Isolation of Probiotics, their Metabolites and Investigation of their Antibacterial Activities

A dissertation submitted to Department of Pharmacy,
East West University, in Partial fulfillment of the requirements for the
Degree of Master of Pharmacy
(M. Pharm)

Submitted by
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Dedicated To My
Parents
&
Honourable
Teachers

Declaration by the Candidate

I, Altaf Hossen (ID-2013-3-79-005), hereby declare that the dissertation entitled "Isolation of Probiotics, their Metabolites and Investigation of their Anti-bacterial Activities" submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirements for the degree of Masters Of Pharmacy. This a genuine & authentic thesis work carried out by me during Fall 2014 –Spring-2015 under the supervision and guidance of Dr. Repon Kumar Saha, Assistant Professor, Department of Pharmacy, East West University.

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Endorsement by the Chairperson

This is to certify that the dissertation, entitled “Isolation of Probiotics,their Metabolites and Investigation of their Antibacterial Activites”, is a thesis work done, by the Altaf Hossen (ID-2013-03-79-005), in partial fulfillment of the requirements for the degree of Masters of Pharmacy. We further certify that all the sources of information and laboratory facilities availed in this connection is duly acknowledged.

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Acknowledgement

All praise is for my Almighty for giving the ability to complete my M. PHRM thesis and the opportunity to study on Clinical Pharmacy and Molecular Pharmacology.

It is my pleasure and proud privilege to express my heartiest regards and gratitude to my respected teacher and supervisor Dr. Repon Kumar Saha, Department of Pharmacy, Assistant Professor, Department of Pharmacy, East West University; for his constant supervision and support, optimistic counseling and continuous backup to carry out the thesis work as well as to prepare this dissertation.

I would like to acknowledge the support of our respected professor Dr. Chowdhury Faiz Hossain, Chairperson, Department of Pharmacy, East West University.

I would also like to extend my thanks to all the research students in the Lab, Lab officers and other staffs of the Faculty of Pharmacy for their help and assistance, friendly behavior and earnest cooperation which enabled me to work in a very congenital and comfortable ambience.

Last but not the least, I would like to thank my family, and friends for their wholehearted inspiration during my thesis work.

Abstract

The present study was carried out to isolation of Probiotics Metabolites and Ant-Bacterial Activity Investigation.

Probiotics were isolated from a local yogurt by using different conformational test such as: gram staining, catalase, resistance to low pH (pH 3.0&1.0), resistance to 1% bile salt (pH 6.8), 6.5% sodium chloride test, antimicrobial test by isolated strains and metabolites, and carbohydrate fermentation test. Total 15 strains were selected from commercial yogurt according to their different size of colony. 8 strains were found as Probiotics by different conformational test from yogurt, but two strains of Lactobacillus were confirmed from six strains. All strains were gram positive, catalase negative . After catalase test, all strains were selected for isolation. Then all strains were tolerated against stomach environment (pH 3.0&1.0), survived against 6.5% sodium chloride.Metabolites of strains were isolated by 60% Ammonium sulphate saturation. There were shown antimicrobial activity of selected strains and isolated metabolites against different pathogenic bacteria and also survived in acidic and neutral environment (pH 3.0 and 6.8). All strains were survived against high concentration of bile salt (1% bile salt) during 4 hour's. 6 of them were ferment in glucose and sucrose, but 2 strains were not ferment in sucrose, only 2 strain was ferment in sorbitol . Finally I was found 6 strains of "Probiotics" and two strains of "Lactobacillus" from total strains by the help of different identical test. Morphological features of "Probiotics" were confirmed using scanning electron microscope (SEM).

In conclusion, isolation of probiotics metabolites can be considered as alternative of anti-biotics, and identification of "Lactobacillus"& probiotics can be used for human healing and animals hervasting.

Key words: Commercial yogurt, Probiotics, Lactobacillus, Metabolites, Bacteriocin, SDS-PAGE analysis.

1.1 Introduction

Yogurt is a food produced by bacterial fermentation of milk. The bacteria used to make yogurt are known as “yogurt cultures”. Fermentation of lactose by these bacteria produces lactic acid, which acts on milk protein to give yogurt its texture and its characteristics tang. Most of human commonly non-Caucasians become lactose intolerant after weaning. These lactose intolerant people can not metabolize lactose due to the lack of essential enzyme β-galactosidase. When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence, cramping and diarrhoea ensue. If lactose passes through from the small intestine, it is converted to gas and acid in the large intestine. When the yogurt is compared with milk, cause the lactose is converted to lactic acid and the yogurt consist of bacterial β-galactosidase enzyme; it is suitable end beneficial to consume by lactose intolerant.

Probiotic microorganisms are often incorporated in food in the form of yoghurt and yoghurt type fermented food. Recently, there are probiotic ice cream, cheese, infant formulas, breakfast cereals, sausages, luncheon meats, chocolate and puddings. Nondairy food also has been manufactured with the addition of the same types of microorganisms. In fact, there are also medical probiotics in the form of capsules and tablets (Hassan Pyar *et al*., 2013)

The genus Lactobacillus is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. Lactobacillus were introduced into dairy products because of the potential advantage of consuming active LAB adapted to the intestine and to produce mildly acidified yoghurts (Schillinger *et al*., 1999)

1.2 Probiotics

The word ‘probiotic’ comes from Greek language ‘pro bios’ which means ‘for life’ opposed to ‘antibiotics’ which means ‘against life’. The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. Nowadays, the term refers to viable, nonpathogenic microorganisms (bacteria or yeasts) that, when ingested, are able to reach the intestine in sufficient numbers to confer health benefits to the host (Schrezenmeir *et al*., 2001). A probiotic microorganism that will be used as a microbial supplement in animal feeding should survive and grow under rumen-like conditions, resist to gastric acidity and the presence of lisozyme, bile salts and pancreatic enzymes. These characteristics can be evaluated in vitro and can be used for strain selection (Salminen S *et al*., 1998). Duodenal acidities and the high concentration of bile in the intestine are the first factors to consider in probiotic selection. Several culture media have been developed and evaluated for the selective enumeration of probiotic Lactic Acid Bacteria in yoghurts (Hassan Pyar *et al*., 2013). A large number of studies have been carried out for the identification of Lactic Acid Bacteria, which include; gram staining, catalase test, long term preservation of isolates, resistance to low ph, tolerance against bile salt, antimicrobial test, antibiotic test, and carbohydrate fermentation. it is well known that, the major selection criteria (antibiotic resistance, resistance to low ph, tolerance against bile salt, bile salt hydrolysis and antimicrobial activity) used for the determination of probiotic properties of lactic acid bacteria isolates (Bassyouni *et al*., 2012).

1.3 Microorganisms in Probiotic Products

Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects. Also probiotics are acceptable because of being naturaly in intestinal tract of healthy human and in foods (Çakır., 2003). The probiotics which are use to feed both man and animals are shown in the Table 1.

***Lactobacillus* species**	***Bifidobacterium* species**	**Others**
L. acidophilus	*B. bifidum*	*Enterococcus faecalis*
L. rhamnosus	*B. animalis*	*Enterococcus faecium*
L. gasseri	*B. breve*	*Streptococcus salivarus* subsp. *thermophilus*
L. casei	*B. infantis*	*Lactococcus lactis* subsp. *lactis*
L. reuteri	*B. longum*	*Lactococcus lactis* subsp. *cremoris*
L. delbrueckii subsp. *bulgaricus*	*B. lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. adolascentis*	*Pediococcus acidilactici*
L. plantarum		*Saccharomyces boulardii*
L. salivarus		*Leuoconostoc mesenteroides*
L. johnsonii		
L. gallinarum		
L.plantarum		
L. fermentum		
L. helveticus		

Table -1.: Microorganisms applied in probiotic products

1.4 Mechanism of Probiotics

Lactobacillus and Bifidobacterium species are the most commonly used probiotics in foods for human consumption given the significant health benefits associated with ingestion of these micro-organisms (Stanton et al. 2003a,b).The ability to resist gastric acidity, adhere to intestinal cells, reduce pathogen adherence, be safe and non-patho-genic and to live and grow as part of a normal, healthy microbiota are characteristics that are considered to make lactobacilli successful probiotics (Saliminen et al. 1996).

1. The mechanisms by which probiotics exert their effects are largely unknown, but may involve modifying gut pH, antagonizing patho-gens through production of antimicrobial compounds, competing for pathogen binding and receptor sites as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and producing lactase.
2. These bacteria are also able to synthesize vitamins, help in digestion and absorption, inhibit growth of exogenous organisms and stimulate the immune system (Marteau et al.2002; Leahyet al.2005).
3. The therapeutic potential of probiotics appears through a number of mechanisms of action, including modulation of the immune response, competitive inhibition of invading microbiota in the gut, modification of pathogenic toxins and host products, and enhancement of epithelial barrier function (Oelschlaeger 2010; Chapman et al.2011).
4. It was postulated that Gram-positive Bacteriocins produced by lactic acid bacteria (LAB) have received great attention in the past decade owing to their potential applications as food preservatives (Cleveland et al. 2001). Production of bacteriocins and bacteriocin-like inhibitory substances (BLIS) from the Bacillusgenus has been studied by sev-eral research groups (e.g. Babasakiet al.1985; Steinet al. 2004; Shelburne et al. 2007; Sutyak et al. 2008; Sutyak Nollet al. 2011).

5. Many bacteriocins and BLIS kill sensi-tive cells by a common mechanism of action through the formation of a transient pore in the cytoplasmic membrane resulting in the leakage of small intracellular compounds and dissipation of proton motive force (PMF) component(s). Antimicrobial proteins may deplete either or both components of PMF(proton motive force) and cause intracellular ATP depletion by either efflux or intracellular ATP hydrolysis (Chen and Montville 1995, S.Riazi,S.E. Dover 2 and M.L. Chikindas2012).
6. Lactic acid bacteria play a key role in maintaining the bal-ance of normal gastro-intestinal microflora (Fuller 1989). One of the key properties of probiotic lactic acid bac-teria is the adhesion of cells to epithelial cells or intestinal mucus. This requires strong interaction between receptor molecules on epithelial cells and bacterial surfaces (Salmi-nen et al. 1996a). Adhesion of probiotic cells prevent the adhesion of pathogens (Salminenet al.1996b) and stimu-late the immune system (Isolauri et al. 1991,1995; Rolfe 2000).
7. The antibacterial activit-ies of the lactobacilli involve numerous mechanisms of action, including the production of H2O2 , metabolites, and antimicrobial substances, including bacteriocins and nonbacteriocin molecules (Servin 2004; Reidet al. 2003).
8. Bifidobacteria, obligate anaerobic rods play an important role in maintaining the healthy function of the colon in a number of ways, including production of short-chain fatty acids, pro-motion of the host immunological activity and production of digestive enzymes and vitamins (Tamura 1983). It is generally accepted that successful delivery and colonization of viable probiotic cells in the host large intestine is essential for pro-biotics to be efficacious (Conway 1996), although a few studies have indicated that non-viable probiotics have similar effects (Ouwehand 1998).

1.5 Selection Criteria for all Probiotics

In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a number of desirable properties. The desired properties of probiotic strains (Mattila *et al*., 2000).

- Acid and bile stability
- Adherence to human intestinal cells
- Ability to reduce the adhesion of pathogens to surfaces
- Colonization of human GI tract
- Antagonism against carcinogenic and pathogenic bacteria
- Production of anti-microbial substances
- Survive the various technological processes of production
- Safety evaluation: nonpathogenic, nontoxic, nonallergic, nonmutagenic
- Desirable metabolic activity and antibiotic resistance/sensitivity
- Clinically validated and documented health effects

The selection criteria are listed in Table 1 briefly.

Probiotic Strain Properties	**Remarks**
Human origin for human usage	Although the human probiotic *Saccharomyces boulardii* is not human origin, this criterion is important for species dependent health effects.
Acid and bile tolerance	Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surface	Important to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization.
Safe for food and clinical use	Identification and characterization of strains accurately, documented safety. No invasion and no degradation of intestinal mucus.
Clinically validated	Minimum effective dosage has to be known for each particular

and documented health effects	strain and in different products.
Good technological properties	Survival in products if viable organisms are required, phage resistance, strain stability, culturable in large scales, have no negative effects on product flavor.

Table-2: Selection criteria for probiotics. (Quwehand *et al.,* 1999 and Çakır, 2003)

The selection criteria can be categorized in four basic groups:

- Appropriatness
- Technological suitibility
- Competitiveness
- Performance and functionality (Klaenhammer *et al*., 1999)

Strains which have these criteria should be used in order to get effective on health and functional probiotic strains. Probiotics are chosed by using the criteria in Table 1. (Saarela *et al*., 2000) proposed the properties of probiotics in three basic groups as;

- Safety aspects
- Aspects of functionality
- Technological aspects

A potential probiotic strains does not need to fulfill all such selection criteria (Quwehand *et al*.,1999).

1.6 Bacteria in Yogurt

It may come as a surprise to some that certain kinds of bacteria, such as those found in yogurt, are actually beneficial rather than harmful. Commonly used bacterial probiotics include various species of Lactobacillus, Bifidobacterium and Streptococcus (Morrow *et al*., 2012). Different types of bacteria in yogurt commonly include:

- *Lactobacillus bulgaricus*
- *Lactobacillus acidophilus*
- *Lactobacillus casei*

- *Streptococcus thermophilus* and
- *Bifidobacteria.*

1.7 Bifidobacteria

Although "Bifidobacteria" produce lactic acid they are not lactic acid bacteria. Bifidobacterium species belong to the Actinomyces subgroup of the gram positive bacteria. Species from the genus *Bifidobacterium* are generally characterized nonspore forming, nonmotile and catalase negative. These bacteria have a rod like appearance. However the rods tend to be "clubbed". Wild strains in particular demonstrate sometimes very irregular rods with branching. Often "Y" shaped rods can be seen. They are strict anaerobes, although some species and strains may tolerate oxygen in the presence of carbon dioxide. Within the genus *Bifidobacterium* pleomorphism exists and it is described as short regular, thin cells with pointed ends, long cells with slight bends with a large variety of branching; single or in chains of many elements; in star-like clusters or disposed in "V" or palisade arrangements (Scardovi *et al*., 1986). In bifidobacteria, glucose catabolism occurs through the fructose 6- phosphate phodphoketolase pathway, which can be used as distinguishing feature of bifidobacteria. During fermentation, acetic and lactic acids are produced in molar ratios 3:2. The optimum temperature for growth is 31-41ºC within the range 25-45ºC. Bifidobacteria are less acid tolerant than lactobacilli and no growth occurs at pH values less than 4.5 (Scardovi, 1986). Nutritional requirements for growth of bifidobacteria are less complex than those of lactobacilli, but in some cases bifidobacteria do require specific factors for optimal growth (Modler *et al*., 1990). Bifidobacteria are natural habitants of GI tract and strains with probiotic properties are mainly of human origin. *Bifidobacterium* species constitute a significant portion of probiotic cultures used in functional food production.

1.8 Streptococcus Thermophilus

Streptococcus species are not typically associated with health benefits and some are highly pathogenic. However, one facultative anaerobic species, *Streptococcus thermophilus* is known to promote health, but it is not a probiotic (it does not survive the stomach) and generally is used in the production of yogurt and the manufacture of several

types of cheese, especially Italian and Swiss cheese. The organism is a modarate thermophile with an optimal growth rate at 45°C.

1.9 Lactobacillus

Lactobacillus represents a highly diverse group of Gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli (Holt G *et al.,* 1994). The growth of Lactobacillus improved under anaerobic conditions. Species within this genus is generally catalase-negative, although a few strains decompose peroxide by a non-heme-containing pseudo-catalase. They have a strictly fermentative metabolism and convert glucose solely or partly to lactic acid. They are classified as homofermentative (producing mainly lactic acid) or heterofermentative (producing carbon dioxide, ethanol, acetic acid and lactic acid). The optimum growth temperature is in the mesophilic range (30- 40ºC), but some strains can grow below 15ºC and some at temperatures up to 55ºC. Differentiation of Lactobacillus species depends on physiological criteria, carbohydrate fermentation, biochemical and molecular characterization (Petrovic *et al*., 2006). Lactobacilli play crucial role in the production of fermented foods: vegetables, meats and dairy products. Lactobacilli represent a significant part of our intestinal microflora, and their friendship with the general state of human health is under serious investigation. Lactobacilli normally predominate in the small intestine, and they are known for their beneficial effect which may antagonize potential pathogens. The lactobacilli are usually more resistant to acidic conditions than are other lactic acid bacteria. Lactobacillus is found to be living in highly acidic environments of pH 4-5 or lower, thereby altering the pH and suppressing pathogens by producing lactic acid (Salminen S *et al*., 1996). Lactobacillus is very heterogeneous genus, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. Physiological characteristics used to identify some species of Lactobacillus.

1.10 Immune System and Probiotics

The effects of immune system are promising. However, the mechanism is not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts (Mombelli *et al*, 2000).

Probiotics affect the immune system in different ways such as;

- Producing cytokines
- Stimulating macrophages
- Increasing secretory IgA concentrations (Çakır, 2003, Scheinbach, 1998 and Dugas, et al. 1999).

Fermented milk containing *Lactobacillus acidophilus* and Bifidobacteria could modulate the immune response in human. Yogurt containing lactobacilli stimulated macrophages and increased secretory IgA concentrations (Scheinbach, 1998).

1.11 Selection criteria for Lactobacillus

- The genus *Lactobacillus* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. (Schillinger U *et al*., 1999)
- *Lactobacillus* is found to be living in highly acidic environments of pH 4-5 or lower, thereby altering the pH and suppressing pathogens by producing lactic acid (Salminen S *et al*., 1996).
- Gram-positive and microscopically appear as long to short rods or even coccobacilli (Holt G *et al*, 1994).
- The lactobacilli are usually more resistant to acidic conditions than are other lactic acid bacteria. Lactobacillus are more stable in Ph 3.0 and against bile salt during 3 and 4 hours respectively (Hatice Yavuzdurmaz *et al*., 2007).
- This genus are generally catalase-negative. (Hassan Pyar *et al*, 2013).
- Lactobacilli tolerate 2%, 4% and 6.5% NaCl concentration. (Estifanos Hawaz, 2014).

- *Lactobacillus* spp. showed high resistance to bile salts (Hatice Yavuzdurmaz *et al*., 2007).
- *Lactobacillus* spp. is either homo- or heterofermentative with regard to hexose metabolism. (Petrovic *et al*., 2006).
- Lactobacillus acidophilus unable to ferment arabinose and sorbitol (Hassan Pyar *et al*, 2013).

Strains	Shape	Catalase	Gas from glucose	6.5% NaCl	Glucose	Maltose	Sucrose	Lactose
Lactobacillus oris	Bacilli	-	+	-	+	+	+	+
Lactobacillus fermentum	Bacilli	-	+	-	+	+	+	+

Ammoniam from Arginine	Xylose	Ribose	Melezitose	Arabinose	Mannitol	Trehalose
-	+	+	-	+	ND	d
+	d	+	-	d	-	d

Melibiose	Raffinose	Galactose	Salicin	Mannose	Fructose	Rhamnose
+	+	+	-	d	+	-
+	+	+	-	w	+	-

Symbols: +: 90 % or more strains are positive, -: 90 % or more are negative, d: 11-89% of strains are positive, w: weak positive reaction, ND: no data available

Table-3: Literature Information of Biochemical Test Results (Roos *et al*., 2005, and Hammes, 1995).

Some major selection criteria will be discussed in details below:

1.12 Gram Staining

The Gram staining method is one of the most important staining techniques in microbiology. It was established by the Danish bacteriologist Christian Gram in 1844. It is almost always the first test performed for the identification of bacteria. In gram's method which is based on the ability of a cell wall in retaining the crystal violet dye during solvent treatment, it is difference in the microbial cell wall that is amplified. The cell walls for gram-negative microorganisms have higher lipid content than gram-possitive cells. Sequentially stained with crystal violet, iodine then destained with alcohol and counter stained with safranin. Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer in gram-possitive bacteria. In a Gram stain test, gram-possitive bacteria retain the crystal violet dye, while a counterstain (commonly safranin or fuchsine) added after the crystal violet gives all gram-negative bacteria a red or pink coloring.

1.13 Catalase activity

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.

$$2H2O2 \rightarrow 2\ H2O + O2$$

Catalase test is performed to isolates in order to see their catalase reactions. Probiotic strains do not give gas bubbles. Lactobacillus are generally catalase-negative (Hassan Pyar *et al*., 2013).

1.14 Acid and Bile Tolerance

Bacteria used as probiotic strains are joined in the food system with a journey to the lower intestinal tract via the mouth. In this food system, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. Then the journey will be going on in the stomach and enter the upper intestinal tract which contain bile. In this stage strains should have the ability to resist the digestion processes. It is reported that time at

the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile (Chou, 199 andÇakır, 2003). To show probiotic sufficiencies, they should reach to the lower intestinal tract and maintain themselves overthere. Because of desirable point the first criteria is looking for probiotic strains is being resistant to acid and bile. Bile acids are synthesized in the liver from cholesterol and sent to the gall – bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli* subspecies, *Klebsiella* spp., and *Enterococcus* spp. in vitro. The deconjugated acid forms are more effective on gram positive bacteria (Dunne *et al.* 1999 and Çakır, 2003).
Lactobacillus acidophilus is the most used probiotic strain in the products like dairy products or capsules. We tried to isolate acid and bile resistant Lactobacillus.

1.15 Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer Kullen *et al*., 1999).

Antimicrobial effects of “Lactic acid bacteria” are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins ((Quwehand *et al.*, 2004 and Çakır, 2003).

Till today there are some researchs on showing that different species produce different antimicrobial substances. Here are some examples of these substances:

- *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance “reuterin”
- *Lactobacillus plantarum* produces a class II bacteriocin “plantaricin S”

- *Lactobacillus acidophilus* produces a class III bacteriocin "acidophilucin A" (Quwehand *et al*., 2004).
- Subspecies of *Lactococcus lactis* produce a class I bacteriocin, "nisin A"
- *Enterococcus feacalis* produces a class I bacteriocin "cytolysin"

Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time. Nisin, produced by L. lactis subsp. lactis is the well known bacteriocin and it is allowed to use in food preparations (Çakır, 2003).

1.16 Metabolites (Bacteriocin and Bacteriocin like substances)

Microorganisms live in a world of chemical signals.They use small molecular weight compounds(≤2,5000amu),known as metabolites,to regulate their own growth and developments,to encourage other organisms beneficial to them and suppress organisms that are harmful.

To control competitors, microbes produce-

- Antibiotic,e.g-penicillin,streptomycin,erythromycin etc.
- Antifungals,e.g-nystatin,amphotericin,cycloheximide etc.
- Antiprotozoal,e.g-monensin,salinomycin,trichostatins etc.

Types of metabolites-

- Primary metabolites: Growth and development,e.g-ethanol,lactic acid,citric acid,certain aminoacid(L-glutamate,L-lysine)
- Secondary metabolites: Defense mechanisms.Producing antibiotic and pigments,e.g-atrophine,erythromycin & bacteriocin(peptide).(Bio-Australis 2008)

Bacteriocins

In recent years bacterial antibiotic resistance has been considered a problem due to the extensiveuse 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 appeared and spread causing difficulties and the restricted use of antibiotics as growth promoters. So, the continue development of new classes of antimicrobial agents has become of increasing importance for medicine (Kumar and Schweiser, 2005; Fisher et al, 2005).In order to control their abusive use in food and feed products, one plausible alternative is the application of some bacterial peptides as antimicrobial substances in place of antibiotics of human application. Among them, bacteriocins produced by lactic acid bacteria have attracted increasing attention, since they are active in a nanomolar range and have no toxicity.Bacteriocins are proteins or complexed proteins biologically active with antimicrobial action against other bacteria, principally closely related species. They are produced by bacteria and are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can potentially illicit allergic reactions in humans and other medical problems (Deraz et al,2005). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activitiy and spectra of action (Saavedra et al,2004). Antibiotics are generally considered to be secondary metabolites that are inhibitory substances in small concentration, excluding the inhibition caused by metabolic by-products like ammonia, organic acids, and hydrogen peroxide. It is likely that most if not all bacteria are capable of producing a heterogeneous array of molecules in the course of their growth *in vitro* (and presumably also in their natural habitats) that may be inhibitory either to themselves or to other bacteria (Jack et al, 1995). Bacteriocin production could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. 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,2006).

Range of activity

Considering the antimicrobial spectrum, producing species, molecular weight, stability, physicalchemical properties and mode of action of bacteriocins, they form a heterogeneous group.There is the classic type, which has a spectrum of activity only against homologous species, and a second type, less common, which shows action against a wide range of Gram-positive microorganisms. One example of this second type is nisin, which is produced by certain strains of *Lactococcus lactis* subsp. *lactis* (De Vuyst, 1994;Rodriguez, 1996; Moreno et al, 2000). Other is pediocin, produced by *Pedicoccus pentosaceus*(Moreno et al, 2006). Nisin, produced by *L. lactis* subsp. *lactis,* is active against Gram-negative bacteria, but only when used at high concentrations or when the target cells have been pre-treated with EDTA (Stevens et al, 1991).Bacteriocins are not frequently active against Gram-negative bacteria. The outer membrane of this class of bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents and dyes from reaching the cytoplasmic membrane (Stevens et al, 1991). However, some studies have already reported bacteriocin activity against this group of bacteria. Examples are plantaricin 35d, produced by *Lactobacillus plantarum* and active against *Aeromonas hydrophila* (Messi et al, 2001); bacteriocin ST151BR, produced by *Lactobacillus pentosus* ST151BR (Torodov and Dicks, 2004) and a bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* active against*Escherichia coli* (Caridi, 2002); thermophylin, produced by *Streptococcus thermophilus* active against *E. coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* among the Gram-negative species and against several *Bacillus* species, *Listeria monocytogenes* and *Salmonella typhimurium* among the Gram-positives (Ivanova et al, 1998). Bacteriocins ST28MS and ST26MS, produced by *Lactobacillus plantarum* isolated from molasses (Torodov and Dicks, 2005) inhibited the growth of *Escherichia coli* and *Acinetobacter baumanii* along with some Grampositive bacteria. Lade et al (2006) have isolated two *Lactobacillus* species (*L. plantarum* and *L. lactis*) from vegetable waste that produced a bacteriocin which inhibitted the growth of *E. coli*.

Classification of Bacteriocins

There is a wide number of bacteriocins produced by different LAB (table 1), and they can be classified according to their biochemical and genetic characteristics (Klaenhammer et al, 1994; González-Martínez et al, 2003).

- *Class I.* – Lantibiotics: small (< 5 kDa) heat-stable peptides acting on membrane structures; they are extensively modified after translation, resulting in the formation of characteristic thioether aminoacids lanthionine and methyllanthionine. These arise via a two-step process, originated from posttransdutional modifications: firstly, gene-encoded serine and threonine are subjected to enzymatic dehydration to give rise to dehydroalanine and dehydrobutyrine, respectively (Sahl and Bierbaum, 1998). A very well known example of this group is nisin (Broadbent et al, 1989). The lantibiotic bacteriocins were initially divided into two subclasses based on structural similarities. *Subclass Ia* included relatively elongated, flexible and positively charged peptides; they generally act by forming pores in the cytoplasmatic membranes of sensitive target species. The prototypic lantibiotic nisin is a member of this group. *Subclass Ib* peptides are characteristically globular, more rigid in structure and are either negatively charged or have no net charge. They exert their action by interfering with essential enzymatic reactions of sensitive bacteria (Deegan et al, 2006).
- *Class II.* – Non-Lantibiotics: bacteriocins of variable molecular weight, but usually small (<10 kDa), heat-stable, containing regular amino-acids. This group was divided into three subgroups:*Class IIa*: peptides active against *Listeria*, the characteristic representants are pediocin PA-1(Venema et al, 1997) and sakacin P. *Class IIb*: formed by a complex of two distinct peptides. These peptides have little or no activity and it appears to be no sequence similarities between complementary peptides. In this group are lactococcin G and plantaricins EF e JK.*Class IIc*: Small peptides, heat-stable, which are transported by leader-peptides. In this subclass are found only the bacteriocins divergicin A and acidocin B.

- *Class III.* – Big peptides, with molecular weight over 30 kDa. In this class are helveticins J (Joerger and Klaenhammer, 1986) and V (Vaugham et al,1992), acidofilicin A and lactacins A and B. Most of the low molecular weight bacteriocins are highly cationic at pH 7.0, and this seems to be a unifying feature of both the lantibiotics and nonlantibiotics (Cintas et al, 2001). Lantibiotics are the most studied and explored industrially. Nisin, a lantibiotic usually produced by *Lactococcus lactis* subsp. *lactis* is used as an additive in foods. All of the variants of nisin are active against Gram-positive bacteria, like *Listeria* sp, *Micrococcus* sp and also on sporulating bacteria, like *Bacillus* sp and *Clostridium* .*SP* (Gonzalo-Martinez et al,2003).

Stability

Some studies of characterization of bacteriocins show that these molecules can be active under certain ranges of temperature and pH. Sensibility to proteolytic enzymes evidences the proteinaceous characteristic of bacteriocins (De Martins et al, 2003). Complete inactivation or significant reduction in antimicrobial activity of the bacteriocins ST28MS and ST26MS produced by *Lactobacillus plantarum* isolated from molasses was observed after treatment with proteinase K, pronase, pepsin and trypsin. These bacteriocins remained stable after incubation for 2h at pH values between 2.0 and 12.0. No decrease in antibacterial activity was recorded after 90 min at 100ºC or 20 min at 121º C (Torodov and Dicks, 2005). The thermotolerance feature might be related to the molecular structure of the bacteriocin, usually composed by small peptides without tertiary structure (Toro, 2005).

Bacteriocin-like Substances

Lactic acid bacteria are capable of producing other substances, known as bacteriocin-like substances (BLS). An example of this class of molecule is reuterin, produced by some strains of *Lactobacillus reuteri* during anaerobic fermentation of glycerol (Rodríguez et al, 2003; Pancheniak et al, 2006). It is water-soluble, active over a wide range of pH values and resistant to proteolytic and lipolytic enzymes, being a suitable compound for food biopreservation. *Lactobacillus plantarum* TF711 isolated from raw Tenerife goat's

cheese dy Hernández et al (2005) produced a bacteriocin-like substance with a molecular weight of 2,5 kDa, which was called plantaricin TF711. It was shown to be active against the Gram-positive bacteria *Bacillus cereus, Clostridium sporogenes* and *Staphylococcus aureus*, as well as against the Enterobacteriaceae *Shigella sonnei* and *Klebsiella pneumoniae*. It was stable to heat and to treatment with surfactants andorganic solvents. Highest antimicrobial activity was found between pH 1 and 9. *Carnobacterium pscicicola* L103 isolated from vacuum packed meat has shown the ability to produce a bacteriocin-like substance, which caused inhibition of six strains of *Listeria monocytogenes* isolated from salmon and from human origin (Schöbitz et al, 2003). No significant growth of the pathogen was observed at 4 ± 2º C during 15 days, when compared to the salmon inoculated only with *L. monocytogenes* (Schöbitz et al, 2006). Three strains of *Lactobacillus* (*L. plantarum, L. fermentum* and *L. acidophilus*) isolated from Turkish dairy products showed inhibitory activity against *Staphylococcus aureus, Escherichia coli* and *Yersinia enterocolitica* due to bacteriocin-like substances. They were resistant to heat; the inhibitory activity of bacteriocin-like substances was not lost after 10 and 20 min at 100º C (Aslim et al, 2005). The antagonistic activity of *Lactobacillus sake*148 is due to the production of sakacin M, a peptide of appox. 4,6 kDa, purified by lyophilization and gel filtration. The partially purified antagonistic activity of *L. sake* 148 was destroyed by treatment with proteolytic enzymes. However, it was resistant to heat, having D-values at 121, 135 and 150° C of 23.8, 17.4 and 15.2 min, respectively.

Molecular weight of Bacteriocin:

Class	Bacteriocin	Molecular weight	Species
1	Niasin	3,5 kDa	*Lactococcus lactis subsp.lactis*
1	Lacticin3147	4,2 kDa	*Lactococcus lactis subsp.lactis*
2	Lactococcin B	5 kDa	*Lactococcus lactis subsp.cremories*
2	Acidocin CH5	H M W	*Lactobacillus*

			acidophilus
2	Lactacin F	6,3kDa	*Lactobacillus acidophilus*
2	Lactobin A	4,8 kDa	*Lactobacillus amylovorous*
2	Lactocin 705	3,4 kDa	*Lactobacillus casei*
2	Leucocin A	3,9 kDa	*Leuconostoc glidum*
2	Mesentericin Y105	3,8kDa	*Leuconostoc mesenteroides*
2	Pediocin F	4,5 kDa	*Pediococcus acidilactica*
2	Pediocin PA-1	4,6 kDa	*Pediococcus acidilactica*
2	Pediocin AcH	4,6kDa	*Pediococcus acidilactica*
2	Pediocin A	2,7 kDa	*Pediococcus pentosaceous*
2	Enterocin A	4,8 kDa	*Enterococcus facium*
2	Lactocin S	3,7 kDa	*Lactobacillus sake*
2	Sakacin P	4,4 kDa	*Lactobacillus sake*
2	Curvacin A	4,3 kDa	*Lactobacillus curvatus*
3	Helveticin J	37 kDa	*Lactobacillus helvaticus*
3	Lactacin B	6,3 kDa	*Lactobacillus acidophylus*

Table-4: Molecular weight of Lactic acid bacteria [Parada,J.et al-2007]

1.17 Charbohydrate Fermentation

Lactic acid bacteria utilize sugars (e.g., glucose) to form lactic acid by either the homo- or heterofermentative pathway.

The homofermentative pathway, results in the transformation of glucose to pyruvate through the Embden–Meyerhof–Parnas pathway (EMP, or glycolysis), eventually yielding lactic acid. NADH produced by the oxidation of glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate is reoxidized to NAD+ in the formation of lactate from pyruvate through the action of lactate dehydrogenases (LDHs). The LDH enzymes vary in their stereospecifi city and can yield d- or l lactic acid or the racemic mixture (dl). Though 1 mole of glucose should produce 2 moles of lactic acid, the actual yield is closer to 1.8 moles of lactic acid. Energetically, glycolysis yields 2 moles ATP per mole glucose.

The heterofermentative pathway, from 1 mole of glucose heterofermentative bacteria produce 1 mole each of lactate, CO2, and either acetic acid or ethanol. In reality, these bacteria produce 0.8 mole lactate from glucose. Unlike homofermentative microorganisms, these bacteria do not have aldolase but possess phosphoketolase, the enzyme responsible for the cleavage of xylulose-5-phosphate to form glyceraldehyde-3-phosphate and acetyl phosphate. Due to the biosynthesis of five-carbon sugars in this pathway (ribulose-5-phosphate and xylulose- 5-phosphate), some strains can utilize the pentoses present in wine such as ribose, xylose, and arabinose. An important consequence of only half of the carbon from glucose going to glyceraldehyde-3-phosphate is formation of only 1 mole of ATP per mole glucose. However, heterofermentative bacteria can gain additional energy though conversion of acetyl-phosphate to acetate.

1.18 The Effect of Different NaCl Concentration

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics, though this has been debated. Probiotic Lactic acid Bacteria act by the production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to gram-positive and gram-negative bacteria. It is well known that, different probiotic bacteria produces different class of bacteriocin (Roos *et al*. 2005 and Hammes, 1995). There are some effects of different Sodium chloride cocentration, such as;

- Sodium chloride (NaCl) reduces the production of bacteriocin.
- Both cell growth and bacteriocin activity are affected by changes in the salt concentration.
- Sodium chloride clearly slowed down the growth of bacteria. low salt concentration (2%, wt/vol) decreased bacteriocin production, while growth is unaffected at this concentration.
- Additionally, when 6% (wt/vol) sodium chloride adds, the minimum biomass concentration necessary to start the production of bacteriocin.
- In 2% and 4% NaCl concentration, more strains of bacillus and coccus (LAB) are positive (Estifanos Hawaz, 2014).
- In 6.5% NaCl concentration, Bacillus (LAB) are positive or negative, but more strains of coccus are negative.

1.19 SDS-PAGE Analysis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. It is almost obligatory to assess the purity of a protein through an electrophoretic method. The technique is also a powerful tool for estimating the molecular wt of protein (weber, *et al.* 1971 & Chambach, & rodbard, D., 1971). Proteins are globular in secondary & tertiary structure deu to disulfide bonds, hydrophobic integrations & hydrophilic interactions with their aqueous environment. Therefore, something must be done to break the secondary & tertiary structure of the proteins in the sample for accurate analysis of peptide size to occur. SDS is a detergent possessing both a hydrophobic end (the dodecyl group) & a hydrophilic end (the sulfate group). The tertiary structure of most proteins often relies upon hydrophilic interaction ar the core of the protein. The hydrophobic end of SDS breaks these interactions through interactions with the hydrophobic side chains of the amino acids. Similarly, a sulfate group can disrupt hydrogen bonding in secondary protein structure. Disulfide bonds holding tertiary or quaternary structure can be broken by using a reducing agent, such as beta-mercaptoenthanol (BME) or dithiothreitol (DTT).

Finally, heating the protein sample also aids the denaturation & unfolding process allowing chemicals like SDS & to interact with the protein.

In addition to denaturing the protein, SDS also serves an addition purpose. Because each protein is coated with SDS molecular & the sulfate group has a negative charge, SDS also serves to give each protein molecule a net negative charge. This means that when an electrical field is applied to the gel in buffer, each protein molecule will move toward the positive electrode. This allows the acrylamide to separate the proteins based on size. You can think of polyacrylamide as a synthetic version of agarose. As the SDS-coated protein molecules move through the gel matrix, smaller molecular wt proteins are able to navigate through the pores in the matrix more quickly than larger ones. Thus, the proteins in the samples are separated by size & relative molecular weight.

2.1 Preparation of Samples

Sample collection

Yogurt was collected from different brand sources such as; Arong sour yoghurt,Rosmela sour and sweet yoghurt, Rajdani sweet yoghurt and Eglo sweet yoghurt. These were immediately transnsfered to the microbial laboratory to apply microbiological examinations for the different values of collected yogurt.

Sample processing and inoculation in culture media

The yogurt samples were filtered by the filter paper and diluted appropriately with 1.0 ml sterile normal saline in each epppendorf tube and then shake using vortex shaker for one to two minutes for appropriate dilution. 100 µl of each samples were taken by the micropipette from each tube, then incorporate into Petri-dish. Samples are spread out into Petri-dish. Then nutrient agar media is to be given in each Petri-dish and kept in incubator for 24-48 hour.

Examination of culture media

Cultures in solid media were visually inspected for growth rate and colony characteristics.

Colony selection

Several types of colony were seen in the Petri-dish. From those 15 colonies were selected by according to their size and shape.

Source	Year	Colony count	Colony code	Colony characteristics	
				Size	Shape
Arong sour	2015	4 types	ATY-1	Small	round
			ATY-2	medium	round
			ATY-3	medium	round
			ATY-4	Large	round
Rosmela sour	2015	4 types	RTY-1	medium	round
			RTY-2	large	round
			RTY-3	large	round
			RTY-4	medium	round
Eglo sweet	2015	3 types	EMY-1	small	round
			EMY-2	medium	round
			EMY-3	large	round
Rosmela sweet	2015	3 types	RMY-1	small	round
			RMY-2	small	round
			RMY-3	medium	round
Rajdani sweet	2015	1 types	DMY	large	round

Table-5: Colony morphology of bacteria.

2.2 Identification and Isolation of Microorganisms

2.2.1 Gram staining

Gram staining is one of the best methods to differentiate between gram positive and gram negative bacteria. Staining techniques are useful tols in biological science. Gram-positive bacteria have a thick mesh like cell wall mde of peptidoglycan (50-90% of cell wall), which strain purple and Gram negative bacteria have a thin layer (10% of cell wall), which strain pink.

Materials

The following materials (equipment and reagent) were used during the test of Gram staining.

A. Equipment

- Bunsen burners
- Slide
- Cotton
- Water bottle
- Cloth pain
- Heat plate
- Microscope

B. Reagent

- Crystal violet
- Methyl blue
- Ethanol, 95%
- Acetone
- Ammonium oxalate
- Iodine
- Potassium iodide
- Sodium bicarbonate
- Safranin O

Procedure

Gram crystal violet solution

Dissolve 20 gm of crystal violet in 100ml of ethanol to make a crystal violet stock solution. Similarly, dissolve 1 g of ammonium oxalate in 100ml of water to make an oxalate solution. A mix 0.03 gm of KOH in 30 ml of water, this is solution B. mixing solutions A and B yields the working solution

Gram iodine solution

Dissolve 1 gm of iodine, 2gm of potassium iodide, and 3 gm of sodium bicarbonate in 300 ml of water.

Gram decolorization solution

Mix equal volume of 95 % of ethanol and acetone.

Gram safranin solution

Dissolves 2.5 gm of safranin O in 100 ml of 95% ethanol to make a stock solution. Working solution is obtained by dilution one part of the stock solution with five parts of water.

Staining procedure

- Transfer the culture to be taken from Petri-dish or a slant culture tube with an inoculation loop on a slide, then irst added a drop of few loopful water on the slide and aseptically transfer a minute amount of colony from the Petri dish. Only a very small amount of culture is needed.
- Spread the culture with an inoculation loop to an even thin film over a circle 1.5 cm in a diameter, approximately the size of a dime.
- Hold the slide with a cloth pin. Air-dry the culture and fix it an over a gentle flame, while moving a slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear.
- Add about 1-2 drop of crystal violet stain over the fixed culture. Let stand for 60 seconds. Pour of the strain and gently rinse the excess stain with a stream of water from faucet or a plastic water bottle.
- Add about 1-2 drops of iodine solution on the smear, enough to cover the fixed culture. Let stand for 30 seconds. Pour of iodine solution and rinse the slide with running water. Shake of he excess water from surface.
- Add a few drops of decolorizer (95 %) so the solution trickles down the slide. Rinse it off with after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide.
- Counter strain with 1-2 drops of the safranin solution for 45 seconds. Wash of the red safranin solution with water.
- The slide may shake to remove most of the water and air-dried. After that, examine the finished slide under a microscope (Gregersen *et al*., 1978)

2.2.2 Catalase test

Principle

Catalage is an enzyme which produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites H2O2. The catalase test is used detect the presence of the enzyme catalase in bacteria. Catalase is produced by certain bacteria, which acts as a catalyst I breakdown of hydrogen peroxide into water and oxygen. This reaction is evident the rapid formation of bubbles (Clark H *et al*., 1952)

Requirements

- Glass rods
- Test tube
- 3% hydrogen peroxide
- Test organisms
- Control organisms

Procedure

- Take 2-3 ml of 3% hydrogen peroxide in test tube
- Take a colony of test organism with sterile wooden or glass rod and immerse it into hydrogen peroxide solution.
- Observe for generation of bubbles. This indicates oxygen production

2.3 Identification and Isolation of Probiotic Properties

2.3.1 Resistance to low pH (pH 3.0 &pH1.0)

Principle of the procedure

Bacteria used as probiotic strains are joined in the food system with a journey to the lower intestinal tract via the mouth. In this food system, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. Then the journey will be going on in the stomach and stay time 3 h, then enter the upper intestinal tract. For entering into the upper intestine probiotic bacteria must survive agains stomach pH (pH of the stomach is 1.5-3.0) (Chou, 199 and Çakır, 2003).

Materials

The following material (equipment and reagent) were used during the test of resistance to low pH.

A. Equipment

- Conical flask
- Falcon tube
- Centrifuge machine
- Water bottle
- Eppendorf tube
- Micropipette
- Tubes
- Petri-dish

B. Reagents

- Sodium dihydrogen phosphate (NaH2PO4)
- Disodium hydrogen phosphate (Na2HPO4)
- Nureint agar media

Preparation of Phosphate Buffer Saline (PBS)

2.40 gm sodium dihydrogen phosphate (NaH2PO4) was taken in a conical flask, and then dissolved in up to 100 ml distilled water. 2.84 gm disodium hydrogen phosphate (Na2HPO4) was also taken in another conical flask, and then dissolved in up to 100 ml distilled water. Then mixed with them and found pH 7.1. Hydrochloric acid was used for adjustment of pH 3.0 & 1.0.

Test procedure

Overnight fresh bacterial subculture with nutrient broth was taken first. Then centrifuged was done at 2000 rpm within 30 minutes at 4°C temperature for separating pellets from supernatant. Then, discarded supernatant by using sterile tubes in aseptic area. Then, pellets were re centrifuged with 10 ml of PBS (pH 7.1) at 2000 rpm within 30 minutes at 4°C temperature. Then, discarded PBS by using sterile tubes in aseptic area. 1 ml PBS (pH 3.0 & 1.0) was added to each strain in each

eppendorf tube and diluted, then incubated at 37°C for 1 h, 2h and 3h. Before incubated, 100 µl each bacteria mixed (pellets and pH 3.0& 1.0 PBS) were taken for 4 times serial dilution, and 100 µl diluted bacteria were taken and spread into the agar media, then incubated at 37°C for 24 h. after 1 h incubation 100 µl taken of each bacteria for 4 times serial dilution, and 100 µl diluted bacteria were taken and spread into the agar media, then incubated at 37°C for 24 h. For 2 and 3 h procedure was repeated. After 24 h incubation, 3 h stayed in stomach isolates were transferred to the bile salt tolerance test. Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test.

2.3.2 Resistance to 1% bile salt

Principle of the procedure

When bacteria survived against stomach pH, then enter the upper intestinal tract which contain bile and the staying time of bacteria in small intestine is suggested to be 4 h (Prasad *et al*. 1998). The experiment was applied at this concentration of bile for 4 h.

Materials

The following material (equipment and reagent) were used during the test of resistance to low pH.

A. Equipment

- Conical flask
- Water bottle
- Eppendorf tube
- Micropipette
- Tubes
- Petri-dish
- Bunsen burners
- Inoculation loop
- Laminar air-flow unit

B. Reagents

- Bile salt
- Nutrient agar media

Preparation of 1% (w/v) bile salt solution

1 gm bile salt was taken in a conical flask, and then dissolved in up to 100 ml distilled water and produced 1% bile salt solution (w/v) , pH were found 6.8

Test procedure

1 ml bile salt solution (1% w/v) for each isolates was taken into the sterilized eppendorf tube in aseptic area, and then mixed with one loop of bacteria isolates (isolates were found after survived against low pH) and shacked for appropriate mixing. Then incubate at 37°C for 2 and 4 hour's. Before incubation, 100 µl each bacteria mixed (bacteria and bile salt) were taken for 4 times serial dilution, and 100 µl diluted bacteria were taken and spread into the agar media, then incubated at 37°C for 24 h. For 2 and 4 h procedure was repeated. After 24 h incubation, 3 h stayed in stomach isolates were transferred to the antimicrobial test. Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test.

2.4 Antimicrobial activity

2.4.1Antimicrobial activity of isolates from subculture against pathogenic bacteria

This test was done for the determination of antimicrobial activity of isolates against different pathogenic bacteria from subcultured. Pathogenic bacteria are: *Bacillus sereus, Bacillus subtilis, Salmonella paratyphi, Vibrio parahemolyticus, Staphylocuccos aureas, E.colli, Shigella dysentriae, Sarcina lutea, Candida albicans* and *Aspergillus niger*.

Apparatus & reagent

The following materials (reagent & equipments) were used during the antimicrobial investigation.

- Filter paper discs (5mm in diameter)
- Petri dishes
- Test tubes
- Sterile forceps
- Inculcating loop
- Bunsen burners
- Micropipette (2-20µl, 100-1000 µl)
- Laminar air-flow unit
- Autoclave
- Incubator
- Nutrient agar media
- Ethanol

Sterilization procedure

Petri dishes & other glass wars were sterilized by autoclaving at a temperature of 121^0c & a pressure of 15Ib/sq inch for 15 minutes. The blank discs were kept in covered Petri dishes & then subjected to dry sterilization for 1hr at 150^0c.

After completion of sterilization, both the autoclave glass & discs were kept in a laminar hood for 30 minutes. UV light was switched on before working in laminar hood to avoid any accidental combination.

Culture media

Nutrient agar media are used for the growth of microorganism. The main requirements for growth of bacteria are as follows:

1. Source of energy such as carbohydrate, protein & nucleic acid
2. Essential trace element such as Mg, Mn, Fe, & Co
3. Optimum pH of media &
4. Option temperature for incubation

Table-6: Composition of Nutrient agar media

Ingredient	Amount
Bactopeptone	0.5g
NaCl	0.5g
Bacto yeast extract	1.0g
Bacto agar	2.0g
Distilled water	100ml
pH	7.2 at 25^0c

Preparation of medium

Nutrient agar media was accurately wt & then reconstituted with distilled water in a screw cap bottles according to specification (2.8% w/v) instantly. Later bottles containing media was then sterilized in an autoclave machine at a temperature of 121^0c & a pressure of 15Ibs/sq inchi for 15 minutes.

Preparation of sample

A test organism was transferred from subculture (this subculture was done in nutrient agar media) to a falcon tube containing 10 ml nutrient broth with the help of an inculcation loop under laminar air flow unit and incubated at 37^0c for 12-18 hrs for the preparation of sample.

Disc preparation

After preparation of sample disc preparation were done. To test the antimicrobial activity, prepared standard discs (isolates) as the concentration of 20µl of each sample.

Placement of disc, diffusion & incubation

The standard antibiotic discs were placed gently on the solidifier agar plates where contains the organism with the help of a sterile forced to ensure complete contact with medium surface. The arrangement of the disc was such that the disc was no closer than 15mm to the plate to prevent overlapping of the zone of inhibiting. This was

sufficient time for the material to diffuse to a considerable area of the medium. Finally, the plates were incubated at 37^0c for 24 hrs.

2.4.2 Metabolites isolation and antibacterial activity against pathogenic bacteria

Metabolites Isolation & Partial purification

The selected 8 strains was propagated in MRS broth (pH=6.8) and was incubated at 37C for 48 hours anaerobically for extraction of bacteriocin , a culture supernatant were obtained by centrifugation (8,000 rpm for 20 min at 4oC). The cell free supernatant was precipitated with ammonium sulphate (60% saturation). The mixture was rotated for 2 hrs at 4oC and later centrifuged at 8,000 rpm for 20 min. The precipitates were resuspended in 0.5 ml of 0.2M potassium phosphate buffer (pH=7.1) for each strains respectively.

Apparatus and Reagents

1. Felcon tube
2. Innoculating loop
3. Bunsen Burner
4. Nutrient Broth Media
5. Autoclave machine
6. Leminer air flow bench
7. Bacterial strains
8. Incubator
9. Ethanol
10. Phosphet Buffer(pH-7.1)
11. Ammonium Sulphet pellet
12. Weighing Machine
13. Centrifuge Machine
14. pH meter

Antibacterial activity of metabolites

This test was done for the determination of antimicrobial activity of isolated metabolites against different pathogenic bacteria from subculture. Pathogenic bacteria are: *Bacillus sereus*, *Bacillus subtilis*, *Salmonella paratyphi*, *Vibrio parahemolyticus*, *Staphylocuccos aureas*, *E.colli*, *Shigella dysentriae*, *Sarcina lutea*, *Candida albicans* and *Aspergillus niger*.

Apparatus & reagent

The following materials (reagent & equipments) were used during the antimicrobial investigation.

- Filter paper discs (5mm in diameter)
- Petri dishes
- Test tubes
- Sterile forceps
- Inculcating loop
- Bunsen burners
- Micropipette (2-20µl, 100-1000 µl)
- Laminar air-flow unit
- Autoclave
- Incubator
- Nutrient agar media
- Ethanol

Sterilization procedure

Petri dishes & other glass wars were sterilized by autoclaving at a temperature of 121^0c & a pressure of 15Ib/sq inch for 15 minutes. The blank discs were kept in covered Petri dishes & then subjected to dry sterilization for 1hr at 150^0c.

After completion of sterilization, both the autoclave glass & discs were kept in a laminar hood for 30 minutes. UV light was switched on before working in laminar hood to avoid any accidental combination.

Culture media

Nutrient agar media are used for the growth of microorganism. The main requirements for growth of bacteria are as follows:

5. Source of energy such as carbohydrate, protein & nucleic acid
6. Essential trace element such as Mg, Mn, Fe, & Co
7. Optimum pH of media &
8. Optimum temperature for incubation

Table-7: Composition of Nutrient agar media

Ingredient	Amount
Bactopeptone	0.5g
NaCl	0.5g
Bacto yeast extract	1.0g
Bacto agar	2.0g
Distilled water	100ml
pH	7.2 at 25^0c

Preparation of medium

Nutrient agar media was accurately wt & then reconstituted with distilled water in a screw cap bottles according to specification (2.8% w/v) instantly. Later bottles containing media was then sterilized in an autoclave machine at a temperature of 121^0c & a pressure of 15Ibs/sq inchi for 15 minutes.

Preparation of sample

Isolated metabolites were resunpended by adding 0.5 ml phosphate buffer (Ph-7.1) and shaking vigorouslly for dissolve of metabolites.For each disc 20µl sample were applied.In Agar plates 500µl pathogenic subculture were spred.

Disc preparation

After preparation of sample disc preparation were done. To test the antimicrobial activity, prepared standard discs (isolates) as the concentration of 20µl of each sample.

Placement of disc, diffusion & incubation

The prepared metabolites discs were placed gently on the solidifier agar plates where contains the pathogenic organism with the help of a sterile forcep to ensure complete contact with medium surface. The arrangement of the disc was such that the disc was no closer than 15mm to the plate to prevent overlapping of the zone of inhibiting. This was sufficient time for the material to diffuse to a considerable area of the medium. Finally, the plates were incubated at 37^0c for 24 hrs.

2.5 Physiological and Biochemical Characterization

2.5.1 Carbohydrate Fermentation Test

Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate. Common end products of carbohydrate fermentation include Lactic acid, Ethyl alcohol and Carbon-dioxide by the probiotics. But different Probiotics ferment in different carbohydrate (Roos *et al*., 2005, and Hammes, 1995). Fermentation reactions are detected by the color change of a pH indicator when acid products are formed. The pH changes when only excess acid is produced as a result of carbohydrate fermentation. Three characteristic reactions can be observed from the fermentation of specific carbohydrate. Based on these reactions, bacteria are classified as:

- Fermentation with acid production only
- Fermentation with gas production only
- Nonfermenter

There is different carbohydrate fermentation test was done by the use of different carbohydrates, such as:

- Glucose
- Sucrose
- Maltose
- Lactose
- Sorbitol

2.5.2 Glucose fermentation test

Principle

Glucose is a monosaccharide that is diretly into the blood stream during digestion. It is a important carbohydrate in biology. Probiotics utilize glucose to form lactic acid by either the homo- or heterofermentative pathway. The homofermentative pathway, results in the transformation of glucose to pyruvate through the glycolysis, eventually yielding lactic acid. The heterofermentative pathway, produced lactate, CO2, and either acetic acid or ethanol (Gottshalk, 1986). The pH and color changes when only excess acid is produced as a result of glucose fermentation.

Apparatus and reagent

The following material (equipment and reagent) were used during the test of glucose fermentation.

- Water bottle
- Screw cap tube
- Micropipette
- Tubes
- Bunsen burners
- Inoculation loop
- Laminar air-flow unit
- Nutrient broth media
- Glucose
- Methyl red pH indicator

Preparation of the medium

2.5 gm nutrient broth, 1.0 gm “glucose” and 0.018gm methyl red pH indicator were taken in a water bottle, and then dissolved in up to 100 ml distilled water and prepared medium and pH were taken by the pH meter.

Sterilization procedure

Media, tubes & screw cap tubes were sterilized by autoclaving at a temperature of 121^0c & a pressure of 15Ib/sq inch for 15 minutes. After completion of sterilization,

Media, tubes & screw cap tubes were kept in a laminar hood. UV light was switched on before working in laminar hood to avoid any accidental combination.

When inoculation loop was used, that was burned by the help of Bunsen burners for every used.

Test procedure

Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test. After sterilization, 5 ml medium was taken in each falcon tube for each bacterial strain in aseptic area, then medium mixed with one loop of bacteria and incubated at 37°C for 24 h. after 24 h observation were done. Negative control and positive control was used for appropriate decision.

2.5.3 Sucrose fermentation test

Sucrose named table sugar or sugar. The molecule is a disaccharide combination of the monosaccharides glucose and fructose. In sucrose fermentation test, produced acid by probiotics. 90 % or more strains of probiotics are positive in sucrose fermentation test (Kavitha et al.,2013). 90 % or more strains of Lactobacilli are positive in sucrose fermentation test (Roos *et al*., 2005, and Hammes, 1995). The pH and color change when only acid is produced as a result of sucrose fermentation.

Apparatus and reagent

The following material (equipment and reagent) were used during the test of glucose fermentation.

- Water bottle
- Screw cap tube
- Micropipette
- Bunsen burners
- Inoculation loop
- Laminar air-flow unit
- Nutrient broth media
- Sucrose

- Methyl red pH indicator

Preparation of the medium

2.5 gm nutrient broth, 1.0 gm “sucrose” and 0.018gm methyl red pH indicator were taken in a water bottle, and then dissolved in up to 100 ml distilled water and prepared medium and pH were taken by the pH meter.

Test procedure

Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test. After sterilization, 5 ml medium was taken in each falcon tube for each bacterial strain in aseptic area, then medium mixed with one loop of bacteria and incubated at 37°C for 24 h. after 24 h observation were done.

2.5.4 Sorbitol fermentation test

Sorbitol is a sugar alcohol with a sweet test which the human body metabolizes slowly. It can be obtained by reduction of glucose, changing the aldehyde group to a hydroxyl group. *Lactobacillus acidophilus* is unable to ferment arabinose and sorbitol (Hassan Pyar *et al*, 2013). The pH and color change when only acid is produced as a result of sucrose fermentation.

Apparatus and reagent

The following material (equipment and reagent) were used during the test of glucose fermentation.

- Water bottle
- Screw cap tube
- Micropipette
- Tubes
- Bunsen burners
- Inoculation loop
- Laminar air-flow unit
- Nutrient broth media
- Sorbitol

- Methyl red pH indicator

Preparation of the medium

2.5 gm nutrient broth, 1.0 gm “sorbitol” and 0.018gm methyl red pH indicator were taken in a water bottle, and then dissolved in up to 100 ml distilled water and prepared medium and pH were taken by the pH meter.

Test procedure

Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test. After sterilization, 5 ml medium was taken in each falcon tube for each bacterial strain in aseptic area, then medium mixed with one loop of bacteria and incubated at 37°C for 24 h. after 24 h observation were done.

2.6 Tolerance Against 6.5% Sodium Chloride

Principle of the procedure

For the physiological and biochemical characterization of bacteria 6.5% sodium chloride tolerance test were done. Both cell growth and bacteriocin activity are affected by different NaCl concentration, but high concentration (6.5%) was selected in this test.

Apparatus and reagent

The following material (equipment and reagent) were used during the test of carbohydrate fermentation.

- Water bottle
- Screw cap tube
- Micropipette
- Tubes
- Bunsen burners
- Inoculation loop
- Laminar air-flow unit
- Nutrient broth media

- Carbohydrate
- Methyl red pH indicator

Preparation of the medium

2.5 gm nutrient broth, 6.0 gm NaCl and 0.018gm methyl red pH indicator were taken in a water bottle, and then dissolved in up to 100 ml distilled water and prepared medium and pH were taken by the pH meter.

Test procedure

Sterile technique (sterilized in an autoclave machine at a temperature of 121°C and pressure of 151bs/sq inch for 15 minutes) was done in this test. After sterilization, 5 ml medium was taken in each falcon tube for each bacterial strain in aseptic area, then medium mixed with one loop of bacteria and incubated at 37°C for 24 h. after 24 h observation done.

2.7 Protein Isolation of Probiotics

2.7.1 Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Electrophoresis was carried out in the discontinues sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (1970) susing 12% (w/v) separation gel & 5% (w/v) stacking gel. Polyacrylamide gels are prepared by the free radical polymerization of acrylamide & the cross linking agent NN methylene bis acrylamide. A 12% running gel (1.5 M Tris-HCL pH 8.8 10% acryl amide + bisacrylamide (Merk), 5% stacking gel (0.5 M Tris-HCL pH 6.9 10% SDS 30% acryl bis) were prepared & polymerized, chemically running gel solution contain 20 µl to the stacking gel solution. A 0.3 g in 3 ml ammonium persulphate (Sigma) was added to both solutions. The technique is a powerful tool for estimating the molecular weights of proteins. Molecular weights of whole cell proteins are analyzed by SDS-PAGE by using a high molecular weight standard marker (Bio-Red Marker).

Materials

Preparation of 10% SDS (pH- 6.6)

- SDS – 10g
- dH_2O -100ml
- Heated to 68^0C for solubility

Reagent for 4X Lower Gel Buffer (1.5 M Tris-Cl, pH 8.8. 0.4% SDS)

- Tris – 18.165g
- HCl -2.5 to 3ml
- 10% SDS – 8ml
- dH_2O – 100ml

Reagent for upper Gel Buffer (1M Tris-Cl, pH 6.8 o.4%SDS)

- Tris – 12.11g
- HCl – 7-8ml
- 10% SDS – 8ml
- dH_2O – 100ml

Ragent for 5X SDS loading Sample Buffer

- 250 mM tris HCl pH 6.8 – 12.5ml
- 10% SDS 10g
- 30% β-mercaptoethanol (or 0.5 DTT)-5ml
- 0.02 bromophenol blue - 52ml

Preparation of 10% Ammonium persulphate (APS)

- APS – 205g
- dH_2O – 25ml
- Store this at 80^0C - 20^0C

Prepartion of 30% Acrylamide

- Acrylamide - 2.92g
- Bisacrylamide - 80mg

- dH_2O - 8ml

Reagent for Separation Solution (Running Gel)

- H0O-3.3ml 30% Acrylamide mix-4ml
- 1.5M Tris (pH 8.8) – 2.5ml
- 10% SDS -0.1ml
- 10% APS -0.1ml
- TENED-0.004ml

Reagent for Stacking Solution

- Tris (FW 121.1-7.57g
- Glycine – 36.032g
- 10% SDS – 2.5ml
- Distilled water –q.s. to 500ml

Reagent for Coomassie Blue Staining

- Coomassie Blue Stain -250mg
- Acetic Acid -2ml
- dH_2O - 8ml
- Methanol

Reagent for Destaing solution

- Methanol – 13.5ml
- Glacial Acetic Acid – 3ml
- dH_2O – 13.5ml

Sample preparation

Sample preparation is crucial for clear & accurate resolution of protein bands. Photographic quality results are routinely possible if samples are carefully prepared. A pure colony was incubated on nutrient broth (25ml) with the help of sterilized wire loop. They were incubated at 37^0C for 24h. After the good growth of bacteria, pellet was collected in eppendorf tube by centrifugation, the supernatant was discarded & pellet was suspended in 5x sample loading buffer. Upon addition of SDS sample

buffer, samples should be immediately mixed & heated to 85^0C for 3 minutes. This treatment is usually sufficient to reduce disulfides, solubilize & dissociate protein without peptide bond cleavage. Sonication is carried out to disrupt the cells & release the inner protein. Common mistakes during sample preparation include using an incorrect protein to sample buffer ratio, delayed heating, overheating, failure to remove insoluble material, & overloading, & under loading of sample. Loading too much protein will result in distorted poorly resolved bands in the overloaded lane & distorted electrophoresis patterns in adjacent lanes. Delayed heating of samples after sample buffer addition or excessive heating can cause electrophoresis artifacts dust to protein degradation & peptide bond cleavage, spectively.

Procedure

- Glass plates were cleaned with dH_2O & ethanol (Laemmli, 1970).
- These alone with the bases were assembled in casting stand.
- Separation & Stacking solution were prepared, mixing all except APS & TEMED.
- To separation mix, APS & TEMED were added, immediately pipette into gel to about 1.5-2.0cm below front/small glass plate.
- Thin layer of ethanol was added to aid edge polymerization & remove air bubbles.
- After the gel has polymerized, APS & TEMED were added in stacking gel, immediately pipette onto gel until flash with top edge of small glass plate.
- The comb was placed & ensured that it was centered. The stacking gel was allowed to polymerize.
- Once polymerized, comb & the bases were removed, 5X tank buffer was taken & squirted into wells to remove bubbles.
- Meanwhile 59µl 5X protein loading buffer was added to protein samples.
- Gel was placed in electrode assembly; gel was placed into tank. Tank was filled with tank buffer.
- Then it was attached to power supply. It was run at 110V for 1hr or until the loading buffer is seen to reach the bottom edge of separation.
- Upon completion of gel run, it was disassembled. Gel was carefully removed from between the glass plates.

- The gel was dipped into Coomassie blue stain left overnight at room temperature.
- The gel was dipped into Coomassie blue & left overnight at room temperature.
- The gel was then washed with dH_2O, followed by destining by destining with destaining with solution.
- After destaining , the gel was stored in 5% glacial acetic acid at 4^0C.

3.1 Identification and Isolation of Microorganisms

3.1.1 Gram Staining

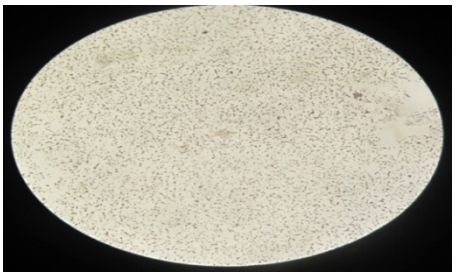
When slide are observed in electron microscope,we shown 8 colony gave blue (violet) colour among 15 colonies.So,they are gram positive(+ve) bacteria and other colonies are discarded.Selected colony were gave new code.

A table are given below which show the result of gram staining results:

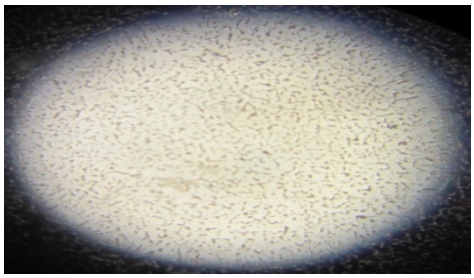
Strain code	Colour	Gram stain	New code
ATY-1	Blue	+	1
ATY-2	Blue	+	2
ATY-3	Red	_	×
ATY-4	Blue	+	3
RTY-1	Blue	+	4
RTY-2	Red	_	×
RTY-3	Red	_	×
RTY-4	Blue	+	5
EMY-1	Blue	+	6
EMY-2	Red	_	×
EMY-3	Red	_	×
RMY-1	Blue	+	7
RMY-2	Blue	+	8
RMY-3	Red	_	×
DMY	Red	_	×

Table-8: Result of Gram staining test

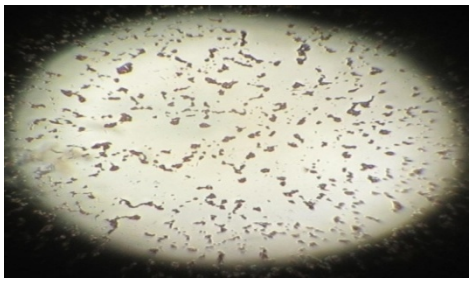
Electron Microscopic image of gram positive bacteria are given below:



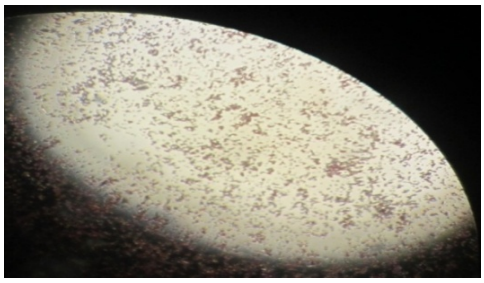
Strain code 1



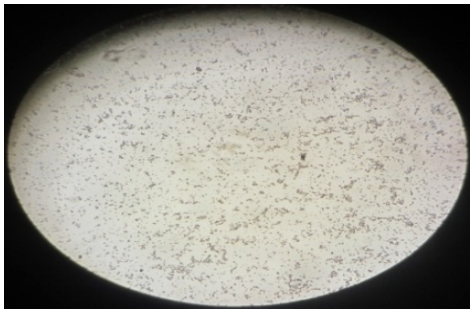
Strain code 2



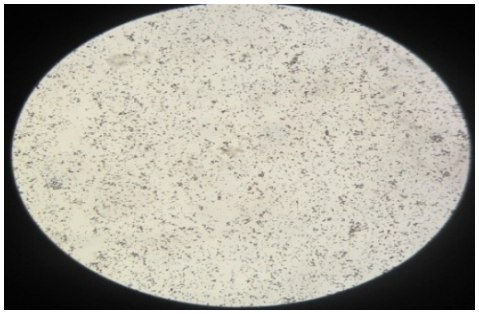
Strain code 3



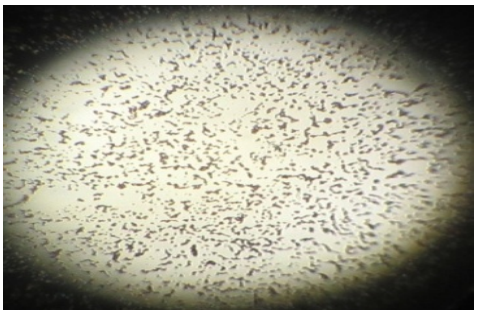
Strain code 4



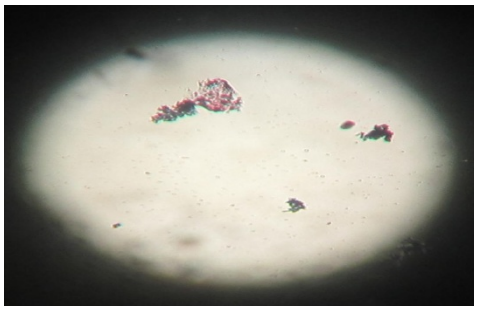
Strain code 5



Strain code 6



Strain code 7



Strain code 8

Fig-1 : Electron Microscopic image of gram positive bacteria

3.1.2 Catalase Test

Gram positive bacterial strains were selected for catalase test.All strains showed negative results when they are exposed in testube containing Hydrogen peroxid(H2O2). But 4 & 8 number strain are prouced small bubble.E.coli are used for positive result.

A table are given below which indicates the catalest test result:

Strain Code	Ctalase
1	–
2	–
3	–
4	–
5	–
6	–
7	–
8	–

Table-9: Represent catalase test

Picture of catalase test-

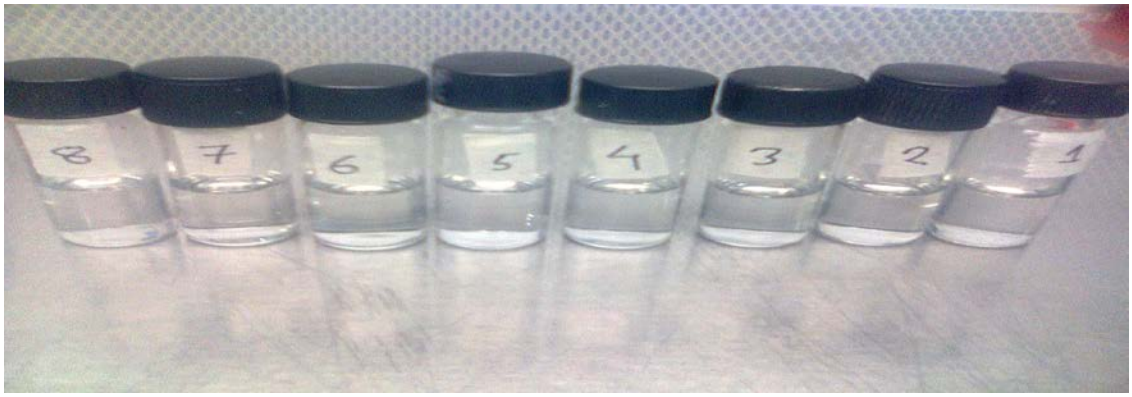


Fig -2: Ctalase reaction of all strains

3.2 Identification and Isolation of Probiotics properties

3.2.1Resistance to Low pH (pH 3.0&pH 1.0)

4 times serial diluation of bacterial samples are done by using sterile phosphate buffer with two pH-3.0& 1.0.Diluation concentration were 1:10,1:100,1:1000 and 1:10000.In case of pH-3.0,all dilluted samples were spred in agar plate and last two concentration (1.1000&1.1000) are used for pH-1.0.After serial diluation all samples containing eppendrope tubes are kepet in incubators for 3hours.Then all samples are spread in agar plate and keept in incubators for 24hours.After 24hours,we shown that bacteria are grown in every concentration.Individual table are given below for pH-3.0 and pH-1.0.Only agar media were used as negative control and Salmonella paratyphi for positive control.

Table for bacteria those survive in pH-3.0-

Strains code	Bacterial Concentration	Hour's	Number of Bacteria
1	1:10	3	37
	1:100		15
	1:1000		9
	1:10000		2
2	1:10	3	8
	1:100		4
	1:1000		1
	1:10000		3
3	1:10	3	2
	1:100		1
	1:1000		7
	1:10000		3
4	1:10	3	47
	1:100		45
	1:1000		30
	1:10000		13

5	1:10	3	6
	1:100		8
	1:1000		3
	1:10000		5
6	1:10	3	25
	1:100		39
	1:1000		3
	1:10000		8
7	1:10	3	7
	1:100		10
	1:1000		1
	1:10000		2
8	1:10	3	35
	1:100		41
	1:1000		7
	1:10000		4

Table-10: Results of resistance to low Ph (pH 3.0) durring 3.0 hour’s

Table for bacteria those survive in pH-1.0-

Strains code	Bacterial Concentration	Hour's	Number of bacteria
1	1:1000	3	5
	1:10000		2
2	1:1000	3	15
	1:10000		9
3	1:1000	3	3
	1:10000		8
4	1:1000	3	10
	1:10000		13
5	1:1000	3	2
	1:10000		9
6	1:1000	3	21
	1:10000		7
7	1:1000	3	7
	1:10000		3
8	1:1000	3	12
	1:10000		5

Table-11 : Bacteria those resistance in pH-1.0.

3.2.2 Resistance to 1% Bile Salt

Bacteria those tolarated pH-1.0,they are considerd for 1% Bile Salt (pH-6.8) tolerenc test.After subculturing of pH-1.0 tolerated bacteria 4 times serial diluation of culture were done by using bile salt and kept for 4 hour's in incubators.Then diluted bacteria were spred in agar plate and agar media and salmonella parathyphi are used respectively for negative and positive control.Only last two concentration (1:1000&1;10000) are consider for spreading.All dish were kept in incubator for 24 hour's.After 1 day bile salt tolerated bacteria were conuted.Following result were found for bile salt tolerated bacteria:

Strains code	Bacterial concentraion	Hour's	Number of bacteria
1	1:1000	4	43
	1:10000		9
2	1:1000	4	31
	1:10000		11
3	1:1000	4	27
	1:10000		6
4	1:1000	4	44
	1:10000		25
5	1:1000	4	22
	1:10000		9
6	1:1000	4	34
	1:10000		5
7	1:1000	4	11
	1:10000		7
8	1:1000	4	23
	1:10000		4

Table-12: Results of resistance to 1% bile salt

3.3 Antimicrobial Activity

3.3.1 Antimicrobial activity of selected strains

Bacteria were considered for antimicrobial activities those passed the gram staining and catalase test.10 pathogenic bacteria were used against selacted strains.All strains had antagonistics effect against patogenic bacteria.Each 20µl of samples were used against 500µl of spreading pathogenic bacteria in agar plate and kept for 24 hour's.Next day zone of inhibition was observed and measure by Varnier scale. Following mm of zone of inhibition are measured:

Pathgenic Bacteria		Zone of Inhibition in mm							
		Strain Code of Selected Bacteria							
Name	Code	1	2	3	4	5	6	7	8
Bacillus sereus	1	9.6 ±4.8	6.8 ±0.6	6.9 ±0.8	7.3 ±0.0	7.4 ±1.0	10.7 ±5.0	6.65 ±1.1	9.6 ±4.4
Bacillus subtilis	3	_	7.4 ±2.7	8.35 ±3.3	7.4 ±0.2	7.0 ±0.6	6.2 ±0.2	6.65 ±0.3	6.1 ±0.0
Salmonella paratyphi	4	7.9 ±0.8	7.5 ±1.6	6.33 ±0.5	6.7 ±1.2	7.4 ±1.4	9.35 ±3.3	13.5 ±8.9	11.5 ±0.0
Vibrio parahemolyticus	6	7.95 ±2.3	6.3 ±0.0	6.4 ±0.0	6.3 ±0.1	6.65 ±1.1	7.85 ±2.5	12.8 ±4.2	10.5 ±4.6
Staphylocuccos aureaus	8	7.4 ±2.6	8.65 ±2.3	7.35 ±0.5	9.15 ±1.7	15 ±0.6	10.9 ±0.9	6.1 ±0.0	7.5 ±0.8
E.coli	9	7.65 ±1.1	7.9 ±0.4	12.85 ±4.2	9.8 ±3.7	12.1 ±0.5	7.7 ±0.4	7.6 ±0.0	6.8 ±0.0
Shigella dysenteriae	10	7.55 ±0.5	7.5 ±2.2	7.1 ±0.0	8.3 ±2.8	7.05 ±1.7	6.4 ±0.0	8.05 ±0.7	6.5 ±0.8
Sarcina lutea	12	7.8 ±0.0	6.25 ±0.3	6.3 ±0.0	7.6 ±0.0	6.15 ±0.1	7.8 ±0.0	9.1 ±0.0	9.2 ±0.0
Candida albicans	15	8.5 ±1.2	9.5 ±2.4	10.95 ±0.3	8.85 ±0.5	7.7 ±1.0	8.2 ±0.2	6.15 ±0.1	7.35 ±2.4
Asergillus niger	16	6.75 ±0.3	7.2 ±0.0	7.2 ±1.8	7.75 ±1.1	7.8 ±1.4	7.7 ±0.0	7.7 ±0.8	6.8 ±0.8

Table-13: Zone of inhibition of isolates against pathogenic bacteria

Iamage of zone of inhibition of pathogenic bacteria by selacted isolates:

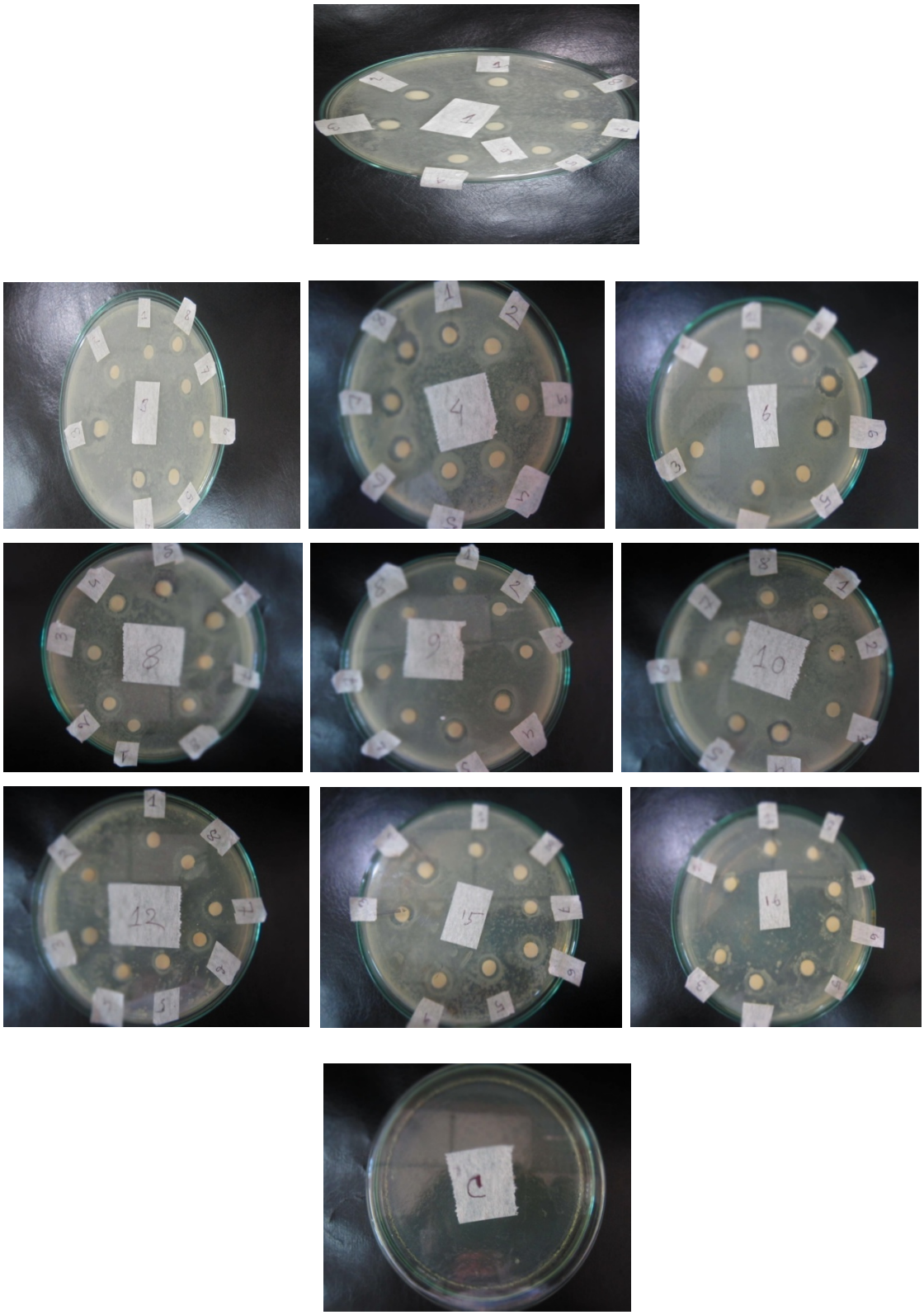


Fig-3: Antimicrobial activity of isolates from subculture against pathogenic bacteria

3.3.2 Antibacterial activity of Bacterial metabolites against pathogenic bacteria

Bacteriocin or bactarial metabolites shows significant inhibitiory action against pathogenic bacteria.Isolated metabolites was diluted by using 500µl phosphat buffer(pH-7.1).Disc contain 20µl metabolites samples and 500µl pathogenic bacteria were spreded in agar plate.Then all dish were kept in incubator for 24 hour's.Nex day zone of inhibition are measered by Varnier scale.Following different data were found:

Pathgenic Bacteria		Zone of Inhibition in mm							
		Strain Code of Isolated Metabolites							
Name	Code	1	2	3	4	5	6	7	8
Bacillus sereus	1	6.85 ±0.1	8.2 ±0.8	6.75 ±1.1	7.9 ±0.0	6.95 ±1.3	8.05 ±0.3	7.6 ±1.0	6.7 ±1.2
Bacillus subtilis	3	7.8 ±0.0	9.8 ±1.5	9.8 ±1.5	7.25 ±0.7	7.8 ±1.8	7.5 ±0.8	7.35 ±1.9	7.9 ±1.4
Salmonella paratyphi	4	7.25 ±0.9	7.25 ±0.9	8.05 ±1.1	8.8 ±0.8	8.65 ±3.8	7.6 ±1.4	8.4 ±1.4	8.4 ±1.6
Vibrio parahemolyticus	6	7.4 ±2.6	7.1 ±2.0	6.95 ±0.3	7.15 ±1.9	7.6 ±2.6	7.35 ±0.1	8.05 ±0.5	7.5 ±2.5
Staphylocuccos aureaus	8	9.05 ±3.0	10.3 ±0.0	8.9 ±0.0	6.85 ±1.1	6.65 ±1.1	7.35 ±1.9	8.1 ±0.4	7.0 ±3.3
E.coli	9	7.6 ±2.2	7.55 ±2.7	6.5 ±0.8	6.45 ±0.5	6.15 ±0.1	6.7 ±1.2	7.7 ±0.9	9.5 ±2.0
Shigella dysenteriae	10	7.6 ±1.4	6.9 ±0.0	_	_	6.2 ±0.2	6.45 ±0.5	6.8 ±0.0	8.5 ±0.7
Sarcina lutea	12	7.6 ±1.8	6.7 ±0.2	7.3 ±1.2	7.35 ±0.9	8.1 ±3.6	7.3 ±1.6	6.95 ±0.5	7.0 ±1.6
Candida albicans	15	8.15 ±1.3	7.8 ±0.6	8.25 ±1.9	7.75 ±2.7	7.25 ±1.3	7.3 ±1.2	8.3 ±0.4	9.1 ±1.0
Aspergillus niger	16	6.5 ±0.8	6.93 ±1.3	7.0 ±0.3	6.1 ±0.0	6.1 ±0.0	6.1 ±0.0	7.35 ±2.1	7.7 ±1.8

Table-14: Inhibitory result of metabolites against pathogenic bacteria.

Image of inhibitory region by bacterial metabolites against pathogenic bacteria:

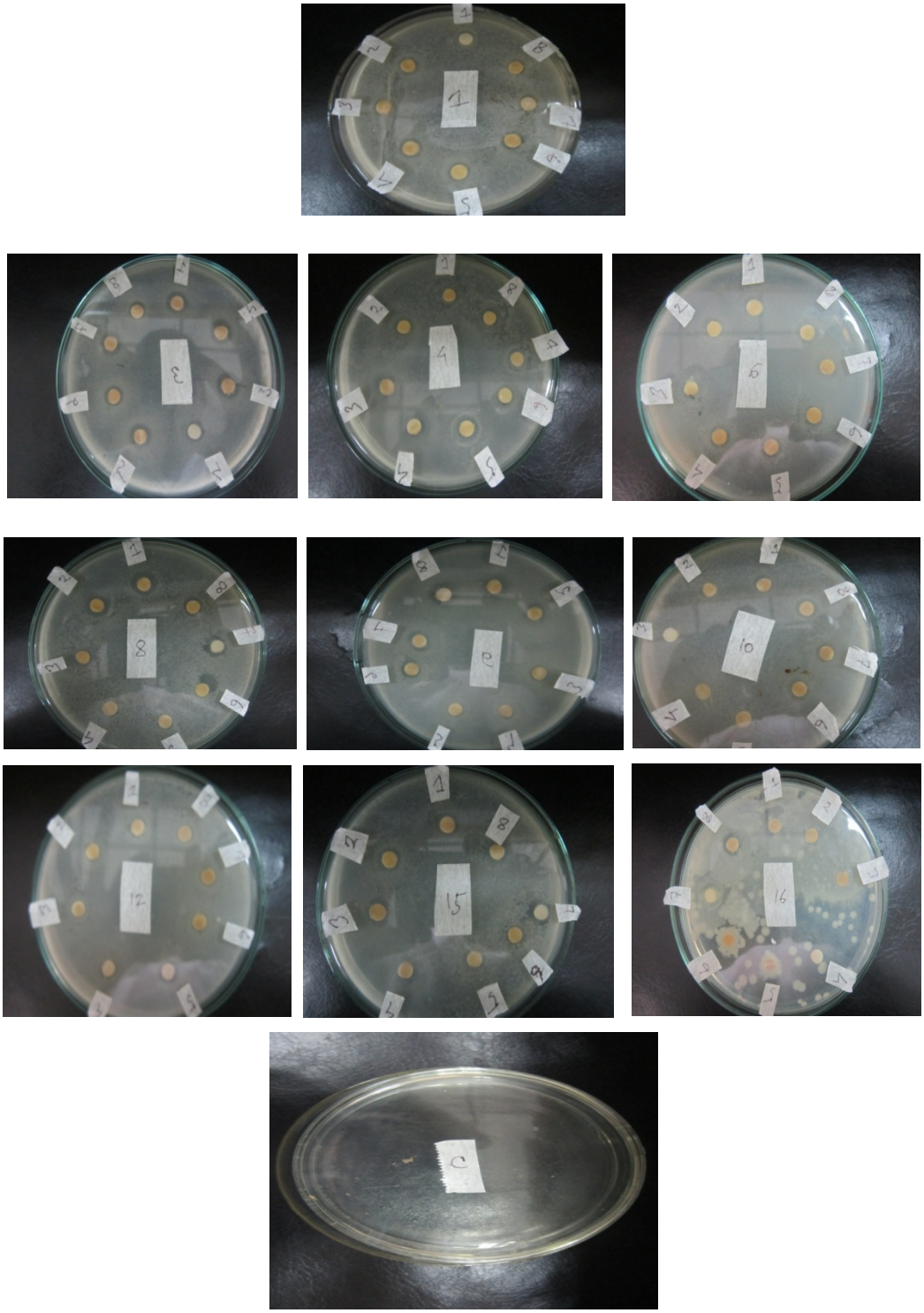


Fig-4: Antimicrobial activity of metabolites against pathogenic bacteria

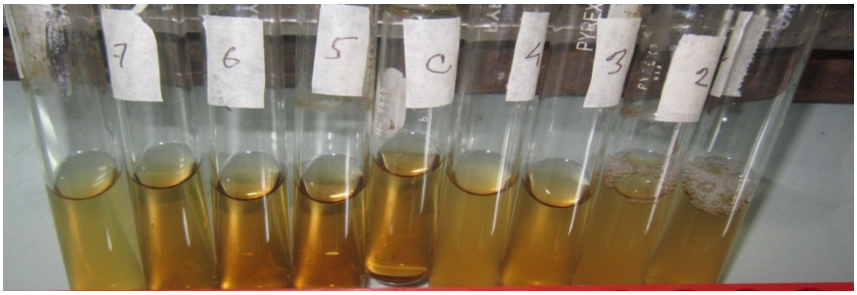
3.4 Carbohydrate Fermentation Test

3.4.1 Glucose Fermentation Test

Methyl red are used for pH indicators.If bacterial isolates ferment the glucose,colour change and pH of test samples are changed.For glucose fermentation folowing data and colour were found:

Strain Code	Fermentation	Color Change	pH
1	+	Yes(yellow)	6.2
2	+	Yes (yellow)	6.2
3	+	Yes (yellow)	6.0
4	+	Yes (yellow)	5.6
5	_	No change	7.2
6	_	No change	7.3
7	+	Yes(yellow)	5.3
8	+	Yes(yellow)	5.7
E. coli	+	Yes(Yellow)	4.8
Only media	_	No change	7.4

Table-15: Result of glucose fermentation test



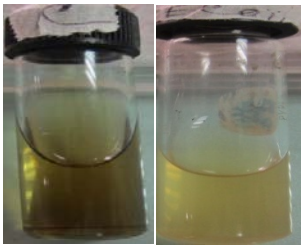
Negative control (Only media)  Positive control *(E. coli)*

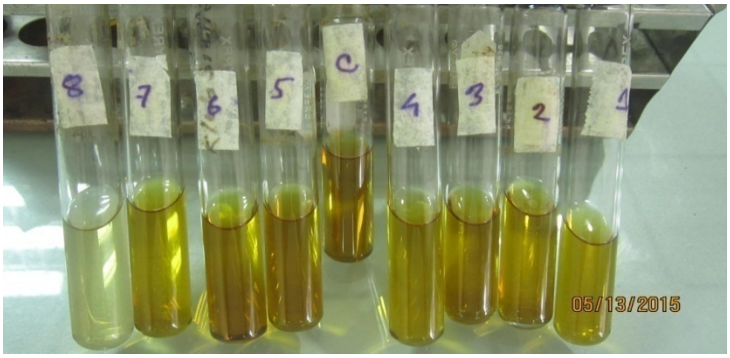
Fig-5: Glucose fermentation test

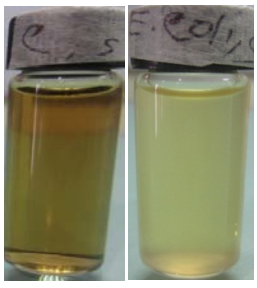
3.4.2 Sucrose Fermentation Test

pH and colour change of samples are occurred in fermentaion of cabohydrates by bacteria due to production of lactic acid,ethanol and carbon-di-oxide.In Sucrose fermentation folowing results were observed:

Strain code	Fermentation	Color change	pH
1	+	Yes (yellow)	7.1
2	+	Yeas(yellow)	7.2
3	+	Yes (yellow)	6.4
4	+	Yes (yellow)	6.1
5	_	No change	7.4
6	_	No change	7.5
7	+	Yes(yellow)	6.2
8	+	Yes (yellow)	6.1
E. coli	+	Yes (yellow)	5.4
Only media	-	No	7.7

Table-16: Result of sucrose fermentation test





Negative control (Only media) Positive control (*E. coli*)

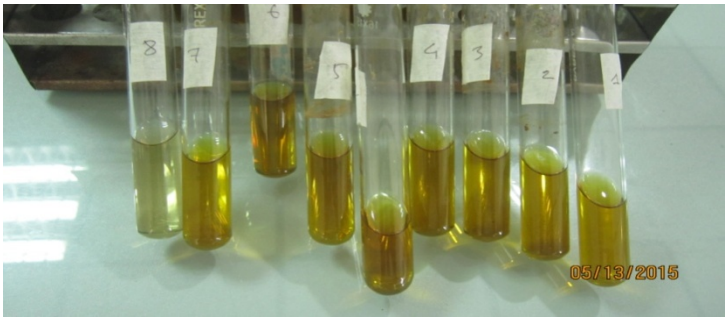
Fig-6 : Sucrose fermentation test

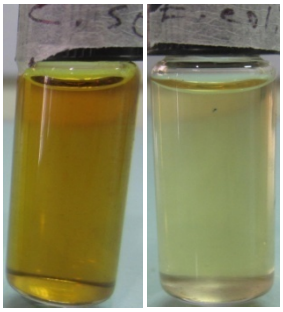
3.4.3 Sorbitol Fermentation Test

Differents probiotics ferment differents carbohydrates.In case of Sorbitol fermentation by isolated strains following test result were recorded:

Strain code	Fermentation	Color change	pH
1	+	Yes(yellow)	6.9
2	-	No	7.7
3	-	No	7.7
4	+	No	6.7
5	-	No	7.7
6	-	No	7.8
7	+	Yes(yellow)	6.4
8	+	Yes (yellow)	4.1
E. coli	+	Yes (yellow)	6.8
Only media	-	No	7.7

Table-17: Result of sorbitol fermentation test





Negative control (Only media) Positive control (*E. coli*)

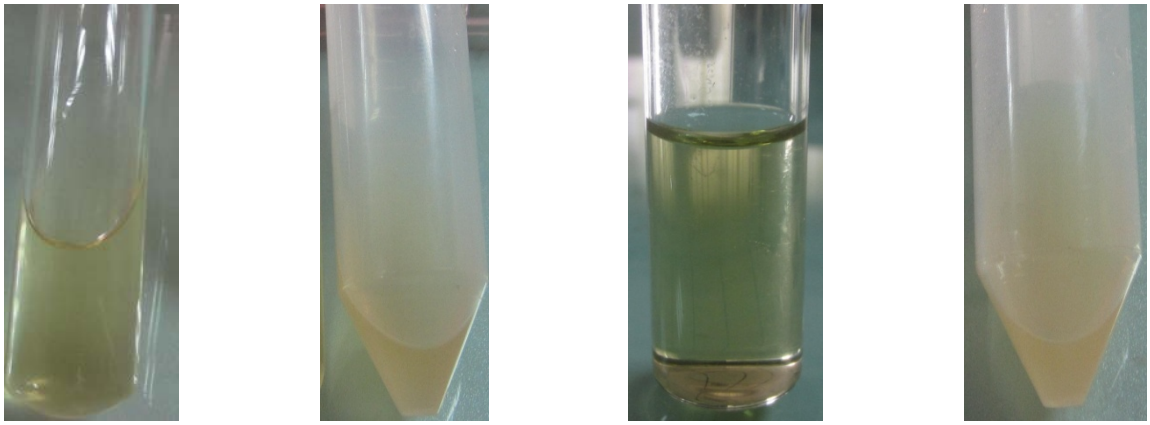
Fig-7 : Sorbitol fermentation test

3.4.4 Tolerance Against 6.5% Sodium Chloride

Cell growth and Bacteriocin are affected by different concentration of NaCl. So, for tolerability test of the isolated strains high concentration of NaCl(6.5%) are used.Following results were found:

Strain Code	Ability to Tolerate	pH
1	+	7.9
2	+	7.9
3	+	7.9
4	+	7.9
5	+	7.9
6	+	7.9
7	+	7.9
8	+	7.9
E. coli	+	7.6
Only media	-	7.9

Table-18: Result of 6.5% sodium chloride tolerance test



Bacteria in NaCl (6.5%) Bacteria in broth Only media Positive control

Fig-8: Sodium chloride (6.5%) tolerance test

3.5 SDS-PAGE Analysis

3.5.1 SDS-PAGE Analysis for Isolated Strains

All strains produced various proteins bands.Figure are given below:

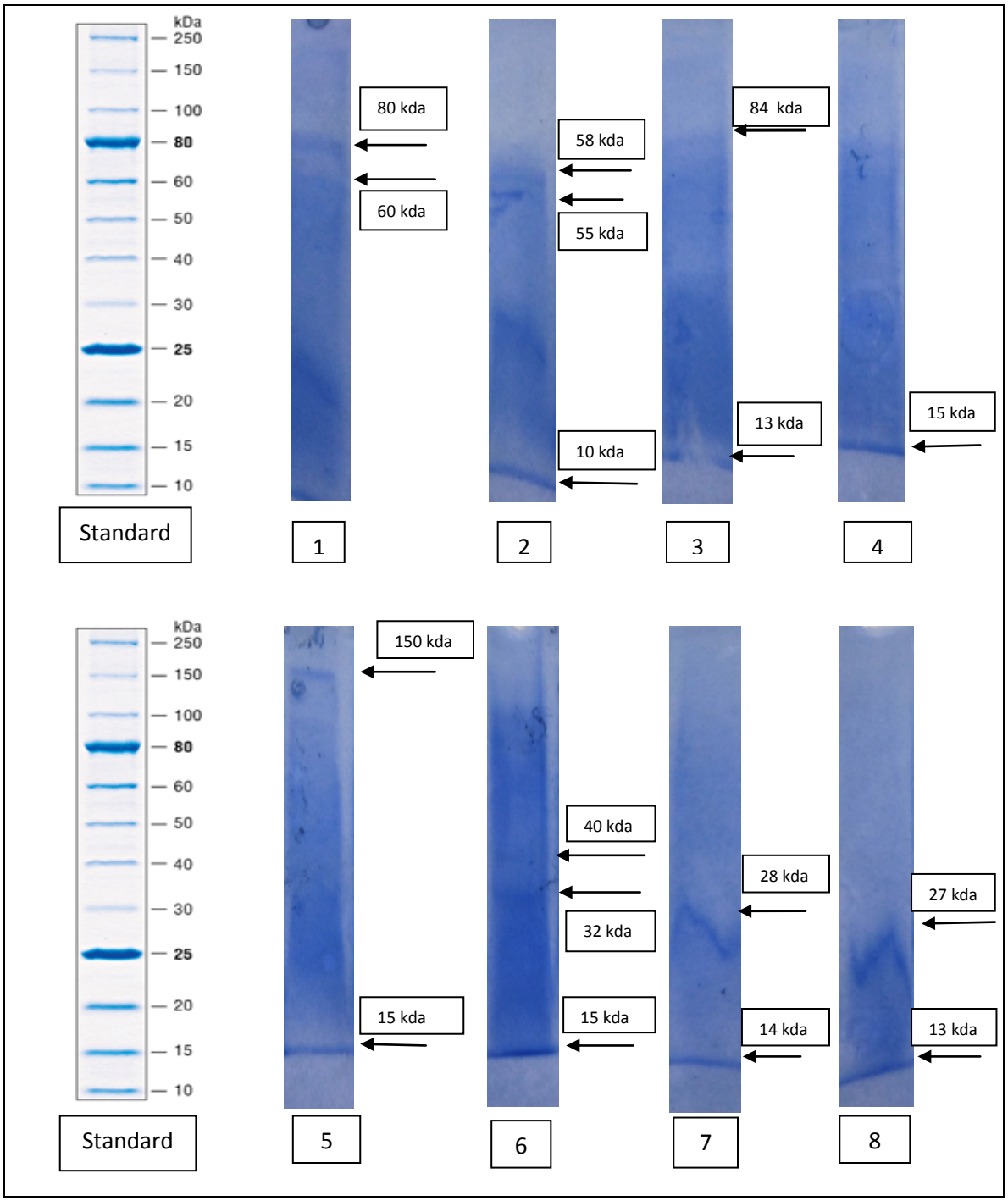


Fig-9: Proteins Bands of Probiotics Bacteria

3.5.2 SDS-PAGE Analysis for Bacterial Metabolites

Metabolites of all strains produced various bands.Figure are given here;

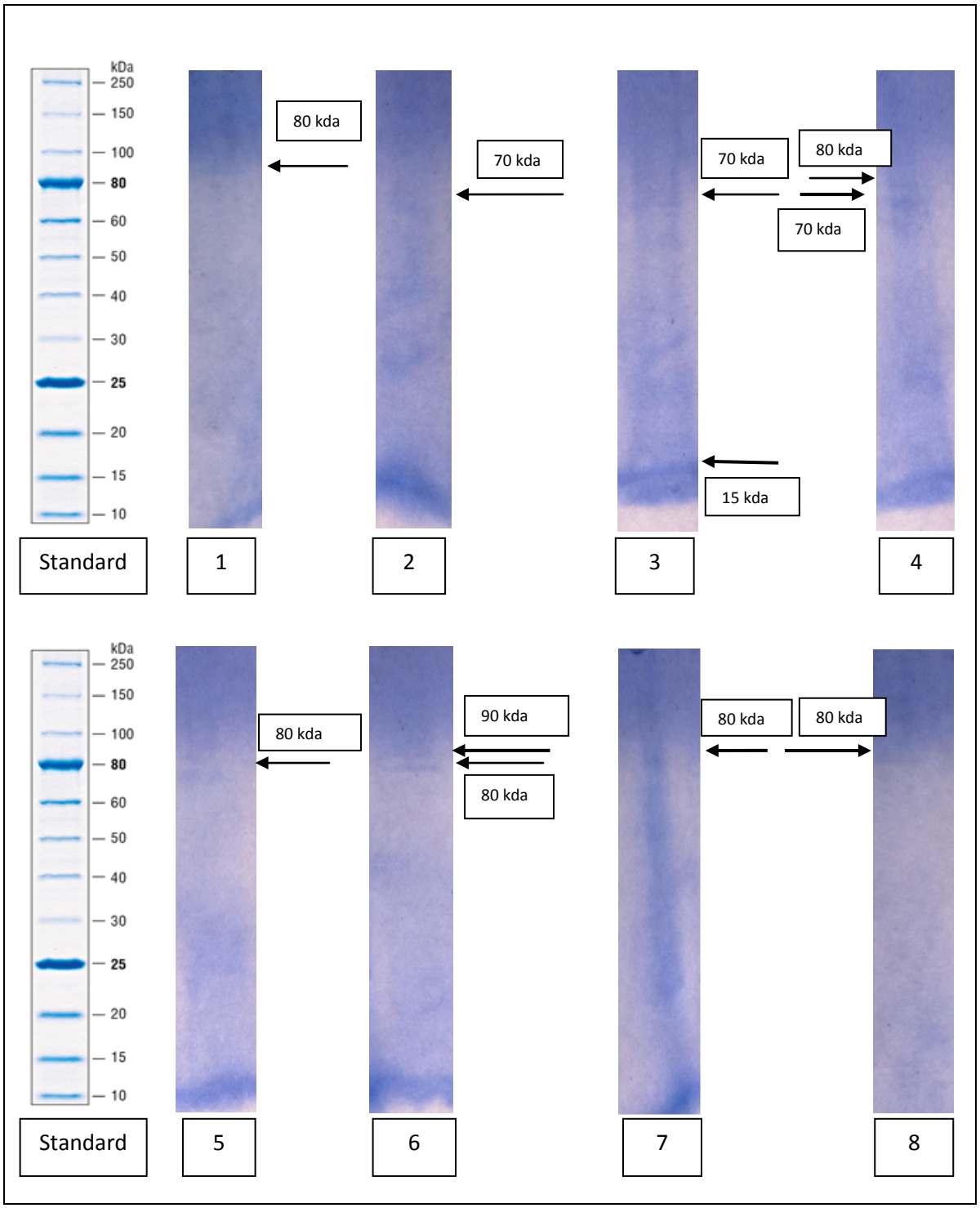


Fig-10 : Proteins Bands of Probiotics Metabolites

Discussion

4 Isolation of Probiotic Bacteria using different methods

A probiotic microorganism that should survive and grow under rumen-like conditions, resist to gastric acidity and the presence of lisozyme, bile salts and pancreatic enzymes (Salminen *et al.,* 1998). Duodenal acidities and the high concentration of bile in the intestine are the first factors to consider in probiotic selection. It is well known that, the major selection criteria (antibiotic resistance, resistance to low pH, tolerance against bile salt and antimicrobial activity) used for the determination of probiotic properties of lactic acid bacteria isolates (Mohsin Shaikh & Gaurav Shah, 2013).

It is well known that Probiotic bacteria are gram positive, catalase negative, survived against stressful environments (stomach pH and high concentration bile salt), active against different pathogenic bacteria and also antibiotic resistance. We can seen the all results of methods and material, seven strains are gram positive but six strains are catalase negative, so we selected the six strains for next experiments.

All strains of probiotic bacteria were found from all experiments. These strains were catalase negative, survived against low pH (pH 3.0& 1.0) during 3 hours and against 1% bile salt during 4 hours, show antimicrobial activity against different types of pathogenic bacteria and isolated metabolites also show antibacterial activity. So that is why we confirmed, 8 strains of Probiotic bacteria were found from all experiment.

Isolation of Lactobacillus using different methods

The genus *Lactobacillus* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. (Schillinger *et al*., 1999). *Lactobacillus* is found to be living in highly acidic environments of pH 4-5 or lower, thereby altering the pH and suppressing pathogens by producing lactic acid (Salminen *et al*., 1996). Gram-positive and microscopically appear as long to short rods or even coccobacilli and (Holt G *et al*, 1994). The lactobacilli are usually more resistant to acidic conditions than are other lactic acid bacteria. Lactobacillus is more stable in Ph 3.0 during 3 hours and more survived against high concentration of bile salt during 4 hours.

This genus is generally catalase-negative. *Lactobacillus* spp. is either homo- or hetero fermentative with regard to hexose metabolism. Lactobacillus acidophilus unable to ferment arabinose and sorbitol (Hassan *et al.*, 2013). Lactobacilli tolerate 2%, 4% and 6.5% NaCl concentration (Estifanos Hawaz, 2014).

We were found strain code 1 and 4 were Lactobacillus from all experiments, following the above characteristics of Lactobacillus. These two strains of Lactobacillus were Gram positive, coccus shaped, catalase negative, more stable in pH 3.0 during 3h and high concentration of bile salt during 4 h than other strains, show antimicrobial activity against different types of pathogenic bacteria,survived against 6.5% NaCl concentration, produce lactic acid from glucose,sucrose. Strain Code 2 and 3 are unable to ferment in sorbitol..

4.1 Identification and Isolation of Microorganisms

4.1.1 Gram staining

Probiotics are known to be gram positive. It means that they give blue-purple color by gram staining. Cultures were grown in appropriate mediums at 37 °C for 24 h under aerobic and anaerobic conditions (kavitha *et al.*, 2013). Probiotics are group of Gram-positive cocci and rods (Hammes *et al*., 2006).

The isolated bacteria were observed by light microscope.Among the 15 strains ,8 strains of the bacteria were gram positive, two bacteria find out as a coccus shaped, occurring singly or in chains. The gram staining results indicated that the isolated bacteria could be identified Probiotics and Lactobacillus. After gram staining test, gram positive bacteria were selected for catalase test. When catalase test were performed, no gas bubble were found.Result are given in table-1 and fig-1.

4.1.2 Catalase test

The catalase test is used detect the presence of the enzyme catalase in bacteria. Catalase is produced by certain bacteria, which acts as a catalyst I,breakdown hydrogen peroxide into water and oxygen. This reaction is evident the rapid formation of bubbles (Clark H *et al*., 1952). Probiotics are catalase negative occurring naturally in variety of niches (Hammes *et al*., 2006).

From the catalase test, all strains were found catalase negative(table-2 and fig-2). It is well known that Probiotics are catalase negative. So, all strain was selected from the catalase test for next experiments.

4.2 Identification and Isolation of Probiotic Properties

4.2.1 Resistance to low pH (pH 3.0 & 1.0)

Resistance to pH 3.0 and 1.0 is often used in vitro assays to determine the resistance to stomach pH. Because the foods are staying during 3 hrs, this time limit was taken into account (Prasad, *et al*. 1998). A probiotic microorganism that will be used as a microbial supplement in animal feeding should survive and grow under rumen-like conditions, resist to gastric acidity (Salminen *et al*., 1998). *Lactobacillus* is found to be living in highly acidic environments of pH 4-5 or lower, thereby altering the pH and suppressing pathogens by producing lactic acid (Salminen *et al*., 1996).

In resistance to low pH test, all strains were survived against pH 3.0 & 1.0 during 3 hours. 1 and 4 strains were more tolerated than other strains, so indicated that, this two strains were Lactobacillus.In all concentration bacteria were survived and growing(table-3 and 4).From table-3 for strain 1,we observed that 37,15,9,2 number of bacteria grow pH-3.0 at different concentration(1:10,1:100,1:1000 and 1:10000 respectively).Similiarly, 8,4,1,3 for strain-2; 2,1,7,3 for strain-3; 47,45,30,13 for strain-4; 6,8,3,5 for strain-5; 25,39,3,8 for strain-6; 7,10,1,2 for strain-7; 35,41,7,4 for strain-8 were found.Incase of pH-1.0 and for strain-1, we found that 5,2 number of bacteria were grown at last two concentration 1:1000&1:10000 respectively.Similiarly, 15,9 for strain-2; 3,8 for strain-3; 10,13 for strain-4; 2,9 for strain-5; 21,7 for strain -6; 7,3 for strain-7 and 12,5 for strain-8 were found(table-4).

4.2.2 Resistance to 1% bile salt

A probiotic microorganism that will be used as a microbial supplement in animal feeding should survive and grow under rumen-like conditions, resist to gastric acidity and the presence of lisozyme, bile salts and pancreatic enzymes. These characteristics can be evaluated in vitro and can be used for strain selection (Salminen *et al.,* 1998). It is reported that time at the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile to show probiotic sufficiency, they should reach to the lower intestinal tract and maintain themselves over there. Because of desirable point the first criteria is looking for probiotic strains is being resistant to acid and bile (Chou, 199 and Çakır, 2003). The lactobacilli are usually more resistant to acidic conditions than are other lactic acid bacteria. Lactobacilli are more stable in Ph 3.0 and against bile salt during 3 and 4 hours respectively (Hatice Yavuzdurmaz *et al*., 2007).

In resistance to bile salt test, 8 strains were survived against 1 % bile salt during 4 hours. Strains 1 and 4 was more tolerated than other strains. This Test also indicated that, 6 strains were probiotics and two strains were lactobacillus.

Incase of strain-1, we found that 43, 9 number of bacteria are survived against bile salt concentration 1:1000 & 1:10000 respectively after 24 hours incubation. Samiliarly, 31, 11 for strain-2; 27, 6 for strain-3; 44, 25 for strain-4; 22, 9 for strain-5; 34, 5 for strain-6; 11, 7 for strain-7 and 23, 4 for strain-8 were counted.

4.3 Antimicrobial Activity of Isolates and their Metabolites Against Pathogenic Bacteria

4.3.1 Antimicrobial Activity of Isolates

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer Kullen *et al*., 1999). Antimicrobial effects of “Probiotics” are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide,

hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins ((Quwehand *et al.*, 2004 and Çakır, 2003).

In antimicrobial activity test, all 8 strains were given action against all pathogenic bacteria. But 1 number strain did not given action against *Bacillus subtilis*. Zone of inhibition were measured by slide calipers. For *Bacillus sereus*, 9.6mm, 6.8mm, 6.9mm, 7.3mm, 7.4mm, 10.7mm, 6.65mm & 9.6mm of zone of inhibition was produced by 1, 2, 3, 4, 5, 6, 7 & 8 starins respectively. Similirly, for *Bacillus subtilis*, 0mm, 7.4mm, 8.35mm, 7.4mm, 7.0mm, 6.2mm, 6.65mm & 6.1mm of inhibition were measured for 1, 2, 3, 4, 5, 6, 7 & 8 strains respectively. For *Salmonella paratyphi*, 7.9mm, 7.5mm, .33mm, 6.7mm, 7.4mm, 9.35mm, 13.5mm & 11.5mm of inhibition were measured for 1, 2, 3, 4, 5, 6, 7 & 8 strains respectively. *Vibrio parahemolyticus*, 7.95mm, 6.3mm, 6.4mm, 6.3mm, 6.65mm, 7.85mm, 12.8mm & 10.5mm of inhibition produced by 1, 2, 3, 4, 5, 6,7 & 8 number of isolated strains respectively. For *Staphylocuccos aureaus*, 7.4mm, 8.65mm, 7.35mm, 9.15mm, 15.0mm, 10.9mm, 6.1mm & 7.5 mm of zone of inhibition were produced by 1, 2, 3, 4, 5, 6, 7 & 8 strains respectively. For *E.coli*, 7.65mm, 7.9mm, 12.85mm, 9.8mm, 12.1mm, 7.7mm, 7.6mm & 6.8mm of inhibition are created by 1, 2, 3, 4, 5, 6, 7 & 8 number of strains respectively. For *Shigella dysentriae*, 7.55mm, 7.5mm, 7.1mm, 8.3mm, 7.05mm, 6.4mm, 8.05mm & 6.5mm of inhibition were found by 1, 2, 3, 4, 5, 6, 7 & 8 number of strains respectively. For *Sarcina lutea*, 7.8mm, 6.25mm, 6.3mm, 7.6mm, 6.15mm, 7.8mm, 9.1mm & 9.2mm of inhibition were found by 1, 2, 3, 4, 5, 6, 7 & 8 number of strain respectively. For *Candida albicans*, 9.5mm, 10.95mm, 8.85mm, 7.7mm, 8.2mm, 6.15mm & 7.35mm of inhibition were observed by 1, 2, 3, 4, 5, 6, 7 & 8 number of strain respectively. For *Asergillus niger*, 6.75mm, 7.2mm, 7.2mm, 7.75mm, 7.8mm, 7.7mm, 7.7mm & 6.8mm of zone of inhibition were produced by 1, 2, 3, 4, 5, 6, 7 & 8 number of bacteria respectively.

Against *Bacillus sereus*, bacterial strain 6 & 1 produced maximum zone of inhibition (10.75mm & 9.6mm). Against *Bacillus subtils*, 3 & 2 number metabolites produced maximum zone of inhibition (8.35mm & 7.45mm). Maximum inhibition of *Salmonella paratyphi* occurred by 7 & 6 number of metabolites and inhibition area are 13.55mm & 9.35mm respectively. Metabolites 7 & 8 produced maximum inhibition (12.85mm &

10.55mm) against *Vibrio parahemolyticus*. For *Staphylococcus aureaus*, metabolites 5 & 6 produced higher inhibition (15.00mm & 10.9mm) than other metabolites. Against *E.coli*, metabolites 3 & 5 produced maximum inhibition (12.85.55mm & 12.15mm) respectively. Higher inhibition of *Shigella dysenteriae* occurred by 4 & 7 number metabolites and inhibited area are 8.3mm and 8.05mm.Metabolites 8 & 7 produced maximum inhibition (9.2mm & 9.1.6mm) against *Sarcina lutea*. For *Candida albicans*, higher inhibition are caused by metabolites 3 & 4 respectively and inhibited area in slide calipers are 10.95mm & 8.55mm.Against *Apergillus niger*, metabolites 5 & 4 produecd maximum zone of inhibition (7.8mm & 7.75mm) than others metabolites.Among all metabolites, 15.0mm is maximum zone of inhibition against *Staphylococcus aureaus* and effective metabolites is 8 number metabolite.

4.3.2 Antimicrobial Activity of Bacterial Metabolites

Antimicrobial effects of “Probiotics” are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Quwehand *et al.*, 2004 and Çakır, 2003).

Isolated bacterial metabolites are considered for antimicrobial test against 10 pathogenic bacteria. We observed that all 8 different metabolites were produced zone of inhibition against given pathogenic bacteria but 3,4 number metabolites have no any activity against *Shigella dysenteriae*. Antibacterial activity of metabolites are given in Table-7 & Figure-4.

For pathogenic bacteria *Bacillus sereus*, 6.85mm, 8.2mm, 6.75mm, 7.9mm, 6.95mm, 8.05mm, 7.6mm, 6.7mm of zone of inhibition produced by 1, 2, 3, 4, 5, 6, 7 & 8 number of metabolites respectively. Against *Bacillus subtils*, isolated metabolites 1, 2, 3, 4, 5, 6, 7, 8 produced 7.8mm, 9.8mm, 9.8mm, 7.25mm, 7.8mm, 7.5mm, 7.35mm, 7.9mm of zone of inhibition respectively. For *Salmonella paratyphi*, 7.25mm, 7.25mm, 8.05mm, 8.8mm, 8.65mm, 7.6mm, 8.4mm of zone of inhibition produced by 1, 2, 3, 4, 5, 6, 7, 8 number of metabolites respectively. Against *Vibrio parahemolyticus*, isolated metabolites1, 2, 3, 4, 5, 6, 7, 8 produced 7.4mm, 7.1mm, 6.95mm, 7.15mm, 7.6mm, 7.35mm, 8.05mm,

7.55mm of zone of inhibition respectively. Against *Staphylococcus aureaus*, isolated metabolites 1, 2, 3, 4, 5, 6, 7, 8 produced 9.05mm, 10.3mm, 8.9mm, 6.85mm, 6.65mm, 7.35mm, 8.1mm, 7.0mm of zone of inhibition respectively. For *E.coli*, 7.6mm, 7.55mm, 6.5mm, 6.45mm, 6.15mm, 6.7mm, 7.75mm, 9.55mm of zone of inhibition produced by 1, 2, 3, 4, 5, 6, 7, 8 number of metabolites respectively. Against *Shigella dysenteriae*, isolated metabolites 1, 2, 3, 4, 5, 6, 7, 8 produced 7.6mm, 6.9mm, 0mm, 0mm, 6.2mm, 6.45mm, 6.8mm, 8.55mm of inhibition respectively. Against *Sarcina lutea*, isolated metabolites produced 7.6mm, 6.7mm, 7.3mm, 7.35mm, 8.1mm, 7.3mm, 6.95mm, 7.0mm of zone of inhibition respectively. For *Candida albicans*, 8.15mm, 7.8mm, 8.25mm, 7.75mm, 7.25mm, 7.3mm, 8.3mm, 9.1mm of inhibition produced by 1, 2, 3, 4, 5, 6, 7, 8 number of metabolites respectively. Against *Apergillus niger*, isolated metabolites 1, 2, 3, 4, 5, 6, 7, 8 produced 6.5mm, 6.95mm, 7.0mm, 6.1mm, 6.1mm, 6.1mm, 7.35mm, 7.7mm of zone of inhibition respectively.

Against *Bacillus sereus*, metabolites 6 & 2 produced maximum zone of inhibition (8.05mm & 8.2mm).Against *Bacillus subtils*, 2 & 3 number metabolites produced maximum zone of inhibition (9.8mm & 9.8mm).Maximum inhibition of *Salmonella paratyphi* occurred by 4 & 6 number of metabolites and inhibition area are 8.8mm & 8.4mm respectively. Metabolites 7 & 5 produced maximum inhibition (8.05mm & 7.6mm) against *Vibrio parahemolyticus*. For *Staphylococcus aureaus*, metabolites 2 & 3 produced higher inhibition (9.05mm & 8.9mm) than other metabolites. Against *E.coli*, matabolites 8 & 7 produced maximum inhibition (9.55mm & 7.75mm) respectively. Higher inhibition of *Shigella dysenteriae* occurred by 8 & 1 number metabolites and inhibited area are 8.55mm and 7.6 mm. Metabolites 5 & 1 produced maximum inhibition (8.1mm & 7.6mm) against *Sarcina lutea*. For *Candida albicans*, higher inhibition are caused by metabolites 8 & 7 respectively and inhibited area in slide calipers are 9.1mm & 8.3mm. Against *Apergillus niger*, metabolites 8 & 7 produced maximum zone of inhibition (7.7mm & 7.35mm) than others metabolites. Among all metabolites, 9.8mm is maximum zone of inhibition against *Bacillus subtils* and effective metabolites are 2 & 3 numbers metabolite.

4.3.3 Comparison between Anti-Bacterial Activity of Isolated Strains and their Metabolites

On average, antibacterial activity of probiotics bacteria is higher than their isolated metabolites. To assure it, we compared antibacterial activity of strains and their metabolites. In case of pathogenic bacteria *Bacillus sereus,* maximum inhibition is 10.75 mm created by strain 6 but metabolite of strain 6 produced 8.05mm of inhibition against same pathogenic bacteria and maximum inhibition is 8.2 mm by strain 2. Strain 1 has no activity against *Bacillus subtils,* but strain 1 metabolites produced 7.8mm of inhibition of this bacteria. Against *Bacillus subtils,* maximum inhibition were observed by metabolites code 2 & 3 and inhibition zone are 9.8mm & 9.8 mm but their respective strain yield 7.45mm & 8.35mm of inhibition which is maximum among all strains. Against *Salmonella paratyphi,* maximum inhibition were done by strain 7&6 respectively and inhibition area are 13.55mm & 9.35 mm. But in case of metabolites, maximum inhibition of *Salmonella paratyphi* were done buy code 4&7 and inhibition area are 8.8mm & 8.4 mm. So, strain 7 is more effective than it metabolite against *Salmonella paratyphi* and difference is 5.15mm. Against *Vibrio parahemolyticus,* 12.85mm of inhibition were created by strain 6 but it metabolite yield only 7.35mm of inhibition and maximum inhibition were done by metabolite code 7(8.05mm). So,strain 6 is more potent than it metabolite. Maximum inhibition(15.0mm) of *Staphylococcus aureaus* were done by strain 5 but it metabolite produced only 6.65mm of inhibition and 9.05mm is highest inhibition against *Staphylococcus aureaus* created by metabolite code 1. Against *E.coli,* highest zone of inhibition is 12.85mm and created by bacterial strain 3.But it metabolite only yield 6.5mm of inhibition.So, strain 3 is more potent than it metabolite. In case of *Shigella dysenteriae,* metabolites 3 & 4 has no any inhibitory activity but their strain produced 7.1mm & 8.3mm of inhibition and 8.3mm is highest inhibition among all strain. But highest inhibition of *Shigella dysenteriae* is 8.55mm created by metabolite code 8. Against *Sarcina lutea,* maximum zone of inhibition (9.1mm) were done by strain 7 but maximum inhibition (8.1mm) were done by metabolite code 5.In case of *Candida albicans,* highest inhibition area is 10.95mm and created by strain 3 but it metabolite yield 8.25mm of inhibition and maximum inhibition of this bacteria were done by

metabolite code 8(9.1mm).Against last pathogenic bacteria *Apergillus niger*, maximum zone of inhibition is 7.8mm and respective strain was 5. In case of metabolites, 8 number code produced 7.7mm of inhibition and which is highest among all metabolites.15mm of zone of inhibition is the highest inhibition among all bacterial strains and metabolites and inhibited bacteria were *Staphylococcus aureaus.*

a. Comparative inhibition against *Bacillus sereus*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	9.6±4.8 mm	6.85± 0.1mm	2.75mm
2	6.8±0.6 mm	8.2±0.8 mm (maximum)	1.4mm
3	6.9±0.8 mm	6.75±1.1mm	0.15mm(minimum)
4	7.3± 0.0mm	7.9±0.0mm	0.6mm
5	7.4± 1.0 (maximum)	6.95±1.3mm	0.45mm
6	10.75±5.0mm	8.05±0.3mm	2.7mm
7	6.65± 1.1(minimum)	7.6±1.0mm	0.95mm
8	9.6±4.4 mm	6.7mm±1.2(minimum)	2.9mm(maximum)

Tab-19:Comparative inhibition of *Bacillus sereus* by probiotic strains & their metabolites

b. Comparative inhibition against *Bacillus subtils*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	_	7.8±0.0mm	7.8mm(maximum)
2	7.45±2.7mm	9.8±1.5 mm (maximum)	2.35mm
3	8.35±3.3 (maximum)	9.8±1.5mm	1.45mm
4	7.4±0.2mm	7.25±0.7mm (minimum)	0.15mm
5	7.0±0.6mm	7.8±1.8mm	0.8mm
6	6.2±0.2mm	7.5±0.8mm	1.3mm
7	6.65±0.3mm	7.35±1.9mm	0.7mm
8	6.1±0.0mm(minimum)	7.9±1.4mm	1.8mm

Tab-20:Comparative inhibition of *Bacillus subtils* by probiotic strains & their metabolites

c. Comparative inhibition against *Salmonella paratyphi* -

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.9±0.8mm	7.25±0.9mm	0.65mm
2	7.5±1.6mm	7.25±0.9mm(minimum)	0.25mm(minimum)
3	6.33±0.5mm(minimum)	8.05±1.1mm	1.72mm
4	6.7±1.2mm	8.8±0.8mm (maximum)	2.1mm
5	7.4±1.4mm	8.65±3.8mm	1.25mm
6	9.35±3.3mm	7.6±1.4mm	1.75mm
7	13.55±8.9 (maximum)	8.4±1.4mm	5.15mm(maximum)
8	11.5±0.0mm	8.4±1.6mm	3.1mm

Table-21: Comparative inhibition of *Salmonella paratyphi* by probiotic strains & their metabolites

d. Comparative inhibition against *Vibrio parahemolyticus*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.95±2.3mm	7.4±2.6mm	0.55mm
2	6.3±0.0mm (minimum)	7.1±2.0mm	0.8mm
3	6.4±0.0mm	6.95±0.3mm (minimum)	0.55mm
4	6.3±0.1mm	7.15±1.9mm	0.85mm
5	6.65±1.1mm	7.6±2.6mm	0.95mm
6	7.85±2.5mm	7.35±0.1mm	0.5mm(minimum)
7	12.85±4.2mm(maximum)	8.05±0.5mm(maximum)	4.8mm(maximum)
8	10.56±4.6mm	7.55±2.5mm	3.01mm

Table-22: Comparative inhibition of *Vibrio parahemolyticus* by probiotic strains & their metabolites

e. Comparative inhibition against *Staphylococcus aureaus*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.4±2.6mm	9.05±3.0mm (maximum)	1.65mm
2	8.65±2.3mm	10.3±0.0mm	1.65mm
3	7.35±0.5mm	8.9±0.0mm	1.55mm
4	9.15±1.7mm	6.85±1.1mm	2.3mm
5	15.00±0.6mm(maximum)	6.65±1.1mm (minimum)	8.35mm(maximum)
6	10.9±0.9mm	7.35±1.9mm	3.55mm
7	6.1mm±0.0 (minimum)	8.1±0.4mm	2.0mm
8	7.5±0.8mm	7.0±3.3mm	0.5mm(minimum)

Table-23: Comparative inhibition of *Staphylococcus aureaus* by probiotic strains & their metabolites

f. Comparative inhibition against *E.coli*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.65±1.1mm	7.6±2.2mm	0.05mm(minimum)
2	7.9±0.4mm	7.55±2.7mm	0.35mm
3	12.85±4.2mm(maximum)	6.5±0.8mm	6.35mm(maximum)
4	9.8±3.7mm	6.45±0.5mm	3.35mm
5	12.15±0.5mm	6.15±0.1mm (minimum)	6.0mm
6	7.7±0.4mm	6.7±1.2mm	1.0mm
7	7.6±0.0mm	7.75±0.9mm	0.15mm
8	6.8±0.0mm (minimum)	9.55±2.0mm (maximum)	2.75mm

Table-24: Comparative inhibition of *E.coli* by probiotic strains & their metabolites

g. Comparative inhibition against *Shigella dysenteriae*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.55±0.5mm	7.6±1.4mm	0.05mm(minimum)
2	7.5±2.2mm	6.9±0.0mm	0.6mm
3	7.1±2.8mm	_	7.1mm
4	8.3±1.7mm(maximum)	_	8.3mm (maximum)
5	7.05±mm	6.2±0.2mm(minimum)	0.85mm
6	6.4±0.0mm (minimum)	6.45±0.5mm	0.05mm
7	8.05±0.7mm	6.8±0.0mm	1.25mm
8	6.5±0.8mm	8.55±0.7mm (maximum)	2.05mm

Table-25: Comparative inhibition of *Shigella dysenteriae* by probiotic strains & their metabolites

h.Comparative inhibition against *Sarcina lutea*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	7.8±0.0mm	7.6±1.8mm	0.2mm(minimum)
2	6.25±0.3mm	6.7±0.2mm (minimum)	0.45mm
3	6.3±0.0mm	7.3±1.2mm	1.0mm
4	7.6±0.0mm	7.35±0.9mm	0.25mm
5	6.15±0.1mm(minimum)	8.1±3.6mm (maximum)	1.95mm
6	7.8±0.0mm	7.3±1.6mm	0.5mm
7	9.1±0.0mm	6.95±0.5mm	2.15mm
8	9.2±0.0mm (maximum)	7.0±1.6mm	2.2mm(maximum)

Table-26: Comparative inhibition of *Sarcina lutea* by probiotic strains & their metabolites

i. Comparative inhibition against *Candida albicans*-

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	8.5±1.2mm	8.15±1.3mm	0.35mm(minimum)
2	9.5±2.4mm	7.8±0.6mm	1.7mm
3	10.95±0.3mm(maximum)	8.25±1.9mm	2.7mm (maximum)
4	8.85±0.5mm	7.75±2.7mm	1.1mm
5	7.7±1.0mm	7.25±1.3mm (minimum)	0.45mm
6	8.2±0.2mm	7.3±1.2mm	0.9mm
7	6.15±0.1mm (minimum)	8.3±0.4mm	2.15mm
8	7.35±2.4mm	9.1±1.0mm (maximum)	1.35mm

Table-27: Comparative inhibition of *Candida albicans* by probiotic strains & their metabolites

j. Comparative inhibition against *Apergillus niger* -

Code	Inhibition by Isolated Bacteria(IIB)	Inhibition by Isolated Metabolites(IIM)	Difference (IIB-IIM)
1	6.75±0.3mm (minimum)	6.5±0.8mm	0.25mm
2	7.2±0.0mm	6.95±1.3mm	0.25mm
3	7.2±1.8mm	7.0±0.3mm	0.2mm (minimum)
4	7.75±1.1mm	6.1±0.0mm (minimum)	1.65mm
5	7.8±1.4mm (maximum)	6.1±0.0mm	1.7mm (maximum)
6	7.7±0.0mm	6.1±0.0mm	1.6mm
7	7.7±0.8mm	7.35±2.1mm	0.35mm
8	6.8±0.8m	7.7±1.8mm (maximum)	0.9mm

Table-28: Comparative inhibition of *Apergillus niger* by probiotic strains & their metabolites

4.4 Physiological and Biochemical Characterization

Physiological and Biochemical Characterization is more important for isolation and identification of Lactobacillus bacteria. This characterization was done by different carbohydrate fermentation test and 6.5 % sodium chloride concentration tolerance test.

4.4.1 Glucose fermentation test

Probiotics utilize glucose to form lactic acid by either the homo- or heterofermentative pathway. The homofermentative pathway, results in the transformation of glucose to pyruvate through the glycolysis, eventually yielding lactic acid. The heterofermentative pathway, produced lactate, CO2, and either acetic acid or ethanol (Gottshalk, 1986).

In glucose fermentation test, 6 strains were fermented in glucose. This result was confirmed by changing color and pH of the media but other 2 strains 5 & 6 are not ferment glucose . The pH of the blank media was found 7.4. The pH of the *E. coli* was found 4.8 and changed the color of media. That is confirmed, this experiment was done appropriately. The pH of strain code 1, 2, 3, 4, 5, 6, 7, 8 was found 6.2, 6.2, 6.0, 5.6,7.2, 7.3, 5.3, 5.7 respectively.

4.4.2 Sucrose fermentation test

In sucrose fermentation test, produced acid by probiotics. 90 % or more strains of probiotics are positive in sucrose fermentation test (Kavitha *et al*., 2013). 90 % or more strains of Lactobacilli are positive in sucrose fermentation test (Roos *et al*., 2005, and Hammes, 1995).

In sucrose fermentation test, 6 strains were fermented in sucrose but 2 strains 5 & 6 are unable to ferment sucrose. This result was confirmed by changing color and pH of the media. The pH of the blank media was found 7.7. The pH of the *E. coli* was found 5.4 and changed the color of media. That is confirmed, this experiment was done appropriately. The pH of strain code 1, 2, 3, 4, 5, 6, 7, 8was found 7.1, 7.2, 6.4, 6.1, 7.4, 7.5, 6.2 and 6.1 respectively.

4.4.3 Sorbitol fermentation test

The result of this test is very important for the isolation of lactobacillus. *Lactobacillus acidophilus is* unable to ferment arabinose and sorbitol (Hassan Pyar *et al*, 2013).

In sorbitol fermentation test, only 3 strain was fermented in sorbitol but other strain were test negative. This result was confirmed by changing color and pH of the media. The pH of the blank media was found 7.7. The pH of the *E. coli* was found 6.8 and changed the color of media. That is confirmed, this experiment was done appropriately. The pH of strain code 1, 2, 3, 4, 5, 6, 7, 8 was found 6.9, 7.7, 7.7, 6.7, 7.7, 7.8, 6.4 and 4.1 respectively.

4.4.4 Sodium chloride (6.5%) tolerance test

The result of this test is also important for the identification of lactobacillus. Lactobacilli tolerate 2%, 4% and 6.5% NaCl concentration. (Estifanos Hawaz., 2014).

In this test, all strains were grown in 6.5 % sodium chloride concentration. So, all strains were tolerated against 6.5 % sodium chloride concentration. This result was confirmed by the turbidity and compared with blank media, bacterial subculture in nutrient broth without sodium chloride. The growth of the positive control was done.

4.5 SDS-PAGE Analysis

Bacteria are simple, single celled microorganisms that can exist in a variety of different shapes. They are identified and classified largely in a series of biochemical tests or through molecular characterization. SDS-PAGE is an important molecular technique used for the identification at species level of whole cell proteins (Leisner *et. al.,* 1994). SDS-PAGE was carried out using 12% (w/v) separating and 5% (w/v) stacking gels. Protein Marker is a mixture of 12 recombinant highly purified proteins, which resolve into clearly identifiable sharp bands from 10-250 kDa when analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Sambrook *et al*., 2001). In the present study, migration of protein bands was observed at the range of 15 to 150 kDa and some common bands of the protein were seen.

4.5.1 SDS-PAGE Analysis for Isolated Strains

From Fig-9, we observed that all isolated probiotics strains produced various protein bands according to molecular weight (kDa).We know that, protein or DNA migrates from higher molecular weight to lower molecular weight in SDS-PAGE. SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field. Molecular weights of whole cell proteins are analyzed by using a high molecular weight standard marker (Bio-Red Marker).Comparing with standard marker, we found that one number strain yield two proteins bands and size of them are 80 kDa and 60 kDa respectively. Strain 2 yield 3 proteins bands and molecular weight of them are 58kDa, 55kDa, 10kDa respectively. Strain 3 produced 2 proteins bands and size them 84kDa, 13kDa respectively. Strain 4 yield only 1 proteins bands and 15 kDa in size. Strain 5 produced 2 proteins bands and their molecular weight are 150kDa ,15kDa respectively .Strain 6 yield 3 proteins bands and molecular weight of them are 40kDa,32kDa & 15kDa respectively. Strain 7 produced 2 proteins bands and size of them are 28kDa,14kDa respectively. Strain 8 yield 2 proteins bands and their molecular weight are 27kDa and 13kDa respectively.

4.5.2 Identification of Probiotics by molecular weight

From SDS-PAGE Analysis we found various proteins bands.Proteinss bands are used to identify genus and species of bacteria.We try to determine bacteria according to molecular weight of all strains.

In strain 1,we found 80 kDa protein band, which is equal to protein band of *Lactobacillus casei*.But in strain 1,we also found 60kDa protein, which also found in *Steptococcus thermophilus*.So, strain 1 may be *Lactobacillus casei* or *Steptococcus thermophilus.* (Mahmood et al-2013).But selection and probiotics characterization test,it may be *Lactobacillus casei*

Strain 2 produced 58kDa,55kDa band. *Lactobacillus acidophilus* CRL 639 contain 58 kDa cell surface protein. So,from bio-chemical test ,strain 2 may be *Lactobacillus*

acidophilus. (B. Kos et al-2003 and from Strain 3 contain 84kDa protein band is same protein produced by *Lactobacillus.salvarius* and is cell wall associated protein (Colum Dunne et al-2004).

Strain 4 contain 15 kDa protein band and *Oenococcus oeni* has the same protein band(Maria João Catalão-2010).

Strain 5 has 150kDa and 15kDa protein band. *Propionibacterium freudenreichii* also contain same protein. So, strain 4 probably *Propionibacterium freudenreichii*. (Hélène Falentin et al.2010).

Strain 6 has 40kDa, 32kDa protein band. S-layer protein of *L.amylovorus* is 40kDa.So, strain may be *L.amylovorus* (Ulla Hynönen et all.-2014).

Strain 7 produced 28 kDa and 14kDa protein band. May be lactobacillus but may be *L.acidophillus*(Mahmood, Talat -2013).

Strain8 contain27kDa and 13kDa protein. *Steptococcus thermophilus* has 27 kDa protein but *Lactobacillus rhamnosus* contain 13kDa protein.So,Strain 8 may be *Steptococcus thermophilus* or *Lactobacillus rhamnosus GG.* (Ija Talja et al-2014).But in biochemical test.it may be *Lactobacillus rhamnosus.*

4.5.3 SDS-PAGE Analysis for Isolated Metabolites

From Fig-10, we observed that all isolated metabolites of probiotics strains produced various protein bands according to molecular weight (kDa).We know that, protein or DNA migrates from higher molecular weight to lower molecular weight in SDS-PAGE. SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field. Molecular weights of metabolites are analyzed by using a high molecular weight standard marker (Bio-Red Marker). Comparing with standard marker, we found that one number metabolite yield 1 proteins bands and size of it is 80 kDa. Metabolite 2 also yield 1 proteins bands and molecular weight of it is 70kDa.Metabolites 3 produced 2 proteins bands and size them 70kDa, 15kDa respectively. Metabolite 4 yield only 2 proteins bands and 80kDa, 70kDa in size. Metabolite 5 produced 1 proteins

bands and molecular weight is 80kDa.Metabolites 6 yield 2 proteins bands and molecular weight of them are 90kDa, 80kDa respectively. Metabolite 7 produced 1 proteins bands and size of them are 80kDa. Metabolite 8 also yield proteins bands and molecular weight is 80kDa and 13kDa.

4.5.4Comparison between probiotics cell proteins and their metabolites

From SDS-PEAGE Analysis, we can compare molecular weight of cell proteins and their metabolites. In case of strain one, we found 2 proteins bands (80kDa & 60kDa) but their metabolites yield only 80kDa protein band. So, difference of cell proteins and metabolites are ±20kDa. Strain 2 has 3 proteins bands of cell with molecular weight 58kDa, 55kDa & 10kDa but their metabolite has only one protein band with 70kDa long. So, metabolite band is 12kDa long than cell protein. Strain has 3 proteins bands with molecular weight 84kDa, 13kDa but their metabolites produced 70kDa, 15kDa proteins bands. So, bands difference of them 14kDa and 2kDa respectively. Strain 4 produced only one protein band with molecular weight 15kDa but its metabolite produced higher bands (80kDa & 70kDa) than it. Strain 5 yield 2 proteins bands with molecular weight 150kDa, 15kDa but their metabolite produced 80kDa in size. So, difference of them is 70kDa long. Strain 6 has three proteins bands with molecular weight 40kDa, 32kDa & 15kDa but it metabolite has two proteins bands with molecular weight 90kDa, 70kDa.Difference first two of them are 50kDa and 32kDa respectively. Strain 7 yield two bands with 28kDa,14kDa in size but it metabolite has only 80kda band. Strain 8 produced 27kDA & 13kDa proteins bands but their metabolite yield 80kDa & 13kDa respectively. Second band are similar for both cell and metabolite.

CONCLUSION

From this reaserch we find out Probiotic Bacteria and genus Lactobacillus. Isolated strains are probiotic in nature and two strains are lactobacillus. We confirmed by performing various test; e.g- Gram staining, Catalase test, Resistanc to low pH (3.0 & 1.0), Bile salt tolerance, Antimicrobial activity test, Carbohydrate fermentation test (Glucose, Sucrose & Sorbitol),Tolerance against 6.5% NaCl. We mainly isolate bacterial metabolites and observe anti-bacterial activity of strains & metabolites and compare them. We found significant inhibition against 10 pathogenic bacteria by using isolated bacteria and their metabolites but strains shows more inhibitory activity than isolated metabolites. Proteins estimation of strains and metabolites are done by SDS-PAEGE Analysis.Various proteins bands were found by comparing Standard marker.According to molecular weight and from various physical,biochemical test,we can said that isolated strains may be *Lactobacillus casei*, *Steptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus.salvarius, Oenococcus oeni, Propionibacterium freudenreichii, Lactobacillus.amylovorus, Steptococcus thermophilus, Lactobacillus rhamnosus* .

Major trait of probiotics is inhibition of other pathogenic bacteria and survive in stomach pH (1.5-3.0) and intestine pH(7.1-7.4).Above information, we can concluded that, isolated bacteria from local Youghart are probiotics and their metabolites has anti-bacterial activity. Future investigation include-

- Antibacterial activity against resistance bacteria
- Metabolites or Bacteriocin purification
- Comparative comparison between metabolites and pharmaceuticals antibiotics
- Biopreservative activity of metabolites etc.

Use of probiotics has long history and are used for various healing purpouse.So,by proper evaluation and investigation, isolated strains can be used as probiotics. For solving anti-biotic resistance and health hazard, now probiotics are main intention of researcher and scientist.Human being is affected all time by different pathogenic bacteria and disease is done. Probiotic bacteria allaways give benifecial effects.

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