Isolation and identification of microbes from various fruit juices made and sold for immediate consumption at home and in the market of Dhaka city

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

Submitted by
Tasnim Mortuza
Student ID: 12126006
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Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Dedicated to Almighty Allah, my supervisor, my beloved family and some loving friends…

Learned from my father,

"Never give up no matter what…keep trying"

Learned from my mother,

"Always be confident and be independent"

Learned from brother,

"If something you desire from your heart truly; you will get it someday somehow"

Learned from friend,

"Time never stops for anyone, just go with the flow"

DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Isolation and identification of microbes from various fruit juices made and sold for immediate consumption at home and in the market of Dhaka city**" submitted by Tasnim Mortuza, has been carried out under the supervision and able guidance of Dr. M. Mahboob Hossain, Coordinator, Microbiology Program, Mathematics and Natural Sciences Department, BRAC University in partial fulfillment of BSc. in Microbiology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

Candidate

_____

Tasnim Mortuza

Certified:

Dr. M. Mahboob Hossain
Supervisor
Coordinator
Microbiology Program
Department of Mathematics and natural Sciences
Dhaka, Bangladesh

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Abstract:

Millions of people around the world are widely consuming fruit juices in every season as it provides an easy and affordable source of nutrients to them. The study was conducted to estimate the microbial quality of various fruit juices made and sold for immediate consumption in home and in the market of Dhaka city. Altogether, 10 fruit juice samples (5 different packed fruit juices and 5 freshly homemade fruit juice) were collected from different areas of Dhaka city and tested for their microbiological quality. Microbial quality was determined by quantifying the total viable count, total *Staphylococcal* count, total *Salmonella* count, total coliform count and total fecal coliform count. In this study, pH of the fruit juices varied from 2.8 to 3.8. The highest count of total viable organism was $2.3x10^6$ cfu/ ml, total *Staphylococcal* count was $1.4x10^4$ cfu/ ml, total coliform count was $1.75x10^4$ cfu/ ml, total fecal coliform was $5.75x10^3$ cfu/ ml and total *Salmonella* count was $3.5x10^4$ cfu/ ml. It was found that, out of 10 fruit juice samples 3 different juice samples (orange, pomegranate, grape) contained *E. coli*, 4 different samples (mango, orange, litchi) contained *Staphylococcus spp.*, 3 different samples (grape, jujube, mango) contained *Bacillus spp.*, 2 different samples (pomegranate and litchi) contained *Pseudomonas spp.*, 4 different samples (grape, jujube, litchi, orange) contained *Moraxella spp.*, 2 different samples (jujube and litchi) contained *Klebsiella spp.*, jujube juice sample contained *Salmonella spp.*, pomegranate juice sample contained *Shigella spp.* and some other different organism such as *Listeria spp., Clostridium spp., Acinetobacter spp., Brevibacterium spp., Acetobacter spp. and Francisella spp.* had been identified from the tested juice samples. In this study, antibiotic susceptibility test was done from collected isolates from juice samples. This study specially highlights the level of microbial loads found in various fruit juice samples.

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Introduction

Introduction:

1.1 Overview

Fruits are a part of our daily consumptions. All over the world, in everyone's diet chart it is always included as a whole fruit, juice, beverage or still drink etc. The world consumed 117.7 billion gallons of industrialized still drinks. Of the total volume, 77% were consumed in 40 countries, with 23.5 million liters in the juice category, 42 million in the category of still drinks, and 35 million in the category of powdered and concentrated juices (Neves, 2012). Fruit juices contain antioxidants, vitamins, and minerals that are essential for human being and they play important role in the prevention of heart diseases, cancer, and diabetes. Fruit juices contain essential nutrients which support the growth of acid tolerant bacteria, yeasts, and molds. In recent years, the increasing consumer awareness has emphasized the need for chemically and microbiologically safe food (Aneja, 2014).

1.1.1 Fruit

A structural part of a plant is fruit which contains seeds, normally fleshy, sweet and edible in the raw states that include: mangoes, oranges, grapes, litchis, and pomegranates etc. They are ripe ovaries or carpels that contain seed (McGee, 2004). Similar in composition to vegetables, fruits contain various phytochemical compounds and a high percentage of water averaging 85%, also in small amount fat; protein and carbohydrate (cellulose and starch) are present (Ihekoronye & Ngoddy, 1985). Most fruits are eaten as desserts and they can be processed into liquid product which includes fruit juices, wines and other preserves like; marmalade, jams, jellies etc. Fruit products are marketed canned, bottled or packaged in tetra-packets.

1.1.2 Fruit juice

The aqueous liquid, puree of the edible portions is juice, or any concentrates of such liquid or puree from one or more fruits or vegetables can be juice either. Fruit juices are mainly used for their nutritional value, refreshing nature also for their medicinal importance. In detoxification of human body and in improvement of blood lipid profile in patients of hypercholesterolemia fruit and vegetable juices play great roles.

Fruit juices are nutritious drinks with great taste and health benefits (Suaads & Hamed, 2008). Fruit juices are important sources of nutrients and contain several important therapeutic

properties that may reduce the risk of various diseases. They contain large amounts of antioxidants, vitamins C and E, and possess pleasant taste and aroma (Abbo et. al, 2006)

1.1.2.1 Nutrition Facts

Table 1: The nutrition facts of juice per cup (*Sources include: USDA)

Juice Amount Per 1 cup (249g)

Vitamin A	2%	Vitamin C	62%
Calcium	0%	Iron	1%
Vitamin D	0%	Vitamin B-6	60%
Vitamin B-12	0%	Magnesium	1%

Calories 136

	% Daily Value*
Total Fat 0 g	0%
Saturated fat 0 g	0%
Polyunsaturated fat 0 g	
Monounsaturated fat 0 g	
Cholesterol 0 mg	0%
Sodium 5 mg	0%
Potassium 105 mg	3%
Total Carbohydrate 33 g	11%
Dietary fiber 0.5 g	2%
Sugar 23 g	
Protein 0.5 g	1%

Figure 1: Nutritional source of various juices.

1.1.3 Health benefits of different fruit juices

Juices are mostly consumed for their perceived health benefits. For example, orange juice is rich in vitamin C, folic acid, potassium, which are an excellent source of bioavailable antioxidant phytochemicals (Franke, 2005) and in people affected with hypercholesterolemia it significantly improves blood lipid profiles (Kurowska, 2000). Prune juice is associated with a digestive health benefit. Cranberry juice has long been known to help prevent or even treat bladder infections, and it is now known that a substance in cranberries prevents bacteria from binding to the bladder. Pomegranate juice reduce dangerous LDL-cholesterol in blood, improve blood flow to the heart in patients with coronary artery disease, reduce thickening of the arteries that supply blood to the brain, lower the level of systolic blood pressure also an antioxidant-rich fruit. This fruit may also be able to help fight cancer which researchers have been looking for. Mango juices are perfect to replenish salts, vitamins and energy after physical exercise. In gall bladder cancer a protective effect of mangoes consumes has been proven. Mango juice also contains a lot of tryptophan, the precursors of serotonin. Litchi juice contains high amount of antioxidants which is effective to prevent early ageing also effective to protect from asthma and a rich source of nutrient that is required for the production of blood. It provides manganese, magnesium, copper, iron and folate that are required for the formation of RBC. The most predominant nutrient in grape juice is manganese. Drinking grape juice helps fight conditions associated with cardiovascular disease, including high blood pressure and plaque buildup. Jujube fruit contains high levels of Vitamin C (higher than in most citrus fruits like the orange), Vitamins B1 (thiamine), B2 (riboflavin), B3 (niacin) and B6 (which are the complex B vitamins), Vitamin A (in 2 forms), and minerals that include calcium, potassium, phosphorus and manganese. It also contains significant levels of copper, zinc, iron and sodium. Perhaps the most significant of all of the jujube benefits is the fact that the fruit contains 18 amino acids.

1.1.4 Manufacture and processing of juice products

Juice is mainly prepared by squeezing fresh fruits by hand or juicers (in home) and by machines (in factories). For example, orange juice is the liquid extract which results from pressing the fruits of the orange tree. Most of the commercial fruit juices are filtered to remove fiber or pulp, but high-pulp fresh orange juice is a popular beverage all over the world. General methods for preservation and processing of fruit juices include canning, pasteurization, concentrating,

freezing, evaporation and spray drying. Many juice preservation strategies include washing and sorting, juice extraction, straining, filtration and clarification, blending, pasteurization, filling, sealing and sterilization, cooling, labeling and packing (Davidson 2001).

The squeezing, macerating or crushing of fruits is done in the process of extracting juice from fruits. A major amount of pulp is obtained by this process or it may be extracted using water. These juices are used either in their natural concentrations or may be concentrated by evaporation or freezing and they are preserved by canning, freezing or drying.

1.1.5 Different reasons of deterioration of fruits and fruit juices

Deterioration of fruits results from so many factors such as physical factors, fruits' own enzyme action, microbial action or combination of all these. When the fruit is soft and juicy, the fruit rot is appropriate to be soft and squashy and some leakage may result. Fruits carry a natural flora of micro-organisms acquired from their environment and certain organisms cause a drying effect, which results in dry, leathery rots with the discoloured surface area. Fruits can be infected by pathogenic microbes which cause wilts, blotching and browning. Once harvested care has to be taken in handling as bruising can allow the entry of harmful organisms, particularly fungi, which soon rot the product. The green mold sometimes seen on oranges is a type of *Penicillium; Botrytis* causes the fuzzy grey growth on strawberries. Juices extracted from fruits are acidic in nature. For the lemon juice, the pH is 2.4 and for tomato juice is 4.2. They also contain high sugar content of about 2% in lemon juice and about 17% in some grape juices. Mold growth is favored on the surface of these juices. Bacteria and yeast grow faster when juices are exposed to high moisture. Removal of solids by extraction and sieving of juices makes the oxidation-reduction potential to become higher which in turn favors the growth of yeast. Lack of vitamin B discourages some bacteria. The character of spoilage depends on the product attacked and the microbe that causes the spoilage. The identification of the type of spoilage will help in finding out the appropriate method for preventing its decay.

Four types of factors determine the colonization of fresh-cut fruits and derivatives by microorganisms such as intrinsic factors, which are dependent on food composition, such as water activity, pH, redox potential, nutrients, structures, and antimicrobial agents; technological treatments, which can modify the initial microbiota; extrinsic factors or environmental conditions of the medium such as temperature, relative humidity, and atmosphere; implicit factors, which

depend on the developing microbiota and the handling of both the raw material and the product during processing and storage (Montville and Matthews, 2001).

Fruits may become contaminated with pathogenic and spoilage microorganisms either during their growing in fields, orchards, vineyards, or greenhouses, or during harvesting, postharvest handling, and distribution (Beuchat 2002). Fresh fruits have a natural protective barrier (skin) that acts effectively against most plant spoilage and pathogenic microorganisms; however, this protection may be eliminated during the processing, thus exposing the fruit flesh to unfavorable environmental conditions as well as to a possible contamination with pathogenic microorganisms including bacteria, viruses, and parasites during the handling, cutting, shredding, and maintenance of the fresh-cut fruit at ambient temperature (Balla and Farkas, 2006).

1.1.6 Causative agent of microbial spoilage and diseases caused by them

Fresh fruits can contain large and diverse populations of bacteria. However, most of the work on produce-associated bacteria has focused on a relatively small number of pathogenic bacteria and, as a result, we know far less about the overall diversity and composition of those bacterial communities found on produce and how the structure of these communities varies across produce types. Moreover, a comprehensive view of the potential effects of differing farming practices is lacked on the bacterial communities to which consumers are exposed. (Jonathan, 2013)

The causative agents of microbiological spoilage in fruits and fruit juices can be bacteria, as well as yeasts and molds. The main spoilage agents can be considered as due to the low pH of most fruits. Some bacteria such as Campylobacter spp., *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus*, *Shigella* spp, *Erwinia* spp., *Enterobacter* spp., *Alicyclobacillus* spp., *Propionibacterium cyclohexanicum*, *Pseudomonas* spp., and lactic acid bacteria can cause spoilage in fruit and fruit juices (Walker and Phillips, 2008). Certain common molds such as *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp., *Alternaria* spp., *Cladosporium* spp., *Paecilomyces* spp., and *Botrytis* spp. have been shown to be involved in the spoilage of fresh fruits (Lund and Snowdon, 2000).

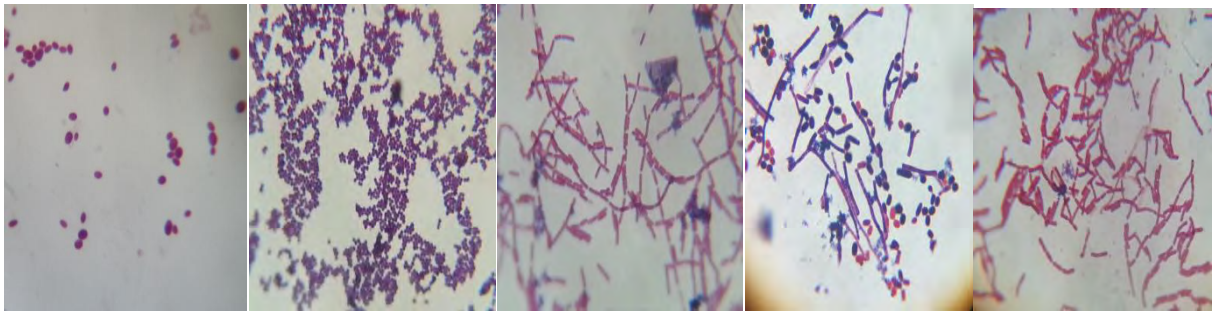


Figure 2: Some bacteria and molds.

E. coli (*Escherichia coli*) is a gram negative bacterium, which lives in the digestive tracts of humans and animals. There are many types of *E. coli*, and most of them are harmless. *E. coli* infection occurs by coming into contact with the feces, or stool, of humans or animals. By drinking water or eating food that has been contaminated by feces anyone can get infected by *E. coli*.

Salmonella spp. are infectious bacteria associated with food borne and gastrointestinal illnesses. *Salmonella* bacteria can be found in food products such as raw poultry, eggs, and beef, and sometimes on unwashed fruit. There are two main diseases caused by *Salmonella spp*. and they are Salmonellosis and typhoid fever. *Salmonella enteritidis* or *Salmonella typhimurium* causes Salmonellosis and Typhoid fever is caused by *Salmonella typhi*. People who eat food contaminated by *Salmonella* can become ill with salmonellosis.

Staphylococcus aureus stains are gram positive and non-moving small round shaped or non-motile cocci. *Staphylococcus* spp. are found in grape-like (staphylo-) clusters. Staphylococcus is one of the five most common causes of infections after injury or surgery. *S. aureus* may occur commonly in the environment. *S. aureus* is transmitted through air droplets or aerosol also by direct contact with objects that are contaminated by the bacteria or by bites from infected persons or animals.

Pseudomonas spp. are gram negative bacteria, morphologically enteric bacilli and vibrios. They have peritrichous flagella which defines them as motile bacteria. *Pseudomonas* spp. are strict aerobes. They are mostly free-living bacteria widely distributed in soil and water. For the most part they are found wherever organic matter is decomposing. *Pseudomonas aeruginosa* can

cause sputum of patients with cystic fibrosis, burns, urinary tract infections also prevalent nosocomial infections, external ear infections etc.

Serratia spp. are gram negative, bacilli shaped, facultative anaerobe, motile bacteria that belongs to the family Enterobacteriaceae. These bacteria grow well on standard media and produce a red to dark pink pigment that aids in identification. (Kayla, 2004). Although *S. marcescens* was considered to be an innocuous, non-pathogenic organism, over the last two decades they have become an opportunist pathogen causing nosocomial infections. A broad range of hospital-acquired infections caused *by S. marcescens* include respiratory tract infections, urinary tract infections (UTI), septicaemia, meningitis, pneumonia, conjunctivitis wound and eye infections, osteomyelitis, keratoconjunctivitis, keratitis, endophthalmitis and endocarditis (Hejazi, 1997).

Above mentioned pathogens can be treated with different medicines and antibiotics. There are other different bacteria in the world those are pathogenic or opportunistic pathogens and causes deadly disease to humans and animals. Some recent studies have shown that so many bacteria have become antibiotic resistant and for that reason the disease they cause become untreated, which is very dangerous for all human being. These are about bacteria but there are huge number of other microorganisms like virus, mold, fungus; they also cause so many diseases to human being which are curable as well as deadly diseases like HIV/ AIDS, cancer etc. Some are treated with medicines and antibiotics or vaccines but most of these microorganisms either themselves yet not been discovered or the disease they cause yet no treatment have been discovered.

1.1.7 Antibiotics for treatment

Antibiotics are medicines that help our body fight bacteria and viruses, either by directly killing or by weakening the organism so that our own immune system can fight and kill them more easily. The vast majority of antibiotics are bacteria fighters; although there are millions of viruses, we only have antibiotics for half-a-dozen or so of them. Bacteria, on the other hand, are more complex and so are easier to kill. Bacteria can live independently but viruses must need a host cell to survive. Bacteria and viruses aren't particularly intelligent. However, it can be possible and unfortunately all too common for bacteria and some viruses to learn how to survive even with antibiotics around. There are several ways that bacteria can become resistant. All of them involve changes in the bacteria's genes.

Bacterial genes when mutate because of chemical or radiation exposure; or sometimes randomly (without specific reason). If bacteria with a changed gene is less susceptible to an antibiotic, and that antibiotic is around, the less susceptible (and more resistant) version of the bacteria is more likely to survive the antibiotic and continue to multiply. This is particularly happening if the amount of antibiotic around is not quite enough to kill all of the bacteria quickly, it can happen if enough of the antibiotic was not taken to keep its level high in our body, or the antibiotic taking was stopped too early. This is why when someone is prescribed an antibiotic they MUST take it exactly as prescribed, and for as long as it was prescribed: they may feel better after only a short time, but still have some bacteria left in body but not enough to make them feel bad, but enough to come back and those bacteria left include the ones that are partly resistant to the antibiotic already and likely to become more resistant. Although there are many different species of bacteria, some bacteria can exchange genes with other bacteria. They can then give the resistance genes they have developed to other, harmful bacteria. There are viruses around that attack bacteria rather than plants, animals, or people. Most of these viruses just kill the bacteria, but sometimes the viruses can copy genes like the antibiotic resistance genes from one kind of bacteria to another and becomes resistant to drug.

1.3 Literature review

Rashed et al. (2012) investigated to resolve the microbiological attributes of the fruit juices collected from different areas around Dhaka city. To check the total bacterial load, coliforms and staphylococci 26 vendor fruit juices and 15 packed juices were examined. Samples were found to harbor viable bacteria within the range between 10^2 -10^7 cfu/ ml. Thirty samples exhibited the presence of staphylococci. Total coliforms were detected in 31 samples within the range of 10^2 - 10^6 cfu/ ml which were further detected as *Escherichia coli* and *Klebsiella* spp. Fecal coliforms were found in 4 vendor fruit juice samples (10^2 cfu/ ml), while in the industrially packed samples, they were completely absent. Drug resistance among the isolates was found against ampicillin, ciprofloxacin, amoxicillin, erythromycin, chloramphenicol, ceftriaxone, piperaciline, trimethoprime-sulfomethoxazole, nalidixic acid and vancomycin. Overall, the study demonstrates that the quality of the both packed and fresh juices was unsatisfactory and hence the products need to be microbiologically controlled in order to ensure the overall health safety.

Tasmina et al. (2010) conducted their study to assess the microbial quality of fresh and commercially packed available juices collected from different locations of Dhaka city. A total of six fresh juice and nine commercially packed juice samples were collected. Standard culture techniques were followed to assess total viable count (TVC), total Staphylococcal count (TSC), total *Bacillus* count (TBC) and total fungal count (TFC) on different culture media. The TVC varied from the range from 10^2 to 10^5 cfu/ ml with the highest of 2.4 x 10^5 cfu/ ml. A large number of Staphylococci and *Bacillus* was also found from several samples. Total coliform and fecal coliform was found in six and five (out of fifteen) samples, respectively. Among total coliforms, *Klebsiella* spp., *Enterobacter* spp. along with *E. coli* were detected. From all the assessment it was determined that the microbial quality of commercially packed juice was fairer than that of fresh juice collected from local market.

Md. Munjur et al. (2014) investigated to resolve the microbiological attributes of the fruit juices collected from different areas around Jessore city. Ten fresh fruit juices and ten commercially packed fruit juices were collected. Standard plate count techniques were followed to assess total viable count (TVC), total coliform count (TCC) and total Staphylococcal count (TSC) on different culture media. Samples were found to harbor viable bacteria within the range between 10^3-10^8 cfu/ ml. 19 samples exhibited the presence of Staphylococci. Total coliforms were detected in 17 samples within the range of 10^3-10^6 cfu/ ml which were further detected as *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. From all the assessment, the study demonstrates that the quality of both packed and fresh juices was unsatisfactory and hence the products need to be microbiologically controlled in order to ensure the overall health safety.

Joy et al. (2006) aimed at examining the quality and safety of freshly squeezed fruit juices, in a metropolitan city (Visakhapatnam) in south India, based on standard techniques (e.g. culturing on selective media), showed that in most localities the street vended fruit juices remained hygienically poor since bacterial loads (Total viable counts and Total coliforms) on the whole are abnormally high (HVC 0.88-33.6×10^4 cfu/ 100 ml; TC 0.8-22.2×10^4 CFUs/ 100 ml). Based on the presence of fecal coliforms (0.4-11.0 cfu/ 100 ml) and fecal Streptococci (0.0-6.6 cfu/ 100 ml), it is concluded that fruit juices in certain areas inside the city (e.g. R.T.C. Complex, Fishermen's colony, Vegetable market) are highly impacted and unfit for human consumption. Overall, it is contended that contamination is mainly due to poor quality of water used for

dilution, prevailing unhygienic conditions related to washing of utensils, maintenance of the premises, and location by the side of a busy road with heavy vehicular traffic or by the side of the waste disposal system and overcrowding. The occurrence of pathogenic *E. coli*, *Streptococcus faecalis*, *Salmonella typhi* and *Salmonella typhimurium* is alarming enough for an immediate action by the suitable agency. It is suggested that regular monitoring of the quality of fruit juices for human consumption must be introduced to avoid any future pathogen outbreaks.

Another study was aimed and done to assess the microbial quality of fruit juices sold for immediate consumption in the markets of Kashmir valley. Twelve fruit juice samples (3 from each apple, orange, pineapple and mango juices) were procured from different markets and tested for their microbiological quality. Microbial quality was determined by enumerating the total viable count. About 25% of the samples (orange juice) did not comply with the standards of microbial quality as per the guidelines for microbiological quality of ready to eat foods while as apple, orange and pineapple juices complied with the standards. The microbial load in orange juice was comparatively higher than that in the apple, pineapple and mango juice which had the microbial load within acceptable limits (Gulzar et al. 2013).

1.2 Aim of the study

The aim of this project is to compare the fruit juices (commercially packed and freshly homemade) and to check microbial quality. If from the samples growth of different microorganism have been found following steps will be done.

- Isolation of microorganisms from juice samples available in Bangladeshi market and at home
- Isolates will be identified with different biochemical test
- To detect the antibiotic resistance pattern of the bacterial isolates found in juice

Chapter *II*

Materials and Method

Materials and methods:

2.1 Study place:

The laboratory works of this research was done in the microbiology research laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Study period:

This research work was carried out from October, 2015 to June, 2016.

2.3 Materials

2.3.1 Equipment

- Laminar airflow cabinet (Model-SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digi system Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Glass wares, laboratory distillation apparatus- fractional distillatory set up, microscope, pH meter petri-dishes, slants, micro-pipettes, Bunsen burner, hot plate, clamp stands, electric balance, etc.

2.3.2 Media

Different types of media were used for selective growth, enrichment culture, and indication of specific properties. Media preparation and sterilization were done according to the protocol and standard recipe.

Figure 3: Different types of mediums were prepared.

2.3.3 Biochemical test media

Different specific biochemical mediums were prepared for different biochemical tests.

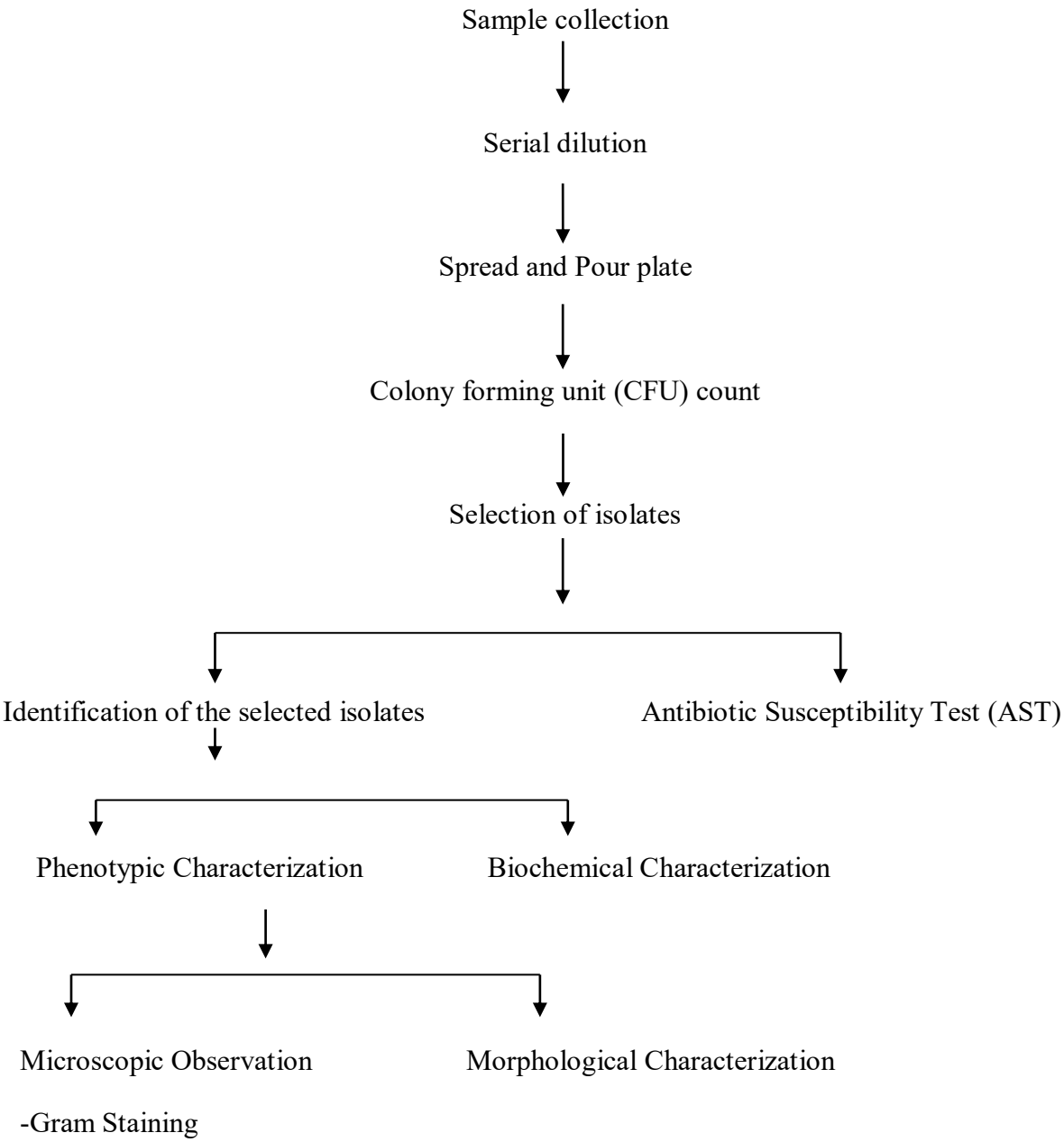
2.3.4 Antibiotic disc

About 32-33 different antibiotic discs were used for identifying antibiotic sensitive and resistant bacteria. Antibiotics those were used are given in Table 2.

Table 2: List of antibiotics and their zone ranges

Serial no.	Antimicrobial agent	Disk code	Disk potency (µg)	Range		
				Resistance (mm)	Intermediate (mm)	Susceptible (mm)
1.	Amikacin	AK	30	14	15-16	17
2.	Amoxicillin	AML	10	13/19	14-17	20
3.	Ampicillin	AMP	10	13/28	14-16	17/29
4.	Azithromycin	AZM	15	13	14-17	18
5.	Aztreonam	ATM	30	15	16-21	22
6.	Cefepime	CPM	30	14	15-17	18
7.	Cefoxitin	FOX	30	14	15-17	18
8.	Ceftazidime	CAZ	30	14	15-17	18
9.	Ceftriaxone	CRO	30	13	14-20	21
10.	Cephalexin	CL	30	14	14-18	19
11.	Chloramphenicol	C	30	12	13-17	18
12.	Ciprofloxacin	CIP	5	15	16-20	21
13.	Clindamycin	DA	2	14	15-20	21
14.	Cloxacillin	OB	5	15	16-19	20
15.	Co-trimethozole/sulfamethoprim	COT	25	10	11-15	16
16.	Doxycycline	DO	30	15	16-19	20
17.	Erythromycin	E	15	13	14-22	23
18.	Gentamicin	CN	10	12	13-14	15
19.	Imipenem	IPM	10	13	14-15	16
20.	Levofloxacin	LEV	5	13	14-16	17
21.	Minocycline	MH	30	14	15-18	19
22.	Nalidixic acid	NA	30	13	14-18	19
23.	Netilmicin	NET	30	15	16-19	20
24.	Nitrofurantoin	NIT	300	14	15-16	17
25.	Norfloxacin	NOR	10	12	13-16	17
26.	Oxacillin	OX	1	10	11-12	13
27.	Penicillin-G	P	10	14/28	12/21-21/28	15/19
28.	Piperacillin-tazobactam	TPZ	110	17	18-20	21
29.	Streptomycin	S	10	11	12-14	15
30.	Tetracycline	TE	30	14	15-18	19
31.	Tobramycin	TOB	10	12	13-14	15
32.	Trimethoprim/ Sulfamethozole	SXT	25	13	14-18	19
33.	Vancomycin	VA	30	14	15-16	17

2.4 Flowchart of the study design:



2.5 Methods

2.5.1 Sample collection

Juice samples were collected from different locations of Dhaka city. Total ten juice samples were collected, where five from different local street shops and the other five from different homemade juice samples from different locations.

Table 3: Name of the fresh juice samples those were used

Fresh juice	Collection area	Sample no.
1. **Orange juice**	Dhanmondi	FO
2. **Pomegranate juice**	Puran Dhaka	FP
3. **Jujube (Boroi) juice**	Mirpur-10	FJ
4. **Mango juice**	Mirpur-14	FM
5. **Grape juice**	Mirpur-13	FG

Table 4: Name of the commercially packed juice sample those were used

Commercially packed juice	Sample no.
1. **Mango juice**	CM1
2. **Mango juice**	CM2
3. **Mango juice**	CM3
4. **Grape juice**	CG
5. **Litchi flavored juice**	CL

From 10 different juice samples the isolates that were found are listed on Table 5.

Table 5: Isolates names

Isolates no.	Original name of the isolates	Given names of the Isolates
1.	Fresh juice Grape- Nutrient Agar- Orange colony1	FG-NO1
2.	Fresh juice Pomegranate- Nutrient Agar- Pink Colony2	FP-NP2
3.	Fresh juice Grape- Mannitol Salt Agar- Red colony3	FG-MSR3
4.	Fresh juice Jujube- MFC Agar- Blue colony4	FJ-MFB4
5.	Fresh juice Pomegranate- XLD Agar- Black colony5	FP-XB5
6.	Fresh juice Jujube- Mannitol Salt Agar- Pink colony6	FJ-MSP6
7.	Fresh juice Pomegranate- MacConkey Agar- Pink colony7	FP-MP7
8.	Fresh juice Grape- Mannitol Salt Agar- Yellow colony8	FG-MSY8
9.	Fresh juice Pomegranate- MFC Agar- Blue colony9	FP-MFB9
10.	Fresh juice Jujube- Mannitol Salt Agar- Yellow colony10	FJ-MSY10
11.	Fresh juice Jujube- Nutrient Agar-Lemon colony11	FJ-NL11
12.	Fresh juice Jujube- XLD Agar- Black colony12	FJ-XB12
13.	Fresh juice Grape-XLD Agar- Grey colony13	FG-XG13
14.	Fresh juice Grape- MFC Agar- Blue colony14	FG-MFB14
15.	Fresh juice Grape- MacConkey Agar- Pink colony15	FG-MP15
16.	Commercially packed juice Litchi- Nutrient Agar- Fluorescent colony16	CL-NFF16
17.	Commercially packed juice Litchi- Mannitol Salt Agar-Grey colony17	CL-MSG17
18.	Commercially packed juice Litchi- Nutrient Agar- Orangish Pink colony18	CL-NOP18
19.	Commercially packed juice Litchi-XLD Agar- Yellow colony19	CL-XY19
20.	Commercially packed juice Litchi-MacConkey Agar- Pink colony20	CL-MP20
21.	Fresh juice Mango- MacConkey Agar- Pink colony21	FM-MP21
22.	Fresh juice Orange- XLD Agra- Yellow colony22	FO-XY22
23.	Fresh juice Mango- Mannitol Salt Agar- Yellow colony23	FM-MSY23
24.	Fresh juice Orange- Mannitol Salt Agar- Yellow colony24	FO-MSY24
25.	Fresh juice Mango- Nutrient Agar- Orange colony25	FM-NO25
26.	Commercially packed juice Mango2- Mannitol Salt Agar-Yellow colony26	CM2-MSY26
27.	Commercially packed juice- Nutrient Agar- Off white colony27	CM1-NO27
28.	Fresh juice Pomegranate-MFC Agar- Blue colony28	FP-MFB28
29.	Fresh juice Orange-XLD Agar- Yellow colony29	FO-XY29
30.	Fresh juice Orange- MacConkey Agar- Pink colony30	FO-MP30
31.	Commercially packed juice Mango2-Mannitol Salt Agar-Yellow colony31	CM2-MSY31
32.	Commercially packed juice Grape- Nutrient Agar- Off white colony32	CMG-NO32
33.	Fresh juice Orange- MacConkey Agar- Pink Colony33	FO-MP33
34.	Fresh juice Pomegranate- Nutrient Agar- Green Fluorescent colony34	FP- NG34

Thirty-four isolates were collected from 10 different juice samples and to make it easier to understand isolates were given short names described in Table 5.

2.5.2 Sample processing:

After collecting the samples, pH was measured; serial dilutions were done from the samples and spread plate, pour plate method was done to see the growth of different microorganisms.

2.5.2.1 pH measurement:

Three beakers were rinsed with water and ethanol, dried and labeled (sample, control and distilled water). After rinsing the pH meter with distilled water it was dipped into the beaker poured with juice sample. After 30 seconds the reading of the pH meter was noted down and the pH meter was rinsed with distilled water and ethanol, dried and switched off. The process was followed for next all samples. As most of the fruit juices showed pH value lower than 7 so the control was set for pH 4. The table below contains examples of substances with different pH values (Decelles, 2002).

Table 6: The pH Scale; Some Examples of substances with different pH values

pH Value	H+ Concentration Relative to Pure Water	Example
0	10 000 000	battery acid
1	1 000 000	gastric acid
2	100 000	lemon juice, vinegar
3	10 000	orange juice, soda
4	1 000	tomato juice, acid rain
5	100	black coffee, bananas
6	10	urine, milk
7	1	pure water
8	0.1	sea water, eggs
9	0.01	baking soda

Table 6: The pH Scale; Some Examples of substances with different pH values		
pH Value	**H+ Concentration Relative to Pure Water**	**Example**
10	0.001	Great Salt Lake, milk of magnesia
11	0.000 1	ammonia solution
12	0.000 01	soapy water
13	0.000 001	bleach, oven cleaner
14	0.000 000 1	liquid drain cleaner

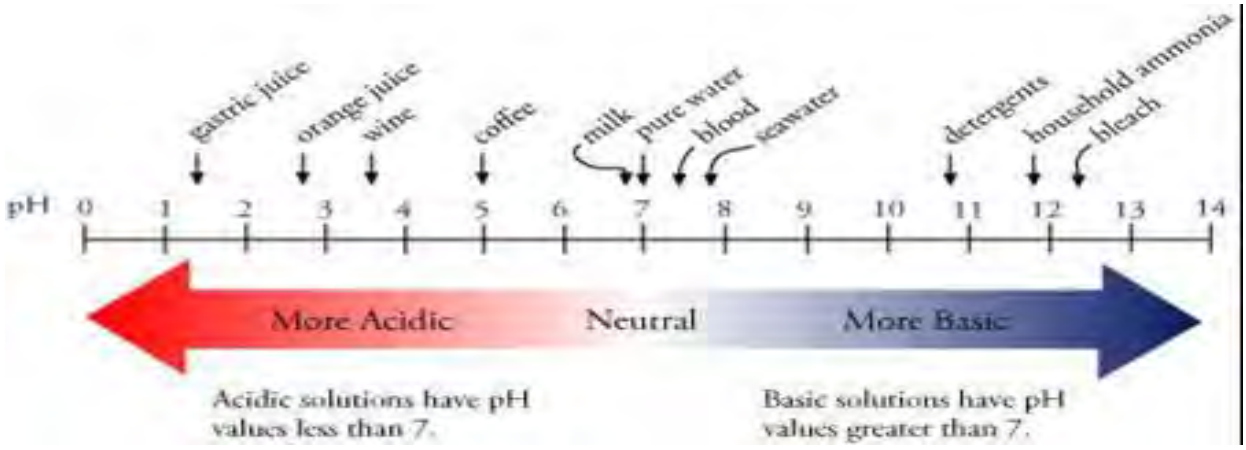


Figure 4: pH scale.

2.5.2.2 Serial dilution:

Test tubes containing 9 ml of physiological saline (0.9% NaCl) were autoclaved before use. Tenfold serial dilutions of the soil sample were prepared in autoclaved saline water. Initially, 1 ml of juice was mixed with 9 ml of saline water in a test tube in order to dilution 10^{-1} and mixed with 9 ml of saline in it by repeated pipetting in order to make tenfold dilution. Again, 1 ml from the 10^{-1} test tube was transferred to 10^{-2} labeled test tube and mixed with 9 ml saline solution in it by repeated pipetting. This action was repeated for the test tubes labeled as 10^{-3}, and 10^{-4}.

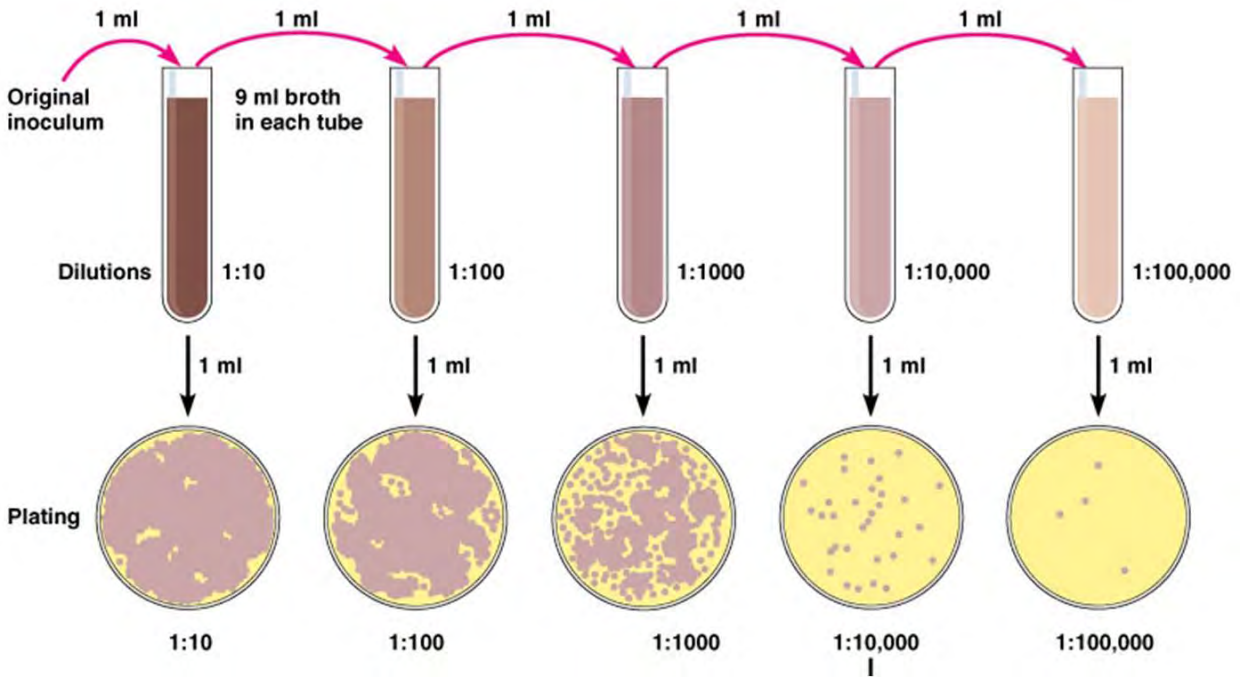


Figure 5: Serial dilution.

2.5.2.3 Spread plate method:

After finishing serial dilution, five Nutrient agar plates were labeled as raw, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, three Mannitol Salt agar plates, three MacConkey agar plates and three XLD agar plates were labeled as raw, 10^{-1}, and 10^{-2}. 0.2 ml from the test tubes labeled raw, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}were added on the respective plates and the drops were spread using spread plate technique with a spreader. All plates were then incubated at 37◦C for 24-48 hours. Finally, the plates showing colonies were counted and noted down for further study.

2.5.2.4 Pour plate:

From each test tube labeled 10^{-1}, 10^{-2} and the raw sample, 2 ml inoculums were added on the respective MFc agar plates. Using pour plate method MFc agar plates were prepared. In the pour plate method, an inoculum was to be added to melted, cooled agar. The agar inoculum mixture was to be then poured into a sterile petri dish. When it would solidify, isolated cells were to be trapped within the agar matrix. These cells would give rise to isolated pure colonies of the bacteria. A colony would be a visible mass of microorganisms growing on a solid medium. A colony would be thought (in general) to have formed from reproduction of a single cell so that all the members of a colony were to be descendent from that original cell.

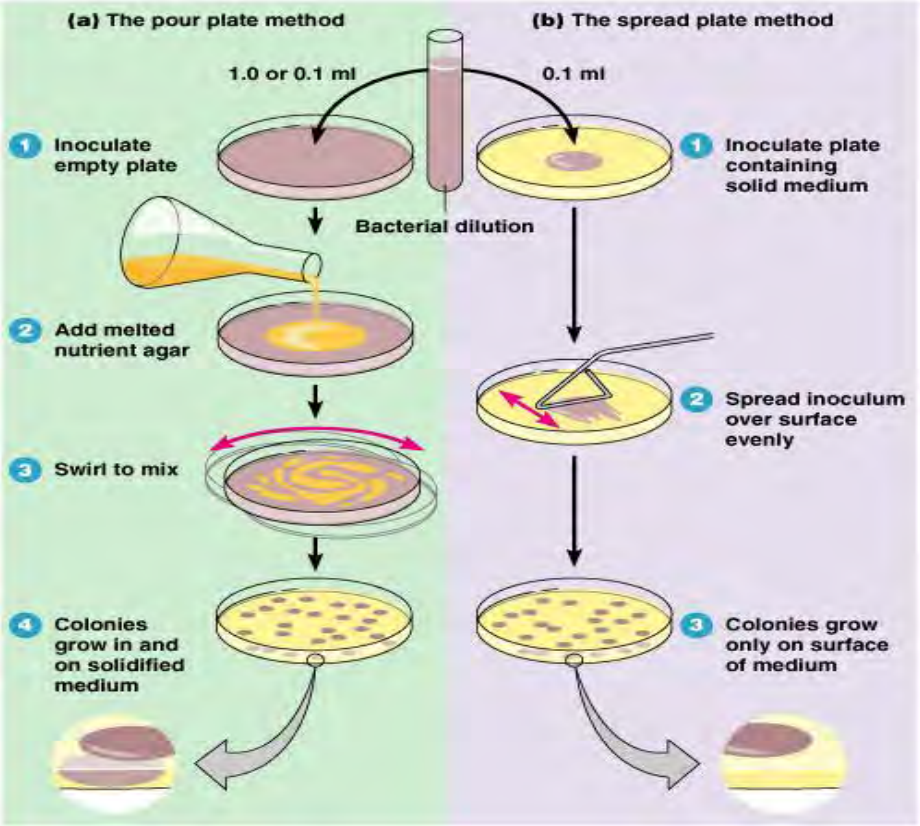


Figure 6: Pour plate and spread plate method.

2.5.3 Morphological characterizations of bacteria

Nutrient agar was prepared and autoclaved at 121◦C, 15 psi. The media was dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a NA plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of three quadrant streak plate method to obtain isolated discrete colonies. The plates were then incubated at 37◦C for 24 hours. After incubation, the growth patterns of the bacteria were evaluated for size, pigmentation, form, margin, elevation and texture (Cappuccino & Sherman, 2005).

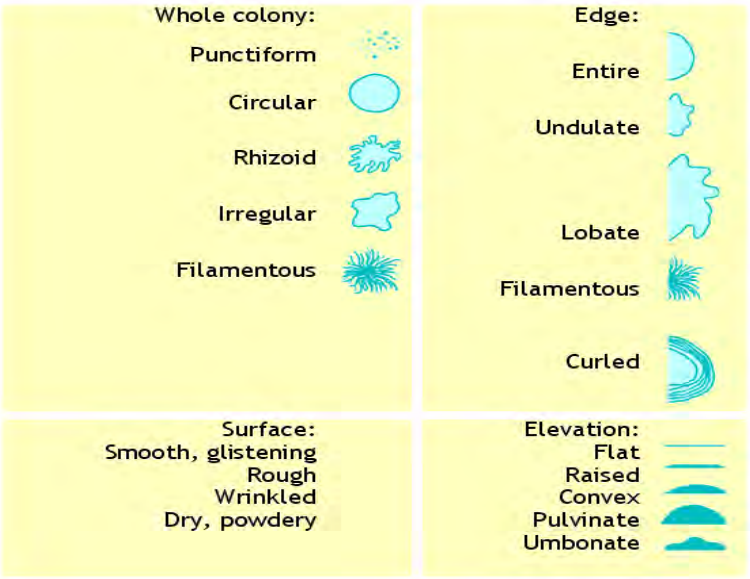


Figure 7: Different colony morphology of bacteria.

2.5.4 Microscopic Observation of the bacteria

The potential bacteria were observed under microscope in order to study their properties.

2.5.4.1 Gram stain

Gram staining was done to differentiate between two principal groups of bacteria: gram positive and gram negative.

2.5.5 Biochemical analysis

2.5.5.1 Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), MIU test (Motility test, Indole test and Urease test), Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Gelatin hydrolysis test, Starch hydrolysis, Blood agar, Eosin methylene blue agar, and Cetrimide agar.

2.5.5.1.1 Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the *Enterobacteriaceae* based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar

iron slants were prepared in the test tubes by autoclaving at 15 psi 121◦C. Using sterile technique; small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened and the tubes were incubated for 24 hours at 37◦C (Cappuccino & Sherman, 2005).

2.5.5.1.2 Indole Production test

Indole production test was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 48 hours at 37◦C. In order to test for indole production, 5 drops of Kovac's reagent was added directly into the tubes (MacWilliams, 2009).

2.5.5.1.3 Methyl red test

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. MR-VP broth of 7 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 hours at 37◦C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hours. After 48-hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red color. (Cappuccino & Sherman, 2005)

2.5.5.1.4 Voges Proskauer test

Voges Proskauer test was done to differentiate further among enteric organisms such as *E.coli, E. aerogenes*, and *K. pneumoniae* by determining the capability of the organisms to produce non acidic or neutral end products such as acetylmethylcarbinol. To the aliquot of MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (reagent B). The tube was gently shaked to expose the medium to

atmospheric oxygen (30 seconds-1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents (McDevitt, 2009).

2.5.5.1.5 Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37◦C (Cappuccino & Sherman, 2005).

2.5.5.1.6 MIU (Motility- Indole- Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. MIU media was prepared by autoclaving at 15 psi 121◦C. the media was cooled to about 50-55◦C and 100 ml of urea glucose solution was added aseptically to 900 ml base medium. After that, 6 ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C (Acharya, 2015).

2.5.5.1.7 Nitrate reduction test

Nitrate reduction test was done to determine the ability or inability of the bacteria to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or beyond the nitrite stage using anaerobic respiration by the enzyme nitrate reductase. Nitrate broth of 6 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37◦C. After incubation, 5 drops of reagent A and 5 drops of reagent B was added to each broth. If there was no red colour development, a small amount of zinc was added to each broth (Cappuccino & Sherman, 2005).
Note: Caution was maintained during the use of powdered zinc since it is hazardous.

2.5.5.1.8 Catalase test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% H_2O_2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation (Reiner, 2010).

2.5.5.1.9 Oxidase test

Oxidase test was done to determine the presence of the enzyme cytochrome oxidase in the bacteria. A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well isolated colony from pure 24-hour culture was picked and rubbed onto filter paper and observed for color change (Shields & Cathcart, 2010).

2.5.5.1.10 Gelatin hydrolysis test

Gelatin hydrolysis test was done to detect the ability of the bacteria to produce gelatinase. All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. Three milliliter from the media was dispensed in glass vials. The glass vials with the medium were then autoclaved at 121◦C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24-hour old culture bacteria was stab inoculated into the tubes with an inoculating needle. The glass vials were then incubated at 37◦C and observed up to for 1 week (Cruz & Torres, 2012).

2.5.5.1.11 Starch hydrolysis test

Starch hydrolysis test was done to determine the ability of the bacteria to hydrolyze starch with the enzyme amylase. Starch agar was prepared and autoclaved at 121◦C, 15 psi. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24-hours old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using gram's iodine (Cappuccino & Sherman, 2005).

2.5.5.1.12 Casein hydrolysis test

Casein hydrolysis test was done to determine the ability of the bacteria to produce the enzyme caseases and hydrolyze casein thereby. Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121◦C, 15 psi. Skim milk powder was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was added into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24 hours (Sturm, 2013).

2.5.5.1.13 Blood agar test

Blood agar test was done to determine the hemolytic capability of the bacteria by producing hemolysins and thereby lyse red blood cells. Blood agar base was prepared in a conical flask and autoclaved at 121◦C, 15 psi. The nutrient agar medium was allowed to cool at 45-50◦C and 5% (vol/ vol) sterile defibrinated sheep blood that had been warmed to room temperature was added and gently mixed avoiding air bubbles. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a blood agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24 hours. After incubation, the plates were observed for gamma, beta and alpha hemolysis (Aryal, 2015).

2.5.5.1.14 Eosin methylene blue agar test:

This test was done to select and isolate Gram negative organisms, and coliforms, and to differentiate among the family of *Enterobacteriaceae*. The main use of this test was to isolate fecal coliforms and to detect for fecal contamination. Using sterile technique, an EMB agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24-48 hours. Slow growing species may require a day or two of additional growth.

2.5.5.1.15 Cetrimide agar test:

This test was used for determining the ability of an organism to produce fluorescein and pyocyanin (Antibiotica). Plates were labeled and marked according to the dish side bottom in

what species would be in each section to observe the growth clearly. Using sterile technique, an Cetrimide agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24-48 hours. Slow growing species may require a day or two of additional growth. (Aryal, 2015)

2.6 Antibiotic resistance and susceptibility analysis

In clinical microbiology laboratory it is an important task to check the performance of antimicrobial susceptibility testing of significant bacterial isolates. The aim of this test is to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. Manual methods that provide flexibility and possible cost savings include the disk diffusion and gradient diffusion methods.

2.6.1 Disk diffusion test:

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test was performed by applying a bacterial inoculum of approximately $1–2\times10^8$ cfu/ mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks were placed on the inoculated agar surface. Plates were incubated for 16–24 hours at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The results of the disk diffusion test were “qualitative,” in that a category of susceptibility (i.e. susceptible, intermediate, or resistant) was derived from the test rather than an MIC. (Jorgensen JH, 2009)

The advantages of the disk method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods. The disadvantages of the disk test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing streptococci, *Haemophilus influenzae*, and *N. meningitidis* through use of specialized media, incubation conditions, and specific zone size interpretive criteria (Wayne, 2009).

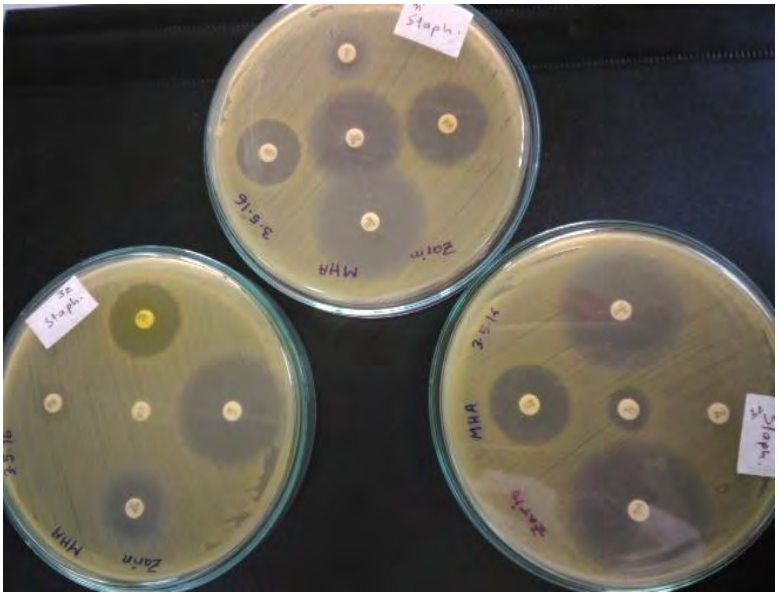


Figure 10: Different antibiotic discs on MHA containing bacterial lawn culture.

Chapter *III*

Result

Result

Although fruit juices are very common and potential for human health, but over their hygiene, safety and quality much concerns have been raised. Many fruit juice company have already started producing different fruit juices and many of them going to market their products in the market, but most of the companies have no concern about the quality of the juice products. On the other hand, while making fruit juices in home people only think about the nutritional benefits other than the quality of the juice. In present study, ten juice samples (Five commercially packed fruit juices such as three different branded mango juices, one litchi juice, one grape juice and five freshly homemade fruit juices such as orange, pomegranate, jujube, mango and orange juices) were examined for microbial analysis.

3.1 pH measurement of various juice samples:

Fruit juices are comparatively rich in organic acids so they have a low pH. Yeasts and molds are capable to grow at pH values of 1.5 and at water activity values below 0.89. The minimum pH values allowing the growth of lactic acid bacteria pH 2.9– 3.5, acetic acid bacteria pH 3.0–4.5, and enteric bacteria pH 3.6–4.5 are higher than those for growth of yeasts and molds. The pH range of juices is shown in Table 1. In the table below have shown that most of the juice samples have pH value within pH 3-4 and few samples have pH value below pH 3, which means mostly lactic acid bacteria, acetic acid bacteria and enteric bacteria will grow within these juice samples.

Overall range of pH is 2-5 for most common fruit juices with the most frequent figures being between 3 and 4. In the study, the highest pH 3.80 was found in the home made pomegranate juice and the lowest pH 2.80 was found in the home made jujube juice.

Table 7: pH value of the samples shown in the table

Samples		pH value
Commercially packed juice	CM1	3.53
	CM2	3.48
	CM3	3.56
	CG	3.45
	CL	3.19
Fresh juice	FO	3.76
	FP	3.80
	FJ	2.80
	FM	2.89
	FG	3.09

From Table 7, pH values of ten different samples were shown in the following bar chart.

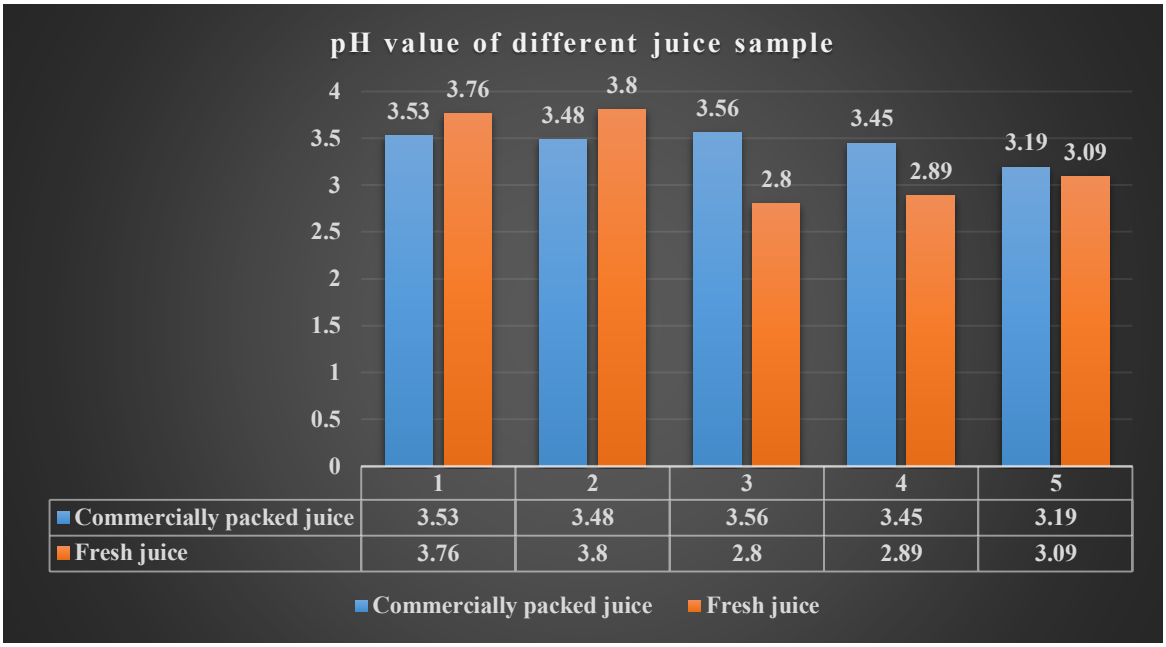


Figure 11: pH of various juice samples.

3.2 Total bacterial count of collected juice samples:

Microbial count of different fruit juices was shown in the table 8 and 9. From the results it is clear that total viable count (microbial load) showed the presences of bacteria in freshly homemade juices are higher than the commercially packed juices. Maximum samples of freshly homemade juices contained higher load of microbes than commercially packed juices. According

to Gulf Standard the total aerobic bacterial count standard is 5.0 x 10^4 (cfu/ ml) (Rashed et al. 2012).

Table 8: Total bacterial count of freshly collected juices

Sample no.	**1. FO**	**2. FP**	**3. FJ**	**4. FM**	**5. FG**
Types of juice / **Different counts**	Orange juice	Pomegranate juice	Jujube (Boroi) juice	Mango juice	Grape juice
Total Viable count (TVC) (cfu/ ml)	$1x10^5$	$5.5x10^5$	$8x10^5$	$2.3x10^6$	$1.05x10^6$
Total Coliform Count (TCC) (cfu/ ml)	$5x10^3$	$1x10^3$	$9.5x10^3$	$5.0x10^3$	$1.75x10^4$
Fecal Coliform Count (FCC) (cfu/ ml)	Nil	$1.8x10^3$	$1.7x10^3$	Nil	$5.75x10^3$
Total Staphylococcal Count (TSC) (cfu/ ml)	$1.2x10^4$	$4.5x10^2$	$1.4x10^4$	$1.15x10^4$	$1.35x10^4$
Total Salmonella count (cfu/ ml)	$1.0x10^4$	Nil	Nil	Nil	$3.5x10^4$

Table 8 showed that total viable count of freshly homemade juices ranged from 1 x 10^5 – 2.3 x 10^6 cfu/ ml, total coliform count ranged from 1 x 10^3- 9.5 x 10^3 cfu/ ml, total Staphylococcal count ranged from 1.15 x 10^4- 4.5 x 10^2 cfu/ ml, total Salmonella count ranged from 0 – 3.5 x 10^4 cfu/ ml and total fecal coliform count ranged from 0 – 5.75 x 10^3 cfu/ ml.

Table 9: Total bacterial count of commercially packed juices

Sample no.	1. CM1	2. CM2	3. CM3	4. CG	5. CL
Types of juice	Mango	Mango	Mango	Grape	Litchi
Different counts	CM1	CM2	CM3	CG	CL
Total Viable count TVC) (cfu/ ml)	$0.5x10^3$	$1.25x10^6$	$1.0x10^5$	$5x10^5$	$5x10^5$
Total Coliform Count (TCC) (cfu/ ml)	Nil	$1.5x10^2$	Nil	Nil	$4x10^1$
Fecal Coliform Count (FCC) (cfu/ ml)	Nil	Nil	Nil	Nil	$3.25x10^1$
Total Staphylococcal Count (TSC) (cfu/ ml)	Nil	$2.5x10^3$	$5x10^1$	Nil	$1x10^3$
Total Salmonella count (cfu/ ml)	Nil	Nil	Nil	Nil	$2.5x10^2$

Table 9 showed that total viable count of commercially packed juices ranged from $0.5 \times 10^3 - 5 \times 10^5$ cfu/ ml, total coliform count ranged from $0 - 4 \times 10^1$ cfu/ ml, total Staphylococcal count ranged from $0 - 2.5 \times 10^3$ cfu/ ml, total Salmonella/ Shigella count ranged $0 - 2.5 \times 10^2$cfu/ ml and fecal coliform count ranged from $0 - 4 \times 10^1$ cfu/ ml.

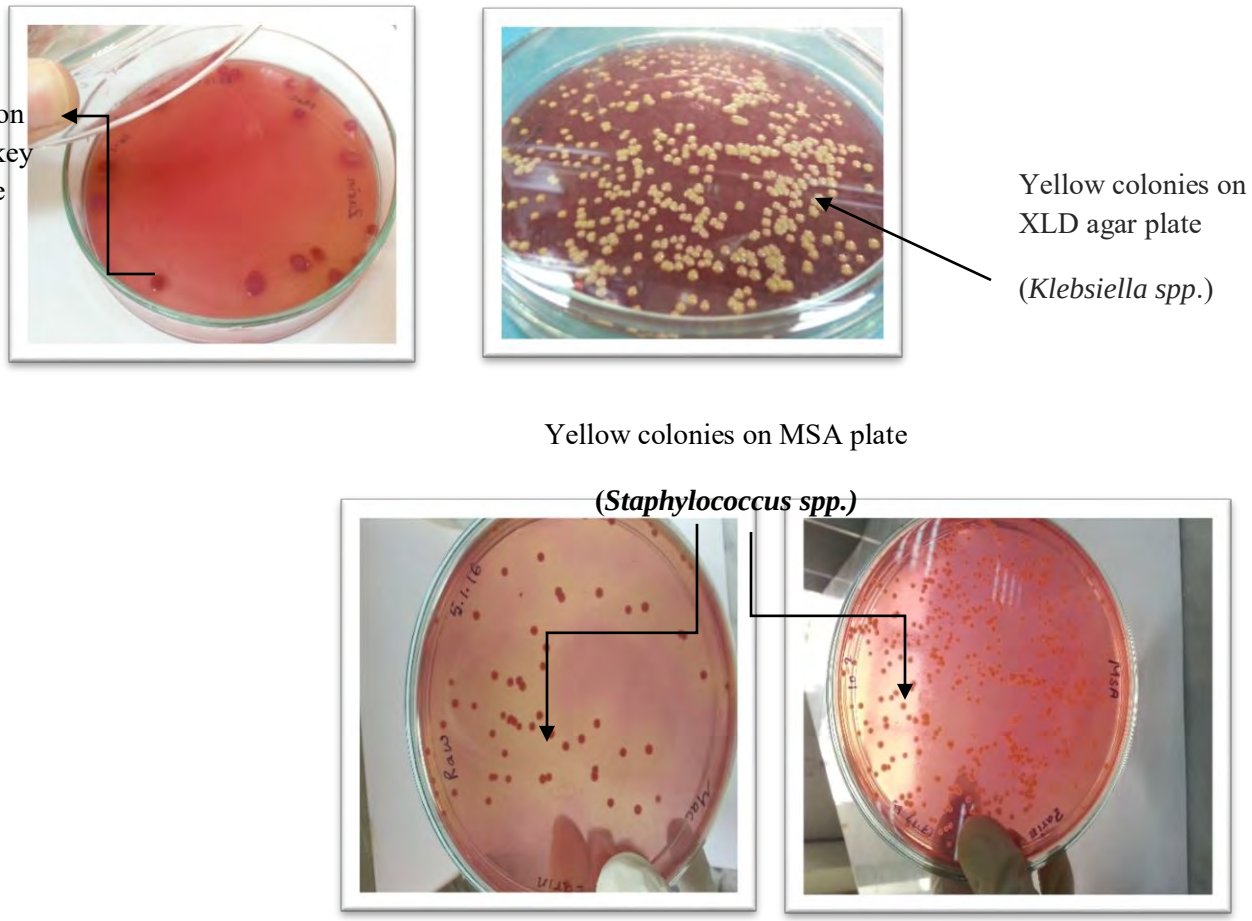


Figure 12: Bacterial growth on different medium.

3.3 Gram reaction and colony morphology:

Gram reaction and colony morphology of different isolates collected from ten different samples were explained in Tables 10. Colour of the colonies, forms of them, margin reactions, elevation, gram reactions of those isolates and their shapes were analyzed and in the following Table 10.

Table 10: Morphological characteristics of bacterial colonies and gram reaction

Bacterial isolates	Colour on Nutrient agar	Configuration	Margin reaction	Elevation	Gram reaction	Shape of isolates
1. FG-NO1	Bright yellow colour	Irregular	Undulate	Raised	Negative	Cocci shape in tetrad
2. FP-NP2	Pink colour	Circular	Entire	Convex	Negative	Cocci in clusters
3. FG-MSR3	Off-white colour	Irregular	Undulate	Convex	Positive	Long rods in chains
4. FJ-MFB4	Off-white colour	Circular	Entire	Raised	Negative	Cocci in grape like brunches
5. FP-XB5	Off-white colour	Circular	Entire	Convex	Negative	Cocci in clusters
6. FJ-MSP6	Off-white colour	Irregular	Undulate	Raised	Negative	Long single rods
7. FP-MP7	Off-white colour	Circular	Entire	Flat	Negative	Coccobacilli
8. FG-MSY8	Off-white colour	Irregular	Undulate	Flat	Negative	Long rods in chains
9. FP-MFB9	Off-white colour	Circular	Entire	Convex	Negative	Cocci in tetrad
10. FJ-MSY10	Off-white colour	Irregular	Undulate	Raised	Negative	Long rods in chains
11. FJ-NL11	Bright yellow colour	Circular	Entire	Convex	Negative	Cocci in clusters
12. FJ-XB12	Off-white colour	Irregular	Undulate	Flat	Positive	Coccobacilli
13. FG-XG13	White (pale) colour	Circular	Entire	Convex	Negative	Short rods
14. FG-MFB14	White (pale) colour	Circular	Entire	Flat	Negative	Short rods
15. FG-MP15	White (pale) colour	Circular	Entire	Flat	Negative	Coccobacilli
16. CL-NFF16	Green fluorescent colour	Rhizoid	Filamentous	Flat	Negative	Long single Rods
17. CL-MSG17	Lemon colour	Circular	Entire	Convex	Positive	Short rods

Table 10: Morphological characteristics of bacterial colonies and gram reaction (Continued)

Bacterial isolates	Colour on nutrient agar	Configuration	Margin reaction	Elevation	Gram reaction	Shape of isolates
18. CL-NOP18	Pink colour	Circular	Entire	Convex	Negative	Diplococci
19. CL-XY19	Light orange colour	Circular	Entire	Raised	Negative	Cocci in grape like brunches
20. CL-MP20	Off-white colour	Circular	Entire	Raised	Negative	Cocci
21. FM-MP21	Light yellow colour	Circular	Entire	Convex	Negative	Cocci in tetrad
22. FO-XY22	Mucoid (transparent)	Circular	Entire	Convex	Negative	Short rods
23. FM-MSY23	Off-white colour	Irregular	Lobate	Flat	Positive	Rods in chains
24. FO-MSY24	White (pale) colour	Circular	Entire	Raised	Positive	Cocci in tetrad
25. FM-NO25	Orange colour	Circular	Entire	Flat	Positive	Short rods
26. CM2-MSO26	Bright yellow colour	Circular	Entire	Convex	Negative	Cocci in tetrad
27.CM1-NO27	Mucoid (transparent)	Irregular	Undulate	Flat	Negative	Long rods
28. FP-MFB28	White (pale) colour	Circular	Entire	Convex	Negative	Coccobacilli
29. FO-XY29	Mucoid (transparent)	Irregular	Undulate	Convex	Negative	Coccobacilli
30 FO-MP30	Light yellow colour	Circular	Entire	Convex	Negative	Coccobacilli
31. CM2-MS31	Bright yellow colour	Circular	Entire	Convex	Positive	Cocci in clusters
32. CMG-MSO32	Lemon yellow colour	Circular	Entire	Convex	Positive	Cocci in tetrad
33. FO-MP33	Mucoid (transparent)	Circular	Entire	Flat	Negative	Short rods
34. FP- NG34	Mucoid	Irregular	Undulate	Flat	Negative	Short rods

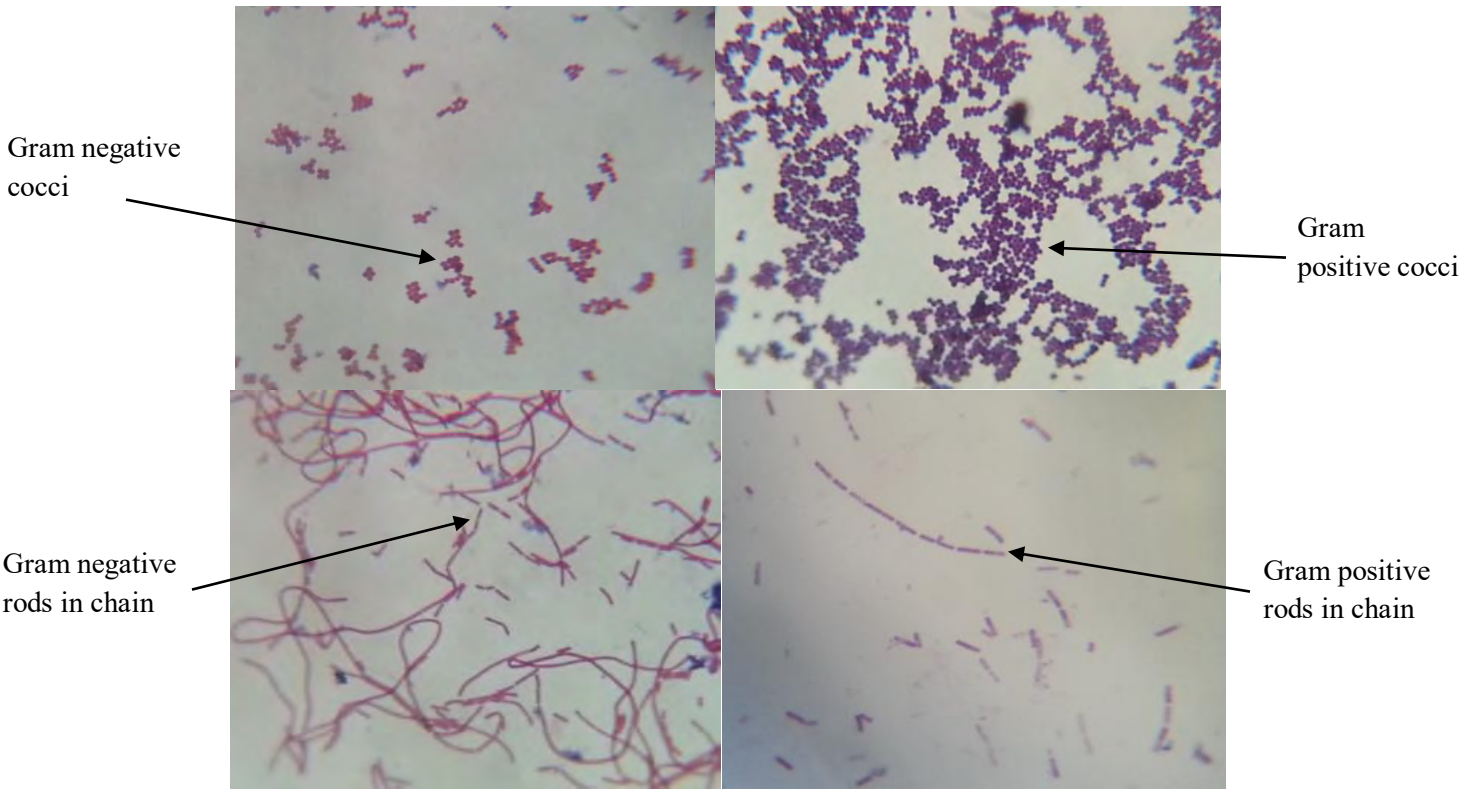


Figure 13: Gram reaction and shapes of different bacteria.

3.4 Biochemical Characteristics of bacterial isolates of different juices

Microorganisms were collected from different culture media according to their growth pattern, morphology, appearance and compared with the morphology of suspected microorganisms. After comparing with suspected organisms the isolates were then sub-cultured and some specific biochemical tests were done for identification. The biochemical tests that were performed are described precisely in materials and method chapter 2 and the biochemical results of the isolates are given below in Table 11.

Table 11: Biochemical characteristics of bacterial isolates of different juices

Isolates no.	Isolates	Oxidase test	Catalase test	Indole	MIU			MRVP		Gelatin	Nitrate reduction	Simmon's citrate	Casein hydrolysis	Starch hydrolysis	Blood agar hemolysis	Eosin methylene blue agar	Cetrimide agar	TSI						Organism Interpretation
					Motility	Indole	Urease	Methyl Red	VogesProskauer									Slant/ Butt	Glucose	Lactose	Sucrose	H_2S production	Gas production	
1.	FG-NO1	+	+	-	+/-	+/-	-	-	+	-	+	-	+	+	+	-	-	R/Y	+	-	-	-	-	*Moraxella spp.*
2.	FP-NP2	-	+	-	+	+/-	+	-	+	+	+	+	+	-	+	+	+	(B)R/R	-	-	-	+	-	*Serratia spp. **
3.	FG-MSR3	-	-	-	+/-	+/-	+/-	-	+	-	+	-	-	-	+	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
4.	FJ-MFB4	+	+	-	+	+/-	+	+	-	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	+	*Moraxella spp.*
5.	FP-XB5	-	+	-	+	+/-	+	+	-	-	+	+	+	-	-	+	-	(B) R/ Y	+	-	-	+	+	*Listeria spp.*
6.	FJ-MSP6	-	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Klebsiella spp.*
7.	FP-MP7	-	+	-	+	-	+/-	+	-	-	+	+	-	+	+	-	-	R/B	-	-	-	+	+	*Shigella spp.*
8.	FG-MSY8	-	-	-	-	+/-	-	+/-	+/-	-	+	-	+	-	-	-	-	R/R	-	-	-	-	-	*Bacillus spp.*
9.	FP-MFB9	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-	(B) R/Y	+	-	-	+	+	*Klebsiella spp. **
10.	FJ-MSY10	-	-	-	-	+/-	-	+/-	+/-	-	+	+	+	+	+	+	-	R/Y	+	-	-	-	-	*Bacillus spp.*
11.	FJ-NL11	-	+	-	+	+	+	+	-	-	+	-	-	-	+	-	-	Y/Y	-	+	+	-	-	*Acetobacter spp.*
12.	FJ-XB12	+	+	-	+	+	+/-	+	-	-	+	+	+	-	-	+	+	B/B				+	+	*Salmonella spp. **
13.	FG-XG13	+	+	-	+	+	+	+/-	+/-	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Brevibacterium spp.*
14.	FG-MFB14	-	+	-	+	-	+/-	+	-	-	+	-	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli **
15.	FG-MP15	-	+	-	+	-	+/-	+	-	-	+	-	-	-	-	+	-	Y/Y	-	+	+	-	+	*Acinetobacter spp.*
16.	CL-NFF16	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R/R	-	-	-	-	-	*Pseudomonas spp.*
17.	CL-MSG17	-	+	-	+	+/-	+/-	+	-	-	+	+	+	-	-	-	-	R/R	-	-	-	-	-	*Micrococcus spp.*
18.	CL-NOP18	+	+	-	-	+/-	-	+	-	-	+	-	-	+	-	-	-	R/R	-	-	-	-	-	*Moraxella spp.*
19.	CL-XY19	-	+	-	+	+	+/-	-	+	-	-	+	+	+	+	+	-	(B) R/Y	+	-	-	+	-	*Staphylococcus spp.*
20.	CL-MP20	+	+	-	+/-	+	+	+	-	-	+	+	-	-	-	+	-	R/R	-	-	-	-	-	*Pseudomonas spp. **
21.	FM-MP21	+	-	-	-	-	-	+	-	-	+	-	-	-	+	+	-	R/R	-	-	-	-	-	*Francisella tularensis*
22.	FO-XY22	+	+	-	+	-	-	+	-	-	+	+	+	-	-	+	-	Y/Y	-	+	+	-	-	*Moraxella cattarrhalis*
23.	FM-MSY23	-	-	-	+/-	-	-	+	-	+	+/-	-	+	-	-	-	+	R/Y	+	-	-	-	-	*Bacillus spp. **
24.	FO-MSY24	-	+	-	+/-	+	+	+	-	-	+/-	-	-	+	-	-	+	R/Y	+	-	-	-	-	*Staphylococcus aureus **
25.	FM-NO25	-	+	-	+/-	-	+/-	-	-	-	+	-	-	+	-	+	-	Y/Y	-	+	+	-	-	*Listeria spp.*
26.	CM2-MSY26	-	+	-	+/-	+	+	+	-	-	+	-	-	-	+	+	+	Y/Y	-	+	+	-	-	*Acinetobacter spp.*

'+' = positive, '- ' = negative; 'Glu' = Glucose; 'Lac' = Lactose, 'Suc' = Sucrose, Y= Yellow, R= Red, B= Black, *showed closer results according to their species

Table 11: Biochemical characteristics of bacterial isolates of different juices (Continued)																								
Isolates no.	**Isolates**	**Oxidase**	**Catalase**	**Indole**	**MIU**			**MRVP**		**Gelatin**	**Nitrate reduction**	**Simmon's citrate**	**Casein hydrolysis**	**Starch hydrolysis**	**Blood agar hemolysis**	**Eosin methylene blue agar**	**Cetrimide agar**	**TSI**						**Organism Interpretation**
					Motility	**Indole**	**Urease**	**Methyl red**	**VogesProskauer**									**Slant/ Butt**	**Glucose**	**Lactose**	**Sucrose**	**H_2S production**	**Gas production**	
27.	CM1-NO27	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	Y/Y	-	+	+	-	-	*Clostridium spp. **
28.	FP-MFB28	-	+	-	+/-	+	+	+	-	-	+	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli **
29.	FO-XY29	-	+	-	+/-	+	+	+	-	-	+	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli**
30.	FO-MP30	-	+	+	+	+	-	+	-	-	-	+	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli **
31.	CM2-MSY31	-	+	+	+/-	+	+	+	-	-	+	-	-	-	+	+	-	Y/Y	-	+	+	-	-	*Staphylococcus spp.*
32.	CMG-NO32	-	+	-	+/-	+/-	+/-	-	-	-	+	-	-	-	-	+	+	Y/Y	-	+	+	-	-	*Staphylococcus spp.*
33.	FO-MP33	-	+	+	+/-	+	-	+	-	-	-	-	-	-	-	+	-	Y/Y	-	+	+	-	-	*Escherichia coli **
34.	FP- NG34	+	+	-	+	-	+	-	-	+	+/-	+	+	-	-	+	+	R/R	-	-	-	-	-	*Pseudomonas spp. **
'+' = positive, '- ' = negative; 'Glu' = Glucose; 'Lac' = Lactose, 'Suc' = Sucrose, Y= Yellow, R= Red, B= Black, *showed closer results according to their species																								

After 20 different biochemical tests, it was found that, out of 10 fruit juice samples *E. coli* were found in 3 different juice samples (orange, pomegranate, grape), *Staphylococcus spp.* were found in 4 different samples (mango, orange, litchi), *Bacillus spp.* were found in 3 different samples (grape, jujube, mango), *Pseudomonas spp*. in 2 different samples (pomegranate and litchi), *Moraxella spp*. in 4 different samples (grape, jujube, litchi, orange), *Klebsiella spp*. in 2 different samples (jujube and litchi), *Salmonella spp.* in jujube juice sample, *Shigella spp.* in pomegranate juice sample and some other different organism such as *Listeria spp., Clostridium spp., Acinetobacter spp., Brevibacterium spp., Acetobacter spp., Francisella spp.* has been identified from juice samples.

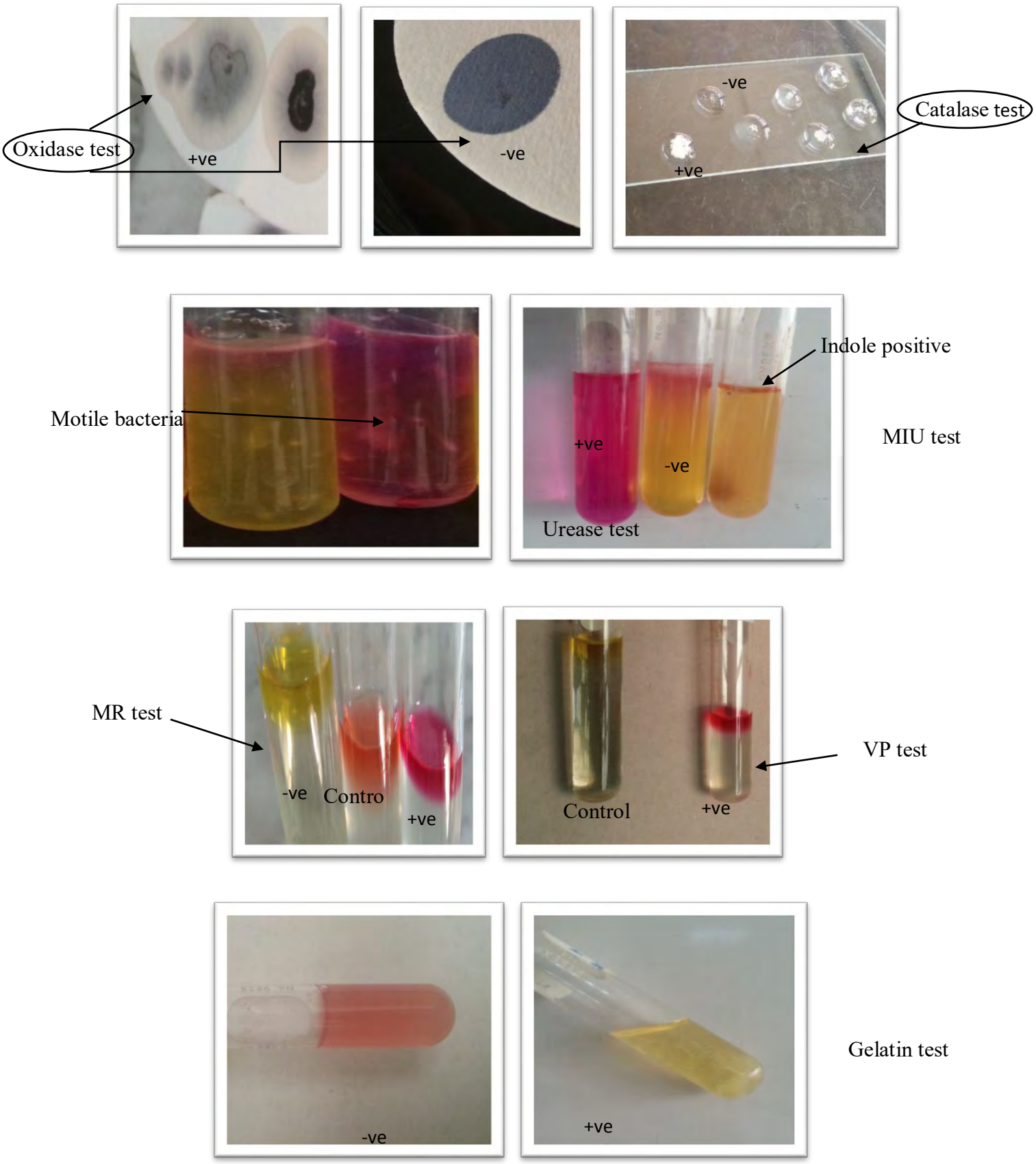


Figure 14: Different biochemical tests performed by different isolates collected.

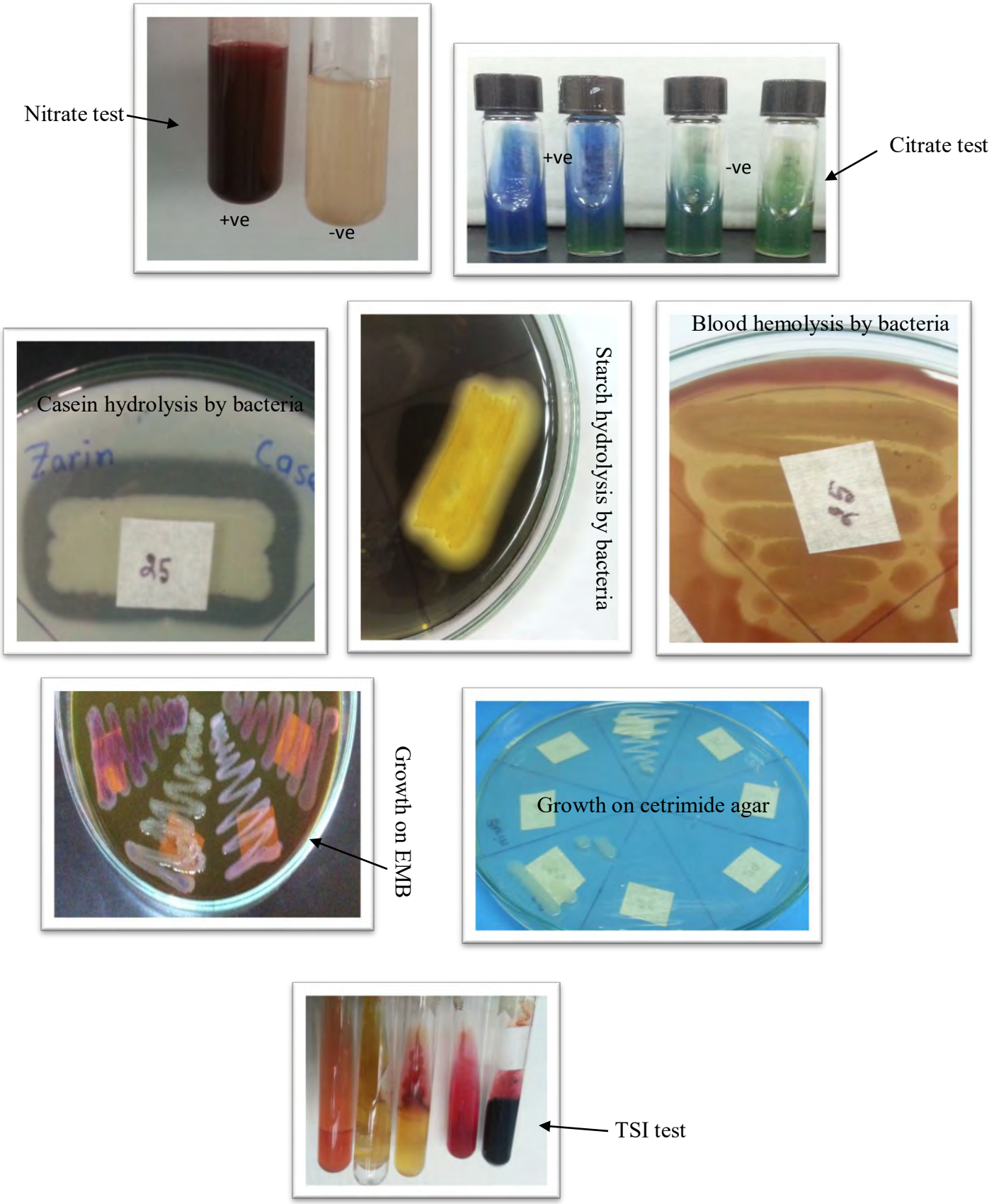


Figure 15: Different biochemical test performed by different isolates collected from juice.

3.5 Antibiotic susceptibility test

From thirty-four isolates total six isolates were selected for antibiotic susceptibility test. Thirty-four antibiotics were used and for each isolates 10-15 antibiotics were analyzed to see the sensitivity and resistance towards antibiotics.

Table 12 was prepared showing the zone of inhibition of six different bacteria according to the zone range for resistance, intermediate and sensitivity to different antibiotics. Some bacteria have shown no clear zone to some antibiotics, which means they are resistant to that particular antibiotic and some have shown very small diameter of clear zone this also indicates resistance towards that antibiotic. If the clear zone diameter is larger than resistant diameter scale and less than susceptible diameter, then this result called intermediate and it means the specific bacteria is neither susceptible nor resistant to that particular antibiotic. Bacteria will be sensitive to the antibiotic if the diameter of clear zone is larger than the susceptibility diameter scale. The interpretation of each bacterium either resistant or susceptible to antibiotic is shown in Table 12.

Table 12: Antibiotic susceptibility test of various organisms isolated from different juice samples												
Antimicrobial agent	**Tested Organisms**											
	E. coli		***Staphylococcus aureus***		***Pseudomonas spp.***		***Salmonella spp.***		***Bacillus spp.***		***Serratia spp.***	
	ZS	***INP***	***ZS***	***INP***	***ZS***	***INP***	***ZS***	***INP***	***ZS***	***INP***	***ZS***	***INP***
1. Amikacin	25	S	-	-	-	-	22	S	-	-	-	-
2. Amoxicillin	-	-	-	-	Nil	R	14	I	-	-	-	-
3. Ampicillin	12	R	-	-	-	-	12.5	R	-	-	15	I
4. Azithromycin	15.5	I	32	S	-	-	18	S	-	-	16	I
5.Aztreonam	-	-	-	-	35	S	-	-	-	-	-	-
6. Cefepime	-	-	-	-	-	-	-	-	-	-	34	S
7. Cefoxitin	17	I	-	-	-	-	-	-	-	-	-	-
8. Ceftazidime	-	-	-	-	37	S	-	-	-	-	34	S
9. Ceftriaxone	-	-	-	-	23	S	28	S	-	-	-	-
10. Cephalexin	-	-	32	S	-	-	-	-	-	-	-	-
11. Chloramphenicol	-	-	33	S	15.5	I	-	-	27	S	23	S
12. Ciprofloxacin	30.5	S	31	S	35	S	-	-	30	S	36	S
13. Clindamycin	Nil	R	30	S	-	-	-	-	26	S	-	-
14. Cloxacillin	Nil	R	-	-	-	-	-	-	-	-	-	-
15. Co-trimethozole/sulfamethoprim	-	-	-	-	-	-	28	S	-	-	-	-
16. Doxycycline	22	S	-	-	9	R	-	-	30	S	15.5	I
17. Erythromycin	-	-	32	S	-	-	-	-	27	S	-	-
18. Gentamicin	20	S	-	-	18	S	-	-	21	S	25	S
19. Imipenem	36.5	S	-	-	25	S	23	S	38	S	-	-
20. Levofloxacin	-	-	28	S	-	-	-	-	-	-	-	-
21. Minocycline	-	-	34	S	-	-	-	-	-	-	-	-
22. Nalidixic acid	-	-	-	-	-	-	25	S	-	-	28	S
ZS= Zone size, INP= Interpretation, S= Sensitive, I= Intermediate, R= Resistant, '-'= Not Done												

Table 12: Antibiotic susceptibility test of various organisms isolated from different juice samples (Continued)												
Antimicrobial agent	**Tested Organisms**											
	E. coli		***Staphylococcus aureus***		***Pseudomonas spp.***		***Salmonella spp.***		***Bacillus spp.***		***Serratia spp.***	
	ZS	**INP**	**ZS**	**INP**	**ZS**	**INP**	**ZS**	**INP**	**ZS**	**INP**	**ZS**	**INP**
23. Netilmicin	23.5	S	-	-	-	-	-	-	-	-	-	-
24. Nitrofurantoin	10	R	22	S	-	-	-	-	-	-	-	-
25. Norfloxacin	-	-	28	S	-	-	-	-	-	-	-	-
26. Oxacillin	-	-	20	S	-	-	-	-	-	-	-	-
27. Penicillin-G	-	-	39	S	-	-	-	-	-	-	-	-
28. Piperacillin-tazobactam	29	S	-	-	-	-	-	-	-	-	28	S
29. Streptomycin	-	-	-	-	17.5	S	-	-	-	-		-
30. Tetracycline	25	S	33	S	9	R	19	I	30	S	-	-
31. Tobramycin	-	-	-	-	-	-	-	-	17.5	S	-	-
32. Trimethoprim/ Sulfamethozole	22.5	S	32	S	Nil	R	23	S	-	-	-	-
33. Vancomycin	-	-	20	S	-	-	-	-	20	S	-	-
ZS= Zone size, INP= Interpretation , S= Sensitive, I= Intermediate, R= Resistant, '-'= Not Done												

Table 12 showed that six different organisms' (*Escherichia coli, Staphylococcus aureus, Pseudomonas spp., Salmonella spp., Bacillus spp.* and *Serratia spp.)* are showing antibiotic susceptibility in different antibiotics. Most of the organisms were sensitive to almost every antibiotic. Some organisms showed resistance to one or two antibiotics, such as *E. coli* showed resistance to Ampicillin, Clindamycin, Cloxacillin and Nitrofurantoin; *Pseudomonas spp.* showed resistance to Amoxicillin, Doxycycline and Tetracycline; *Salmonella spp.* showed resistance to only Ampicillin but not every organism is resistant to those antibiotics, some showed much large clear zone to these antibiotics which meant they are sensitive and it can be said that it varies from organism to organism. Other three organisms *Staphylococcus spp., Bacillus spp*. and *Serratia spp.* showed no resistance to any of the antibiotics those were used.

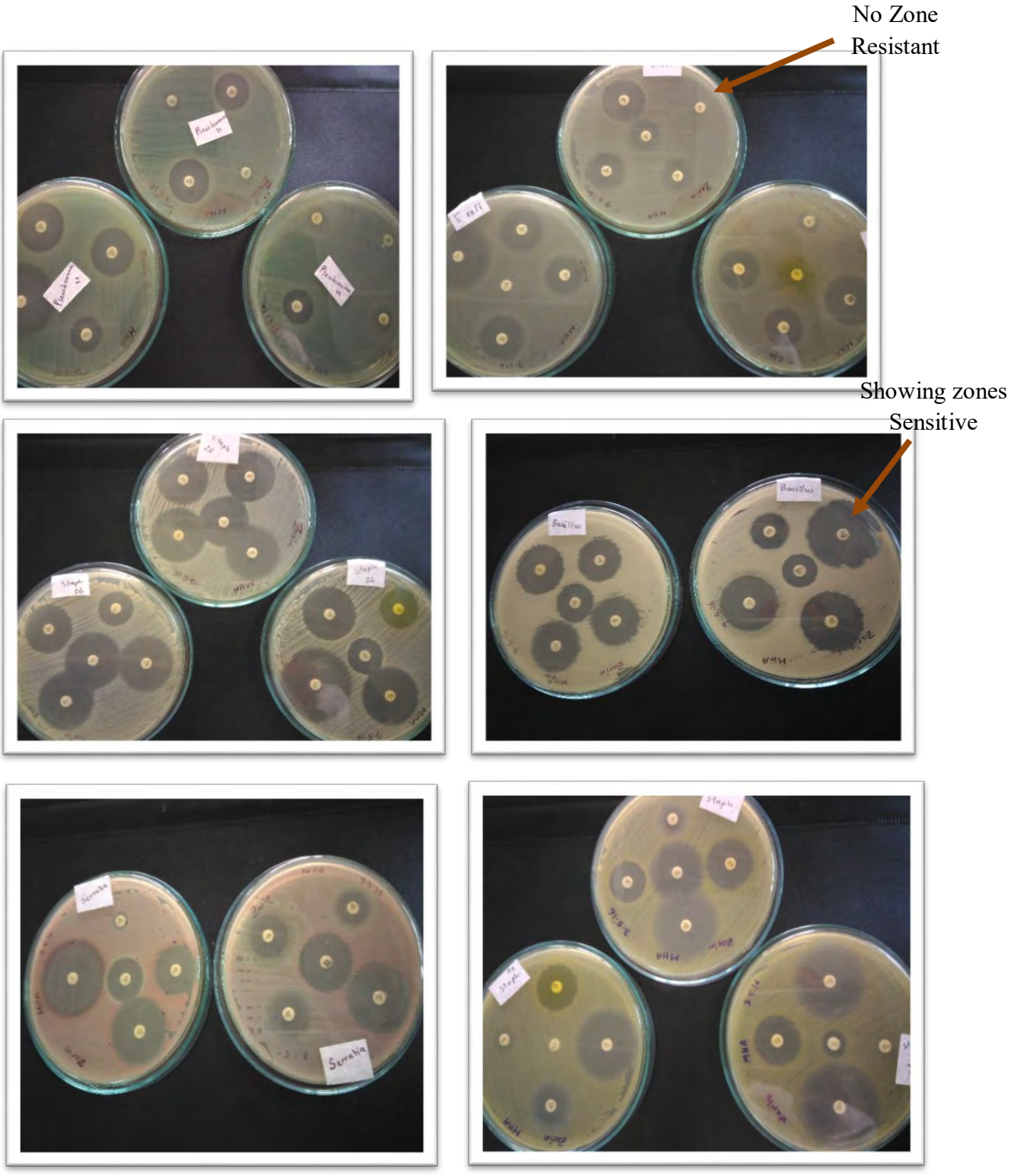


Figure 16: Six different organism showing antibiotic susceptibility and resistance to different antibiotics on Mueller Hinton agar plates.

Chapter *IV*

Discussion

Discussion:

In many developing countries including Bangladesh, millions of people are widely consuming fruit juices in every season as it provides an affordable source of nutrients to them. Packed fruit juices being good in taste and available at low price and at the same time they are liked by the consumers. (Ohiokpehai, 2003). Many fruit juice company are producing and marketing different fruit juices, but most of the companies have no concern about the quality of the juice products. Most of them think commercially and they are only concerned about the marketing (with colorful advertisement) of their products. Though they might maintain a lot of hygiene in their factory to avoid contamination (which is a good sign) but in most of the factory they use preservatives or harmful chemicals to lower the microbial growth in juice. In long time effect these harmful preservatives and chemicals can cause a thousand times more powerful disease to human being than the microbes, or cause mutation inside our body which eventually kills people ten times faster than normal diseases. Factors which determine the colonization of juices by microorganisms include pH, redox potential, water activity, nutrients, structures, antimicrobial agents, temperature, relative humidity, and atmosphere (Raybaudi, 2009). In the present study the frequencies of occurrence of molds and yeasts were more as compared to bacterial genera which are attributed to low pH values and high sugar content (A. Rivas, 2006).

4.1 Colony morphology, phenotypic and biochemical traits of the isolates

From different medium after incubation of 24 hours, some different morphological characteristic showing colonies from nutrient agar, typical pink, circular, convex colonies from MacConkey Agar (considered as coliforms), black colonies from XLD agar (considered as *Salmonella spp.*), blue colonies from MFc agar (considered as fecal coliform) and yellow colonies from Mannitol Salt Agar (considered as *Staphylococcus spp.)* were initially isolated. Isolates from MacConkey- and MFc agar media were observed as Gram negative, single, short rods, compared to the characteristic of coliforms whereas isolates from MSA were Gram positive in a cluster arrangement which were typical for *Staphylococcus spp.* and from XLD agar media again some Gram negative rod were also observed compared to the characteristics of *Salmonella spp.* Based on the biochemical characteristics, isolates were confirmed as *E. coli*, *Klebsiella spp.*, *Salmonella spp.*, *Staphylococcus spp.*, *Serratia spp.* and some other different organisms.

4.2 Total viable count (TVC)

Most of the fruit juice samples showed equal or much higher heterotrophic count than Gulf standard. The highest bacterial count ($2.3x10^6$ cfu/ ml) for freshly homemade fruit juice sample was found in a mango juice (sample FM), collected from Mirpur and the lowest count was ($1.0x10^5$ cfu/ ml) found in an orange juice (sample FO) collected from Dhanmondi area (Table 3). On the other hand, the highest total bacterial count ($1.25x10^6$ cfu/ ml) for packed fruit juice sample was found in a mango juice (sample CM2, Table 4) and the lowest count was observed to be $0.5\ x10^3$ cfu/ ml in another mango juice (sample CM1, Table 4). Variations in TVC of both types of fruit juices may be due to the unhygienic maintenance during preparing the juice. Rahman et al. (2011) reported that total viable bacterial count in most of the fresh juice samples was higher than the commercially packed juice, as the highest count was found as $2.4x10^4$ cfu/ ml and $3.2x10^3$cfu/ml in fresh and packed juice, respectively which was found to be lower than this study. Tasnim et al. (2010) also found the load of viable bacteria in processed juice samples within the standard limit in the average of 10^3 cfu/ml. Bagde and Tumane (2011) found that total bacterial counts in juice samples ranged between $2.0\ x10^6$ to $1.0\ x\ 10^5$ cfu/ ml in Nagpur, India. Munjur et al. (2014) reported that the highest bacterial load ($3.7x10^8$ cfu/ ml) for fresh fruit juice sample was found in a grape juice, which was found to be higher than this study.

4.3 Prevalence of coliforms and fecal coliforms

The presence of coliform in fruit juice is not allowed by safe food consumption standard (Andres et al., 2004). Most of the fruit juices in this study were found to be unfavorable for consumption because many of them showed the presence of coliforms (*E. coli* and *Klebsiella spp.)*. The highest coliform count for fresh homemade fruit juice and packed juice samples were $1.75x10^4$ cfu/ ml (sample FG, Table 3) and$1.5x10^2$ cfu/ ml (sample CM2, Table 4), respectively. And the highest fecal coliform count for fresh homemade fruit juice and packed juice samples were $5.75x10^3$ cfu/ ml (sample FG, Table 3) and$3.25x10^1$ cfu/ml (sample CL, Table 4), respectively. In Bangladesh, Ahmed et al. (2009) showed the presence of *E. coli* ranging from 43 to > 2400/ 100 ml in different types of vended squeezed fruit juices in Dhaka city, Also Munjur et al. (2014) reported that the highest and lowest coliform count for fresh fruit juice samples were $8.2x10^6$ cfu/ ml and $1.53x10^3$ cfu/ ml respectively. In India, the fruit juices were heavily contaminated by *E. coli* (Bagde and Tumane, 2011). Moreover, 3 fresh fruit juice samples (FP, FJ and FG) and 1 packed juice (CL) exhibited the presence of fecal coliform in the present study (Table 3 and 4).

4.4 Prevalence of *Staphylococci spp.*

A few reports have shown the prevalence of *Staphylococci* in fruit juice samples (Ahmed et al., 2009; Tambekar et al., 2009). Coagulase-positive *Staphylococci* may cause human disease through the production of toxins. Effective levels of toxin formation require a high number of microorganisms (approximately 10^5-10^6 micro-organisms per ml of food) (IDF, 1994). In this study, *Staphylococci spp.* was found in some tested samples. The highest total *Staphylococcal* count for homemade fruit juice sample ($1.4x10^4$ cfu/ ml) was found in a jujube juice (sample FJ, Table 3). On the other contrary, the highest total *Staphylococcal* count for packed fruit juice sample ($2.5x10^3$ cfu/ ml) was found in a mango juice (sample CM2, Table 4).

4.5 Prevalence of *Salmonella spp.*

In fruit samples there were no report about Salmonella prevalence in fruit juice samples. In this study, some *Salmonella spp.* were found in some tested samples but the number was very low. The highest *Salmonella* count for homemade fruit juice sample ($3.5x10^4$ cfu/ ml) was found in a grape juice (sample FG, Table 3). On the other contrary, the highest total staphylococcal count for packed fruit juice sample ($2.5x10^2$ cfu/ ml) was found in a litchi juice (sample CL, Table 4). Out of 10 fruit juice samples 3 had shown the growth of *Salmonella*.

Interestingly, coliform, fecal coliform and Salmonella were absent in three packed juice samples (samples CM1, CM3 and CG) in this study (Table 3), and hence these samples were considered to be safe. Notably, these samples were prepared under good sanitation practices and stored in appropriate storage conditions. Besides, the results of this study (Tables 3 and 4) showed the safer consumption of commercially packed juice than the fresh homemade juice. This might be due to the usage of automated machine directing aseptic processing as well as for the application of some preservatives. But some preservatives of higher concentrations can be harmful for our health (Bashar and Sabita, 2007). Therefore, further studies on the optimization of preservative concentrations should be performed.

4.6 Antibiotic susceptibility test

Rashed et al. (2012) used a new aspect on their investigation comparative to the previous related ones is the study of antibiogram of the pathogenic isolates found in the juice samples. They found the *E. coli* isolates highly resistant against ciprofloxacin (61%), nalidixic acid (71%) and

ceftriaxone (57%). *Klebsiella spp*. showed higher resistance against ampicillin (74%), ciprofloxacin (86%), piperaciline (88%), amoxicillin (72%), ceftriaxone (97%) and nalidixic acid (61%). Staphylococcus spp. showed resistance against ampicillin (93%), piperaciline (75%), amoxicillin (92%) and vancomycin (63%). Such drug resistance properties may render these pathogens cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

In this study, *E. coli* showed resistance against Ampicillin, Clindamycin, Cloxacillin, and Nitrofurantoin; *Pseudomonas spp.* showed resistance against Amoxicillin, Doxycycline, Tetracycline and Trimethoprim/ Sulfamethozole; *Salmonella spp.* showed resistance against only Ampicillin and rest of the organisms *Staphylococcus aureus, Bacillus spp*. and *Serratia spp.* did not show any resistance to any antibiotics that were used.

The present research study has been carried out to investigate and compare the microbial quality of fresh homemade fruit juice collected from different houses and commercially packed juice sold in the local public shops. Where, in the study they exhibited the presence of *E. coli, Salmonella spp., Staphylococcus spp., Bacillus spp, Serratia spp., Pseudomonas spp., Micrococcus spp,* and some other species. Probiotic microorganisms may also be isolated from the packaged fruit juice. The average counts for bacteria of the packaged fruit juice samples examined were generally below the maximum allowable limit in foods to be marketed for consumption (10^3 cfu/ ml) but fresh homemade fruit juices were above the limit. However, the average ranges obtained for the bacteria indicated a public health concern as they showed counts far above this limit in both packed and fresh homemade juices. These high counts indicate heavy bacterial contamination of both the packaged and fresh homemade fruit juice during handling since they are liquid, which could have contributed to the development as well as multiplication of these contaminants. Also, contamination can occur within fruits and materials used for the production of the juice as well as poor sanitation, extraction, raw material contaminations (often from insect damage), lack of both proper heat sterilization and adequate quality control during processing of fruit juice. The study has also shown that these packaged fruit juices are not sterile and thus can favour the growth of microorganisms when conditions become favourable, which could pose a public health risk to their consumers.

4.7 Conclusion:

From the data presented in the current study, it could be hard to claim that, consumption of fresh homemade juice was safe than commercially packed juice because almost all types of fresh homemade and commercially packed juice samples collected from different areas of Dhaka city were not satisfactory as *Escherichia coli, Klebsiella spp., Enterobacter spp., and Staphylococcus spp.,* were found in large numbers from samples. There is a generalized belief among the people that, automated machines and some preservatives are used during processing of commercial fruit juices. Despite all these issues, a large number of coliforms, and *Staphylococci* count were detected from both commercially packed fruit juices and fresh homemade juice in this current study, which clearly indicates poor plant management and personnel hygiene. A combination of regular monitoring and proper training could be an appropriate choice in fruit juice industries to minimize the health risks. In addition to this, not only government authorized institution like BCSIR and BSTI but also some strongly active administrative organization like mobile court should be given more authorization to undertake precautionary investigations to check the microbial and chemical quality of fruit juices. Besides, government and non-government institutions should create public awareness about the contamination and adulteration of fruit juices more intensely with the help of social media. So that people can take initiative for increasing awareness among them for checking batch manufacturing date before consume juice products.

Chapter V

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Appendices

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Xylose-Lysine-Deoxycholate Agar

Component	Amount (g/L)
Yeast extract	3.00
L-lysine	5.00
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.00
Sodium deoxycholate	2.50
Sodium thiosulfate	6.80
Ferric ammonium	0.80
Phenol red	0.08
Agar	15.00

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.00
Lactose	10.00
Bile salts	1.50
Crystal violate	0.001
Neutral red	0.03
Agar	15.00

M-FC Agar

Component	Amount (g/L)
Tryptose	10.00
Proteose peptone	5.00
Yeast extract	3.00
Lactose	12.50
Bile salts mixture	1.50
Sodium chloride	5.00
Aniline blue	0.10
Agar	15.00

Physiological Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Methyl red Vogus Prekaure (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Eosine Methylene Blue Agar

Component	Amount (g/L)
Peptone	10.00
Dipotassium phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin yellow	0.14
Methylene blue	0.065
Agar	13.50

Cetrimide Agar

Component	Amount (g/L)
Pancreatic digest of gelatin	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000
Final pH	(at 25°C) 7.2±0.2

Mueller Hinton Agar

Component	Amount (g/L)
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH	(at 25°C) 7.3±0.1

Appendix – II

Reagents

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5Nacetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N, N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N)and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)[a] to form a colourless solution and stored at 4°C.

MacFarlane turbidity standard no. 5

Sulfuric acid	0.18 M
Barium chloride	0.048 M
Distilled water	1000 ml

Appendix – III

Gadgets

List of gadgets that were used during the study

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini - 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
Microscope	A. Krüssoptronic, Germany
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Refrigerator (4^{O}C) Model: 0636	Samsung

Appendix- IV

List of abbreviation

TVC	Total Viable Count
TSC	Total Staphylococci Count
TFC	Total Fecal Count
MSA	Mannitol Salt Agar
NA	Nutrient Agar
EMB	Eosin Methylene Blue Agar
CA	Cetrimide Agar
XLD	Xlylose Lysine Deoxycholate Agar
Mac	MacConkey Agar
MIU	Motility Indole Urea
TSI	Triple Sugar Iron Agar
ml	Milliliter
µl	Microliter
mg	Milligram
gm	Gram
Kg	Kilogram
e.g.	For example
et al.	And others
pH	Negative logarithm of hydrogen ion concentration
CFU	Colony Forming Unit
spp.	Species
%	Percentage
°C	Degree Celsius
BCSIR	Bangladesh Council of Scientific and Industrial research
BSTI	Bangladesh Standards and Testing Institution
Sec	Second
mm	Millimeter
µm	Micrometer
MHA	Mueller Hinton Agar