Antimicrobial Activity of Lactic Acid Bacteria against Pathogens causing Food Borne Diseases

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

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DECLARATION

I hereby certify that this thesis project entitled **"Antimicrobial Activity of Lactic Acid Bacteria against Pathogens causing Food Borne Diseases"** is submitted by me, **Dipanjana Datta** (ID – 12136007), to the Department of Mathematics and Natural Sciences under the supervision of **Dr. M. Mahboob Hossain**, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. This dissertation was done as a part of my requirement for the degree of B.Sc in Biotechnology. I also declare that this work is entirely based on the original results I have found. Materials and knowledge that I have consulted from the published works accomplished by other researchers have been properly cited and acknowledged within the text of my work.

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Dedicated to Shrabana Datta, my beloved sister and friend.

Thank you for being a source of inspiration and strength and shaping me into who I am today.

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List of Abbreviations

CFS	Cell Free Supernatant
GRAS	Generally Recognized as Safe
IBD	Inflammatory Bowel Disease
IMViC	Indole, Methyl Red, Voges-Proskauer, Citrate Utilization
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LPS	Lipopolysaccharide
MH	Mueller Hinton
MIU	Motility Indole Urease
MRS	De Man, Rogosa and Sharpe
MRSA	Methicillin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NADH	Nictonamide adenine dinucleotide
TCA	Tricarboxylic acid
TSI	Triple Sugar Iron

Abstract

Food borne diseases are quite prevalent in the current situation of the world. Adulteration and contamination of food lead to occurrence of several food borne diseases, which can be fatal, especially for infants. Moreover, the rise of new and antibiotic resistant pathogenic strains has lead to the search for other alternative solutions. Probiotics can be an excellent solution to treat many common food borne diseases. Probiotics are described as live microorganisms which help in the maintenance of the health and well being of the hosts by improving the intestinal microbial balance. Lactic Acid Bacteria (LAB) are known to have many health benefits and are mostly used as probiotics. As they can cause inhibition of food pathogens by the reduction of pH due to lactic acid production, hydrogen peroxide production and production of antimicrobial compounds such as bacteriocin. In this study lactic acid bacteria were isolated from several food items and screened for antimicrobial activity against the pathogens such as *Bacillus subtilis, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus, E. coli* and *Salmonella typhi*. Fourteen isolates were obtained, among which only five were identified as Lactic Acid Bacteria by biochemical tests. The LAB isolates showed inhibitory activity against all the pathogens in the agar spot test but no zone of inhibition was observed in the case of agar diffusion test even after the addition of ammonium sulphate to precipitate proteins present in the supernatant. This suggests that the antimicrobial activity might not be due to bacteriocin production. Instead it might have been due to the production of lactic acid and hydrogen peroxide. In conclusion, LAB is still effective as probiotics against food borne diseases as they have antimicrobial properties but more sophisticated methods and equipment should be used to isolate and purify bacteriocin.

Chapter 1: Introduction

1. Introduction

1.1 Background

One of the major crises the world is facing these days is related to food crisis. Whereas, one of them is due to the scarcity of foods, another is a vast array of diseases contaminated via food. Ever year 40,000 people die by food borne diseases (WHO, 2015). Although the number of sufferers from food borne diseases is higher in the under developed and developing countries, the number is nonetheless significantly large in the western countries such as the USA (Mead et al., 1990). The common symptoms of these diseases include vomiting, bloating, stomach ache, flatulence, excessive fluid discharge through feces, bloody fecal discharge and so on. Some severe ones include neurological disruption and even paralysis. Along with the severe ones, even the vomiting or diarrhea becomes lethal if not treated well, as they cause dehydration and shock. The diseases are botulism, cholera, dysentery, shigellosis, giardiasis, amibiasis, botulism etc. These diseases are due to a wide range of pathogens, which include bacteria, virus, protozoa and parasites (Acheson, 1999). The common ones include *Salmonella spp.*, *Vibrio cholera*, *Campylobacter*, *Helicobacter pylori*, *E. coli*, *Giardia lamblia* and many more (Newell et al., 2010).

The pathogens come in contact to the food in both preparation time and in during preserving time. Improper food preparation include cooking with polluted water that contain fecal bacteria, improperly washed vegetables, fruits and especially raw meat and fish with blood. Some of the pathogens die while cooking, whereas some persist and cause diseases in the enteric and other parts of the body. Moreover, cooking for a short time also lets the pathogens to grow and thrive in food products. Foods in restaurants and particularly food from street side shops cause the most cases of food borne diseases (Rane, 2011).

When it comes to the spread of pathogens by ill preservation, it involves storing the food at wrong temperature, storing both raw and cooked food together, improper pasteurization of milk, not storing food in proper temperature and etc. In fact, dairy products and meat products are the most common sources for spreading diseases. Additionally, in the coastal areas, the shellfishes also cause food borne diseases as the shell and the crust on their flesh can harbor harmful potential pathogens. Every year many people die or at least get affected by food poisoning related to shell fishes (Levine

and Griffin, 1993). Food items which are eaten raw, for example, hors d'oeuvres, sushi etc can cause many illnesses among people (Gwida et al., 2014). Failure to maintain a clean environment during cooking and following hygienic habits such as not cleaning hands properly before eating also aggravates the whole situation.

Whereas, most of the food borne illness can be prevented from spreading by adopting healthy hygienic habits, it is not always possible to do so. Especially, if an individual is dependent on eating outside or for children, it becomes harder for them to maintain a pathogen free healthy meal (Jones and Angulo, 2006) and hence many of them fall sick. The treatment for the less severe diseases includes replacing the lost fluid with oral or intra venous saline. The diseases that initiate high fever require antibiotics and for severe toxic reaction, anti toxins are often required. Oral medication is an effective and benign way to treat the disease. However, it takes time to eliminate the pathogens from the body. Antibiotic treatment, though fast acting, is currently a least recommended treatment option (Nyenje and Ndip, 2013). Due to rapid emergence of resistant pathogens, antibiotics are becoming less and less effective. Most common food borne bacteria are becoming resistant to commonly available antibiotics, such as MRSA (Levy, 1998). Moreover, antibiotics kill all the bacteria irrespective of their functions in the body. This results in destroying the gut flora that can prevent pathogen invasion. Thus, need for alternative treatment is crucial.

Probiotics can become an excellent solution to treat many of the common food borne diseases. These are harmless to the body and become part of the natural gut flora opposed to the antibiotics. In addition, probiotics improve digestion of the one consuming these. Moreover, some of the probiotics show certain anti-microbial activity against a few enteric pathogens (Sartor, 2004). This can be a new approach for tackling many food borne maladies. What makes the probiotics suitable is the fact that these can be incorporated into dairy products and then consumed without any hassle. Furthermore, probiotics add to the nutritional value of the dairy product from the plain milk.

Lactic acid bacteria have antagonistic effects on food borne pathogenic and spoilage microorganisms (Schillinger and Lucke, 1989), e.g. inhibition of *B. subtilis* which contaminates bread and causes spoilage (Vogel et al., 1999). Survival of *E. coli* O157:H7 in dairy products is a potential health hazard because of the link with dairy cattle and raw milk (Saad et al., 2001). Earlier studies had shown that, some *Lactobacillus* strains had

an inhibitory activity on *E. coli* (Rodriguez *et al.*, 1989). Psychrophilic *Pseudomonas* species spoil foods by their lipolytic and proteolytic activities (Unluturk and Turantas, 1998). Hydrogen peroxide produced by *Lactobacillus* species inhibits *Pseudomonas* species (Daeschel, 1989). The antibacterial effect of neutralized supernatant fluid of a *L. casei* strain inhibits *S. aureus*, *B. subtilis*, *E. coli* and *Salmonella typhimurium* (Vignolo et al., 1993). These can be very well used as probiotics. Most importantly, these can be isolated from nature and then produced in a large-scale production cost effectively. Therefore, it can be one of the best deemed alternative for treating food borne illness and also for increasing general immunity (Cadirci and Citak, 2005).

1.2 Probiotics- an Alternative Solution

Probiotics are described as live microorganisms which help in the maintenance of the health and well being of the hosts by improving the intestinal microbial balance (Asahara et al., 2004). In recent years, multiple reports have described beneficial effects by probiotics against several infectious diseases such as intestinal infections, inflammatory bowel diseases (IBD) and allergic reactions caused by certain food products (Ljungh and Wadström, 2006). To create a probiotic potential within the host, two factors are taken into consideration. Primarily, probiotic microorganisms must have the ability to tolerate the extremely low pH and the detergent effect of the bile salts and reach the site of action in a physiological state (Chou and Weimer, 1999). Second, they should adhere to the intestinal mucosal cells and proliferate (Aslim et al., 2007). By colonizing the intestinal lining they can achieve best result as they affect the intestinal immune system, displace enteric pathogens, provide antioxidants and antimutagens and also cause other effects by cell signaling. Although most probiotic strains confer beneficial effects in the colon and the small intestine, there are some studies which provide data about dead cells of probiotic strains exerting beneficial immunological effect (Mottet and Michetti, 2005).

Probiotic microorganisms are most commonly used in fermented dairy products (Ouwehand et al., 2002) but recently they are being used in fermented vegetable or meat products as well. The probiotic strains need to be safe for consumption and that is why most probiotics belong to Lactic Acid Bacteria (LAB), such as *Lactobacillus spp.*, *Bifidobacterium spp.* and *Enterococcus spp.* (Klein et al., 1998). It has been studied that the intake of LAB for one month treatment has resulted in the up-regulation of 334 genes

and down-regulation of 9 genes involved in inflammation, apoptosis, cell-cell signaling, cell adhesion and differentiation (Di Caro et al., 2005).

1.3 Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria constitute a group of genus that has the following common features: cocci, rods and a basic composition of DNA below 50 mol% G+ C. They are typically Gram positive, mesophilic, can grow within 5°C to 45 °C under aerobic, anaerobic or microaerobic conditions and are asporogenous. In addition, they are oxidase and catalase negative, cannot reduce nitrate to nitrite and are incapable of producing indole or hydrogen sulphide. This group consists of numerous genera: *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Streptococcus, Enterococcus, Aerococcus, Bifidobacterium and Pediococcus* (Carr et al., 2002; Axelsson, 2004; Doyle and Meng, 2006).

Lactic acid producing bacteria (LABs) are known to have various beneficial functions such as anti-tumour activity, reduction of serum cholesterol, alleviation of lactose intolerance, stimulation of the immune system, enhancement of resistance against pathogens and prevention of diarrhea's (Aimutis, 1999; Reid, 2006). Based on these functions, different kinds of LAB have been developed as probiotics, and the market volume of probiotics has rapidly increased (Brashears et al., 2003; Hamilton- Miller, 2003).

In recent years LAB have been playing important role in the food and feed fermentation and preservation either as the natural microflora or as starter culture added under controlled conditions. This is due to the fact that they have been recognized as GRAS (Generally Recognized as Safe) microorganisms. *S. thermophilus* is a lactic acid bacterium of major importance in food industry e.g. the manufacture of yogurt. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) (Daeschel, 1989). LABs also produce anti-microbial compounds including H_2O_2, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001). The most common anti-microbial agent produced by LAB is bacteriocin (Deegana et al., 2006).

1.4 Antimicrobial Agents

In recent years bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatment of human and animal diseases (Roy, 1997; Lipsitch et al., 2000; Yoneyama and Katsumata, 2006). As a consequence, multiple resistant strains have appeared and have spread causing difficulties and the use of antibiotics as growth promoters has been restricted. Therefore, the continued development of new classes of anti-microbial agents has become of increasing importance for medicine (Kumar and Schweiser, 2005; Fisher et al., 2005). Application of some bacterial peptides as anti-microbial substances can be one plausible alternative for abusive use of antibiotics in food and feed products.

1.4.1 Antimicrobial Property by Lactic Acid Production

Lactic acid produced by lactic acid starter culture bacteria functions as a natural anti-microbial component, having a generally recognized as safe status. Lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae* (Doores, 1993). Among other organic acids, lactic acid is recognized as a bio-preservative in naturally fermented products. The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane of organisms, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (Ray and Sandine, 1992).

1.4.2 Antimicrobial Property by Hydrogen Peroxide Production

Hydrogen peroxide is a thermodynamically unstable compound and destroys bacterial enzymatic activity (Collins and Aramaki, 1980). The inhibition of the growth of one bacterial species by the H_2O_2 produced by another species is a well-recognized mechanism of bacterial antagonism. Lactobacilli, as well as other lactic-acid-producing bacteria, lack heme and thus do not utilize the cytochrome system (which reduces oxygen to water) for terminal oxidation. Lactobacilli utilize flavoproteins, which generally convert oxygen to H_2O_2. This mechanism, together with the absence of the heme protein catalase, generally results in the formation of H_2O_2 in amounts which are in excess of the capacity of the organism to degrade it. The H_2O_2 formed may inhibit or kill other members of the microbiota, particularly those which lack or have low levels of

H_2O_2-scavenging enzymes, such as catalase peroxidase (Thompson and Johnston, 1950; Dahiya and Speck, 1968; Wheater et al., 1952).

1.4.3 Antimicrobial Property by Bacteriocin Production

Bacteriocins are ribosomaly synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect (Garneau et al., 2002). Use of either the bacteriocins or the bacteriocin-producing LAB like starter cultures for food preservation has received a special attention (Sabia et al., 2002). Nisin, produced by *Lactococcus lactis*, is the most thoroughly studied bacteriocin to date and has been applied as an additive to certain foods worldwide (Delves-Broughton et al., 1996). Moreover, bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract (Cintas et al., 1995; De Vuyst and Vandamme, 1994).

Four major classes of bacteriocins have been listed**:** (I) Lantibiotics which are small (< 5 kDa) heat stable peptides that act on membrane structures, (II) Non lantibiotics which are small (< 10 kDa) heat stable peptides, (III) Large heat-labile proteins and (IV) Complex bacteriocins (Klaenhammer, 1993; González-Martínez et al, 2003). The majority fall into classes I and II (Deegana et al., 2006). These ribosomally synthesized proteinaceous compounds are bactericidal only toward Gram-positive bacteria, which can be explained by the additional protective layer of Gram-negative composed of phospholipids, proteins and lipopolysaccharides (L.P.S) (Dortu and Thonart, 2009; Abee et al., 1995; Bromberg et al., 2004). It is generally accepted that bacteriocins exert their inhibitory action by formation of pores in the cytoplasmic membrane of Gram-positive bacteria. These cells differ in their sensitivity mainly because of difference in membrane composition and fluidity. Self-evidently, bacteriocin producers exhibit specific immunity against their bacteriocin. This is accomplished by the production of dedicated immunity (Lucke, 2000).

Bacteriocins that have often been mooted as potentially food-grade to improve food safety can reduce the prevalence of foodborne diseases and also help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties (Gálvez et al., 2007).

This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources (Deegan et al., 2006).

1.5 Current Scenario in Bangladesh

Unsafe food represents a major threat to public health in Bangladesh. Each year millions of citizens suffer bouts of illness following the consumption of unsafe food. Aside from acute effects arising from food contaminated by microbial pathogens, long term health impacts may result from consumption of food tainted by chemical substances and toxins (Afzal, 2014). In Bangladesh, the predominant group of *E. coli* associated with childhood diarrhea is enterotoxigenic *E. coli*, accounting for approximately 20% of all diarrheal cases (Qadri et al., 2005). The application of modern biotechnology to food production presents new opportunities and challenges for human health.

The number of studies conducted in Bangladesh indicating the possibility of anti-microbial property by Lactic Acid Bacteria against food pathogens is too low. Most research includes the possibility of LABs in the food preservation and studies inclining towards the probiotics field have mostly isolated Lactic Acid Bacteria from yogurt (Chowdhury and Islam, 2016; Rashid et al., 2007). As there is a lack of substantial work on the development of probiotics by Lactic Acid Bacteria against food pathogens by bacteriocidal activity, this research was inspired to be carried out.

1.6 Objectives

- As there is an increase in the emergence of new pathogens and antibiotic resistant pathogens, the purpose of this study is to find new and more antimicrobial compounds among the local species of Lactic Acid Bacteria.
- To find new strains of Lactic Acid Bacteria that produce anti-microbial compounds.
- To compare the well diffusion assay and spot-on-lawn method and to determine the most reliable method for detection of anti-microbial activity against other pathogenic bacteria.

Chapter 2: Materials and Method

2. Materials and Method

This research work was carried out at the Microbiology and Biotechnology Laboratory of the Department of Mathematics and Natural Sciences, BRAC University.

2.1 Materials

2.1.1 Samples used:

- Lactic acid bacteria (LAB) sourced from fruits, vegetables and dairy products:
  - Banana
  - Cabbage
  - Carrot
  - Tomato
  - Pointed gourd
  - Yogurt
  - Radish
  - Local cheese
  - Honey
  - Cauliflower
- Indicator strains from laboratory stock, ICDDR,B:
  - *Bacillus subtilis*
  - *Bacillus cereus*
  - *Streptococcus pneumonia*
  - *Staphylococcus aureus*
  - *E.coli*
  - *Salmonella typhi*

2.1.2: Reagents (for media preparation and other purposes):

- ✓ 0.9% Sodium chloride solution (normal saline)
- ✓ Malachite Green

- ✓ Crystal Violet
- ✓ 1 M Sodium hydroxide
- ✓ Kovac's Reagent
- ✓ 3% H_2O_2 solution
- ✓ Nitrate test Solution A and Solution B
- ✓ Barrit's Reagent A and B
- ✓ Gram's iodine
- ✓ Safranin
- ✓ 95% ethyl alcohol
- ✓ $CaCO_3$ powder
- ✓ Sterilized sheep's blood
- ✓ Zinc powder
- ✓ Methyl red solution
- ✓ Oxidase test reagent

2.1.3 Equipment:

- Laminar airflow cabinet
- Incubator
- Vortex machine
- Autoclave machine
- Anaerobic Jar
- Glasswares, microscope, pH meter, petri dishes, vials, test tubes, pipettes, micro-pipettes, Bunsen burner, centrifuge machine, electric balance etc.

2.1.4 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. For biochemical tests, specific media were prepared.

2.1.4.1 Important agar media and broth

- **Nutrient agar medium**

  Nutrient Agar is a common microbiological growth medium. Nutrient agar typically contains 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, 97.2% distilled water.

- **De Man, Rogosa and Sharpe (MRS) agar and broth**

  De Man, Rogosa and Sharpe Agar and broth were designed to encourage the growth of the `lactic acid bacteria' which includes species of the following genera: *Lactobacillus, Streptococcus, Pediococcus and Leuconostoc. It typically contains* 1.0 % peptone, 1.0 % beef extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80 (also known as Tween 80), 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate and the desired amount of distilled water. It can be used as a solid medium by adding 1.0% agar.

- **Mueller Hinton (MH) agar**

  Mueller and Hinton developed Mueller Hinton Agar (MHA) which is more commonly used for the routine susceptibility testing of non-fastidious microorganism by the Kirby-Bauer disk diffusion technique. It contains 0.2% beef extract, 1.75% casein hydrolysate, 0.15% starch and 1.7% agar.

2.2 Method

2.2.1 Sample collection

Various types of fruits, vegetables and dairy products were chosen as samples for the isolation of Lactic Acid Bacteria (LAB) which were sourced from banana, tomato, carrot, pointed gourd, cabbage, cauliflower, radish, yogurt made from raw cow milk, local cheese and honey. Each of the samples was purchased from the local market named Karwan Bazar in Dhaka and carried in an aseptic bag or container to the laboratory. The fruits and vegetables were chopped up, mixed with sterile water and allowed to ferment in closed conical flasks filled to the top with water for two weeks.

2.2.2 Isolation of Lactic Acid Bacteria from Samples:

Two ml of each of the sample solutions was individually added to 10 ml MRS broth and incubated for 24 hours at 37°C. From each of the MRS broth solutions 200 µl was spread plated onto MRS agar plates and incubated further. The different bacterial colonies based on their morphology were selected in a way to ensure no two colonies displayed the same characteristics. These were four-way streaked on MRS agar plates to obtain single colonies of pure isolates. The plates were sealed with parafilm, refrigerated at 4°C and were sub cultured every two weeks.

Table 2.1: Fifteen isolates based on their morphology were selected.

Sample	Number of isolates	Designation of isolates
Banana	1	D1
Cabbage	1	D2
Pointed gourd	1	D3
Tomato	1	D4
Carrot	1	D5
Yogurt	3	D6, D8, D9
Radish	1	D7
Local cheese	3	D10, D11, D12
Honey	2	D13, D14
Cauliflower	1	D15

2.2.3 Screening of Lactic Acid Bacteria

Isolates (Table 2.1) were screened to obtain Lactic Acid Bacteria based on Gram staining, catalase test and growing isolates in agar medium namely MRS with 0.3% $CaCO_3$. Isolates which were Gram positive, catalase negative and showed a clear zone around the colonies in the MRS media with 0.3% $CaCO_3$ were selected and incubated anaerobically at 37°C for 48 hours (Maragkoudakis et al., 2006). The clear zone around the colonies might indicate the production of lactic acid or other organic acids by the bacterial colonies (Chang et al., 2013). The plates were anaerobically sub cultured

frequently and refrigerated at 4°C. The strains D7, D10, D11, D12 and D15 were selected and used throughout the study.

2.2.4 Evaluating Antimicrobial properties of different bacterial strains:

2.2.4.1 Agar spot test

The agar spot test was a modification of that described by Schillinger and Lucke (1989). Colonies of each isolates were picked and stabbed on MRS agar medium and incubated anaerobically for 24 hours at 37°C. The plates were later overlaid with 10 ml of Nutrient soft agar (0.5%). The overlay agar was seeded with 10^4 cfu/ml of the pathogenic bacteria to be tested for sensitivity (*Escherichia coli, Bacillus subtilis, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus and Salmonella typhi*). After incubation for 18- 24 h at 37 °C the plates were checked for inhibition zone of inihibition. Inhibition was scored as positive if the diameter of the clear zone around the colonies of the producer strain was 10 mm or larger.

2.2.4.2 Agar diffusion test

Strains exhibiting antagonistic activities against pathogenic bacteria were investigated for their antimicrobial compounds such as bacteriocins or bacteriocin like substances. The agar bioassay described by Herreros et al. (2005) was used to screen for bacteriocin producing isolates.

2.2.4.2.1 Determination of antimicrobial property by crude bacteriocin

The pathogenic bacteria or indicator strain were incubated overnight in nutrient agar. Each of the indicator strain was added to normal saline solution until the concentration was 0.3 McFarland. Using a sterilized cotton swab, nutrient plates were spread with the indicator strain saline suspension and within 15 minutes, wells of 5 mm were cut using a sterilized cork borer. 60 µl of cell free supernatant from each LAB isolate were added to the well. Cell free supernatant was prepared as follows: Strains were grown for 24 h at 37 °C in 10 ml of MRS broth and then centrifuged at 9500 rpm for 25 min. The pH of the supernatant of each sample was adjusted to 6.5 with 1N NaOH to rule out inhibition by organic acid. The pH adjusted supernatant was filtered through a sterile 0.22 µm syringe filter and 60 µl of the aliquot was added to the wells. Clear MRS broth was added to the wells as control. To ensure proper diffusion of supernatant and to inhibit growth of test

organism during the diffusion process, plates were kept in a 4°C refrigerator for 30 min after which the plates were incubated aerobically for 24 hours at 37°C and the antimicrobial property by crude bacteriocin present in the CFS was tested.

2.2.4.2.2 Determination of antimicrobial property by precipitated bacteriocin

Since some bacteria secrete bacteriocins in minute amounts, ammonium sulphate was added for precipitation of protein. Ammonium sulphate was added to the CFS till 40% saturation and then the solutions were incubated at 4°C for 45 minutes with mild shaking. Afterwards the solutions were centrifuged at 9500 rpm for 25 minutes twice and the supernatants were decanted until only 1 ml of CFS remained in the test tube. The pellet was recombined with the remaining CFS and the peptide concentrates were refrigerated at 4°C. Wells were filled with 60 µl of the reconstituted pellet and MRS broth was used as a control. The MH agar plates were refrigerated for 30 minutes at 4°C and later incubated overnight aerobically at 37°C.

2.2.5 Identification of Bacterial strains

2.2.5.1 Morphological characterization of the bacteria

Using sterile technique, MRS plates were streaked to obtain isolated discrete colonies. The plates were then incubated at 37°C for 24 hours. After incubation, the bacterial colonies were evaluated for size, pigmentation, form, margin, elevation and texture (Cappuccino and Sherman, 2005).

2.2.5.2 Microscopic Observation of the bacteria

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

2.2.5.2.1 Gram stain

Using sterile technique, a drop of saline was placed on the slide and a small amount of a bacterial colony was then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The smear was flooded with crystal violet and let stand for 1 minute. Then, the smear was gently washed with tap water. It was then flooded again with the Gram's iodine mordant and let stand for one minute followed by gentle wash with tap

water. After that, the smear was decolorized with 95% ethyl alcohol and gently washed with tap water. Finally, it was counterstained with Safranin for 45 seconds and gently washed with tap water. The slide was then blot dried with bibulous paper and examined under oil immersion (Cappuccino and Sherman, 2005).

2.2.5.2.2 Spore stain

Using sterile technique, a drop of saline was placed on the slide and a small amount of a bacterial colony was then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was flooded with malachite green while placed over a water bath and allowed to steam for 2 to 3 minutes. The stain was prevented from drying out by constant application of the dye. The slides were removed, cooled and washed under running tap water. The smear was then counterstained with Safranin for 30 seconds and washed with tap water. The slide was then blot dried with bibulous paper and examined under microscope (Cappuccino and Sherman, 2005).

2.2.5.3 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 and Sherman, 2005). The biochemical tests performed were:

- Carbohydrate fermentation (dextrose, sucrose, fructose, lactose, mannitol, L-arabinose, L- rhamnose, galactose and maltose)
- Triple sugar iron agar test
- IMViC test (Indole production test, Methyl red test, Voges- Proskauer test)
- Citrate utilization test
- Urease test
- Nitrate reduction test
- Catalase test
- Oxidase test

- Motility test
- Gelatin hydrolysis test
- Mannitol Salt Agar
- Starch hydrolysis
- Blood agar
- Growth at 45°C
- Anaerobic growth

2.2.5.3.1 Carbohydrate Utilization test

Phenol red dextrose, sucrose, fructose, lactose, maltose, L-arabinose, L-rhamnose and galactose broths of 6 ml were prepared by autoclaving at 15 psi 121°C for 15 minutes (Autoclave, SAARC) in separate test tubes. Using sterile technique, a small amount of the experimental bacteria from 24 hour old pure culture was inoculated into the broths by means of loop inoculation. All the tubes were incubated for 24 hours at 37°C (Cappuccino and Sherman, 2005).

2.2.5.3.2 Triple Sugar Iron Agar test

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from a 24 hour old pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37°C (Cappuccino and Sherman, 2005).

2.2.5.3.3 Indole Production test

Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi, 121 C. Using sterile techniques, a small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes 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).

2.2.5.3.4 Methyl red test

Methyl Red- Voges Proskauer broth of 7 ml in each test tubes were prepared by autoclaving at 15 psi 121°C. Using sterile technique, a small amount of the experimental bacteria from a 24 hour old pure culture was inoculated into the tubes 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 hour. 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 and Sherman, 2005).

2.2.5.3.5 Voges Proskauer test

To the aliquot of MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (Barrit's reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (Barrit's reagent B). The tube was gently shaken to expose the medium to atmospheric oxygen (30 seconds to 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).

2.2.5.3.6 Citrate utilization test

Simmons citrate agar slants of 2 ml in each vial 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 vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37°C (Cappuccino and Sherman, 2005).

2.2.5.3.7 Motility- Indole- Urease (MIU) test

Motility- Indole- Urease media was prepared by autoclaving at 15 psi, 121°C. The media was cooled to about 50- 55°C and 100 ml of urease reagent 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, a small amount of the experimental bacteria from a 24 hour 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).

2.2.5.3.8 Nitrate reduction test

Nitrate broth of 6 ml in each test tube was prepared by autoclaving at 15 psi, 121°C. Using sterile technique, a small amount of the experimental bacteria from a 24 hour 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 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 color development, a small amount of zinc was added to each broth (Cappuccino and Sherman, 2005).

Note: Caution was maintained during the use of powdered zinc since it is hazardous.

2.2.5.3.9 Catalase test

A microscopic slide was placed inside a Petri dish. Using a sterile inoculating loop, a small amount of bacteria from a 24 hour old culture was placed onto the microscopic slide. One 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). Bubble formation indicates a catalase positive test.

2.2.5.3.10 Oxidase test

A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and was allowed to 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 and Cathcart, 2010). Colour change to dark purple indicates a positive result.

2.2.5.3.11 Gelatin hydrolysis test

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 1 week (Cruz and Torres, 2012).

2.2.5.3.12 Mannitol Salt Agar (MSA) test

Using sterile technique, a plate of MSA agar was streaked by picking a loopful colony of 24 hour old pure culture to obtain isolated colonies. The plates were then incubated at 37◦C for 24 hours (Shields and Tsang, 2013).

2.2.5.3.13 Starch hydrolysis test

Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using Gram's iodine (Cappuccino and Sherman, 2005).

2.2.5.3.14 Blood agar test

Blood agar base medium was prepared in a conical flask and autoclaved at 121◦C, 15 psi. The blood agar medium was allowed to cool to 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).

2.2.5.3.15 Growth at 45 °C and anaerobic condition

De Man, Rogosa and Sharpe agar was prepared in conical flasks and was autoclaved at 121°C, 15 psi. The media were then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, nutrient agar plates were 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 45°C for 24-48 hours. The MRS plates placed in anaerobic jar were incubated at 37°C for 24 hours (Cappuccino and Sherman, 2005).

2.2.5.3.16 Lipid Hydrolysis

For lipid hydrolysis test Tributyrin agar base was prepared by adding 23 grams to 990 ml distilled water followed by 10 ml Tributyrin addition to the flask. The media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes followed by transfer of media to glass petri dishes. After solidification, the plates were streaked with the samples and incubated at 37°C for 24 hours.

2.2.6 Preservation of bacteria

Three milliliter of T1N1 agar was inoculated through stabbing each bacterium from nutrient agar plate. The vial was incubated for 5 hours to allow the bacteria to acquire log phase. Two hundred microlitre of sterile glycerol was next added and the vial sealed with parafilm and stored at room temperature.

Chapter 3: Results

3. Results

In this study, 15 isolates were collected from the fermented food samples (Figure 3.1) and cultured on MRS agar anaerobically for 48 hours at 37°C. The isolates were further sub cultured to obtain pure colonies and were further screened for the presence of Lactic Acid Bacteria (LAB) based on the morphological and biochemical characteristics.

Figure 3.1: Comparison of some of the samples before fermentation (left picture) and after fermentation (right picture)

3.1 Primary Screening of Lactic Acid Bacteria

Bacterial isolates which were observed to be Gram positive, catalase negative and showed clear zone around the colonies in the medium MRS with 0.3% $CaCO_3$ were selected as Lactic Acid Bacteria. The strains chosen for further study were D7, D10, D11, D12 and D15 (Table 3.1).

Table 3.1: The results of Primary Screening of Lactic Acid Bacteria from total isolates

Strain Designation	Gram Stain	Catalase test	MRS-$CaCO_3$
D1	-	+	No clear zone
D2	-	+	No clear zone
D3	-	+	No clear zone
D4	-	+	No clear zone
D5	-	+	No clear zone
D6	-	+	No clear zone

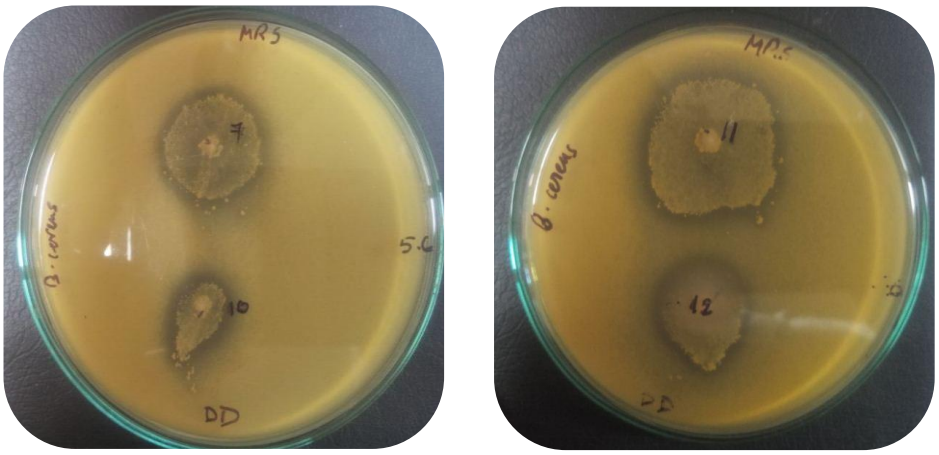
Table 3.1: The results of Primary Screening of Lactic Acid Bacteria from total isolates (continued)

Strain Designation	Gram Stain	Catalase test	MRS-$CaCO_3$
D7	+	-	Clear zone
D8	-	+	No clear zone
D9	-	+	No clear zone
D10	+	-	Clear zone
D11	+	-	Clear zone
D12	+	-	Clear zone
D13	-	+	No clear zone
D14	-	+	No clear zone
D15	+	-	Clear zone

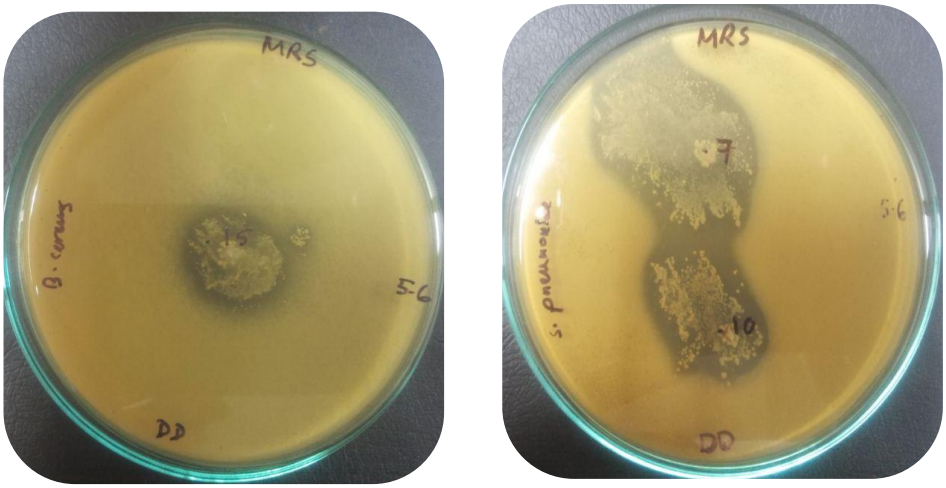
3.2 Antimicrobial Assay by Agar Spot test

The antimicrobial activity of the five isolates was initially determined against the pathogens by agar spot assay. It was found that all the five isolates showed zones of inhibition against the pathogenic strains (Figures 3.2 – 3.4). The LABs showed biggest zones of inhibition against *S. pneumonia* and smaller zones against the other pathogens. Most of the zones were irregular in shape and some had either merged (b1, b2, f2) while others had zones whose perimeters were difficult to distinguish due to less growth of the indicator strains (e1-e3) and excessive dispersal growth of LAB around the plates during agar overlay (b3, d2, e2, f1- f3).

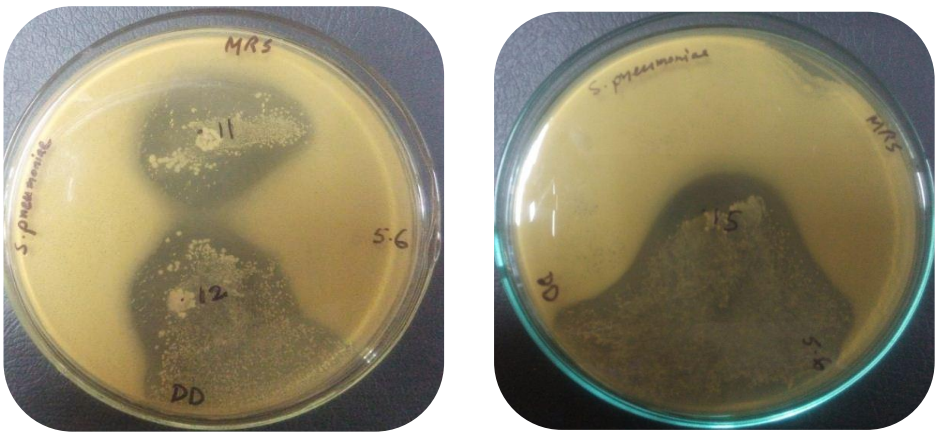
In the investigation it was observed that the isolates D11 and D12 which were obtained from cheese and the isolate D15, which was obtained from cauliflower had consistently showed higher inhibition compared to the other strains D7 (obtained from radish) and D10 (obtained from cheese). The isolates D11, D12 and D15 yielded bigger zone of inhibitions against *B. cereus, S. pneumonia* and *B. subtilis*. However, the isolates D7 and D10 showed higher inhibition against *S. aureus* compared to other isolates. All the isolates had almost the same inhibition zone diameter against *E. coli*. Overall, it was seen that all the isolates were most effective at inhibiting *S. pneumonia, E. coli, S. aureus* and *S. typhi*. Therefore isolates collected from cheese and cauliflowers were more effective against food pathogens.



a 1 a 2

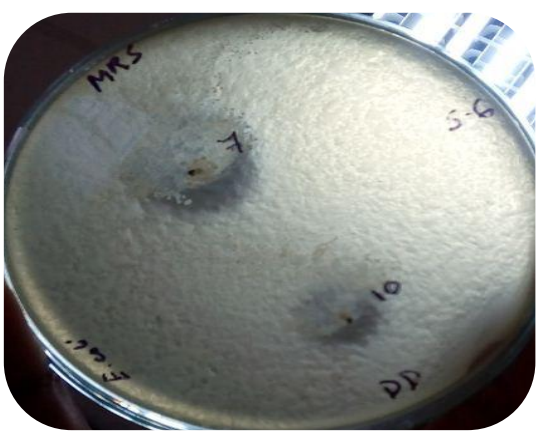


a 3 b 1



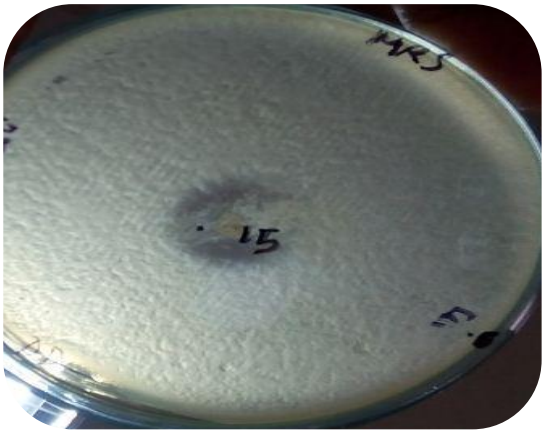
b 2 b 3

Figure 3.2: Results of Agar Spot Test of the five isolates D7, D10 (a 1, b 1), D11, D12 (a 2, b 2) and D15 (a 3, b 3) against the pathogens *B. cereus* (a 1-a 3) and *S. pneumonia* (b 1-b 3)

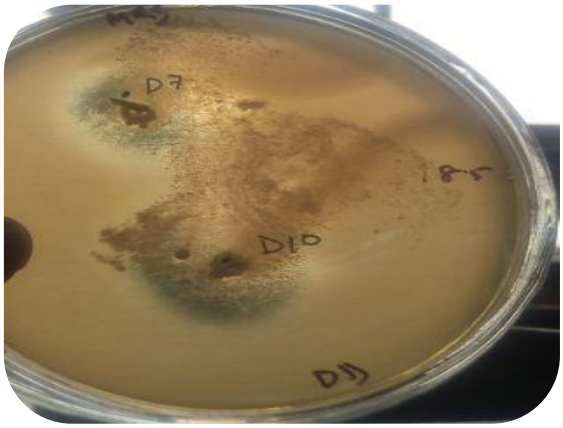


c 1

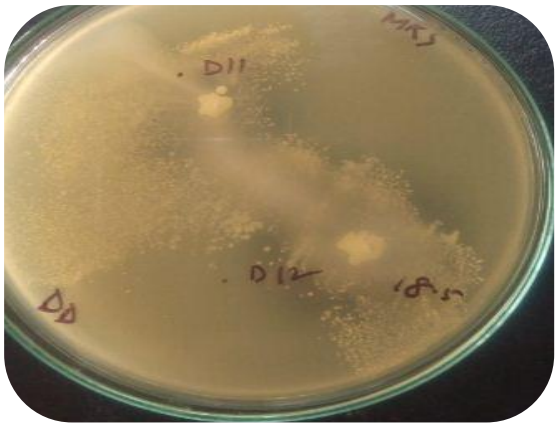
c 2



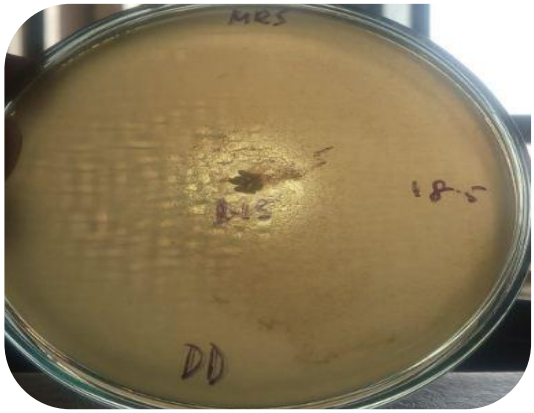
c 3



d 1



d 2



d 3

Figure 3.3: Results of Agar Spot Test of the five isolates D7, D10 (c 1, d 1), D11, D12 (c 2, d 2) and D15 (c 3, d 3) against the pathogens *E. coli* (c 1-c 3) and *S. aureus* (d 1-d 3)

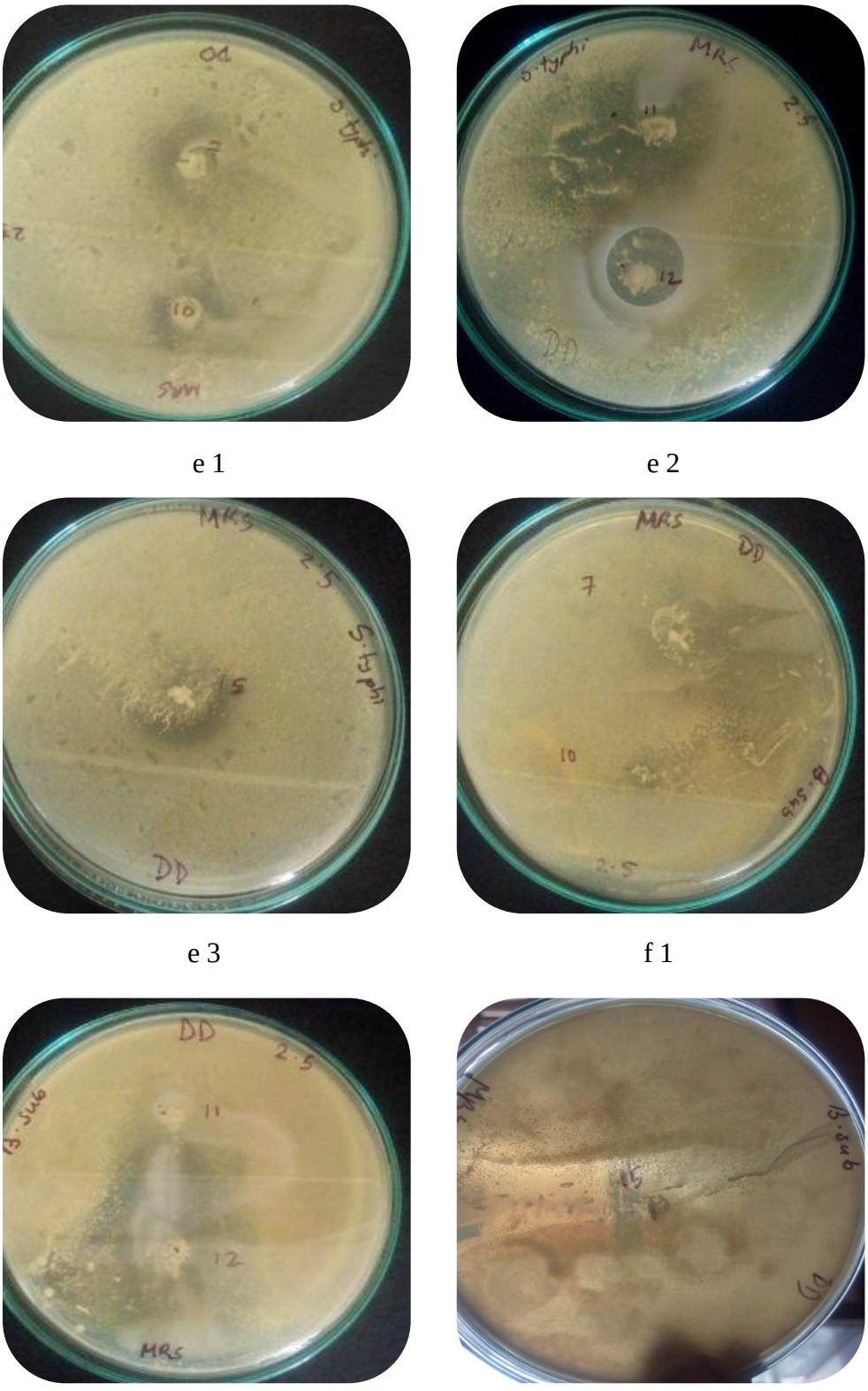
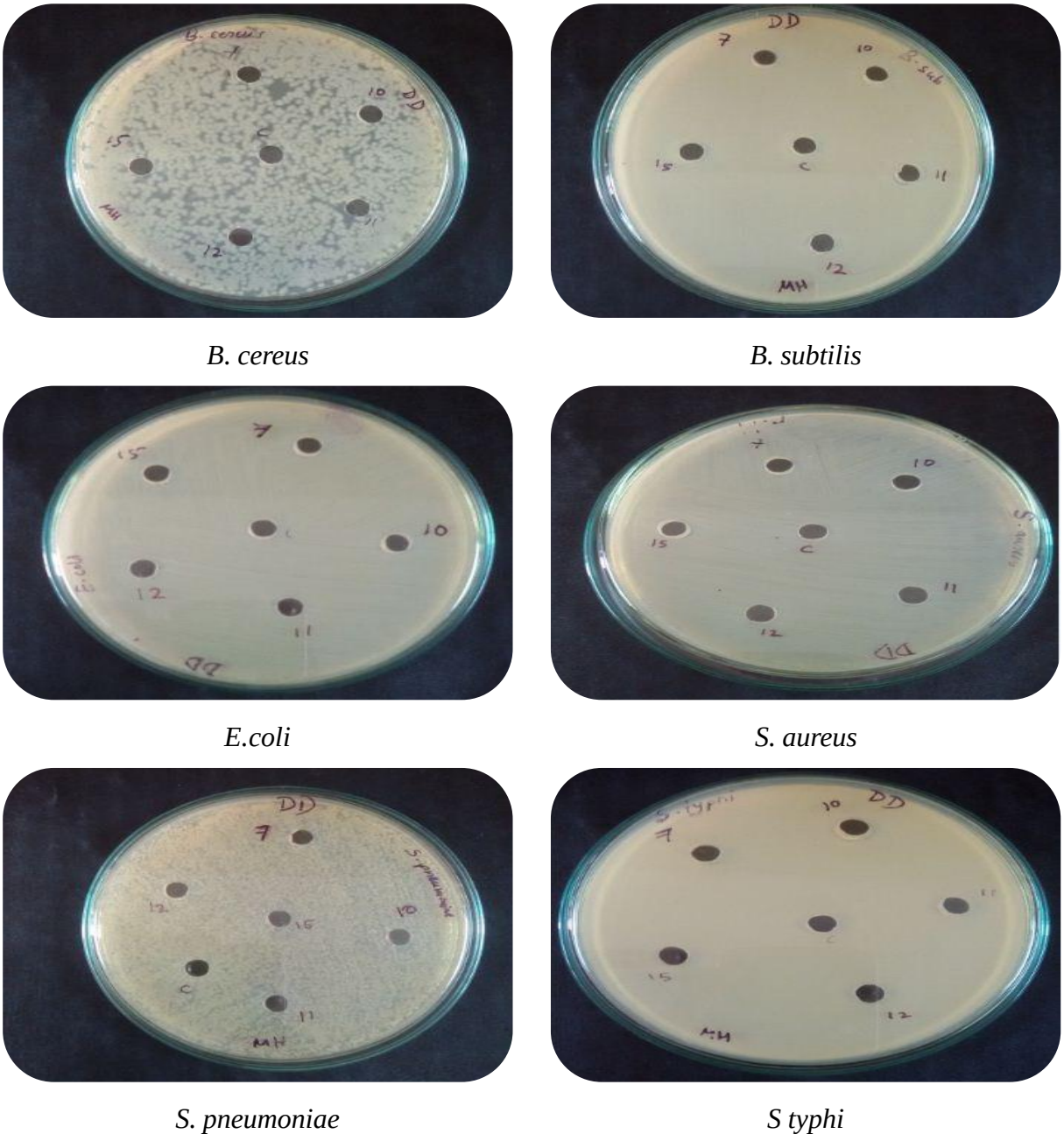


Figure 3.4: Results of Agar Spot Test of the five isolates D7, D10 (e 1, f 1), D11, D12 (e 2, f 2) and D15 (e 3, f 3) against the pathogens *S. typhi* (e 1-e 3) and *B. subtilis* (f 1-f 3)

3.3 Determining antimicrobial Assay by Agar diffusion method

The five LAB strains which showed inhibitory effect against the pathogenic strains were analyzed for the production of antimicrobial compounds. Supernatants obtained from all the strains did not exhibit inhibition zones around the wells after pH adjustment to 6.5. Ammonium precipitation of bacteriocin also did not display any inhibitory zones (Figure 3.5).



B. cereus *B. subtilis*

E.coli *S. aureus*

S. pneumoniae *S typhi*

Figure 3.5: Antimicrobial assay of the five isolates by agar diffusion against pathogenic strains using precipitated protein solution. No zone of inhibition was observed.

3.4 Identification of Bacteria

The five isolates were streaked on MRS agar plates and their distinct colony morphologies were subsequently analyzed. The isolates were viewed under microscope after Gram staining (Figure 3.6) and spore staining (Figure 3.7). Biochemical tests were also performed and assumptive identification of the bacterial strains made through the use of ABIS software online (Figures 3.8- 3.9). Isolates identified were assumed to be *Lactobacillus apodemi, Pediococcus spp., Lactobacillus delbrueckii subsp. lactis, Lactobacillus fermentum and Lactococcus lactis* (Table 3.2).

Table 3.2: Biochemical test results of five LAB isolates and their identification through the use of ABIS online software

Isolate name	Gram's Stain		MIU			TSI				Catalase	Oxidase	Starch Hydrolysis	Gelatin Hydrolysis	Spore stain	Carbohydrate fermentation										Growth at 45°C	Methyl Red	Voges Proskauer	Nitrate reduction	Blood Hemolysis	Motility	Citrate utilization	Lipid Hydrolysis	Presumptive organisms
	Stain	Shape	Motility	Indole	Urease	Butt	Slant	H_2S	Gas						Dextrose	Dextrose with gas	Lactose	Galactose	Mannitol	Maltose	L-arabinose	L-rhamnose	Frusctose	Sucrose									
D7	+	Rod	-	-	-	A	A	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-	*Lactobacillus apodemi*
D10	+	Cocci	-	-	-	A	K	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	*Pediococcus spp.*
D11	+	Cocco bacilli	-	-	-	A	A	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-	*Lactobacillus delbrueckii subsp. lactis*
D12	+	Rod	-	-	-	A	A	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-	*Lactobacillus fermentum*
D15	+	Cocci	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	*Lactococcus lactis*

*(-) = Negative *(+) = Positive *K = Alkaline reaction *A = Acidic reaction

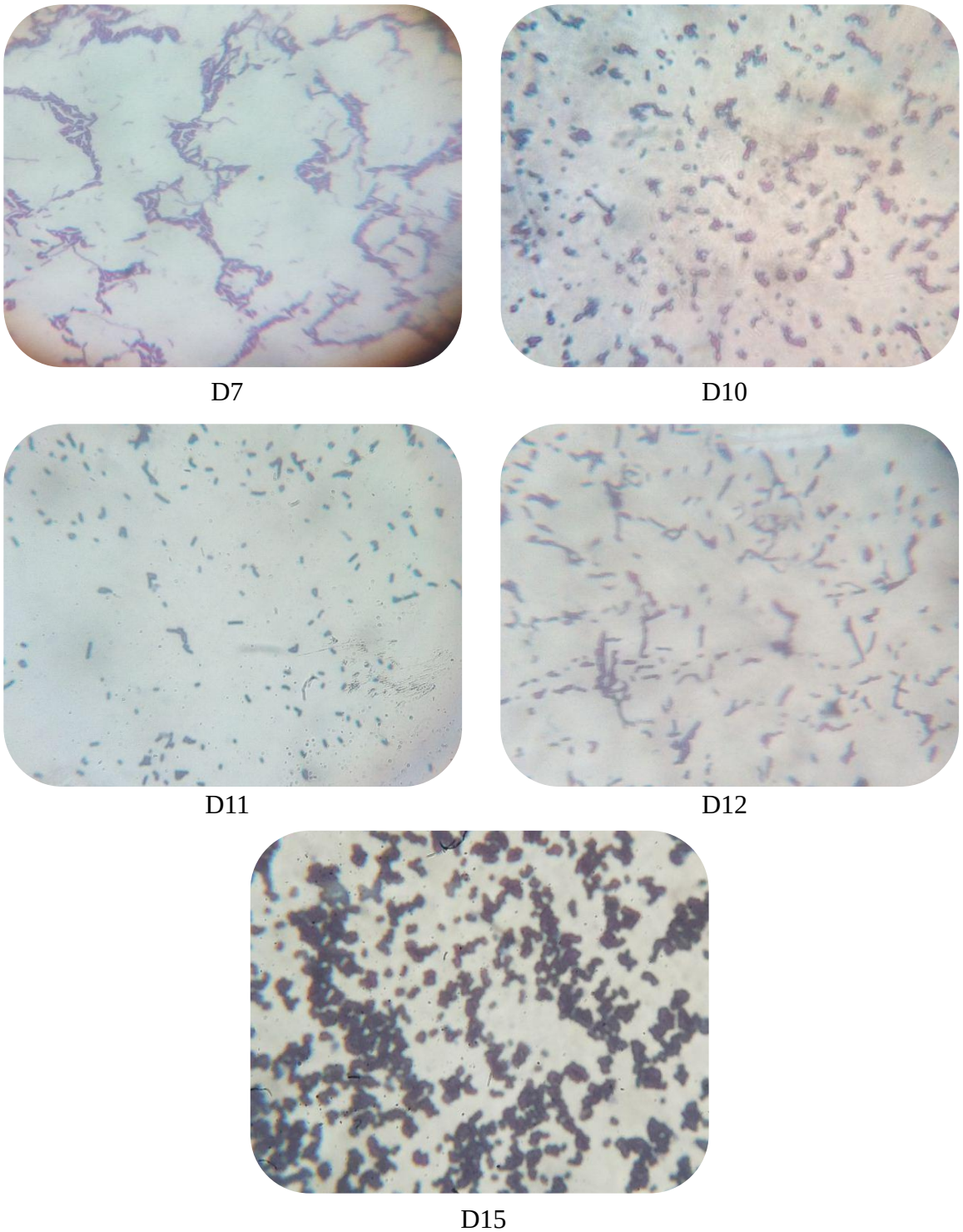
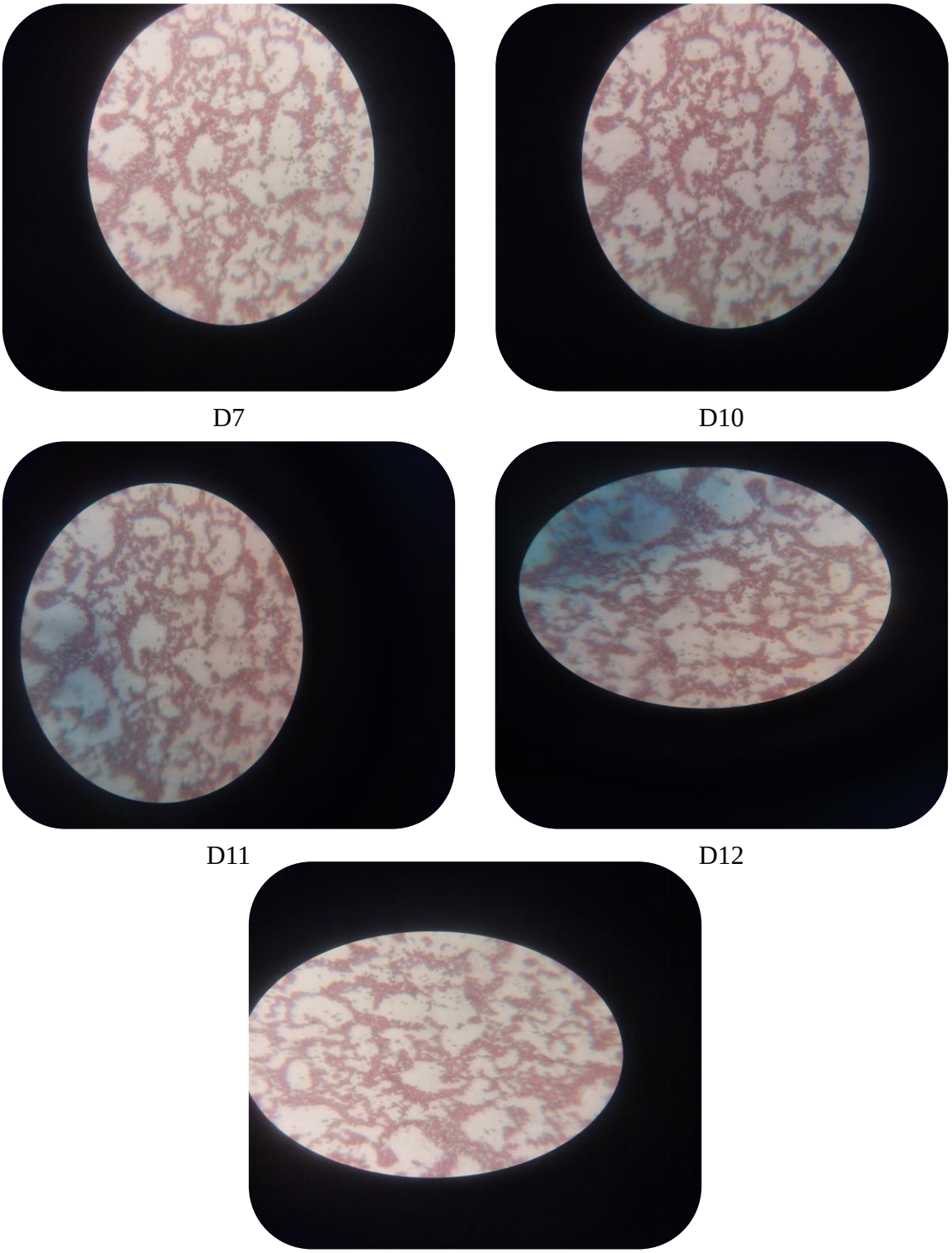


Figure 3.6: Gram staining results of the five LAB isolates



D7 D10

D11 D12

D15

Figure 3.7: Spore staining showing absence of spores in all the LAB samples

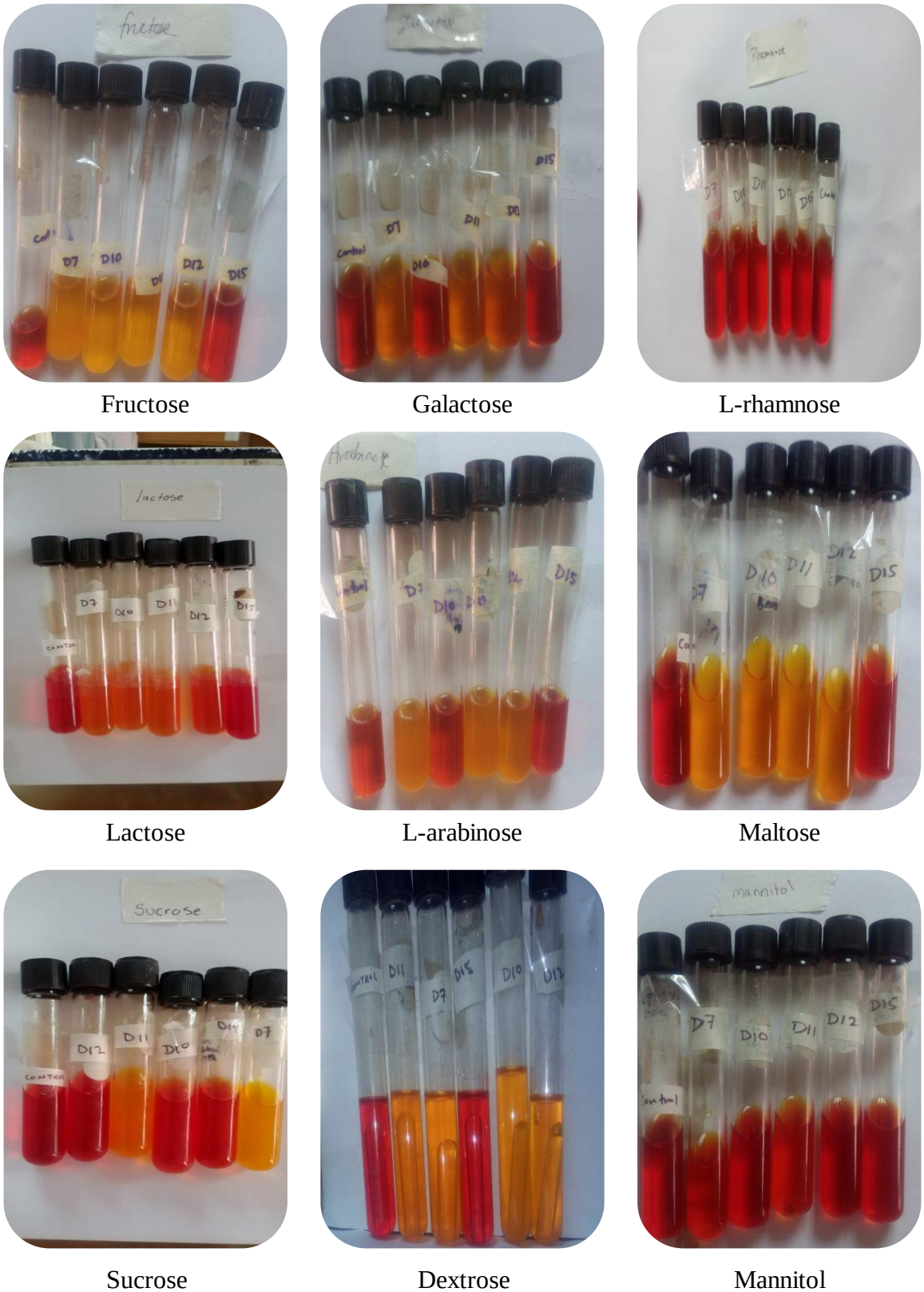
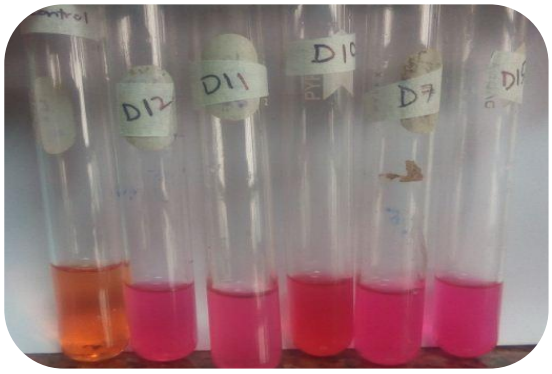
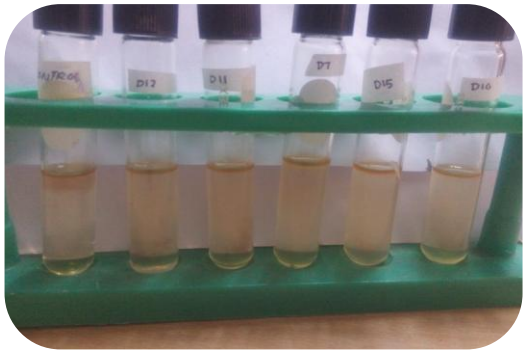


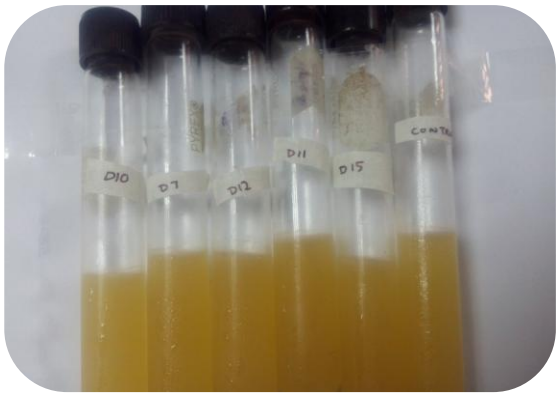
Figure 3.8: Results of fermentation of different sugars by LAB samples



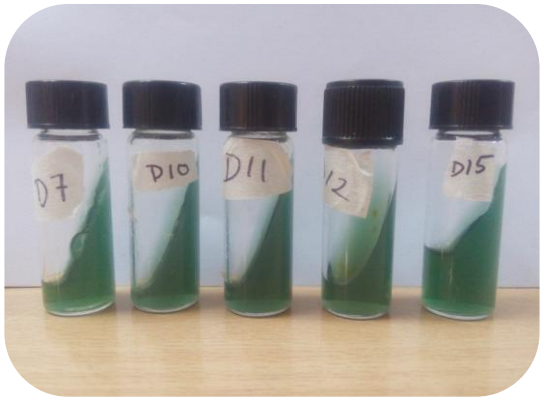
Methyl Red test



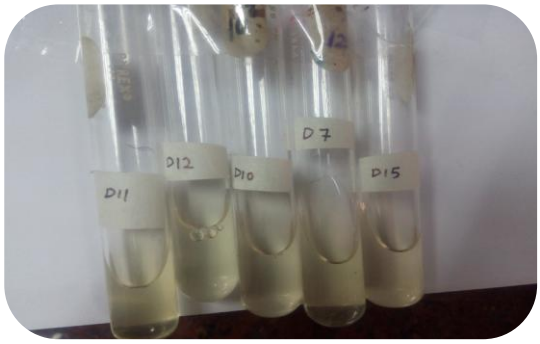
Indole test



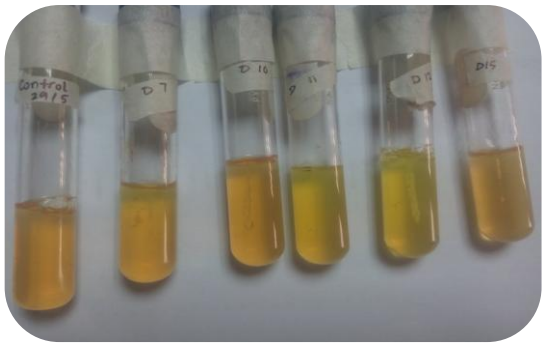
Gelatin test



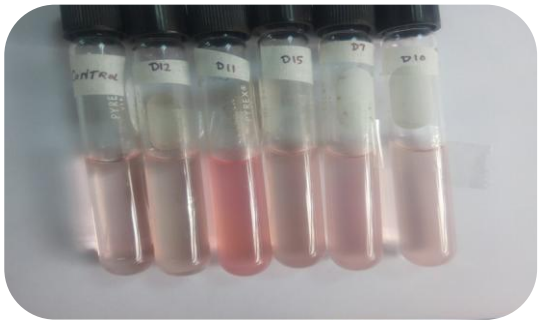
Citrate test



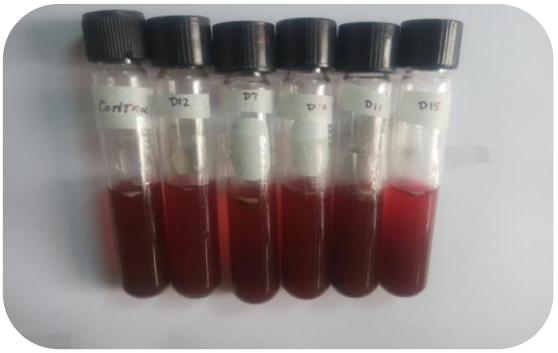
VP test



MIU test

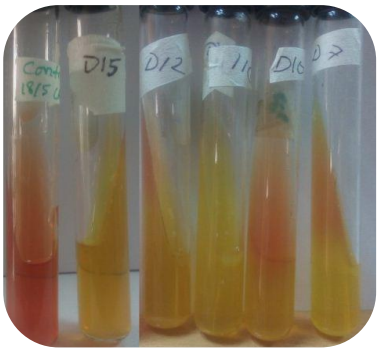


Nitrate reduction test
(before zinc)



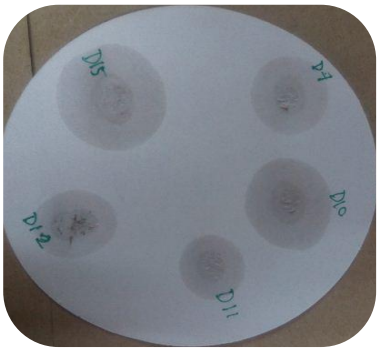
Nitrate reduction test
(after zinc)

Figure 3.9: Biochemical tests performed with LAB samples

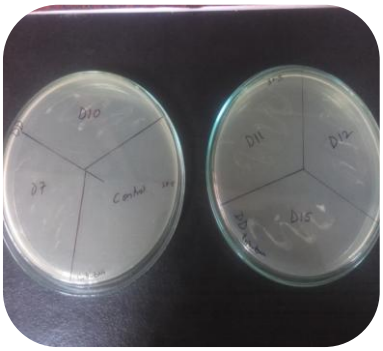


TSI test

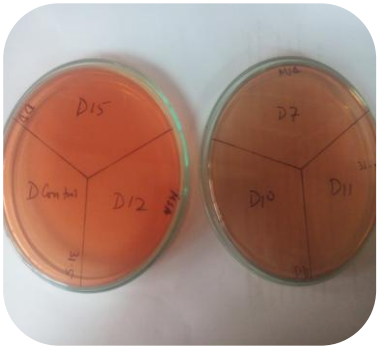
Catalase



Oxidase

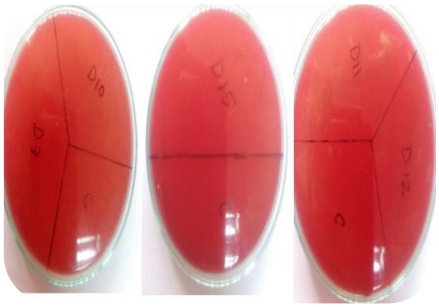


Lipid test

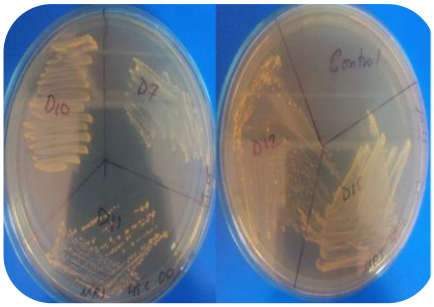


Mannitol salt

Starch test



Blood agar test



Growth at 45°C

Figure 3.9: Biochemical tests performed with LAB samples (continued)

Chapter 4: Discussion

4. Discussion

In the present investigation, the five LAB isolates obtained from various fermented food samples were identified *as Lactobacillus apodemi, Pediococcus spp*., *Lactobacillus delbrueckii subsp. lactis, Lactobacillus fermentum* and *Lactococcus lactis* which were designated as D7, D10, D11, D12 and D15 respectively (Table 3.2). The five isolates obtained from various food items showed zones of inhibition when the bioassay was carried out by agar spot test method for antimicrobial activity against food borne pathogens. However, when the agar diffusion method was followed to find the antimicrobial activity of the five isolates against the pathogens, no zone of inhibition was observed for all the isolates. This could mean that the antimicrobial activity of the isolates might not have resulted from bacteriocin, rather it might have been caused by the presence of lactic acid or hydrogen peroxide. Lactic Acid Bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations. Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). Since bacteriocins are proteins produced against closely related species (Klaenhammer, 1988), there is a probability that either no bacteriocins have been produced in the supernatant or the bacteriocins that might have been produced by the five isolates were of too low concentration to be effective against the particular chosen indicator pathogenic strains since the isolates and the indicator strains are not closely related species. Another alternative might be that the LAB stopped secreting bacteriocin after their log phase was exceeded which caused the absence of inhibition zone in agar diffusion method.

In the case of agar spot assay, the plates were incubated aerobically which might have lead to the formation of hydrogen peroxide and lactic acid. Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Ammor et al., 2006). In the case of agar

diffusion assay, the isolates were grown in MRS broth anaerobically which prevented the formation of hydrogen peroxide and the pH of the supernatant was adjusted to 6.5 by 1N NaOH to rule out possibilities of inhibition caused by lactic acid (Hwanhlem et al., 2011).

Since no zone of inhibition was observed in agar diffusion method for all the isolates, it could mean that bacteriocin might not have been present even after using the supernatant containing the resuspended pellet which was formed by the addition of ammonium sulphate to saturate the proteins present in the supernatant. In the study reviewed by Anas et al., (2008) bacteriocin was concentrated by precipitating with 5% Tricarboxylic acid (TCA) which yielded zone of inhibitions in agar diffusion assay whereas no zone of inhibitions was observed in this study where precipitation was done by using ammonium sulphate till 40% (v/v) which might lead to a possibility of the bacteriocin not being properly precipitated. Moreover, precipitating by ammonium sulphate reduces the working volume but they do not provide a high degree of purification (Guyonnet et al., 2000). Isoelectric focusing and/or multiple chromatographic separations, including cation exchange, gel filtration, hydrophobic interaction and reverse-phase liquid chromatography are necessary to achieve significant purification of bacteriocins. Usually the yields obtained are still low. An ideal protocol for bacteriocin production should be one that is applicable to large-scale purification, leading to bacteriocin yields higher than 50% and purity around 90% (Schöbitz et al., 2006).

In this study, it was observed that agar spot assay showed signs of inhibitions compared to agar diffusion assay which showed no zone of inhibition for all the isolates. This suggests that live cells are better and more effective at showing antimicrobial activity than supernatants. Similarly, this result is not surprising due to the fact that, generally, the technique spot on the lawn always reveals antagonistic activity with a higher proportion compared to that observed by the well diffusion assay (Moraes et al., 2010). Although the well-diffusion assay and the spot technique are the most widely used techniques to screen for bacteriocin-producing strains, they have been criticized of being tedious and time-consuming, and they may yield false results (Davidson and Parish 1989; Benkerroum 1992; Kang and Fung 1998). Therefore, when such techniques are used, they should be followed by other tests to rule out the possible effect of interfering inhibitory metabolites and to confirm the proteinaceous nature of the active substance.

In conclusion, it can be stated that Lactic Acid Bacteria can be used as probiotics against food borne pathogens as this research have shown that LAB produce inhibitory properties. Lactic Acid Bacteria can be used as live cells as probiotics since they have a "Generally Recognized as Safe" status although inhibition by bacteriocin might be more effective as well. Further research needs to be done to find the antimicrobial property by bacteriocin produced by LAB. A bigger and more diverse sample collection should be chosen for the study and the number of isolates should be increased to alleviate the possibility of obtaining bacteriocin producing strains of LAB. Since bacteriocins are proteins produced against closely related species (Klaenhammer, 1988), to determine optimal parameters for the bacteriocin production, it is necessary to determine the ideal conditions of growth of the lactic strains and the composition of the culture medium. Moreover, bacteriocin should be precipitated and further purified by more sophisticated methods such as protein dialysis membrane and equipment to get better results.

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Appendix I

Media compositions

Nutrient Agar

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

MRS Agar (oxoid)

Component	Amount (g/L)
Peptone	10.0
Lab-Lemco Powder	8.0
Yeast Extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1.0 ml
Di-potassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Tri-ammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Magnesium sulphate 4 H_2O	0.05
Agar	10.0

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

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0
Component	**Amount (g/L)**
Peptone	10.0
Sodium chloride	5.0

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

Nitrate Reduction Broth

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

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

Sugar Fermentation Broth

Component	Amount (g/L)
Sugar	5.0
Trypticase	10.0
Sodium chloride	5.0
Phenol red	A very small amount until the broth turns red

T1N1

Component	Amount (g/L)
Tryptone	1.0
Sodium chloride	1.0
Agar	0.75

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

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

Tributyrin Agar (Himedia)

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Yeast extract	3.0
Agar	15.0
Final pH	7.5±0.2
Tributyrin (FD081)	10 ml

Mueller-Hinton Agar (Himedia)

Component	Amount (g/L)
Beef, infusion	300.0
Casamino acids	17.5
Starch	1.5
Agar	17.0

Lactobacillus MRS Broth (Himedia)

Component	Amount (g/L)
Dextrose	20.0
Protease peptone	10.0
Beef extract	10.0
Yeast extract	5.00

Sodium acetate	5.00
Ammonium citrate	2.00
Dipotassium phosphate	2.00

Appendix II

Reagents

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.

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 temeperature.

Malachite green (100 ml)

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was 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)

35 % hydrogen peroxide

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)

5N acetic 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.

Ethyl Alcohol (95%)

95 ml of ethyl alcohol (100%) was added to 5 ml of distilled water. This solution was stored at room temperature.

Appendix III

Instruments

Instrument	Manufacturer
Electric Balance	Scout, SC4010 USA
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Water Bath	Daihan Scientific Companies, Korea
Table Top Centrifuge	Digisystem, Taiwan
Microscope	A. Krüssoptronic, Germany
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Microcentrifuge tubes	Tarsons Products, Pvt Ltd, Kolkata