PREVALENCE OF BACTERIAL CONTAMINATION IN MIXED VEGETABLE SALAD IN DHAKA CITY

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By

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ABSTRACT

The research work was undertaken with a view to isolate and identify bacteria from mixed vegetable salad sold within Dhaka city with special emphasis on *E. coli*, *Salmonella.* and *Staphylococcus* .The samples were analysed to isolate and identify the bacterial species by continual cultural, morphological, staining and biochemical tests, the microbial load such as Total viable count (TVC) & Total coliform count (TCC) and the prevalence of bacteria and antimicrobial-resistant pattern of isolates was investigated by the disc diffusion method. A total number of 120 mixed vegetable salad samples were collected from different restaurants, food corners and street vendors of different locations of Dhaka City. *Escherichia coli* showed metallic sheen on EMB agar and produced rose pink colonies on MacConkey agar, positive to Indole, MR and catalase test but negative to VP test. It was revealed as gram negative, small rod shaped, arranged in single or pair shaped under microscope. *Salmonella spp.* were identified by observing black smooth colony on SS agar and pinkish red smooth colony on BGA agar. They showed positive to MR, catalase test and negative to Indole and VP test and revealed gram negative, short rod shaped in gram staining. *Staphylococcus* species showed β haemolysis, fermented mannitol salt agar and produced yellowish colonies. The catalase, MR, VP and indole test was positive and these cocci arranged in grapes like clusters. The prevalence was 10%, 5% and 4.1% for *E. coli, Salmonella* and *Staphylococcus* respectively. The antimicrobial sensitivity test indicated that all bacterial species were resistant to Ampicillin, Amoxicillin, Cefixime and Tetracycline.

Key word: *E. coli, Salmonella, Staphylococcus,* Isolation, Identification, Prevalence

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LIST OF ABBREVIATIONS	
AMP	Ampicillin
AMX	Amoxicillin
AFSSA	French Agency for Food Safety
APHA	American Public Health Association
BA	Blood agar
BAX	B-cell lymphoma associated X
BGA	Brilliant Green Agar
°C	Degree Celsius
cfu/g	Colony forming unit/gram
CFM	Cefixime
CTR	Ceftriaxone
Conc.	Concentration
EFSA	European Food Safety Authority
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EMB	Eosin Methylene Blue agar
et al.	Associated
FAO	Food and Agriculture Organization
GEN	Gentamicin
H_2O_2	Hydrogen per oxide
HCL	Hydrogen chloride
I	Intermediate
ISO	International Organization for Standardization
KCL	Potassium chloride
KH_2PO_4	Potassium hydrogen phosphate
MC	MacConkey agar
MDR	Multi drug resistant
MPV	Minimally processed vegetables
MSA	Mannitol salt agar

LIST OF ABBREVIATIONS

MR	Methyl-Red
ml	Millilitre
NA	Nutrient agar
NaCl	Sodium Chloride
Na_2HPO_4	Di-sodium hydrogen phosphate
NB	Nutrient broth
No.	Number
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	quantitative PCR
RTE	Ready to eat'
R	Resistant
S	Streptomycin
S	Susceptible
SS	Salmonella-Shigella agar
SAU	Sher-e-Bangla Agricultural University
SD	Standard deviation
SEA	Staphylococcal enterotoxin A
SEI	Staphylococcal enterotoxin I
SEG	Staphylococcal enterotoxin G
STEC	Shiga toxin-producing *E. coli*
SPP.	Species
spvR	Successive Partial Variation Relaxation
TVC	Total viable Count
TCC	Total Coliform Count
TE	Tetracycline
VP	Voges-Proskauer broth
Vol.	Volume
WHO	World Health Organization

LIST OF ABBREVIATIONS	
≥	Greater than or equal
µg	Microgram
%	Percentage
/	Per
+Ve	Positive
-Ve	Negative

CHAPTER -1

INTRODUCTION

Vegetables are important components of a healthy and balanced diet. They provide an extraordinary dietary source of nutrients, micronutrients, vitamins, and fibre for humans and are thus vital for health and well-being (Slavin and Lioyd,2012). Within the past decade and because of intensified public awareness of the benefits of healthy food, attention on vegetables as a vital dietary component has significantly increased their consumption. Moreover, health agencies in many countries as World Health Organization (WHO), European Food Safety Authority (EFSA), Food and Agriculture Organization (FAO), and French Agency for Food Safety (AFSSA) encourage their consumption to protect against a range of illnesses such as cancers and cardiovascular diseases (Olaimat and Holley, 2012).

Vegetables consist of a range of plant parts (leaves, roots, tubers, fruits, and flowers). A vegetable is a tender plant part that is not sweet and may be flavoured or spiced with condiments before consumption. These plants or plant parts may be eaten raw as salad or added to some cooked foods like rice (Abdullahi and Abdul, 2010).

Fresh vegetables easily get exposed to potential microbial contamination during harvesting, washing and transportation, packaging, storing and selling. In addition, the processing of vegetables as salad may alter or increase the number and type of pathogens present on the surface of the product. With this aspect, exposure to pathogens, vegetables have been associated with the outbreaks of food borne disease in many countries.

Common vegetables utilized as salad consists of cucumber, onions, tomatoes, lettuce, carrots, spring onions, green pepper, radish, and other ingredients which include olives (Meldrum *et al.,* 2009). Nowadays, salads have become much popular among people because of health-related benefits. The major part of salads consists of raw vegetables. They offer a great variety of fibre contents, vitamins, minerals, and other phytochemicals that functions like a good supply of antioxidants as well as phy-tonutrients for the enhancement of human health (Aycicek *et al.,* 2006).

Ready to eat' (RTE) is defined as the status of the food is ready for immediate consumption at the point of sale. Ready-to-eat salads (RTES) are low-calorie convenience foods of good dietary value. RTES technology implies procurement of raw

vegetables from different local markets, cutting, sorting, washing, drying, packaging in permeable plastics, and retailing in a cold chain regime. The increased consumption of RTE fresh vegetables, in particular, leafy greens, which are used in salad mixtures that are consumed raw has increased the chance of foodborne illnesses associated with these products in different regions of the world. These are increasingly being recognized as important vehicles for the transmission of human pathogens that were traditionally associated with foods of animal origin (Castro-Rosas *et al.,* 2012).

Foodborne illness is a major public health concern worldwide in terms of the number of persons affected and economic cost. An estimated 600 million almost one in 10 people in the world fall ill after eating contaminated food and 420 000 die every year, resulting in the loss of 33 million healthy life years (Aycicek *et al.,* 2006). Most people will experience a foodborne disease at some point in their lives. This highlights the importance of making sure the food we eat is not contaminated with potentially harmful microorganisms, toxins, or chemicals. Food can become contaminated at any point during production, distribution, and preparation. Everyone along the production chain, from producer to consumer, has a role to play to ensure the food we eat does not cause diseases (WHO, 2016).

The predominant bacterial types found on vegetables are lactic acid bacteria, *Corynebacterium, Enterobacter*, *Proteus, Micrococcus*, *Enterococcus, Pseudomonas* and spore-formers. They may also possess different types of moulds, such as *Alternaria, Fusarium*, and *Aspergillus* growing on their surface (Sagoo *et al.,* 2001). Vegetables can be contaminated by enteric pathogens if the animal or human wastes and polluted water are used for fertilization and irrigation.

Raw vegetables may be bruised during processing and distribution resulting in the release of plant nutrients which may serve as the essential organic and inorganic substrates for microorganisms. A variety of microorganisms including pathogens can be introduced from the surface of vegetables during the processing of fresh vegetable salad. A large number of pathogenic microorganisms including *Salmonella, Escherichia* coli, *Staphylococcus*, *Bacillus anthracis, Mycobacterium* spp., *Brucella* spp., *Listeria monocytogenes*, *Yersinia enterocyte*, *Clostridium perfringens*, *Klebsiella* spp. and *Mycobacterium paratuberculosis* have been reported to be associated with contamination of vegetables (Sagoo *et al.,* 2001) Many bacterial diseases such as diarrhoea, anthrax, salmonellosis, listeriosis, Crohn's disease and arthritis have been

reported to be caused through consumption of contaminated vegetable salad by pathogenic microorganisms (Fröder *et al.,* 2007)

Vegetables can be contaminated from different environmental sources, such as soil, water, insects, air, birds, animal or equipment during cultivation and marketing. Pathogens present in contaminated foods may harbour virulence genes, toxins and enzymes, which aids in the pathogenesis of infectious diseases (Faour-Klingbeil *et al*., 2006).

Many foodborne illness outbreaks in numerous countries have been associated with the consumption of contaminated fresh vegetables. Hence, many consumers question the quality and safety of these foods. Problems linked with pathogens in fresh produce, including the associated public health and trade implications, have been previously reported in many countries worldwide (Castro-Rosas *et al.,* 2012) The quality of RTE leafy greens have been surveyed in the United Kingdome (UK) (Sagoo *et al*., 2001) and Brazil (Cray and Moon, 1995). Most of the reported foodborne outbreaks originate from Europe, North America, Australia, and New Zealand, as these locations have well-developed epidemiological surveillance systems. In developing countries, paucity of data on food safety together with inaccessibility to safe water, lack of agricultural infrastructures, and limitations to implementing good agricultural practices are persistent challenges (Cray and Moon, 1995). Foodborne disease outbreaks originating in prepared raw green vegetable salads were more likely to occur on commercial food service premises than outbreaks from other sources, with restaurants and hotels accounting for almost 75% of outbreaks. Several outbreaks have been associated with the consumption of the products from salad bars (Gómez *et al.,* 2013).

Food habit in Bangladesh has been drastically changed in recent years. Nowadays the tendency of eating out is increasing although the food typically handled, arranged, and sold at roadside eateries and other open sites are generally unhygienic. Factors encouraging the people to eat outside of the home may be the participation of females in the job sector, lifestyle-changing tendency, overtime working, away from home when voyaging, fast urbanization and craving for advanced education and research.

In Bangladesh, food borne enteric disease is responsible for one-third of childhood deaths each year from diarrhoeal diseases (Gómez *et al.* ,2013). Coliforms are usually indicators of intestinal contaminants from man and animals. This may not be too

surprising, as most often the source of watering the gardens is usually sewage from domestic sources and runoff water, which is mostly used for irrigation purposes in some communities. If the counts are high, then they pose dangers to consumers ((Abdullahi and Abdul, 2010). On the other hand, human pathogens such as *Escherichia coli* and *Salmonella spp.* are among the greatest concerns during food-related outbreaks. Several cases of typhoid fever outbreaks have been associated with eating contaminated vegetables grown in or fertilized with contaminated soil or sewage (WHO, 2016).

Dhaka is a densely populated area in Bangladesh and is occupied by several restaurants, food corners and street vendors. It is well known that most food vendors in Dhaka city use the same tools for meat and vegetable processing and bare hands for serving vegetable salad and other foods. So, it is essential to determine the restaurant's workers contribution to the microbial load of mixed vegetable salads in and around restaurants and other food corners of Dhaka city. *Staphylococcus, E. coli*, and *Salmonella* are very common and great indicators of unhygienic food handling, temperature misuse, and cross-contamination (Goburdhun *et al.*, 2019).

Microbial studies on mixed vegetable salad in Bangladesh generally revealed the association of a huge pathogenic load. Several reports showed the growth and proliferation of pathogenic bacteria causing enteric diseases (Goburdhun *et al.,* 2019).

Besides such spoilage threat, a major consideration in the medication of diseases has to be brought into the development of drug-resistance of the pathogens against commonly used antibiotics (Gómez *et al.,* 2013).

This study was performed to assess the bacteriological quality of mixed vegetable salad collected from restaurants, food corners and street vendors in Dhaka to determine the presence of pathogenic bacteria with special emphasis on *Staphylococcus, Salmonella,* and *E. coli* in mixed vegetable salad and find out the antibiotic-resistant profile of bacteria.

So, keeping the above facts in mind, the present study was designed with the following objectives

- ❖ Isolation and identification of, *E. coli*, *Salmonella* and *Staphylococcus* bacteria from mixed vegetable salad
- ❖ Prevalence of bacterial load in supplied mixed vegetable salad
- ❖ Antimicrobial resistance pattern (AMR) of identified bacteria.

CHAPTER -2

REVIEW OF LITERATURE

The review of literature has been conveniently brought to obtain the distinct evidence of key information is conducted by other workers relevant to the research study undertaken. For better presentation, the literature related to the present study has been reviewed under the following subheadings.

2.1. Isolation and identification of *E. coli, Salmonella* and *Staphylococcus* bacteria

2.1.1 Isolation, Identification and Characterization of *Escherichia. Coli*

Merchant and Packer (1967), described that *E. coli* is a gram-negative rod-shaped, varying from coccoid bipolar shape to long filamentous form.

Ali *et al.*(1998); identified the colony characteristics of *E. coli*. The authors found metallic seen on EMB agar, rose pink colony on McConkey agar and pinkish colony on SS agar.

Thomas (1998), performed some biochemical tests for *E. coli* and *E. coli* ferment lactose, reduced nitrate and MR.

Parma *et al.*(2012); described that entero haemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin-producing *E. coli* (STEC) associated with a broad spectrum of diseases that includes diarrhoea, haemorrhagic colitis and a life-threatening haemolytic-uremic syndrome (HUS). Regardless of serotype, Shiga toxins (Stx1 and/or Stx2) are uniformly expressed by all EHEC and exploitable targets for laboratory diagnosis of these pathogens.

Margot *et al,* (2013); developed various real-time PCR-based methods enabling the detection of Shiga toxin genes that have been developed and used for applications in food microbiology. The present study was conducted to evaluate the reliability of seven commercially available real-time PCR systems for the detection of stx1 and stx2 subtypes.

Reuben and Makut (2014); investigated the occurrence of *E. coli* O157:H7 in vegetable salad sold in Lafia metropole is, Nigeria. 40 vegetable salad samples (Spinach, cabbage, cucumber, and bitter leaf) used for the study were collected from markets and farms in the Lafia metropolis. Strains of *Escherichia coli* O157:57 was isolated bacteriologically employing cultural techniques (involving enrichment on modified E.C broth

supplemented with novobiocin (mEC+n) and selective plating on Cefixime Tellurite sorbitol McConkey Agar), biochemical characterization (Microbact 12E) and serological assays (Oxoid diagnostic kit, latex R30959601).

Kim *et al.*(2014); determine the presence of six virulence factors (*stx* 1, *stx* 2, *lt, st, eae*A, and *ial*) in *E. coli* isolated from fresh vegetable products which provide information on risk assessment of pathogenic *E. coli* in Korea from 416 no. of collected samples including vegetable salad mix, sprouts, baby leaf vegetables, and unpasteurized fruit and vegetable juices which commercially available in Korea. A total of 30 no. of samples were positive for *E. coli* strains, resulting in an overall prevalence of 7.2%. Of the 120 *E. coli* isolates, only one isolate (0.8%), which was obtained from unpasteurized fruit and vegetable juices, was confirmed to possess the *eae* A gene but lacked *stx* genes. This study showed that some fresh vegetable product samples were contaminated with enteropathogenic *E. coli*.

Abubakari *et al.* (2015); experimented with a total of 270 vegetable salads that were aseptically collected from vendors and transported on ice to the laboratory to determine the presence of total *coliforms* and *E. coli* (*E. coli* O157:H7) using standard microbiological methods. This study was carried out to assess the distribution of *E. coli* O157:H7 in the vegetable salad from restaurants and street food vendors from the Kumasi Metropolis.

Mritunjay and Kumar (2017), evaluated the microbiological quality of mixed vegetable salad consumed in Dhanbad city, India. A total of 480 no. of samples from 8 different restaurants were examined for the microbial quality in terms of aerobic mesophilic, psychotropic counts, yeast, mould and total coliform levels. *E. coli* O157:H7, *E. coli* was detected in 16.7% of the total samples. Pathogenic microorganisms such as *E. coli* O157:H7 were detected in the sample

Akbari *et al.* (2018); worked on a total of 30 no. of vegetable salad samples purchased from (4) zones of the district and then transported to the Spanish Laboratory of the University for the Development Studies, Ghana for analysis. Standard microbiological methods that were following American Public Health Association (APHA) were used to determine the presence and levels of bacteria in the vegetable salad samples. *Escherichia coli* were detected in 96.7% of vegetable salad samples with levels ranging from 0 to 7.56 log10 cfu/g.

2.1.2 Isolation, Identification and Characterization of *Salmonella spp.*

Froder *et al.* (2007); investigated that the increasing demand for fresh fruits and vegetables and convenience foods caused an expansion of the market share for minimally processed vegetables. Among the more common pathogenic microorganisms that can be transmitted to humans by these products are *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella.* The study aimed to evaluate the microbial quality for the selection of minimally processed vegetable salad. A total no. of 181 samples that minimally processed. Leafy salads were collected from retailers in the city of Sao Paulo, Brazil. Counts of total *coliforms*, *faecal coliforms, Enterobacteriaceae,* psychotropic microorganisms, and *Salmonella* were conducted for 133 samples. *L. monocytogenes* was assessed in 181 no. of samples by using the BAX System and by plating method to enrich the broth onto Palcam and Oxford agars. The suspected *Listeria* colonies were submitted to classical biochemical tests. Populations of psychotropic microorganisms $>10^6$ cfu/g were found in 51% of the 133 samples, and *Enterobacteriaceae* populations between 10^5 and 10^6 cfu/g were found in 42% of the samples. *Faecal coliform* concentrations were higher than 10^2 cfu/g (Brazilian standard) that found in 97 (73%) of the samples, and *Salmonella* was detected in 4 (3%) of the samples. Two of the *Salmonella*-positive samples had $<10^2$ cfu/g concentrations of *faecal coliforms.*

Abadias *et al.* (2008); was researched the fresh and minimally-processed fruit and vegetables, and sprouts in several retail establishments in the Lleida area (Catalonia, Spain) during 2005–2006 to determine the microbial contamination. Particular potential pathogenic bacteria were present under these commodities. A total of 300 samples including 21 no. of ready-to-eat fruits, 28 no. of whole fresh vegetables, 15 no. of sprout samples and 237 no. of mixed vegetable salads containing from one to six vegetables were purchased from 4 restaurants. They were tested for mesophilic and psychotropic aerobic counts, yeasts and moulds, lactic acid bacteria, Enterobacteriaceae, presumptive *E. coli* and *Listeria monocytogenes* counts as well as for the presence of *Salmonella*, *E. coli* O157:H7, *Yersinia enterocolitica* and thermotolerant *Campylobacter*.

Meldrum *et al.* (2009); reported the purpose of this study was to establish the microbiological safety of vegetable salad and sauces served in kebab take-away restaurants. Comparison with published microbiological guidelines revealed that 4.7% of 1213 no. vegetable salad samples were of unsatisfactory microbiological quality due

to *Escherichia coli* and/or *Staphylococcus aureus* levels at $\geq 10^2$ cfu/g. Another 0.3% of salad samples were of unacceptable quality due to *S. aureus* at $\geq 10^4$ cfu/g (2 samples) or the presence of *Salmonella* Kentucky (1 sample). Cucumber was the most contaminated vegetable salad with regards to unsatisfactory levels of *E. coli* (6.0%) or *S. aureus* (4.5%). Five percent of 1208 sauce samples were of unsatisfactory microbiological quality due to *E. coli*, *S. aureus* at $\geq 10^2$ cfu/g and *Bacillus cereus* at $\geq 10^4$ cfu/g. A further 0.6% of sauce samples were of unacceptable quality due to *Bacillus* spp. (*Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*) at $\geq 10^5$ cfu/g or the presence of *Salmonella*.. More samples of chilli sauce (8.7%) were of unsatisfactory or unacceptable microbiological quality than any other sauce type. The results emphasize the need for good hygiene practices in kebab take-away restaurants handling these types of ready-to-eat products.

De Giusti *et al.* (2010); found that *Salmonella* was not detected in any vegetable salad samples using both cultural and molecular methods. On the contrary, 2/265 (0·75%) of the whole vegetable salad samples were positive for *Salmonella*, with the molecular method and in one sample the presence of *Salmonella* was also confirmed by the cultural method.

Sant *et al.* (2011); provided that minimally processed vegetable salad may be important vehicles of *Salmonella* spp. and cause disease. This study aimed at detecting and enumerating *Salmonella s*pp. in MPV marketed in the city of São Paulo, Brazil. A total of 512 samples of packages collected in retail stores were tested for *Salmonella*, total coliforms and *Escherichia coli* as an indication of the hygienic status. *Salmonella* spp. was detected in four samples, two using the detection method and two using the counting method, where the results were 8.8×10^2 cfu/g and 2.4×10^2 cfu/g. The serovars were *Salmonella typhimurium* (three samples) and *Salmonella enterica* sub-sp. *enterica* O:47:z4,z23 (one sample). Fourteen samples (2.7%) presented counts of *E. coli* above the maximum limit established by the Brazilian regulation for MPV (10^2 cfu/g). Therefore, tightened surveillance and effective intervention strategies are necessary in order to address consumers and governments concerns on the safety of vegetable salad.

Ahmed *et al.* (2012); investigated that *Salmonella* isolated by the conventional cultural method based on pre-enrichment, enrichment and plating on some selective and

different media. Suspected colonies were then confirmed by the different biochemical tests.

2.1.3 Isolation, Identification and Characterization of *Staphylococcus spp.*

Freeman *et al.* (1979); stated that *Staphylococcus aureus* is a lactic acid fermenter and majorities are coagulase positive. They produce only acid not gas from glucose fermentation.

Uzeh *et al.* (2009); stated that mixed vegetable salad and salad ingredients like carrots, cucumber, cabbage, and lettuce were analysed for their microbiological quality. The microorganisms isolated from vegetable salads and salad ingredients include *Citrobacter fruendii, Proteus vulgaris, Proteus mirabilis, Staphylococcus aureus, Mucor, Aspergillus fumigatus, Trichoderma, Neurospora crassa* and *Aspergillus niger. Corynebacterium* was isolated from only salad samples collected from open markets. *Pseudomonas aeruginosa* and *Rhizopus* were isolated from only salad ingredients. More micro-organisms occurred in vegetable salad obtained from the open markets than in salad obtained from fast food outlets.

Seo *et al.* (2010); experimented on *a* minimally processed vegetable salad that is often contaminated with enterotoxigenic strains of this bacterium. The paper reports the results of a 3-year survey (2006–2008) on the occurrence of *S. aureus* in minimally processed vegetables and sprouts. Of 345 examined samples, 40 samples (11.6%) were contaminated with *S. aureus*. A total of 25 enterotoxigenic *S. aureus* strains were bio typed and their resistance to antibiotics was examined. Most isolated strains produced Staphylococcal enterotoxin A (SEA) (*n*=23) followed by Staphylococcal enterotoxin I (SEI) and Staphylococcal enterotoxin G (SEG) and mainly belonged to the human biotype (88%). At least 96.1% of the analysed strains showed antibiotic resistance properties, while 56% of the analysed strains exhibited multiple antibiotic resistance to the antibiotics tested. Two of the analysed strains were resistant to methicillin. Moreover, a strain that had multi-resistance to 6 antibiotics was found. The results indicate that enterotoxigenic, antibiotic-resistant strains of *S. aureus* are widely proliferated in minimally processed vegetables and sprouts.

Abdullahi and Abdul (2010); stated that *Staphylococcus aureus* was the predominant bacteria isolated from the vegetable salad. The counts were obviously above the recommended standards for mixed vegetable salad, especially coliforms which should

be less than 10 coliform bacteria per gram. It is recommended that hygiene officials are required to take an inspection of what is offered to consumers and specify acceptable handling practices.

Moayed *et al.* (2013); investigated that in India out of 50 samples, 86 bacterial pathogens s were *E. coli* (38.3%), followed by *Enterobacter aerogenes* (20.9%), *Pseudomonas* (16.2%), *Staphylococcus aureus* (15.1%), *Salmonella* (5.8%) and *Shigella* (3.4%) were isolated. The authors suggest strong washing of vegetable salad with sanitary running water before consumption. This study mentioned that the main bacterial pathogen was *E. coli,* followed by *Staphylococcus aureus.*

Sabbithi *et al.* (2014); had investigated the microbiological quality of salads served along with street foods of Hyderabad. They had collected 163 vegetable salad samples from four different zones of Hyderabad among which 53 were carrot and 110 were onion samples. About 74% and 56% had *Staphylococcus aureus* in carrots and onions, respectively. Fifty-eight percent of carrots and forty-five percent of onions samples contained *Salmonella*, 68% of carrots and 24% of onions had *Yersinia*.

Amoah*,* (2014); found that *S. aureus* and *Salmonella* are among the bacteria commonly reported to be associated with contamination of vegetable salads, The *S. aureus* and *Salmonella spp.* are transmitted into food mainly through improper food handling, temperature abuse and cross-contamination during food preparation. The (KNUST) Kwame Nkrumah University of Science and Technology and the (UEW-K) University of Education, Winneba, Kumasi campus have identified among the areas of Kumasi metropolis with 6 high population of food vendors and restaurants The research indicated that most food vendors in Kumasi use the same knife and chopping board for meat and vegetables as well as bare hands for serving ready to eat vegetable salad and other foods. The need to determine the contribution of canteen workers to the microbial contamination of ready to eat mixed vegetable salads in KNUST and UEW-K and their environs using *S. aureus* and *Salmonella spp.,* pathogens that are very good indicators of improper food handling, temperature abuse and cross-contamination.

Saifullah *et al.* (2018); had identified 54 samples out of 100 samples contaminated with *S. aureus*. Out of 54 *S. aureus* isolates, 32 (59%) were coagulase-positive and 22 (40%) were coagulase-negative. Enumeration of coagulase-positive *S. aureus* samples revealed that only10% (n=3) of the total collected samples were within the level of

acceptance<20 cfu/g while 40% (n=12) sample was almost on the threshold of borderline (20<10^4 cfu/gm). Whereas 50% (n=17) samples were at Unacceptable level (>10^4 cfu/gm). Furthermore, higher *S. aureus* contamination of fresh food samples was observed during the early summer season.

2.2 Prevalence of bacterial load in mixed vegetable salad

Saddik *et al. (*1985); had tested thirty-six samples for *Staphylococcus aureus. Salmonella* was isolated from two samples of green leafy vegetables (greens) and one sample of mixed vegetable salad that most likely contained greens. *Shigella* was isolated from one sample of greens, one sample of parsley, and three samples of mixed salads. Most samples of raw vegetables and salads were at either room or outside temperature just before sampling. Eighty percent of the samples had aerobic colony counts of more than 10^6 cfu/g. Three of 36 samples contained 1×10^3 *S. aureus*/g.

Khan *et al.* (1992); stated that vegetable salad is mostly consumed fresh and act as an effective source for the transmission of various pathogens. Fresh samples of cucumber, carrot and lettuce were collected from different markets in Dhaka metropolitan city. Bacterial loads were found to be $7 \cdot 1 \text{x} 10^4$ to $6 \cdot 34 \text{x} 10^8$ colony-forming unit (cfu/100gm). *Escherichia coli, Klebsiella, Enterobacter*, and *Serratia* were amongst the coliforms (lactose fermenters) while P*seudomonas, Shigella* and *Acinetobacter* were non-lactose fermenters associated with the vegetable salad samples.

Tambekar and Mundhada (2006), total 50 no. of vegetable salad samples were analysed and 86 no. of bacterial pathogens were isolated. Among them, *Escherichia coli* was found to be predominant (38.3%), followed by *Enterobacter aerogenes* (20.9%), *Pseudomonas* sp. (16.2%)*, Staphylococcus aureus* (15.1%), *Salmonella* sp. (5.8%) and *Shigella* sp. (3.4%). The presence of *Escherichia coli, Enterobacter aerogenes* and *Pseudomonas* sp. is observed on all samples of vegetable salad. This study revealed the potential hazard of street vended vegetables salad. Therefore, needs vigorous washing of vegetables with safe running water before consuming to reduce the number of microorganisms. Vegetables mostly get contaminated with pathogenic microorganisms during growing in fields or orchards and harvesting, post-harvesting handling, processing and distribution.

Geimba *et al.* (2004); were isolated *Salmonella* strains from foods involved in food borne disease outbreaks in the Rio Grande do Sul State, Brazil, during 1999 and 2000

were studied. Strains were serotyped and submitted to PCR analysis to verify the prevalence of the *Salmonella* plasmid virulence (spvR) regulatory gene. Among the 75 no. of isolates, 73 (97%) were classified as *Salmonella enterica* serovar Enteritidis. All of the *Salmonella* strains isolated in 1999 were classified as serotype Enteritidis, whereas in 2000 two isolates were serotyped as *Salmonella* Derby and *Salmonella Typhimurium*. Regarding the prevalence of the spvR gene, 62 strains (82.7%) were PCR positive, and a positive correlation ($P < 0.05$) between the strains of *Salmonella Enteritidis* and the presence of the spvR gene was demonstrated, which suggests that this gene is a characteristic of the *Salmonella Enteritidis* analysed.

Izumi *et al.* (2004); was conducted a study to evaluate bacterial flora in vegetable salad items and for isolation, identification, characterization and antibiogram studies of the organisms obtained. For this, a total of 90 samples from mixed vegetables which are commonly used for salad such as tomato, lemon, green chilli, coriander leaf, carrot and cucumber were collected from five different markets located in Mymensingh city. All the vegetables were highly contaminated with bacterial flora. Range of microbial count of tomato was log 6.276 cfu/ml to 6.543 cfu/ml, lemon was log 5.493 to 6.261 cfu/ml, green chili was log 5.205 to 5.64 cfu/ml, coriander leaf was log 7.055 to 7.759 cfu/ml, carrot was log 6.786 to 7.221 cfu/ml and cucumber was log 5.469 to 6.845 cfu/ml respectively.

Froder *et al.* (2007); a total of 181 samples of minimally processed leafy salads were collected from retailers in the city of Sao Paulo, Brazil. Counts of total coliforms, faecal coliforms, *Enterobacteriaceae*, psychotropic microorganisms, and *Salmonella* were conducted for 133 samples. *L. monocytogenes* was assessed in 181 samples using the BAX System and by plating the enrichment broth onto Palcam and Oxford agars. Suspected Listeria colonies were submitted to classical biochemical tests. Populations of psychotropic microorganisms >10^6 cfu/g were found in 51% of the 133 samples, and Enterobacteriaceae populations between 10^5 and 10^6 cfu/g were found in 42% of the samples. Faecal coliform concentrations higher than 10^2 cfu/g (Brazilian standard) were found in 97 (73%) of the samples, and *Salmonella* was detected in 4 (3%) of the samples. Two of the *Salmonella*-positive samples had <10^2 cfu/g concentrations of faecal coliforms. L. monocytogenes was detected in only 1 (0.6%) of the 181 samples examined. This positive sample was simultaneously detected by both methods. The other *Listeria* species identified by plating were *L. welshimeri* (one sample of curly

lettuce) and *L. innocua* (2 samples of watercress). The results indicate that minimally processed vegetables had poor microbiological quality, and these products could be a vehicle for pathogens such as Salmonella and L. monocytogenes.

Abadias *et al.* (2008); was done a microbiological analysis in order to identify and enumerate *faecal coliforms*, *Escherichia coli*, *Enterococcus* and *Pseudomonas*. These results indicated that, for tomatoes and endives, the average load was $1.5x10^4$ cfu/g of *Enterococcus* $1.3x10^3$ cfu/gm of *Pseudomonas* and $1.7x10^2$ cfu/g of *faecal coliforms*. In mixed salad products, the load was $9.3x10^2$ cfu/g for *Enterococcus*, $1.03x10^1$ cfu/g for *Pseudomonas* and $9.9x10^1$ cfu/g for *faecal coliforms*.

Meldrum *et al.* (2009); revealed that 4.7% of 1213 no. of vegetable salad samples were of unsatisfactory microbiological quality due to *Escherichia coli* and/or *Staphylococcus aureus* levels at $\geq 10^2$ cfu g^{-1}. Another 0.3% of salad samples were of unacceptable quality due to *S. aureus* at $\geq 10^4$ cfu g^{-1} (2 samples) or the presence of *Salmonella* Kentucky (1 sample). Cucumber was the most contaminated salad vegetable with regards to unsatisfactory levels of *E. coli* (6.0%) or *S. aureus* (4.5%).

Khiyami *et al.* (2011); Microbial quality of minimally processed vegetable salads (Tabbouleh, Fattoush, Hummus, Mutable and Caesar) being served in restaurants and homes in Riyadh were evaluated to ascertain that they are safe for human consumption and are free from potential food-borne pathogens. The samples were assessed for the presence of total aerobic bacterial plate count, total *coliforms, Escherichia coli, Salmonella*, and *Shigella*. The total aerobic plate count for salad prepared in the restaurants was around 2 -4.5 $x10^5$ cfu/g, however, in homemade salads, the count was 2-8 x 10^4 cfu/g. The total coliform counts in restaurants salad were around 2-8 x 10^4 cfu/g as compared to 2-4.8 x 10^3 cfu/g of homemade salads. All salads, except Caesar, recorded *E. coli* and *Enterobacter aerogenes,* while *Shigella* and *Salmonella* were present in a few samples.

Itohan *et al. (2011)*; The vegetable salad showed a wide variation in total viable count ranging from 1.6 x 10^6 to 2.9 x 10^8 cfu/g at 37°C. Cucumber samples had the lowest bacterial load. The highest total viable count for cucumber samples was gotten from samples sourced from Lugbe with a load of 2.4 x10^7 cfu/g while those sourced from Kuchingoro had the lowest load of 1.6 x 10^6 cfu/g.

Nigad *et al.* (2011); said the retail markets and super shop vegetables showed no difference in terms of *Coliform* and *faecal coliform* (more than 1100 cfu/100 ml). The range of microbial count of Tomato was 9.0×10^5 cfu/g to 3.8×10^4 cfu/g, Cucumber was 5.5×10^5 cfu/g to 1.9×10^5 cfu/g, Carrot was 1.2×10^4 to 2.6×10^5 cfu/g. Eight types of vegetables which are commonly used for salad i.e., Tomato, Cucumber, Carrot, Green chilli, Lemon, coriander leaf, Pepper mint, Beet root were collected from two Open markets and two Super shops of Chittagong City. All the vegetables were highly contaminated with *Coliform* and *faecal Coliform* (> 1100 cfu/100ml). The range of microbial count of Tomato was 9.0×10^5 cfu/ml to 43.8×10^5 cfu/g, Cucumber was 5.5×10^6 cfu/g to 1.9×10^6 cfu/g, Carrot was 1.2×10^4 to 2.6×10^6 cfu/g, green chilli was 1.0×10^4 to 4.0×10^5 cfu/g, Lemon was 1.5×10^5 to 1.2×10^6 cfu/g, Coriander leaf was 5.87×10^7 to 1.8×10^4 cfu/g, Peppermint was 2.2×10^4 to 7.7×10^5 cfu/g and it was 5.0×10^3 to 5.4×10^4 cfu/g for Beet root.

Nawas *et al.* (2012); this study aimed at examining the microbial quality of restaurant salad and the water used for salad preparation and their role as a source of antibiotic-resistant bacteria. Samples were collected from 15 no. of different restaurants located in Chittagong city. The range of Total Viable Count was 1.86×104 to7.28×105 cfu/g and 1.60×104 cfu/ml to 4.38×10^5 cfu//ml for salad and water respectively. Total *coliform* and *faecal coliform* count > 1100 cfu/100 ml were found in 73.33% of salad and 33.33% water samples. *Salmonella* was present in 46.67% of restaurants vegetable salad and water. Vibrio was present in 66.67% of vegetable salad and 53.33% of water.

Rahman and Noor (2012), worked on the microbial quality of common vegetable salad (carrot, cucumber, tomato and lettuce) collected from the Dhaka metropolis was analysed to detect the presence of bacterial pathogens. The occurrence of large numbers of *faecal coliforms* (1.0×10^4-4.09×10^6 cfu/g), *Escherichia coli* (1.0×10^6 - 5.0×10^8 cfu/g), *Staphylococcus aureus* (2.0×10^5 - 5.95×10^7 cfu/g), and *Listeria* spp. (1.5×10^6 - 6.5×10^7 cfu/g) were detected in all the tested samples. *Faecal coliforms, E. coli, Staphylococcus aureus* and *Listeria spp*. were found to be the most frequently proliferating pathogens in the vegetable salad samples. *Klebsiella* spp. was found only in tomatoes (3.0×10^5). A load of *Vibrio, Salmonella* and *Shigella* were found to be nil. However, upon enrichment, the number of these pathogenic bacteria was found significantly higher. Vibrio was estimated upon enrichment within a range of 2.0×10^4 - 8.3×10^7 cfu/g in carrot, lettuce and tomato samples, while *Salmonella* and *Shigella* were

found within a range of 1.0×10^3 - 3.1×10^7 cfu/g and 3.0×10^4 - 4.8×10^8 cfu/g respectively, in all samples.

Leon *et al.* (2013); stated that the presence of *coliform* bacteria, *faecal coliforms*, *Escherichia coli*, diarrhoeagenic *E. coli* pathotypes and *Salmonella* were determined in vegetable salads from restaurants in Pachuca city, Mexico. The vegetable salad was purchased from three types of restaurants: national chain restaurants (A), local restaurants (B) and small restaurants (C). Two restaurants for each A and B, and three for C, were included. Forty ready-to-eat samples were purchased at each A and B restaurant and 20 at each C restaurant among the total of 220 analysed samples, 100, 98.2, 72.3, 4.1 and 4.1% had *coliform* bacteria, *faecal coliforms*, *E. coli* and *Salmonella*, respectively. Identified pathogenic bacteria included enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC). The EPEC, ETEC and STEC were isolated each from 1·4% of samples. No *E. coli* O157:H7 were detected in any STEC-positive samples.

Denis *et al.* (2016); fresh fruits and vegetables have been linked to various foodborne illnesses outbreaks in different regions of the world, including in Canada. In light of rising concerns over the microbial safety of these commodities, the Canadian Food Inspection Agency conducted retail surveys to obtain information on the occurrence of bacterial pathogens in a wide range of produces available in the Canadian marketplace (local vs. imported, organic vs. conventional). Samples were collected across Canada over four years consisting of leafy vegetables, leafy herbs, green onions, cantaloupes, tomatoes and berries. These samples were analysed in ISO 17025-accredited laboratories for various bacterial pathogens (*Salmonella, Escherichia col*iO157, *Shigella, Campylobacter* and *Listeria monocytogenes*).

Mritunjay and Kumar (2017), stated that a total of 480 samples of 8 different mixed vegetable salads from the restaurant were examined for overall microbial quality in terms of aerobic mesophilic, psychotropic counts, yeast, mould and total coliform levels. *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. were detected by real-time polymerase chain reaction (qPCR) and subsequent isolation. Results showed that all the samples were found positive for total coliform; however, *E. coli* was detected in 16.7% of the total samples. Pathogenic microorganisms such as *E. coli* O157:H7, *L. monocytogenes* and *Salmonella spp*. were detected in 1.3, 3.5 and 4.0%, respectively, of the total samples.

Kuddus *et al.* (2017); aimed to determine microbial and parasitic contamination in a mixed vegetable salad served in restaurants of Hail city, KSA. A total of 25 different vegetable salads were collected aseptically from different restaurants of Hail city, Saudi Arabia which were analysed for total heterotrophs and coliforms along with parasites. The highest number of viable count was found in sample number R-2 (4.91 log cfu/g) and the least microbial loads (2.25 log cfu/g) were observed in sample number R-9. A significant microbial load was recorded in the samples.

Godwin *et al. (*2018) ; reported that in Ghana, *Escherichia coli* was present in 96.7%, *Bacillus cereus* was present in 93.3% of vegetable salad samples whereas *Salmonella* spp. and *Shigella* spp. were present in 73.3% and 76.5% of vegetable salad samples respectively. Intestinal parasites were also detected in 16%of unwashed samples and not in standard washed samples. There was a significant difference between vegetable salad after three successive washing. This study revealed the potential hazard of mixed vegetable salads.

Toe *et al.* (2018); determine the prevalence of *E. coli* with virulence genes in mixed vegetable salads sold in collective catering in Abidjan. A total of 436 strains of *E. coli* were isolated from 306 no. mixed vegetable salads and then identified biochemically and molecularly. It appears from the study that vegetable salads sold in collective catering in Abidjan are at risk for contamination by *E. coli* pathovars.

Waturangi *et al.* (2019); demonstrated that salad vegetables and fruits sold in Jakarta were contaminated with pathogenic *E. coli* including EPEC, EAEC, and ETEC. These bacteria were detected in four out of five regions of Jakarta. Despite the low prevalence of pathogenic *E. coli*, it indicates that the presence of pathogenic *E. coli* was evenly spread in many vegetables salad and fruits sold in Jakarta.

Iftekhar *et al.* (2020); stated that aerobic plate count was ranged from 7.73 ± 0.61 to 9.04 ± 0.26 log cfu/gm, *Staphylococcus* spp. from 4.64 ± 0.61 to 6.42 ± 0.53 log cfu/gm, *Salmonella* spp. from 4.75 ± 0.08 to 5.27 ± 0.53 log cfu/gm, and *E. coli* from 4.98 ± 0.20 to 6.66 ± 0.80 log cfu/gm in RTE salad from different restaurants of in and around of BAU campus, Mymensingh.

2.3. Antimicrobial Resistance Pattern (AMR) of identified bacteria

Osterblad *et al.*(1999); stated that antibiotic-resistant bacteria may readily penetrate the gastrointestinal tract of a vegetable consumer. Therefore, restaurants salad can be a

potential source of bacteria resistant to antibiotics. One hundred and thirty-seven vegetable samples were studied, Escherichia coli was rare. Sensitivity testing was undertaken only for isolates with different biotypes and antibiograms. No resistance was found to Cefotaxime, Aztreonam, Imipenem, Gentamicin, Nalidixic acid or Ciprofloxacin.

Bager and Helmuth (2001); stated that antimicrobial resistance is a global problem which becomes a worldwide public health issue now-a-day. Foods and environmental sources contain bacteria that are resistant to one or more microbial drugs used for both human and veterinary health sectors. The *E. coli* was found to be sensitive to Gentamicin, Ofloxacin, Ampicillin, Neomycin, Chloramphenicol, Nitrofluraton and resistant to Sulfisoxazole, Tetracycline, Streptomycin.

Farzana *et al.* (2004); examined the antibiotic sensitivity of *Staphylococcus aureus* and found that 96.10% of organisms were susceptible to Erythromycin and only 1.3% was moderately susceptible and 2.60% resistant to Erythromycin. They reported that the susceptibility of the other antibiotics in decreasing order against *Staph. aureus* was found to be Methicillin (80.52%), Ampiclox (77.92%), Augmentin (76.62%), Tetracycline (70.13%), Ampicillin and Penicillin (58.44%), Robinin (36.36%), Lincomycin (18.18%) and Cotrimoxazole (7.79%) whereas resistance pattern of the Cotrimoxazole, Robinin, lincomycin, Ampicillin, Penicillin, Augmentin, Ampiclox, Methicillin, Tetracycline and Erythromycin was in decreasing order 81.81%, 63.63%, 57.17%, 25.97%, 24.67%, 23.37%, 18.18/%, 10.39%, 2.60% and 2.60% isolates, respectively.

Su *et al.* (2004); found that different types of *Salmonella* serovars are resistant to some conventional antibiotics such as Chloramphenicol, Sulfamethoxazole, Ampicillin Trimethoprim, Quinolones, Cephalosporins in many countries of the world.

Khan *et al.* (2005); reported the antibiogram study and plasmid profile analyses to find out the correlation of the recently isolated *Salmonella spp.* in Bangladesh. Antibiogram study revealed that the isolates were highly sensitive to ciprofloxacin, cephalexin and kanamycin but resistant to cloxacillin, erythromycin, cloxacillin.

Alcaine *et al*. (2007); observed that *Salmonella* is one of the leading organisms that cause food borne illness in various countries around the world. They reported that treatment of Salmonellosis in both animals and humans has become more difficult with

the emergence of multi-drug-resistant (MDR) *Salmonella* strains. Food borne infections and outbreaks with MDR *Salmonella* were also increasingly reported.

Oluyege *et al.* (2009); reported 85% of the resistant isolates where multiple drug-resistant was highest (89.1%) resistance was to the Amoxicillin.

Nigad *et al. (2011)*; stated that vegetable salads from different restaurants were highly contaminated by *coliform* and *faecal coliform* and Multiple drug resistance was observed in 98.06% isolates with resistance to 2-7 no. of antibiotics. 96.07% of isolates were found resistant to Ampicillin in the same study.

Immaculate Jeyasanta *et al.* (2012); revealed that Ciprofloxacin and Chloramphenicol are the best antibiotics to treat *E. coli* infection.

Raza *et al. (*2012); stated that *Salmonella* was susceptible to Ciprofloxacin {100%), Chloramphenicol (93.6%), Ofloxacin (95.7%), Ceftriaxone (95.7%).

CHAPTER -3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area

The present study was conducted during the period of January' 2021 to August '2021, in the Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University (SAU), and. samples were collected from 10 different areas of Dhaka city.

Fig 1: Map showing sample collection point in Dhaka city

3.1.2 Sample collection and transportation

A total of 120 Mixed vegetable salad (tomato, cucumber and carrot) samples of 3 categories from different restaurants, roadside food corners and street vendors from different places of the metropolitan area (Farmgate, Bashundhora area, Uttara, Mirpur, Kalshi, Matikata, Manikdi, Kochukhet, Mohammadpur, Agargaon) in Dhaka city, Bangladesh. The samples were selected randomly from different places considering their keeping condition. During the collection of samples, precautionary measures were maintained avoid to touch and a sterilized polythene bag was used to carry samples separately. The samples were then brought to the laboratory under the Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh.

3.1.3 Solid culture media

Nutrient agar (NA), MacConkey agar (MC), Salmonella-Shigella agar (SS), Eosin Methylene Blue agar (EMB) Brilliant- Green agar (BGA), Mannitol salt agar (MSA), Blood agar (BA)were used for this study.

3.1.4 Liquid culture media

The liquid media used in the study were Nutrient broth (NB), Peptone broth, Methyl-red and Voges-Proskauer broth (MR-VP).

3.1.5 Chemicals and reagents

 The chemicals and reagents used for the study were 0.1% peptone water, Phosphate buffered saline (PBS) solution, reagents for Gram's staining (Methylene blue, Gram's iodine, Safranin, Acetone alcohol, etc.), Kovac's reagent, 3% Hydrogen peroxide, Concentrated HCL, Mineral oil, Normal saline solution, Human plasma, 80% glycerine and other common laboratory reagents and chemicals.

3.1.6 Glass wares and other appliances

The glass wares and appliances were used during the course of the experiment. These were as follows: Conical flask, beaker, cotton, glass slides, cover slip, micro pipette and tips, compound microscope, Petri dishes, Glass rod spreader, Test tube stand, mortar and pustule, centrifuge machine, Bacteriological incubator, refrigerator, sterilizing instruments, laminar air flow, centrifuge tubes and machine, ice boxes, electronic

balance, syringe and needle, different bacteriological media, autoclave, inoculating loop, electric homogenizer, spirit lamp, tray, measuring cylinder, electric balance.

3.1.7 Experimental design
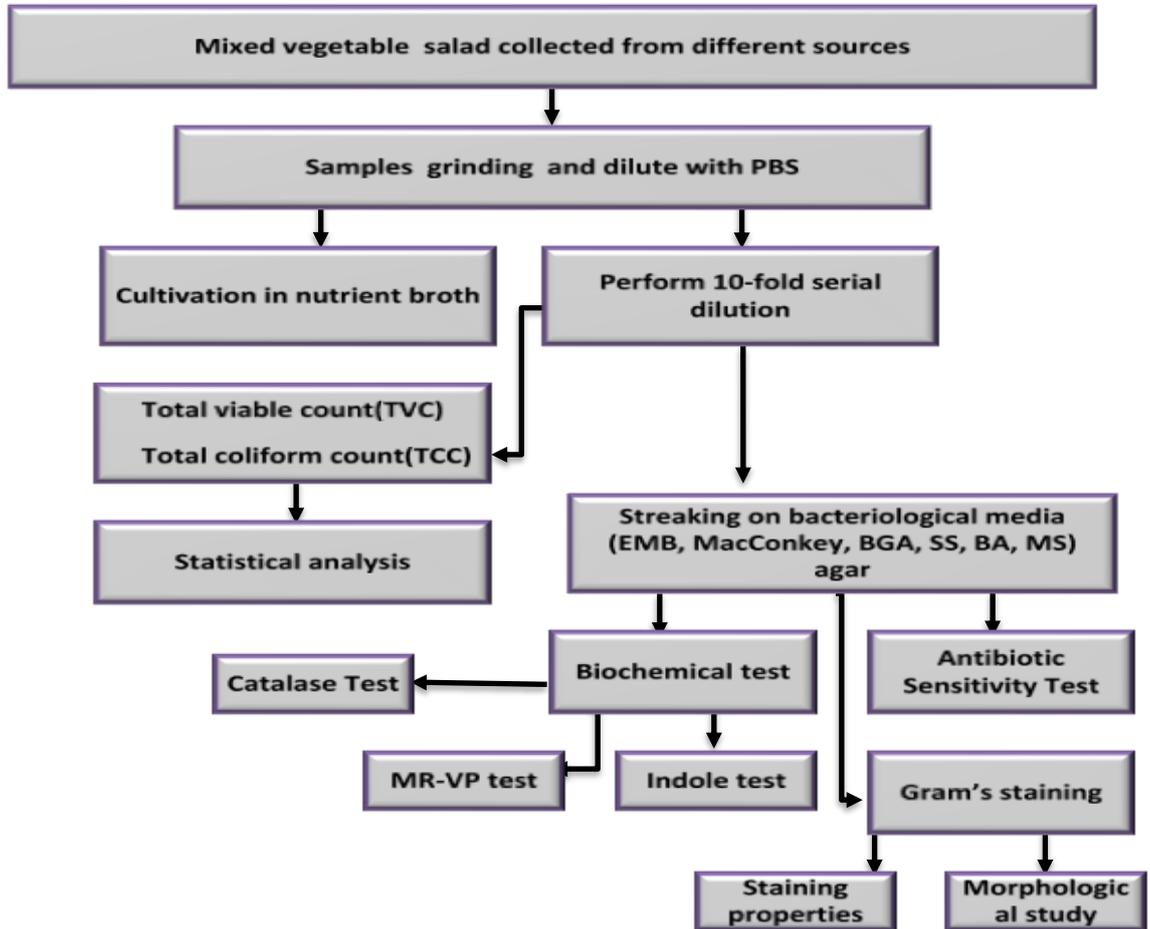
Fig 2. Flow chart of the experimental design at a glance

3.2 Preparation of culture media

All the media used in this experiment were prepared according to the procedure of the manufacturer described by (Merchant and Packer,1967).

3.2.1 Nutrient broth

Nutrient broth (NB) was prepared by dissolving 13 grams of dehydrated nutrient broth (Hi-media, India) into 1000 ml of distilled water and was sterilized by autoclaving at 121^0C under 15lb pressure for 15 minutes. Then the broth was dispended into the tubes (10 ml/tube) and stored at 4^0C in the refrigerator until used. The media was used to grow the organisms from the samples by incubation at an incubator at 37^0C for 24 hours.

3.2.2 Nutrient agar

Twenty-eight grams of nutrient agar (NA) medium was suspended in 1000 ml of distilled water in a conical flask and heated to a boil for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was put into a water bath of 45^0C to cool down its temperature. Then 10-15 ml of medium was poured into each sterile petri-dish and allowed to solidify. After solidification of the medium in the Petri-dishes, these were allowed for incubation at 37^0C overnight to cheek their sterility and then stored at 4-8^{0}C in a refrigerator for future use.

3.2.3 MacConkey agar

 Fifty grams powder of MacConkey agar (MC) agar base was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121^0C maintaining a pressure of 15 lb/ sq. inch for 15 minutes. After autoclaving, the medium was put into a water bath of 45^0C to decrease its temperature. After solidification of the medium in the Petri dishes, the Petri dishes were allowed for incubating at 37^0C overnight to check their sterility and then stored in a refrigerator and were used for the identification of Enterobacteriaceae organisms.

3.2.4 Salmonella-Shigella agar

Sixty-gram powder of Salmonella-Shigella agar (SS) base (Hi- media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121^0C After autoclaving, the medium was put into a water bath of 45^0C to decrease its temperature. Then 10-15 ml of medium was poured into each sterile Petri dish and allowed to solidify. After solidification of the medium in the Petri dishes, these were allowed for incubation at 37^0C overnight to check their sterility and then stored in a refrigerator for future use.

3.2.5 Eosin Methylene Blue Agar

Thirty-six grams of Eosin Methylene Blue agar (EMB) (Hi- media, India) was added to 1000ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121^0C. After autoclaving, the medium was put into a water bath of 45^0C to decrease its temperature.

After solidification of the medium in the Petri dishes, these were allowed for incubation at 37^0C overnight to check their sterility and then stored in a refrigerator for future use. This medium was used as a selective medium for the identification of *E. coli* and the growth of other organisms.

3.2.6 Brilliant Green Agar

Thirty grams of Eosin Methylene Blue agar (EMB) (Hi- media, India) was added to 1000ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C. After autoclaving, the medium was put into a water bath of 45^0C to decrease its temperature. After solidification of the medium in the Petri dishes, these were allowed for incubation at 37^oC overnight to check their sterility and then stored in a refrigerator for future use. This medium was used as a selective medium for the identification of *Salmonella and* the growth of other organisms.

3.2.7 Mannitol Salt Agar

One hundred and eleven grams of dehydrated mannitol salt medium was suspended in 1000 ml distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving. The autoclaved materials were allowed to cool to a temperature of 45°C in a water bath and distributed to sterile Petri dishes. After solidification Petri dishes were placed in an incubator for sterility test.

3.2.8 Blood agar

This medium was used for the observation of haemolytic reaction and for encouraging the growth of *Staphylococcus sp. Bacillus spp. Pasteurella spp.* and antibiotic sensitivity tests. Forty grams Blood agar (BA) base (Hi-media, India) powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving. The autoclaved materials were allowed to cool to a temperature of 45^0C in a water bath. Defibrinated 5% sheep blood was then added to the medium aseptically and distributed 5% sheep blood was then added to the medium aseptically and distributed to sterile Petri dishes and allowed to solidify. After solidification of the medium, the plates were allowed to incubate at 37^0C overnight to check their sterility and then stored in a refrigerator for future use.

3.2.9 Phosphate Buffered Saline

Solution For the preparation of phosphate-buffered saline, 8 grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 grams of potassium chloride (KCL) and 0.2 gm of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled water, the solution was heated to dissolve completely. The solution was then sterilized by autoclaved and stored for future use.

3.2.10 Methyl-Red Voges-Proskauer broth

A quantity of 3.4 grams of Bacto Methyl-Red Voges-Proskauer (MR-VP) medium was dissolved in 250 ml of distilled water dispended in 2 ml amount in each test tube and then the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37^0c for overnight to check their sterility and then stored in a refrigerator for future use.

3.3 Samples collected for bacterial isolation and identification

Proper care was taken during the sampling procedure to prevent contamination of the sample. After carrying by sterilized zip lock bag, the samples were transferred into a sterilized petri dish separately. A portion of 25 gm of each mixed vegetable salad sample was aseptically weighed.
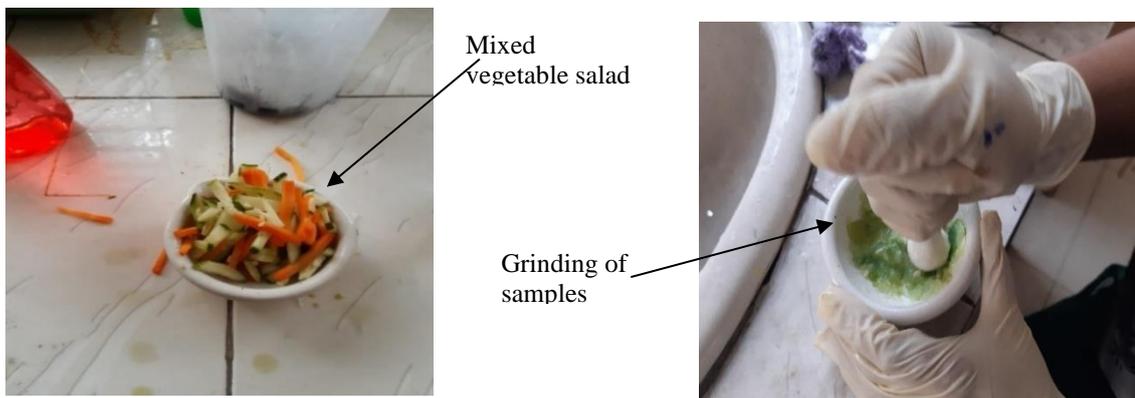
Fig 3: Prepare sample for microbiological analysis

The mixed vegetable salad sample was taken to mortar and ground with the help of a pestle and washed with distilled water. Then 0.5 ml of each sample was taken in sterile test tubes having 4.5 ml of 0.1% peptone solution to get 1:10 dilution. After performing tenfold dilutions were used to perform isolation and identification of different bacteria. The sample was placed in nutrient broth. After 24 hours of incubation at 37^0C, each

plate was examined for the identification of organisms and stored in the refrigerator for further study.

3.3.1 Isolation of different bacteria from the sample

Rinsed samples directly or after performing tenfold dilutions were used to perform isolation and identification of different bacteria. The sample was placed in nutrient broth. After 24 hours of incubation at 37^{0}C, each plate was examined for the identification of organisms and stored in the refrigerator for further study.

After the collection of samples, 100 microliters of the processed sample was inoculated into Nutrient agar media and EMB agar media by spread plate technique. The inoculated media was incubated at 37^{0}C. for overnight in an incubator. Different types of bacterial colonies were counted and isolated in pure culture.

3.3.2 Determination of Total Viable Count (TVC)

For the determination of total viable counts, 0.25 microliter of each 10-fold dilution was transferred and spread on plate count agar using a sterile pipette for each diluent. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. The plates were then kept in an incubator at 37^{0}C for 24-48 hours. Following incubation, plates exhibited 30-300 colonies. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count, Total viable count was expressed as the number of the organism of colony-forming units per gram (cfu/ml) of samples.

Fig 4: Perform a 10-fold serial dilution

3.3.3 Determination of Total Coliform Count (TCC)

For the determination of coliform bacteria TCC method was employed. For the TCC method, MacConkey agar and broths were used for bacterial propagation.

3.3.4 Gram's staining

The representative bacterial colonies were characterized morphologically using Gram's stain according to the method described by (Merchant and Packer,1967).

The procedure was as follows: A small colony was picked up from NA plates with a bacteriological loop, smeared on a separate glass slide and fixed by gentle heating. Crystal violates was then applied on each smear to stain for two minutes and then washed with running water.

Staining with crystal violet

Iodine treatment

Treat with alcohol

Treat with safranin

Microscopic observation

Fig 5: Perform Gram Staining

Few drops of Gram's iodine were then added to act as mordent for one minute and then again washed with running water. After washing with water, acetone alcohol was added which act as a decolourizer. After 10 seconds washed with water and safranin was added as counter stain and allowed to stain for 1- 2 minutes. The slides were then washed with water, blotted and dried in air and then examined under a microscope with a high-power objective (100X) using immersion oil.

3.3.5 Securing pure culture of Isolated bacteria

For identification, bacterial pure culture is important. From primary culture, the individual colony of different types were selected for securing pure culture according to the following procedure-

i) 0.25 micro litter of diluent taken into the different agar media and spread the diluent by using separate sterile spreader Incubate at 37^0C for 24 hours. After viable and coliform count the Petri dishes with colony were then used for isolation of pure culture of different bacteria. A single colony was then picked up by a sterile inoculating loop and sub culture is performed in another different media. After the plate has been inoculated and labelled, Incubate at 37^0C for 24 hours.

ii) The colonies are examined for the specific size, the shape of the specific organism colony. Several representative colonies of different types Again, gram staining was performed.

3.4 Identification of isolated bacteria

3.4.1 Culture on different media

A sterilized platinum loop was used for streaking the culture on EMB agar, MacConkey agar, Salmonella-Shigella (SS) agar, Brilliant green agar, mannitol salt agar to get

Streaking on EMB agar

Streaking on SS agar

Fig 6: Culture on different media

isolates in pure culture. All incubated media were kept at 37^0C overnight in an incubator.

3.5 Biochemical test

Isolated organisms with specific characteristics on SS, MC, EMB, BGA, MS agar were subjected to biochemical tests. Standard methods were followed to conduct this test according to the procedure of (Cowan & Steel, 1985).

3.5.1 Catalase test

This test was used to differentiate bacteria, which produce the enzyme catalase. It is used to distinguish the catalase-positive Micrococci such as *Staphylococcus* from that non-catalase *Streptococcus and Enterococcus genera.* To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3% H_2O_2) was added to the smear. The slide was observed for bubble formation. The formation of bubbles within a few seconds was the indication of the positive test while the absence of bubble formation indicated a negative result (Chesbrough, 1985).

Take inoculum on slide

Add H_2O_2

Observe colour changes
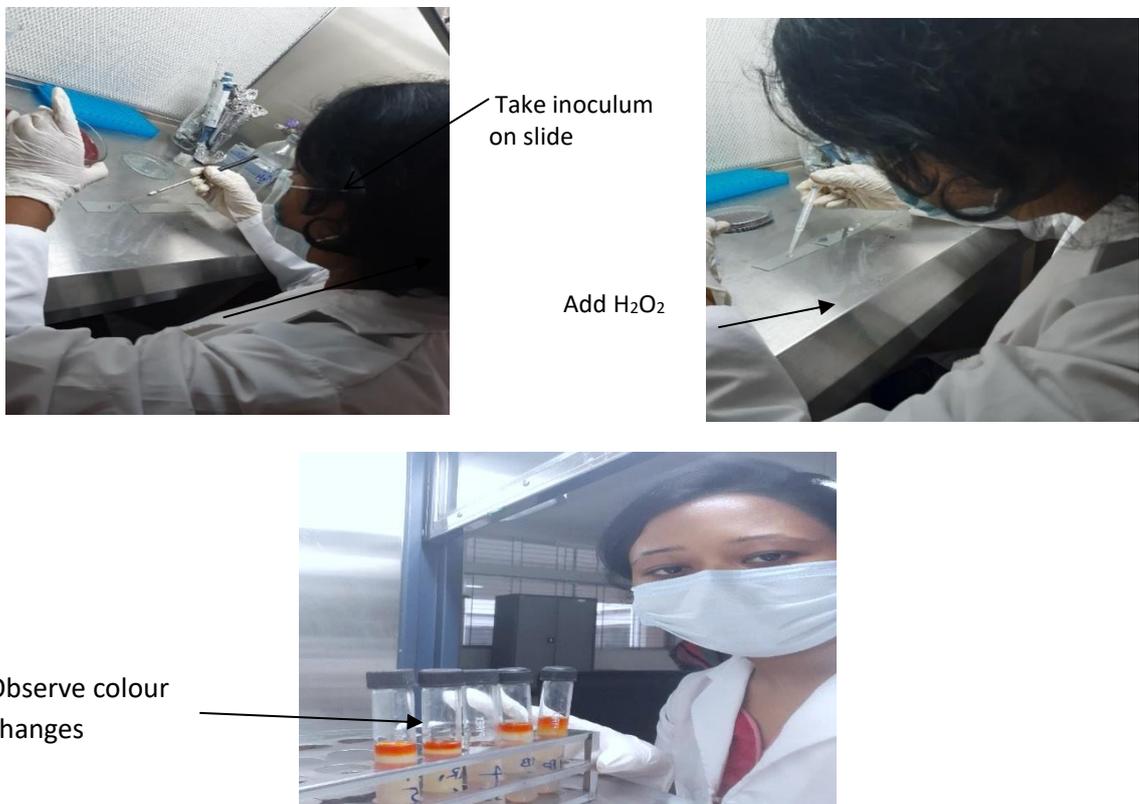
Fig 7: Biochemical Test

3.5.2 Indole test

2ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added, Shaked well and examined after 1 minute. The red colour in the reagent layer indicated indole. In the negative case, there is no development of red colour (Chesbrough, 1985).

3.5.3 Methyl red test

For the methyl red test, the tubes containing 2ml of MR-VP broth were inoculated with the isolated organism. Inoculated the broth for 37^0C for 48-72 hours. At the end of the time add 5 drops of methyl red reagent directly to the broth. The development of a stable red colour on the surface of the medium indicates sufficient acid production to lower the P^H and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substance, an intermediate orange colour between yellow and red may develop, this does not indicate a positive test. Yellow colour negative for MR test.

3.5.4 Voges-Proskauer test

2ml of MR-VP broth were inoculated with the isolated organism. Inoculated the broth for 37^0C for 48-72 hours. A very small amount of creatine was added and mixed. 3ml of sodium hydroxide was added and Shaked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of pink colour for positive cases. In case of negative results, there was no development of pink colour (Chesbrough, 1985).

3.6 Maintenance of stock culture

For the maintenance of stock culture, a simple 1ml of 80% sterilized glycerol was used in 1ml of nutrient broth (NB) culture was mixed and stored at 20^0C.

3.7 Isolation and identification of bacterial flora

For the isolation and identification of bacterial flora, the procedure suggested by Carter *at el.,*1995 was followed throughout the experiment.

3.7.1 Isolation and identification of *Escherichia coli*

For isolation of *Escherichia coli*, the samples were first inoculated on MC agar and incubated at 37^0C for 24 hours. To identify *E. coli* and other coliforms lactose fermenting red colonies from the MC agar were sub-cultured on EMB agar. Colonies on EMB agar with metallic sheen were suspected as positive for *E. coli* and were confirmed by biochemical test and E. *coli* was positive MR tests.

3.7.2 Isolation and identification of *Salmonella spp.*

Processed samples were inoculated on MC and incubated at 37^0C for 24-48 hours. Lactose non-fermenter colourless colonies from MC were sub-cultured on SS agar and BGA agar media. Translucent, round and colourless colonies on SS agar were suspected to be *Salmonella* and/or *Shigella*. Salmonella produces a characteristic blackish sheen on ss agar media. On BGA salmonella produce a yellowish colour colony which were late confirmed by the biochemical test.

3.7.3 Isolation and identification of *Staphylococcus spp.*

Isolation of *Staphylococcus* was based on morphology and cultural characteristics. The colonies of *Staphylococci* are round, glistening, convex, smooth and opaque on mannitol salt agar They are Gram-positive cocci arranged in a cluster. The coagulase test was performed for the identification of the pathogenic *Staphylococcus aureus* from non-pathogenic ones.

3.8 Antibiotic sensitivity test

Three isolates randomly selected from five areas were tested for antimicrobial drug susceptibility against seven commonly used antibiotics by disc diffusion or the Kirby-Bauer method. Selection of 3 to 5 isolated colonies from the SS, BGA, EMB, MS and BA agar plate. Touched the top of the colony with a loop and the growth is transferred into the nutrient broth. The broths were streaked onto Muller-Hinton agar plates by using a sterile glass spreader homogenously. Then placed the antibiotic disc onto Muller-Hinton agar and incubated at 37°C for 24 hours. Examined the plates and measured the diameter of zones of inhibition mm from the edge of the disc to the edge of the zone using a meter ruler.

3.8.1. Antimicrobial discs

The lists of commercially available antimicrobial discs (Oxoid, UK) with their concentrated are listed below (Table 4). The discs are applied to the plates as soon as possible but no longer than 15 minutes after inoculation. The disc was placed individually with sterile forceps and then gently pressed down onto the agar. In general, no more than 12 discs on a 150-mm plate and no more than 4 discs on a 100-mm plate were placed.

Table 1. Antimicrobial disc concentration

SL No.	Antimicrobial agents	Symbol	Disc conc. (µg/disc)
1	Ampicillin	AMP	25 µg
2	Amoxicillin	AMX	30 µg
3	Cefixime	CFM	5 µg
4	Ceftriaxone	CTR	30 µg
5	Gentamicin	GEN	10 µg
6	Streptomycin	S	10 µg
7	Tetracycline	TE	30 µg

3.8.2 Recording and interpreting results

After placing the discs on the plate, the plates were inverted and incubated at 37ºC for 16-18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimetres. The measurements were made with a ruler under the surface of the plate without opening the lid. The zones of growth inhibition were compared with the zone-size interpretative (Table-2,3,4) provided by the Clinical and Laboratory Standards Institute (CLSI, 2007). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretative standards provided by (Wilson *et al.*,2007).

Table 2: Zone diameter (in mm) interpretative standards for *E. coli* (according to the CLSI,2007)

Sl no.	Name of antibiotic	Disc con. (µg/disc)	Resistant	Intermediate	Sensitive
1	Ampicillin	25 µg	≤13	14-16	≥17
2	Amoxicillin	30 µg	≤13	14-17	≥18
3	Cefixime	5 µg	≤19	16-18	≥15
4	Ceftriaxone	30 µg	≤19	20-22	≥23
5	Gentamicin	10 µg	≤12	13-14	≥15
6	Streptomycin	10 µg	≤11	12-14	≥15
7	Tetracycline	30 µg	≤11	12-14	≥15

Table 3: Zone diameter (in mm) interpretative standards for *Salmonella spp* (according to the CLSI,2007)

Sl no.	Name of antibiotic	Disc con. (µg/disc)	Resistant	Intermediate	Sensitive
1	Ampicillin	25 µg	≤13	14-16	≥17
2	Amoxicillin	30 µg	≤13	14-17	≥18
3	Cefixime	5 µg	≤19	16-18	≥15
4	Ceftriaxone	30 µg	≤19	20-22	≥23
5	Gentamicin	10 µg	≤12	13-14	≥15
6	Streptomycin	10 µg	≤15	12-14	≥11
7	Tetracycline	30 µg	≤15	12-14	≥11

Table 4: Zone diameter (in mm) interpretative standards for *Staphylococcus spp.* (according to the CLSI,2007)

Sl no	Name of antibiotic	Disc con. (µg/disc)	Resistant	Intermediate	Sensitive
1	Ampicillin	25 µg	≤13	14-16	≥17
2	Amoxicillin	30 µg	≤13	14-17	≥18
3	Cefixime	5 µg	15	16-18	19
4	Ceftriaxone	30 µg	≤22	23-27	≥28
5	Gentamicin	10 µg	≤12	13-14	≥15
6	Streptomycin	10 µg	≤11	12-14	≥15
7	Tetracycline	30 µg	≤14	15-18	≥19

Disc placement

Measure zone diameter
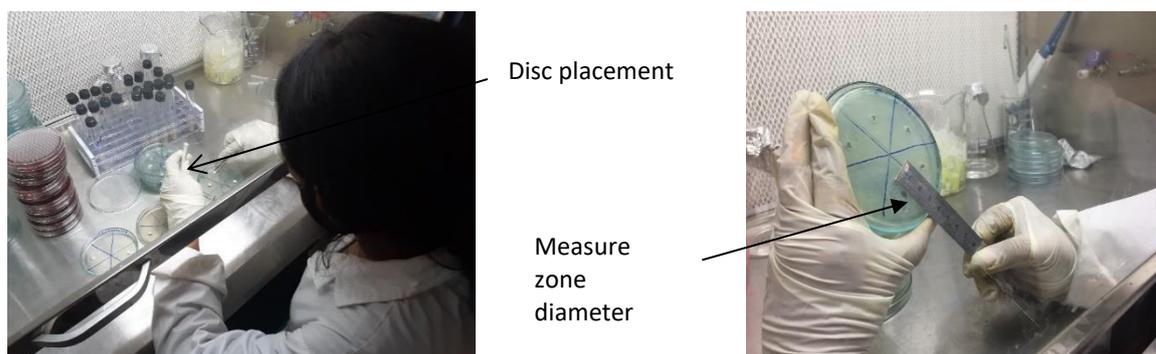
Fig 8: Antibiotic sensitivity test

3.9 Data analysis

The analysis was carried out by descriptive statistics (finding means and standard deviations) and (ANOVA) of mean microbial counts among the ten zones were also determined by checking for significant differences through SPSS software. A (P-value) of ≤0.05 was considered to be statistically significant. Microsoft excels software was also used.

CHAPTER-4

RESULTS & DISCUSSION

Raw vegetables are widely consumed in the form of salads in most countries. They are considered to be suitable and convenient meals for today's lifestyles. In Egypt, RTE vegetable salad (known as a green salad) is one of the most popular and widely consumed dishes in the Egyptians' daily food. RTESs are considered high-risk food because they do not require heating, and may not be cleaned or washed properly before consumption (Health protection agency,2009).

Contamination of mixed vegetable salad sold in restaurant premises, food corners and by street vendors rendering them unacceptable for human consumption has become a global health problem. In this piece of work, 120 vegetable salad samples were examined. This work included 120 samples distributed as 40 mixed vegetable salads from the restaurant, 40 mixed vegetable salads from the food corner and 40 mixed vegetable salads from the street vendor. All these samples yielded the growth of aerobic bacteria with varying densities. It has been documented that aerobic organisms reflect the exposure of samples to any contamination and generally the existence of favourable conditions for microorganisms' multiplication.

4.1 Determination of TVC and TCC

The total viable count (TVC) of mixed vegetable salad samples collected from the different restaurants are presented in (Table 5). The mean ±SD value of TVC was log 6.07±0.69 (cfu/gm). The bacterial load was highest at Kalshi log 6.81±0.81(cfu/gm) followed by Matikata, Mohammadpur, Kochukhet, Uttara, Farmgate, Bashundhora area, Mirpur, Agargaon and Manikdi (log 6.70±0.95, 6.63±0.91, 6.44±0.95, 6.24±0.95, 6.21±0.21,6.10±0.91, 5.90±0.95, 4.64±0.95, 4.61±0.91 cfu/gm) respectively. The lowest bacterial density at Manikdi is log 4.61±0.91cfu/gm. The P-value of TVC was (P<0.001) which is statistically highly significant so the null hypothesis should be rejected.

The total coliform count (TCC) of mixed vegetable salad samples collected from different restaurants are presented in (Table 5). The mean ±SD value of TCC was log 6.20±0.59 (cfu/gm). The coliform count was highest at Kalshi log 6.63±0.85 (cfu/gm) followed by Matikata, Mohammadpur, Bashundhora area, Agargaon, Uttara, Kochukhet, Farmgate, Mirpur and Manikdi (log 6.44±0.95, 6.42±0.95, 6.41±0.95,

6.32±0.91, 6.30±0.95, 6.24±0.95, 6.04±0.95, 5.81±0.95, 5.40±0.91 cfu/gm) respectively. The lowest bacterial density at Manikdi is log 5.40±0.91cfu/gm. The P-value of TCC was (P<0.001) which is statistically highly significant so the null hypothesis should be rejected.

Table 5: Total Viable Count (TVC) and Total Coliform count (TCC) of bacteria isolated from Restaurants' samples

Sl. no	No. of sample	Area of sample collection	TVC Mean ±SD (cfu/gm)	TCC Mean ±SD (cfu/gm)
1	4	Uttara	6.24±0.95	6.30±0.95
2	4	Agargaon	4.64±0.95	6.32±0.91
3	4	Kochukhet	6.44±0.95	6.24±0.95
4	4	Farmgate	6.21±0.21	6.04±0.95
5	4	Bashundhora area	6.10±0.91	6.41±0.95
6	4	Manikdi	4.61±0.91	5.40±0.91
7	4	Matikata	6.70±0.95	6.44±0.95
8	4	Kalshi	6.81±0.81	6.63±0.85
9	4	Mirpur	5.90±0.95	5.81±0.95
10	4	Mohammadpur	6.63±0.91	6.42±0.95
Total	40		6.07±0.69	6.20±0.59

The total viable count (TVC) of the mixed vegetable salad sample collected from different food corner are presented in (Table 6). The mean ±SD value of TVC was log 5.42±0.69 cfu/gm). The bacterial load was highest at Mohammadpur log 6.63±0.91(cfu/gm) followed by Bashundhara area, Uttara, Farmgate, Mirpur, Kalshi, Kochukhet, Matikata, Manikdi, (log 5.74±0.95, 5.70±0.95, 5.65±0.81, 5.60±0.95, 4.92±0.81, 4.90±0.95, 4.73±0.95, 4.72±0.95 cfu/gm) respectively. The lowest bacterial density at Agargaon is log 4.70±0.95 cfu/gm. The P-value of TVC is (P<0.001) which is statistically highly significant so the null hypothesis should be rejected (Table-6). The total viable count (TCC) of Ready-to-eat (mixed salad) street vendor samples collected

from different food corners are presented in (Table 6). The mean ±SD value of TCC was log 5.23±0.59 (cfu/gm). The coliform count was highest at Mohammadpur log 6.42±0.95 (cfu/gm) followed by Mirpur, Bashundhora area, Matikata, Kochukhet, Uttara, Manikdi, Kalshi, Farmgate, (log 6.42±0.95, 5.70±0.95, 5.44±0.95,5.34±0.95, 5.30±0.81, 4.63±0.85, 4.31±0.12 cfu/gm) respectively. The lowest bacterial density at Agargaon log 4.04±0.95 cfu/gm. The P-value of TCC was (P<0.001) which is statistically highly significant so the null hypothesis should be rejected (Table-6).

Table 6: Total Viable Count (TVC) and Total Coliform count (TCC) of bacteria isolated from Food Corner samples

Sl. no	No. of sample	Area of sample collection	TVC Mean ±SD (cfu/gm)	TCC Mean ±SD (cfu/gm)
1	4	Uttara	5.70±0.95	5.31±0.81
2	4	Agargaon	4.70±0.95	4.04±0.95
3	4	Kochukhet	4.90±0.95	5.34±0.95
4	4	Farmgate	5.65±0.81	4.31±0.12
5	4	Bashundhora area	5.74±0.95	5.70±0.95
6	4	Manikdi	4.72±0.95	5.30±0.81
7	4	Matikata	4.73±0.95	5.44±0.95
8	4	Kalshi	4.92±0.81	4.63±0.85
9	4	Mirpur	5.60±0.95	5.81±0.95
10	4	Mohammadpur	6.63±0.91	6.42±0.95
Total	40		5.42±0.69	5.23±0.59

The total viable count (TVC) of mixed vegetable salad samples collected from the different street vendors are presented in (Table 7). The mean ±SD value of TVC was log 7.04±0.48 (cfu/gm). The bacterial load was highest at Farmgate was log 7.84±0.12 (cfu/gm) followed by Kalshi, Mirpur, Agargaon, Matikata, Mohammadpur, Bashundhora area Kochukhet, Manikdi, Uttora (log 7.54±0.81, 7.52±0.81, 6.95±0.57,

6.94±0.57, 6.83±0.95, 6.82±0.95, 6.80±0.95, 6.61±0.81, 6.60±0.81 cfu/gm) respectively. The lowest bacterial density at Uttara is log 6.60±0.81 cfu/gm. The P-value of TVC is (P<0.001) which was statistically highly significant so the null hypothesis should be rejected

The Total coliform count (TCC) of tomato samples collected from a different street vendor are presented in (Table 7).The mean ±SD value of TCC was log 6.70±0.57(cfu/gm).The coliform count was highest at Farmgate log 7.70±0.95 (cfu/gm) followed by Mirpur, Kalshi, Agargaon, Kochukhet, Bashundhora area, Mohammadpur, Manikdi, Uttora (log7.34±0.12, 7.31±0.12, 6.81±0.95, 6.80±0.95, 6.34±0.50, 6.32±0.50, 6.30±0.50, 6.05±0.50, 6.04±0.50 cfu/gm respectively. The lowest bacterial density at Uttara is log 6.04±0.50 cfu/gm. The P-value of Tomato TCC was (P<0.001) which is statistically highly significant so the null hypothesis should be rejected

Table 7: Total Viable Count (TVC) and Total Coliform count (TCC) **of** bacteria isolated from Street Vendor samples

Sl. no	No. of sample	Area of sample collection	TVC Mean ±SD (cfu/gm)	TCC Mean ±SD (cfu/gm)
1	4	Uttara	6.60±0.81	6.04±0.50
2	4	Agargaon	6.95±0.57	6.81±0.95
3	4	Kochukhet	6.80±0.95	6.34±0.50
4	4	Farmgate	7.84±0.12	7.70±0.95
5	4	Bashundhora area	6.82±0.95	6.32±0.50
6	4	Manikdi	6.61±0.81	6.05±0.50
7	4	Matikata	6.94±0.57	6.80±0.95
9	4	Mirpur	7.52±0.81	7.34±0.12
10	4	Mohammadpur	6.83±0.95	6.30±0.50
Total	40		7.04±0.48	6.70±0.57
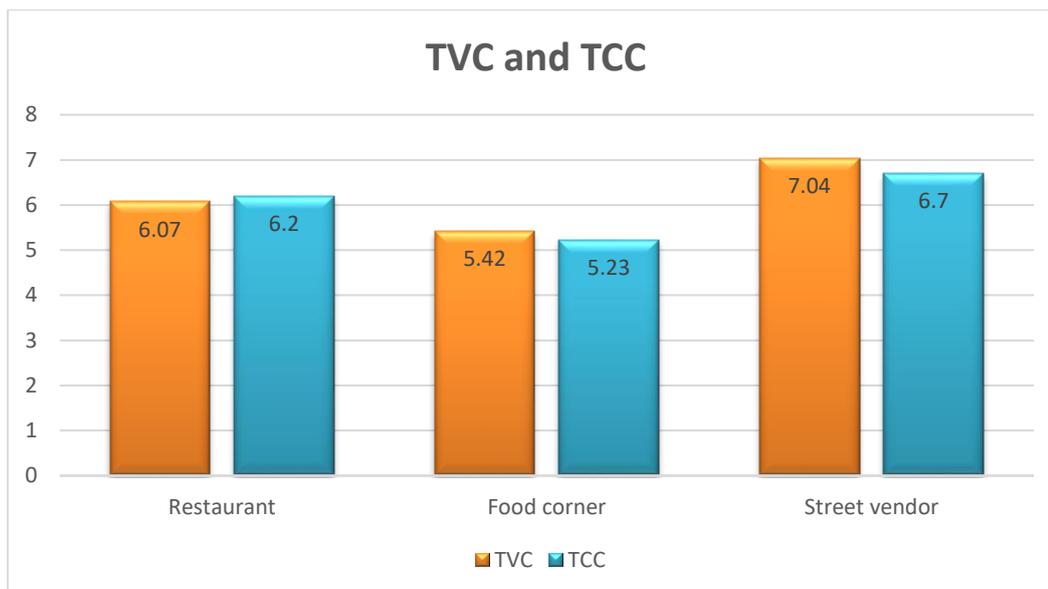
Fig 9: Total Viable Count (TVC) and Total Coliform Count (TCC) of bacteria

The microbiological quality of mixed vegetable salad mostly depends on the bacterial load, particularly the coliforms. High total viable count (TVC) indicates unsafe conditions and therefore the occurrence of possible contamination. The results of total viable count (TVC) and total coliform count (TCC) of various samples have given in Table (5-7). The TVC and TCC of mixed vegetable salad samples of different areas are varied. Such variations occurred due to differences in hygienic conditions maintained by the waiter of different restaurants, food corners and street sellers of vendors. The variation in the bacterial count was more or less identical at the good hygienic condition of other sources.

There was a great effect of hygienic condition on the total viable count (TVC).The mean ±SD values of TVC were log 6.07±0.69, log 5.42±0.69, log 7.04±0.48 for Restaurant, food corner and street vendor respectively. This variation may be due to variation in maintained sanitary, handling and preservation condition of vegetable salad. (Doores,1983) suggested to pay attention to maintaining a microbiologically stable environment to achieve high quality in raw vegetables and processed products.

In case of the restaurant sample, the mean ±SD values of TVC was log 6.07±0.69. The highest TVC was found at Kalshi area where it was log 6.81±0.81 and the lowest was at Manikdi where it was log 4.61±0.91 cfu/gm which indicated that the restaurants'waiter maintained good hygienic condition during handling and serving vegetable salad while others did not maintain good hygienic condition.

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The mean ±SD values of the TVC of mixed vegetable salad from different food corners was log 5.42±0.69 cfu/gm. The TVC value was highest in the mixed vegetable salad from the food corner at Mohammadpur log 6.63±0.91 cfu/gm. The lowest TVC was observed at Agargaon log 4.70±0.95 cfu/gm which indicated that the waiter of the food corner maintained good hygienic condition during handling and serving mixed vegetable salad while others did not maintain good hygienic condition. The obtained TVC value of the mixed vegetable salad sample from the food corner of the present study is lower than the findings of (Abadias *et al.,* 2008). He reported that the total bacterial population was log 10.57 cfu/gm.

The mean ±SD values of the TVC of mixed vegetable salad from the different vendors were log 7.04±0.48 cfu/gm. The TVC value was highest in the mixed vegetable salad from street vendors at Farmgate was log 7.84±0.12 cfu/gm. The lowest TVC was observed at Uttara log 6.60±0.81 cfu/gm which indicated that the sellers of street vendors maintained good hygienic conditions during handling and serving vegetable salad while others did not maintain good hygienic conditions.

In this study, the total coliform count (TCC) was different in the mixed vegetable salad sample of ten different places. The mean ±SD values of the TCC of examined 120 sample was log 6.20±0.59, 5.23±0.59 and 6.70±0.57 cfu/gm for the mixed vegetable salad of restaurant, food corner and street vendor respectively. This was in line with the findings of (Faour-Klingbeil *et al.,2016*) in Lebanon who reported that the mean TCC levels ranged from 2.90 to 7.38 log cfu/g, with counts above 10^7 cfu/g recorded for 17% of their samples. Furthermore, in accordance with the present results (Khalil and Gomaa, 2014) in Egypt recorded a wide range of Total Coliform Count (TCC) for conventional vegetable salad samples (3.63–7.17 log cfu/g). On the other hand, (Weldezgina and Muleto, 2016) in Ethiopia and (Nyenje *et al.,2012)* in South Africa observed narrower ranges of high TCC from 6.94 to 8.06 and from 6.3 to 6.8 log cfu/g, respectively. A much lower TCC range of 2.95–3.75 log cfu/g was reported by (Buyukunal *et al.*, 2015) in Turkey. The difference in the TCC mean values may be attributed to the different areas of vegetable cultivation and different irrigation sources. It has been noted that plate count of aerobic coliform microorganisms found in food is one of the microbiological indicators for food quality, and most foods are regarded as harmful when they have large populations of these microorganisms, even if the organisms are not known to be pathogens (Aycicek *et al.,2006*). The acceptable TCC

limit of mixed vegetable salad by some countries for export purposes should not exceed 6.69 log cfu/g (Beuchat.,1995).

Indicator bacteria may be associated with an increased likelihood of the presence of pathogens. They are useful in the assessment of food product safety because they tend to be present in higher numbers than most pathogens and are relatively quick and easy to identify (Frimpong *et al.*, 2015). Another higher TCC mean value was reported in a study done in India by (Mritunjay and Kumar,2017) where a total of 480 samples of eight different raw mixed vegetable salads from the restaurant had a mean TCC of 6.1 log cfu/g, ranging from 2.0 to 9.6 log cfu/g.

In this study, The TCC mean value for the 40 samples purchased from restaurants was 6.20±0.59 log cfu/g and food corner was 5.23±0.59 cfu/gm in concordance with the results in the present study, (Pamuk *et al.,2013)* in Turkey reported that 55.1% of the mixed salad samples from different private restaurants, cafes, and shopping centres were found to have total coliform counts of more than 6 log cfu/g, and (Jeddi *et al.,*2014) in Iran reported that the count of these bacteria in salads ranged from 5.5 to 7.4 log cfu/g. This agreed with the findings of (Nguz *et al.,*2005) who showed that mixed vegetables were still found to harbour high levels of TCC (log 7.05±0.5 cfu/gm) High loads of coliforms in vegetables at retail levels can be directly influenced by intense use of untreated manure during preharvest and extensive handling during postharvest (Aycicek *et al.,*2006).

On the contrary, The TCC mean value for the 40 street-vended mixed vegetable salad was 6.70±0.57 log cfu/g this was nearly similar to the results recorded by (Ameko *et al.,2012).* In Ghana, where raw mixed vegetable salads obtained from five randomly selected vendors had a mean coliform count of $\log^{10}$ 4.70 cfu/g (5×10^4 cfu/g). A higher count was reported earlier by (Kubheka *et al.*,2001) in South Africa, as the 55 salads collected from street vendors had a TCC mean value of 5.9 (±0.6) log cfu/g and the count ranged between 2.7 and 8.9 log cfu/g.

In the present study, the TCC mean value of mixed vegetable salad from restaurants was significantly higher than that of food corner. This may be the result of the greater number of people be involved in the handling and preparation of this type of food in restaurants. In restaurants, the salads are stored, and leftovers may be used for several days. The leftovers could serve as a good source for contaminating freshly prepared

salads (Jeddi *et al.,*2014). According to (Frank-Peterside and Waribor,2006) bacterial load on vegetables increase with time during storage. It is advisable to separate the leftovers from freshly prepared salads to prevent cross-contamination. The leftovers could be kept for sale the next day, only if they are kept under cold storage and then reheated above 70°C before being sold. However, as salads are generally not heated, it is advisable to discard any leftovers (Alimi,,2006).

In agreement with the current findings, (Khater *et al.,2013)* in Egypt documented that salad samples obtained from restaurants had a higher TCC value than the food corner ones (7.05 and 4.94 log cfu/g, respectively). On the other hand, salad of food corner prepared instantly on the demand of customer's order so there are fewer possibilities to keep leftover for the next day. In this study, we found a higher load in street vendor salad samples although it prepared instantly and lack of storage facilities but the water and instrument, they used for the preparation is unhygienic it contrasts to these results, in Togo, (Soncy *et al.,2015)* reported that the microbial loads of salad samples from the street vendors in Lomé were higher than that of the studied Domino restaurant.

4.2 Isolation of selected bacteria from different media

4.2.1 Identification of *E. coli* on the basis of cultural properties

4.2.1.1 Culture on nutrient broth

All the *Escherichia. coli* isolates produced turbidity in nutrient broth (Fig:10)

— Turbidity

Fig 10: Presence of *E. coli* in Nutrient broth

4.2.1.2 Culture on Nutrient agar

The experimental samples were streaked on nutrient agar to reveal the growth of *E. coli* after 24 hrs. of incubation at 37°C aerobically and were indicated by the growth of smooth, circular, white to greyish-white colony (Fig: 11)

Smooth,
white
colony
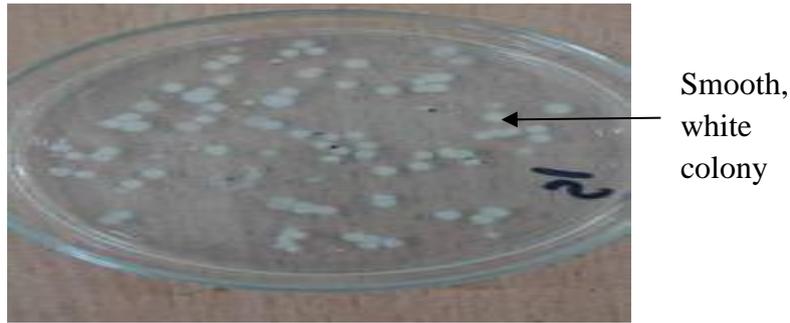
Fig 11: Colony of *E. coli* showing smooth, circular, white colony on Nutrient agar

4.2.1.3 Culture on MacConkey agar

MC agar plates were streaked separately with the organism and revealed the growth of bacteria after 24 hrs. of incubation at 37°C aerobically and were indicated by the growth of bright pink to the red coloured colony due to fermentation of lactose by *E. coli.*
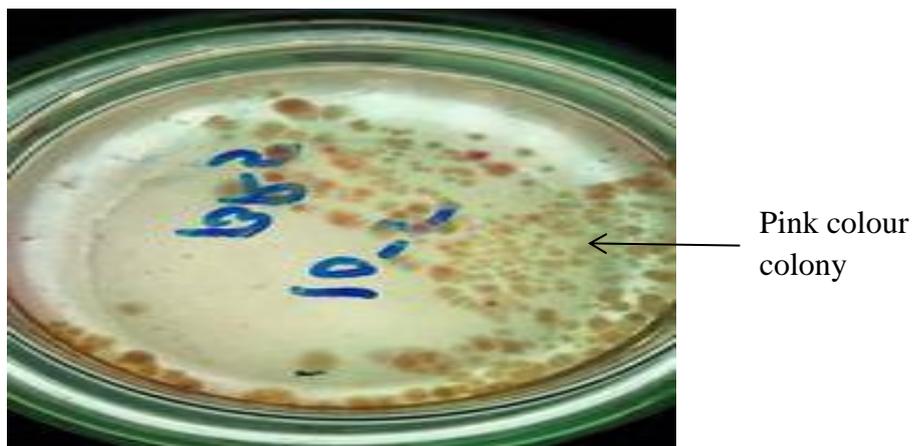
Pink colour
colony

Fig 12: Colony of *E. coli* showing bright pink colour on MacConkey (MC) agar

But other gram-negative enteric bacteria that do not ferment lactose appear colourless on Mc agar and the agar surrounding the bacteria remains relatively transparent (Fig:12).

4.2.1.4 Culture on EMB agar

EMB agar plates were streaked separately with the organism and incubated at 37°C for 24 hrs. The growth was indicated smooth, circular, green colour colonies with a metallic sheen. EMB agar is used to differentiate coliform enteric bacteria from other enteric bacteria due to the production of acid. In acidic conditions, the dyes produce a dark purple complex which is usually associated with a green metallic sheen which is an

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indication of the growth of *E. coli, colonies* of other no lactose fermenters appear as translucent or pink.

Fig 13: Green-metallic sheen of *E. coli* on EMB agar

4.2.2 Identification of *E. coli* by Gram's staining

The microscopic examination of Gram's-stained smears from NA, MC agar and EMB revealed that the isolated bacteria were Gram-negative, pink coloured, small rod-shaped organisms arranged in single, pairs or short-chain (Fig:14).
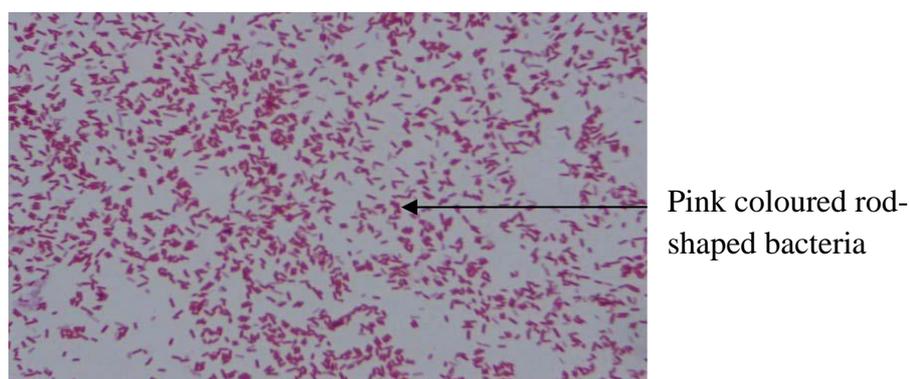
Fig 14: Gram-negative, pink coloured, long rod shape E. coli under the light microscope. (100x)

Table 8: Morphological and cultural properties of *E. coli*

Feature	Appearance
Nutrient agar	Smooth, circular, white to greyish white colonies were found.
Eosin Methylene Blue agar	Smooth, circular, black colour colonies with metallic sheen were produced.

Feature	Appearance
MacConkey agar	Rose pink lactose fermented colonies were formed.
Staining Properties	Gram-negative, pink coloured, small rod-shaped organisms arranged in single, pairs or short-chain was observed.

4.3 Identification of *Salmonella spp.* on the basis of cultural properties

4.3.1 Culture on Nutrient Broth

All the *Salmonella* isolates produced turbidity in nutrient broth (Fig:15).

— Turbidity
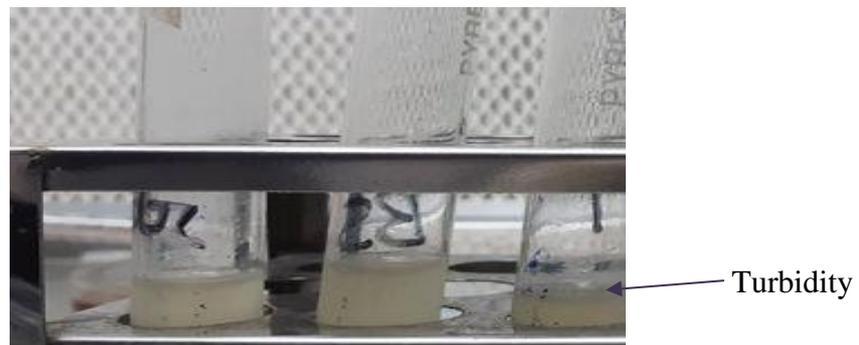
Fig 15: Presence of *Salmonella spp* in Nutrient broth

4.3.2 Culture on Salmonella-Shigella agar

On SS agar, the isolated *Salmonella sop* produced opaque, translucent, colourless, smooth and round colonies with black centres as they are lactose non-fermenter (Fig:16).
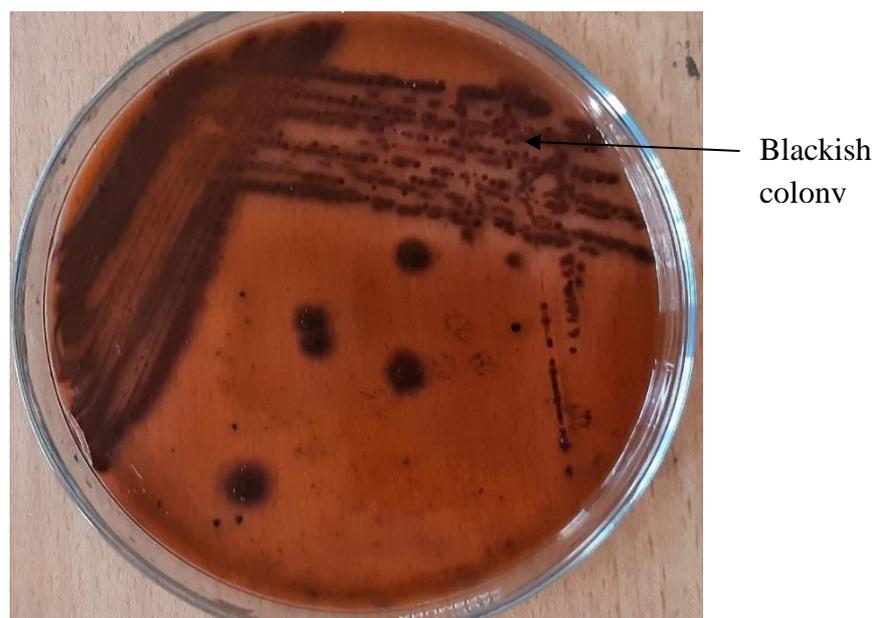
— Blackish colony

Fig 16: Blackish colonies of *Salmonella* on SS agar

4.3.3. Culture on Brilliant Green Agar

On Brilliant Green Agar, typical *Salmonella* colonies appear as pinkish-white or red colonies by a red halo in the medium, On the other hand, differentiation is quite pronounced as lactose or sucrose fermenting organisms which are inhibited or overcome inhibition produce yellow-green colonies with a green halo (Fig:17).
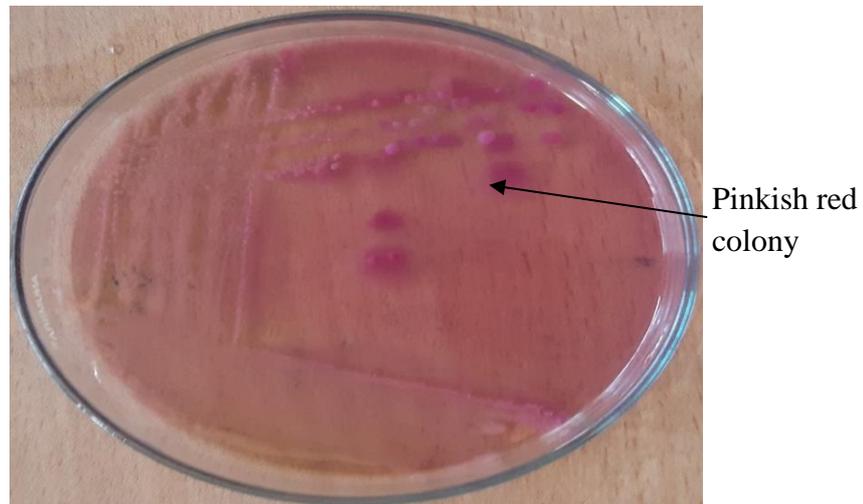
Fig 17: Pinkish red colonies of *Salmonella spp*. on Brilliant -Green agar

4.3.4 Identification by Gram's Staining

The microscopic examination of Gram's-stained smears from SS agar, BGA revealed Gram-negative, pink colour, small rod-shaped organisms arranged in single or paired (Fig.18).

Fig18: Gram-negative, pink colour, small rod-shaped organisms arranged in single or paired under the light microscope (100x)

Table 9: Morphological and cultural properties of *Salmonella spp.*

Feature	Appearance
Nutrient agar	Smooth, circular, white to greyish white colonies were found.
SS agar	Opaque, translucent, colourless, smooth, round colonies were found.
BGA agar	Pinkish red, smooth, round colonies are found
Staining property	Gram-negative, pink colour, small rod-shaped organisms arranged in single or paired was observed.

4.4 Identification of *Staphylococcus spp on* the basis of cultural properties

4.4.1 Culture on Nutrient Broth

All the *Staphylococcus* isolates produced turbidity in nutrient broth (Fig:19).
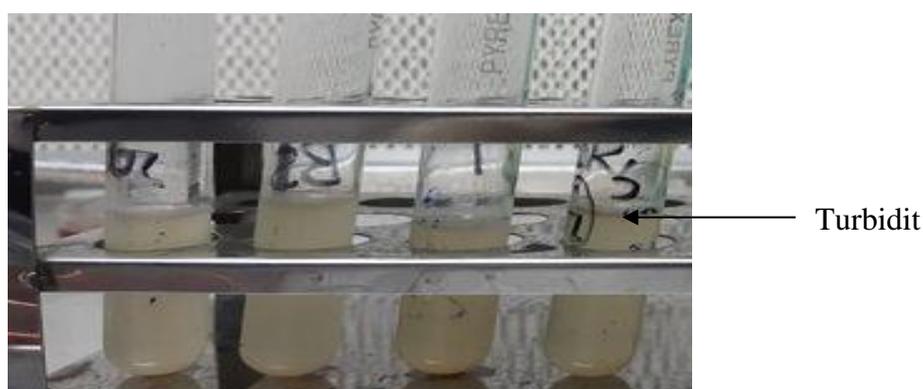
Fig 19: Presence of *Staphylococcus spp.* in Nutrient broth

4.4.2 Culture on Nutrient Agar

The NA plates streaked separately with the organisms revealed the growth of bacteria after 24 hrs. of incubation at 37 ^{0}C aerobically and were indicated by the growth of Gray, white or yellowish colonies (Fig 20).
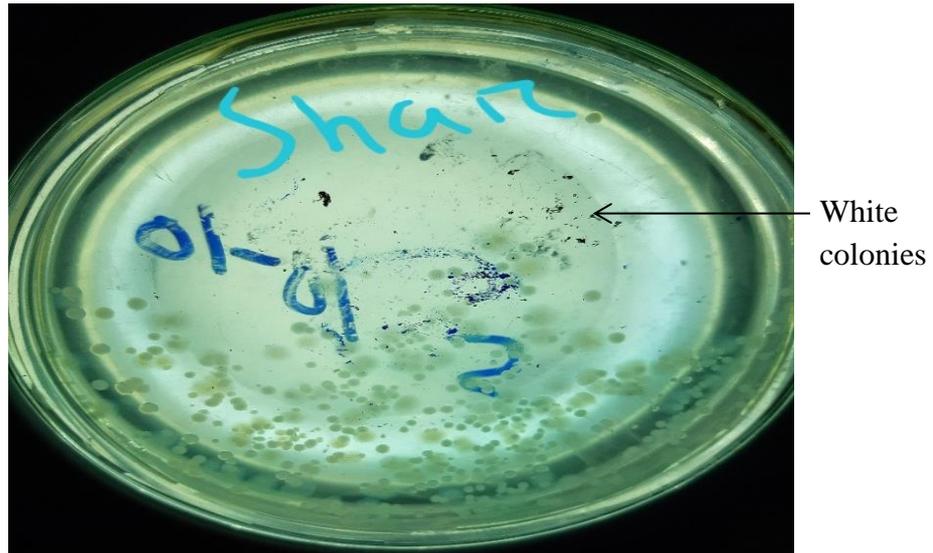
Fig 20: White colonies of *Staphylococcus spp.* on nutrient agar

4.4.3 Culture on Blood Agar

The BA plates were streaked separately with the organism and incubated at 37^0 C aerobically for 24 hrs large, creamy white, beta haemolytic colonies were produced by Staphylococcus *aureus* which is the typical characteristics of *Staphylococcus aureus* on blood agar (Fig. 21).
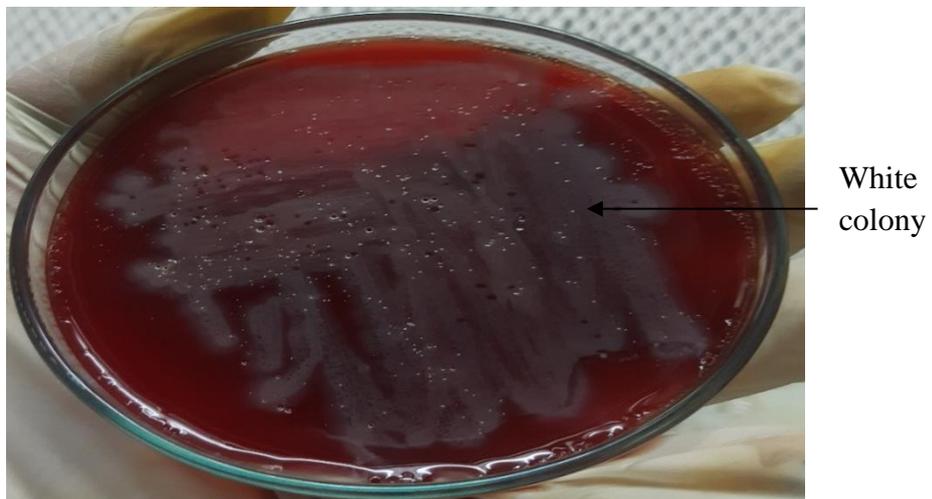
Fig 21: White colony with β haemolysis *Staphylococcus aureus* on 5% sheep blood agar

4.4.4 Properties on Mannitol Salt Agar

The MSA plates were streaked separately with the organism and incubated at 37^0C for 24 hrs. The growth revealed a yellow colour colony (Fig.22).
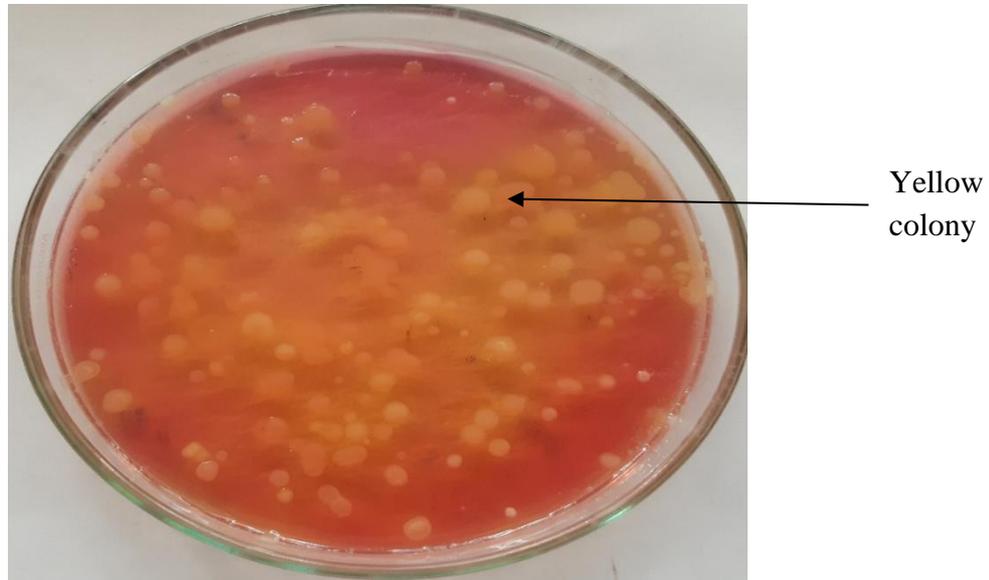
Yellow colony

Fig 22: Yellowish colony of *Staphylococcus aureus* on mannitol salt agar

4.4.5 Identification of *Staphylococcus aureus* by Gram's Staining

Gram's-stained smears from NA, BA and MSA were examined microscopically which revealed Gram-positive, cocci arranged in grapes like clusters (Fig:23).

Gram positive cocci
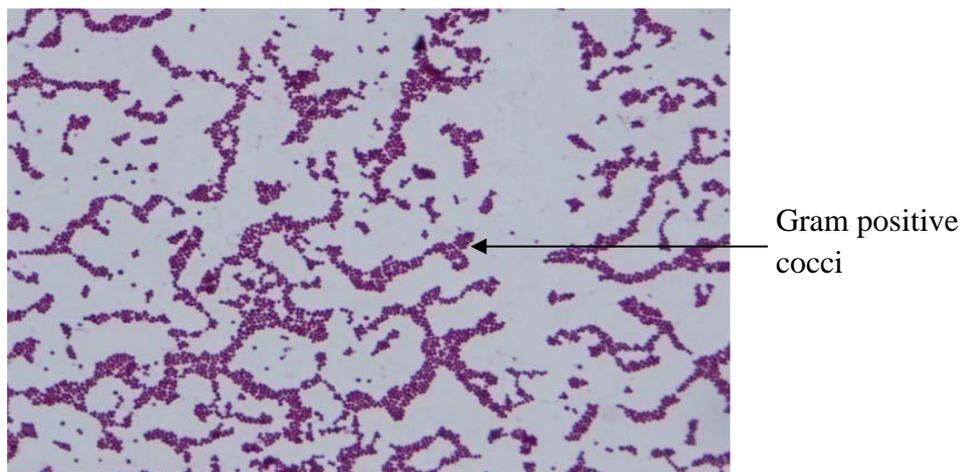
Fig 23: Gram-positive, cocci, arranged in grapes like a cluster of *Staphylococcus aureus* under the light microscope (100x)

Table 10: Morphological and cultural properties of *Staphylococcus aureus*

Feature	Appearance
Nutrient agar	Smooth, circular, white to greyish white colonies were found.
Blood Agar	White to golden yellow colonies were found
Mannitol salt agar	Yellow colour colonies were found
Staining Property	Gram-positive, cocci arranged in grape-like clusters observed

4.5 Identification of different bacteria by Biochemical Test

4.5.1 Identification of *E. coli* by MR-VP test:

Escherichia. Coli gave colour change in MR test and give no colour to VP test (Fig:24).

Colour changes

No colour changes
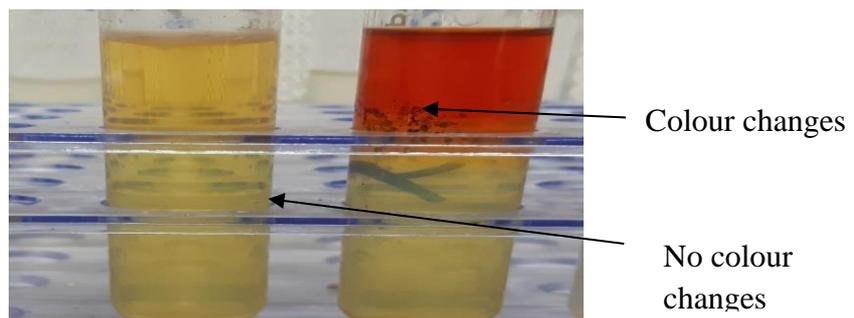
Fig 24: MR-VP test of *E. coli*

4.5.1.1 Identification of *E. coli* by Catalase test:

Escherichia. Coli gave a positive result to the Catalase test (Fig:25).

Bubble formation
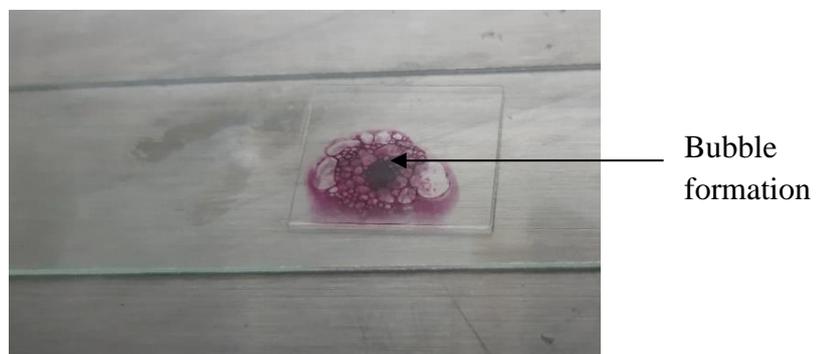
Fig 25: Catalase test of *E. coli*

4.5.1.2 Identification of *E. coli* by Indole test:

Escherichia. Coli gave a positive result to the Indole test (Fig:26).

Red ring
formation
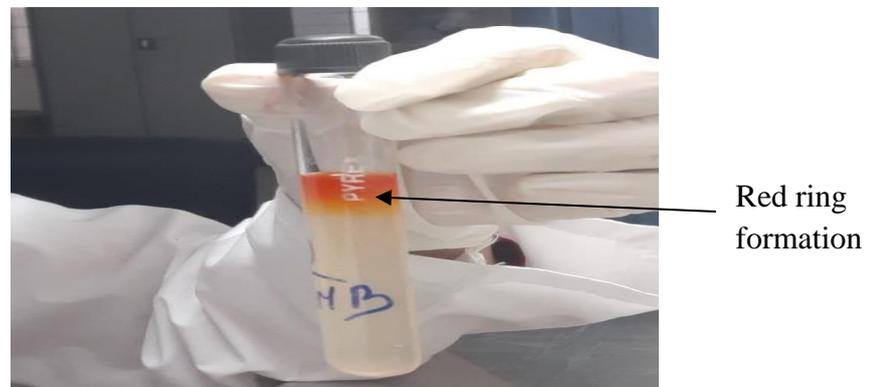
Fig 26: Indole test of *E. coli*

4.5.2 Identification of *Salmonella spp. by* Biochemical Test

4.5.2.1 Identification of *Salmonella spp.* by MR-VP Test:

Salmonella. show a positive reaction to MR test and negative to VP test (Fig:27).

Colour change

No colour
changes
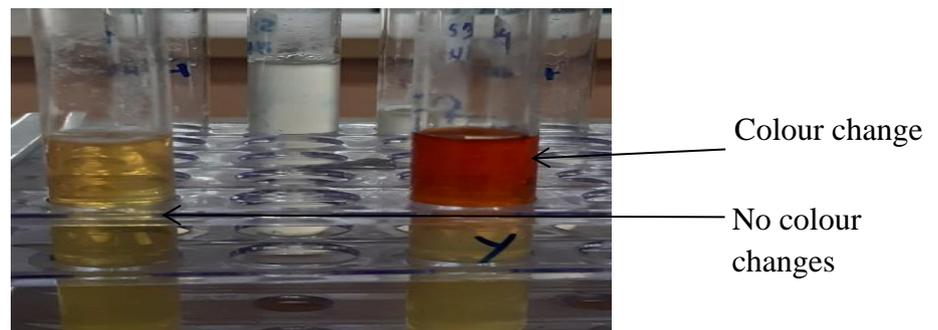
Fig 27: MR-VP test of *Salmonella*

4.5.2.2 Identification of *Salmonella spp*. by Catalase Test:

Salmonella. spp. show positive catalase test. (Fig:28).

Bubble
formation
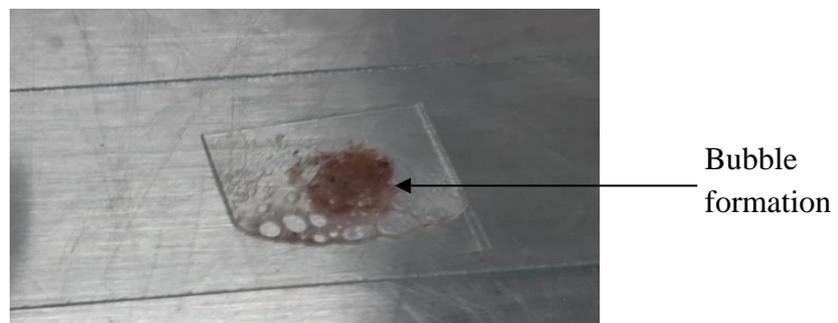
Fig 28: Catalase test of *Salmonella*

4.5.2.3 Identification of *Salmonella spp*. by Indole Test:

Salmonella produced negative reaction to Indole (Fig:29).

Orange colour formation
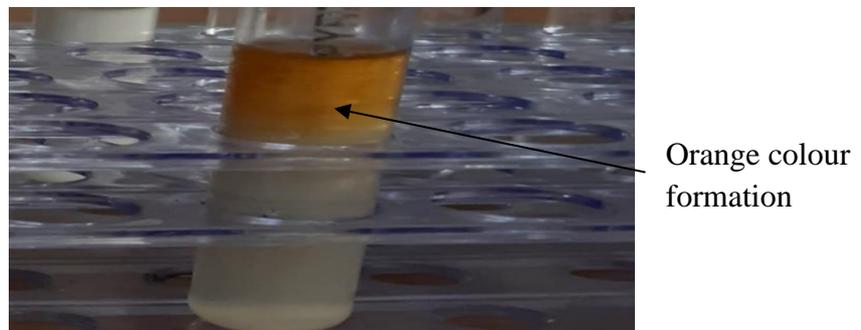
Fig 29: Indole test of *Salmonella*

4.5.3 Identification of *Staphylococcus aureus* by Biochemical Test

4.5.3.1 Identification of *Staphylococcus aureus* by MR-VP Test:

Staphylococcus aureus showed a positive reaction to the MR-VP test. (Fig:30).

MR positive
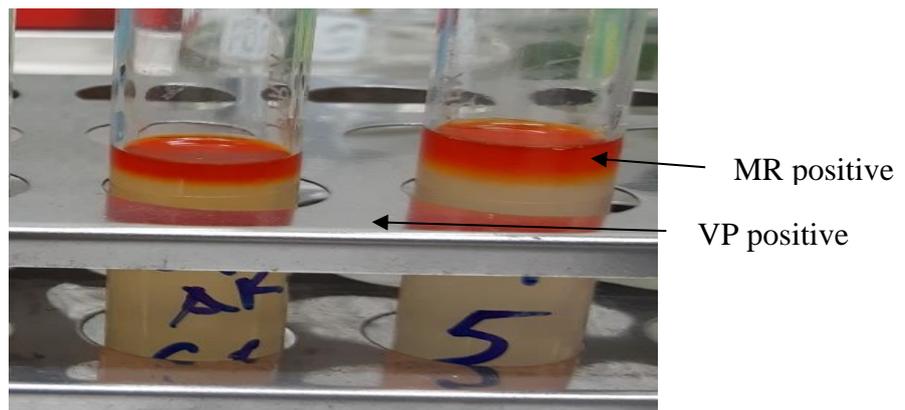
VP positive

Fig 30: MR-VP test of *Staphylococcus aureus*

4.5.3.2 Identification of *Staphylococcus aureus* by Catalase Test:

Staphylococcus aureus showed a positive reaction to the Catalase test. (Fig:31).

Bubble formation
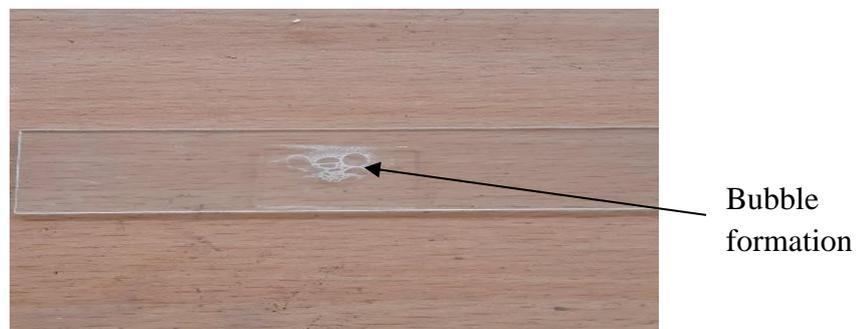
Fig 31: Catalase test of *Staphylococcus aureus*

4.5.3.2 Identification of *Staphylococcus aureus* by Indole Test:

Staphylococcus aureus showed a positive reaction to the Indole test. (Fig:32).

— Red ring form

Fig 32: Indole test of *Staphylococcus aureus*

Table 11: Results of Biochemical Tests of isolates

Name of the organisms	Catalase Test	MR Test	VP Test	Indole Test
E. coli	+ve	+ve	-ve	+ve
Salmonella spp.	+ve	+ve	-ve	-ve
Staphylococcus spp.	+ve	+ve	+ve	+ve

4.6 Prevelance of bacteria

Salmonella is a common foodborne pathogen that causes food contamination, which has resulted in higher economic losses and poses a significant threat to public health. *Salmonella* has been implicated in disease outbreaks associated with the consumption of fresh and mixed vegetable salad (Health Protection Agency, 2009).

Salmonella is a non-lactose fermenter usually associated with water contamination. Contamination with these organisms could arise from washing vegetables with contaminated water or handling of vegetables by infected workers. In this study, *Salmonella* was isolated from all samples. Its presence in food is of serious safety concern. According to the (WHO,2006) effect of microbiological hazards such as *Salmonella* on food safety is now a major public health concern worldwide. According to (Ibrahim,1996) isolated *Salmonella* from lettuce, cucumber and parsley. *Salmonella* has also been isolated from vegetable salad in waakye a street food in Ghana.

Salmonella and *Shigella* spp. have been frequently found in salads and dairy products. It is a principal-agent of bacterial dysentery.

The presence of *E. coli* in the mixed vegetable salad analysed is indicative of faecal contamination. *E. coli* are part of the normal flora of the human intestines. Some strains of *E. coli* have been linked to diarrhoea, gastroenteritis and urinary tract infections (Ameco *et al.,* 2012)*.*

The detection of *Staphylococcus* is of serious public health importance because of its ability to cause a wide range of infections especially food-borne intoxication (Tambekar *et al.,*2006). Contamination with *Staphylococcus* has been linked to carriage in nasal passages of food handlers or by infected workers. The presence of *S. aureus* and some Gram-negative rods have been reported to contaminate some vegetable salad such as carrots, cucumber, tomato and radishes (Beuchat, 1995). Examination of the presence of pathogens in food products contributes to food safety (Table 13).

Table 12. Guidance on the interpretation of results for specific foodborne pathogens in ready-to-eat food in general (colony-forming unit (cfu)/g)

Criterion	Satisfactory (cfu)/g)	Borderline (cfu)/g)	Unsatisfactory*
Escherichia coli	< 20	20 to $< 10^2$	$> 10^2$
S. aureus	10^2 to $< 10^4$	Unsatisfactory	$> 10^4$
Salmonella	Not Detected in 25 g	Not applicable	Detected in 25 g

*Potentially injurious to health and/or unfit for human consumption(cfu)/g

source: Health Protection Agency, 2009. Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market**.**

Table 13. Prevalence of isolated bacteria in vegetable salad samples

Criteria	No. of the sample examined	Number of bacteria Isolated					
		E. coli	Prevalence	Salmonella	Prevalence	Staphylococcus	Prevalence
Restaurant	40	2	5%	2	5%	0	0%
Food corner	40	4	10%	1	2.5%	2	5%
Street vendor	40	6	15%	3	7.5%	3	7.5%
Total count	120	12		6		5	
Net prevalence	-	-	10%	-	5%	-	4.1%

The presence of *E. coli, Salmonella* has been observed in samples from all sources and *Staphylococcus* was absent in restaurant samples (Table 13).
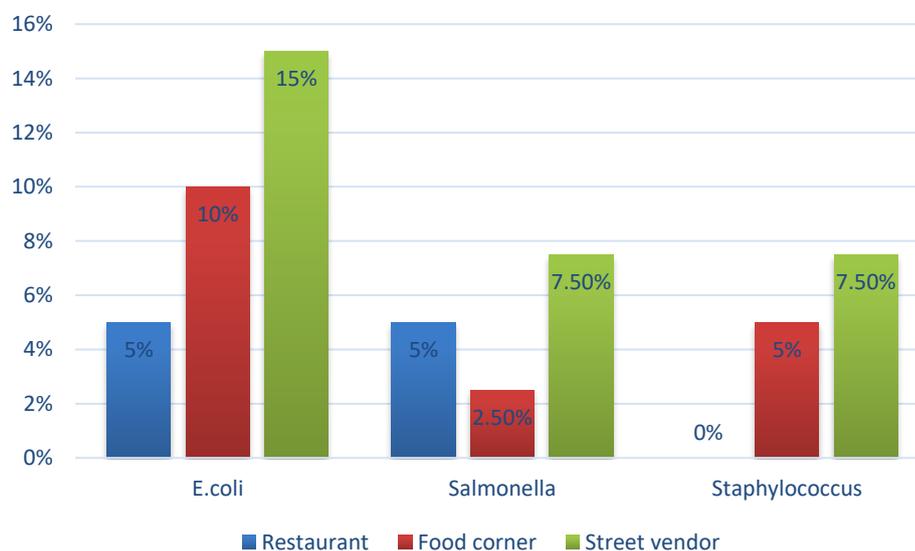
Fig 33: Prevalence of bacteria in the vegetable salad in Dhaka city

In this piece of work, the prevalence of *Salmonella* was 5%, 2.5% and 7.5%. for mixed vegetable salad from restaurant, food corner and vendor respectively.
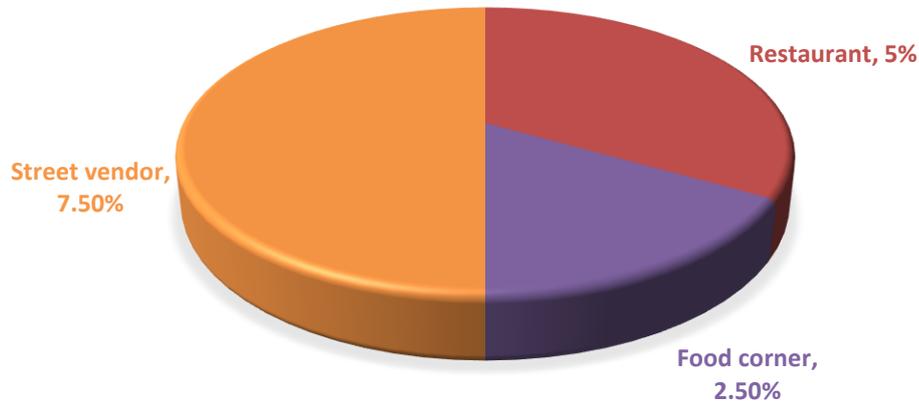
Fig 34: Prevalence of *Salmonella spp*. in vegetable salad sample

According to (Weldezgina and Muleta, 2016) *Salmonella* was detected in the mixed vegetable sample was 20.7%, in Ethiopia, according to (Gomez-Aldapa *et al.,* 2013) 6.8% in Mexico and according to (Toe *et al.,2017)* 2.6% in Ivory cost respectively. In contrast to the present findings, *Salmonella* was not detected in their samples (Soncy *et al.,2015)* in Togo, (Allen *et al.,2013)* in Canada (Caponigro *et al.2010),* in Italy and earlier (Sagoo *et al.,*2008) in the UK. It has been documented those negative results may suggest that levels of occurrence of these pathogens on the sampled population of a mixed vegetable salad may be below the sensitivity of the used detection method and could be minimally affected by season and other considered factors (Caponigro *et al.,2010).*

It is worth mentioning that in this study, 5% of restaurant salad samples, 2.5% of food corner salad samples and 7.5% of street vendor salad samples were unsatisfactory regarding *Salmonella levels*. Unsanitary handling of street foods by some of the vendors has been commonly found to be the source of contamination. This could be attributed to the fact that most of the street vendors do not take the needed precautions to avoid contamination of the raw salads during preparation and sale, as they are usually unaware of food contamination causes. In addition, in many developing countries, street food vending activities are not usually protected or regulated by the governments. Furthermore, it has been previously noted that stands used by street vendors are usually of inefficient construction, running water is not easily accessible, hand and dishwashing are performed in the same bucket, sometimes without soap. Waste water is usually

discarded right there in the streets, and garbage is likewise conveniently discarded right next to the stands, providing attraction, food, and harbourage for insects and rodents. In many cases, toilets are not available, thus forcing the vendors to eliminate their body wastes also in areas close by and to return to their vending sites without washing their hands. Such conditions and practices are likely to lead to cross-contamination of street food, thus adequate measures for treatment and cleaning of raw materials, environment and utensils together with hygienic practices of vendors must be strictly implemented to ensure good quality of fresh vegetables and mixed vegetable salad and significantly reduce their contamination (Alimi,,2006).

The prevalence of *E. coli* was 5%, 10% and 15%. for mixed vegetable salad from the restaurant, food corner and vendor respectively whereas according to (Abakari *et al*.,2018)   Escherichia coli were detected in 96.7% of salad samples.
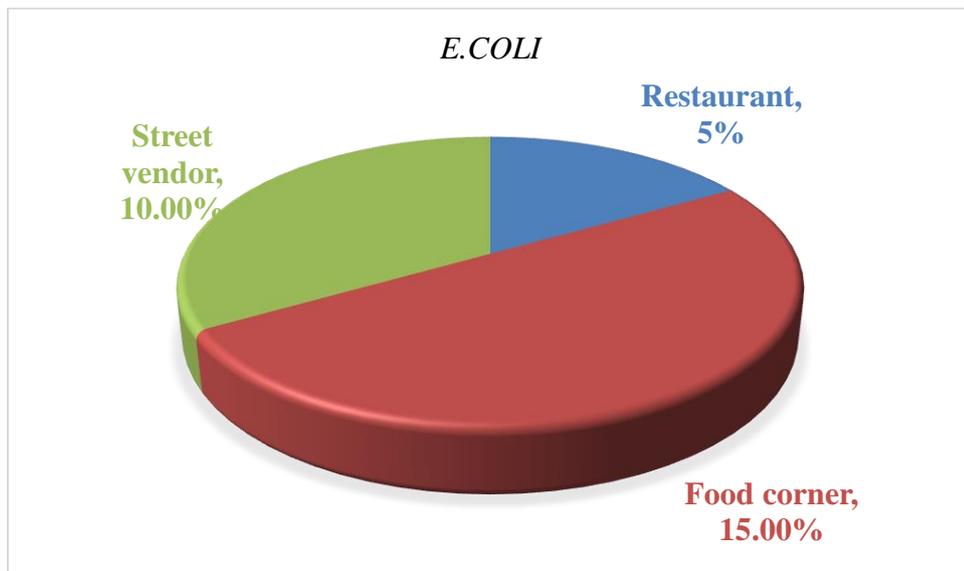
Fig 35: Prevalence of *E. coli* in vegetable salad sample

According to guidelines from the (Health Protection Agency, 2009) *E coli* load in 25 g of mixed vegetable salad $> 10^2$ cfu/g is categorized as unwholesome for human consumption however the vegetable salad will be categorized as satisfactory if the load in 25 g is < 20 cfu/g, therefore, all the samples from the restaurant, food corner and vendor were considered satisfactory for consumption. According to (Coulibaly-Kalpy *et al.,* 2017), who reported 94% of some mixed vegetable salad including pre-cut salads samples contaminated with *E. coli* as Satisfactory for consumption in their study carried out in Cote d'Ivoire.

Staphylococcus is a common pathogen usually carried by food handlers (Castro-Rosase *et al.,*2012). In this study, the prevalence of *Staphylococcus aureus was* 0%, 5% and 7.5%. for mixed vegetable salad from the restaurant, food corner and vendor respectively According to (Guimarães *et al.,* 2015) prevalence of *Staphylococcus spp.* in salad vegetables was 5.6% and 77.8%.

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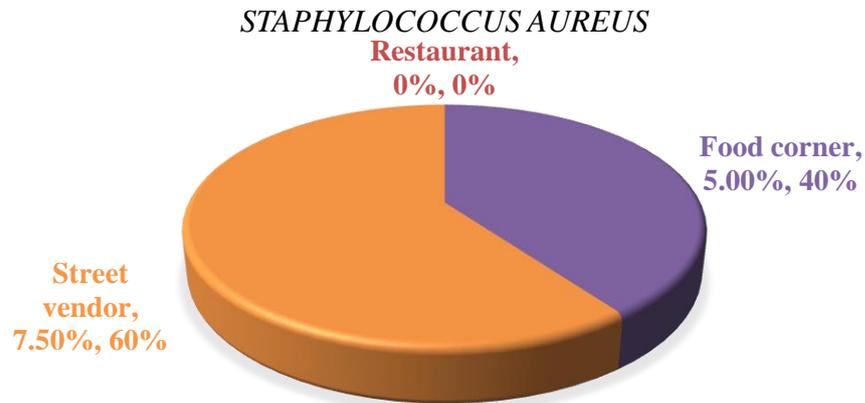
Fig 36: Prevalence of *Staphylococcus aureus* in vegetable salad samples

If the *Staphylococcus* levels are higher than 10^4 cfu/gm in freshly cut mixed vegetable salad, then it is potentially hazardous. In this study, *Staphylococcus* is not found in restaurant samples but found in both food corner and street vendor samples which are unsatisfactory.
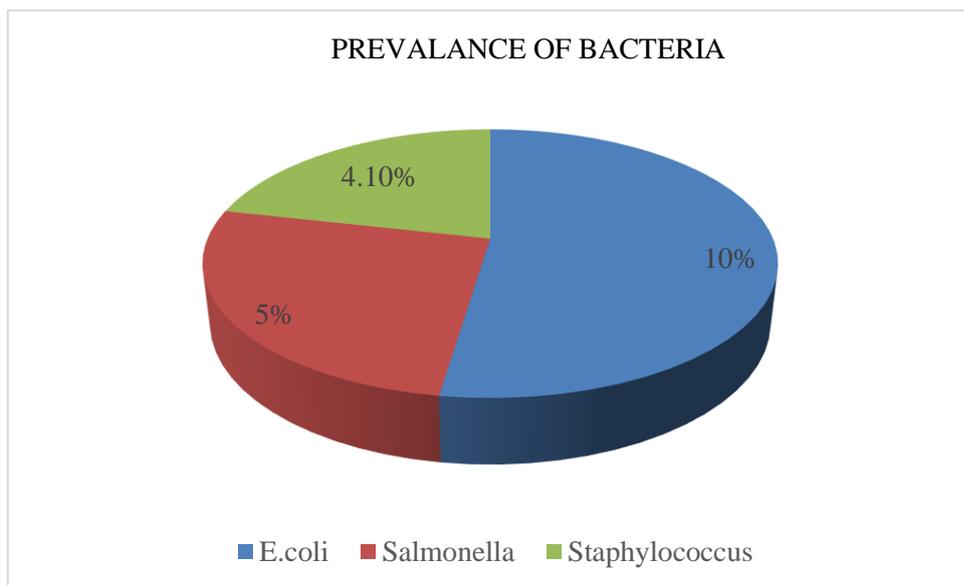
Fig 37: Net prevalence of bacteria in vegetable salad samples

In this study, about a total of 120 samples the net prevalence of bacteria was 10%,5% and 4.1% for *E. coli*, *Salmonella spp*. and *Staphylococcus aureus* respectively.

4.7 Antibiotic Sensitivity Test

Drug resistance is a serious problem these days and that is becoming riskier and riskier for global public health. Our study of antibiogram revealed that most of the isolates were susceptible to some antibiotics, whereas resistance towards several antibiotics indicated the risk of the emerging resistant isolates causing health hazards difficult to eradicate by those antibiotic therapies.

Table 14: Zone diameter (in mm) of the isolated sample of, *E. coli*, *Salmonella spp.*& *Staphylococcus aureus*

Antibiotics	*E. coli*	*Salmonella*	*Staphylococcus aureus*
Ampicillin	8	8	8
Amoxicillin	7	7	7
Cefixime	7	7	7
Ceftriaxone	23	15/30	21
Gentamicin	17	20	11/22
Streptomycin	16	8/24	12
Tetracycline	10	8/12	7/14

Table15: Antibiotic sensitivity test against *E. coli, Salmonella* and *Staphylococcus* mixed vegetable salad

Name of bacteria	Samples	AMP	AMX	CFM	CTR	GEN	S	TE
E. coli	RT$_4$	R	R	R	S	S	S	R
	RT$_{10}$	R	R	R	S	S	S	R
	F$_9$	R	R	R	S	S	S	R
	F$_{13}$	R	R	R	S	S	S	R

Name of bacteria	Samples	AMP	AMX	CFM	CTR	GEN	S	TE
	F_{20}	R	R	R	S	S	S	R
	F_{31}	R	R	R	S	S	S	R
	V_2	R	R	R	S	S	S	R
	V_{10}	R	R	R	S	S	S	R
	V_{19}	R	R	R	S	S	S	R
	V_{33}	R	R	R	S	S	S	R
	V_{37}	R	R	R	S	S	S	R
	V_{40}	R	R	R	S	S	S	R
Salmonella	RT_9	R	R	R	S	S	R	R
	RT_{14}	R	R	R	S	S	R	R
	F_{26}	R	R	R	S	S	R	R
	V_{11}	R	R	R	R	S	R	R
	V_{21}	R	R	R	R	S	R	I
	V_{23}	R	R	R	R	S	R	I
Staphylococcus	F_{22}	R	R	R	R	S	I	R
	F_{29}	R	R	R	R	S	I	R
	V_{15}	R	R	R	R	S	I	R
	V_{25}	R	R	R	R	R	I	I
	V_{32}	R	R	R	R	R	I	I

[RT= Restaurant, F= Food corner, V= Vendor, R= Resistant, I= Intermediate, S= Sensitive]

Zone of diameter
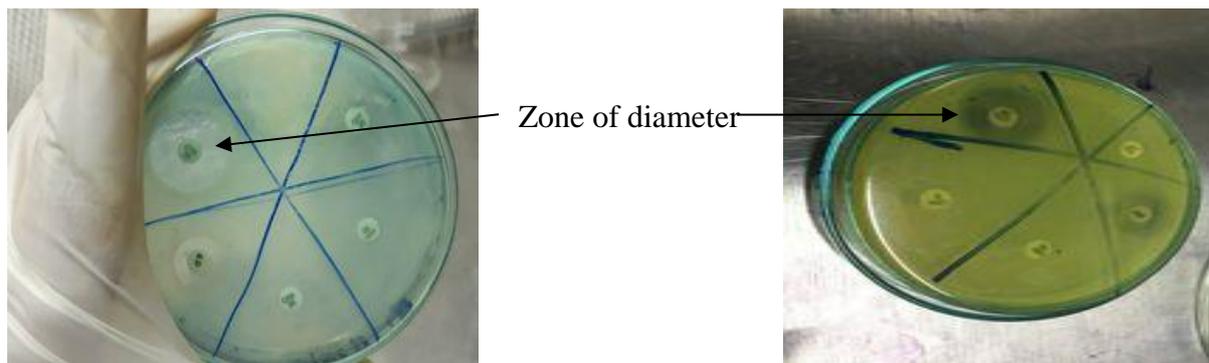
Fig 38: Antibiotic sensitivity test

A total of 12 isolates of *E. coli* from mixed vegetable salad samples were further used to determine the antibiotic sensitivity pattern. All isolated bacteria showed significantly 100% resistance to Ampicillin, Amoxicillin, Cefixime and Tetracycline. 100% sensitive to Ceftriaxone, Gentamycin and Streptomycin. According to (Salmanov *et al.*, 2021*) E. coli* was most sensitive (95%) to ertapenem (100%), cefotaxime (99.1%), ceftazidime (99.4%), Fosfomycin (98.7%), imipenem (98.9%), piperacillin/tazobactam (97.3%), and gentamycin (94.5%) but least susceptibility (70%) was observed for moxifloxacin (54.2%), cefuroxime (65.8%), amoxicillin. No strains of *E. coli* resistant to ertapenem were found. Resistance to third-generation cephalosporins was observed in 14.2% *E. coli* isolates.
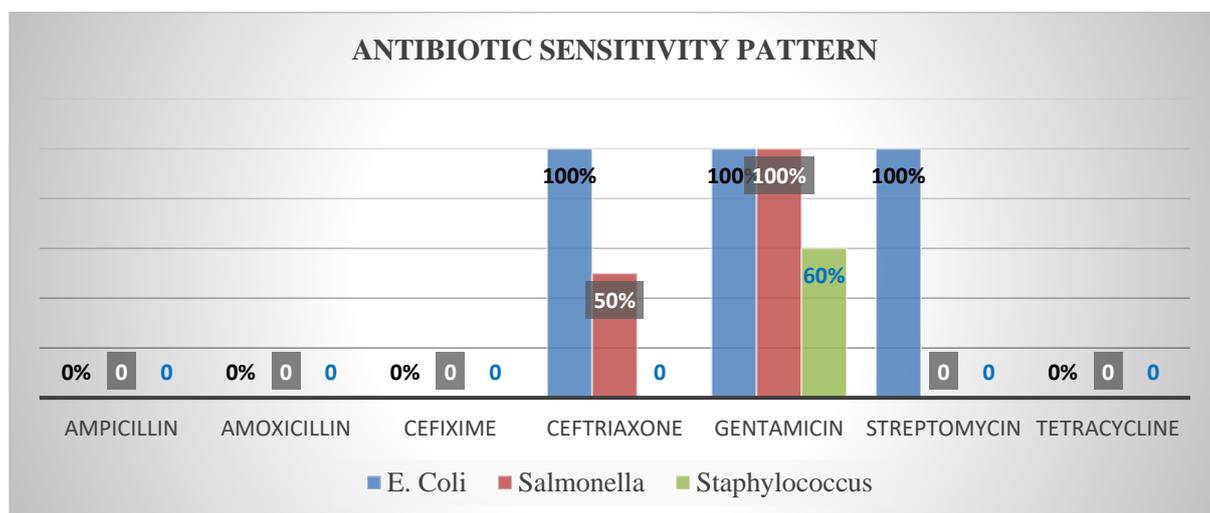
Fig 39: Antibiotic sensitivity pattern of vegetable salad

A total of 6 isolates of *Salmonella* from mixed vegetable salad samples were 100% resistant to Ampicillin, Amoxicillin, Cefixime and Streptomycin. 100% sensitive to Gentamycin. 50% sensitive to 50% resistant to Ceftriaxone, 66.66% Resistant to 33.33% intermediate resistant to Tetracycline. According to (Nawas *at el.*,2012),

Salmonella from salad showed resistance against Amoxicillin (75%), Cephradine and Cephalexin (68.75%). 85.71% *Vibrio* isolated from salad and water were resistant to Amoxicillin respectively. Multiple drug resistance was seen in 39 and 51 isolates of *Salmonella* and *Vibrio* isolates, respectively.
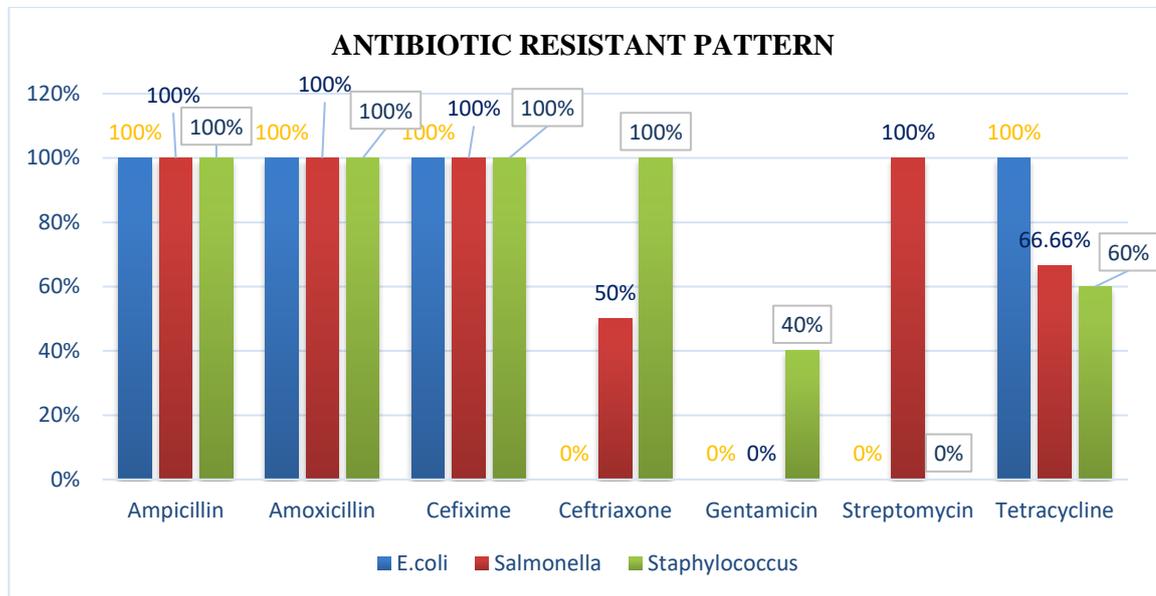
Fig 40: Antibiotic-resistant pattern of vegetable salad

A total of 5 isolates of *Staphylococcus* from mixed vegetable salad samples were 100% resistant to Ampicillin, Amoxicillin, Cefixime and Ceftriaxone, 60% sensitive to 40% resistant to Gentamycin. 100% intermediate resistant to Streptomycin. 60% Resistant and 40% intermediate are resistant to Tetracycline.

According to (Ahmed, 2014) the majority of the vegetable salad isolates showed susceptibility against ciprofloxacin, tetracycline, kanamycin. However, most of the pathogenic isolates were resistant to ampicillin. The results suggest the necessity to follow the hygienic practices in salad preparation and salad might have an important role as a source of multiple antibiotic-resistant bacteria.

CHAPTER-5

CONCLUSION

The present study was undertaken during the period of January-August 2021 to know the bacteriological status of mixed vegetable salad from the restaurant, food corner and street vendors in Dhaka city, Bangladesh. For this purpose, a total of 120 samples were collected from 10 different areas as well as to suggest public health importance based on the present hygienic condition of mixed salad vegetables by using standard bacteriological techniques. Among 120 samples, 12 samples were found positive for *Escherichia coli, 6* samples for *Salmonella spp and 5* samples for *Staphylococcus spp.* The highest TVC was (log 7.04 ± 0.48 cfu/gm) and TCC was (log 6.04 ± 0.57 cfu/gm) in street vendor, moderate value TVC (log 6.07 ± 0.69 cfu/gm) and TCC (log 6.20 ± 0.59 cfu/gm) in Restaurant and lowest value TVC (log 5.42 ± 0.69 cfu/gm) and TCC (log 5.23 ± 0.59 cfu/gm) in food corner respectively. In statistical analysis, it was found a 5% level of significance. All examined samples were contaminated and yielded the growth of aerobic coliform bacteria with varying densities which could be attributable to improper handling, unhygienic salad preparation and processing, poor sanitary practices, an unhygienic constructed vehicle with permeable and uncleaned interiors. It could be concluded that the prevalence of *E. coli* was highest in street vendor samples (15%), in food corners (10%) and in the restaurant (5%) respectively. Prevalence of *Salmonella* highest in street vendors was (7.5%), in the restaurant (5%) and in food corner (2.5%) respectively. In the case of *Staphylococcus* highest in street vendors (7.5%) and in food corner (5%) respectively. In restaurant samples, no *staphylococcus* was found. Net prevalence of *E. coli, Salmonella* and *Staphylococcus* was 10%, 5% and 4.1% respectively. The pathogenic bacteria isolated from the study are of public health importance and their high levels of resistance to commonly used antibiotics. The antibiotic sensitivity pattern of the isolates was performed against the positive results. The antibiotic sensitivity tests indicated that the isolated *E. coli* were highly resistant to Ampicillin, Amoxicillin, Cefixime and Tetracycline and sensitive to Ceftriaxone, Gentamycin and Streptomycin. *Salmonella was* highly resistant to Ampicillin, Amoxicillin, Cefixime, Streptomycin and sensitive to Gentamycin, moderate sensitive to resistant to Ceftriaxone and intermediate resistance to Tetracycline, whereas *Staphylococcus* was highly resistant to Ampicillin, Amoxicillin, Cefixime and Ceftriaxone and intermediately resistant to Streptomycin, Gentamycin and Tetracycline

RECOMMENDATION

The present findings highlight the importance of training restaurants' staff regarding sanitary methods of salad preparation, together with avoiding long storage duration and usage of leftovers. Moreover, they emphasize on the particular attention that should be paid to the hygienic handling of raw vegetables to ensure the microbiological standards for managing vegetables are effectively followed. That also indicates the need for regular inspection and close supervision of handling practices and preparation methods of street-vended salads The current study also indicate that the vegetable salad samples were largely populated with various microorganisms leading to serious public health hazards. The pathogenic bacteria present in the commonly consumed vegetable salad showed resistance against the regular antibiotics which is significant from the viewpoint of public health. The high bacterial load and presence of these organisms especially *E. coli* in the mixed vegetable salad samples could serve as an indicator for the need to promote awareness about the possible health hazards that could be due to poor handling of these vegetables. There is, therefore, the need for regulatory bodies to ensure that microbiological standards are established and practised by sellers and workers for the handling and distribution of mixed vegetable salad.

LIMITATIONS

Due to the Corona situation, sample collection was challenging. Others confirmatory test was not performed due to limited funds.

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Appendix-I

A. Formula of various bacteriological media

1. Nutrient agar

Ingredients	Gm/litre
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.5
Agar	15.00

2. Nutrient broth

Ingredients	Gm/litre
HM peptone B#	1.5
Yeast extract	1.50
Peptone	5.0
Sodium chloride	5.0

3. MacConkey Agar

Ingredients	Gm/litre
Peptic digest of animal tissue	1.5
Bile salts	1.5
Sodium chloride crystal violet	0.001
Neutral red	0.03
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatine	17

Ingredients	Gm/litre
Lactose	10
Agar	15

4.Blood Agar

Ingredients	Gm/litre
Bacto tryptose	10.00 gm
Beef extract	1.50 gm
Sodium chloride	2.50 gm
Glocose	0.15 gm
Dextrin	0.25 gm
Para-aminobenzoic acid	10.00 gm
Agar	7.50 gm
Distilled water	500 ml
Defibrinated blood	50 ml

5.EMB Agar

Ingredients	Gm/litre
Peptic digest of animal tissue	10.0
Lactose	5.0
Sucrose	5.0
Dipotassium phosphate	2.0
Agar	13.5
Eosin Y	0.4
Methylene blue	0.065
Agar	13.50

Ingredients	Gm/litre
Final p^H (at 25^{0}C)	7.2 ± 0.2

6. Salmonella-Shigella agar

Ingredients	Gm/litre
Peptone	5.00
HM peptone B#	5.00
Lactose	10.00
Bile salts mixture	8.50
Sodium citrate	10.00
Sodium thiosulphate	8.50
Ferric citrate	1.00
Brilliant green	0.33
Neutral red	0.025
Agar	15.000
Final pH (at 25^{0}C)	7.0 ± 0.2

7. Brilliant Green Agar

Ingredients	Gm/litre
Proteose peptone	10.00
Yeast extract	3.00
Lactose	10.00
Sucrose	10.00
Sodium chloride	5.00
Phenol red	0.08
Brilliant green	0.0125

Ingredients	Gm/litre
Agar	20.00

8. Mannitol salt Agar

Ingredients	Gm/litre
Proteose peptone	10.0
Beef extract	1.00
Sodium chloride	75.00
D- mannitol	10.00
Phenol red	0.025
Agar	15.00
Final pH (at 25°C)	7.4 ± 0.2

9. Muller- Hinton Agar

Ingredients	Gm/litre
Beef infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH (at 25°C)	7.3 ± 0.1

10. MR-VP broth

Ingredients	Gm/litre
Bacto MR- VP	1.7
Distilled water	100 ml

11. Phosphate buffered saline solution

Ingredients	Gm/litre
Sodium chloride	8.0
Disodium hydrogen Phosphate	2.8
Potassium chloride	0.2
Distilled water	1000 ml
Potassium dihydrogen phosphate	0.2

B. Preparation of reagents

1. Gram stain

a. Crystal violet solution (Solution A)

Ingredients	Gm/litre
Crystal violet (85 % dye content)	4.0gm
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

parts of solution B.

b. Lugol's iodine

Ingredients	Gm/litre
Iodine crystal	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml.

Allow standing 24 hours for the iodine to dissolve.

c. Decolourizer

95 % alcohol (ethyl)

d. Counterstain

Safranin stock solution

Safranin	2.5 gm
Alcohol (95 %)	100 ml

To 10.0 ml of stock solution, add 100.0 ml distilled water.

2. Methyl blue

Methylene blue	2.5 gm
Alcohol (95 %)	100.0 gm

3. Leishman's stain

Leishman's stain powder	0.15 gm
Absolute methyl alcohol (acetone free)	100 ml

4. Acetone-alcohol decolouriser

Acetone	500 ml
Ethanol/Methanol, absolute	475 ml
Distilled water	25 ml

5. Crystal violet Gram stain

Crystal violet	20 gm
Ammonium oxalate	9 gm
Ethanol/Methanol, absolute	95 gm
Distilled water	1000 ml

6. Kovac's reagent for Indole preparation

Ingredients	Gm/litre
p- dimethyl aminobenzal dehydrade	5 gm
Amyl alcohol	75 gm
Conc. Hcl	25 gm

7. Phosphate buffer solution

Ingredients	Gm/litre
Sodium chloride	8.0 gm
Potassium chloride	0.2 gm

Ingredients	Gm/litre
Disodium hydrogen phosphate	2.89 gm
Potassium hydrogen phosphate	0.2 gm