Assessment of Bacterial Contamination in Flaxseed and Their Antibiotic Resistance

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of the Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
November, 2024

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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of Fall, 2024 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on December 19, 2024.

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Ethics Statement

For the completion of this study, samples from selected venues were collected following all the necessary precautions. All the experiments were done in the BRAC University Laboratory. It should also be noted that no animal or human models were used or harmed in this study.

Abstract

The study was on the microbial contamination of flaxseed in Dhaka City from October 2023 to June 2024 aimed to identify the presence of harmful microorganisms. Flaxseed were collected from local markets and grocery stores. 56 samples were analyzed in a laboratory by maintaining the standard protocol to determine the presence of targeted bacteria, *Escherichia coli, Pseudomonas aeruginosa, klebsiella pneumonia, Staphylococcus aureus,* and their antimicrobial resistance level. The study's findings revealed that 87.5% of the flaxseed samples showed signs of microbial contamination, raising concerns about the potential health risks of consuming flaxseed. Among the isolated organism 28.57% *Escherichia coli,* 22.45% *Pseudomonas aeruginosa,* 18.37% *Klebsiella pneumoniae* and 30.61% *Staphylococcus aureus.* Multidrug-resistant isolates included 64.29% of *E. Coli*, and 36.36% of *P. aeruginosa,* Of the *S. aureus* isolates, none (0%) were multidrug-resistant, however all (100%) of the *K. pneumoniae* isolates were. While it is positive that *S. aureus* did not exhibit multidrug resistance, the high prevalence of this bacteria still poses a significant risk for foodborne illnesses. Additionally, the fact that *K. pneumoniae* had a lower number of isolates did not diminish the severity of its 100% multidrug resistance. It was particularly alarming and indicated the need to overcome the situation by continuing further research and developing new antibiotics to combat these resistant microbial organisms.

Keywords: Flaxseed; Multidrug resistance; *Escherichia coli; Pseudomonas aeruginosa; Klebsiella pneumoniae; Staphylococcus aureus.*

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Jannatun Fabiha (19336014)

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List of Acronyms

E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
K. pneumoniae	*Klebsiella pneumoniae*
S. aureus	*Staphylococcus aureus*
MAC	MacConkey
MSA	Mannitol salt agar
EMB	Eosin Methylene Blue
MHA	Mueller Hinton agar
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
AST	Antibiotic Susceptibility Test
CLSI	Clinical and Laboratory Standards Institute
MDR	Multidrug resistance

Chapter 1

1.1 Introduction:

Herbal seeds are extensively used in traditional and organic health care and skin care formulations. However, consumers often display a concerning lack of awareness and consideration regarding the potential risks of microbial contamination associated with these herbal plant seeds.

1.2 Background:

The popularity of herbal seeds has surged in recent years, many consumers under the belief that these are inherently safer and more beneficial for their skin and health (Geng, 2023) (Corazza et al., 2009). However, this widespread trust in herbal plant seeds may be misplaced, as these natural ingredients can pose significant risks of microbial contamination that are often overlooked (Turcov et al., 2022) (Rubin & Brod, 2019). The production and manipulation processes of herbal seeds are not immune to microbial contamination, which can be introduced through various environmental factors and inadequate hygienic practices employed during their cultivation, harvesting, and processing. While consumers may assume that natural products are inherently free from harmful microorganisms, the reality is that insufficient quality control measures throughout the supply chain can lead to the presence of pathogenic bacteria in the final products, posing serious health risks to users (Araujo & Maria, 2012).

1.3 Contamination in flaxseed

Flaxseed has been highly regarded for people's health including as a superfood. Preliminary studies show that they may help fight heart disease, stroke, diabetes, and breast cancer. (Vaisey-Genser, n.d) In the nutrition world, it is considered a staple and renowned for being an excellent source of healthy omega-3 fatty acids, lignans, and fiber. A 2010 Canadian Journal of Cardiology study showed that the greatest use of omega-3 fatty acids is an excellent nutritional therapy that will demonstrate cardiovascular benefits in the long run. (The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid, 2010) The study also showed that dietary flaxseed has a

cardio-proactive effect by improving vascular relaxation responses. Flaxseed is often promoted as a nutritional aid it can boost your fiber intake and add a bit of protein but not a sustainable amount. Flaxseed prevents constipation. Besides, soluble fiber in flaxseed dissolves easily in water, softening stools and increasing gut motility. This soluble fiber flexes the intestinal walls and triggers a contractionary increase in bowel bulk ultimately improving digestion. The soluble fiber content of flaxseed gives it a lower blood sugar property. (Vaisey-Genser, n.d) It balances digestion and hence the release of glucose into the bloodstream. Moreover, flaxseed is a rich known source of lignans. Lignans have antioxidant and phytoestrogen both of those properties reduce the risk of breast cancer and improve health. Another hidden power of flaxseed is its ability to control cholesterol levels, a heart disease biomarker. Flaxseed has also been proven to lower stroke risk due to its plant-based ala fatty acids as an end product. (Bloedon et al., 2008)

Flaxseeds are bursting with nutritional goodness. It is a versatile ingredient; you can have it in whole or ground or opt for flaxseed oil. Despite its numerous health benefits, the microbial quality of flaxseed remains a significant concern, particularly in developing nations like Bangladesh, where agricultural and processing practices may introduce various microbial contaminants (Hossain et al., 2019) (Khairuzzaman et al., 2014) (Akond et al., 2013).

The increasing demand for nutritionally-enhanced food products has made flaxseed a highly sought-after ingredient, as it is a rich source of the essential omega-3 fatty acid alpha-linolenic acid, which has been associated with various health benefits (Kabašinskienė et al., 2015). However, the microbial quality of flaxseed can be influenced by a range of factors during its production and processing stages, which is crucial to ensure the safety and stability of the final products. The pre-harvest stage of flaxseed production is a critical juncture where the crop's exposure to various environmental factors can introduce microbial contaminants. Factors such as the composition and quality of the soil, the presence of animal excrement in the cultivation area, and the microbiological status of the irrigation water used can all contribute to the introduction of undesirable microorganisms that may subsequently colonize the flaxseed (Gollop et al., 2024). Similarly, the subsequent handling and processing of the harvested flaxseed can also serve as a vector for additional microbial contamination.

Indeed, the various post-harvest operations, including the collection, classification, packaging, and transportation of the flaxseed, can provide additional opportunities to introduce new microbial

agents that may further compromise the product's microbiological quality. Improper storage conditions, such as high humidity or temperature, can also lead to the proliferation of undesirable microbes.

1.4 Food and seeds mediated Outbreak

Escherichia coli is a rod-shaped, Gram-negative bacterium in the Enterobacteriaceae family. They commonly inhabit the lower intestinal tract of warm-blooded animals, including humans. *E. coli* is frequently released into the environment through feces or wastewater effluent. (*Escherichia coli* Infection, 2022). Recent research suggests *E. coli* can survive and reproduce outside the intestinal tract, challenging its use as a sole indicator of fecal contamination. (Ishii & Sadowsky, 2008*). E. coli* populations are affected by ambient environmental conditions, influencing their long-term survival. Different strains of *Escherichia coli* are found in soil, manure, and water, indicating the bacterium can persist in terrestrial and aquatic habitats. The 1996 *Escherichia coli* O157:H7 outbreak in Japan highlighted the risks of foodborne illnesses arising from contaminated products. Similarly, in February 2003, *E. coli* O157 infections affected individuals in Minnesota and Colorado. Shiga toxin-producing *Escherichia coli* outbreaks associated with ready-to-eat salads have been reported since 1995. More recently, in 2011, a severe outbreak occurred in Germany, where 3,816 people were infected with STEC O104:H4, and 845 patients developed hemolytic uremic syndrome, resulting in 54 deaths. The responsible strain was characterized by its high pathogenic potential, affecting diverse populations, including children, the elderly, and healthy adults. *Pseudomonas aeruginosa* exhibits high intrinsic and acquired resistance mechanisms that enable it to counter most antibiotics. Treatment of *P. aeruginosa* infections has become a significant challenge due to the bacterium's ability to resist many currently available antibiotics. (Lin et al., 2018) This pathogen exhibits resistance to various classes of antibiotics, including aminoglycosides, quinolones, and β-lactams. Its outer membrane acts as a selective barrier that restricts antibiotic penetration, with a permeability about 12- to 100-fold lower than that of *E. coli.* Intrinsic resistance in *P. aeruginosa* is primarily attributed to the production of antibiotic-inactivating enzymes. The Aminoglycoside family of antibiotics inhibits bacterial protein synthesis by binding to ribosomal 30S subunits. (Galbraith et al., 1984) However, aminoglycoside-

modifying enzymes can be produced to deactivate the drug. Aminoglycosides target ribosomes and undergo mutations to prevent drug binding, leading to reduced accumulation of the drug within the cell. These specific enzymes can be produced by a single bacterial strain or encoded on a single plasmid. Low levels of aminoglycoside activity, barely detectable by common assays, may still confer clinically significant aminoglycoside resistance. (Zembower et al., 1998) Mutations affecting ribosomal structure are more valuable as tools to understand bacterial physiology than as causes of clinically important antibiotic resistance. The "small colony" variant of *P. aeruginosa* is a common mutation that confers aminoglycoside resistance. *Pseudomonas* mutants often have mutations affecting cellular energy systems, such as deficient cytochromes and nitrite reductase. Quinolone drugs interfere with bacterial topoisomerases, enzymes that nick and uncoil DNA helices during DNA replication. (Drlica et al., 2007) Resistance to quinolones does not develop rapidly through a single-step mutation. β-lactam antibiotics block bacterial cell wall biosynthesis by targeting penicillin-binding proteins involved in peptidoglycan synthesis. Polymyxins, a group of polypeptide antibiotics, bind to lipopolysaccharides on the outer membrane of Gram-negative bacteria, increasing cell membrane permeability and enhancing antibiotic uptake. However, *P. aeruginosa* produces β-lactamases and aminoglycoside-modifying enzymes, which can break down or modify these antibiotics. *P. aeruginosa* also has an inducible ampC gene encoding the hydrolytic enzyme β-lactamase, which can cleave the amide bond of the β-lactam ring, leading to the inactivation of β-lactam antibiotics. Acquired antibiotic resistance leads to the development of multidrug-resistant strains, increasing the difficulty in eradicating this microorganism and resulting in more persistent infections. (Bush & Bradford, 2016; González-Bello et al., 2019) *Klebsiella pneumoniae*, a Gram-negative, non-motile, facultatively anaerobic bacterium, is a critical multidrug-resistant pathogen (Piperaki et al., 2017). It is ubiquitous in various environmental reservoirs, including water and soil, and frequently causes serious infections such as urinary tract infections and pneumonia. Infections with this wild-type *K. pneumoniae* strain are characterized by substantial bacterial proliferation in the respiratory tract, a robust inflammatory immune response, and a gradual progression to systemic disease that often leads to mortality. (Shon et al., 2013) The key components in *Klebsiella pneumoniae* pathogenesis include capsular antigens, adherence factors, lipopolysaccharides, and siderophores. The capsular antigen is the primary virulence factor, forming a thick, hydrophilic capsule that gives *K. pneumoniae* colonies their mucoid appearance. Specific antibodies are used to classify *K. pneumoniae* isolates based on

their capsular serotypes. *Staphylococcus aureus* is a Gram-positive, spherical, non-motile, non-spore-forming facultative anaerobe that generates enterotoxins. Its genome consists of a circular chromosome with genes regulating virulence and antibiotic resistance. (Kim et al., 2014) The cell wall is composed of 50% peptidoglycan, with varying structures impacting the ability to induce disseminated intravascular coagulation. Staphylococcal surface proteins share structural characteristics, including a secretory signal sequence, positively charged amino acids, a hydrophobic membrane-spanning domain, and a cell-wall-anchoring region. (Foster et al., 2019) These proteins play a vital role in the capacity of staphylococci to colonize host tissue. Staphylococci produce numerous toxins, classified based on their mechanisms of action. Cytotoxins create pores and provoke proinflammatory changes in mammalian cells, contributing to sepsis syndrome manifestations. Pyogenic-toxin superantigens are structurally related and trigger extensive T-cell proliferation and cytokine release. These bacteria also generate various enzymes that destroy tissue, facilitating the spread of infection. β-Lactamase inactivates penicillin, and coagulase converts fibrinogen to fibrin, contributing to bacterial virulence. (Ward & Kunkel, 1983) The emergence of penicillin-resistant *Staphylococcus aureus* strains in the early 1940s coincided with the introduction of antibiotics. These resistant strains produced a β-lactamase enzyme that hydrolyzed and inactivated the critical β-lactam structural feature of antibiotics.

Objective: To isolate and identify microbial bacteria in flaxseed and analyze their antibiotic resistance.

Chapter 2

Materials and Methods

2.1 Sample collection:

The study was conducted by selecting 7 distinct areas (Uttara, Khilgaon, Basabo, Moghbazar, Mirpur, Mohakhali, Badda) within Dhaka city between October 2023 and June 2024. Flaxseed samples were procured from a variety of local and heavily trafficked bazaars in these areas. Four target microorganisms, namely *Escherichia Coli, Pseudomonas aeruginosa, Klebsiella pneumonia,* and *Staphylococcus aureus,* were isolated from the flaxseed samples.

2.2 Sample preparation:

Collected herb samples from various parts of Dhaka city, cleaned properly with distilled water, and grinding them using a home grinder transferred aseptically in a clean plastic bag and stored at room temperature. It should be made sure samples are carried in an undamaged container to avoid contamination.

2.3 Sample Enrichment:

In this step, ground seeds powder enriched in Phosphate buffered saline or PBS solution for the growth of bacteria for enumeration and identification process. In an autoclaved falcon tube add 1 g ground seed powder with 9ml Phosphate buffered saline and leveled with individual sample code. Keep this mixer for 24 hours at 37 degrees Celsius in a shaker incubator.

2.4 Agar Media Preparation

The preparation of agar media is a crucial step in microbiology lab work. To properly prepare the agar media, it is important to follow a standardized procedure to ensure the growth of bacteria in a controlled environment. (Potato Dextrose Agar (PDA)- Principle, Uses, Procedure &

Characteristics, 2015) The MacConkey agar is selective for gram-negative bacteria, as it contains crystal violet and bile salts that inhibit the growth of gram-positive bacteria. Other media used in this lab report include Salmonella Shigella Agar, xylose lysine deoxycholate agar, mannitol salt agar, and Cetrimide agar (Gonelimali et al., 2018). The ingredients, including agar, peptone, and sodium chloride, must be accurately measured and mixed in a sterilized flask (The Ultimate Guide to Good Laboratory Practices (GLP), 2021). The mixture is then heated to dissolve the components before being sterilized in an autoclave. Once the agar media has been prepared, it is poured into petri dishes and allowed to solidify before use. To identify different types of bacteria, the prepared agar media were inoculated with test strains and incubated at an appropriate temperature (Ulrikh et al., 2022). After incubation, the plates were examined for colony growth and specific characteristics such as color, morphology, and hemolytic activity.

2.5 Serial dilution:

Bacterial samples often require dilution to reduce their concentration to a manageable and countable level (Castillo-Fernandez et al., 2015). This can be done using various methods, depending on the specific needs and constraints of the experiment or application. One common method is the ten-fold dilution factor, where 1 ml of the original sample is diluted in 9 ml of fresh diluent. This approach allows for a rapid reduction in the bacterial concentration while minimizing the number of dilution steps required. However, it is important to note that there are other techniques and strategies available for the concentration and conditioning of original samples, especially when it comes to microscale techniques. In this project I use the ten-fold dilution method in combination with fluorescence calibration measurements and absorbance measurements for colony-forming units to ensure accurate and reliable results, taking into account factors such as sampling theory and variation in the calibration materials.

2.6 Spreading on agar plate:

Agar is a gelatinous substance that is widely used in microbiology laboratories for culturing bacteria and other microorganisms. Spread the 10-fold diluted sample of 100 microliters onto an

agar plate using a sterile glass spreader as swiftly as possible to prevent contamination. Following spreading, place it in an incubator at 37 degrees Celsius for 24 hours.

2.7 Bacterial Identification by Colony Morphology:

Bacterial identification is another fundamental aspect of microbiology lab work. After obtaining bacterial cultures, various methods can be employed to identify the specific species present. (Vivas & Gutiérrez, 2019) These may include Gram staining, biochemical tests, and molecular techniques such as polymerase chain reaction and sequencing. By utilizing a combination of these methods, the identity of the bacteria can be determined, providing valuable information for research and medical purposes. (A Complete Guide to Microbial Identification, 2021)

Media	Organisms	Color of colony
MacConkey agar	*Escherichia Coli, Klebsiella pneumoniae*	Red, large mucoid dark pink/ pink yellow
Mannitol salt aga	*Staphylococcus aureus,*	Yellow, white
Cetrimide	*Pseudomonas aeruginosa*	Blue-green/yellow-green
KPC	*Klebsiella pneumoniae*	Deep blue

Table 1: Bacterial Colony morphology

2.7.1 Subculture of *Escherichia Coli* in Eosin Methylene Blue (EMB):

As a common inhabitant of the lower intestine in warm-blooded organisms, the ubiquitous bacterium *Escherichia coli* is known to play a role in both beneficial and pathogenic processes in the human body. While some *E. coli* strains are harmless commensals, others can cause acute infections or even contribute to the development of chronic diseases. To reliably identify and differentiate *E. coli* from other enteric bacteria, microbiological techniques such as the use of selective and differential media like Eosin Methylene Blue (EMB) agar. This agar

contains dyes that are toxic to Gram-positive bacteria, thereby inhibiting their growth and allowing for the selective isolation of Gram-negative bacteria, particularly members of the Enterobacteriaceae family. The procedure for confirming the presence of *E. coli* on Eosin Methylene Blue agar involves inoculating the sample onto the agar surface and incubating it at 37°C for 16-24 hours. The colonies grown on Eosin Methylene Blue agar exhibit a distinct metallic green sheen, which is a characteristic feature of the bacterial species. It is produced by the large quantities of acid generated during the metabolic process of lactose utilization. Whereas the majority of *E. coli* isolates can readily and efficiently ferment lactose, leading to the production of copious amounts of acid, a small subset of atypical or variant strains may display delayed or diminished lactose fermentation, resulting in less pronounced or absent metallic sheen. (Ali et al., 2021; Braz et al., 2020)

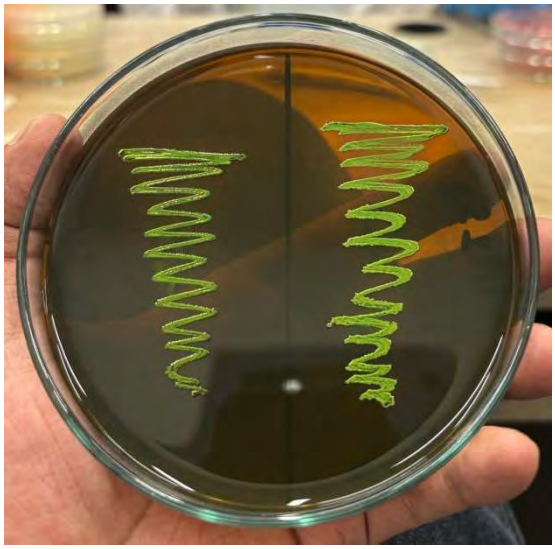

Figure 1: *Escherichia Coli* in Eosin Methylene Blue agar

2.7.2 Subculture of *Pseudomonas aeruginosa* in Cetrimide Agar:

Pseudomonas aeruginosa, a ubiquitous Gram-negative bacterium, is renowned for its versatility and ability to thrive in diverse environments. The ability of this opportunistic pathogen to produce a vibrant, greenish-blue pigment when grown on cetrimide agar and exposed to ultraviolet (UV)

light is a captivating phenomenon that has long piqued the interest of microbiologists and researchers. Vibrant, blue-green pigment that gives the colonies a striking and distinctive appearance when viewed under UV illumination. The production of this characteristic pigment, known as pyocyanin, is a hallmark feature of *Pseudomonas aeruginosa* and is closely linked to its adaptability and survival strategies in diverse environments. Pyocyanin, a secondary metabolite synthesized by *Pseudomonas aeruginosa*, is a water-soluble, blue-green pigment with unique redox properties that contribute to the bacterium's pathogenicity and ability to outcompete other microorganisms.

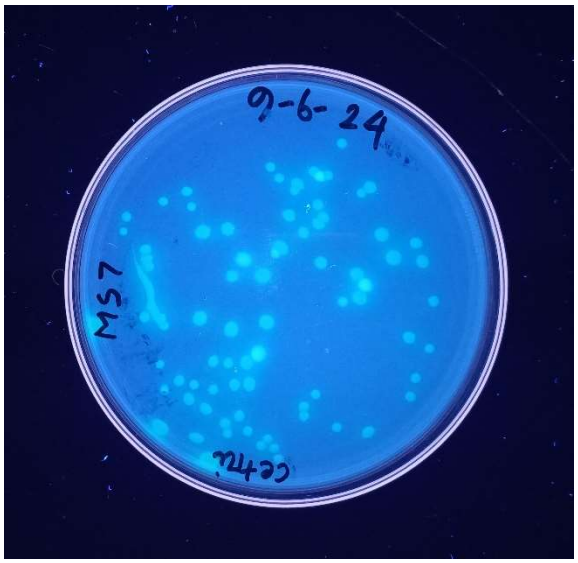

Figure 2: *Pseudomonas aeruginosa* colony in cetrimide agar

under UV light

2.8 Molecular Identification of the Bacteria

2.8.1 DNA Extraction:

To extract DNA, previously sub-cultured organisms from an NA plate were collected using a loopful and suspended in 400 microliters of TE buffer in an Eppendorf tube. The mixture was vortexed for 15 seconds, then centrifuged at 13,000 RPM for 10 minutes at 25°C. The supernatant was discarded, and the pellet was resuspended in another 400 microliters of TE buffer. After vortexing for 15 seconds, the sample was placed in a water bath at 100°C for 10 minutes to lyse the cells. Next, the mixture was allowed to cool at room temperature for 5 to 10 minutes. It was then centrifuged one more time at 13,000 RPM for 10 minutes at 25°C. Finally, the supernatant was transferred to a 1.5 microliter MCT tube and stored at -20°C for further use.

2.8.2 Polymerase Chain Reaction:

The Polymerase Chain Reaction (PCR) technique was utilized to identify and confirm the presence of desired DNA segments from microbial samples. (Ralph & McClelland, 2003) To initiate the process, 13µL mixtures of PCR components were prepared in sterile tubes for each organism. These mixtures consisted of 6µL of Master Mix, 1µL each of forward and reverse primers, 3µL of nuclease-free water, and 2µL of extracted DNA. The DNA templates were added immediately after combining the other components. Subsequently, the tubes underwent a brief centrifugation, either in a microplate spinner or a microcentrifuge, to eliminate any air bubbles. The prepared samples were then loaded into the PCR instrument, with the settings adjusted according to the target gene. Finally, upon completion of the PCR procedure, the amplified products were stored at -20°C in a culture freezer, prior to being subjected to agarose gel electrophoresis.

Primer	Sequence (5-3)	Target	Amplicon size (bp)	Condition	Reference
ECO F:	GACCTCGGTTTAGTTCACAGA	*E. coli*	585	3min at 95°c; 45 sec at 94°c; 45 sec at 58°c; 60 sec at 72°c; 3 min at 72°c; 30 cycle	(Patel et al., 2022)
ECO R:	CACACGCTGACGCTGACCA				
PA-SS-F:	GGGGGATCTTCGGACCTCA	*P. aeruginosa*	956	2 min at 95°c; 20 sec at 94°c; 20 sec at 72°c; 40 sec at 72°c; 1 min at 72°c; 25 cycle	(Spilker et al., 2004)
PA-SS-R:	TCCTTAGAGTGCCCACCCG				
KP Pf-F:	ATTTGAAGAGGTTGCAAACGAT	*K. pneumoniae*	130	10 min at 94°c; 30 sec at 94°c; 20 sec at 57°c; 20 sec at 72°c; 35 cycle	(Albasha et al., 2020)
KP Pr1-R:	GCAATGGCCATTTGCGTTTGCGTTAG				
Nuc F:	GCGATTGATGGTGATACGGTT	*S. aureus*	275	4 min at 94°c; 1 min at 94°c; 1 min at 55°c; 1 min 72°c; 10 min at 72°c; 32 cycle	(Karimzadeh & Ghassab, 2022)
Nuc R:	AGCCAAGCCTTGACGAACTAAAGC				

Table 2: Primers and PCR conditions for this study

2.8.3 Agarose Gel Electrophoresis:

Agarose gel electrophoresis is an effective analytical technique for the separation and investigation of nucleic acids. The high gel strength of agarose enables the efficient separation of large DNA fragments, typically ranging from 100 base pairs to 25 kilobases in size. While pulse field gel electrophoresis is utilized for even larger DNA fragments, polyacrylamide gel electrophoresis is more suitable for the separation of smaller DNA segments. The agarose matrix can be chemically modified through hydroxyethylation, resulting in low melting agarose, which reduces the packing density and pore size of the gel. Ethidium bromide is employed as a loading dye to enhance the density, color, and migration patterns of DNA fragments, facilitating the estimation of their size based on a DNA standard. To conduct the experiments, an agarose gel is prepared by boiling the required agarose powder with 50X TAE buffer and distilled water. After cooling, ethidium bromide, an intercalating agent used as a fluorescent tag, is added. The previously amplified PCR products are then loaded into the agarose gel using sterile pipette tips. The DNA samples are electrophoresed using 1X TAE as the running buffer, which allows the negatively charged DNA molecules to migrate toward the positive electrode. Agarose gel electrophoresis is typically performed using 5µl of a 100 bp ladder and 100 V of electricity. The applied electric current causes the DNA to move through the strongly cross-linked agarose substrate, with the negatively charged DNA phosphates migrating toward the positive pole. MIDORI Green is used to stain the DNA, enabling the tracking of its progress through the gel. After the DNA has been separated, it is stained with a substance that specifically binds to DNA molecules, either reflecting a certain color in visible light or fluorescing under UV light. The band sizes are then determined by comparing the sample DNA to the DNA ladder.

2.8.3.1 Confirmation of microbial organisms through Agarose Gel Electrophoresis

Confirmation of *Escherichia coli*

The desired isolates were determined by using Polymerase Chain Reaction. After performing PCR to visualize and identify the target DNA bands, Agarose Gel Electrophoresis was conducted to confirm the organisms by comparing them to the targeted base pair 585 in 100 bp ladder. Out of 49 samples 14 samples isolates *Escherichia coli* positive.

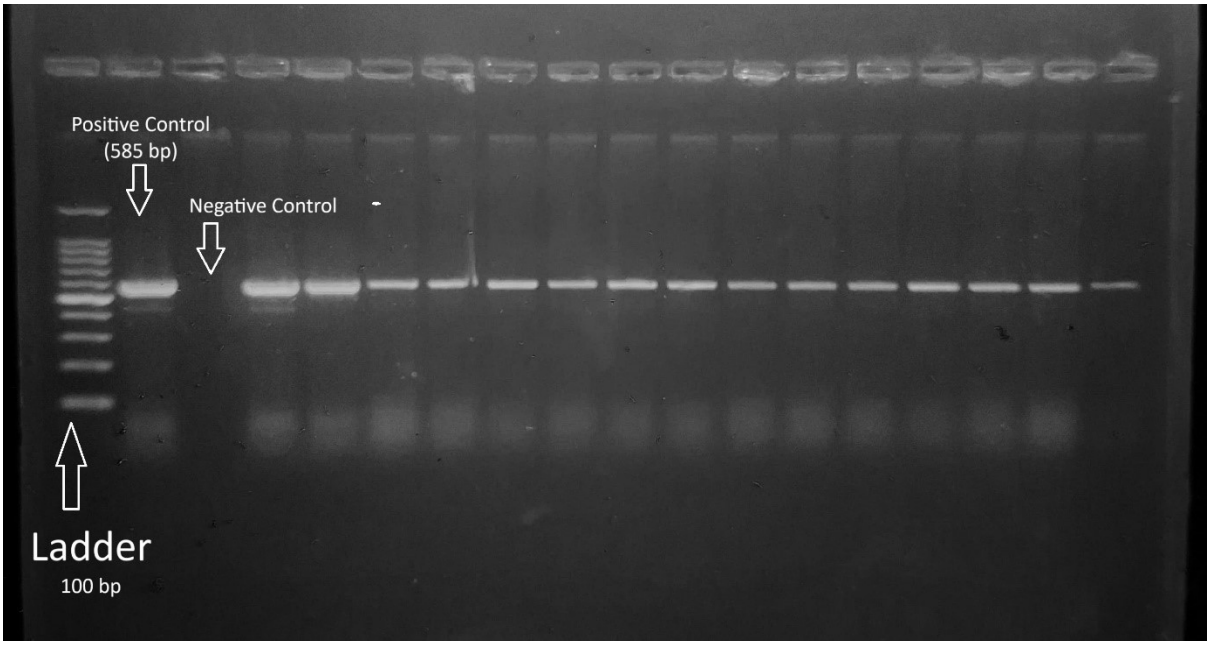

Figure 3: Agarose gel electrophoresis & visualization of *Escherichia coli* DNA bands

Confirmation of *Pseudomonas aeruginosa*

Here ladder was a 100 bp DNA marker. Lane 1 is laddering lane 2 was positive control, lane 3 was negative control. From lane 4-18 different samples were loaded. Amplicon size 956 bp, 11 isolated

detected

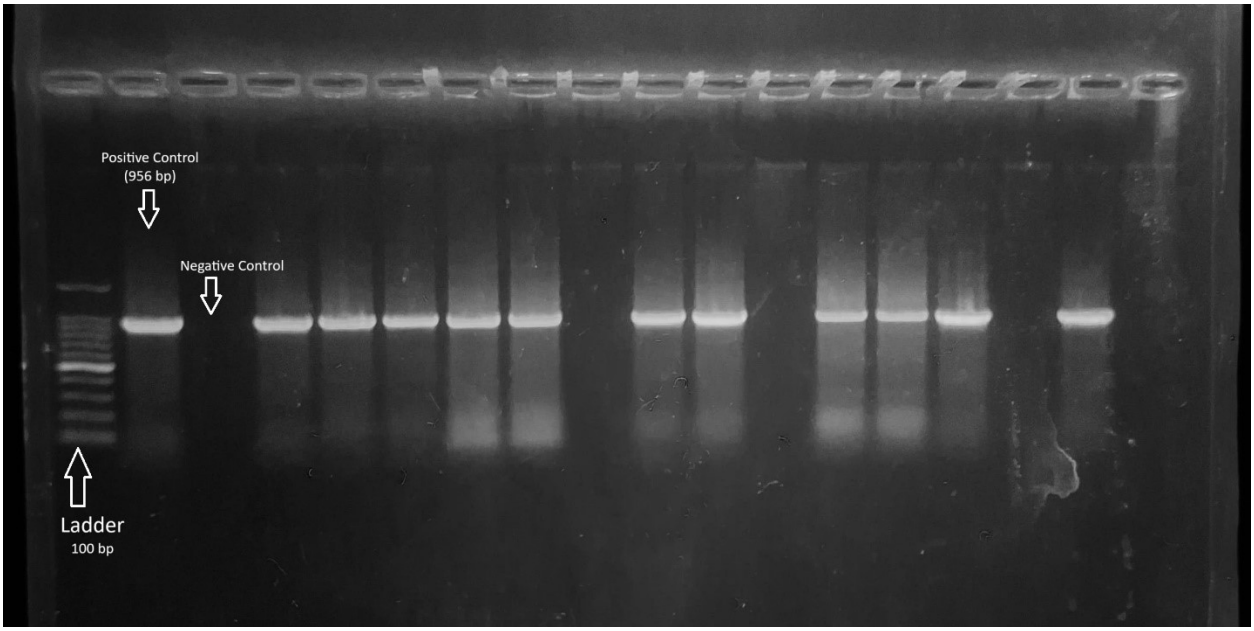

Figure 4: Agarose gel electrophoresis & visualization of *Pseudomonas aeruginosa* DNA bands

Confirmation of *Klebsiella pneumoniae*

9 isolates were detected as *Klebsiella pneumoniae* from the 133bp is the band size. The first lane contains the 1kb ladder. Then from lane 2 to lane 18 contains the sample products.

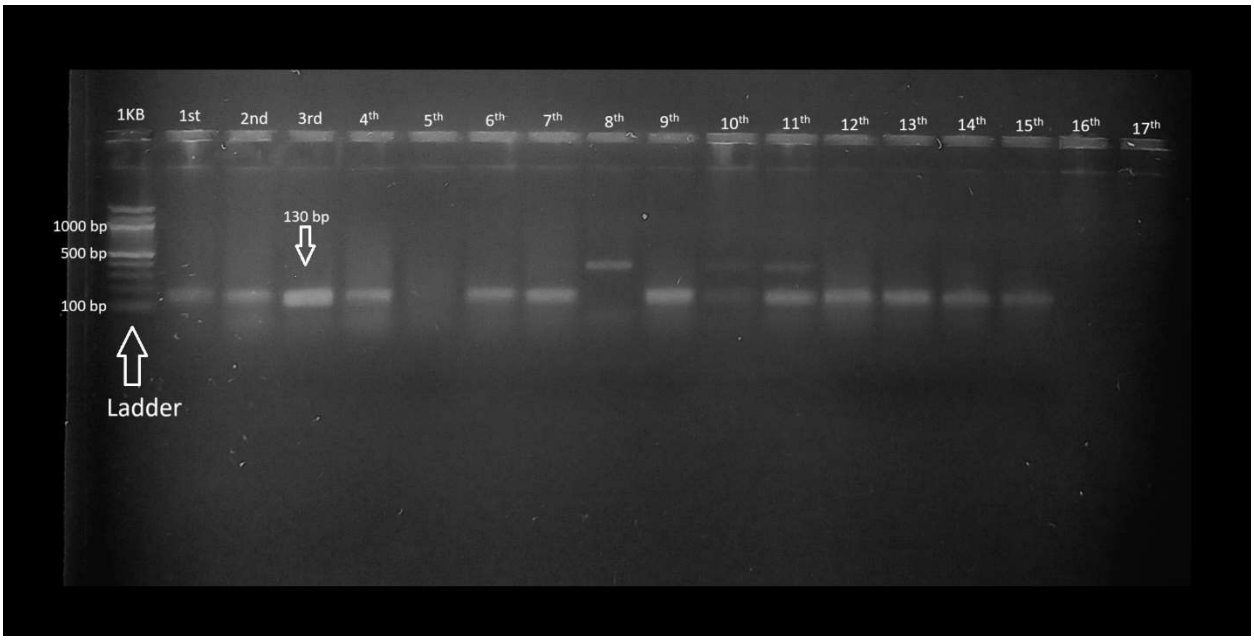

Figure 5: Agarose gel electrophoresis & visualization of *Klebsiella pneumoniae* DNA bands

Confirmation of *Staphylococcus aureus*

15 isolates were detected as Staphylococcus aureus. Here ladder was a 100 bp DNA marker. Lane 1 is laddering lane 2 was positive control, lane 3 was negative control. From lane 4-18 different sample were loaded. Amplicon size 275 bp

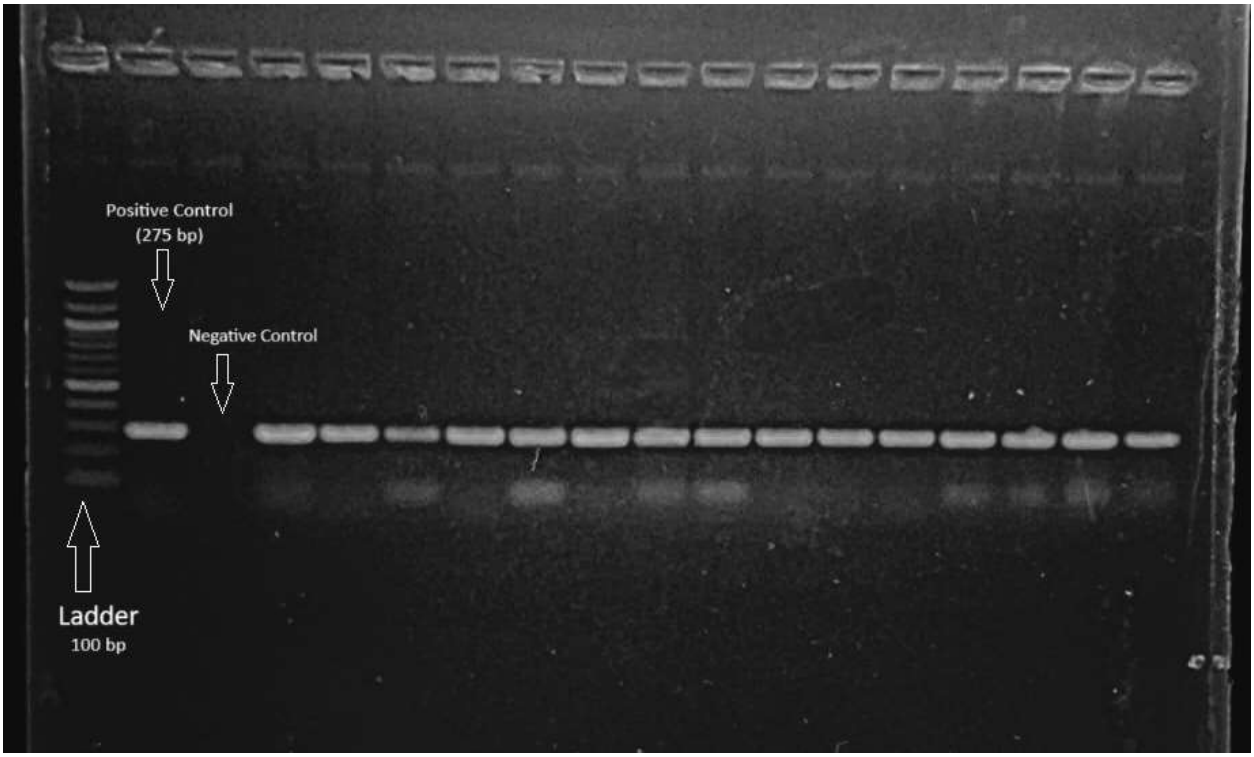

Figure 6: Agarose gel electrophoresis & visualization of *Staphylococcus aureus* DNA band

2.9 Antibiotic Susceptibility Testing (AST):

To determine the effectiveness of antibiotics against specific bacterial pathogens I used the antibiotic susceptibility test (AST). The Clinical and Laboratory Standards Institute (CLSI) standardized Muller-Hilton Agar (MHA) medium used as the growth substrate for bacterial cultures. This medium provides optimal growth conditions for a wide range of bacterial species and allows for the clear visualization of antibiotic-induced zones of inhibition around the antibiotic disks. Consistent plate preparation is maintained properly as any irregularities in the thickness or distribution of the agar can compromise the reliability and reproducibility of the test results, leading to inaccurate interpretations of the bacterial susceptibility patterns (Kuper et al., 2009). Before inoculation, bacterial cultures are typically grown to an exponential phase and then diluted in a physiological saline solution to achieve a standardized cell density, often corresponding to a 0.5 McFarland standard. After that sterile cotton swabs were used to dip in the saline solution and then lawn on the freshly prepared MHA agar plates. The selection of relevant antibiotic disks was placed on the lawned plates carefully using sterile forceps. by maintaining a proper distance so that the inhibition zones did not overlap each other. Finally, the plates were labeled and incubated for 18-24 hours at 37 °C.

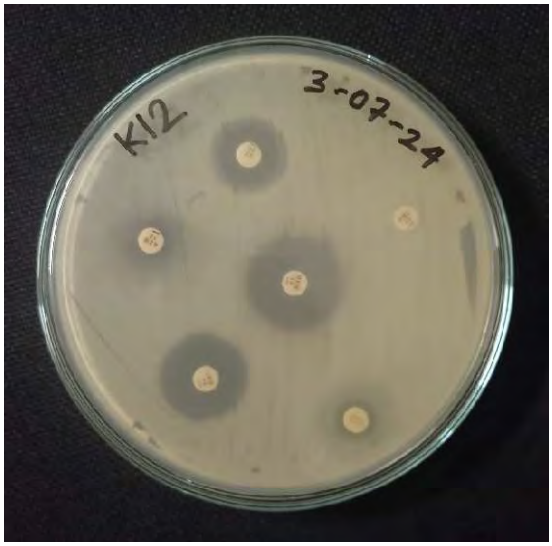

Figure 7: Antibiotic Susceptibility Testing (Kirby-Bauer disk diffusion method)

2.9.1 Zone interpretive chart for *Escherichia coli:*

Antibiotic group	Antibiotics	Disc code	Disc potency (µL)	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Aminoglycosides	Amikacin	AK	30	≥ 17	15-16	≤ 14
	Gentamicin	GEN	10	≥ 15	13-14	≤ 12
	Kanamycin	KAN	30	≥ 18	14-17	≤ 13
	Streptomycin	S	10	≥ 15	12-14	≤ 11
Carbapenem	Imipenem	IMI	10	≥ 23	18-22	≤ 19
	Meropenem	MEM	10	≥ 23	18-22	≤ 19
B-lactam	Ampicillin	AMP	10	≥ 17	12-16	≤ 13
Fluoroquinolone	Ciprofloxacin	CIP	5	≥ 21	16-20	≤ 15
Macrolides	Erythromycin	E	15	≥ 23	14-22	≤ 13
Phenicol	Chloramphenicol	C	30	≥ 18	13-17	≤ 12
Polymyxin	Colistin	CT	10	≥ 17	12-16	≤ 11
Tetracycline	Tetracycline	TE	30	≥ 15	12-14	≤ 11

Table 3: Concentration and diffusion zones of the antibiotics *(Escherichia coli)*

2.9.2 Zone interpretive chart for *Pseudomonas aeruginosa:*

Antibiotic group	Antibiotics	Disc code	Disc potency (µL)	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Aminoglycosides	Amikacin	AK	30	≥17	15-16	≤14
	Gentamicin	GEN	10	≥15	13-14	≤12
Fluroquinolone	Ciprofloxacin	CIP	5	≥21	16-20	≤15
	Norfloxacin	NOR	10	≥17	13-16	≤12
	Levofloxacin	LEV	5	≥19	16-18	≤15
Carbapenem	Imipenem	IPM	10	≥23	18-22	≤19
	Meropenem	MEM	10	≥ 23	18-22	≤ 19
Polymyxins	Colistin	CT	10	≥ 18	13-17	≤ 12
Monobactams	Aztreonam	ATM	30	≥ 22	16-20	≤ 15
Beta-lactams	Cefepime	FEP	30	≥ 18	15-17	≤ 14
Penicillinase + β- lactamase	Piperacillin + Tazobactam	TZP	100/10	≥ 21	18-20	≤ 17

Table 4: Concentration and diffusion zones of the antibiotics *(Pseudomonas aeruginosa)*

2.9.3 Zone interpretive chart for *Klebsiella pneumoniae:*

Antibiotic group	Antibiotics	Disc code	Disc potency (µL)	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Aminoglycosides	Amikacin	AK	30	≥ 17	15-16	≤14
Ketolides	Azithromycin	AZM	15	≥ 18	14-17	≤ 13
Cephem	Cefixime	CFM	5	≥ 19	16-18	≤ 15
Tetracycline	Tetracycline	TE	30	≥15	12-14	≤ 11
Carbapenem	Imipenem	IMP	10	≥ 23	18-22	≤ 19
	Meropenem	MEM	10	≥ 23	18-22	≤ 19
Fluroquinolone	Ciprofloxacin	CIP	5	≥ 21	16-20	≤ 15
Cephalosporin	Ceftriaxone	CRO	30	≥ 25	22-24	≤ 21
Penicillinase + β- lactamase	Piperacillin + Tazobactam	TZP	100/10	≥ 21	18-20	≤ 17
Penicillin	Amoxycillin	AMC	30	≥ 18	14-17	≤ 13
	Amoxiclav	AML	30	≥ 18	14-17	≤ 13

Table 5: Concentration and diffusion zones of the antibiotics *(Klebsiella pneumoniae)*

2.9.4 Zone interpretive chart for *Staphylococcus aureus:*

Antibiotic group	Antibiotics	Disc code	Disc potency (µL)	Sensitive (mm)	Intermediate (mm)	Resistance (mm)
Aminoglycosides	Kanamycin	K	30	≥ 18	14-17	≤ 13
Amphenicol	Chloramphenicol	C	30	≥ 18	13-17	≤ 12
Clindamycin	Clindamycin	CD	2	≥ 21	15-20	≤ 14
Macrolides	Azithromycin	AZM	15	≥ 18	14-17	≤ 13
	Erythromycin	E	15	≥ 23	14-22	≤ 13
Fluoroquinolone	Ciprofloxacin	CIP	5	≥ 21	16-20	≤ 15
	Fleroxacin	F	5	≥19	16-18	≤15
	Levofloxacin	LEV	5	≥ 17	14-16	≤ 13
Tetracycline	Doxycycline	DO	30	≥ 16	13-15	≤ 12
Oxazolidinones	Linezolid	LZ	30	≥21	-	-

Table 6: Concentration and diffusion zones of the antibiotics *(Staphylococcus aureus)*

Chapter 3

Results

3.1 Culture Positive sample of flaxseed based on area:

Seven locations within Dhaka city were surveyed, and 56 flaxseed samples were analyzed in the laboratory. Of these, 49 samples tested positive for the target substance using polymerase chain reaction analysis. The subsequent line graph illustrates the percentage of positive samples across the different locations around Dhaka city.

Area Name	Collected Sample	Positive Sample	Positive Sample %
Uttara	8	7	87.5%
Khilgaon	8	8	100%
Basabo	8	6	75%
Moghbazar	8	8	100%
Mirpur	8	7	87.5%
Mohakhali	8	5	62.5%
Badda	8	8	100%
Total	56	49	87.5%

Table 7: Positive sample of flaxseed based on area.

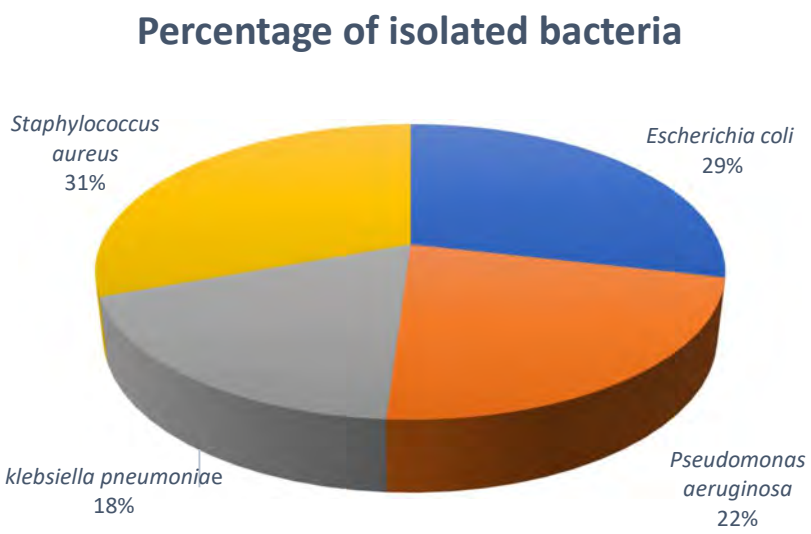

Figure 8: A pie chart showing the percentage of the presence of each mentioned bacterial organism

3.2 Antimicrobial Susceptibility Test (AST):

Antibiotic Susceptibility Testing was performed on all four organisms, following the Kirby Bauer disc diffusion method and the latest CLSI guidelines (M100: Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition).

3.2.1 AST for *Escherichia coli:*

The antibiotic susceptibility profiles of 14 *Escherichia coli* isolates are presented below:

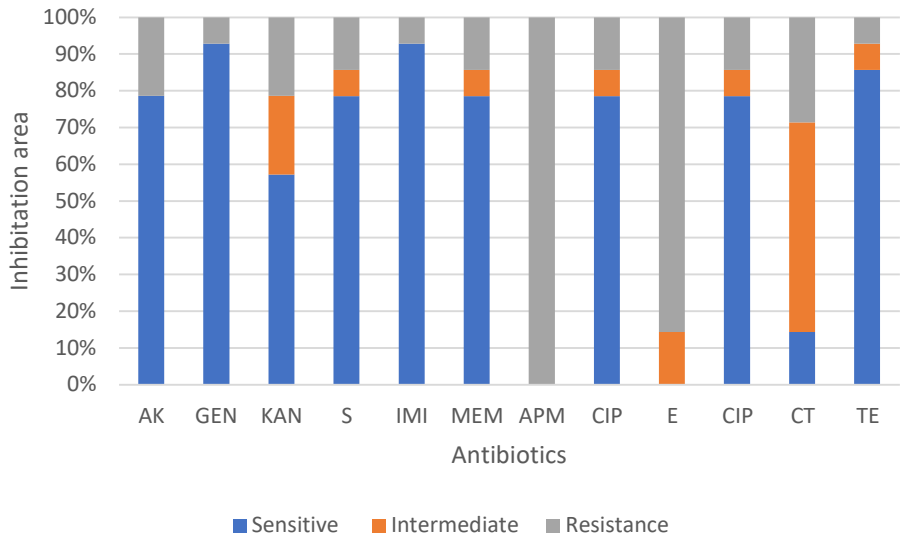

Figure 9: Bar chart of the percentage of Antibiotic Susceptibility Testing of *E. coli*

3.2.2 AST for *Pseudomonas aeruginosa:*

The antibiotic susceptibility profiles of 11 *Pseudomonas aeruginosa* isolates are presented

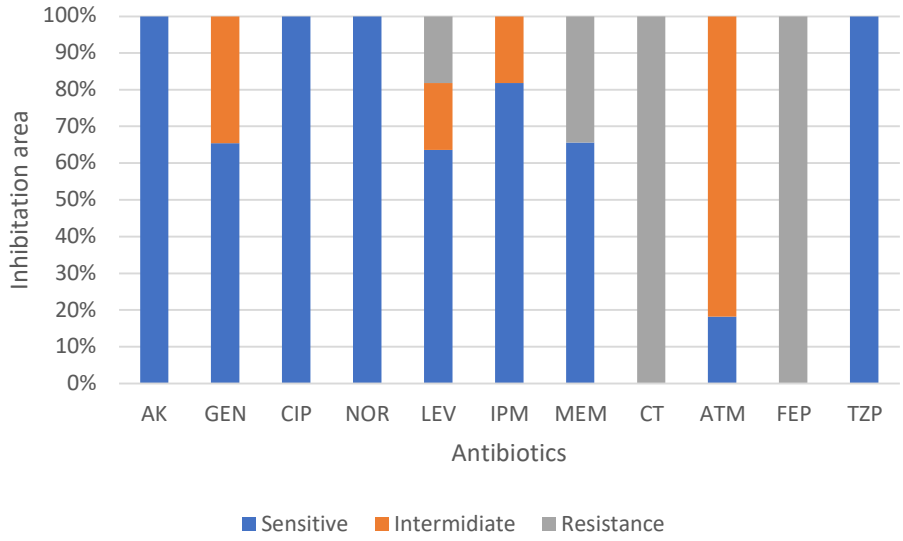

Figure 10: Bar chart of the percentage of Antibiotic Susceptibility Testing of *Pseudomonas aeruginosa*

3.2.3 AST for *Klebsiella pneumoniae:*

The antibiotic susceptibility profiles of 9 *Klebsiella pneumoniae* isolates are presented below:

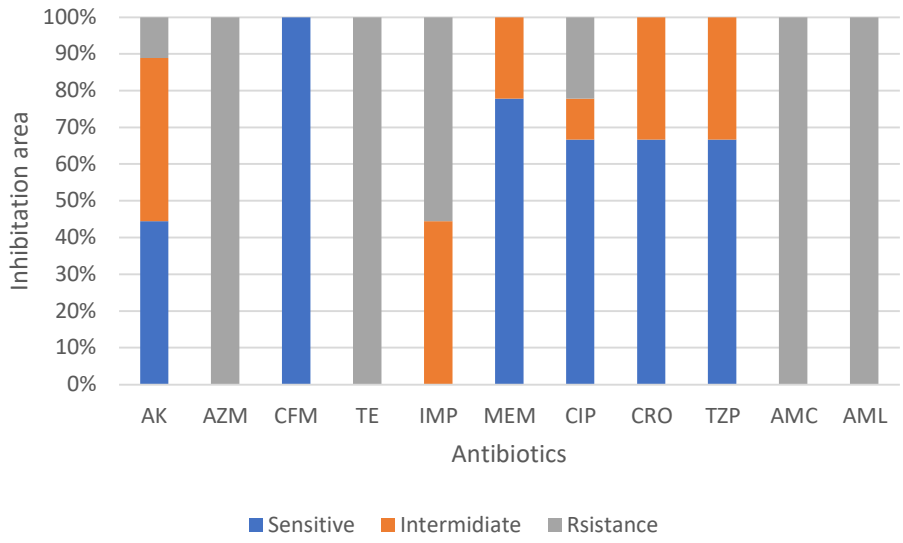

Figure 11: Bar chart of the percentage of Antibiotic Susceptibility Testing of *Klebsiella pneumoniae*

3.2.4 AST for *Staphylococcus aureus:*

The antibiotic susceptibility profiles of 15 *Staphylococcus aureus* isolates are presented below:

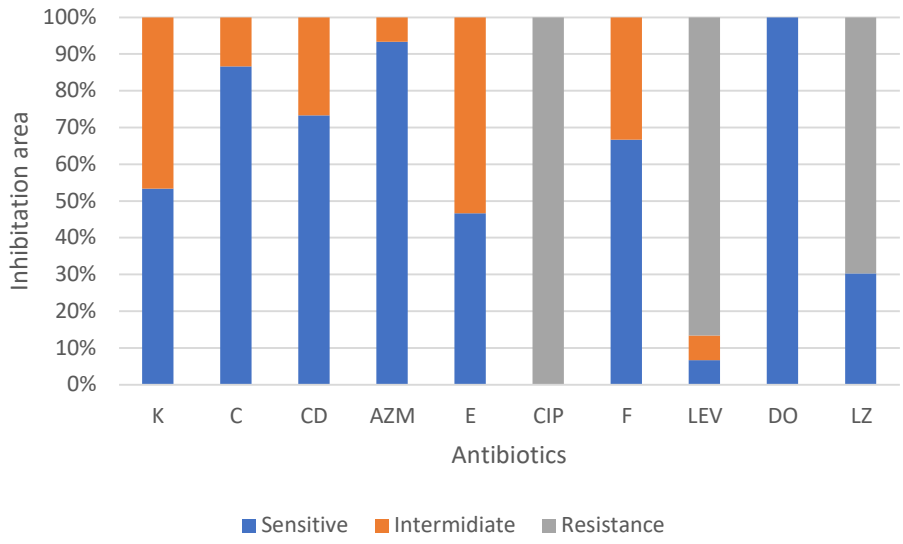

Figure 12: Bar chart of the percentage of Antibiotic Susceptibility Testing of *Staphylococcus aureus*

3.3 Multidrug Drugs Resistant (MDR):

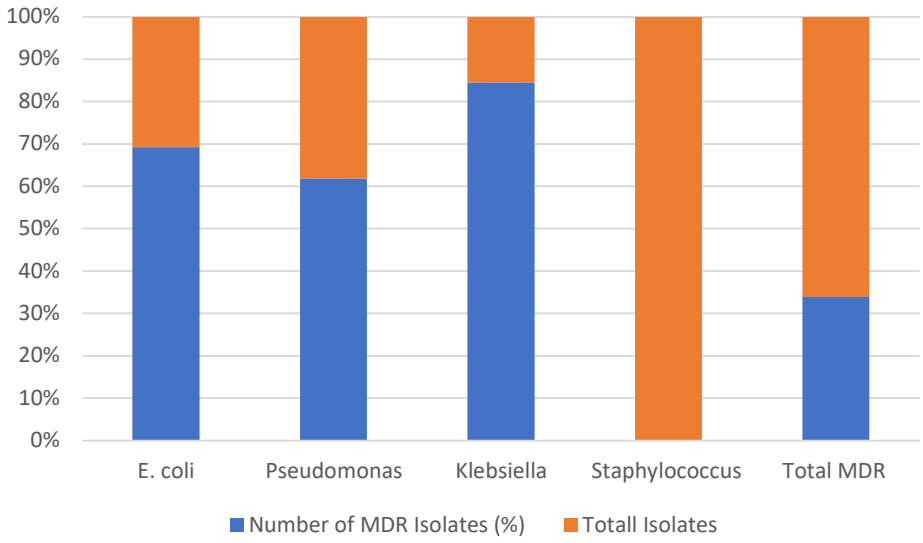

Figure 13: Bar chart comparing the Multidrug-resistant and total isolate number

Chapter 4

Discussion

Flaxseed is cultivated globally, with particularly high production in nations such as Kazakhstan, Russia, China, and India, as well as across regions including South America, North America, Europe, and Africa. (Abida, 2022). Production reached 875,995 tons, with Russia accounting for 75%. Russia has expanded its flax cultivation to 1.1 thousand hectares by 2020 as a leading flax producer. (Arora, 2024). Despite a 19% decrease in flaxseed production from 1.73 million tons in 2022 to 1.41 million tons in 2023, Russia remains the world's top flaxseed producer by 2023 the country supplied 1.48 million tons of flaxseed to the global market, holding over 56% of the total market share. China is the largest buyer of Russian flaxseed, further strengthening Russia's market position. (Goral, 2024) The head of the Kazakhstan Grain Union predicts a 25-50% increase in flaxseed production in 2024 due to low profitability and growing Chinese demand, with output potentially rising from 450,000 to 550,000 tons. (Goral, 2024) Bangladesh is not a major linseed producer, with most of the global production concentrated in countries like Canada, China, Russia, and the United States (Hu et al., 2020). However, Bangladesh imports linseeds from countries like India, Australia, and Argentina, and it is crucial to understand the processes of handling, harvesting, and importing to tackle microbial contamination issues. CDCs report outbreaks in a standard form from January 2003. Foodborne disease outbreaks are incidents involving similar illnesses from common food consumption. (Scallan et al., 2010). According to the World Health Organization, approximately 600 million people globally experienced foodborne illnesses in 2010 (Lorenzo et al.2017). Between 2003-2017, China experienced 19,517 foodborne disease outbreaks. These outbreaks led to 235,754 illnesses, 107,470 hospitalizations, and 1,457 deaths (Li et al., 2020). A regional survey in the Northern Great Plains was conducted in 2008-2009 to measure microbial levels in flaxseed. The study examined how cleaning flaxseed affected microbe counts, including total aerobic bacteria, mold, yeast, coliforms, Escherichia coli, and Enterobacteriaceae. (Vegi et al., 2016). Another study on sprouted chia and flax seed powders found 10 linked to Salmonella outbreaks in Canada and the US. Contaminated samples showed higher levels of Enterobacteriaceae and coliforms, suggesting low Salmonella levels can cause foodborne outbreaks. (Tamber et al., 2016). The microbial quality of various seeds and flaxseed flour sold in southern Portugal was examined by counting aerobic microbes. The study found that

organic flaxseed had higher levels of aerobic microbes, molds, and yeast compared to non-organic flaxseed (Silva et al., 2022). In Bangladesh, the linseed industry encounters challenges such as inadequate storage facilities and lack of proper sanitation practices linked to microbial contamination, which can result in outbreaks and pose risks to public health similar to outbreaks in other countries. The primary intent of this research was to isolate and identify the presence of pathogenic bacterial species, including Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus, in flaxseed samples were collected from various locations within Dhaka, Bangladesh. In total, bacterial pathogens were detected in 49 out of 56 flaxseed samples, indicating an 87.5% rate of microbial contamination. Similarly, the Uttara and Mirpur regions exhibited the same percentage of contamination. According to the percentage of contaminated samples, Khilgaon, Moghbazar, and Badda appear to be the most heavily affected areas, with 100% of the samples testing positive for the presence of these pathogens. Conversely, Basabo and Mohakhali displayed relatively lower levels of microbial contamination, with 75% and 62.5% of the samples affected, respectively. PCR analysis confirmed probable bacterial species. Based on the isolate counts, Staphylococcus aureus was the predominant organism, accounting for 30.61% of the isolates. The second most prevalent microbial pathogen in the flaxseed samples was Escherichia coli, comprising 28.57% of the isolates. Klebsiella pneumoniae had the fewest isolates, at 18.37%, while Pseudomonas aeruginosa was the second least common, at 22.45%. The Kirby-Bauer disk diffusion method was used to test for antibiotic susceptibility, and antibiotic selection followed the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) for each microbial strain. By absorbing genetic material from different sources, E. coli can develop new skills. As a result, the bacteria can inhabit in different environments, and the ones that are most adapted to those environments then become more proliferate. (Michele & Ricardo, 2011). In this study Escherichia coli isolates were tested for susceptibility to 12 individual antibiotics and 8 different antibiotic classes. E. coli exhibits high levels of resistance to the β-lactam and macrolide antibiotic groups, with the highest percentages of resistance observed for ampicillin (100%) and erythromycin (85.71%). Colistin shows an intermediate resistance level of 57.14%. Additionally, Kanamycin, Streptomycin, Meropenem, Ciprofloxacin, Chloramphenicol, Tetracycline, and even erythromycin have a relatively low percentage of intermediate resistance levels. The aminoglycoside antibiotic Gentamicin and the carbapenem group of antibiotic Imipenem demonstrate the highest susceptibility rates, with

92.86% of the isolates being sensitive to these agents. Second bacterium of this study for Pseudomonas aeruginosa, 7 different types of antibiotic groups and 11 different antibiotics were evaluated. Among them, the Polymyxins and Beta-lactams groups exhibited 100% resistance. Conversely, the Aminoglycosides, Fluoroquinolones, and Penicillinase + β-lactamase groups demonstrated the highest sensitivity, with some antibiotics from these groups, such as Amikacin, Ciprofloxacin, Norfloxacin, and Piperacillin + Tazobactam, showing 100% sensitivity. The Monobactams group's antibiotic Aztreonam exhibited the highest intermediate level at 81.8%. Third Organism Klebsiella pneumoniae has shown a marked increase in antibiotic resistance. Susceptibility testing of 11 antibiotics across 9 groups revealed that Penicillinase, Tetracycline, and Ketolides groups exhibit 100% resistance. Only the Cephem group antibiotic Cefixime shows 100% susceptibility. However, this is not enough to effectively combat this microbial organism, as most antibiotics demonstrate intermediate or resistant profiles. For instance, Imipenem exhibits 44.4% intermediate and 55.56% resistance, with no sensitivity. The only glimmer of hope lies in the Cephem group antibiotic Cefixime (100% susceptible) and the Carbapenem group antibiotic Meropenem (77.78% susceptible). Additionally, the Fluoroquinolone, Cephalosporin, and Penicillinase+β-lactamase groups show moderate sensitivity at 66.67% susceptible isolates. The analysis of the antibiotic susceptibility profile of the fourth and last microbial organism, Staphylococcus aureus, examined its response to 10 antibiotics from 7 different groups. The Tetracycline group displayed a 100% susceptibility rate, while the Macrolides group antibiotic Azithromycin showed the second highest percentage of sensitivity at 93.33% of isolates. Additionally, the Amphenicol and Oxazolidinones groups of antibiotics exhibited relatively high percentages of sensitive isolates, although slightly lower than the Tetracycline and Macrolides groups. Both Chloramphenicol and Linezolid had the same percentage of sensitive isolates at 86.67%. In contrast, Erythromycin and Kanamycin demonstrated intermediate levels of sensitivity at 53.33% and 46.67% of isolates, respectively. Notably, the Fluoroquinolone group antibiotics exhibited the highest resistance, with Ciprofloxacin at 100% and Levofloxacin at 86.67% resistance among the isolates. In this study, also examined the prevalence of multidrug-resistant microbial contaminants in flaxseed samples, as flaxseed has been identified as a potential source of antibiotic-resistant bacteria. The results demonstrate that 64.29% of E. coli and 36.36% of Pseudomonas aeruginosa isolates were multidrug-resistant. However, the situation is more concerning when 100% of the Klebsiella pneumoniae isolates were multidrug resistant. In contrast,

Staphylococcus aureus showed no multidrug resistance which is a more favorable finding compared to the high levels of resistance observed in the other pathogens studied in this thesis research.

Chapter 5

Conclusion

To conclude, this study shows the high prevalence of multidrug-resistant microbes in flaxseed samples is a significant concern, as these strains exhibit resistance to multiple antimicrobial agents, limiting treatment options and increasing the risk of severe outcomes. Furthermore, the study highlighted the importance of educating the public about the potential dangers of microbial contamination in herb seeds and the need for proper storage and handling to minimize these risks. The results of this research have the potential to influence public health policies and regulations related to the sale and distribution of flaxseeds in Dhaka City, ultimately contributing to a safer and healthier environment for consumers.

Chapter 6

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