1000 Genomes and Bravo supports its rarity, a characteristic commonly associated with pathogenic variants in Mendelian conditions. The rarity of the variant supports its classification under the PM2 criterion (ACMG). [13]

The use of multiple computational prediction tools further strengthens this clinical result. SIFT and PolyPhen-2 both predicted the variant to be damaging, with SIFT producing a score of 0 and PolyPhen shows a score of 0.927, indicating a likelihood of functional impairment. The affected lysine residue at position 120 showed high evolutionary conservation, as demonstrated by a PhyloP score of 8.87. This suggests that alterations at this site are poorly tolerated and may have significant functional consequences. Furthermore, the Grantham score, which quantifies the physiochemical difference between amino acids to assess the effect of a substitution, gave a score of 53 indicating a moderately radical change, supporting a potential impact on protein structure, function and DNA binding capacity. While some predictive results were unavailable, the consistent deleterious findings among the available computational tools support the ACMG/AMP PP3 criterion, thereby supporting the likely pathogenic classification of the variant.[14]

This classification aligns with the interpretations carried out by Franklin database. Comprehensive literature searches using databases such as NCBI/PubMed, Google scholar, Google and Mastermind, showed no prior studies or cases on the c.358A>C (p.Lys120Gln) variant on HNF1A. The absence of published data further emphasizes the novelty of this variant and the importance of functional and clinical studies to clarify its clinical significance.[12]

However, this study has several important limitations. First, the study is limited by the availability of data from a single patient, the findings should be interpreted with caution and require further validation in larger cohorts. Second, no segregation analysis was performed due to the unavailability of genetic samples from family members, limiting the ability to confirm inheritance patterns or establish genotype/ phenotype correlations. Additionally, functional validation assays such as transcriptional activity or protein expression studies were not conducted, limiting the ability to assess the biological impact of the identified HNF1A c.358A>C (p.Lys120Gln) variant. Although multiple in-silico tools predicted deleterious effects and these predictions require experimental support.

Furthermore, treatment response data, particularly regarding sulfonylurea sensitivity, which is often informative in HNF1A MODY cases, were not available for the SN10 patient. Future studies including familial samples, longitudinal clinical data and functional assays are essential to confirm the clinical relevance of this novel HNF1A variant.[12]

Based on ACMG/AMP 2015 standards and guidelines, the combination of three moderate (PM1, PM2, PM5) and two supporting (PP2, PP3) criteria led to classification of the HNF1A c.358A>C (p.Lys120Gln) variant as Likely Pathogenic.[12]

In conclusion, this study verified and characterized a novel missense variant in the HNF1A gene, c.358A>C (p.Lys120Gln), using a combination of bioinformatic tools, database analysis, literature search and standardized ACMG/AMP classification criteria. Although the variant is absent from population and clinical databases and no experimental evidence is currently available, its location in a conserved functional domain, damaging *in silico* predictions, and structural context provide significant support for pathogenic potential. According to the applied ACMG criteria (PM1, PM2, PM5, PP2, PP3), the variant is classified as Likely Pathogenic. While no functional data are available at present, together with the clinical features suggestive of MODY, the findings support a significant role of the HNF1A c.358A>C (p.Lys120Gln) variant in monogenic diabetes and justify further investigation through familial segregation and functional assays.[12,13]

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Isolation, identification, antimicrobial susceptibility testing and public awareness of *Escherichia coli* on raw beef, pork and chicken meat in Western and Southern provinces of Sri Lanka

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Abstract

Escherichia coli (*E. coli*) is a major foodborne pathogen with significant health and economic implications due to its virulence and antibiotic resistance. This cross-sectional study assessed the prevalence, antimicrobial resistance, and public awareness of *E. coli* in raw beef and chicken from the Western province and pork from the Southern province of Sri Lanka. Meat samples were collected under aseptic conditions and cultured on MacConkey agar. Presumptive *E. coli* colonies were identified using Gram staining and biochemical tests (indole and citrate utilization). Antimicrobial susceptibility was tested against Gentamicin, Chloramphenicol, and Erythromycin. Additionally, a questionnaire-based survey evaluated public knowledge and practices regarding *E. coli* and meat safety. The prevalence of *E. coli* was 33.3% in beef, 10% in pork, and 50% in chicken samples. All *E. coli* isolates from meat samples exhibited 100% resistance to Erythromycin. Susceptibility to Gentamicin was 76.9% in beef, 83.4% in pork, and 50% in chicken isolates. For Chloramphenicol, susceptibility was 100% in beef, 50% in pork, and 83.4% in chicken isolates. Survey results from 100 beef, 123 pork, and 429 chicken consumers revealed that 56%, 27%, and 47.1% respectively were unaware that *E. coli* is a foodborne pathogen, while only about half recognized undercooked meat as a source of infection. This study highlights the need for improved household hygiene, increased public awareness, and better handling practices by meat retailers. The observed antibiotic resistance underscores the necessity for stricter antibiotic usage guidelines. These findings provide baseline data for future interventions to enhance meat safety in Sri Lanka.

Keywords: *Escherichia coli,* foodborne pathogen, beef, pork, chicken, antimicrobial susceptibility testing

1. Introduction

Foodborne diseases are a global health issue due to the rise in demand for foods of animal origin with the increase of the world population. The risk of foodborne infections has grown tremendously over the past two decades because of the emerging foodborne pathogens hence, food safety and prevention of foodborne outbreaks is a public health concern.[1] Some of the major foodborne bacterial pathogens related to meat are *Salmonella* spp., *Escherichia coli (E. coli), Campylobacter jejuni* and *Listeria monocytogenes*.[2] Among them, *E. coli* infection is considered one of the important health issues[3].

Beef and pork are considered to be the key sources of foodborne transmission of *E. coli* and pork[4-8]. Chicken meat also poses a significant risk *E.coli* through food borne transmission.[9,10] In 2019, the most consumed meat in the world was poultry (14.7 kilogram/capita/year) followed by pork (11.1 kilogram/capita/year) and beef (6.4 kilogram/capita/year).[11] In a study conducted in Sri Lanka, the most preferred types of meat were chicken (84 %) followed by mutton (44 %), beef (33 %) and pork (24 %). Since chicken is not restricted by ethno-religious

beliefs and is regarded as a nutritious white meat, its consumption may be higher.[12] Sri Lanka is a nation with multi-ethnicity and religion, therefore the growth of the meat industry and beef and pork consumption is highly influenced by ethnoreligious views.[13]

E. coli is a rod-shaped, gram-negative and facultative anaerobic bacterium that belongs to the family of Enterobacteriaceae in the class of Gammaproteobacteria. It was first discovered and isolated in 1885 by T. Escherich during his study of intestinal microbes in infants and it was initially named Bacterium coli commune.[14] Majority of the *E.coli* strains are part of the intestinal microbiota, harmlessly colonizing the gastrointestinal tract. However, some *E. coli* strains have developed pathogenicity due to much phylogenetic diversity with certain lineages acquiring diverse combinations of virulence genes.[15] Several highly adapted *E. coli* clones have gained unique virulence properties, allowing them to adapt to new habitats and causing a wide range of diseases.[16] The virulence factors are acquired through transposons, bacteriophages and pathogenicity islands.[17] The first time *E. coli* was associated with human outbreaks was in 1982 due to the consumption of undercooked meat and beef patty was one of the common ingredients.[18] More than 40 non-O157 Shiga toxin-producing *E. coli* (STEC) outbreaks were discovered between 2000 and 2010. Almost half of them were caused by food poisoning, but many more were spread from person to person, particularly in child day care centre, waterborne transmission or contact with animals in public were responsible for a few epidemics.[19]

The five main foodborne diarrheagenic *E. coli* pathotypes based on virulence factors, invasiveness, toxin production, patterns and effect of bacterial attachment to host cells are Enteropathogenic *E. coli* (EPEC), *STEC*/Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), and Enterotoxigenic *E. coli* (ETEC).[20] The most widely recognized pathotype associated with foodborne illnesses is the STEC, representing the predominant serotype *E. coli* O157:H7.[21] STECs are estimated to be responsible for 2.8 million acute illnesses worldwide.[22] The global prevalence (Figure 1) in cattle is 5.68%, with a higher prevalence in African (31.2%) and Northern American regions (7.35%).[23]

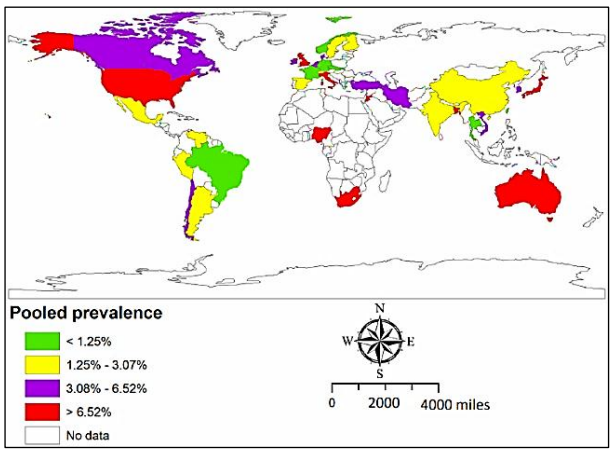

Figure 1. Estimated prevalence of *E. coli* O157 in cattle in different countries.[23]

Pathogenic *E. coli* ingested by the animal is excreted in feces leading to faecal contamination of the environment (Figure 2). Musca domestica (housefly) is a significant insect vector for STEC infection in farms and other vectors include birds, rodents and ruminants.[24]

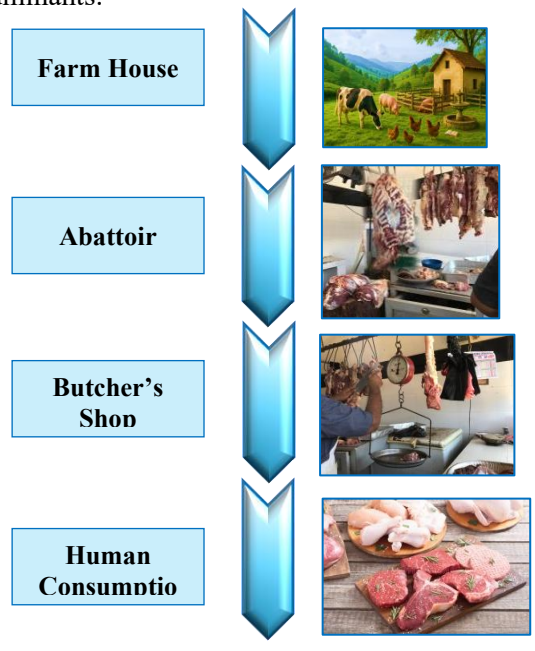

Figure 2. Mode of transmission of *E. coli*

Cross contamination can occur in the abattoir during evisceration. Handling money with bare hands, unwashed cutting board, knives and unhygienic practices can be reason for contamination at butcher's shop. The contamination of the carcass at the slaughterhouse can also occur by faecal shedding or by hides.[25, 26] Human exposures to *E. coli* is mainly through consumption of contaminated food or via direct contact.[27]

The production of Shiga toxins (Stx) by the stx1 and stx2 genes carried by lysogenic phages is the major virulence factor of STEC.[28] Intimin is essential for bacterial adherence to epithelial cells, resulting in a histopathological lesion called "attaching and effacing" (A/E lesion) controlled by locus of enterocyte effacement (LEE) which is a huge pathogenicity island leading to type III secretion system, Tir, and other secreted proteins.[29] STEC strains interact with the gut through long polar fimbriae (LPF), forming A/E lesions (Figure 3-A); Stx is produced in the intestine and transported in blood causing inhibition of protein synthesis and host inflammatory response leading to clinical manifestations.[30]

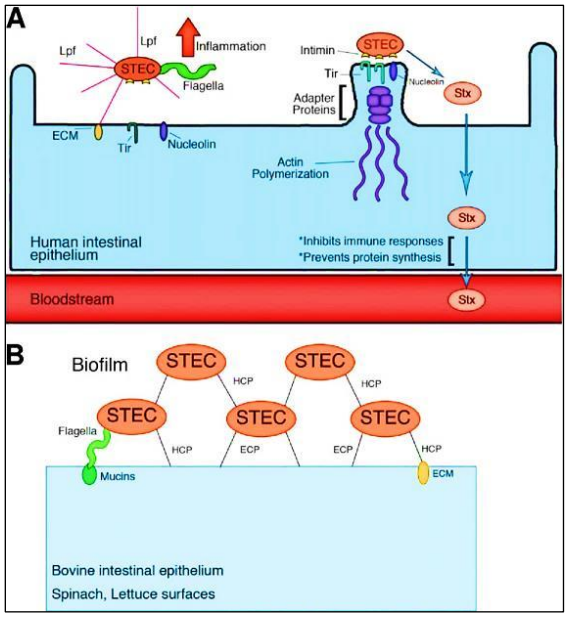

Figure 3. A- Colonization of STEC in intestine, B- Biofilm formation.[30]

Some infected people may be asymptomatic, while others may experience symptoms including fever, abdominal cramps, bloody diarrhea or even life-threatening conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura.[31] STEC strains may also adhere and colonize other surfaces, like the bovine intestine forming biofilm using adhesins such as haemorrhagic coli pilus (HCP), *E. coli* common pilus (ECP) and flagella (Figure 3-B). In swine, ETEC strains with fimbriae F5, F6, and F41 mostly colonise the posterior jejunum and ileum while ETEC with fimbriae F4 colonises the jejunum and ileum.[32]

In a study conducted contemporaneously across Great Britain, *E. coli* O157 was common in British beef cattle; the estimated herd-level prevalence was high in Scotland (23.6%) than in England and Wales (21.4%).[33] Similar studies conducted in other countries such as in India, the occurrence of *E. coli* O157:H7 in beef was 25.46% 25.8% in exported Malaysian beef and 11.1% in Thailand beef and 29.70% in Iran therefore contamination of beef meat with pathogenic *E. coli* continues to be a health concern worldwide.[34-36]

In Sri Lanka, the prevalence of STEC was found to be 53% in cattle calves.[37] In recent years, studies have been conducted on the prevalence of *E. coli* in chicken meat (20%), small fish (70%) and large fish (5%), but not on raw beef and pork.[38,39] However in a study conducted on ready-to-eat meat-based food products in Colombo, Sri Lanka including beef the prevalence of *E. coli* was 59%.[40]

Preventive and control strategies to minimise the risk of food/cross-contamination of *E. coli* include using safe water, hygienic conditions, vaccination for cattle and public awareness. Targeting super-shedders can also have a significant advantage.[26] Further methods to reduce contamination include pre-harvest interventions such as probiotics, vaccines (Epitopix SRP®), bacteriophages (Finalyse®) and post-harvest interventions such as physical interventions (knife trimming,

steam), using acid antimicrobials and oxidizer antimicrobials.[41] Preventative strategies for pigs include immunoprophylaxis (live attenuated and live wild type avirulent *E. coli*, subunit vaccines (purified F4 fimbriae), breeding of resistant pigs and diet management.[42]

Sri Lanka has implemented different measures in food safety such as the HACCP system, Food Act no.26 of 1980, including its amendments in 1991[43-45]. Understanding the potential factors for microbial meat contamination along the whole meat supply chain is required to identify targets for interventions and to minimise the number of meat-borne *E. coli* outbreaks.

This study focuses on the isolation and identification of *E. coli* from raw beef, pork and chicken samples from different regions of Sri Lanka, perform antimicrobial susceptibility test and determine the level of public awareness, as well as assess the knowledge, attitude and practice of beef, pork and chicken consumers via a questionnaire-based survey.

2. Methodology

2.1 Sample collection and preparation. The cross-sectional study was conducted between November 2021 and January 2022. A total of 21 beef and 20 chicken samples were collected from the Western Province, while 21 pork samples were obtained from the Southern Province of Sri Lanka. The study populations were all beef, chicken and pork carcasses which were fresh, unprocessed and slaughtered in the abattoir. Processed and spoiled samples were excluded. The samples were collected in sterile zip lock bags from the butcher's shop and were transported at 4°C in an icebox. The samples were then finely chopped using a mortar and pestle. All the procedures were done under aseptic conditions.

2.2 Pre-enrichment. Approximately 5 g of the meat was distributed into 50 mL falcon tube containing 30 mL of buffered peptone water using a sterile spoon spatula. The tubes were vortexed until the samples were homogenised and were incubated at 37°C for 4 hours.

2.3 Initial Culture. Isolation and identification of *E. coli* were performed following the flow chart in the Bergey's Manual of Determinative Bacteriology (Figure 4).[46] The inoculated peptone water was taken from the incubator and vortexed. It was used to streak plate using a sterile inoculation loop between 2 Bunsen burners and spread plate using a cotton swab inside a biosafety cabinet onto MacConkey agar Petri plate. The Petri plates were sealed with parafilm and were kept inside the incubator at 37°C for 24 hours.

2.4 Sub-culturing of presumptive E. coli colonies. After 24 hours of incubation, the MacConkey agar petri plates were observed for colony morphology. According to the colony morphology of *E. coli* on MacConkey agar: circular, moist, flat bright pink/red smooth colonies of entire margins were selected and were sub-cultured onto another new MacConkey agar Petri plate which was divided into 4 sections. The plates were sealed with parafilm and were incubated at 37°C for 24 hours.

2.5 Gram's staining. Colonies were picked using a sterile inoculation loop from sub-cultured MacConkey agar petri plates. Thin smears were prepared on a clean, dry glass slide using the inoculation loop and were air-dried and heat-fixed. The glass microscope slides were placed onto the staining tray and were stained according to the order and duration in Table 1. After the addition of each reagent, the slides were washed with distilled water. The slides were then placed on a clean surface and were left to air dry. Finally, the Gram's stained microscope slides were observed under different magnifications using a compound light microscope. A drop of immersion oil was added onto the smear and a coverslip was placed for observation at 100X magnification.

Table 1. Reagents used for Gram's staining

Gram's Reagents	**Duration (s)**
Crystal Violet	60
Gram's iodine	60
Gram's decolourizer	20
Safranin	60

2.6 Biochemical tests. The positive control used for the indole and citrate utilization test was the reference strain, *E. coli* (ATCC 25922) and the negative control was the uninoculated indole/citrate tube. Indole and citrate utilization tests were performed according to standard procedures.[47]

2.6.1 Indole Test. 5 mL test tubes containing 4 mL of Trytophan broth was inoculated by stirring with presumptive *E. coli* colonies which were picked using an inoculation loop from the sub-cultured MacConkey agar. The inoculated tryptophan broth was incubated at 37°C for 24 hours. After incubation, 0.5 mL of Kovacs reagent was added to the broth to observe cherry-red ring formation at the meniscus.

2.6.2 Citrate utilization test. Citrate utilization test was only carried out when the indole test was positive. A presumptive *E. coli* colony was picked using an inoculation loop from the sub-cultured MacConkey agar Petri plate and the citrate agar was streaked according to the conventional tube method. The tubes were incubated at 37°C for 48 hours. Results were observed to confirm the presence or absence of *E. coli* in the beef, pork and chicken samples.

2.7 Identification of E. coli. Result interpretation was guided using the taxonomic characteristics from Bergey's Manual of Determinative Bacteriology (Figure 4*)*.[46]

2.8 Antimicrobial Susceptibility Testing. Antibiotic susceptibility testing was performed using the disk diffusion method according to CLSI guidelines.[48] Luria Bertani (LB) broth was prepared according to the manufacturer's instructions (Himedia, India). An *E. coli* colony was picked using an inoculation loop from the sub-cultured MacConkey agar petri plate and was dipped into LB broth. The inoculated broth was incubated at 37°C for 24 hours. After incubation, 1 mL of the cultured LB broth was poured into an empty falcon tube. The turbidity of the LB broth was compared to the prepared 0.5M McFarland standard using a Wickerham card. If the turbidity of the inoculated LB broth was higher, fresh LB broth was poured into the falcon tube containing inoculated LB broth, until the turbidity of the inoculated LB broth was similar to that of 0.5M McFarland standard. Mueller-Hinton agar (MHA) was prepared according to the manufacturer's instructions (Himedia, India). It was cultured by spread plating using a cotton swab that was dipped into inoculated LB broth which had similar turbidity to that of 0.5M McFarland standard. The Petri plates were divided into 4 sections. Gentamicin 10µg/disc (Himedia, India), chloramphenicol 30µg/disc (Himedia, India), erythromycin 15µg/disc (Himedia, India) and a negative control filter paper dipped in autoclaved distilled water were placed in the MHA Petri plate using sterile forceps. The plates were sealed with parafilm and were kept in the incubator at 37°C for 24 hours. After incubation, the diameters of the zones of inhibition were measured and were compared to the zone size interpretative chart to determine whether the samples are sensitive, intermediate or resistant.

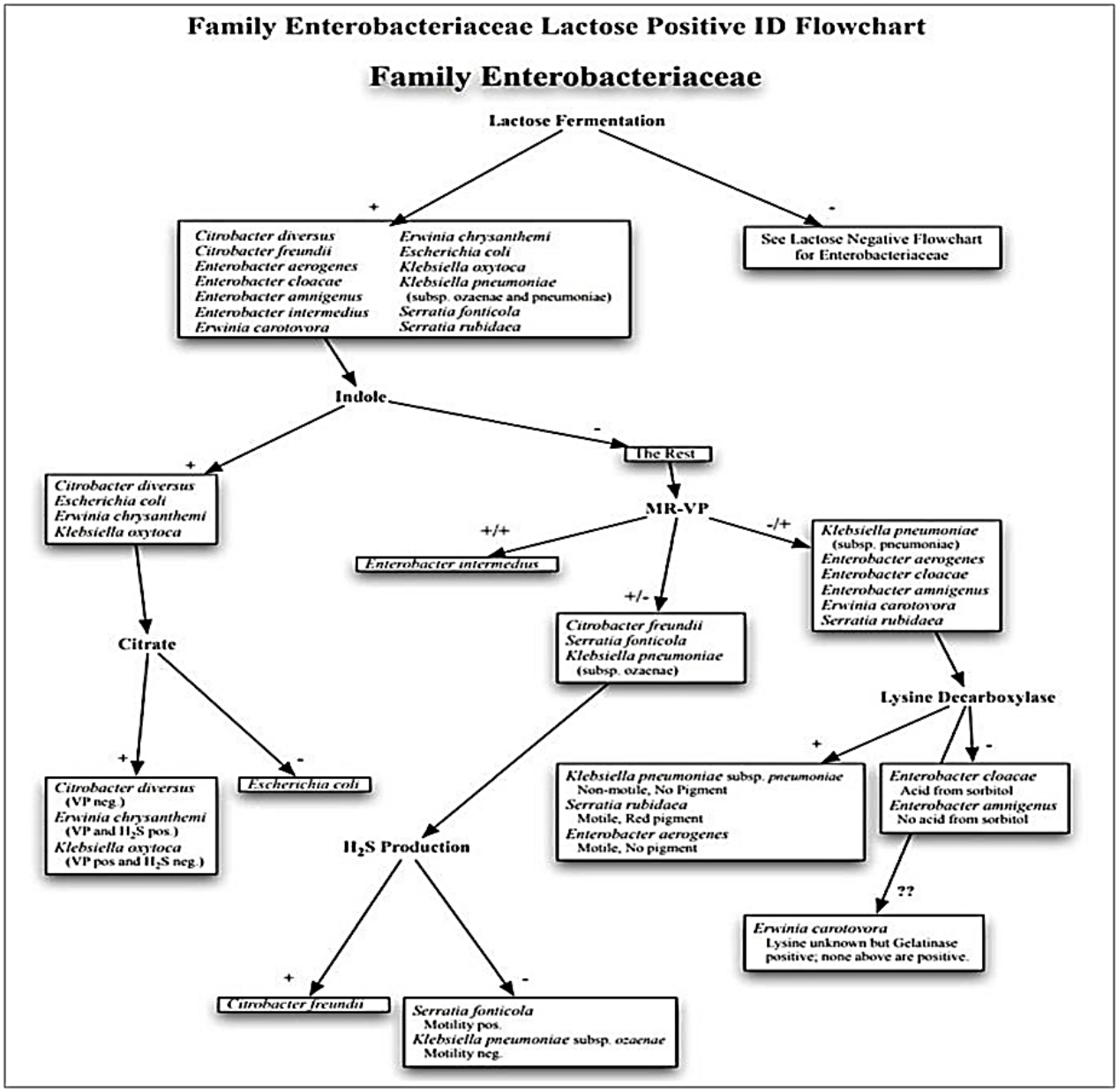

Figure 4. Flowchart for the Identification of Lactose-Positive Enterobacteriaceae based on Biochemical Tests.[46]

2.9 Data Analysis. A self-reported questionnaire-based survey was employed to assess the knowledge, attitude and practice of the beef, pork and chicken consumers. The participation was entirely voluntary, and no personal or identifying information was collected. As the study posed minimal risk and was conducted for academic purposes without involving sensitive topics, formal ethical approval was not sought. Consent was implied through completion of the form. Participants were regular consumers of meat, aged ≥18, and residents of Sri Lanka. Participants who were vegan, <18 years and incomplete responses were excluded. The questionnaire was created using Google forms and was shared via social media. The survey data were analyzed using Microsoft Excel 2010 and SPSS version 20. Descriptive statistics, including frequencies and percentages, were generated to summarize participant responses.

3. Results

3.1 Culture plates. Pink/red, flat, bright, circular, moist smooth colonies of entire margins were observed in the culture plates as shown in Figure 5 and 6. Colourless colonies were observed too.

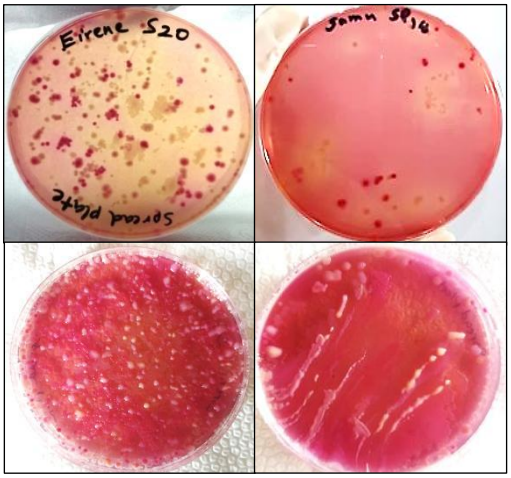

Figure 5. Initial culture plates

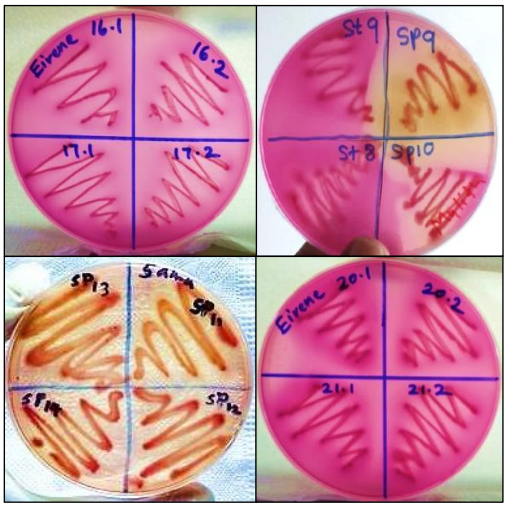

Figure 6. Sub-cultured Plates

3.2 Microscopic Observation. Pink, rod shaped, gram-negative bacteria arranged singly or in pairs (Figure 7) were observed in all samples under 100X magnification using compound light microscope.

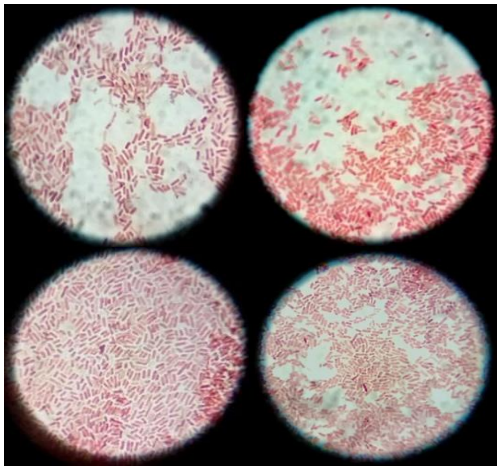

Figure 7. Gram-negative bacteria (100X)

3.3 Biochemical tests. A cherry red ring formation (Figure 8) was observed soon after adding Kovacs reagent indicating a positive result. Absence of a cherry red ring formation with the solution being yellow indicated a negative indole test result.

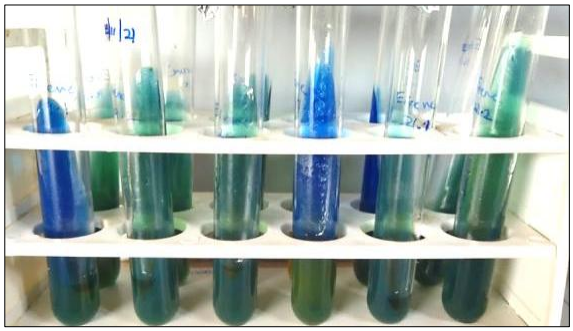

Figure 8. Indole Test

Citrate utilization test (Figure 9) was done only for the samples which had positive indole result. A negative citrate tested resulted in no colour change while a colour change from green to blue of the citrate agar indicated a positive test.

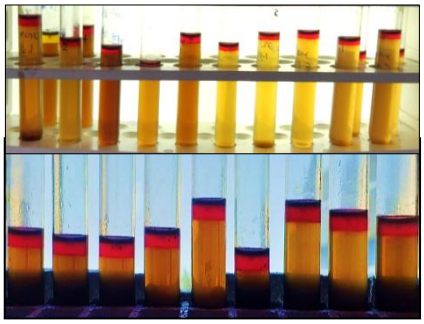

Figure 9. Citrate Utilization Test

3.4 Antimicrobial susceptibility test (AST). AST was done for all meat samples with a positive citrate utilization test as shown in Figure 10. The zone of inhibition was recorded and categorised as sensitive (S), intermediate (I), and resistant (R) as illustrated in Figure 11. Zone of inhibition was observed for gentamicin and chloramphenicol. Erythromycin and negative control (filter paper soaked in autoclaved distilled water) had no zone of inhibition.

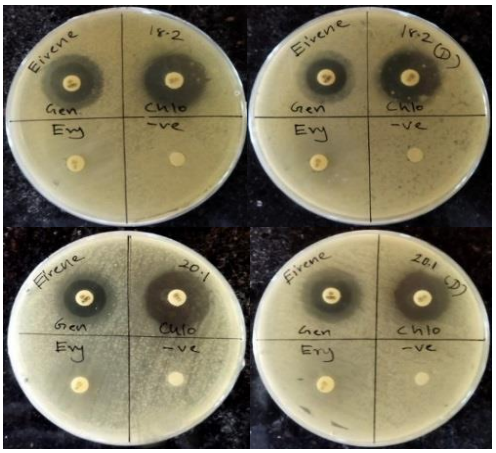

Figure 10. AST Plates

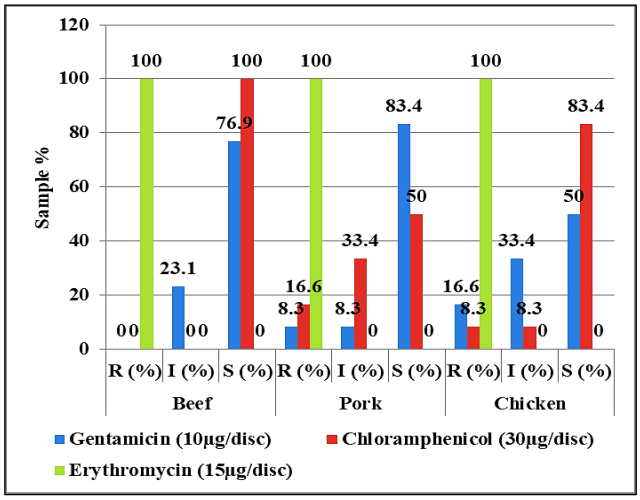

Figure 11. Zone of Inhibition for each meat sample

3.5 Data Analysis. Table 2 shows the knowledge, attitude and practice of the meat consumers. X is the specific meat sample which could be beef, pork or chicken.

4. Discussion

This is the first study conducted in Western Province and Southern Province, Sri Lanka to isolate, identify, perform antimicrobial susceptibility test and determine the level of public awareness of *E. coli* in raw beef, pork and chicken meat. The prevalence of *E. coli* in the beef (B) and chicken (C) meat bought from butcher's shop in the Western province, Sri Lanka is 33.3%, 50% and for pork (P) meat is 10% from Southern Province, Sri Lanka, respectively in our study. In a previous study conducted in South China, beef had the highest prevalence (13.32%) of contamination by *E. coli*, followed by pork (6.90 %) and chicken (3.28%).[49] In Ghana, the prevalence of *E. coli* in beef and chicken was 86.67% and 80%, in Northern Egypt, it was 6.7%, 16.7% in South Korea, 42.3%, 75.9%, and for pork 39.2% respectively[50-52]. In a study conducted in Italy, STEC isolates were obtained in pork samples indicating a stx-positive rate of 7.1%, and 2.8%.[53] According to reported prevalence rates of stx-positive *E. coli* isolates in live swine, slaughtered swine, and retail pork samples around the world varied from 4.4%-68.3%, 22%-86.3%, and 0.10%-80%, respectively, which depended on the sample classifications, detection methods, and the sanitation of the slaughterhouses and retail markets.[54]

The differences in prevalence and contamination rates of meat isolated from various countries could be due to sample types, seasonal influences, and detection methods utilized or due to the hygienic measures used in the whole beef, pork and chicken meat supply chain which varies among countries and also from the farm to abattoir to butcher's shop to the consumer.

Cross-contamination is an important source of carcass contamination in abattoir which could happen by faeces during evisceration, cattle transport or in the lairage, handlers' hands, and knives.[55] As a result of simultaneous handling of money and meat, *E. coli* was detected in meat and in money samples with a 100% contamination of

Table 2. Data Analysis of the questionnaire based survey

Variables	**Values**	**Beef**		**Pork**		**Chicken**	
		Frequency	**%**	**Frequency**	**%**	**Frequency**	**%**
Place of buying *X*	Butcher's shop	65	65	75	61	175	40.7
	Supermarket	39	39	75	61	249	58.0
	Local farms	1	1	24	19.5	24	5.5
	Small retail shops	3	3	20	16.3	66	15.3
	Other	4	4	14	11.4	7	1.6
Priority criterion when purchasing *X*	Quality (purity, taste and nutritive value)	38	38	66	53.7	236	55.0
	Price	10	10	42	34.1	120	27.9
	Freshness	45	45	92	74.8	290	67.5
	Other	8	8	7	5.7	7	1.6
Methods of consumption	Boiled	20	20	13	10.6	80	18.6
	Fried/deep fried	30	30	52	57.7	194	45.2
	Cooked	82	82	98	79.7	359	83.6
	Roasted	12	12	25	20.3	92	21.4
	Baked	11	11	33	26.8	68	15.8
	Smoked	9	9	9	7.3	31	7.2
	Raw	2	2	0	0	5	1.1
	Other	4	4	5	4.1	0	0
Knows that undercooked meat is a source of *E.coli*	Yes	52	52	52	42.3	173	40.3
	No	48	48	71	57.7	256	59.7
Thinks that cooked meat is always safe to eat	Yes	38	38	67	54.5	218	50.8
	No	62	62	56	45.5	211	49.2
History of meat *X* poisoning	Yes	11	11	9	7.3	25	5.8
	No	89	89	114	92.7	404	94.2
Symptoms	Headache	13	13	35	28.5	48	11.1
	Fever/chills	19	19	26	21.1	56	13.0
	Diarrhea	65	65	81	65.9	193	44.0

	Abdominal cramps	48	48	70	43.1	241	56.1
	Nausea and vomiting	63	63	74	60.2	262	61.0
	Other	11	11	14	11.4	0	0
Thinks that *X* slaughtered in abattoir is always safe to eat	Yes	37	37	10	8.1	51	11.9
	No	63	63	33	26.8	378	88.1
Heard of *E.coli* as a foodborne pathogen	Yes	44	44	10	8.1	227	52.9
	No	56	56	33	26.8	202	47.1
Knows that *E.coli* can be transmitted through the consumption of contaminated *X* meat	Yes	34	34	43	35	119	27.7
	No	66	66	79	64.2	310	72.3

currency from the same butcher.[56] Similar results were reported in another study with 100% prevalence of *E. coli* in money samples from meat sellers.[57]

Culturing in MacConkey agar helped to differentiate between lactose and non-lactose fermenting bacteria, since *E. coli* is lactose fermenting bacteria with its colonial morphological features which include pink, bright, flat, deeper central depression, dome-shaped appearance.[58] Gram's staining was done to determine the shape, size and arrangement of the bacteria. All the samples were gram-negative because the microscope slides were inoculated from the sub-cultured MacConkey agar plates (Figure 6), which were selective for the growth of gram-negative bacteria. Gram-negative bacilli were arranged singly or in pairs under microscopic observations and these were suspected to be *E. coli* as observed in Figure 7.

According to Bergey's manual of determinative bacteriology (Figure 4), if the Indole test was positive, it was presumed that the isolated organism could be *E. coli,* *Klebsiella oxytoca, Erwinia chrysanthemi*, or *Citrobacter diversus*.[46] However, a negative citrate utilization test further narrows down the identification, confirming the presence of *E. coli*. This biochemical distinction is important given that the indole-positive microorganisms have also been isolated from raw meat in previous studies. For instance, in a study conducted in Egypt, the prevalence of *E. coli* was 54.0% and *Klebsiella* spp. was 6.0% in beef meat.[59] In another study to assess the presence of Enterobacteriaceae in raw meat, the prevalence of *K. oxytoca* was 27.4% and *E. coli* was 12.1%.[60] In a study in Ghana the prevalence in beef meat was 8.2% for *C. diversus* and 17.3% for *K.oxytoca.*[61] In a study conducted among 500 pork samples, 68% were positive for coliforms including *Citrobacter* spp. and *Klebsiella* spp.[62] *E. chrysanthemi* is a phytopathogenic bacterium which is not typically found in meat samples but the presence of it could indicate cross-contamination. These findings highlight the need for confirmatory biochemical tests to accurately distinguish *E. coli* from other closely related species.

All *E. coli* isolates (100%) were resistant to Erythromycin, as shown in Figure 10, where no zone of inhibition was observed around the Erythromycin discs on the ABST plates. Similar results were obtained in another study.[60] B - 76.9%, P - 83.4%, C - 50% of the *E. coli* isolates were susceptible to Gentamicin. Figure 11 reveals that Chloramphenicol had the highest (100%) antimicrobial activity in *E.coli* isolates from beef, followed by C - 83.4% and P - 50% The findings were similar to a study done in Bangladesh, where the *E.coli* isolates were susceptible to Chloramphenicol (83%), Gentamicin (73%) and resistant (83%) to erythromycin.[63] However, colonies were observed inside the zone of inhibition for Chloramphenicol. This could indicate the presence of different strains of *E. coli* which are resistant to the antibiotic. Contrastingly, without the bacteria having developed a resistance phenotype, it could also indicate the presence of tolerant and persistent bacteria.[64] B - 23.1%, P - 8.3%, C - 33.4% were intermediately resistant to Gentamicin, whereas for Chloramphenicol, it was B - 0%, P - 33.4% and C - 8.3% for *E. coli* isolates In a previous study, B - 2.2%, P - 0.4%, C - 30.1% of the *E. coli* isolates were intermediately resistant to Gentamicin, Chloramphenicol and Erythromycin.[65] A study from 2002-2011 conducted among Italian swine herds showed an increase in resistance to Erythromycin (92.4–100%) and Gentamicin (63.6–85.7%).[66]

100, 123, 429 respondents in beef, pork and chicken participated in the self-reported questionnaire-based survey respectively, which was used to determine the public awareness including knowledge, attitude and practice of beef and pork consumers comprising hygiene and food safety as seen in table 2, 65% of the beef consumers and 40.7% of chicken consumers buy from butcher's shop and 39% and 58.7% buy from supermarkets. 61% of pork consumers buy from supermarket and butcher shop. Similar to our results, in a study conducted in Hungary, butcher shops (45.36%) are where most pork meat is purchased, followed by hypermarkets and supermarkets (28.56%).[67] A study reported that beef samples from wet markets had high contamination rate (89.50%) than in hyper markets (35.35% and 20%).[68]

When purchasing beef, 45% of the participants considered freshness as the priority criterion compared to quality (38%) as seen in Table 2. For pork and chicken, 74.8% and 67.5% of respondents prioritized freshness, while 53.7% and 55% prioritized quality, respectively. Similarly in a study conducted in Venezuela, freshness of the meat was an important attribute when buying/consuming meat.[69] In a research conducted in four European nations (France, UK, Germany and Spain), participants did not consider packed beef products as fresh, and it is likely that the perceived healthiness of branded and labelled beef is linked to its perceived quality; contrastingly, fresh meat is typically unbranded and unlabeled, so consumers assess its healthiness mostly based on its appearance and labelled, branded, fresh. Lean beef was seen as healthy compared to further processed packed beef.[70] Pork consumers' primary quality criterion is cleanliness, followed by moderate fat layer, freshness, colour, texture and smell when buying pork.[71] In another recent study conducted in Spain and Brazil, participants defined the traits for purchasing beef based on the intrinsic (colour, freshness, fat distribution) and extrinsic aspects (price and expiration date); freshness provided the buyer the impression of a good hygienic quality product; in Brazil, frozen packaged beef meat was seen as a lower-quality product and was purchased poorly, with customers assuming that freezing reduces freshness resulting in loss of quality but in comparison, Spanish consumers consider packaged beef as convenient and safe.[72]

Table 2 shows that majority of the consumers' preferred method of consumption for beef, pork and chicken is cooked 82%, 79.7%, 83.6%; boiled 20%, 10.6%, 18.6%; fried/deep fried 30%, 57.7%, 45.2%; roasted 12%, 20.3, 21.4%. Grilling or broiling beef

patty samples at 65°C resulted in higher reduction of overall bacterial and *E. coli* O157:H7 populations than at 60°C, however this reduction was not seen in pan-fried samples, moreover, broiling and grilling when combined, may have significant cooking temperature effect on *E. coli* O157:H7 reduction in comparison to panfrying.[73] This suggests that although frying is a commonly preferred method, it may be less effective in eliminating *E. coli* compared to grilling or broiling at higher temperatures. The pan-cooking method for pork meat was associated with an appetizing and nutritive product that is tasty/salty, juicy and soft/tender, with pepper and toasted flavours, and with a spicy aroma. In contrast, ohmic cooked pork meat was associated with golden and green colour, intense, spicy, and beer aroma, toasted flavour, and beer and cumin flavour, related to the brine solution used prior to cooking. Toasted flavours and aromas could be attributed to the formation of poly-cyclic aromatic hydrocarbons or polycyclic aromatic hydrocarbons, which are produced in greater quantity in cooking methods such as smoking, grilling and roasting. Interestingly, it was found that in ohmic cooking, the formation of these compounds could occur despite the meat not being exposed to temperatures above 100°C during cooking, suggesting that ohmic cooking is indeed a promising alternative for processing meat products with attributes such as toasted aroma, toasted flavour, and golden appearance. Moreover, pan-cooked pork meat was significantly more preferred than the other cooking methods.[74]

In the current study as stated in Table 2, 62%, 50.8% thought that cooked beef, chicken is not always safe to eat and 63%, 88.1% think that beef, chicken slaughtered in the abattoir is not always safe to eat whereas 54.5% of the pork consumers think that cooked pork is always safe to eat and 76.7% think that pork slaughtered in the abattoir is always safe to eat. More than half of the respondents (52%) were aware that undercooked beef meat can be a source of *E. coli*, while 59.7% of chicken consumers and 57.7% of pork consumers were unaware of this risk. A previous study revealed that *E. coli* isolates of various serotypes were present even in cooked meat, with 26.67% of chicken kofta and 20% of beef kofta samples testing positive. Among the bacterial isolates, *Enterobacteriaceae* was found to be the most prevalent group in chicken.[75] From the research survey, it was observed that B - 66%, P - 64.2%, C - 72.3% of the participants did not know that *E. coli* can be transmitted through contaminated meat. Majority of the participants had no history of B - 89%, P - 92.7% and C - 94.2% meat poisoning. Diarrhea (65%, 65.9%, 44%) and nausea/vomiting (63%, 60.2%, 61%) were selected as the common symptoms associated with contaminated beef, pork and chicken consumption and other symptoms included abdominal cramps (48%, 43.1%, 56.1%), headache (13%, 28.5%, 11.1%) and fever/chills (19%, 21.1%, 13%), respectively. The results were similar to a study conducted in Grampian and North Wales where they frequently selected vomiting, and cramps were the secondly placed symptom; 54% of the respondents also had heard about *E. coli*.[76] In a research done in Uganda, 50% of the participants reported that they have heard of foodborne illnesses; all participants agreed that they can contract disease from consuming pork; symptoms included worms (26%) and stomach ache (20%), diarrhea (16%), and fever (13%).[71]

More than half (52.9%) of the chicken respondents knew *E.coli* as a foodborne pathogen but majority of the beef (56%) and chicken (26.8%) participants were unaware and stated that only because of this questionnaire that they learned about *E.coli* and undercooked meat as a source.

Livestock-derived food demand is expected to surge globally by 14% per person and by a total 38% between 2020 and 2050; this demand growth is predicted to be greatest in South Asia (49%) and also in sub-Saharan Africa (55%) with the fastest growth in beef and pork.[77] Ground-breaking researches are being conducted to find safe alternatives to the

conventional beef and pork meat production due to the demand in meat and rising global population. Globally, there are around 32 cultured meat companies, with an emphasis on cultured beef (25%) and pork (19%) while 31% of these businesses are present in Asia.[78] In 2018, 2 companies developed cell-cultured pork utilizing stem cell technology and a successful prototype of pork sausage was produced using fat and muscle cell culture from live pig samples.[79] 3D bio-printed beef was recently made using bovine satellite cells and adipose-derived stem cells.[80] Lab-grown 'clean meat' or the cultured meat is also progressively making its way from academic laboratories towards the factory production line. Further researches conducted on these novel and 'non-traditional' beef, pork and chicken products, may have the potential to minimize the ethical concerns involving animal slaughter and diminish the environmental and health hazards related to conventional meat production, such as antibiotic resistance, food-borne and zoonotic infections.

The objectives of this study were successfully achieved. The isolation and identification of *E. coli* from raw beef, pork, and chicken meat confirmed its presence in all three types of meat, with varying prevalence. Antimicrobial susceptibility testing revealed high resistance to Erythromycin among all *E. coli* isolates. Furthermore, the public awareness survey highlighted significant gaps in knowledge regarding food safety, particularly among meat consumers. These findings collectively emphasize the need for improved hygiene practices and consumer education in meat handling and preparation.

5. Conclusion

Public awareness is low regarding *E. coli* and its potential risk as a foodborne pathogen despite meat being its major source. In this study, *E. coli* was isolated from raw beef, pork, and chicken samples, confirming its presence across all meat types. The isolates were fully resistant to Erythromycin and were susceptible to Gentamicin and Chloramphenicol. The questionnaire-based survey further revealed that a significant proportion of consumers were unaware of the risks associated with undercooked meat and poor handling practices. Awareness initiatives and a synchronized effort is required to mitigate or to effectively prevent the danger posed by *E. coli* at various levels in the entire beef, pork and chicken meat supply chain from farmers to consumers, as well as ensure that antimicrobials are used appropriately in both veterinary and human treatment regimes. Furthermore, public awareness should be raised about foodborne illnesses caused by *E. coli*, emphasizing the safe practices and consumption of beef, pork and chicken products, as well as the selection and safe use of antimicrobials.

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Detection of Leptospira DNA by Real Time PCR (qPCR) in urine samples from clinically suspected patients

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Abstract

Leptospirosis is a widely distributed zoonotic disease with a significant public health threat globally. It is caused by 11 pathogenic species and five intermediate species of Leptospira belong to the order Spirochaetales, family Leptospiraceae, genus Leptospira. Human disease is acquired via direct or indirect contact with the infected urine from the reservoir hosts, mainly rats. The patients infected with Leptospira present with a wide range of severities, ranging from mild to severe life-threatening diseases. Due to the limited laboratory facilities available in the country, the usual practice is to suspect leptospirosis clinically and to manage it accordingly. The retrospective laboratory confirmation is obtained via serology. The present study aims to assess the presence of leptospirosis DNA in urine samples collected from ten clinically suspected patients of leptospirosis as an acute phase diagnostic test. The study was done on previously collected, de-identified, and preserved urine samples from patients who managed leptospirosis, and samples retained in the MMU, Faculty of Medicine, Ragama. QIAamp viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA from urine samples. The extracted DNA samples were then tested for Leptospira positivity using Real Time PCR. A targeted DNA molecule was amplified and simultaneously quantified using real-time PCR (Polymerase Chain Reaction). A total of ten urine samples were tested and Leptospira DNA was detected in two samples indicating the potential for using urine samples as a specimen for the acute phase diagnosis of leptospirosis.

Key words: Leptospirosis, Molecular diagnosis, urine-based testing, Real-Time PCR, Public health

1 Introduction

Leptospirosis is a widely distributed zoonotic bacterial infection with a significant global health threat. The disease accounts for 2.9 million disability-adjusted life years (DALYs) annually, and the world reports about 1.03 million new cases each year with an average of 58,900 deaths. The disease is more prevalent in tropical and subtropical regions, where the resources are limited for patient management and diagnosis. Leptospirosis is caused by a spiral shape 11 pathogenic and 5 intermediate pathogenic bacteria belonging to the genus Leptospira, family Leptospirosis. Figure 1 depicts Leptospira from an electron microscope. Infection affects both animals and humans. Rodents are the main animals affected by the disease, although other animals such as dogs, cattle, pigs, and wildlife can harbor bacteria. These kinds of animals also act as the reservoir hosts. The infected animals excrete pathogenic Leptospira in their urine for a prolonged time. The first step in the transmission of an infection to humans is the contamination of soil and water by

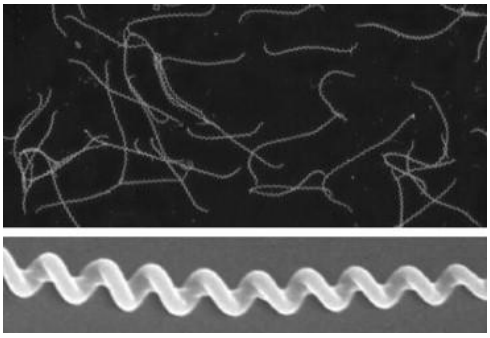


Figure 1: Leptospira bacteria[2]

an infected animal's urine. The bacteria find its way to human body through direct or indirect contact with contaminated water or soil, more easily through skin breaches and contact with unbroken mucous membranes (such as those in the mouth, nose, or eyes) and conjunctiva.1 Clinical manifestations of leptospirosis range from minor flu-like symptoms to severe illness affecting the kidneys, liver, lungs, and nervous system. Respiratory distress syndrome, liver

failure, and kidney damage are evident in the affected patients.

The non-specific initial clinical manifestations are challenging to clinicians as they mimic other common infective and non-infective diseases in the country. Leptospirosis is diagnosed by a combination of laboratory testing, clinical features, and epidemiological risk factors.3 The facilities for the acute phase diagnosis are limited in the country, especially in disease prevalent areas. The usual practice is to manage patients clinically and to obtain laboratory confirmation retrospectively mainly by serological tests such as the enzyme-linked immunosorbent assay (ELISA) and the microscopic agglutination test (MAT) which detect antibodies against Leptospira in a patient's serum. Due to limited availability of acute phase testing and the disease severity on presentation, patients with leptospirosis are likely to receive broad spectrum antibiotics instead of targeted therapy. Therefore, establishing acute phase testing will improve the care minimizing adverse effects. When diagnostic facilities are available and leptospirosis is confirmed, it can be treated with narrow spectrum antibiotics such as penicillin or ceftriaxone, which efficiently eliminate the bacteria from the bloodstream.4

Leptospira DNA can be detected in clinical samples such as blood and urine, using polymerase chain reaction (PCR) assays. The disease must be confirmed in a laboratory. For the acute phase of disease diagnosis, real-time PCR is employed (qPCR).5 This molecular biology technique measures and amplifies specific DNA sequences in real time. Scientists can accurately determine the amount of DNA in a sample by using Real Time PCR, which detects DNA amplification as happens (in real time) during the PCR process. Adding fluorescent dyes or probes to the reaction mixture is a real-time PCR technique. A fluorescence dye or probe is used for the detection of amplified DNA. The fluorescence signal emitted is directly proportional to the concentration of DNA amplified. When comparing the fluorescence signals to those generated by standards or controls, researchers can determine with precision the initial concentration of DNA present in the sample.

Establishing diagnostic facilities will help to prevent disease transmission. Integrating environmental management, public health initiatives, and personal protective measures together is considered as the most effective way to prevent leptospirosis. Some preventive measures include staying out of water sources where animal feces may be present and refrain from swimming, wading, or submerging yourself, wearing protective gear, such as waterproof boots and gloves. However, preventing occupational exposure is challenging in the country. The main risk groups include paddy cultivators, chana cultivators, manual workers and people involved in gem mining. Given that rodents frequently carry Leptospira, health authorities must take action to control rodent populations.6

The main aim of this project is to establish a real time PCR (qPCR) as a method to detect Leptospira nucleic acid in acute phase urine samples collected from patients clinically suspected of having leptospirosis. Specific objectives, to establish QIAamp Viral RNA Mini Kit, to extract leptospirosis nucleic acid from urine samples; to assess the day of urine nucleic acid positivity for leptospirosis in compared to days of illness; to evaluate the urine qPCR result in compared to renal function of the patient.

2 Methodology

Study site: The laboratory work was carried out at MMU, Faculty of Medicine, Ragama.

Study samples: The study was done on retained urine specimens in the MMU, samples were collected from patients admitted to the hospital with clinically suspected Leptospirosis. The ten samples were collected from the patients during the acute stage of the disease and two positive controls, and one negative control also used in this project.

Study design: The study was carried out as a prospective descriptive study to detect Leptospira DNA in urine samples collected from patients clinically managed as leptospirosis by a pre-validated real time PCR.

Samples processed were collected from the patients clinically managed as leptospirosis and the duration of illness was less than 7 days.

Method of sample collection and transport: A urine sample was collected into a sterile container and was transported into the laboratory in ice.

DNA extraction from urine specimens. Total DNA from urine (200µl) were extracted using QIAamp viral RNA Mini Kit according to the manufacturer's instructions.7,8

2.1 Primer design. Pathogenic Leptospira specific primers were selected from the fragment of the secY gene homologous to the Leptospira interrogans S10-spc-a locus (Genbank accession number AF115283). The primers amplified a 202 bp fragment between the locus positions 15 744 and 15 946 of secY gene that is located within the S10-spc-α locus containing genes for ribosomal proteins and it encodes preproteintranslocase for Leptospira (Table 1).9

Table 1: Optimal reaction conditions for real time PCR

Reagents	Optimized concentration range	Final concentration
Double distilled water		
Forward primer SecY IV F	80-250 nM	2.5 mM
Reverse primer Sec Y IV R	80-250 nM	2.5 mM
SYBR Green qPCR Master mix	1X-5X	1X
Template DNA	<100 ng	variable

2.2 Optimal reaction conditions for real time PCR. The amplification process was carried out after an initial denaturation at 950C for 10 minutes. Thermal process of amplification for 40 cycles was carried out including steps of denaturation at 950C for 15 seconds, annealing at 540C for 30 seconds, extension at 720C for 30 seconds and with an extended final incubation at 720C for 8 minutes. After cooling at 300C for 1 minute, melting curve (Tm) analysis from 65-940C with readings every 0.5 0C was performed according to the manufacturer's instructions. The cut off for the analysis was set at Threshold Cycle (Ct) value 35 and the approximate total time taken to run the entire program was about 2 hours. All samples were tested twice for maximum reproducibility. All the data was analyzed using the software provided by the Esco e Swift Spectrum 48 fluorescence qPCR detection system (Table 2).9,10

Table 2: Optimal thermal conditions for real time PCR

Program	Cycles	Target Temperature	Incubation time
Initial Denaturation		95^{0}C	15minutes
Denaturation	40 Cycles	95^{0}C	15 seconds
Annealing		54^{0}C	30Seconds
Extension		72^{0}C	30Seconds
Final Extension		72^{0}C	8 Minutes
Melting Temperature		65^{0}C-95^{0}C	

3 Results

Table 3: Table of Fluorescence Values

Fluorescence												
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13
24.06	11.66	11.14	13.90	22.16	7.68	16.56	15.44	8.52	21.96	22.66	14.08	12.14
24.00	11.64	11.08	13.94	22.16	7.68	16.56	15.50	8.50	21.98	22.66	14.08	12.14
23.96	11.62	11.04	13.98	22.16	7.70	16.56	15.50	8.50	21.98	22.66	14.08	12.14
23.92	11.60	11.00	14.02	22.16	7.74	16.56	15.50	8.50	21.98	22.68	14.08	12.14
23.90	11.58	10.98	14.06	22.16	7.80	16.56	15.50	8.50	22.00	22.72	14.08	12.14
23.88	11.56	10.96	14.10	22.16	7.86	16.56	15.50	8.50	22.04	22.80	14.10	12.14
23.86	11.56	10.94	14.14	22.16	7.86	16.56	15.50	8.52	22.08	22.94	14.14	12.14
23.86	11.56	10.94	14.16	22.16	7.86	16.56	15.50	8.54	22.12	23.08	14.20	12.14
23.86	11.56	10.94	14.20	22.16	7.88	16.56	15.50	8.56	22.16	23.28	14.28	12.14
23.84	11.54	10.94	14.24	22.16	7.90	16.58	15.50	8.56	22.18	23.62	14.42	12.14
23.84	11.54	10.94	14.30	22.16	7.92	16.60	15.50	8.56	22.20	24.22	14.70	12.14
23.86	11.54	10.94	14.34	22.16	7.94	16.60	15.50	8.56	22.22	25.06	15.18	12.14
23.88	11.54	10.94	14.36	22.18	7.96	16.60	15.50	8.56	22.24	26.32	15.88	12.14
23.88	11.54	10.94	14.38	22.20	7.96	16.60	15.50	8.56	22.22	27.86	16.90	12.14
23.88	11.54	10.94	14.40	22.18	7.96	16.60	15.50	8.56	22.20	30.12	18.14	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.18	32.58	19.94	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.16	35.04	21.94	12.12
23.88	11.54	10.94	14.40	22.16	7.96	16.60	15.50	8.56	22.14	37.22	23.98	12.12
23.88	11.54	10.94	14.42	22.16	7.96	16.60	15.50	8.58	22.12	39.16	26.02	12.12
23.88	11.54	10.94	14.44	22.16	7.96	16.60	15.50	8.60	22.12	41.04	27.88	12.12
23.86	11.54	10.94	14.46	22.16	7.96	16.60	15.50	8.64	22.12	42.80	29.60	12.12
23.86	11.54	10.94	14.50	22.16	7.96	16.60	15.50	8.68	22.16	44.42	31.18	12.12
23.86	11.54	10.94	14.56	22.16	7.96	16.62	15.50	8.72	22.20	45.84	32.70	12.12
23.86	11.54	10.94	14.62	22.16	8.02	16.64	15.50	8.74	22.24	46.94	34.08	12.12
23.86	11.54	10.94	14.68	22.16	8.08	16.66	15.50	8.76	22.26	47.78	35.20	12.12
23.86	11.54	10.94	14.72	22.16	8.08	16.66	15.50	8.76	22.28	48.52	36.10	12.12
23.86	11.54	10.94	14.76	22.16	8.08	16.66	15.52	8.76	22.30	49.14	36.92	12.12
23.86	11.54	10.94	14.80	22.16	8.08	16.68	15.54	8.76	22.32	49.68	37.62	12.12
23.84	11.54	10.94	14.82	22.16	8.08	16.70	15.56	8.78	22.34	50.18	38.16	12.12
23.82	11.54	10.94	14.82	22.16	8.08	16.70	15.60	8.80	22.36	50.68	38.62	12.12
23.80	11.54	10.94	14.82	22.16	8.08	16.70	15.64	8.82	22.38	51.02	39.08	12.12
23.80	11.54	10.94	14.84	22.16	8.08	16.72	15.64	8.84	22.38	51.30	39.54	12.12
23.78	11.56	10.94	14.86	22.16	8.08	16.74	15.64	8.84	22.38	51.54	40.00	12.12
23.78	11.58	10.94	14.90	22.16	8.08	16.76	15.64	8.84	22.38	51.78	40.40	12.12
23.78	11.68	10.96	14.94	22.14	8.08	16.78	15.64	8.84	22.38	52.00	40.76	12.12
23.76	11.94	10.98	14.96	22.14	8.08	16.80	15.66	8.84	22.38	52.20	41.06	12.12
23.76	12.34	11.00	14.98	22.16	8.08	16.86	15.74	8.86	22.38	52.38	41.38	12.14
23.76	13.02	11.04	15.02	22.18	8.10	16.92	15.88	8.88	22.38	52.56	41.70	12.16
23.74	14.08	11.10	15.06	22.20	8.16	16.98	16.10	8.92	22.42	52.64	42.04	12.20
23.76	15.34	11.18	15.10	22.24	8.22	17.06	16.54	8.98	22.52	52.70	42.38	12.26

Table 4: Table of Temperatures and Time Used by Real Time PCR

Program	Cycles	Target Temp.(C) /Melting End Temp.(C)	Incubation Time	Temp. Transition Rate(C/s)	Secondary Target Temp.(C) /Melting Start Temp.(C)	Step Size(C)	Step Delay (Cycles)	Grad Temp.(C)	Sample Mode
Cycle/Temp.1	1	95.0	0:12:00	4.0	-	-	-	-	None
Cycle/Temp.2	40	95.0	0:00:15	4.0	-	-	-	-	None
		60.0	0:00:20	4.0	-	-	-	-	None
		72.0	0:00:20	4.0	-	-	-	-	Single
Cycle/Temp.3	1	72.0	0:05:00	4.0	-	-	-	-	None
Melting1	-	95.0	0:00:30	-	65.0	0.5	-	-	Step
End	-	4.0	-	4.0	-	-	-	-	-

Table 5: Table of Patient Details

SN	Sample	Group	Property	Date of illness	Diagnosis	Age in years	Gender	Serum creatinine (mg/dl)
A1	1	1	S	5	Leptospirosis	41	Male	1.4 (high)
A2	2	1	S	8	Leptospirosis	44	Male	2.6 (high)
A3	3	1	S	7	Leptospirosis	32	Male	0.9
A4	4	1	S	7	Leptospirosis	56	Male	2.2 (high)
A5	5	1	S	8	Leptospirosis	37	Female	3.1 (high)
A6	6	1	S	8	Leptospirosis	41	Male	0.8
A7	7	1	S	6	Leptospirosis	46	Male	1.0
A8	8	1	S	7	Leptospirosis	45	Male	1.1
A9	9	1	S	8	Leptospirosis	39	Female	2.0 (high)
A10	10	1	S	8	Leptospirosis	58	Female	1.65 (high)
A11	11	1	P	9	Leptospirosis	67	Male	1.0
A12	12	1	P	10	Leptospirosis	69	Male	1.4(high)
A13	13	1	N	4	Leptospirosis	41	male	2.3(high)

4 Discussion

Direct detection of *Leptospira* DNA by conventional or real-time PCR is done in clinical laboratories to facilitate diagnosis and management of leptospirosis in the acute phase of the illness. Such facilities are minimally available in the country even in disease-prevalent areas due to high cost, there is a need for laboratory facilities, and qualified, experienced human resources. However, establishing such facilities will result in better patient management with a reduction in mortality rate. The present study aimed to re-evaluate a pre-validated real-time PCR for the detection of *Leptospira* DNA in urine samples while obtaining hands-on experience to perform the technique.[7,8]

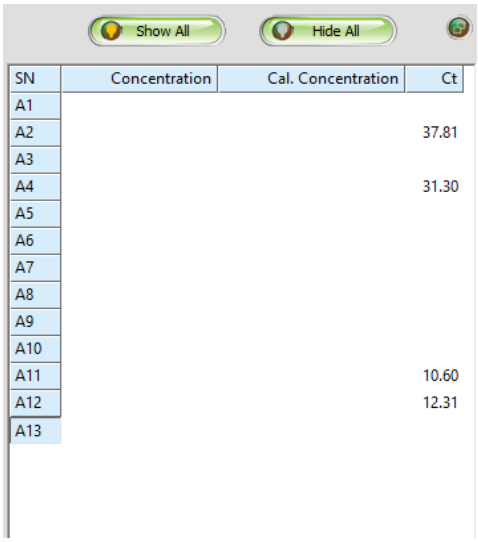
A total of ten samples were tested using real-time PCR. Two of the samples tested positive with a Ct value of 37.81(sample 2) which is not a higher detection of *Leptospira* DNA because the Ct value is above 35 and other sample's Ct value was 31.30 (sample 4) this Ct value is a higher detection of *Leptospira* DNA because the Ct value is below 35. The two positive controls have Ct values of 10.60 and 12.3 these Ct values are higher detection of *Leptospira* DNA because Ct values are below 35. The results for the remaining samples and the negative control were negative.

Two tested positive samples were collected on day 7 and day 8 of the illness. The tested negative samples were collected before day 10 of the illness. A possible explanation for negative results includes degradation of DNA while storing in the laboratory, inadequate sample volume, intermittent excretion of pathogen, and the presence of DNA in undetectable

Figure 2: Samples and Fluorescence curves

levels. DNA degradation might happen if the samples were not stored properly or are subjected to extremely high or low temperatures; The patient may have a concentration of the target organism or

nucleic acid below the PCR test's detection limit. This could happen if the patient is responding well to treatment or at an early or late stage of the infection; When collecting samples, it is crucial to consider how the infection is progressing. It is possible that the target nucleic acids will not be detectable if the sample is taken too early or too late during the infection. Furthermore, substances that can inhibit PCR reactions can be found in certain biological samples. The sample may naturally contain these inhibitors, or they may be added during collection or processing; False negative results can be caused by mistakes made during the PCR procedure itself, such as poor reagent quality, broken thermocyclers, or incorrect reaction setup by the operator. The test might not detect it if the patient has a slightly different strain or if the target sequence has mutated; A mismatch between the primers or probes used in the PCR test may arise from the considerable



genetic variation exhibited by certain pathogens;

And a false negative test result could result from the patient taking antibiotic medication prior to collection of the specimen which would lower the pathogen's detectable amounts.[11]

Figure 3: Concentration and Ct Values

High serum creatinine values were noted in the two patients from whom *Leptospira* DNA was tested positive. However, negative results were noted in 6 patients with impaired renal function as suggested by elevated serum creatinine. No statistical analysis was done to evaluate the significance of impaired renal function and the detection of *Leptospira* DNA in urine due to limited sample numbers.[9,10]

5 Conclusion

According to the test results obtained in the research, that urine samples can be used as specimen for diagnosing leptospirosis in the patients.

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Future direction towards human in-vivo organoid transplantation

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Abstract

Stem-cell derived organoids possess the potential to offer an unlimited source of transplanted tissues which would aid in overcoming concerns related to shortage of viable organs. Furthermore, organoids have proven to be exceptional in ex-vivo models for toxicity studies, drug screening and disease modelling due to their ability to respond to stress by expressing injury biomarkers from specific cell populations. The advancement in prolonged culturing of the organoids has allowed comparatively enhanced complexity and maturity of brain organoids. Hence, brain organoids have become important tools in studying how neuropsychiatric and neurodevelopmental diseases such as schizophrenia or autism spectrum disorder affect the mechanism of brain cell interactions. Moreover, organoids are utilized to test the hypothesis whether most lung diseases are due to stem cell dysfunction and if in the occasion of stem cell defect, new cell types could be the target for drugs hence helping in finding treatments to diseases such as cystic fibrosis. Ongoing research is trying to come up with methods of transforming and transplanting cells or tissue that could serve as a cure for certain diseases leading to the development of novel therapeutic tools. Although organoids have several benefits, most organoids that have been yielded exhibit partial components of corresponding tissue. Furthermore, regulating cell type, spatial organization and cell-cell, cell-matrix interaction have collectively become a challenge. For further enhancement of their utility in therapeutics, bioengineering strategies can be utilized to navigate cell composition and their three-dimensional (3D) organization. However, revolutionary advancements in organoid research have led to multiple in-vivo clinical applications with promising outcomes.

Keywords: Organoids, organoid transplantation, adult stem cells, pluripotent stem cells, induced pluripotent stem cells

1. Introduction

Organoids are three-dimensional and miniature organs produced in vitro that mimic key structural and functional aspects of real organs.[1] Moreover, they are developed via multiple regulated processes. Different stem cell types, such as induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), and adult stem cells (ASCs), can be utilized to create organoids. iPSCS are created by reprogramming adult somatic cells, such as skin fibroblasts, back to a pluripotent state by introducing specific transcription factors.[2] Once created, iPSCs can be directed to differentiate into specific cell lineages by exposure to defined culture conditions and signaling molecules. Likewise, the inner cell mass of blastocyst-stage embryos gives rise to ESCs, which have the capacity to differentiate into any type of cells.[3] Similar, to iPSCs, ESCs can be developed in particular ways that encourage organoid self-organization. In contrast, ASCs are found in specific tissues and play a crucial role in maintaining and repairing those tissues.[4] ASCs may divide and create organoids that preserve the properties and capabilities of their original tissue by being isolated from organs like the liver or intestine, and cultured in a supportive three-dimensional matrix containing vital growth factors.[5] In the process of producing organoids utilizing this method, ESCs or iPSCs– which are collectively known as pluripotent stem cells (PSCs) – are the primordial cells which are used to grow these

organoids. The cells are cultured within a medium to initiate aggregation and promote directed differentiation. The cell clusters are then placed within a matrix to provide structural support for the cells to form a structure similar to that of endogenous tissues which develop from various germ layers of the human body (the endoderm, mesoderm and ectoderm) by interpolating various organoid differentiation protocols which generate different models for different organs.[6]

The 3D culture mediums, such as Matrigel, mimics the extracellular matrix and offers structural support, enabling appropriate spatial arrangement. Proceeding with differentiation, which involves exposing the cells to particular growth factors and signaling molecules that direct their development into the desired cell types and promote the self-organization of organoid structures. In the last stages of maturity and maintenance, the organoids are cultivated in ideal conditions with a constant supply of vital nutrients, allowing for their development and operation, making them useful models for researching human development, disease processes, and applications of regenerative medicine.[7]

Adult stem cells (ASCs), when used to produce ASC-derived organoids, do not require the step of aggregation to differentiate into a specific cell type as they are grown from tissue resident stem cells. The cells are extracted from the organ tissue and dissociated into cells from which they are placed within a medium to initiate organoid formation by providing growth factors optimal for their growth and development and to optimize cell activity to produce specific organoid models such as intestines, stomach, pancreas and taste buds.[6] Major stem cells utilized for the derivation of each organoid are summarized in Table 1.

Within the ex vivo environment, organoids emphasize heterogeneity and can undergo poor morphogenesis during self-assembly, along with the lack of stromal, vascular and immunological components. Thus, the further development of organoids will progress by the deep intellect of human organogenesis as well as the way in which these cells manage their cellular and physical microenvironment. Numerous attempts were made, by tissue engineering – by reproducing mechano-chemical cues by engineered hydrogels and micro-devices – to the stem cell niche to gain high spatiotemporal control for both cell-cell and cell-matrix interactions.[1] In this review, processes and strategies of organoid development, cases of in-vivo organoid transplantation in humans and the impact of organoids in the future are discussed.

2. Retinal Pigment Epithelium Transplantation

Human trials have proved that Retinal Pigment Epithelium (RPE) cells can be transplanted from Retinal pigment epithelium of fetal, post-mortem adult, autologous, iPSC-derived and ESC-derived origin. iPSCs offer an unlimited supply of autologous cells and do not require the use of immunosuppressants, they may carry patients' own genetic vulnerabilities contributing to disease processes, however this can be avoided by the usage of ESCs.[8]

Table 1. Summary of the type stem cells utilized for the derivation of each organoid

Organoid	Stem cell type
Retinal Pigment Epithelium	Embryonic stem cell
Intestinal	Hematopoietic stem cell Intestinal stem cell
Colonic mucosal	Adult stem cell
Brain	Human pluripotent stem cell

RPE cells possess several functions essential for vision such as, adsorption of excessive light, transportation of nutrients to and from the neuroretina, protection against photooxidation, regeneration of 11 cis-retinal for the visual cycle, phagocytosis of shed photoreceptor outer segments and, constitutes the outer part of the blood-retinal barrier. Dysfunction of the RPE leads to; age-related macular degeneration (AMD), proliferative vitreoretinopathy and retinitis pigmentosa (RP).[8]

The three techniques for subretinal RPE transplantation developed are surgical placement of RPE as an intact cell sheet (with or without scaffold), injection of RPE as a cell suspension, and macular translocation.

The delivery of RPE as patch through injection is less traumatizing however, it comes along with associated complications such as subretinal hemorrhage and proliferative vitreoretinopathy. Moreover, cell clumping, poor attachment and disorganization of RPE upon injection are the drawbacks. Macular translocation is a less surgically straight-forward technique that involves rotating the retina away from a subretinal pathology to an area of healthy RPE and is complicated by cataract, retinal detachment and diplopia.[8]

In a phase 1 and 2 clinical trial, Schwartz and associates injected human ESC-derived RPE subretinally via a small 38-gauge retinotomy in 18 patients, nine with AMD and nine with Stargardt's Macular Dystrophy (SMD). The procedure resulted in improved visual acuity in the majority of patients. No ocular or systemic safety issues were recorded aside from surgery associated complications, such as vitreous inflammation, cataract and endophthalmitis.[8]

In phase 1 and 2 study of the California project to cure blindness, an engineered patch which is an ESC-derived RPE monolayer attached to a synthetic parylene substrate, was implanted in 16 patients with advanced non-neovascular AMD with a median age of 78 years. It was reported that mild to moderate subretinal hemorrhages and macular holes were the adverse events, with one patient developing ischemic colitis, possibly linked to immunosuppression.[8]

The London Project to Cure Blindness phase 1 trial, performed sub-retinal implantation of differentiated ESC-derived RPE, but the scaffold was made of polyester membrane coated with human vitronectin to one eye in each of two patients with severe exudative AMD. The procedure exhibited survival of the RPE patch and a visual acuity gain of 29 and 21 letters in the two patients over 12 months. Further preclinical safety studies did not reveal tumorigenicity or notable proliferative capacity of the ESC-derived RPE cells. Additionally, undifferentiated ESCs were not detected in the final differentiated RPE product.[8]

Although the number of patients included in the study is too low to conclude on the clinical efficiency of the RPE patch, the report provides valuable data about the surgical technique, stability of the transplant, and the safety of the ESC-derived cells. However, ESCs raise ethical concerns and are, in contrast to iPSCs, neither autologous nor unlimited in supply.[8]

3. Intestinal organoids

Intestinal organoids have been created as a cure for chronic inflammatory disorders of the gastrointestinal (GI) tract such as Inflammatory Bowel Disease (IBD) which also includes other disease such as Crohn's disease (CD), all of which causes inflammation of the mucosal layer of the both small and large intestines which causes other painful symptoms such as GI bleeding, abdominal pain, obstruction and many other complications such as cancer formation and could even lead to patient death.[9]

Previous treatments of IBDs include therapies involving administration of monoclonal antibodies and kinases which target the inflammatory cytokines (examples like Tumour Necrosis Factor a, interleukin-23 and 17) or any immune inflammatory responses. Another treatment method would be the removal of damaged areas through surgery. However, by utilising advanced treatment techniques such as

stem cell therapy and organoid transplantation, it may generate a long-term restoration of the mucosal lining and better healing.[9]

The first trial of cells used were hematopoietic stem cells (HSCs) to cure refractory CD by replacement of the patient's immune system by autologous hematopoietic stem cell transplantation (HSCT). The trial was partly successful, however only certain patients are permitted for the treatment as adverse effects were experienced in some patients during the trial.[9]

Another stem cell that is used is intestinal stem cells (ISC) for the treatment of IBD. ISCs are extracted from the lowest part of the intestinal crypt, which were then produced into a culture in vitro using innovative organoid technology as shown in Figure 1.

The ISCs showed continuous growth within a 3D-culture environment with the aid of non-ISC cells and growth factors, and the availability of an extracellular matrix which imitate true ECM and the components found within, mimicking the environment found in vivo. Organoids were developed with the above culture system with defined factors and type I collagen, which was then delivered colonoscopically into a human patient with CD in July of 2022.[10] Follow up of the trial and patients has not been disclosed yet (April 2025).

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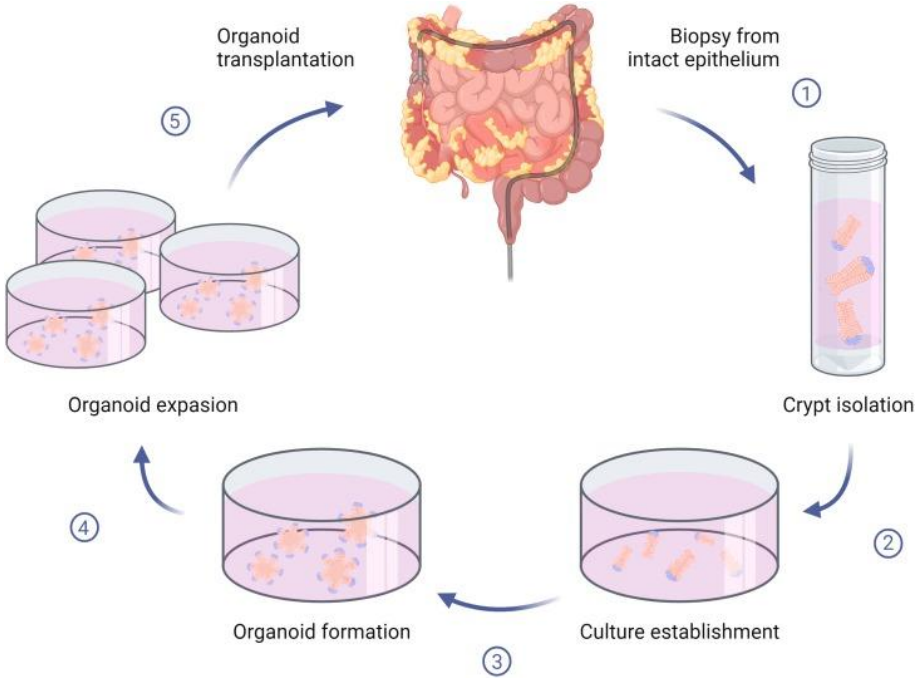

Figure 1. Intestinal organoids formation and insertion for regenerative therapy [Adapted from[11]]

4. Colonic mucosal organoids

Ulcerative Colitis (UC) is a chronic illness characterized by inflammation of the colon, prevalent among young adults, resulting in symptoms such as fatigue, bloody diarrhea and abdominal pain. It can spread through the colon after beginning in the rectum, furthermore, affecting the joints, skin and eyes.[12] Research have demonstrated that patient-derived colonic mucosal organoids can be utilized as regenerative therapy for ulcerative colitis.

The development begins by collecting a small sample of healthy colon tissue from the patient using a colonoscopy. This is a gentle, non-surgical method. The cells from the tissue are then grown in a lab for about one month. During this time, they form spherical organoids measuring approximately 0.1 to 0.2 millimeters in diameter, like miniature versions of the colon lining. Once ready, the organoids are transplanted back into the patient's colon using another colonoscopy.[13] Since the cells come from the same person, there is no risk of rejection. The method is also less invasive because it avoids major surgery.

Patient-derived colonic mucosal organoid transplantation has shown exceptional results in pre-clinical tests, where mice exhibited healing of the colon lining and improvement in symptoms. In the first human trial, the transplant patient had a better prognosis sufficient to go home the progressive day. Since the results were promising, the research team looks forward to treating up to eight patients and monitoring their progress for one year to ensure safety and efficacy of the treatment.[12]

Although this novel treatment demonstrated promising results, several challenges remain. Currently, only one patient has been treated, therefore the sample number included in the study is too low to conclude on the efficacy of colonic mucosal organoids. Furthermore, organoids developed were very small (0.1– 0.2 mm), limiting repair to small areas and making it difficult to treat widespread damage. The process also depends on using healthy tissue from the same patient, which may not be possible if there are no healthy mucosa available, moreover culturing organoids requires approximately a month, therefore making it unsuitable for urgent treatment. Long-term effects in humans are yet unknown, and there's a small risk of unexpected alterations in lab-grown cells. Since stem cells cannot be cultured alone for direct use, the method is limited to localized repair and is unsuitable for extensive or systemic disease.[13] Due to the novelty of the treatment, more time and effort is required to completely understand the long-term risks and adverse effects. As a result, more research is essential before it becomes a standard treatment.

5. Brain organoids

Brain organoids are 3D structures derived from human pluripotent stem cells (hPSCs) that recapitulate key aspects of early human brain development. These organoids self-organize into layered, functional tissues resembling specific brain regions such as the cortex, midbrain and hippocampus.[14] They are cultured under defined conditions, including extracellular matrices and bioreactors, where brain organoids offer unprecedented opportunities to study human-specific neurodevelopmental processes. Two main strategies are employed to generate brain organoids: unguided and guided approaches.[15]

Unguided methods leverage the intrinsic self-organizing properties of hPSCs, allowing them to spontaneously differentiate into a variety of brain-like structures, including forebrain, midbrain, and retinal regions. Although this method results in high cellular diversity, it often suffers from considerable inter-organoid variability. In contrast, guided methods utilize external signals such as growth factors and small molecules to direct hPSCs differentiation toward specific brain regions like the cortex or midbrain. While this approach yields more homogeneous and reproducible organoids with reduced heterogeneity, it may restrict the development of natural cytoarchitecture.[15]

Recent innovations have led to the development of "assembloids," in which region-specific organoids are fused to model inter-regional neural interactions, such as interneuron

migration and synaptic connectivity.[16] Additional advancements, including microfilament scaffolding and miniaturized bioreactors, have further enhanced the structural stability and scalability of organoid cultures. Each methodology serves distinct research goals, Unguided methods are suitable for studying whole-brain development and cellular diversity, while guided methods provide consistent models of specific brain regions. Assembloids, meanwhile, enable the study of complex neural circuits.[16]

Therapeutically, brain organoids serve as powerful platforms for modeling neurodevelopmental and neurodegenerative diseases such as microcephaly, epilepsy, Alzheimer's disease (AD), and autism spectrum disorders (ASD).[17] Patient-derived organoids support personalized medicine through individualized drug screening and genomic analysis. For example, in ASD, organoids derived from patients have revealed abnormal neurogenesis, altered synapse formation, and disrupted neural connectivity, helping to elucidate the developmental origins of the disorder. In Rett syndrome, organoids generated from iPSCs with Methyl-CpG Binding Protein 2 (*MECP2*) gene mutations show impaired neuronal maturation, reduced dendritic complexity, and gene expression abnormalities. Organoids infected with the Zika virus mimic microcephaly by exhibiting reduced proliferation of neural progenitors and smaller organoid size. For AD, brain organoids display hallmark features such as amyloid-β plaques and tau pathology, providing a model to study disease progression and test therapeutics. Midbrain organoids from Parkinson's disease (PD) patients exhibit dopaminergic neuron degeneration and mitochondrial dysfunction. Similarly, Huntington's disease (HD) organoids demonstrate mutant huntingtin protein aggregation, neuronal loss, and gene dysfunction, offering platforms for testing gene-editing strategies.[17]

A promising future direction involves the transplantation of brain organoids into living organisms. This in vivo approach aims to integrate human-derived neural tissues into host brains to repair damage, restore function, or replace degenerative cells. Studies have shown that transplanted cortical organoids can survive, vascularize, and form functional synaptic connections in rodent models. However, significant challenges persist, including poor vascularization, limited cell-type diversity (notably the absence of microglia and oligodendrocytes), and central necrosis due to hypoxia.[16] Ethical considerations, particularly those related to cognitive capacity in chimeric animals, demand stringent oversight. To address these issues, ongoing research focuses on enhancing functional integration, vascular support, and immune compatibility. The use of assembloids combining various brain regions and cell types shows particular promise in promoting neural maturation and interconnectivity.[16] While the path to clinical application of brain organoid transplantation is complex, it holds transformative potential for treating a range of neurological disorders, provided that scientific, ethical, and translational challenges are addressed with diligence and care.

6. Current Challenges in organoid transplantation

Organoid technology has rapidly advanced, yet it still faces several critical challenges that limit its effectiveness as a cell culture model. One major limitation is the lack of vascularization, which restricts nutrient and oxygen diffusion, thereby limiting organoid growth and leading to necrotic cores in thicker tissues.[7] To address this, researchers have explored the use of microfluidic devices that replicate blood flow, as well as liver sinusoidal cells that promote vascularization in liver organoids.[18] Another significant challenge is the poor reproducibility of organoid cultures due to inconsistencies in size, shape, and cellular composition, which complicates data analysis and study design.[7] Standardizing culture protocols and integrating automated tools to regulate the microenvironment have been proposed as solutions to enhance consistency[20]. Additionally, organoids often fail to fully mimic the complexity and functionality of mature organs due to limited cellular diversity, incomplete maturation, and insufficient interaction with surrounding tissues.[19] To

improve this, co-culturing organoids with supportive cell types, introducing mechanical stimuli, and utilizing biomaterials that provide biochemical and mechanical signals have been recommended.[7] Ethical and safety concerns, particularly regarding ESC derived organoids and immune rejection, also present significant barriers.[8] Efforts to address these issues include gene editing techniques, such as Clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9), to modify Human Leukocyte Antigen (HLA) genes in allogeneic organoids for immune compatibility, as well as refining surgical techniques and microsurgical tools for safer transplantation procedures.[21] Furthermore, the use of Matrigel, derived from mouse sarcoma, limits the generation of human-transplantable organoids, necessitating the development of synthetic biomimetic scaffolds as an alternative.[4] Although significant progress has been made in overcoming these challenges, further research and clinical trials are essential to establish the long-term efficacy, safety, and scalability of organoid-based therapies. Such advancements made towards the 3D generation of organoids, have paved the way towards clinical applications.

7. Conclusion

The ability to create miniature, functional models of organs using stem cells is not simply a remarkable scientific achievement, it has proven to be a novel approach for the next generation of personalized and regenerative medicine. Furthermore, organoids would be crucial for personalized treatment in the future, as patient derived tissues enable toxicity screening and medication testing that is specific to each patient's genetic background. It is promising that organoids would be able to successfully replace damaged tissues or organs as bioengineering techniques advance. It is also evident that they possess the potential to become feasible for transplantation upon overcoming obstacles such as vascularization and immunological compatibility. Moreover, lab-grown tissue may be used to treat conditions such as intestinal disorders or liver failure that presently require donor organs, avoiding long transplant waiting periods. The use of organoids in disease modeling also creates new opportunities for research into illnesses that are otherwise hard to reproduce in animals and to also provide a more ethical approach for research in medicine and disorders, particularly in genetic or neurological disorders. In addition, they aid in providing insights on preventing diseases before symptoms appear, accelerating treatment discoveries, bringing upon a great impact in the future of medicine.

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Comprehensive Kinetic Study of Phenanthrene and Naphthalene Biodegradation by Soil Fungi from Urban Areas Using Mycoremediation Approaches

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Abstract

Soil contamination by polycyclic aromatic hydrocarbons (PAHs) poses serious environmental and health risks. PAHs are ubiquitous pollutants composed of multiple aromatic rings and are hazardous to the environment due their mutagenic and carcinogenic effects. Among PAHs, naphthalene and phenanthrene are focused on this research due to their prevalence in contaminated environments. Bioremediation denotes the application of microorganisms to eradicate or diminish the level of hazardous waste at contaminated sites. In this research, mycoremediation technique is employed where fungal species are utilized to degrade PAHs. This study aimed to investigate the phenanthrene (PHE) and naphthalene (NAP) biodegradation potential of different fungal strains isolated from soil samples of three distinct urban areas namely Galle (6°02'.78"N 80°12'35.26"E), Jaffna (9°40'0"N 80°02'0"E) and Colombo (6°28'.0"N 79°58'0"E). Morphologically different fungal strains were isolated using serial dilution technique. Isolated ones were screened for their capacity to breakdown PHE and NAP in bushnell hass agar supplemented with both PAHs at 30°C for 3 days. Molecular identification was conducted, and accession numbers were obtained from NCBI. The best degraders underwent kinetic analysis. Cytotoxic experiments were carried out using Brine shrimp lethality assay to assess the toxicity, and a phylogenetic tree was created to determine evolutionary relationships. Eight morphologically different fungal strains were isolated followed by a series of screening processes to screen for their ability to degrade PAHs, NAP and PHE. Kinetic Analysis demonstrated only two different fungal strains G1-1 (Galle) and J1-2 (Jaffna) as the best PAH degraders among the eight strains. These fungal strains were identified as *Trichoderma harzianum* isolate (PV225528) and *Talaromyces verruculosus* (PV225531) based on ITS sequence data. *Trichoderma harzianum* (PV225528) showed the highest degradation rate for NAP and *Talaromyces verruculosus* (PV225531) showed the highest degradation rate for PHE. Toxicity Assay using *Artemia salina* indicated reduced toxicity compared to 100 ppm PAHs supplemented seawater after the degradation of PAHs by *Trichoderma harzianum* PV225528 and *Talaromyces verruculosus* PV225531. The study highlights the significance of fungal bioremediation as a sustainable and effective approach for degrading PAHs in contaminated soils.

Keywords: Phenanthrene, Naphthalene, PAHs, Biodegradation, *Trichoderma harzianum, Talaromyces verruculosus*

1. Introduction

1.1 Soil pollution

Soil pollution refers to contamination of soil by higher than normal concentration of pollutants such as heavy metals, PAHs, microplastics, and toxic organic chemicals such as pesticides. It is a growing environmental concern that has adverse effects on ecosystems and human health[1]. Human activities such as urbanization, improper waste disposal, industrialization and use of agrochemicals are primary sources of soil pollution[2].

1.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are organic compounds that are composed of two or more fused aromatic rings of carbon and hydrogen atoms. They are present in crude oil and are primarily produced by the partial combustion of fossil fuels and coals. In general, PAHs are solids that are colorless, white or pale yellow-green and they have a mild pleasant odor including examples such as anthracene, naphthalene and phenanthrene. They are ubiquitous in the environment and pose potential mutagenic, carcinogenic and toxic effects on both humans and other living organisms. Based on the numbers of rings present in the compound, they are classified into light light-molecular weight (LMW) and high molecular weight (HMW) PAHs. LMW have two or three aromatic rings while HMW consists of four or more aromatic rings[3]. PAHs are commonly found in urban areas due to vehicular emissions and poor dispersion of pollutants[4]. The US Environmental Protection Agency (US EPA) has listed 16 PAHs as priority pollutants due to their dangerous properties. Humans are exposed to PAHs via inhalation, ingestion or even dermal contact. Several adverse health effects such as diabetes, oxidative stress, infertility, oxidative stress and poor fetal development are associated with exposure to PAHs[5].

1.3 Naphthalene (NAP)

NAP is classified as a HWM PAH and is commonly found in coal, mothballs, disinfectant and during polyvinyl chloride production. Various sources of naphthalene emissions include industrial processes, combustion and the distillation and crystallization of coal tar fractions. It has a molecular formula of $C_{10}H_8$ and is the most volatile PAH. It is a ubiquitous pollutant[6]. Naphthalene can effectively reduce the individual density and population of soil fauna. High levels of NAP can destroy RBCs in humans resulting in hemolytic anemia. Studies in animals have shown that inhaling air contaminated with naphthalene results in nose and lung tumors. Due to this, the US Department of Health and human services and the International Agency for Research on Cancer have concluded that NAP causes cancer[7].

1.4 Phenanthrene (PHE)

PHE is classified as a LWM PAH and has a molecular formula of $C_{14}H_{10}$. The incomplete combustion of various organic materials such as wood and fossil fuels releases PHE. Once PHE is released, it adheres itself to airborne particles and settles on the surface of soil and water. Urban populations are exposed to PHE by ingesting contaminated food and water, inhaling contaminated air and dermal contact. It is used in making dyes, production of pharmaceutical products, plastics and explosives. A forward mutation was found in human lymphoblast cells that were metabolically activated and also exposed to 9 ug/mL of PHE[8]. PHE pollution decreases the microbial community's diversity and increased PHE levels limits microbial metabolism resulting in decline in soil fertility. Prolonged exposure to PHE is associated to adverse health effects including carcinogenic effects[9].

1.5 PAHs distribution in urban areas

Urban areas serve as hotspots for PAH contaminants due to increased anthropogenic activities such as industrial activities, vehicular emissions, and improper waste disposal. PAHs contaminants enter into soils through atmospheric deposition and direct spillage of petroleum. With rapid urbanization, soil contamination by PAHs has become a growing concern, highlighting the need for effective remediation techniques. Their degradation is essential to reduce soil pollution and in preventing long term ecological damage[10].

1.6 Bioremediation

Bioremediation uses microorganisms to reduce hazardous waste concentrations in contaminated sites and it is carried out in non-sterile environments with various microorganisms, with fungi playing a central role in degradation[11]. Degradation of the pollutants is facilitated by the microorganism's enzymatic metabolic pathways. Factors such as type of the soil, pH, concentration of contaminants and the ability of microorganism to bind to the pollutants influences the effectiveness of bioremediation[12]. It is a sustainable approach to control environmental pollution and manage waste. Mycoremediation

is a form of bioremediation in which enzymes produced by fungi instead of bacteria are used to break down pollutants and restore balance to the ecosystem. There is a wide variety of organisms that are capable of degrading LMW PAHs such as NAP and PHE and acenaphthene. Various fungal species such as *Penicillium, Aspergillus, Trichoderma* and *Rhizopus* are effective in degrading a wide range of pollutants including PAHs. Fungi produce lignolytic enzymes such as laccase, lignin peroxidase and manganese peroxidase which enables fungi to degrade the PAHs. These enzymes work in a broad and non-specific way making them promising strategies in the removal of PAHs. In addition to extracellular lignolytic enzymes, Cytochrome P-450 Monooxygenase has been shown to aid in PAH breakdown. Certain lignolytic fungi have the ability to break down both LMW as well as HMW PAHs. Mycoremediation is an eco-friendly, cost-effective and sustainable approach for preventing the rising issue of soil pollution[13]. This study aimed to investigate the phenanthrene (PHE) and naphthalene (NAP) biodegradation kinetics of different fungal strains isolated from soil samples of three distinct urban areas in Sri Lanka.

Methodology

2.1 Sample collection

A 50g of soil samples were aseptically collected from distinct locations across the country, i.e., Jaffna-J (9°40'0"N 80°02'0"E), Colombo-C (6°28'0"N 79°58'0"E) and Galle-G (6°02'.78"N 80°12'35.26"E). Samples were collected from a depth of 5-10 centimeters in urban areas using sterile rules and transferred into sterile zip-lock bags. Samples were collected and transferred under ambient conditions and stored at 4°C in the refrigerator.

2.2 Spread plate technique

A total 5g of each soil sample was weighed using an analytical balance and transferred into individually labelled conical flasks. Each soil sample was washed separately with 50 ml of 0.9% NaCl solution by mixing gently ensuring an even distribution of soil particles. The supernatant was followed by a tenfold serial dilution was performed. Potato Dextrose Agar (PDA) medium was prepared, autoclaved and poured into sterile petri dishes that had been previously sterilized by keeping it under the UV light. After the medium solidified, 100 µL of the diluted soil suspension from the selected dilution factors 10^{-1}, 10^{-4} and 10^{-8} were pipetted onto the surface of PDA plates. Then the inoculum was evenly spread across the plate using a sterile glass spreader. The petri plates were then incubated at 30°C for 3 days.

2.3 Isolation of fungal strains and starvation

Fungal colonies with distinct morphologies were selected from different PDA plates and sub-cultured onto fresh PDA plates to obtain pure cultures. The petri plates were then incubated at 30°C temperature for 5 days. Bushnell Haas Broth (BHB) along with Bacteriological Agar (BA) was prepared, autoclaved and poured into sterile petri plates that were partitioned into four divisions and allowed to set. The purified fungal strains were starved by inoculating them into the center of each partition in the Bushnell Hass Agar (BHA) medium. The plates were incubated under 30°C for 3 days to determine their survival and adaptability.

2.4 PAHs spiking

BHA media was prepared, autoclaved and poured into sterile petri plates that were partitioned into four divisions and allowed to set. The petri plates were divided, with eight designated to NAP and eight for PHE. A 100-ppm solution of NAP and PHE was prepared separately. On their respective plates, using a cotton swab, PHE and NAP solution were swabbed. Four divisions were created in each petri plate, with two sections inoculated with the same sample in the center while the other two sections were inoculated with a different sample. The plates were then incubated for three days at 30°C.

2.5 DNA Extraction

Fungal cultures were scraped and crushed using mortar and pestle separately. Then the samples were transferred into 1.5 mL microcentrifuge tubes which were vortexed for 1-3 seconds and incubated at 65°C for 15 minutes. Then 3 µl of RNase Solution was added to each microcentrifuge tube containing the cell lysate and was mixed by inverting the tube containing the samples 2–5 times. And incubated at 37°C for 15 minutes. The samples were then allowed to cool to room temperature for 5 minutes

before proceeding. A total of 200 µl of Protein Precipitation Solution was added and vortexed vigorously at high speed for 20 seconds and then centrifuged for 3 minutes at 14,000 rpm. The precipitated proteins formed a tight pellet. To another set of 1.5 ml microcentrifuge tubes 600 µl of room temperature isopropanol were pipetted. The supernatant containing the DNA was carefully removed, transferred to the tubes containing the isopropanol and was mixed by inversion until thread-like strands of DNA were formed. The samples were centrifuged at 14,000 rpm for 1 minute at room temperature. The supernatant was decanted carefully. Then 600µl of 70% ethanol was added and was inverted several times to wash the DNA. The samples were centrifuged at 13,000–16,000 rpm for 1 minute at room temperature. The ethanol was drawn out using a micropipette. A total of 100µl of DNA Rehydration Solution was added to the samples and were incubated at 65°C for 1 hour. Periodically the solution was mixed by gently tapping the tube. The solution was incubated overnight at 4°C. The DNA was stored at 2–8°C.

2.6 PCR

After extracting DNA from the selected fungal strains that could degrade the PAHs, PCR was performed. For each sample, 8.5 µL of Nuclease-free water, 12.5 µL of Go Taq® green master mix, 1 µL from each fungal primers ITS1 forward primer (5'TCC GTA GGT GAA CCT GCC G3') and ITS4 reverse primer (5'TCC TCC GCT TAT TGA TAT GC 3') was added to each PCR tube containing 2 µL of the extracted DNA samples. The PCR samples were loaded into the PCR machine and the following conditions were set: initial denaturation for 5 mins at 94 °C, denaturation at 94° C for 30 seconds, annealing for 1 min at 52 °C, extension for 1 min at 72 °C for 35 cycles and final extension for 5 mins at 72 °C. Once the conditions were set, the machine was allowed to run.

2.7 Gel electrophoresis

A 1.5% agarose gel was prepared, heated and then transferred to a biosafety cabinet to cool down. Once cooled, 4 µL of ethidium bromide (EtBr) was added to the solution. The gel solution was poured into a cassette and allowed to set. It was then placed in the chamber and a 1X TAE running buffer was added which was prepared. Using a micropipette, 10 µL of 1KB DNA ladder was loaded into the first well followed by 10 µL of PCR samples into the other wells. The electrophoresis was run at 65 volts for 40 mins. The gel was then visualized under UV light to visualize the bands.

2.8 DNA sequencing

Sequencing of the DNA was performed via the sanger sequencing method and the sequences were submitted to the NCBI BLAST tool to determine the identity of each isolate. The resultant accession numbers were obtained from NCBI GenBank. Then the phylogenetic tree was constructed using mega software.

2.9 Kinetic Analysis

Kinetic analysis was performed. For eight days, 16 test tubes were prepared for each sample, eight tubes designated for NAP while the other eight were designated for PHE. This procedure was repeated for all other samples. BHB media was prepared and autoclaved. Once autoclaved, the media was divided into two different portions, one portion was supplemented with 100 ppm of NAP solution while the other portion was supplemented with 100 ppm of PHE solution. A total of 10 mL from both the media portions were transferred to their corresponding test tubes. A total of 500 µL of methylene blue solution was added to each test tube followed by inoculation of samples. After 24 hours, the first test tube was taken from each sample set (NAP and PHE) and centrifuged. The supernatant was measured using a spectrophotometer to determine the absorbance value. This procedure was repeated every 24 hours for each sample for eight days.

2.10 Statistical and Phylogenetic Analysis

Anova single factor test was conducted to determine whether there are significant differences in the degradation rate of PHE among the three fungal strains, i.e., C1-3, G1-1 andJ1-2.

The phylogenetic tree of the selected strains was designed using MEGA11 software version 11.0.13.

2.11 Toxicity Assay

Brine shrimps *(Artemia salina)* cysts were allowed to hatch for 48 hours in 500 mL filtered seawater. Two test tubes were taken for each sample, one for PHE and the other for NAP. BHB was prepared and autoclaved. Once autoclaved, the media was divided into two different portions, one portion was supplemented with 100 ppm of NAP solution while the other portion was supplemented with 100 ppm of PHE solution. A total of 10 mL from both the media portions were transferred to their corresponding test tubes. The test tubes were inoculated and incubated at room temperature for eight days. On the 8th day the samples were centrifuged and transferred into petri plates. Ten nauplii were transferred to each Petri plate using a Pasteur pipette. The mortality readings were recorded hourly for four hours, followed by observation at 24 hours and 48 hours.

2.11 Microscopic analysis

Fungal colonies were selected and subjected to morphological characterization using the Scotch tape method, as previously described[14]. A small piece of transparent adhesive tape (Sellotape) was gently pressed onto the surface of the fungal colony to collect fungal structures, including hyphae and conidia.

The tape was then mounted onto a clean glass microscope slide containing a drop of lactophenol cotton blue, a staining and mounting medium that enhances visualization by staining chitinous components of the fungal cell wall. The slide was examined under a light microscope at 40× magnification to observe key morphological features such as hyphal organization, spore type, arrangement, and reproductive structures. These characteristics were used to aid in the preliminary identification and differentiation of fungal taxa.

2. Results and Discussion

3.1 Sample collection and Isolations

Soil samples were collected from three distinct regions as pollution is abundant in such areas and also to cover geographical diversity Soil sample collection from distinct regions also provides a diverse microbial profile as different fungal species have varying sensitivity to pollutants. The ten-fold dilution series reduced the overgrowth of colonies thereby allowing for the isolation of individual distinct colonies (figure 1).

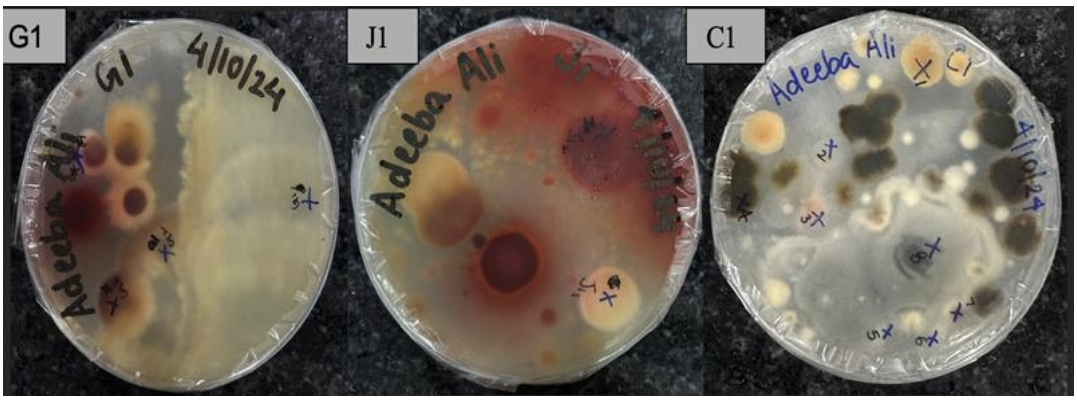

Figure 1: Morphologically different Fungal colonies from Galle (G1), Jaffna (J1) and Colombo (C1).

3.2 Subculturing

Pure fungal cultures were observed after sub culturing. PDA is effective for fungal isolations as it provides an optimal nutrient-rich environment for fungal growth (Figure 2).

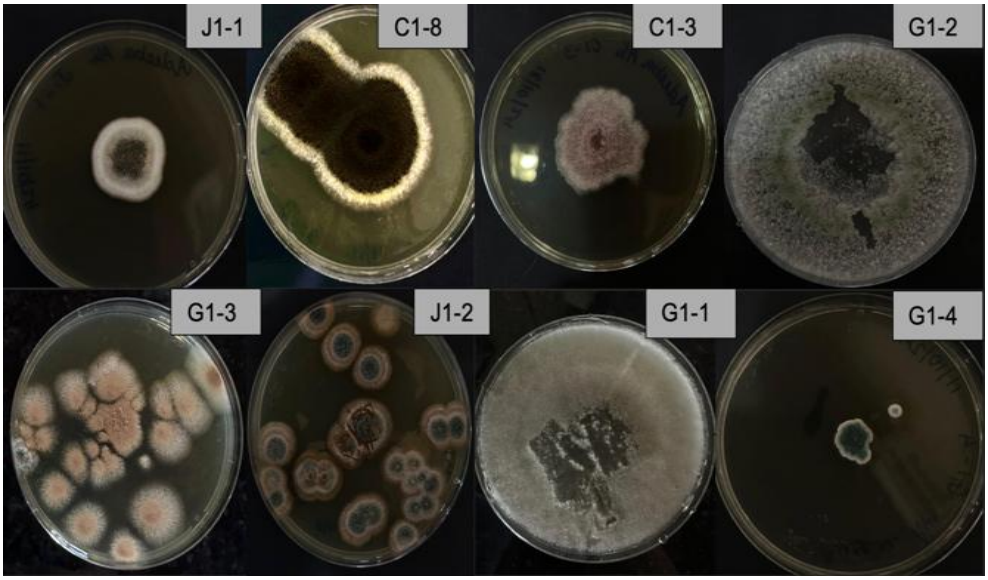

Figure 2: Isolated fungal strains grown on PDA plates

3.3 Primary screening

Starvation forces Fungi to degrade hydrocarbons (Figure 3) and helps researchers to understand metabolic pathways involved in breaking down PAHs and potential applications in bioremediation. Primary screening was carried out using BBH medium in order to evaluate the degradation of PAH by each fungal strain. BBH media contain minimal nutrients necessary for survival but lack carbon sources, which inhibits the growth of fungal strains and allows them to enter a state of stress-induced dormancy[15]. During the PAH spiked plate assay, sample G1-2 only showed a growth in Naphthalene and all the other sample grew on both Naphthalene and Phenanthrene spiked plates.

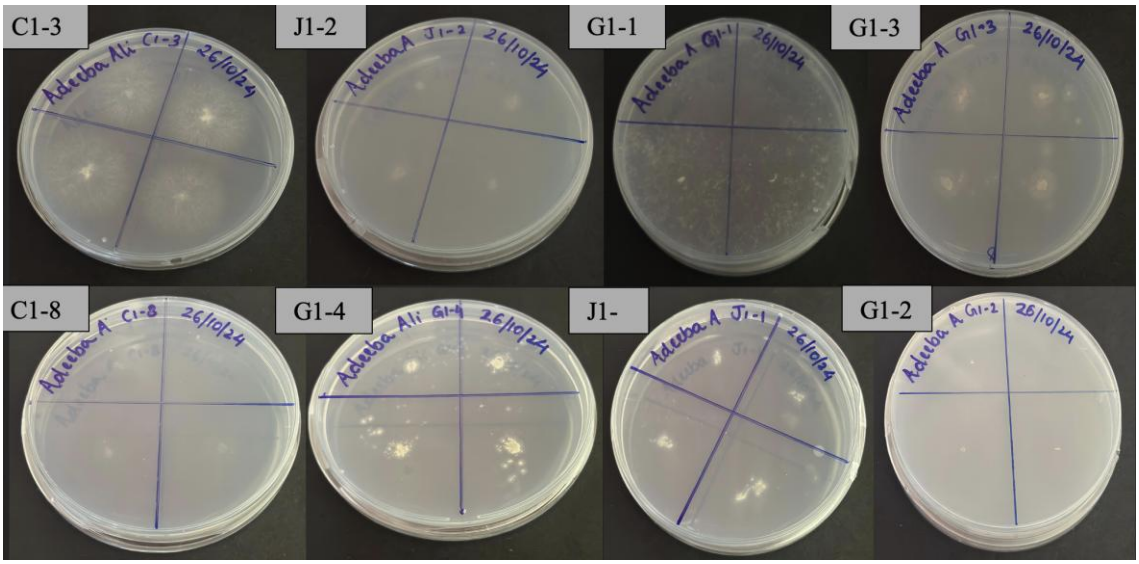

Figure 3: Primary screening of isolates from Galle (G1), Jaffna (J1) and Colombo (C1). The samples were incubated leading to fungal growth on plates.

Their observed growth illustrates the potential application of these fungal strains in PAH degradation. This highlights their adaptive response and enzymatic degradation. The results indicate maximum growth of fungal strains indicating their ability to utilize PAHs efficiently. Growth differences between the two PAHs indicates that the fungal strains may have distinct metabolic pathways or substrate preferences[16].

3.4 Molecular Analysis

C1-3 was identified as *Fussarium verticillioides* isolate with accession number of PV225534. It is a plant pathogen affecting mainly maize and it belongs to the section *Liseola* of the fusarium genus and phylogenetically defined as a member of the *Fusarium fujikuroi* species complex[17]. G1-1 was identified to be *Trichoderma harzianum* isolate with an accession number PV225528. It belongs to genus *Trichoderma* and is used as a bioagent used as a fungicide and also promotes plant growth. JI-2 was identified to be *Talaromyces verruculosus* with accession number PV225531. It belongs to the genus *Talaromyces.*

3.5 Kinetic Analysis

In this research, the half-life of fungi is the time taken by half of the fungal population to degrade the PAHs. In order to break down recalcitrant pollutants fungi primarily use cytochrome P450 monooxygenases and extracellular enzyme system that includes manganese and lignin peroxidases [18]. A shorter half-life indicates the fungi's ability to break down PAHs more quickly and efficiently. For naphthalene, out of the three fungal strains, C1-3 (*Fusarium verticillioides*) has a better degrading activity due to its lower half-life compared to the other two strains. Although previous studies have not reported half- life values of *Fusarium* species, their findings on higher degradation percentage support the observed rapid degradation by *Fusarium verticillioides* in the present study. Given the remarkable effectiveness in breaking down 1% naphthalene, one study also indicated that *Fusarium* species have a strong capacity of degrading PAHs. *Fusarium* demonstrated upto 98% degradation over 9 days using methylene blue assay[19]. The same applies for PHE, C1-3 appeared to have a shorter half-life compared to other two strains resulting in a quick degrading activity. This in line with earlier research that demonstrated the high phenanthrene breakdown efficiency of *Fusarium solani* as vertified by HPLC analysis, underscoring their significance potential for mycoremediation in PAH contaminated environments[20].Therefore, the fungal strain C1-3 with the shortest half-life is most efficient in degrading PAHs compared to the other two strains J1-2 and G1-19 (table 1).

Table 1: Half-life values of different fungal strains during the degradation of both PAHs. The values represent the time taken for 50% of degradation of each PAH by the respective fungal strains.

Sample	Half-life	
	NAP	PHE
Fusarium verticillioides	2.855 $days^{-1}$	3.215 $days^{-1}$
Trichoderma harzianum	2.253 $days^{-1}$	2.186 $days^{-1}$
Talaromyces verruculosus	4.194 $days^{-1}$	4.194 $days^{-1}$

3.6 Statistical Analysis

The results showed significant differences ($p<0.05$) in the degradation of PHE among the three fungal strains (figure 6). Since, the p-value is extremely lower, the null hypothesis is rejected indicating that at least one of the fungal strains has a significantly different degrading rate compared to the other two strains. This might be due to production of higher levels of lignolytic enzymes, a more efficient metabolic pathway to degrade PAHs and rapid fungal growth.

3.7 Degradation rate of PAH

However, during the NAP degradation, the p value appeared to be 0.59056 indicating no significant differences ($p>0.05$) in the degradation of NAP among the three fungal strains. Since, the p-value is extremely high, the null hypothesis is accepted indicating that the lack of variation in degradation rates could be due to production of similar levels of lignolytic enzymes and metabolic capacities and also the strains could be substrate specific.

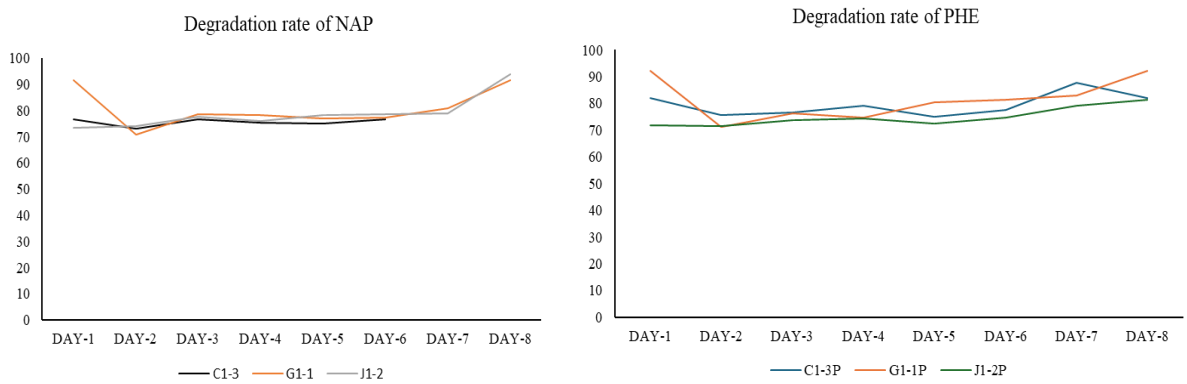

Figure 4: Degradation rate of NAP and PHE over the course of 8 days, expressed in terms of percentage.

The graph (figure 4) illustrates the degradation rate of NAP and PHE over a course of 8 days by three fungal isolates, i.e., C1-3: PV225534 *Fusarium verticillioides,* G1-1: PV225528 *Trichoderma harzianum* and JI-2: PV225531 *Talaromyces verruculosus.*

The following degradation pattern was observed. C1-3 and G1-1 showed a non-linear pattern in both the graphs (figure 8) suggesting the production of secondary metabolites or delayed production of PAHs degrading enzymes. The secondary metabolites might interfere with the degradation of PAHs and either slow or speed up the degradation rate. J1-2 suggest a linear degradation pattern with consistent degradation with minimal fluctuations and stable degradation in both the graphs (figure 4). All three stains are effective biodegradation agents as they have degradation percentage above 70% in both graphs (figure 8). However, in the case of NAP J1-2 is the most effective stain as its' degradation percentage is more than 90% whereas in the case of PHE, G1-1 is the most effective stain as its' degradation percentage is more than 90%. In, Gao *et al* 2019., reported *Talaromyces verruculosus* DJTU-SJ5 strain isolated from rhizosphere soil of *Taxus mairei* showed higher degradation of PAHs than other strains.[21] A study also reported that *Pleurotus ostreatus* and *Irpex lacteus* degraded 65%-80% of PHE out of 400 mg kg^{-1} after 28 days of incubation and *Trichoderma asperellum* could degrade 74% of PHE out of 1000 mg kg^{-1} after 14 days of incubation[22,23]. This indicates that the *Trichoderma* species have a better degrading ability than the white rot fungi which are said to be excellent degraders of PAHs.

3.8 Toxicity Assay

During the first hour, both NAP and PHE showed no mortality, suggesting that both do not have immediate lethal effects. During the 2^{nd} to 4^{th} hour in PHE there is an increase in the mortality rate in all three strains whereas there is a significant decrease of nauplii in C1-3. In NAP, for J1-2 and G1-1 the mortality rate stays the same during 2^{nd} to 4^{th} hour indicating NAP doesn't significantly affect mortality during this short-term exposure, whereas for G1-1 there is a slight increase in mortality rate. After 24 hours, for PHE in C1-3 there is a significant increase in mortality rate reaching to 2 nauplii whereas in J1-2 and G1-, there is a slight increase in mortality rate. For NAP, after 24 hours there is a slight increase in mortality rate for all three strains, this indicates that NAP causes lower mortality rate compared to PHE after 24 hours. The final observation was taken after 48 hours, for PHE in C1-3 the mortality rate reached 0 indicating that the compound is very toxic. PHE has a significantly higher level of toxicity due to a higher level of mortality rate particularly in C1-3. On the contrary, NAP has a lower level of toxicity due to gradual increase in mortality rate over the course of 48 hours. C1-3 is more sensitive to pollutants as it has a more significant increase in mortality rate.

Table 2: Viability of brine shrimp nauplii (*Artemia salina*) exposed to PHE, NAP and fungal degraded by-products over time.

Time	C1-3		J1-2		G1-1	
	PHE	NAP	PHE	NAP	PHE	NAP
1st hour	10	10	10	10	10	10
2nd hour	8	10	10	10	10	10
3rd hour	7	9	10	10	10	9
4th hour	6	9	9	9	10	9
24 hours	2	7	8	8	10	8
48 hours	0	6	7	7	10	7

3.9 Phylogenetic tree

C1-3 and G1-1 share a common ancestor whereas J1-2 has an ancestor that does not share a common ancestor with C1-3 and G1-1 (figure 5). According to previous researches *Trichoderma* and *Fusarium* which are members of the class Sordariomycetes order Hypocreales, have a same evolutionary origin, indicating their close taxonomic relationship within fungal lineage[24]. Although both of them share the common ancestor, studies have shown that *Talormyces* is in within the phylum Ascomycota and class of Eurotiomycetes, proving that it evolved from another ancestor[25]. *Trichoderma lentiforme voucher* has a totally different ancestor which is not common to any of the other three fungal strains obtained from NCBI GenBank. Although, Fusarium *verticillioides* and *Trichoderma harzianum* are different species they share a common ancestor but *Trichoderma lentiforme voucher* and *Trichoderma harzianum* being the same species do not share a common ancestor.

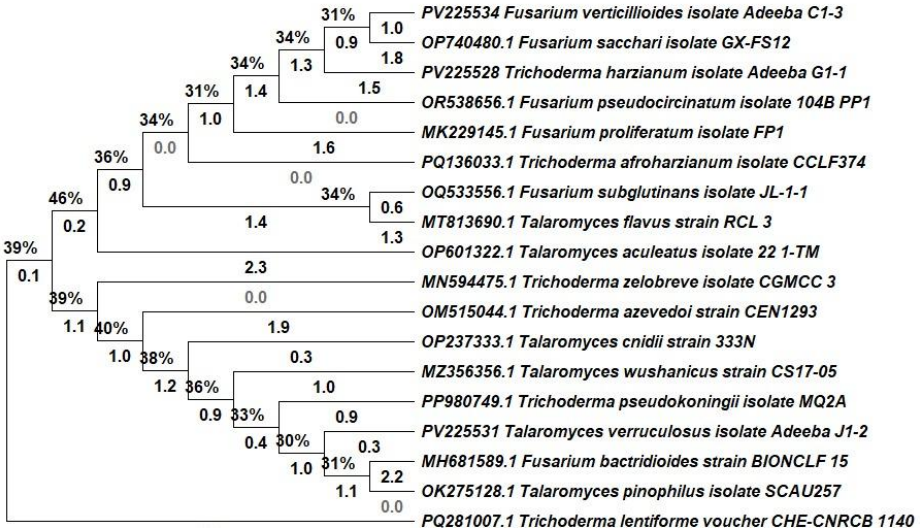

Figure 5: Phylogenetic tree illustrating the evolutionary relationship among the three fungal strain

4. Conclusion

The current study highlighted the biodegradation abilities of three different fungal species, i.e., *Fusarium verticillioides* PV225534, *Trichoderma harzianum* PV225528 and *Talaromyces verruculosus* PV225531 isolated from contaminated soil samples collected from three different locations across the country. The fungal strain C1-3, *Fussarium verticillioides* PV225534 was assumed as the most efficient strain in degrading PAHs compared to the other two strains J1-2 and G1-1 due to its shortest half-life. However, *Talaromyces verruculosus* PV225531 showed the best degradation capability in the case of NAP as the degradation percentage is more than 90% whereas *Trichoderma harzianum* PV225528 showed the best degradation

capability in the case of PHE as it's degradation percentage above 90%. The toxicity count revealed C1-3 being more sensitive to the pollutant as it has a more significant increase in mortality rate. According to the findings in this research fungal strains such as *Talaromyces verruculosus* PV225531 and *Trichoderma harzianum* PV225528 can be used as bioremediation agents in the future. These fungal strains can be incorporated into a biofilter to degrade and remove contaminants such as PAHs from air, water or soil aiding in pollution control making them agents in bioremediation.

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The role of AR in influencing consumer purchase decisions in e-commerce in the cosmetic industry

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Abstract

The effects of augmented reality (AR) on consumer behaviour in Sri Lanka's cosmetics industry are qualitatively assessed in this study through six major themes: the need for touch, authenticity and effectiveness, repeat purchases and reviews, immersion and enjoyment, interactivity and vividness, and customization. The results show that while AR features such as try-on simulations and recommendation personalization add convenience, none can ever replace the tactile evaluation of texture and formulation, especially in the case of foundations and lipsticks. Credibility will be eroded when AR representations are not real with influencer endorsements misleading. The other side of the coin is that AR strengthens brand allegiance through repeat purchases and positive reviews, and customization and interactivity empower consumers. These findings speak to the augmentation of online shopping through AR, but never its substitution for tactile evaluation of the product. Based on the research findings, this study recommends enhancing AR technology to simulate texture and consistency more accurately, integrating consumer-generated content and real-time reviews to increase authenticity and trust, and offering AR-linked product samples or flexible return policies to reduce consumer hesitation.

Keywords: Augmented Reality, E – commerce, Purchasing Decisions, Need for touch

1. Introduction

1.1 Background of the study

Augmented Reality (AR) technology advances rapidly to change business operations especially in e-commerce (Pettersen & Asheim, 2022). The integration of digital data through AR technology improves user interaction and it is currently applied extensively in educational and entertainment sectors as well as online shopping (Eldokhny & Drwish, 2021).

AR enables users to virtually test products which creates a connection between physical and digital shopping environments (Rauschnabel et al., 2022). Users can test makeup virtually through this technology which leads to better purchase confidence and satisfaction (Whang, Song, Choi, & Lee, 2021). The shopping experience gets improved by virtual fitting spaces along with instant product displays.

Retailers achieve success by connecting their online and offline systems to increase distribution channels and provide individualised convenience to customers. The realistic testing experience of AR makeup on smartphones demands additional research regarding its effects on consumer purchasing behaviour (Dhianita & Rufaidah, 2024).

1.2 Research Problem

The rapid revolution of e-commerce has transformed the way consumers interact when it comes to making purchase decisions or buying products. However, the online shopping experience in the cosmetic industry face various challenges, as consumers are not able to touch and feel or understand the product attributes such as colour and texture via e-commerce (Reinartz, Wiegand, & Imschloss, 2019). Such problems create customer uncertainties that lead to dissatisfaction since businesses must handle excessive returns that reduce profit and damage customer trust.

New technologies such as Augmented Reality (AR) offers promising solution by allowing consumer to visualise and virtually try on cosmetic products thus bridging the experimental gap in e – commerce. Despite its potential, research is still being carried out to understand impact of AR on consumer purchase behaviour within the cosmetic industry (Jessen et al., 2020).

1.3 Research Aim

The aim of this research is to explore the role of AR in influencing consumer purchase decisions in e-commerce in the cosmetic industry.

2. Methodology

This research adopted a qualitative research method, using semi-structured interviews as the primary research strategy to explore consumer perceptions of augmented reality (AR) in the cosmetic industry.

Primary data was gathered through interviews with 10 selected participants, while secondary data was sourced from relevant academic journals and articles to support the analysis.

The study employed the purposive sampling method, allowing the researcher to purposefully select participants with relevant experience or interest in cosmetic product purchasing.

For data analysis, thematic analysis was used to identify key patterns and insights from the participant responses, aligning with the exploratory nature of the study.

3. Analysis and Findings

3.1 Participants' Background

Participants of this study belong to the age group ranging from 21 to 39 years. Customers showed preference for obtaining cosmetics by combining online shopping with traditional in-store methods because this dual approach provides them maximum flexibility in their purchasing process. The majority of the participants were female.

Table 1. Participants' Background

Part.	Age	Designation	Buys Online/ In-store/ Both	Frequency of purchase
A	21	Lecturer, social worker and school counsellor.	Both	Once in 6 months
B	32	Housewife and Home baker	In-store	Very Rarely
C	23	Student	Both	Every 2 or 3 months
D	35	Marketing Manager	Online	Every month
E	39	Bank Manager	In-store	3 – 4 months
F	26	Graphic Designer	Both	Once every 2 months
G	21	Student	Both	Once in 6 months
H	29	Makeup Artist	Both	Every 2 – 3 months
I	23	Makeup Artist	Both	Every few moments
J	25	Makeup Artist	Both	Every month

3.2 Need For Touch (NFT)

The Need for Touch (NFT) plays an essential role in beauty product purchases, because participants base their decisions on texture. Online shoppers find it difficult to purchase foundation and lipstick because they cannot physically test colours and textures (Wang, 2024). The touch substitute quality of AR improves trust with its try-on capabilities and colour filters, yet insufficient touch detection hinders the assessment of product texture and finish together with weight estimation. The inability to properly assess beauty products remains a critical issue in online shopping for beauty (Zhang, Wang, Cao, & Wang, 2019).

Respondent A stated, *"It's really important, especially for things like foundation and lipstick. The shades often look different online than in real life. I also like testing textures. Some products feel too heavy on the skin, and you can't tell that from an online listing."*

The selection of shades and consistency of texture play essential roles in cosmetic shopping according to Respondent A. Online listings do not provide touch-based assessments, which make it difficult for

customers to determine their skin tone match. AR technology improves the representation of shades and texture simulation which decreases purchase uncertainty and resolves the Need for Touch (NFT) in online shopping.

3.3 Authenticity and Effectiveness

The purchasing decisions of cosmetics consumers depend on both Effectiveness and Authenticity. A product reaches effectiveness by performing the promised functions which include durable wear and skin hydration and health benefits (Dacko, 2017). The brand should authenticate itself by presenting valid product statements through clear marketing practises. Online shoppers depend on product descriptions and customer reviews and company reputation to develop trust in online shopping platforms. Customers doubt product features that need tactile testing since virtual evaluations are impossible (Krishna, Luangrath, & Peck, 2024).

Respondent B stated, *"I would still prefer to physically test them as I have tried a few websites that has implemented virtual try-ons and I was not quite happy with it."*

The British Broadcasting Corporation (BBC) considers AR-driven virtual try-ons to be unreliable because they display inaccurate colours and poor lighting effects and unconvincing textures. The benefits of AR for online shopping exist but do not substitute the need for actual product testing. Customer satisfaction depends on realistic product representations because colour and application inaccuracies result in unhappy customers who return products (Wang, Ko, & Wang, 2021; Pandey & Pandey, 2025).

3.4 Repeat Purchases and Positive Reviews

Consumer trust together with brand loyalty in the cosmetic market depends on customers' repeat purchases followed by positive feedback. Customers tend to buy again when their products exceed their quality benchmarks and fulfil their satisfaction needs (Rita et al., 2019). Since customers cannot test products in online shopping (Filho et al., 2023) positive reviews serve as proof that helps new buyers make decisions. The virtual try-ons enabled through Augmented Reality (AR) improve online shopping, yet real user testimonials still dominate purchase decisions (Daroch et al., 2021).

Respondent E stated, *"If the AR trial mirrors my expectations and the product performs as promised, I would feel more motivated to share my experience with others and support the brand with a positive review. AR might enhance my confidence in a product by providing more accurate and personalized experience."*

Augmented reality (AR) technology has a direct impact on customer trust as well as repeat buying patterns and verbal recommendations among consumers. Consumer confidence improves through accurate AR product performance, and this drives more customers to buy and recommend the brand to others. Custom AR trials improve the accuracy of product perception which builds stronger customer confidence in their purchases. Positive reviews stem from trust that keeps building up brand reputation which in turn brings in new buyers.

Research demonstrates that product authenticity perception rises when AR provides virtual try-on experiences which enable customers to see products prior to purchase (Yang & Lin, 2024). The use of realistic AR simulations creates both certainty and satisfaction according to Voicu, Sirghi and Toth (2023).

3.5 Immersion and Enjoyment

Digital shopping receives its most valuable experience from Augmented Reality (AR) technology, which brings immersion and enjoyment together. Immersion emerges from user engagement levels during AR use together with enjoyment as the emotional and value-based satisfaction from the experience (Dieck, Cranmer, Prim, & Bamford, 2023). AR technology at cosmetic companies lets customers experience interactive virtual try-ons combined with tutorial content that gives them an interactive journey beyond traditional buying practices. Research evidence shows brand loyalty strengthens when customers have positive and deep shopping experiences. Higher purchase rates and better customer retention statistics emerge due to this phenomenon (Hudson, Barkat, Pallamin, & Jegou, 2019).

Respondent F stated, *"Being able to virtually try on products using AR would definitely make the shopping experience more fun and engaging. As a makeup artist, being able to experiment with different products, shades and looks in real time adds an element of creativity and exploration to the process. I could quickly see how a lipstick or eyeshadow would look on my face without having to try it physically. This would make the shopping experience feel more interactive and exciting, I think."*

According to Respondent F AR technology improves customer shopping experiences because people find it more captivating and fun. The introduction of virtual try-ons enables customers to make online purchases through an interactive creative experience. Makeup artists use AR to evaluate colour choices and hairstyles on clients by virtually test-driving options before delivering individualized customer service. AR applications interact with users in real time and therefore extend brand contact periods which in turn generate higher sales conversion rates.

The research by Schultz & Kumar (2024) proves that shopping with AR tools provides customers with dual sensory benefits that facilitate direct involvement in their product choices. Real-time product style interaction as described in the statement provides tailored shopping that improves customer perception of shopping value. The experiential retailing theory validates this research outcome since customers normally buy items while engaging in interactive product exploration sessions (Wu & Kim, 2022).

3.6 Interactivity and Vividness

Compelling consumer interactions of deep quality emerge from the conjunction of vividness elements and interactivity in Augmented Reality (AR) shopping experiences. Digital content interactivity defines the personal product interaction possibilities that customers can achieve when they customize their product exploration process. Vividness creates a distinct visual experience that generates items, which look genuine to actual products (Roy, Singh, Sadeque, Harrigan, & Coussement, 2023). The real-time functionality of AR lets cosmetic shoppers assess multiple products together with colours and textures simultaneously thus creating an advanced interactive shopping experience. When retailers enhance both interactivity features and vividness of online experiences, customers develop stronger bonds with products that leads to higher purchase intent and brand loyalty (Hollebeek & Macky, 2019).

Respondent B stated, *"Being able to rotate, zoom in or apply virtual cosmetic products before purchasing is definitely a game changer. I would really appreciate the ability to see the product from every angle and get a close up view of how it would look on my skin."*

Virtual tools allow consumers to enhance their experience by testing cosmetic products through rotation and zoom functions and virtual skin application. Users can authenticate their interaction with product visuals by interacting from different perspectives through the user-valued features. Through interactive product features, consumers enhance their purchasing trust and obtain improved satisfaction levels.

Product realism perception stands as a vital component that depends on the clarity and richness of digital content according to consumer perception. Research shows that AR technology enables consumers to establish confident product choices and satisfied purchasing experiences because it provides clear and detailed visual outputs (Ngo, Tran, An, & Nguyen, 2025). The study indicates that AR technology delivering close-up views in multiple viewing angles enables consumers to perceive products realistically better than traditional static images (Poushneh & Vasquez-Parraga, 2017).

3.7 Customisation in Online Shopping

Through online shopping customers can personalize their shopping products based on their distinct preferences. Retailers allow consumers to personalize their shopping experience by offering options that allow specification changes along with colour and size selection and virtual product fitting and interactive features (Yu, Xie, & Lu, 2024). The cosmetic industry offers customers opportunities to view products on their skin tone along with colour selection and formula mixing features which improve product-personal connection. Customization leads to

enhanced customer satisfaction along with better shopping satisfaction by enhancing purchase confidence thus generating stronger customer commitment and revisits (Jain & Sundström, 2021).

Respondent C stated, *"If I could, you know, try combining different lip liners and lipsticks virtually to see how they complement each other, I think I'd feel more confident in my ability to create the exact look I'm going for. You know, and this can give me more flexibility assuring that I'm purchasing something that fits my desired outcome. This level of customization would likely increase my confidence in purchasing as I would know exactly what I'm getting."*

According to Respondent C product customization stands as a fundamental key to create consumer confidence during the shopping process. Virtual product combination features enable online consumers to test multiple lip liner and lipstick combinations which provides them with testing flexibility. By allowing customers to select customized products they achieve control because the chosen items match their desired aesthetic result. The consumer becomes more confident about their purchase because the product matches their exact needs.

Customization serves as an online shopping tool for market-based industries like cosmetics to appeal to customer preferences (Jussani, Vasconcellos, Wright, & Grisi, 2018). Customers who personalize their products acquire satisfaction along with control thus boosting their confidence as they prepare to buy. Customers enhance their interaction by testing different product combinations as shown by lip liner and lipstick blending (Adawiyah, Purwandari, Eitiveni, & Purwaningsih, 2024).

4. Conclusion

This research identified three areas that significantly influence consumer behaviour in AR-driven cosmetic e-commerce which are the Need for Touch, Authenticity and Effectiveness, and Repeat Purchases and Positive Reviews.

Although AR technology enhances visual experiences through try-ons and shade matchers, it cannot replicate physical touch, which limits consumer trust in online purchases (Wang, 2024; Zhang et al., 2019). Respondents noted this gap, explaining that textures often feel different than they appear online. While tools like TikTok filters improve shade selection confidence (Gatter et al., 2021), they don't solve the issue of texture evaluation. Brands like Sephora address this by offering both digital and physical testing. Haptic feedback may eventually bridge this gap, but until then, the absence of tactile experience remains a challenge in AR-driven cosmetic shopping (Scholz & Duffy, 2018).

Consumers' purchasing decisions in the cosmetic industry rely heavily on a product's authenticity and effectiveness. Effectiveness refers to how well the product performs its intended functions, such as long wear or skincare benefits (Dacko, 2017), while authenticity depends on clear, honest marketing and accurate product representation. Moreover, respondents expressed distrust toward AR-based try-ons, noting that visual simulations often fail to reflect real colours, lighting, or texture (Krishna, Luangrath, & Peck, 2024). Negative experiences, such as with overrated products like SHEGLAM, highlight how misleading AR previews and influencer-driven hype can create customer dissatisfaction (Kim et al., 2022). Further, AR offers visual convenience but cannot fully replace physical testing, especially for texture and application feel (An et al., 2022). As a result, consumer trust depends on transparent marketing and accurate product visuals, rather than overpromised digital features (Pandey & Pandey, 2025).

Repeat purchases and positive reviews are strong indicators of consumer trust and brand loyalty in the cosmetic industry. Customers are more likely to repurchase and recommend products when their expectations are met or are exceeded (Rita et al., 2019). Since physical testing isn't possible online, peer reviews serve as a major decision-making tool (Filho et al., 2023).

Augmented Reality (AR) enhances online shopping by offering virtual try-ons, helping users visualize products in real-time. When AR experiences align with actual product outcomes, they increase consumer confidence

and drive both repeat purchases and positive feedback (Daroch et al., 2021; Yang & Lin, 2024). This trust-building process strengthens brand credibility and encourages organic promotion through customer advocacy (Park & Lin, 2020).

Makeup artists especially value AR's ability to simulate real application experiences, reducing uncertainty in areas like shade matching and texture. Ultimately, accurate and personalized AR simulations lead to long-term customer relationships and consistent brand loyalty (Dacko, 2017).

5. Recommendations

Based on insights gained from this research, the following recommendations have been developed.

5.1 Enhance AR Technology to Simulate Texture and Consistency

Online AR helps customers predict product aesthetics but fails to reproduce texture and consistency properties which results in product purchase uncertainty across foundations and lipsticks and skincare items. Consumers depend on unreliable reviews when making choices. Textured simulation technology powered by AI systems should be developed by brands to demonstrate foundation coverage along with blend ability and completion. The application process can be shown through interactive animations for various skin types. Future users of haptic feedback technology will be able to experience skin textures which will increase their confidence level in online purchases and decrease product returns.

5.2 Integrate Consumer-Generated Content and Real-Time Reviews

Since AR cannot duplicate material texture and product consistency consumers depend on feedback from reviews and actual demonstrations. Brands should connect consumer-produced content and instant AR review feedback systems to establish trust with their audience. The assessment of product coverage and finish becomes possible through influencer partnerships, which utilize AR to compare real versus virtual products. Consumers engage more with video reviews and live try-ons, as well as unedited content found on TikTok Instagram and YouTube. Live AR shopping events unite digital and physical shopping while reducing the problem of deceiving product previews.

5.3 Offer AR-Linked Sample and Flexible Return Policies

The inability of AR technology to address customers' doubts about product texture and consistency prevents them from making online cosmetic purchases. Brands must provide customers the possibility to acquire small trial sizes through a sample-based purchase system following an AR try-on experience. Return policies connected to AR technology should offer customers free or discounted returns through the "Texture Guarantee" when products fail to meet texture and coverage expectations. Such strategies help decrease customer uncertainty while creating trust and ensuring future sales.

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The adoption of AI into online delivery services: A study based on the Sri Lankan supermarket industry

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Abstract

This research aims to examine the factors that impact Artificial Intelligence (AI) in online delivery services within the Sri Lankan supermarket industry. This study's findings are valuable for organisations aiming to gain a competitive advantage over rivals by leveraging AI-integrated tools to improve customer relationship management, a crucial aspect in customer satisfaction and retention. A quantitative method was adopted using a structured questionnaire to obtain the responses of 100 customers from Colombo using online supermarket delivery services. Primary data findings reveal that personalisation, speed, accuracy and human-like interaction have a strong positive correlation with the adoption of AI. Specifically, "human-like interaction" is recognised as the most significant factor that influences AI adoption, based on multiple linear regression analysis. Therefore, supermarkets need to be particularly mindful of these factors, especially "human-like interaction," when incorporating AI into their online delivery services to ensure successful adoption by customers. The finding reveal that the customers are willing to adopt AI. The customers agreed that integrating AI into online supermarket delivery services would enhance their overall shopping experience by improving personalisation, speed and accuracy.

Keywords: Artificial intelligence, Online delivery services, Supermarket industry

1. Introduction

1.1. Background of the Study

Digitalisation has become crucial in the modern era, significantly impacting how people live and businesses operate. It encompasses high-end tools like artificial intelligence, machine learning and other advanced technologies, which has aided to enhance business operations contributing to positive organisational changes (Bukhari, Daim, Alzahrani, Gillpatrick & Santiago, 2024). Digitalisation has evolved to where consumers form perceptions about a company based on their online presence. Thereby, the reliance on digital platforms has drastically increased, making online presence imperative for all businesses, specifically, the food retail industry, which is important to all (Bukhari et al., 2024).

Particularly, speed and selection are important customer requirements when shopping online. Therefore, retailers are constantly finding ways to engage customers through technology (Bukhari et al., 2024). Consequently, the integration of AI in online delivery services is ideal, since it has the potential to cater to different customers through quick and personalised services (Lee, 2020).

Several retail outlets have utilised AI in their online platforms (Ying 2022; Lu, Cai, & Gursoy, 2019). Sri Lankan supermarkets are yet to adopt AI into their online shopping services. Therefore, this research aims to study the adoption of AI in online delivery services in the Sri Lankan supermarket industry.

1.2 Overview of the industry

According to Nadarajah, Chanaka, and Achchuthan (2014), supermarkets are a large store that offers a wide variety of consumable and household items organised in different shelves. The retail sector has been a major contributor to the GDP, providing one third of national GDP, while providing 14% of employment in Sri Lanka (Habaragoda, 2021).

The most prominent Sri Lankan supermarkets are Cargills Food City, Arpico and Keells Super

(Nadarajah et al., 2014). There is a robust competition among the supermarkets (Karunaratna, 2021). Customers prioritise service quality, which is regarded as a critical factor to foster satisfaction and loyalty (Ahmad, Ihtiyar, and Omar, 2014). Hence, retailers are constantly finding ways to create a competitive edge over rivals (Ahmad et al., 2014).

1.3 Rationale

Supermarkets are becoming a prominent shopping destination and are constantly evolving as an attempt to keep up with changing trends (Vithanage, Wattage, Kariyawasam, Wilson, & Khanal, 2023; Rishi & Singh, 2012). This has led to high competition, motivating supermarkets to explore various techniques to form a competitive edge over its rivals (Karunaratna, 2021). The benefit of technology enables personalised services fostering customer satisfaction (Grewal, Roggeveen, & Nordfält, 2017). Many studies prove positive impacts concerning the integration of technology on the overall performance in retail (Bukhari et al., 2024). This is due to AI-based solutions being developed in a manner to meet a variety of customer requirements (Grewal et al., 2017). Sri Lankan supermarkets, however, have not yet adopted AI into the online shopping services.

The insufficient use of technology presents a concerning challenge for traditional businesses in acquiring vital supply and demand data (Souiden, Ladhari & Chiadmi, 2019). This difficulty is compounded by the increasing preference of shoppers, who are inclined to virtual channels which often incorporate tools like chatbots (Souiden at al., 2019). Thus, numerous consumers highly prefer online shopping due to its convenience (Wang, Ji & Zhao, 2024; Njomane & Telukdarie, 2022). Some customers visit physical stores to see products, yet buy them online to acquire discounts (Wang, Ji, & Zhao, 2024). The retail industry is evolving from traditional to contemporary online retailing (Ailawadi & Farris, 2017; Grewal et al., 2017) by actively investigating and employing technology to improve customer experience (Bukhari et al., 2024).

It is noteworthy that using AI enables customers to buy the ideal product based on their tastes (Wang, Ji, and Zhao, 2024). For example, by learning about customer preferences, AI chatbots may interact with consumers and provide tailored services (Kalisetty, 2025; Cohen, 2018; Yang, Zhang, & Yan, 2022). This demonstrates that online retailing systems can be simplified and revolutionalise organisations to adapt to a digitalised era. Meanwhile, the COVID crisis has shown a change in consumer's purchasing habits with e-commerce becoming increasingly popular (Creazza et al., 2022). Thus, customers anticipate a prompt and easy method for shopping (Spencer, 2024). Hence, e-commerce is perceived to help retailers win within this digitalised context (Melacini, Perotti, Rasini, & Tappia, 2018). Therefore, it is important to study the adoption intentions of Sri Lankan customers towards AI integration into the online delivery services in the Sri Lankan supermarket industry.

1.4 Research Aim

This study aims to evaluate the adoption of AI in online delivery services in the Sri Lankan Supermarket industry.

1.5 Research Objectives

1. Identify the factors that lead to adoption of AI in online delivery services

2. Identify the significant factors that lead to adoption of AI in online delivery services within the Sri Lankan supermarket industry.

3. To make recommendations on the adoption of AI in online delivery services within the Sri Lankan supermarket industry.

1.6 Scope

This study will be based on a survey of one hundred customers of Sri Lankan supermarkets based in Colombo.

1.7 Significance

The supermarkets can benefit by understanding the adoption intentions of AI in online delivery services and the significant factors that drive AI adoption.

This study will add to the limited research concerning the adoption of AI in the Sri Lanka.

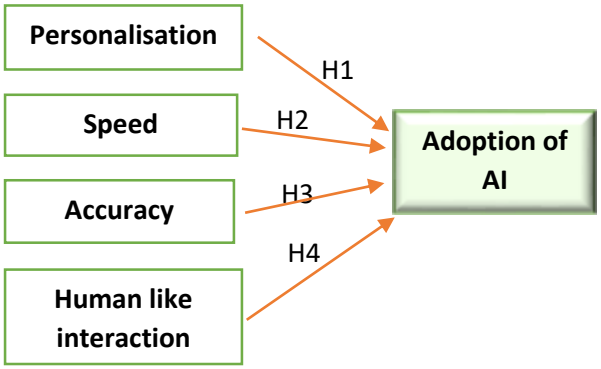
2. Methodology

2.1 Research Method

This study used the quantitative research method, since it is suitable to study the adoption intentions of a large population.

2.2 Conceptual Framework

The independent variables are personalisation, speed, accuracy and human like interaction as illustrated in Figure 1.



Independent variable Dependent variable

Figure 1. Conceptual Framework

2.3 Hypotheses

$H1_A$ - There is a relationship between personalisation and AI adoption in online delivery services.

$H1_0$ - There is no relationship between personalisation and AI adoption in online delivery services.

$H2_A$ - There is a relationship between speed and AI adoption in online delivery services.

$H2_0$ - There is no relationship between speed and AI on online delivery services.

$H3_A$ - There is a relationship between accuracy and AI adoption in online delivery services.

$H3_0$ - There is no relationship between accuracy and AI adoption in online delivery services.

$H4_A$ - There is a relationship between human like interaction and AI adoption in online delivery services.

$H4_0$ - There is no relationship between human like interaction and AI adoption in online delivery services.

2.4 Population and Sample

The population of the study are the customers who make purchases using online delivery services. A sample of 100 customers were selected based on the convenience sampling technique.

2.5 Data Collection

This research used Microsoft Forms, an online survey to collect the data. The survey used a 5-point Linkert Scale based questionnaire.

2.6 Data Analysis

Quantitative data gathered through the structured questionnaire was analysed using SPSS software. Correlation and multiple linear regression analysis were conducted.

3. Analysis and Findings

3.1 Demographic Data

Table 1. Demographic Data

	Description	Percentage (%)
Gender	Male	43.00%
	Female	46.00%
	Prefer not to say	11%
Age	20-29	62.89%
	30-39	17.53%
	40-49	19.59%
Highest Educational Qualification	High school diploma	15.31%
	College diploma	16.33%
	Degree	30.61%
	Masters	31.63%
	PhD	6.12%
Occupation	Student	14.29
	Self employed	20.41%
	Employed	58.16%
	Unemployed	6.12%
	Retired	1.02%

As show in Table 1, the majority of the respondents (63%) are young, in the age category of 20-29, with higher educational qualifications and are employed.

3.2 Data Validity and Reliability

The Cronbach Alpha test is suitable to assess the reliability of the scale when a questionnaire is created utilising many Likert scale statements (Taber, 2017; Chetty & Datt, 2015).

Cronbach's alpha score should be greater than 0.7 in order to be acceptable (Taber, 2017).

Table 2. Cronbach Alpha

Variables	Cronbach's Alpha
Personalisation	.757
Speed	.859
Accuracy	.892
Human like interaction	.849

Cronbach's Alpha values for every variable show high reliability, surpassing the 0.7 threshold. This verifies that the questionnaire items consistently measure their respective constructs, guaranteeing the data's validity for additional analysis.

3.3 Pearson Correlation

Table 3. Pearson Correlation

Independent variable	Pearson Correlation	Sig Value
Personalisation	.540	<.001
Speed	.655	<.001
Accuracy	.724	<.001
Human like interaction	.749	<.001

Correlation analysis is the process of examining the relationship between variables. Thereby, a positive correlation ranges from 0 to +1, whereas a negative correlation is below 0 (Gogtay & Thatte, 2017). In this instance, Table 3 illustrates that all the variables are above 0.5 which implies a strong correlation. This demonstrates that there's a strong positive correlation between the dependent and independent variables.

Moreover, an acceptable Sig. value less than 5% (0.05) indicates a statistically significant correlation between the independent variable and the dependent variable (Kwak, 2023). In this case, all the variables (as indicated on Table 3) are less than 0.01, which means there is a statistically significant correlation between all the aforementioned independent variables and the adoption of AI.

3.4 Hypotheses Validation

Based on the correlation analysis, all the hypotheses are accepted as shown in Table 4.

Table 4. Hypotheses

Hypotheses	Accepted/Rejected
$H1_A$ - There is a relationship between personalisation and AI in online delivery services.	Accepted
$H2_A$ - There is a relationship between speed and AI in online delivery services.	Accepted
$H3_A$ - There is a relationship between accuracy and AI in online delivery services.	Accepted
$H4_A$ - There is a relationship between human like interaction and AI in online delivery services.	Accepted

The correlation analysis reveals that personalisation, speed, accuracy and human like interaction are positively correlated to AI adoption, thus supporting the hypotheses formulated in this study.

These findings coincide with previous studies. AI for instance is believed to have a strong impact on personalisation (Christian, Anene, Ewuzie & Iloka, 2023). This plays a crucial role for AI to be accepted (Kumar, Rajan, Venkatesan, & Lecinski, 2019), resulting in companies embracing this factor in AI adoption (Liu, Zhang & Caesarius, 2024).

Additionally, speed is considered to be AI's key strength (Nosta, 2024). This makes it a fundamental component of AI (Chowdhury & Sadek, 2012).

Interestingly, accuracy is prioritised by many companies motivating them to adopt AI (Akter et al., 2021; Kaplan & Haenlein, 2019). In the same way, customers adopt AI-integrated tools due to its precision (Seeger & Heinzl, 2018).

Furthermore, "human like interaction" is improving online services from its human-like qualities such as emotions, cognitive, critical and social intelligence (Kaplan & Haenlein, 2019).

3.5 Multiple Linear Regression

3.5.1 R square

R square is identified to be 0.730, which means that 73% of the variation of "adoption of AI" is explained by the variables: personalisation, speed, accuracy and human like interaction.

3.5.2 Multiple Linear Regression Results

Table 5. MLR Results

	Beta Value	Sig Value
Personalisation	.135	.163
Speed	-.064	.626
Accuracy	.242	.093
Human like interaction	.603	<.001

Based on the regression analysis, the most significant variable is 'human like interaction' with a Sig. value less than .001, as indicated in Table 5. The beta value is .603. This indicates that enhancing the human-like qualities of AI interactions will contribute to a strong and positive impact on AI adoption.

3.6 Descriptive Statistics

Table 6. Mean Values

Variables	Mean
Personalisation	**3.9**
1. AI-driven recommendations in online supermarkets enhance my shopping experience	3.8
2. I am more likely to use an online supermarket, if AI suggests products based on my past purchases.	4.0
3. Personalised promotions and discounts offered by AI influence my shopping decisions.	4.1
4. AI personalisation makes my online shopping journey feel more tailored and convenient	4.0
5. I trust AI to understand my shopping preferences better over time.	3.9
Speed	**3.9**
1. I prefer using online supermarkets with AI features, because they speed up the shopping process.	4.0
2. AI-powered search and filtering options help me find products faster than manual browsing	4.0
3. Faster checkout processes, enabled by AI, encourage me to shop online more frequently.	3.9
4. I am more likely to use an online supermarket, if AI reduces the time spent on order placement.	4.0
5. AI's ability to quickly suggest alternative products when items are unavailable enhances my shopping experience.	4.0
Accuracy	**3.9**
1. I trust AI in online supermarkets to provide accurate product recommendations.	3.9
2. AI-powered chatbots and customer service tools provide reliable and precise responses to my queries.	3.8
3. AI-based systems help reduce errors in order processing and delivery.	3.9
4. I am more likely to adopt AI in online supermarkets, if it ensures accurate substitutions for out-of-stock products.	3.8
5. I prefer AI-powered systems in online supermarkets, because they minimise the chances of	3.9

incorrect charges or billing issues.	
Human like interaction	**3.9**
1. I feel more comfortable interacting with AI chatbots, if they provide responses similar to human customer service agents	4.0
2. A human-like AI assistant makes my online shopping experience more engaging.	4.0
3. I am more likely to use an AI-powered online supermarket, if the chatbot understands natural language and responds conversationally.	4.0
4. AI with human-like interaction enhances my trust in online supermarkets.	3.9
5. The ability of AI to express empathy and understanding influences my decision to adopt it in online supermarkets.	3.8
Adoption of AI	**4.0**
1. AI-driven features in online supermarkets positively impact my willingness to shop online.	3.9
2. I am more likely to use an online supermarket that integrates AI for recommendations, search, and customer support.	4.0
3. AI-based automation in online supermarkets enhances my overall satisfaction.	4.1
4. If an online supermarket offers AI-powered features, I am more likely to shop online.	4.0
5. I would like to use AI tools when shopping online.	4.1

The overall mean values for the independent variables fall within the acceptable range, generally around 4.0, which implies customers consider these variables to be important in their intention to adopt AI in online shopping services. These findings are supported by previous studies on AI adoption.

Personalisation is perceived to boost engagement and revenue (Davenport & Ronanki, 2018). It is known for enhancing customer's overall shopping experience, which encourages them to make more purchases (Adam, Wessel, & Benlian, 2021). Additionally, AI's human like traits showcase the ability to enhance customer experience fostering satisfaction (Murtarelli, Gregory, & Romenti, 2020).

Brands need to ensure AI is updated with information that goes in line with customer requirements (Peham, 2023). It is recommended to update chatbots with large datasets of customer information to enable them to make informed decisions and boost customer satisfaction (Sherman, 2023).

Some users find it unappealing to interact with chatbots (Gnewuch, Morana, Adam & Maedche, 2018). Thus, conversational chatbots are perceived to boost customer satisfaction (Hsu and Lin, 2023). Moreover, to improve user impressions of human similarity, chatbots can also be given human names, photos, personalities, and be updated to react like humans (Hu, Lu, & Gong, 2021; Folstad, Nordheim, & Bjorkli, 2018).

Customers shopping experience are enhanced and found to be convenient especially when AI-powered tools are integrated (Haleem, Javaid, Qadri, Singh, & Suman, 2022). AI-integrated solutions are therefore anticipated to change the e-commerce sector, guaranteeing an upward trajectory for all retailers (Lu, Cai & Gursoy, 2019).

4.Conclusion

The correlation analysis indicates that all the variables (personalisation, human-like interaction, speed and accuracy) have a strong positive correlation with the dependent variable "adoption of AI". The regression analysis indicates that "human like interaction" is the most significant factor influencing the adoption of AI. The majority of the respondents agreed that AI will enhance their online shopping experience and were willing to adopt.

Given that human-like interaction is found to be a significant predictor of AI adoption, supermarkets should take this into account. Natural language processing (NLP) is a type of AI tool known to facilitate communication with customers, playing a major role in resolving complex problems (Stryker & Holdsworth, 2024). Interestingly, the NLP function is integrated into AI chatbots, which has transformed it to communicate like humans (Chakraborty, Kumar Kar, Patre, and Gupta, 2024), portraying traits like empathy (Mari, Mandelli, & Algesheimer, 2024).

With regard to AI's ability to enhance "personalisation", AI-chatbots can ideally be employed, as they help to provide personalised experiences, which are crucial to boost customer satisfaction (Pawaskar & Nattuvathuckal, 2024; Chandra et al., 2022; Przegalinska et al., 2019). Notably, AI-chatbots help to resolve customer queries and update customers with pertinent product information to ensure smooth order processing (Samuels, 2023). Augmented Reality (AR) is versatile in terms of providing personalised recommendations, which is essential to address customers with the right product that aligns with their expectations (Necula and Pavaloaia, 2023; Samuels, 2023). Similarly, companies use machine learning (ML) extensively because, it has the potential to enhance "personalisation", which is useful for creating successful marketing campaigns (Haleem et al., 2022). AI-personalised suggestions based on user purchasing patterns are instrumental in creating customer-targeted marketing strategies (Hayes & Downie, 2024).

Therefore, AI's ability to enhance personalisation has motivated supermarkets to adopt AI chatbots, in its online delivery services to enhance their marketing goals (Chakraborty et al., 2024). Despite these benefits, there are situations where the quality of AI suggestions may fall short of expectations. In this case, it is advised to update AI with a large amount of data to guarantee that customer requirements are met (Peham, 2023; Booch et al., 2021).

Speed is crucial to create positive customer relationships in a contemporary digital world (Chacko, 2020). Quick replies demonstrate the company's dedication to its customers, which boosts brand satisfaction, loyalty, and reputation (Hyken, 2023; Chacko, 2020). Fast speed is anticipated by most customers (Spencer, 2024; Hyken, 2023; Chacko, 2020). Thus, AI is crucial in terms of enhancing speed, as it has the ability to think faster than humans (Chen et al., 2022; Chacko, 2020). For instance, AI-chatbots provides quick assistance by effectively resolving customer queries (Pawaskar & Nattuvathuckal, 2024; Chacko, 2020; Przegalinska et al., 2019). The 24/7 availability of responding to customer queries reduces workload of companies and lessens their dependence on human agents (Chacko, 2020). Likewise, AI tools are capable of addressing different customer queries simultaneously (McClune, 2024).

Accuracy in AI refers to matching predictions with a specific set of facts or anticipated results, giving it a sense of dependability and trustworthiness. Thus, AI has the ability to enhance the concept of "accuracy", since it carefully ascertains complicated data to formulate informed decisions (Sakhvidi & Saadat, 2024). This has been possible due to its strong algorithms which accurately assess customer data (Bawack, Wamba, & Carillo, 2021; Deng, Tan, Wang, & Pan, 2019). Specifically, machine learning (ML) has the potential to solve problems, since its systems are trained to evaluate and understand information without the need of constant updates (Haleem et al., 2022).

In conclusion, AI adoption brings a lot of advantages to the online shopping experience of Sri Lankan supermarkets. The customers surveyed are willing to adopt AI enabled online delivery services to benefit from personalisation, speed and accuracy improvements related to AI adoption. Therefore, Sri Lankan supermarkets should seriously consider adopting AI into their online delivery services.

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Understanding the Impact of Brain Drain of Academics and Professionals on the Sri Lankan Economy

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Abstract

This research examines the increasing issue of brain drain among academics and professionals in Sri Lanka, focusing on the diverse motivations behind skilled emigration and its implications for the country's socio-economic landscape. Utilizing an inductive approach, the study incorporates qualitative data from semi-structured interviews and focus group discussions with professionals across various sectors, including healthcare, education, engineering, and information technology. Key findings reveal that economic factors, especially wage disparities and limited career advancement, are primary drivers of migration. Participants expressed frustration with stagnant salaries and insufficient job prospects in Sri Lanka. Additionally, other influential factors such as the desire for professional development, concerns about political stability, quality of life, and significant social networks play critical roles in shaping migration decisions. The analysis illustrates that the emigration of skilled individuals has profound consequences for critical sectors, exacerbating talent shortages and hindering innovation and productivity. This outflow intensifies existing social inequalities, particularly affecting marginalized communities that rely on skilled professionals for essential services. To address these challenges, the study calls for urgent policy interventions targeting the root causes of brain drain. Recommendations include enhancing domestic career opportunities, improving institutional support, and fostering diaspora engagement. By providing valuable insights into the brain drain phenomenon, this research aims to retain skilled professionals and leverage their potential for national development.

Keywords: Brain Drain, Skilled Migration, Economic Factors, Talent Retention, Policy Interventions

1. Introduction

1.1 Background of the study

Brain drain has long posed a challenge in Sri Lanka, particularly within technology, healthcare, and education sectors. The World Bank indicates that over the past few decades, the nation has witnessed a notable outflow of its skilled labor force, adversely impacting economic development and hindering progress (World Bank, 2017). This exodus compromises Sri Lanka's capacity to enhance service delivery, foster innovation, and maintain competitiveness in the global market. The education system has notably suffered, as many scholars, particularly those with postgraduate degrees, seek opportunities abroad due to poor research facilities, limited professional advancement, and political instability (Perera, 2015). The brain drain extends beyond individual talent loss, negatively influencing economic growth. For instance, data from the World Bank shows that Sri Lanka's labor force participation rate was only 50.7% in 2020, indicating that many skilled workers are employed abroad (World Bank, 2020). The departure of these professionals deprives the nation of crucial expertise and productivity needed for economic advancement. Furthermore, the loss of skilled individuals exacerbates social inequalities, disproportionately affecting marginalized communities reliant on professionals for essential services. The urgent need for policy interventions targeting the root causes of brain drain becomes paramount. Initiatives such as the 'Diaspora for Development' program aim to harness the expertise of Sri Lankan expatriates for national development, highlighting the

potential for positive outcomes even amidst the challenges posed by emigration.

Sri Lanka has historically been a hub for developing skilled professionals across various fields, including medicine, engineering, and information technology. However, a continuous outflow of talent has emerged due to limited career opportunities, political instability, and inadequate infrastructure (Wickramasinghe, 2020; Perera, 2019). The phenomenon of brain drain ultimately hampers economic development by depriving the country of essential human capital necessary for progress. The migration of skilled professionals reflects deeper socio-economic issues within the country. Major factors driving this trend include the lack of career growth prospects and persistent political instability, which undermine confidence in Sri Lanka's future (Jayasuriya, 2017). Additionally, insufficient investment in vital sectors further exacerbates the outflow of talent, trapping the nation in a cycle of underdevelopment (Hettige & Huq, 2015).

1.2 Rationale

In recent years, the brain drain situation in Sri Lanka has worsened, creating substantial hurdles for the country's economic advancement and growth prospects (Hettige & Huq, 2015). The flight of skilled professionals and academics has resulted in a talent shortage in essential sectors, which adversely impacts innovation, productivity, and overall competitiveness. Furthermore, the loss of human capital deprives Sri Lanka of necessary resources to tackle critical socio-economic challenges, including poverty, healthcare, and education. Therefore, it is important to understand the factors that lead to the brain drain of skilled professionals and academics.

1.3 Research Aim

The aim of this study is to examine the various factors that lead to the brain drain phenomenon in Sri Lanka

1.4 Research Objectives

1. To identify and assess the primary causes of brain drain affecting skilled professionals and academic researchers in Sri Lanka.

2. To explore the effects of brain drain on essential sectors such as healthcare, education, engineering, and information technology.

3. To develop a strategy to help promote the retention of human capital within the country.

2. Methodology

The philosophical foundation of this study is interpretivism, emphasizing the understanding of human experiences from the subjective interpretations of individuals. This philosophy is well-suited for exploring skilled professionals' perspectives on brain drain and the social interactions that shape their motivations and decisions. The study utilizes an inductive approach, allowing insights and theories to emerge from the qualitative data collected rather than testing existing hypotheses. A qualitative method focused on in-depth interviews and focus group discussions was facilitated thorough an examination of participants' perspectives.

2.1 Population and Sampling

The population for this study comprises of academics and professionals across various sectors, including education, healthcare, engineering, and technology. This population consists of individuals with specialized skills and expertise, who are particularly susceptible to brain drain. The purposive sampling method was used to select participants with relevant characteristics. The participants were approached through professional networks, social media platforms, and referrals to create a targeted participant pool consisting of individuals with advanced degrees and significant experience in their fields. A total of 10 participants were interviewed to gain insights into their views on migration and domestic career opportunities.

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2.2 Participants' Background

1. Participant A: A seasoned professional who has made a significant impact in the tech industry since relocating to Australia in 2014.

2. Participant B: An award-winning business strategist recognized for her expertise in developing effective strategies that foster growth and innovation. After leaving Sri Lanka, she has established herself in Australia.

3. Participant C: A software engineer with 20 years of industry experience in Sri Lanka, migrated to Canada in 2024.

4. Participant D: Previously the finance manager for Hemas Group, moved to Australia in 2015.

5. Participants E: A dedicated state sector nurse with 15 years of experience, migrated with her family to Australia in 2020.

6. Participants F: A sworn translator (English-Sinhala-English) currently serving in the Public Administration Ministry. Participant F processing her documents for migration to Canada.

7. Participant G: A CIMA-qualified professional who graduated from the University of Colombo, worked in the mercantile sector in Sri Lanka for seven years before her migration to Australia in 2015.

8. Participants H: After serving for 10 years as a psychiatrist in Sri Lanka, moved to Australia in 2020.

9. Participants I: A chef who migrated to Australia in 2016.

10. Participants J: A former government school teacher with 5-6 years of experience, initially moved to the UK with her family before relocating to Australia for permanent residency.

2.3 Data Collection

Data was gathered through semi-structured interviews with skilled professionals who have either emigrated or chosen to remain in Sri Lanka. Semi-structured interviews provide flexibility, allowing interviewers to explore predetermined topics while enabling participants to express their own experiences and insights. This adaptability is crucial for capturing the diverse perspectives surrounding brain drain (Kvale & Brinkmann, 2009).

2.4 Data Analysis

The qualitative data obtained from these methods was analyzed using thematic analysis, which identifies, analyzes, and reports patterns or themes within the data (Braun & Clarke, 2006). This approach helps researchers organize and interpret qualitative data to uncover significant insights and connections to broader research questions. The data analysis involved thematic analysis of the qualitative data collected from interviews and focus groups.

2.5 Limitations

Several limitations to the methodology must be acknowledged. The subjective nature of qualitative data can introduce variation in participants' interpretations, influenced by social desirability bias (Patton, 2002). The purposive sampling method, while effective for in-depth insights, may limit the representativeness of the sample and generalizability of findings (Creswell & Poth, 2018).

3. Analysis and Findings

The analysis revealed several recurring themes reflecting participants' motivations to migrate. These include economic incentives, professional development opportunities, socio-political factors, pursuit of a higher quality of life and the influence of social networks and family ties.

3.1 Economic Incentives

A prominent theme identified is the influence of economic factors on participants' decisions to emigrate. Many respondents cited significant wage disparities and better job opportunities in destination countries as key motivators for their migration. Participants expressed frustration with stagnant salaries and limited opportunities for career advancement within Sri Lanka, aligning with existing literature that emphasizes the role of economic incentives in migration decisions (World Bank, 2020). For instance, a teacher (Participant J) noted how financial constraints hindered their ability to provide quality education, stating that moving to Australia offered not just better pay, but an

improved environment for professional growth and student learning.

Participant J stated, "*As a teacher in a government school for over five years, I continually faced the frustration of stagnant wages and limited resources for my students. Despite my passion for education, the financial constraints made it difficult for me to provide the quality of learning my students deserved. Moving to Australia represented not just a chance for better pay and working conditions, but also an opportunity to create a more conducive environment for both my professional growth and the future of my students."*

3.2 Professional Development

Participants frequently discussed the lack of growth opportunities in Sri Lanka, highlighting their desire for better training, mentorship, and resources to enhance career prospects. Many reported that access to advanced research facilities and collaborative environments abroad was pivotal in their decision to migrate, reflecting the fact that the pursuit of professional growth was in important factor in the decision to migrate.

Participant H stated, "*As a psychiatrist who spent a decade working in Sri Lanka, I found that my ability to help patients was often limited by the resources available and the stigma surrounding mental health. Moving to Australia has given me access to better facilities and a supportive professional environment where mental health is prioritized. I want to provide the best possible care for my patients, and I believe that here, I can truly make a difference in their lives*."

Participant E stated, "*As a dedicated nurse working in Sri Lanka for 15 years, I often felt the weight of not having the professional support I needed to excel in my role. The healthcare system was stretched thin, and adequate resources were frequently lacking, including essential medications, which made it incredibly challenging to provide the high standard of care my patients deserved. While I love my profession and the opportunity to help others, the limited support, the scarcity of medicine, and the lack of opportunities for continuing education were frustrating. This experience motivated me to relocate to Australia, where I can access professional development and work in an environment that truly values the contributions of skilled healthcare professionals*."

3.3 Political and Social Stability

Concern for political and social stability also emerged as a critical factor influencing migration decisions. Participants cited political uncertainty, deteriorating governance, and social unrest in Sri Lanka as reasons for their desire to leave. These sentiments align with findings of Kumar and Silva (2022), indicating that perceptions of instability foster insecurity about the future, prompting professionals to seek stability in more developed countries. Participants articulated the importance of a stable environment for personal and professional growth, underlining that political factors weigh heavily in their migration choices.

3.4 Quality of Life

The analysis reveals that quality of life considerations significantly influence migration decisions. Respondents expressed that migration offered better living conditions, access to quality healthcare, education, and a favorable social environment. This observation echoes research by De Silva and Nawaratne (2018), indicating that perceptions of a higher quality of life in destination countries significantly impact individuals' decisions to emigrate. Concerns regarding their children's education were prevalent among participants, as illustrated by one who emphasized the importance of providing better educational opportunities for their children in Canada. This desire underscores how family considerations are intricately tied to professional migration decisions.

Participant C stated, "*I worry deeply about my children's education and future; I want them to have access to the best opportunities and learning environments possible, which I believe they can find in Canada. I want them to grow up with the resources and support needed to thrive in their careers, something that has become increasingly challenging in Sri Lanka."*

3.5 Social networks and family ties

The influence of social networks and family ties also emerged as a noteworthy theme. Many participants highlighted the role of familial connections and social networks in facilitating their migration process. Research supports that such networks offer essential support and information regarding job opportunities and emotional backing during the transition (De Silva & Nawaratne, 2018). The presence of relatives or friends in the destination country often made the prospect of migration more feasible and appealing, reinforcing the interconnectedness of social support and migration.

3.6 Impact of the brain drain on the Sri Lankan Economy

3.6.1 Health Care Sector

The outflow of health care professionals such as doctors and nurses has created shortages in skilled medical personnel, especially in rural areas, leading to increased workloads on the remaining staff, diminished quality of care, and delayed access to services. It also impacts public health outcomes negatively.

The government invests heavily in training medical professionals; therefore, their departure causes a loss of return on public investment. The healthcare system struggles to meet demand, which can reduce overall productivity and increase healthcare costs (Sirisena & Gamage, 2019).

3.6.2 Education Sector

Sri Lanka faces a shortage of qualified teachers and academics, particularly in science, technology, engineering, and mathematics (STEM) fields. Many educators migrate for higher salaries and better working conditions abroad. This reduces the quality of education and hampers the development of a skilled workforce in Sri Lanka. Lower education quality affects the skill level of future professionals, limiting innovation and productivity growth. It also reduces Sri Lanka's competitiveness in the global economy (Perera & Jayawardena, 2017).

3.6.3 IT Sector

Sri Lanka's IT sector has potential for rapid growth, but brain drain of software developers, IT managers, and specialists limits this growth. Skilled IT workers often migrate to countries with more advanced tech industries and higher wages**.** This causes a talent and skill gap, slowing innovation and reducing the sector's contribution to GDP and export earnings. It also reduces the ability of local firms to compete internationally (Fernando & Silva, 2018).

3.6.4 Engineering Sector

Many engineers seek opportunities abroad, attracted by higher pay and better infrastructure. The loss of engineers affects infrastructure projects, construction, and industrial development. The shortage can delay key infrastructure projects and industrial expansions, hindering economic growth and foreign investment opportunities (Wickramasinghe, 2020).

3.7 Policy interventions

The talent deficit resulting from the emigration of skilled professionals, particularly in vital sectors like healthcare and education is a concern for Sri Lanka. The Sri Lanka Bureau of Foreign Employment (SLBFE, 2021), indicates a growing number of skilled migrants departing from the country in these critical areas. The outflow of talent undermines local industries and economic growth, highlighting an urgent need to address the underlying drivers of migration.

The findings emphasize the necessity for comprehensive policy interventions to address the root causes of migration and enhance the socio-economic environment in Sri Lanka. By understanding the multifaceted reasons driving skilled individuals to emigrate, policymakers can develop strategic initiatives aimed at retaining talent and fostering a more conducive environment for professional growth and social equity within the country.

4. Conclusion

This study revealed systemic issues, such as economic instability and insufficient professional support, motivating skilled professionals to emigrate. Furthermore, it highlighted the negative consequences of brain

drain on sectors like healthcare and education, underscoring the urgency for solutions addressing these challenges.

To mitigate brain drain and promote knowledge retention in Sri Lanka, several recommendations are proposed: enhancing domestic career development opportunities through collaboration between government and private sectors; improving institutional support for professionals; implementing targeted immigration policies to attract expatriates back to the country; fostering collaboration and networking to create a supportive ecosystem; investing in research and innovation; promoting diaspora engagement; and establishing regular monitoring and evaluation of policy interventions.

In conclusion, the research highlights the need for a multifaceted approach to tackle brain drain in Sri Lanka. By addressing economic, social, institutional, and policy-related challenges through evidence-based strategies, the country can leverage its human capital effectively, fostering a conducive environment for talent retention and sustainable development in the years to come.

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The Role of Green Supply Chain Management in Organisational Performance - A Case Study of Hayes Tea Estate

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Abstract

Green Supply Chain Management (GSCM**)** serves as a vital strategy for enhancing environmental sustainability, while simultaneously improving operational performance across industries. In the Sri Lankan tea plantation sector, three major barriers hinder the effective implementation of GSCM practices: limited financial resources, inadequate training programs, and insufficient infrastructure. This research examines the impact of GSCM on organizational performance using Hayes Tea Estate as a case study. The tea plantation industry urgently requires research aimed at overcoming these implementation barriers, as constrained resources, inefficient waste management, and high operational costs hinder both sustainability efforts and organizational effectiveness. This study addresses the problem by analyzing how GSCM can be implemented at Hayes Tea Estate to improve organizational performance. It also offers recommendations on how to effectively utilize GSCM practices to achieve improved outcomes. The literature on GSCM highlights the foundational 3R1D principles (Reduce, Reuse, Recycle, and Decompose), emphasizing their potential impact on supply chain operations. A qualitative methodology was employed, using judgmental sampling to select ten employees. The study used thematic analysis to interpret data collected through semi-structured interviews with managerial staff. These interviews explored existing GSCM practices, current challenges, and opportunities for improvement. The findings revealed that Hayes Tea Estate has made reasonable progress in areas such as packaging reuse and energy savings. However, challenges persist in composting practices, stemming from inadequate staff training and limited waste management systems. Issues such as damaged packaging, ineffective composting techniques, and insufficient recycling infrastructure continue to hinder the estate's sustainability efforts. The study concludes that enhancing GSCM practices, through the adoption of more durable packaging, expanded use of renewable energy, and improved waste management, can significantly improve operational efficiency, reduce environmental impact, and provide Hayes Tea Estate with a sustainable competitive advantage.

Keywords: Green Supply Chain Management (GSCM), 3R1D Principle, Tea Industry, Organisational Performance

1. Introduction

1.1. Background of the study

Green Supply Chain Management (GSCM) has garnered significant attention in recent years as businesses increasingly recognize the need to balance economic growth with environmental responsibility. GSCM practices have been shown to positively influence organizational outcomes across economic, social, and environmental dimensions (Wu & Chang, 2015). This is particularly relevant in agriculture-dependent sectors, such as the tea industry, where sustainability concerns are deeply intertwined with production and supply chain activities.

GSCM involves the integration of environmentally responsible practices throughout the supply chain, from the sourcing of raw materials to production, distribution, and final disposal (Khan, Ajmal, Jabeen, Talwar, & Dhir, 2022). These practices aim to minimize waste, reduce carbon emissions, and promote efficient resource utilization while maintaining product quality and operational effectiveness. Moreover, GSCM contributes to achieving a balance between economic performance and environmental protection by reducing environmental risks and improving ecological efficiency across the entire supply chain (Zhu et al., 2008).

Tea is one of the most widely consumed beverages globally, and its cultivation is typically concentrated in regions with specific environmental conditions such as high altitudes, moderate temperatures, and consistent rainfall (Bai et al., 2024). However, tea production can exert significant environmental pressures, including soil erosion, excessive water use, and heavy reliance on pesticides. As the tea industry continues to expand, there is growing pressure to adopt sustainable practices to mitigate these adverse effects.

In this context, GSCM offers a comprehensive approach to embedding sustainability throughout the tea supply chain. Implementing eco-friendly agricultural techniques, reducing production waste, conserving natural resources, and minimizing carbon emissions in logistics can enhance organizational performance. These improvements not only reduce operational costs but also strengthen brand reputation and align with consumer preferences for sustainably sourced products (Saeed, Rasheed, Waseem, & Tabash, 2021).

Tea plantations differ from industrial sectors such as manufacturing and technology due to their direct dependence on natural resources like soil, water, and climatic conditions. Therefore, the adoption of sustainable supply chain practices is not optional but essential. According to Zhang et al. (2022), GSCM in agriculture must address both the immediate environmental impacts and the long-term sustainability of resource use. In tea plantations, sustainable practices directly influence crop quality, yield, and long-term soil health, underscoring the urgent need for effective GSCM implementation.

1.2. Company Background

Hayes Tea Estate, located in the historically significant Morawaka region of Sri Lanka, is a prominent entity within the nation's tea plantation industry. It operates alongside other major players such as Mathurata and Browns Plantations, contributing to the country's reputation for producing high-quality tea (Hayes, 2024). Hayes specializes in the cultivation and processing of premium black, green, and specialty teas, primarily targeting business-to-business (B2B) markets both domestically and internationally.

The estate operates within a challenging industry landscape characterized by high production costs, particularly those associated with labour and energy. Notably, firewood constitutes a major portion of the estate’s energy expenditure, underscoring the need for cost-effective and sustainable energy alternatives. Despite these operational challenges, Hayes maintains a strong market position, with an annual production output of approximately 657 metric tons (R. D. Ashoka, personal communication, September 29, 2024). Its ability to remain profitable highlights the

estate's strategic focus on innovation and operational efficiency.

In addition to its commercial success, Hayes Tea Estate plays a vital role in regional socioeconomic development by providing employment opportunities to members of the local community. This integration of traditional tea cultivation practices with contemporary operational strategies positions Hayes as a benchmark for excellence within Sri Lanka's tea industry (Hayes, 2024).

1.3. Research Problem

Despite the well-documented advantages of Green Supply Chain Management (GSCM), including enhanced environmental sustainability, cost reduction, operational efficiency, and improved organizational performance, its adoption within Sri Lanka's tea plantation sector remains limited. The tea industry, due to its reliance on manual labour, minimal automation, and restricted access to recycling and logistics infrastructure, faces significant barriers to implementing green practices (Zhang et al., 2022).

Additionally, many tea plantations operate under financial and technological constraints, lacking the necessary capital, training, and access to green technologies required to adopt advanced GSCM strategies (Martínez-Falcó et al., 2023). These limitations hinder the sector's ability to transition toward environmentally sustainable practices despite the growing global demand for eco-friendly and ethically sourced products.

This study aims to address these challenges by examining the implementation of GSCM practices at Hayes Tea Estate. Specifically, it investigates how GSCM contributes to enhancing organizational performance through the application of the 3R1D principles: Reduce, Reuse, Recycle, and Dispose. The research seeks to provide actionable insights, not only for Hayes but also for the broader tea industry, which is under increasing pressure to align with sustainable operational standards.

By bridging the existing knowledge gap, this study aspires to support the advancement of sustainability practices in Sri Lanka's tea sector and promote more environmentally responsible supply chain management.

1.4. Rationale

The existing literature reveals a significant gap in understanding Green Supply Chain Management (GSCM) practices within sensitive and resource-dependent industries such as tea plantations in Sri Lanka. While many studies have examined general GSCM principles or their applications in industrialized sectors, including manufacturing, logistics, and large-scale agriculture, these investigations often overlook the unique environmental and operational challenges faced by tea plantations. Such challenges include a heavy reliance on manual labour, specific local ecological conditions, and economic dependencies intrinsic to the region (Martínez-Falcó et al., 2023; Zhang et al., 2022).

Of particular concern is the limited integration of the 3R1D principles of Reduce, Reuse, Recycle, and Decomposition, within tea plantation supply chains. Although these principles are well-established in large-scale agriculture and food production, their applicability and effectiveness in smaller-scale tea plantation contexts remain underexplored. Moreover, operational strategies such as minimizing packaging waste, encouraging the use of recyclable materials, and fostering environmentally conscious supplier relationships, as highlighted by Ivalua (2023), have not been adequately investigated in the Sri Lankan tea industry.

From a practical perspective, this research is motivated by the urgent need to address sustainability challenges within the tea plantation sector, which plays a crucial role in both the economic and environmental stability of the region. This study aims to explore how GSCM practices can enhance operational efficiency and promote sustainability in this vital industry.

1.5. Research Aim

The aim of this study is to identify the role of green supply chain management in organisational performance: a case study of Hayes Tea Estate.

1.6. Significance

This study, which investigates the relationship between Green Supply Chain Management (GSCM) and organizational performance at Hayes Tea Estate in Sri Lanka, offers valuable contributions to industry stakeholders, policymakers, and academic researchers. By addressing key challenges such as resource constraints, water usage, and waste management, the research highlights the importance of integrating the 3R1D principles of Reduce, Reuse, Recycle, and Decomposition, to enhance both sustainability and operational performance within tea plantations.

From an academic perspective, this study fills a critical gap in the literature by focusing specifically on the application of GSCM practices in tea plantations, an area that has received limited attention compared to other agricultural or industrial sectors. The findings aim to contribute to the development of tailored sustainability strategies that are sensitive to the unique environmental and operational characteristics of resource-dependent agricultural industries.

2. Methodology

This study employs an inductive research design, which focuses on generating novel theoretical insights grounded in empirical observations (Bryman, 2016). This approach is particularly appropriate for investigating the role of Green Supply Chain Management (GSCM) in shaping organizational performance at Hayes Tea Estate, as it allows theory to emerge directly from the data.

A case study strategy is adopted to enable a comprehensive exploration of the complex, real-world phenomena associated with GSCM implementation. This approach facilitates an in-depth analysis of Hayes Tea Estate as a single, bounded system, allowing for rich, context-specific insights into the application of the 3R1D sustainability principles within its operational framework (Yin, 2018).

The study utilizes qualitative research methods, which provide a detailed understanding of participants' perspectives and behaviors through non-numerical data (Saunders, Lewis, & Thornhill, 2019). Data collection was conducted via semi-structured interviews with ten managerial staff at Hayes Tea Estate, enabling the capture of in-depth, nuanced responses reflecting individual experiences and perceptions of GSCM practices.

3. Analysis and Findings

Table 1. Participants' Background

Respondent	Experience	Designation
1	15 years	Operations Manager
2	10 years	Field Supervisor
3	07 years	Assistant Manager
4	02 years	Manager
5	07 years	Deputy Manager
6	05 years	Executive
7	25 years	Manager
8	05 years	Deputy Manager
9	03 years	Manager
10	05 years	Assistant Manager

3.1 Reuse

3.1.1 Reusing Packaging Materials

Most respondents identified material reuse as a critical sustainability practice. Tea sacks, cardboard boxes, and paper wraps are reused multiple times before being discarded. As Respondent 1 noted, "*Used tea sacks are repurposed multiple times before disposal*."

These practices not only support environmental sustainability but also generate long-term financial benefits through cost savings. Kumar et al. (2021) argue that material reuse within supply chains lowers both environmental impact and operational costs. The circular economy model promotes reusing packaging to reduce dependency on virgin materials and extend the resource life cycle (Ellen MacArthur Foundation, 2020).

Gupta et al. (2022) emphasized that reuse practices can also lead to lower carbon emissions by reducing manufacturing and transportation needs. Hayes Tea Estate enhances its environmental branding by

incorporating these practices (Subramanian & Gunasekaran, 2021).

However, challenges remain. Respondent 4 explained, "*Some old sacks or boxes weaken after multiple uses, making them unsuitable for repurposing*." Zhang et al. (2022) corroborated this finding, noting that material degradation over time undermines reuse efficiency. Consequently, Hayes is transitioning to more durable, regulation-compliant packaging (Sarkis, Wortmann, & Sheng, 2021), while implementing quality control protocols and employee training to improve reuse outcomes (Jabbour et al., 2020).

3.1.2 Equipment and Machinery Reuse

Several respondents highlighted the reuse of machinery components as a cost-effective strategy. Respondent 3 remarked, "*We reuse old machinery parts instead of outright replacements*." This approach reduces waste and ensures the longevity of industrial equipment. Govindan et al. (2022) found similar benefits in factory settings, including extended equipment life and reduced environmental impact.

Hayes has also retrofitted older machinery with energy-efficient features, increasing operational performance without requiring full replacements (Dubey et al., 2020). Wu et al. (2021) confirm that extending equipment lifecycles aligns with sustainable manufacturing standards.

Nevertheless, machinery reuse presents logistical challenges. Respondent 6 explained, "*Not all equipment components are in a reusable condition, requiring additional effort to assess and refurbish parts*." Predictive maintenance programs and partnerships with equipment suppliers are suggested to address these challenges (Jabbour et al., 2020; Kumar et al., 2022).

3.1.3 By-product Utilisation

Hayes Tea Estate actively converts tea processing by-products into compost, biofuels, and secondary products. Respondent 2 stated, "*Tea dust and steam that would otherwise be discarded are processed into compost*." This initiative reduces waste and enhances soil health, promoting sustainable farming (Singh & Trivedi, 2020; Sarkar et al., 2021).

Additionally, repurposed tea dust and stems are used in lower-grade tea blends, reducing product loss (Subramanian & Gunasekaran, 2021). Respondent 5 noted, "*Lower-grade tea blends are produced using repurposed tea dust and stems, reducing overall wastage*." These practices support circular economy goals (Ellen MacArthur Foundation, 2020).

Yet, composting presents technical hurdles. Respondent 7 explained, "*Maintaining the right balance of by-products for composting can be difficult*." Composting performance depends on managing organic ratios, microbial activity, and moisture levels (Zhu et al., 2021; Sarkis et al., 2021). Collaborative networks and controlled composting technologies are recommended for improvement (Govindan et al., 2022).

3.2 Reduce

3.2.1 Energy Reduction

Energy conservation is central to Hayes Tea Estate's sustainability strategy. Respondent 1 reported, "*We installed energy-efficient machinery that operates at optimal power levels*." These upgrades reduce operational costs and carbon emissions (Jabbour et al., 2020; Govindan et al., 2022).

Solar panels have also been installed to reduce grid dependency. Respondent 3 stated, "*We have installed solar panels on some buildings to reduce reliance on grid electricity*." However, older machinery continues to impede total energy efficiency. As Respondent 7 noted, "*While new machinery reduces energy consumption, some older equipment still operates inefficiently*." Zhu et al. (2021) found that full technological conversion requires significant financial investment.

3.2.2 Water Conservation

Water management is critical due to the tea industry's reliance on irrigation. Rainwater harvesting and drip irrigation are employed to optimize water use. Respondent 4 stated, "*Rainwater harvesting systems have significantly reduced our reliance on groundwater*."

Research by Subramanian and Gunasekaran (2021) supports these approaches for improving water efficiency and reducing operating costs. However, storage limitations remain. Respondent 6 shared, "*Storage capacity remains a limitation during prolonged dry periods*." Infrastructure upgrades and wastewater reuse are suggested to improve resilience (Govindan et al., 2022; Sarkis et al., 2021).

3.2.3 Reduction in Chemical Usage

Hayes has transitioned to organic fertilisers and integrated pest management (IPM), reducing reliance on synthetic chemicals. Respondent 5 commented, "*Switching to organic fertilisers has significantly improved soil health and crop yields*." Martinez-Falcó et al. (2023) highlight how such practices enhance biodiversity and nutrient retention.

Composting supports this initiative, creating natural fertilisers from organic waste (Sharma et al., 2021). While effective, this transition faces challenges such as delayed results and higher costs. Respondent 8 noted, "*Organic fertilisers take longer to show results, making the transition difficult*." Zhu et al. (2021) and Govindan et al. (2022) recommend planning support and government incentives for implementation.

3.3 Recycle

3.3.1 Waste Management and Recycling

Hayes employs structured waste segregation and collaborates with external recycling firms. Respondent 6 shared, "*We have a structured waste segregation system in place... to facilitate efficient recycling*." External partnerships have proven effective in waste diversion (Jabbour et al., 2020).

Organic waste is composted on-site, contributing to fertilizer production and landfill reduction (Sarkar et al., 2021). Respondent 1 stated, "*We compost tea dust and stems to produce organic fertilizer*."

Despite success, material degradation poses reuse challenges. Respondent 8 stated, "*Recycled sacks become weak over time, forcing us to purchase new ones*." Proper employee training is also essential for sorting and recycling effectiveness (Jabbour et al., 2020).

3.3.2 Cost Savings from Recycling

Recycling initiatives at Hayes have resulted in significant cost reductions. Respondent 1 reported that packaging reuse has lowered procurement costs, consistent with findings by Subramanian and Gunasekaran (2021). Composting reduces fertiliser costs and enhances soil health (Sharma et al., 2021).

Recycling has also enhanced the estate's market image. Wu et al. (2021) found that sustainability attracts environmentally conscious consumers. However, Respondent 7 noted, "*Recycling demands investment in equipment and training*." Zhu et al. (2021) and Sarkis et al. (2021) stress the importance of infrastructure and policy support.

3.4 Decomposition

3.4.1 Composting Practices

Hayes operates dedicated composting pits for organic waste decomposition. Respondent 10 explained, "*We have dedicated composting pits where we decompose organic waste under controlled conditions*." This reduces methane emissions and supports sustainable agriculture (Sarkar et al., 2021; Sharma et al., 2021).

3.4.2 Challenges in Decomposition

Respondent 8 pointed out, "*If the ratio of green to brown matter is not maintained, composting slows down.*" Composting performance relies on maintaining ideal material ratios and environmental conditions (Gupta et al., 2022). Cold weather can further delay decomposition (Dubey et al., 2020).

3.5 Organisational Performance

3.5.1 Operational Performance

GSCM has enhanced operational efficiency through logistics optimization, waste reduction, and energy-efficient systems. Respondent 3 shared, "*Logistics optimisation and packaging waste reduction allowed the team to enhance supply chain operations*."

Digital tracking systems have also improved resource monitoring. Respondent 7 noted, "*The adoption of digital tracking systems allows us*

to monitor waste management in real-time." Govindan et al. (2022) link these technologies to improved supply chain transparency and agility.

3.5.2 Cost Reduction

Cost savings have been realized across multiple areas. Respondent 1 stated, "*Switching to energy-efficient equipment has significantly reduced our electricity bills*." Similarly, organic fertiliser usage has decreased dependency on synthetic inputs (Sarkis et al., 2021).

However, expansion of these initiatives requires funding. Respondent 3 noted, "*We need additional funding to expand our renewable energy projects and waste management systems*." Public-private partnerships and sustainability grants are recommended (Zhu et al., 2021).

4. Conclusion

Strengthening Reuse Practices for Weak Packaging Durability

Hayes Tea Estate should invest in high-quality, reusable packaging materials to enhance resource efficiency. Improving the durability of packaging materials will extend their lifespan, reduce maintenance costs, and minimize waste output (Govindan et al., 2022). The estate should also develop predictive maintenance systems for equipment refurbishment programs to maximize efficiency. By repairing tools before they reach their operational limits, the estate can reduce the frequency of new purchases and optimize equipment management (Dubey et al., 2020). Additionally, the estate should expand by-product utilization by enhancing composting operations and finding alternative uses for tea dust and stems. This strategy will improve resource utilization and lower disposal costs (Singh & Trivedi, 2020).

Enhancing Reduction Strategies for High Dependence on Non-Renewable Energy and Inefficient Water Management

To decrease reliance on non-renewable energy sources, Hayes Tea Estate should increase investments in renewable energy, particularly by expanding solar panel installations and exploring the use of renewable biofuels. Long-term investments in renewable energy, combined with cost-reduction strategies, are projected to yield significant benefits (Zhu et al., 2021). The estate should also improve water conservation by expanding rainwater harvesting facilities and adopting advanced irrigation systems. Implementing these measures can promote sustainable water usage and reduce drought-related risks (Sarkis et al., 2021). Furthermore, employee training programs should be enhanced to educate workers on sustainable farming techniques and the principles of green supply chain management (GSCM) (Jabbour et al., 2020).

Optimizing Recycling Efforts for Poor Waste Segregation Practices

Effective waste management at Hayes Tea Estate requires improved segregation at the source, which can be achieved through employee awareness initiatives and the introduction of color-coded bins. These efforts can lead to enhanced recycling outcomes and proper waste disposal practices (Chen et al., 2020). Strengthening partnerships with recycling companies will also help improve the estate's capacity to process and dispose of non-biodegradable waste appropriately (Subramanian & Gunasekaran, 2021). A comprehensive waste management system that recycles materials internally will further support sustainability goals (Sharma et al., 2021).

Advancing Decomposition Practices for Inefficient Composting Methods

Investments in controlled composting facilities will result in more efficient decomposition processes and improved soil quality. Upgraded facilities can create optimal conditions for the decomposition of organic waste, thereby producing higher-quality compost (Gupta et al., 2022). Compost quality can also be improved by systematically tracking the ratios of green and brown waste. Adopting this method enhances the efficiency of decomposition and reduces overall operational waste (Sarkar et al., 2021). Additionally, the implementation of incentive programs can encourage farmers throughout the estate to participate in composting and organic farming. These strategic initiatives not only increase employee

engagement but also promote sustainable agricultural practices (Govindan et al., 2022).

Implementing these strategic recommendations will enable Hayes Tea Estate to enhance its sustainable practices, achieve greater cost savings, and strengthen its competitive market position. Future research should aim to expand these agricultural sustainability initiatives across all tea plantations to facilitate the development of standardized GSCM practices within the industry.

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The Challenges for Sri Lankan SMEs in Entering the Clothing Industry Export Market

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Abstract

Research on small and medium-sized enterprises (SMEs) within the apparel sector has extensively explored their roles and contributions. However, there remains a notable gap in the literature concerning the specific challenges faced by Sri Lankan apparel SMEs when entering export markets. This study seeks to address this gap by examining how these enterprises navigate and sustain themselves within the industry, with particular emphasis on the barriers to export entry, the interrelationships among these challenges, and the identification of the most critical obstacles. The research identified four key thematic challenges: limited financial capacity, trade regulations and barriers, insufficient internal capabilities, and inadequate marketing knowledge. A purposive sampling strategy was employed, and data were collected through qualitative interviews with eight participants actively engaged in the SME apparel industry in Sri Lanka's Western Province. Thematic analysis was used to interpret the data and uncover recurring patterns. Findings indicate that financial constraints represent the most significant impediment to international expansion. Trade and regulatory barriers also exert considerable influence. Internally, managerial knowledge gaps and worker absenteeism were highlighted as major limitations, while marketing knowledge appeared to be the least prioritized area among Sri Lankan apparel SMEs. Based on these insights, the study offers practical recommendations for both SMEs and policymakers, including the development of targeted marketing strategies and the creation of a more supportive ecosystem to facilitate SME growth and export readiness.

Keywords: SMEs, Export Market, Clothing Industry

1. Introduction

1.1 Background of the study

Small and medium-sized enterprises (SMEs) constitute the backbone of the global economy, accounting for approximately 90% of all businesses and nearly 50% of global employment. In developing countries, SMEs are responsible for creating seven out of every ten formal jobs. Officially recognized SMEs contribute up to 40% of GDP in these regions, and when informal enterprises are included, this figure increases significantly. Projections indicate that 600 million new jobs will be required by 2030 to accommodate the expanding global workforce.

In Sri Lanka, SMEs contribute over 52% of the national GDP. However, their participation in international trade remains limited. The World Trade Report (2016), based on data from 25,000 SMEs in developing countries, revealed that manufacturing and service SMEs contribute only 8.5% to total export revenues, compared to 34% in developed economies (Lakshman, Kumarasinghe, & Weerasinghe, 2023). In 2022, Sri Lanka's total exports reached USD 14.8 billion, ranking it as the 89th largest exporter globally, with an increase of USD 2.24 billion since 2017 (OEC, 2024). The apparel sector, in particular, stands out as the country's second-largest export industry and a key driver of export growth (OEC, 2024).

Despite this, SMEs in Sri Lanka contribute only around 5% to total exports, a figure significantly lower than that of other Asian economies such as China, where SME export participation exceeds 40% (Lakshman et al., 2023). This disparity highlights Sri Lanka's underperformance in integrating SMEs into global trade, particularly when compared to other emerging economies in the region.

The limited involvement of SMEs in exports is attributed to a range of internal challenges, including limited motivation to export, inadequate technological capabilities, low risk tolerance, a shortage of skilled labor, financial constraints, and limited firm size and experience. Additionally, external barriers such as fluctuating market demand, regulatory complexities, global competition, environmental uncertainty, and restricted access to information and resources further hinder SME export participation (Lakshman et al., 2023).

1.2 Industry Overview

This study focuses on the apparel industry, which accounts for 39.74% of Sri Lanka's total export earnings and employs approximately 15% of the country's industrial workforce (Export Development Board [EDB], 2024). The SME sector was selected due to its central role in the national economy, comprising 75% of all enterprises and contributing 20% to total exports, 45% to employment, and 52% to GDP (Alles, 2023).

1.3 Research Problem

Sri Lankan SMEs are currently facing significant challenges and are widely regarded as among the least prepared for an uncertain future (Alles, 2023). One of the most pressing issues is limited access to finance. SMEs are often denied bank loans and must rely on internal resources or family funding for initial capital.

Recent fiscal policy changes have further exacerbated these difficulties. The corporate tax rate has increased to 30%, affecting SMEs with annual earnings below LKR 500 million, including those engaged in export activities (PWC, 2023). This tax burden reduces profitability and limits reinvestment capacity, thereby hindering efforts to expand production, innovate, improve product quality, and invest in marketing and branding (Dayarathna et al., 2018).

Given these constraints, it is essential to investigate the specific challenges faced by SMEs in the apparel sector as they attempt to enter and sustain operations in the export market.

1.4 Rationale

Existing literature has paid insufficient attention to the distinct factors influencing SME export entry, as opposed to post-entry performance. Haddoud, Onjewu, Nowinski, and Jones (2021) argue that international business research often fails to differentiate between the determinants of initial market entry and those affecting long-term survival. Furthermore, Kasema (2022) notes that most export-related studies focus on firms in developed economies, overlooking the unique challenges faced by SMEs in developing countries. These include market analysis, location selection, timing of entry, and resource limitations.

In the Sri Lankan context, SMEs in the apparel industry face several pressing challenges, including a shortage of skilled labor, resource constraints, regulatory barriers, and financial limitations. Maintaining a seamless production process is particularly difficult due to high employee turnover, as workers frequently leave in search of better-paying opportunities. These challenges are exacerbated by the country's current economic instability.

Addressing these gaps is essential to support the growth and sustainability of Sri Lankan SMEs in the global marketplace.

1.5 Research Aim

This study aims to examine the challenges faced by Sri Lankan SMEs in entering the export market, with a specific focus on the clothing industry.

1.6 Scope

This research is confined to SMEs operating within Sri Lanka's clothing industry. Data will be collected from a sample of 10 interviewees. While the findings will provide valuable insights into the apparel sector, they may not be generalizable to the broader SME landscape either within Sri Lanka or internationally.

1.7 Significance

This study offers valuable insights for policymakers seeking to design targeted support programs for SMEs and make informed investment decisions. For SME owners and managers, the findings will facilitate proactive planning, informed decision-making, and collaborative problem-solving to enhance export readiness.

Understanding the barriers faced by Sri Lankan apparel SMEs is crucial for improving competitiveness, fostering innovation, and diversifying the industry. By addressing these challenges, the study aims to increase SME participation in international trade, thereby contributing to economic growth and job creation.

Given the strategic importance of the apparel sector and the intensifying global competition, this research can help SMEs tap into underexplored markets and strengthen their position in the global value chain.

2. Methodology

2.1 Research Design

2.2 Research Approach

This study adopts an inductive research approach, which is well-suited for exploring complex, context-specific phenomena. By analyzing qualitative data collected through in-depth interviews, the study seeks to identify recurring patterns and emergent themes that reflect the lived experiences of participants (Azungah, 2018). This approach allows for theory development grounded in empirical evidence rather than testing pre-existing hypotheses.

2.3 Research Method

A qualitative research method has been employed to explore the challenges faced by Sri Lankan SMEs in the clothing industry when entering the export market. This method facilitates a comprehensive understanding of participants' real-life experiences. Specifically, semi-structured interviews were chosen to allow for both consistency across interviews and the flexibility to probe deeper into individual perspectives, thereby enriching the data collected.

2.4 Sample

The study involved a purposive sample of 10 participants, including SME owners, employers, and directors within the Sri Lankan clothing industry.

2.5 Data Collection Methods

The primary data collection technique for this study is semi-structured interviews, which are considered more effective than other interview formats for eliciting rich, detailed responses (Ruslin, Mashuri, Rasak, Alhabsyi, & Syam, 2022). This method provides a balance between structure and flexibility, enabling the researcher to explore key themes while allowing participants the freedom to express their views in depth. An interview guide comprising open-ended questions was used to facilitate discussion and encourage participants to share their personal experiences and insights.

2.5 Data Analysis

The data was analyzed using thematic analysis, a widely recognized method in qualitative research for identifying, analyzing, and interpreting patterns within textual data (Naeem, Ozuem, Howell, & Ranfagni, 2023). This systematic approach ensures that the findings are grounded in the data and provide meaningful interpretations of the challenges faced by SMEs.

3. Analysis and Findings

The analysis draws upon thematic patterns identified in the literature and reinforced through empirical evidence gathered during the interviews.

The sample was purposefully selected to include individuals actively engaged in the Sri Lankan apparel industry, ensuring relevance and depth in understanding the challenges faced by SMEs in this sector.

3.1 Lack of Financial Capabilities

Financial constraints emerged as a dominant theme across the interviews. As noted by Dubey and Das (2022), financial resources are a critical competitive advantage for SMEs, yet many lack the necessary capital to support

business operations and international expansion. Participants consistently emphasized the importance of financial strength in areas such as procuring raw materials, upgrading machinery to meet export standards, and investing in innovation to access new markets.

Participant P2 remarked:

"If I plan for the April summer season, I should have the necessary raw materials ready by January. That's a big challenge - it's costly. We need to be ready to spend immediately, and we cannot afford delays. It's actually difficult."

Similarly, P7 highlighted:

"We face many financial issues, especially in acquiring machinery suitable for export-quality production. Our current equipment does not meet the required standards."

These observations align with Paplikar (2023), who underscores the role of financial resources in enabling access to strategic investments, product development, and market expansion. Participant P2 further elaborated:

"Seasonality is crucial for international market reach. We must offer fresh innovations and timeless fashion. To do this, we need adequate human capital, financial resources, and material availability."

The collective responses indicate that financial limitations are a significant barrier to internationalization for Sri Lankan clothing SMEs. Most participants expressed difficulties in securing funding for various operational needs.

SMEs often rely on personal or familial financial support due to limited access to formal credit channels. This was echoed by several participants who shared their experiences with Sri Lankan banks.

P8 explained:

"When I started this business, no one supported me. I presented my proposal to the bank, but they rejected it. They said I needed a minimum income of LKR 5 million to qualify for a loan. They don't support SMEs at the inception stage."

P4 added:

"I've obtained several loans, but the process is extremely time-consuming. Some approvals took up to one and a half years. Even small loans require extensive documentation and long waiting periods."

P3 described a similar experience:

"Due to financial issues, we collaborated with other companies to fulfill bulk orders. We've had some exposure to exports, but not directly. I approached banks and financial institutions, but the process is exhausting. The paperwork is extensive, and even after submission, it takes months to get basic information like interest rates. In some cases, we waited nearly a year for a decision. It's not a pleasant experience."

Interestingly, P1 offered a contrasting perspective:

"I'm a bit cautious about loans. We built the business through bootstrapping - reinvesting profits slowly over time."

P1's approach reflects a preference for self-financing and aligns with Gunatilake (2016), who found that a lack of understanding of banking procedures can hinder SME growth and expansion.

3.2 Trade Regulations and Barriers

The Sri Lankan apparel industry has faced considerable operational challenges due to recent changes in trade policy. According to the International Trade Administration (2022), the imposition of a 15% Value Added Tax (VAT) on imported textiles and a 5% VAT on imported fabrics has led to significant price increases. This policy shift has directly impacted the cost structure of SMEs operating in the sector. Six out of eight participants in this study reported substantial hikes in raw material costs as a result of the newly introduced VAT.

Participant P7 noted:

"VAT issues do not directly affect our company, but when we source raw materials locally, we observe price increases because those materials are subject to VAT."

Similarly, P5 stated:

"Raw material costs have risen dramatically. However, we are constrained to sell at current market prices. If we export, we must absorb the additional costs. VAT is causing financial strain across the industry."

Ranasinghe (2023) warns that rising taxes and electricity tariffs may lead to the closure of many small businesses in export-oriented sectors. Specifically, SMEs are at risk due to a 15% to 30% increase in export revenue tax and a 65% rise in electricity costs. These findings were corroborated during the participant recruitment phase, where the researcher observed that several clothing SMEs had already ceased operations due to escalating costs of materials and utilities.

Participant P6 shared:

"Raw materials are increasingly scarce. We face issues with colour matching and quantity. For instance, matching lace with the same colour thread has become difficult. There are also other challenges related to limited imported supplies in the domestic market."

P6's remarks highlight the difficulties in sourcing essential accessories for production. These challenges align with findings from Economy Next (2022), which reported that Sri Lankan apparel manufacturers face delays in raw material shipments due to currency shortages, import clearance delays, and port congestion.

The evidence presented suggests that trade regulations and logistical barriers significantly hinder Sri Lankan clothing SMEs' ability to access necessary inputs. A supportive regulatory environment is therefore essential to foster export growth and enhance local competitiveness. Such an environment should include streamlined procedures for international market entry, legislative stability, and the implementation of innovative, export-oriented programs tailored to SME needs.

Participant P2 expressed concerns regarding institutional support:

"There is a lack of support. For example, large-scale businesses can easily find export buyers through the Export Development Board. But for small-scale businesses like ours, unless we have personal contacts, we must visit the Board, submit our profiles, and explain our products. While they do offer some assistance, the process is slow and cumbersome."

P2's experience reflects the findings of the Asian Development Bank (ADB, 2019), which identified institutional inefficiencies as a barrier to SME export readiness.

Logistics companies increasingly rely on advanced technologies and rapid adaptation. SMEs, however, often struggle to keep pace with these developments, particularly in establishing e-commerce platforms and negotiating competitive rates with third-party logistics providers.

Participant P1 elaborated:

"Shipping is a major challenge. Using local shippers or third-party services like DHL or Aramex is very expensive—about 20 to 25 dollars per 900 grams. We occasionally ship abroad to customers who have migrated, but the cost is high. Even third-party rates are steep, and we must account for documentation weight. The entire supply chain is costly and complex."

These insights collectively illustrate how trade regulations, institutional inefficiencies, and logistical constraints pose significant barriers to export participation for Sri Lankan clothing SMEs.

3.3 Lack of Internal Capabilities

Safari and Saleh (2020) argue that the organisational decision-making structure plays a pivotal role in SME internationalisation, with managerial and psychological constraints often emerging as significant barriers to entering foreign markets. Consistent with this view, several participants in this study, as well as the researcher's observations, identified a lack of market entry knowledge as a major obstacle for Sri Lankan apparel SMEs seeking to engage in export activities.

Participant P8 stated:

"It's the knowledge. People don't understand how to get started or where to begin. They lack basic knowledge and the initial steps. There are professionals for that. It's important to reach out and acquire fundamental knowledge first."

This highlights the importance of understanding foundational procedures before initiating export operations. Similarly, Madushanka and Sachitra (2021) found that SME export entry and performance are significantly influenced by managerial mindset, including attitudes toward risk, market knowledge, commitment, and awareness of export challenges.

Participant P1 shared:

"We haven't pursued any foreign orders, to be honest. My objective is to build the brand." "I'm a marketer, but I tend to be risk-averse. Marketers usually take risks and move forward, but I'm cautious."

These responses suggest that leadership mindset and strategic priorities significantly influence export decisions. Participant P4 also noted that limited knowledge of export procedures affects SMEs' ability to enter international markets.

In addition to knowledge gaps, workforce-related challenges were frequently cited. Chandra et al. (2020) emphasize that an effective workforce is essential for maintaining competitiveness in export operations. However, developing countries often face difficulties in attracting, training, and retaining skilled labor. All participants in this study reported that absenteeism and high turnover rates among workers have severely disrupted daily operations.

Participant P3 explained:

"I work with a mostly semi-skilled local workforce. The machinery we use requires less technical expertise than in competitive markets. I try to manage production with proper timing and technique, but frequent absenteeism, mostly due to personal reasons, makes optimization difficult."

Chandra et al. (2020) further note that shortages in production, marketing, logistics, and compliance staff can directly impact the internal functioning of apparel firms.

P3 added:

"When skilled workers don't show up, it affects daily output. Over a month, this becomes a major issue in meeting delivery deadlines. No buyer wants to work with a supplier who consistently delays orders."

This illustrates how persistent absenteeism can reduce production efficiency and jeopardize client relationships. The Sri Lankan garment sector predominantly employs female workers, 70% of whom often become inactive due to factors such as marriage, maternity leave, and childcare responsibilities.

Participant P1 elaborated:

"Absenteeism is a problem in our factory. Most of our workers are women, so when their children fall ill or have exams, they miss work. It's definitely an issue in the blue-collar category. In contrast, our office staff, mostly young people, so they don't pose such problems. The office feels like a family, not in terms of relatives, but in terms of culture."

This underscores the gendered nature of workforce challenges in the apparel sector, particularly in production environments.

Moreover, Safari and Saleh (2020) argue that poor leadership skills exacerbate internal organisational issues, including inefficient management practices, weak decision-making capabilities, and internal corruption. In contrast, transparent and inclusive leadership can mitigate such challenges.

Participant P8 described their approach:

"We hold monthly team meetings to discuss any issues and provide solutions. We maintain transparency with the team. We don't hide problems; we address them together. When management and staff are aligned, issues don't escalate."

This aligns with Hofmann and Strobel (2020), who emphasize that internal transparency enhances employee satisfaction by facilitating access to information and encouraging feedback.

3.4 Lack of Marketing Knowledge

Abubakari et al. (2022) argue that successful export marketing outcomes for SMEs depend not only on marketing knowledge, but also on the ability to effectively apply that knowledge through relevant competencies. This was reflected in the responses of participants P1 and

P2, who emphasized the importance of cultural sensitivity in developing overseas marketing campaigns and tailoring products to resonate with international audiences.

Participant P1 noted:

"Creativity is key. For example, someone in Europe or the USA might not relate to a model who looks Sri Lankan or Indian. You may need to use the right person for the campaign."

This aligns with Abubakari et al. (2022), who highlight the importance of cultural considerations in selecting marketing visuals that appeal to target markets. Similarly, Madushanka and Sachitra (2021) found that successful local marketing campaigns can indirectly attract international orders, thereby facilitating export entry.

Participant P2 shared:

"I haven't specifically targeted overseas customers in my campaigns. I currently boost posts targeting a Sri Lankan audience. However, I still receive inquiries from abroad, from India, from the UK, because people are watching. But I haven't responded to those requests yet. When they ask for product catalogs, I can't proceed because we don't specialize in seasonal manufacturing for international markets."

This illustrates how even domestically focused marketing can generate international interest, though a lack of readiness and strategic planning may prevent SMEs from capitalizing on such opportunities.

Davcik et al. (2021) emphasize that marketing capacity is shaped by an organisation's strategic planning and the resources available to conduct market research and implement distribution strategies. Participant P3 reflected on the impact of resource constraints:

"I currently handle marketing myself. I have the experience and qualifications, and I've been doing it for the past three years. We had a dedicated marketing person, but due to COVID and the market downturn, we had to eliminate that position."

This supports Davcik et al.'s (2021) assertion that the absence of a structured marketing plan and adequate funding can hinder the implementation of new services and the retention of skilled professionals. Only a few participants in this study had experience with marketing campaigns, and even fewer operated e-commerce platforms.

Madushanka and Sachitra (2021) also report that most Sri Lankan clothing SMEs struggle to develop e-commerce capabilities, despite the potential to reach broader local and international audiences.

4. Conclusion

This study identified several critical barriers hindering the internationalisation of Sri Lankan clothing SMEs. Among these, financial limitations emerged as the most significant, with participants consistently citing difficulties in securing adequate funding for essential export-related activities such as raw material procurement, hiring skilled professionals, investing in marketing, and upgrading machinery.

Marketing capabilities were the least emphasized theme among participants, revealing a potential gap in marketing knowledge, experience, and strategic commitment. This deficiency may represent a substantial obstacle for SMEs attempting to navigate and compete in international markets.

The findings underscore the importance of addressing both structural and capability-based challenges to support the successful internationalisation of SMEs. This research contributes to a broader understanding of the export entry process and offers practical insights for policy development and SME support initiatives.

5. Recommendations

SME decision-makers should adopt robust financial management strategies to improve working capital management, capital structuring, and accounting systems. This will strengthen their financial resilience and readiness for export activities.

Digital platforms offer cost-effective avenues for reaching global audiences. As of early 2024, Sri Lanka had 12.34 million internet users (56.3% penetration) and 7.5 million social media users (34.2% of the population) (Kemp, 2024). With over five billion global social

media users expected to rise to six billion by 2028, SMEs should invest in consistent digital marketing campaigns across platforms like Facebook and Instagram. Organic content and paid partnerships can significantly expand brand visibility and customer reach.

Effective management is essential for organisational success and competitiveness. According to Orji, Emmanuel, and Egwuatu (2023), rare, valuable, inimitable, and non-substitutable (VRIN) resources—such as managerial skills—can provide SMEs with a sustainable competitive advantage. Training in digital marketing, employee development, and strategic planning can enhance SME performance and long-term viability.

Trade authorities should introduce supportive regulations and initiatives tailored to the needs of export-oriented SMEs. These may include financial assistance programs, simplified export procedures, and capacity-building workshops. Government-led training sessions on export readiness, digital marketing, and international compliance standards could empower SMEs to compete more effectively in global markets.

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