Isolation, Identification and Characterization of Probiotic Bacteria, Development of Bio-Yogurt and Its Effects of Feeding on Mice.

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Isolation, Identification and Characterization of Probiotic Bacteria, Development of Bio-Yogurt and Its Effects of Feeding on Mice.

A thesis submitted to

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DECLARATION

This thesis is the original research work of the author, except as otherwise stated. The material presented has not been submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.

Date: 10 November, 2016

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Dedication

Dedicated
To
My beloved Parents

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The Author

ABSTRACT

Widespread interest in probiotic bacteria that can be observed nowadays results from their medicinal properties reported both for human and animal subjects. Probiotics are generally recognized as preparation of or a product containing viable single or mixed cultures of safe, defined microorganisms in sufficient numbers that are used as functional food components with claimed health-promoting effects. Yogurt is one of the potential source of probiotic bacteria as well as an ancient famous nutritious food which have variety of beneficial effect on human. With this perspective, the aim of the present study was the isolation of probiotic bacteria from regional yogurt, their physiological and biochemical identification, study of their probiotic properties along with development of yogurt using the isolates and *in vivo* mice trial regarding body weight gain, blood cholesterol level and antibiotic resistance of gut microflora. In the present study, a total of nine isolates of which three isolates of each *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Bifidobacterium* spp. were obtained from three different yoghurt samples of Khulna City Corporation area, Khulna, Bangladesh. According to their morphological, physiological and biochemical assay, it was observed that all the nine isolates of three species were gram positive, endospore negative, catalase negative which are the characteristic of typical probiotic bacteria. Carbohydrate/sugar fermentation profiles experiment using eight important sugars ensured the presumptive identification of these three species. The NaCl tolerance test results have shown that all the isolates had excellent tolerance against 1-5% NaCl, moderate in 6-8% and in 9%-10% NaCl, low or no growth were observed after 24 hours of incubation at 37^0 C. They showed very good tolerance against 0.1% and 0.3% phenol and moderate or low tolerance against at 0.4% phenol after 24 hours of incubation at 37^0 C, respectively. All the nine isolates were passed to bile salt tolerance test at 0.05%, 0.1%, 0.15%, 0.2% and 0.3% during 4, 8 and 24 hours of incubation. They showed resistance to artificial gastric juice environment at pH 2.2 and 6.6. These nine isolates showed antimicrobial activity against *Shigella flexeri*, *Shigella dysenteriae*, *Vibrio cholerae* and *Salmonella typhi*. *Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp. also showed *antimicrobial activity against Staphylococcus epidermis, Pseudomonas spp. and E. coli.* After confirming their probiotic properties, yogurt was developed using all the nine isolates as inoculums. Developed yogurt was tested for pH and their probiotic dose and subjected to mouse trial for conducting efficacy and safety study. After 28 days *in vivo* mice trial, all three treatment groups showed significant body weight gain and decreased blood cholesterol level along with increased antibiotic resistant gut microflora in comparison to control group. All these result showed that these nine isolates had probiotic properties and the probiotic yogurt produced using the isolates had a beneficial effects on mice. However, most of the results from the experiments have shown that there were variations in probiotic properties of the nine isolates belonging to three species. Molecular identification of isolated Probiotic bacteria is needed for technological interest and probiotic product development both for human and poultry.

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ABBREVIATIONS

AC = Ash content.

AOAC = Association of Official Analytical Chemists

CFU= Colony forming unit.

CRD = Completely Randomized Design.

FAO = Food and Agriculture Organization.

FDA = Food and Drug Administration.

FGM = fat globule membrane.

GRAS = Generally Recognized as Safe

ICDDR, B = International Centre for Diarrhoeal Disease Research, Bangladesh.

IDF = International Dairy Federation.

LAB = Lactic acid bacteria.

Lb = Lactobacillus

MC = Moisture content

MRS = De Man, Rogosa and Sharp.

SAS = Statistical analysis system.

SE = Standard mean.

SEM = Standard error mean.

SPSS = Statistical Pakage for Social Science.

TA = Titratable acidity.

TS = Total solid.

CHAPTER: ONE

INTRODUCTION

INTRODUCTION

Increasing industrialization, urbanization and mechanization in the world have led to dramatic changes in dietary pattern and lifestyle of the human being which in turn has caused an increase in the occurrence of a variety of diseases and health. Therefore, in recent years, people seeking a healthier life style prefer diets having low- or reduced-fat and fat free foods; this has led to the development of functional foods. Functional foods can be defined as foods 'that can beneficially affect one or more target functions in the body, beyond adequate nutritional effect, in a way relevant to an improved state of health and well being and reduction of risk of disease' (Stanton et al.,2005). Dairy products, particularly those containing probiotics, prebiotics and synbiotics are most popular in this category of foods. Widespread interest in probiotic bacteria that can be observed nowadays results from their medicinal properties reported both for human and animal subjects. Probiotics have a long history of human use as cultured dairy products and are traditionally consumed in several parts of the world. The FAO/WHO defines probiotics as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'. The probiotic concept has been defined by Fuller (1989) to mean "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yoghurt, soy yoghurt or as dietary supplements.

From health point of view, ingestion of live cells of certain species and strains like probiotic bacteria in adequate amounts is believed to confer several beneficial physiological effects on the host (reviewed by Tannock, 2004). Such as managing lactose intolerance, improving immune system, prevention of colon cancer, reduction of cholesterol and triacylglycerol plasma concentrations, lowering blood pressure, reducing inflammation, reduction of allergic symptoms, beneficial effects on mineral metabolism, particularly bone density and stability, suppression of pathogenic microorganisms (antimicrobial effect), reduction of helicobacter pylori infection, prevention of osteoporosis, prevention of urogenital infections (Çakır 2003, Scherezenmeir and De Vrese 2001, Dunne, et al. 2001, Dugas, et al. 1999).

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rod shaped, catalase-negative and fastidious organisms, considered as 'Generally Recognized as Safe' (GRAS) organisms (Patil et al, 2010) and can be safely used for medical and veterinary applications (Fuller, 1989). These type of bacteria usually found in decomposing plants and lactic products. In humans they are present in the gastrointestinal tract and the vagina, where they are symbiotic and make up a small portion of the gut flora. They produce lactic acid as the major metabolic end-product of carbohydrate fermentation. This trait has, throughout history, linked LAB with food fermentations, as acidification inhibits the growth of spoilage agents and harmful bacteria. Proteinaceous bacteriocins are produced by several LAB strains and provide an additional hurdle for spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to the organoleptic and textural profile of a food item. Some lactic acid bacteria are used for the production of yoghurt, cheese, sauerkraut, pickels, beer, wine, cider, kimchi, chocolate and other fermented foods, as well as animal feeds, such as silage. The genera that comprise the LAB are at its core *Lactobacillus, Bifidobacteria ,Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus* as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weisella.*

Lactic acid bacteria are sometimes termed probiotic and are used as health adjuncts in food to provide a wide variety of health benefits which meet the criteria of probiotic bacteria (Metchnikoff, 1908). These bacteria, mainly *Lactobacilli* and *Bifidobacteria*, may have several therapeutic functions, including antimicrobial activity, ant icholesterol activity, improved lactose utilization, and anticarcinogenic activity which help to maintain a healthy and equilibrated intestinal microbiota and reducing incidence of intestinal infection (Chou and Weimer, 1999),(Mahrous, 2011).

The choosing criteria for the in vitro selection of lactic acid bacteria to be used as health promoting probiotic ingredients in food and pharmaceuticals preparations should possess the abilities of antibiotic tolerance due to their possible use to reconstitute the intestinal micro flora of patients suffering from antibiotic associated colitis as well as the production of lactic acid that inhibits the growth of other microorganism allow them to be established in the intestinal tract (Hoque et al, 2010). They also resistant to the enzymes in the oral cavity (e.g., lysozyme)

and should also have the ability to resist the digestion process in the stomach and the intestinal tract such as bile and gastric juice resistance (Chou and Weimer, 1999).

In Bangladesh yogurt is perhaps the oldest fermented milk product known and consumed by large segments of our population as a part of their daily diet. In most of the areas of Bangladesh different types' traditional yogurts are found but their probiotic role is in limited extend. Although yogurt is the potential source of beneficial lactic acid bacteria, a small number of evidence has been found so far regarding extensive study of its probiotic properties. Incorporation of probiotic live microorganism (isolated from indigenous yogurts) in market yogurts can positively enhance health status of larger segment of communities of Bangladesh, having with probiotic foods. Therefore, the present study was undertaken with the following objectives:

- Isolation of LAB in pure culture.
- Identification by morphological characteristics and biochemical assay
- Study on the probiotic properties.
- Development of Bio-yogurt.
- *In vivo* mice trial for measurement of body weight, blood cholesterol and antibiotic resistance of gut microflora.

CHAPTER: TWO

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The word 'probiotic' comes from Greek language 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much (Gismondo, et al. 1999, Guarner, et al. 2005). In 1908 a Russian researcher Ellie Metchnikoff, who has a nobel prize, firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (*Lactobacillus* spp.). Therefore, these bacteria affect the gut microflora positively and decrease the microbial toxic activity (Hattingh and Viljoen B C, 2001; Gismondo, et al. 1999; Çakır 2003; Chuayana et al. 2003).

The term 'probiotic' firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word 'probiotic' was used in different meaning according to its mechanism and the affects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined 'probiotic' as 'substances and organisms which contribute to intestinal microbial balance'. In 1989, the meaning use today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. Then this definition was broadened by Havenaar and Huis in't Veld in 1992 including mono or mixed culture of live microorganisms which applied for animal and man (Çakır 2003, Guarner, et al. 2005, Sanders 2003, Fuller 1989).

According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used as probiotics; but certain yeasts and bacilli may also be helpful. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt.

Probiotics are also challenging for the industrial applications. The probiotic concept is open to lots of different applications in a large variety of fields relevant for human and animal health. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have beneficial effects on the health of host. They can contain one or several species of probiotic bacteria. Most of products which destine human consumption are produced in fermented milk or given in powders or tablets. These capsules and tablets do not used for medicial applications. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. Lots of studies suggest that probiotics have beneficial effects on microbial disorders of the gut, but it is really difficult to show the clinical effects of such products. The

probiotic preperations use for traveller's diarrhoea, antibiotic associated diarrhoea and acute diarrhoea which is showned that they have positive therapeutic effect (Gismondo,et al. 1999, Çakır 2003).

More than 400 bacterial species exit in human intestinal tract. It is an enormously complex ecosystem that includes both facultatively anaerobic and anaerobic microorganisms (Naidu, et al. 1999). The numbers of genera is nearly steady, because they each have their own growth niches (Fooks, et al.1999). The composition of the gut microflora is constant but can be affected by some factors such as; age, diet, environment, stress and medication. To have a healthy intestine the balance of the bacteria must be maintained but this is difficult as the lifestyles change. Lots of factors may change the balance away from potenially beneficial or health promoting bacteria like lactobacilli and bifidobacteria to potentially harmfull or pathogenic microorganisms like clostridia, sulphate reducers and *Bacteroides* species. It makes the host more susceptible to the illnesses. In this case the prevalence of the beneficial bacteria must be supported. Using of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks, et al. 1999). Probiotics are suggested as food to provide for the balance of intestinal flora. 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).

Table 2.1: The probiotic micro-organisms which are use to feed both man and animals

Micro-organisms	Strain	Company(products
Acidophilus Species		
L. acidophilus	LA-1/LA-5,NCFM, DDS-1,SBT-2062	Chr. Hansen, Rhodia, Nebraska Cultures Snow Brand Milk Products
L. rhamnosus	GG,GR-1,LB21,271	Valio,Urex Biotech,Essum AB, Probi AB
L. gasseri		
L. casei		Shirota Yakult (Yakult)
L. reuteri	SD2112/MM2	Biogaia
L. delbrueckii subsp. Bulgaricus	Lb12	
L. crispatus		
L. plantarum	299v,Lp01	Probi AB
L. salivarus		
L. johnsoni		
L. gallinarum		

Micro-organisms	Strain	Company(products
L. plantarum		
L. fermentum	RC-14 Urex Biotech	
L. helveticus		
L. lactis	L1A	Essum AB
Lactobacillus paracasei	CRL 431	Chr. Hansen
Bifidobacterium Species		
B. bifidum	Bb-11	Chr. Hansen
B. animalis	Bb-12	
B. breve		
B. infantis	Shirota® Immunitas®	Yakult Danone
B. longum	BB536 SBT-2928 UCC 35624	Morinaga Milk Industry Snow Brand MilkProducts UCCork
B. lactis	L1A	Essum AB
B. adolocentis	ATCC 15703	
B. essencis	Danone®(Activia)	
Other Microorganisms		
Enterococcus faecalis, Streptococcus salivarus sub. Thermophilus, Lactococcus lactis subsp. Lactis, Lactococcus lactis subsp. Cremoris, Propionibacterium freudenreichii, Pediococcus acidilactici, Leuoconostoc mesenteroides		

(Ref: Soccol et al, 2010)

Potential Benefits

Experiments into the benefits of probiotic therapies suggest a range of potentially beneficial medicinal uses for probiotics. For many of the potential benefits, research is limited and only preliminary results are available. It should be noted that the effects described are *not* general effects of probiotics. Recent research on the molecular biology and genomics of *Lactobacillus* has focused on the interaction with the immune system, anti-cancer potential, and potential as a biotherapeutic agent in cases of antibiotic-associated diarrhoea, travellers' diarrhoea, pediatric diarrhoea, inflammatory bowel disease and irritable bowel syndrome (Ljungh et al, 2009).

All effects can only be attributed to the individual strain(s) tested. Testing of a supplement does not indicate benefit from any other strain of the same species, and testing does not indicate benefit from the whole group of LAB (or other probiotics). So, while a number of reported effects have been only partially established, some can be regarded as well established and clinically well documented for specific strains.

Table 2.2: Claimed beneficial effects and therapeutic application of probiotic bacteria in humans

Beneficial effects:	• Reduction of lactose-intolerance. • Maintenance of normal intestinal microflora • Enhancement of the immune system • Reduction of serum cholesterol levels • Anticarcinogenic activity • Improved nutritional value of foods
Therapeutic applications:	• Suppression of pathogenic microorganisms (antimicrobial effect). • Prevention of urogenital infection • Alleviation of constipation • Protection against traveller's diarrhoea • Prevention of infantile diarrhoea • Reduction of *Helicobacter pylori* infection. • Reduction of antibiotic-induced diarrhoea • Prevention of hypercholesterolaemia • Protection against colon/bladder cancer • Prevention of osteoporosis • Beneficial effects on mineral metabolism, particularly bone density and stability. • Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence). • Lowering blood pressure. • Reduction of allergic symptoms.

(Ref: Fuller, 1989)

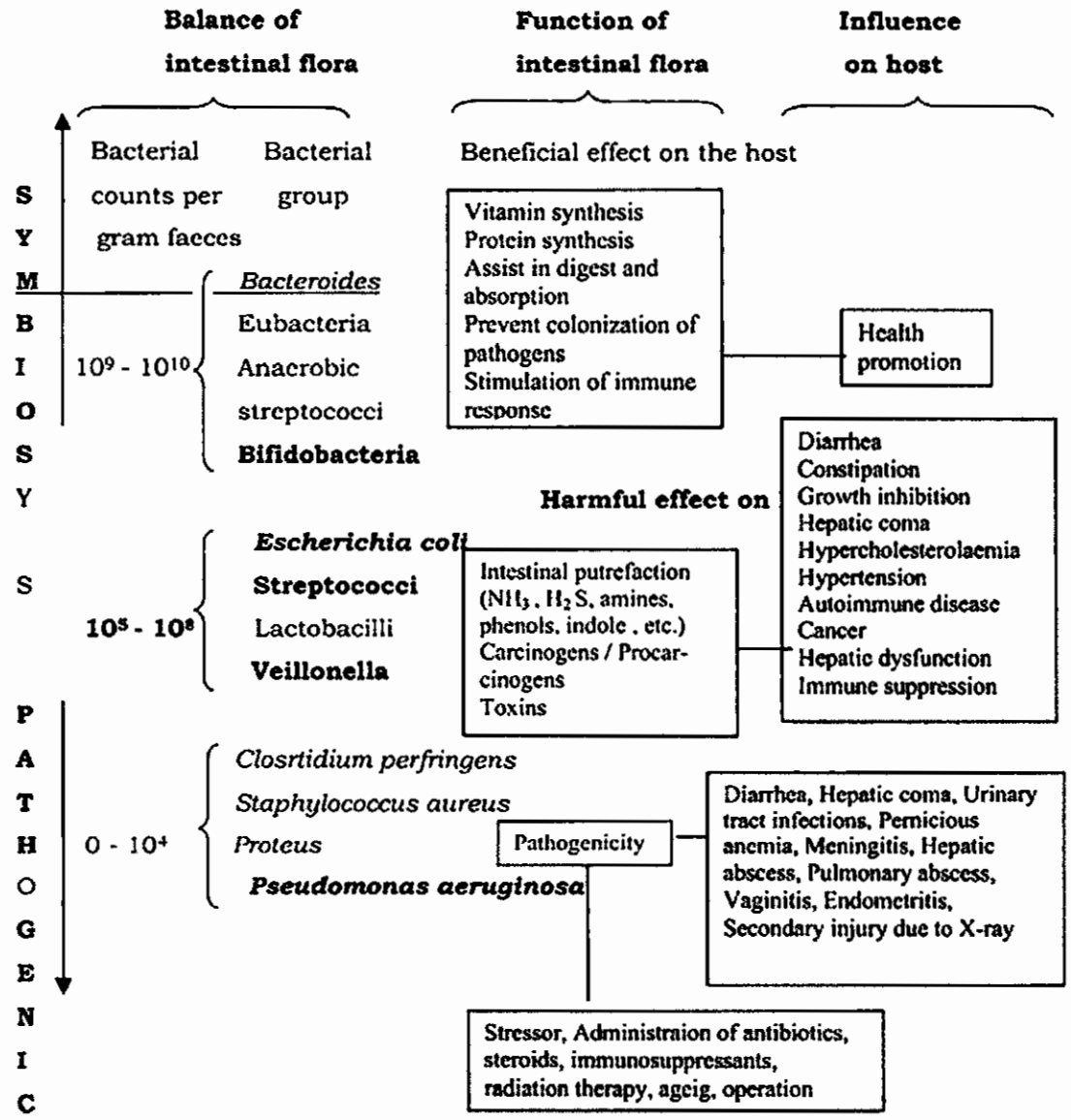

Figure 2.1: The interrelationship between intestinal bacteria and human health (Ishibashi and Shimamura, 1993).

Managing Lactose Intolerance

The inability to digest lactose adequately by certain people is due to the absence of b-d-galactosidase in the human intestine and this leads to various degrees of abdominal discomfort (Kim a Gilliland, 1983). As lactic acid bacteria actively convert lactose into lactic acid, ingestion of certain active strains may help lactose intolerant individuals tolerate more lactose than what they would have otherwise (Sanders, 2000). Some lactic acid bacteria used as starter cultures in milk and fermentation, and probiotic bacteria such as L. acidophilus and B. bifidum produce b-d-

galactosidase. This enzyme hydrolyses lactose, which results in increased tolerance for dairy products (Kim a Gilliland, 1983).

Anti Carcinogenic Activity and Prevention of Colon Cancer

The antitumour action of probiotics is attributed to the inhibition of carcinogens and procarcinogens, inhibition of bacteria that convert pro-carcinogens to carcinogens, activation of the host's immune system and reduction of the intestinal pH to reduce microbial activity (Hattingh and Viljoen, 2001). In laboratory investigations, some strains of LAB (*Lactobacillus bulgaricus*) have demonstrated anti-mutagenic effects thought to be due to their ability to bind with heterocyclic amines, which are carcinogenic substances formed in cooked meat. Animal studies have demonstrated that some LAB can protect against colon cancer in rodents, though human data is limited and conflicting. Most human trials have found that the strains tested may exert anti-carcinogenic effects by decreasing the activity of an enzyme called β-glucuronidase (which can generate carcinogens in the digestive system). Lower rates of colon cancer among higher consumers of fermented dairy products have been observed in one population study (Sanders, 2000).

Cardiovascular Disease Risk through Lowering Serum Cholesterol

There are claims that consumption of fermented milk significantly reduces serum cholesterol (Gilliland, Nel-son and Maxwell, 1985; Gilliland, 1989). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks. The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Claims are strong that certain L. Acidophilus strains and some bifidobacteria species are able to lower cholesterol levels within the intestine. Cholesterol co-precipitates with deconjugated bile salts as the pH declines as a consequence of lactic acid production bythe lactic acid bacteria (Marshall, 1996). Another theory is that L. acidophilus deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than are conjugated bile acids. As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977b). A third hypothesis is that reduction of cholesterol may also be due to a co-precipitation of cholesterol with deconjugated bile salts at lower pH Lowering blood pressure values as a result of lactic acid production by the bacteria (Kailasapathy a Rybka, 1997).

Improving Immune Function and Preventing Infections

LAB are thought to have several presumably beneficial effects on immune function. They may protect against pathogens by means of competitive inhibition (i.e., by competing for growth) and there is evidence to suggest that they may improve immune function by increasing the number of

IgA-producing plasma cells, increasing or improving phagocytosis as well as increasing the proportion of T lymphocytes and Natural Killer cells. Clinical trials have demonstrated that probiotics may decrease the incidence of respiratory tract infections and dental caries in children. LAB foods and supplements have been shown to aid in the treatment and prevention of acute diarrhea, and in decreasing the severity and duration of rotavirus infections in children and travelers' diarrhea in adults (Reid et al, 2003). It was demonstrated that dietary consumption of B. lactis HN019 and L. rhamnosus HN001 resulted in measurable enhancement of immune parameters in the elderly (Arunachalam et al., 2000). Probiotic modulation of host immunity is a very promising area for research. Supportive data is emerging, such as those carried out in humans showing that probiotic microorganisms can enhance NK cell activity in the elderly (Gill et al., 2001) and non-specific host defenses can be modulated (Donnet-Hughes et al., 1999)

Helicobacter Pylori

LAB are also thought to aid in the treatment of *Helicobacter pylori* infections (which cause peptic ulcers) in adults when used in combination with standard medical treatments. However more studies are required into this area.

Antibiotic Associated Diarrhea

Antibiotic-associated diarrhea (AAD) results from an imbalance in the colonic microbiota caused by antibiotic therapy. Microbiota alteration changes carbohydrate metabolism with decreased short-chain fatty acid absorption and an osmotic diarrhea as a result. Another consequence of antibiotic therapy leading to diarrhea is overgrowth of potentially pathogenic organisms such as *Clostridium difficile*.

Probiotic treatment can reduce the incidence and severity of AAD as indicated in several meta-analyses. However, further documentation of these findings through randomized, double blind, placebo-controlled trials are warranted.

Efficacy of probiotic AAD prevention is dependent on the probiotic strain(s) used and on the dosage. Up to a 50% reduction of AAD occurrence has been found. No side-effects have been reported in any of these studies. Caution should, however, be exercised when administering probiotic supplements to immunocompromised individuals or patients who have a compromised intestinal barrier.

Reducing Inflammation

LAB and supplements have been found to modulate inflammatory and hypersensitivity responses, an observation thought to be at least in part due to the regulation of cytokine function. Clinical studies suggest that they can prevent reoccurrences of inflammatory bowel disease in

adults, as well as improve milk allergies. They are not effective for treating eczema, a persistent skin inflammation. How probiotics counteract immune system overactivity remains unclear, but a potential mechanism is desensitization of T lymphocytes, an important component of the immune system, towards pro-inflammatory stimuli (Braat et al, 2004).

Improving Mineral Absorption

It is hypothesized that probiotic lactobacilli may help correct malabsorption of trace minerals, found particularly in those with diets high in phytate content from whole grains, nuts, and legumes.

Preventing Harmful Bacterial Growth Under Stress

In a study done to see the effects of stress on intestinal flora, rats that were fed probiotics had little occurrence of harmful bacteria latched onto their intestines compared to rats that were fed sterile water.

Irritable Bowel Syndrome and Colitis

B. infantis 35624, sold as Align, was found to improve some symptoms of irritable bowel syndrome in women in a recent study.[39] Another probiotic bacterium, *Lactobacillus plantarum* 299v, was also found to be effective in reducing IBS symptoms.[40] Additionally, a probiotic formulation, VSL#3, was found to be safe in treating ulcerative colitis, though efficacy in the study was uncertain. *Bifidobacterium animalis* DN-173 010 may help.[42] For maintenance of remission of ulcerative colitis, Mutaflor (*E.coli* Nissle 1917) there are 3 controlled, randomized, double blind clinical studies which have proven equivalence of Mutaflor and mesalazine (5-ASAs) (Ruis et al, 2004).

Managing Urogenital Health

Several in vitro studies have revealed probiotics' potential in relieving urinary tract infections and bacterial vaginosis. Results have been varied on these studies, and in vivo studies are still required in this area to determine efficacy.

Mechanism of Probiotics

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel, et al. 1998). There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly (Rolfe 2000, Çakır 2003).

1. Production of inhibitory substances: Production of some organic acids , hydrogen peroxide and bacteriocins which are inhibitory to both gram-positive and gram-negative bacteria.

2. Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.

3. Competition for nutrients: Despite of the lack of studies in vivo, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.

4. Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvants and increase humoral immune response.

5. Degradation of toxin receptor: Because of the degredation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* protects the host against *C. difficile* intestinal disease.

Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks, et al. 1999).

Selection Criteria for 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 selection criteria are listed in Table 1.2 briefly. Some of them will be disscussed in more details. A potential probiotic strains does not need to fulfill all such selection criteria

Table 2.3: Selection criteria for probiotics

Probiotic Strain Properties	Remarks
Human origin for human usage	Although the human probiotic *Saccharomyces boulardii* is not human origin, this criteria 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	Imortant 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 accuratly, documented safety. No invasion and no degradation of intestinal mucus.
Clinically validated and documented health effects	Minumum effective dosage has to be known for each particular strain and in different products. Placebo-controlled, double-blinded and randomized studies have to be run.
Good technological properties	Survival in products if viable organisms are required, phage resistance, strain stability, culturable in large scales, oxygen resistance, have no negative effects on product flavour.

(Ref: Quwehand, et al. 1999, Çakır 2003)

The selection criteria can be categorized in four basic groups. Appropriatness, technological suitibility, competitiveness, performance and functionality (Klaenhammer and Kullen, 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 1.2. (Saarela et al., 2000) proposed the properties of probiotics in three basic groups as; safety aspects, aspects of functionality and technological aspects.

Some major selection criteria will be discussed in details below.

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 and Weimer 1999, Çakır 2003). To show probiotic sufficiency, they should reach to the lower intestinal tract and maintain themselves over there.

Antimicrobial Activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer and Kullen 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 (Çakır 2003). Till today there are some researchs on showing that different species produce different antimicrobial substances, e.g. *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuterin; subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A.

Safety Aspects of Probiotics

Today, there are evidences that probiotic strains used as commercial bacteria are safe to use in applications. The safety of the probiotic products is appraised with the phenotypic and genotypic characteristics and the statistics of used microorganisms (Çakır 2003). Safety aspects of probiotic bacteria include the following requirements.

1. Strains for human use are preferred to be human origin.
2. They are isolated from healthy human gastrointestinal tract.
3. They have to be non-pathogenic.
4. They have to no history of relationship with diseases like, infective endocarditis or gastrointestinal tract disorders.
5. They do not deconjugate bile salts.
6. They should not carry transmissible antibiotic resistance genes

Molecular Identification of Probiotic Strains

Methods used for detection of probiotics in human gastrointestinal tract are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations they are used for identification. With the developing technology about the molecular typing it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for selection, enumeration and biochemical characterization (fermentation profiles, salt-pH-temperature tolerances) but it is not efficient to classify a culture taxonomically. Molecular characterization methods are powerful even between closely related species. There are number of alternative taxonomic classification methods well known including hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers (Klaenhammer and Kullen 1999). Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools (Bulut 2003). Comparison between these methods, the most powerful and accurate one is sequencing. Characterization of microorganisms according to their 16S rDNA regions sequencing was firstly proposed by Woese in 1987. The application of 16S or 23S rRNA-targeted oligonucleotide probes is the best and most reliable approach to identify bacteria on a phylogenetic basis.

Foods as a Source of Potential Probiotic Strain

There are many foods that contain probiotics, in addition to the numerous supplements available over-the-counter containing the microorganisms.

Human milk: After birth, breast milk is the best food for infants because it fullfills all the nutritional requirements for them during months. Also breast milk protects the newborn against infectious diseases. This effect seems a result of the action of some breast milk components, like different antimicrobial compounds, immunoglobulins, immunocomponent cells (Martin, et al. 2003) and also breast milk contains prebiotic substances which stimulate the growth of the benefical bacteria neonate gut (Martin, et al. 2004). In a general view human milk contains fat, protein, carbohydrate, minerals and bacteria.

Buttermilk: Buttermilk is gross for some people to drink by itself, but using it in recipes or adding it to fruit smoothies will be a major benefit because of the probiotics it contains.

Tempeh: This is a chewy soy product with a meat-like texture. Tempeh, when made, uses whole soybeans that are given enough time to ferment, forming a beneficial mold that helps hold the soy together. It is used in vegetarian dishes and contains an abundance of probiotics.

Miso: Miso is a Japanese seasoning produced by fermenting beans or grains. It is used in soups, sauces, and spreads.

Sauerkraut: sauerkraut is essentially fermented or pickled cabbage. During the time that the cabbage is fermenting, probiotics are formed.

Kim chi: this is another cabbage product. It smells awful, but contains a good dose of beneficial probiotics. It is like sauerkraut, but stronger in flavor and smell.

Brewer's yeast: brewer's yeast is a by-product of beer and contains probiotics.

Kefir: kefir is a beverage that contains probiotics; it is made from cow's milk, goat's milk, or sheep's milk, or from plant milks, like rice milk or soy milk. There are lactose and non-lactose varieties.

Yogurt as Probiotic Carrier Food

The growing popularity of yogurt over the years, has largely been increased due to its perceived health benefits. Yogurt is one of the best-known foods that contain "probiotics" which is a living microorganism, upon ingestion in sufficient amount, exerts beneficial effects on the normal microbial population of the gastrointestinal tract (Bourlioux et al., 2003). Different microbial species are considered as probiotics. The major strains *Lactobacillus acidophilus, L. casei* and various *Bifidobacterium* species , *B. longum, B. bifidum* are the most dominant species in human small and large intestine, that can inhibit the growth of pathogenic organisms, through production of organic acids and bacteriocins (Mazza, 1998). Among the most important food products, supplemented with variable probiotic strains, like *Lactobacilli* and *Bifidobacterium* strains yogurt is one of the most common, available and famous product. Yogurt is a coagulated milk product, that results from the fermentation of lactic acid in milk, by LAB bacteria *Lactobacillus bulgaricus* and *Strepetococcus thermophilus* (Pelczar et al., 1986). Its traditionally manufactured by fortifying whole or skimmed milk by evaporation or addition of skim milk powder, heating to 85-95 C° for 10-30 min and inoculating with LAB Bacteria and then incubating at 42-45 C.

Dairy products such as yogurt has been recognized as excellent sources of vitamins and minerals including riboflavin, phosophorus and calcium (Mazza, 1998). Up to date, many research studies focused on the role of probiotic Including (LAB) bacteria in the yogurt for the enhancement of the gastrointestinal function through increasing minerals absorption, reduction in lactose intolerance (lactase deficiency) and through consumption of *L. acidophilus* culture yogurt (Vesa et al., 2000, Martini et al., 1991).

CHAPTER: THREE

MATERIALS & METHODS

MATERIALS AND METHODS

Collection of Sample

Yoghurt samples were collected from three local Ghosh Dairy Shops viz. 1.Tala Adi Ghosh Dairy, 2. Satkhira Ghosh Dairy and 3. New Satkhira Ghosh Dairy of Khulna City Corporation areas. The samples were stored carefully at low temperature refrigerator (- 4°C) to protect it from contamination and deterioration.

Media Preparation and Isolation of Bacteria

The bacteria *Lactobacillus spp.* and *Bifidobacterium spp.* were isolated from yogurt sample using modified MRS agar media (De Man *et al.,* 1960) viz. MRS-Salicin and MRS- Cysteine and MRS-NNLP. Salicin (0.5%) and 0.05% cysteine were added to MRS to improve the specificity of the medium for isolation of *Lactobacillus acidophilus* and *Lactobacillus delb.* ssp. *bulgaricus,* respectively (Hartemink et al., 1997; Lankaputhra et al., 1995; Shah, 2000). On the other hand, MRS-NNLP agar media (Moriya et al., 2006) was used for isolating *Bifidobacterium spp* where 0.3% lithium chloride, 0.01% neomycin sulphate, 0.02% parmomycin sulphate and nalidixic acid (15µ/ml) was added to MRS agar media. Cysteine (0.05%) was also added for creating an anaerobic condition for *Bifidobacteria.*

The pH of the medium for isolation of *L. acidophilus, Lactobacillus delbrueckii ssp. bulgaricus* and *Bifidobacterium spp* was adjusted to 6.5 ± 2, 6.5 ± 2 and 5.2 respectively.

Table 3. 1: Compositions of MRS broth media (for 1 L)

Nitrogen source	polypeptone (10 g) meat extract (10 g) and yeast extract (5 g)
Carbon source	glucose (20 g)
Salts	K_2HPO_4 (2 g) Ammonium citrate (2 g) $MnSO_4 \cdot 4H_20$ (0.05 g)

	Magnesium Sulfate (0.10 g) Sodium Acetate (5.00 g)
Growth factor	Tween-80 (1 ml)
The pH was adjusted to 6.4± 0.2 before autoclaving at 121°C for 15 min	

Source: De man, Rogosa and Sharpe, 1960.

Preparation of Peptone Water

Peptone water (0.15%) was prepared by dissolving 1.5 g of peptone (Oxoid, West Heidelberg, Australia) in 1 L of distilled water, adjusting the pH to 7.0, followed by autoclaving at 121^0C for 15 min.

Isolation of Bacteria

Lactobacillus and *Bifidobacterium* were isolated from collected yoghurt samples using modified MRS agar medium at specific pH described as above. All the media were autoclaved at 121° C for 15 minute. Lithium chloride, neomycin sulphate, paramomycin sulphate and nalidixic acid were sterilized using a 0.42 µm pore membrane and then added to the sterilized MRS medium in a laminar flow at 50° C .

One gram of each sample was dissolved in 9 ml of 0.15% peptone water and diluted up to ten logarithmic (10^{-10}) fold. The diluted sample was then inoculated into the modified MRS agar plate by spread plate technique. The following criteria of pH level, incubation temperature and incubation time shown in the Table-2 was ensured for the isolation of *Lactobacillus* and *Bifidobacterium spp.*

Table 3. 2: Inoculum and conditions for growth of LAB isolates

Name of probiotic bacterial species	pH value	Incubation time (hour)	Incubation temperature (^{0}C)	Media Used
Lactoacillus acidophilus	6.5	24	37	MRS-Salicin agar
Lactobacillus delbrueckii ssp.bulgaricus	5.2	72	45	MRS-Cysteine agar
Bifidobacterium spp.	5.2	72	45	MRS-NNLP agar

Source: Linn *et al.*, 2008.

Many fastidious organisms are removed in the presence of acetate, citrate and Tween 80, because of anaerobic condition. In the present experiment, the growth of Lactobacillus and bifidobacteria were ensured by streaking on MRS agar plate by 5 times and observing the colony morphology and biochemical tests. Finally the single colony of *Lactoacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Bifidobacterium spp.* was isolated. The isolated culture was maintained in MRS broth at pH 6.5 (International Dairy Federation, 1998).

Identification of Probiotic Bacteria

Isolated bacteria were identified as *Lactobacillus spp* and *Bifidobacterium spp.* by observing their morphological characteristics such as colony morphology, bacterial shape and biochemical tests viz. catalase acttivites, gram stain, endospore test, 0.1% - 0.4% bacteriostatic phenol tolerance test and 1-10% NaCl tolerance tests. Finally, the species of the *Lactoacillus* and *Bifidobacterium* were identified using sugar fermentation test.

Catalase test was performed in order to observe their catalase reactions. 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.

$$2H2O2 \xrightarrow{\text{Catalase}} 2\,H2O + O2$$

For catalase test, overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h, 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Absence of gas bubble indicated the absence of catalase enzyme. The isolates, which did not give gas bubbles, were considered as catalase negative.

Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. LAB is 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 anaerobic condition. Cells from fresh cultures were used for gram staining. Harley and Prescott (2002) protocol was used for gram staining. The individual colony was picked up aseptically from the agar plate with loops, smeared on to a clean dry slide and heat-fixed. Then heat-fixed smear was flooded with crystal violet solution for 30 sec and rinsed with water for 5 sec. Then grams iodine solution was used to cover over the slide for 1 minute and rinsed with tap water for 5 sec. The slide was then decolorized with 95% ethanol for 15 to 30 sec and rinsed with 5 sec. Finally safranin was used as counter stain for 60-80 sec and rinsed with water. Then under light microscope gram positive bacteria were detected.

Endospore Test

The Endospore test of the isolates was performed by Schaeffer-Fulton method described by Harley and Prescott (2002). LAB is known to be non spore forming. It means that they give red color by Endospore test. LAB is non spore forming and by using loop, single colony of isolated probiotic bacteria was aseptically transferred on a slide which was air dried and heat fixed. The slide was covered with filter paper and then soaked with malachite green and then heated to steaming the stain for 5 to 6 minutes. Paper towel was removed and allowed to cool the slide and was rinsed with water for 30 sec. Finally, counter stained with safranin for 60 to 80 sec and

rinsed with water for 30 sec and then examined under light microscope. Vegetative cells stained as red and both endospore and free spore stained as green.

Sugar Fermentation Test

For species identification of lactic acid forming bacteria, sugar fermentation test was performed. In sugar fermentation test, dextrose of the MRS broth was replaced by different types of sugar such as ribose, sorbitol, mannitol,sucrose, fructose, cellobiose, salicin and lactose.

Materials:

- Phenol red (0.01 %)
- 10% Sugar solution
- An overnight culture in MRS broth
- Durham tube
- MRS broth

MRS broth (pH 6.5) was taken into screw cap test tube and phenol red (0.01 g per L) was added into the tube as pH indicator. The medium was autoclaved at 121°C for 15 min. After autoclaving, 1 ml different sugar solutions (10%) (filtered/sterilized) were inoculated into different tubes. Then 200 µl overnight liquid cultures were inoculated into the broth. Incubation was performed anaerobically at 37°C for 24 h. If fermenting bacteria are grown in a liquid culture medium containing the carbohydrates, they may produce organic acids as by-products of the fermentation. These acids are released into the medium and lower its pH. If a pH indicator such as phenol red or bromocresol purple is included in the medium, the acid production will change the medium from its original color to yellow.

Gases produced during the fermentation process was detected using a small, inverted tube, called a Durham tube (named after Herbert Edward Durham, English bacteriologist, 1866–1945), within the liquid culture medium. After adding the proper amount of broth, Durham tubes were inserted into each culture tube. During autoclaving, air was expelled from the Durham tubes, and they filled with the medium. Gas was produced, the liquid medium inside the Durham tube were displaced entrapping the gas in the form of a bubble.

NaCl Tolerance Test (1-10%)

For the determination of NaCl tolerance of isolated lactobacillus and bifidobacteria, 10 test tubes containing MRS broth were adjusted with different concentrations (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture and incubated at 37^0C for 24 h. After 24 h of incubation, their growth were determined by observing their turbidity. Maximum growth were indicated as triple positive sign (+++), moderate growth as double positive sign (++), low growth as single positive sign (+) and no growth as negative sign (-).

Phenol Tolerance Test (0.1-0.4%)

For the determination of phenol tolerance of isolated lactobacillus and bifidobacteria, 4 test tubes containing MRS broth were adjusted with different concentrations (0.1-0.4%) of phenol. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture and incubated at 37^0C for 24 h. After 24 h of incubation, their growth were determined by observing their turbidity. Maximum growth were indicated as triple positive sign (+++), moderate growth as double positive sign (++), low growth as single positive sign (+) and no growth were indicated as negative sign (-).

Bile Tolerance Test

Growth rate of bacterial cultures was determined in MRS broth containing different levels (0.05, 0.1, 0.15, 0.2 and 0.3%) of Oxgall. Final pH of the medium was adjusted to 6.5 and was autoclaved. Freshly prepared cultures were inoculated (1%) into medium and incubated at 37^0C for 24 h under anaerobic condition. Optical densities were measured spectro-photometrically at 620 nm after 4, 8 and 24 h for measurement of survival rate of the isolates.

Assay for Gastric Juice Tolerance

Preparation of Artificial Gastric Juice (for 1L)

NaCl (2 g), pepsin (3.2 g) was adjusted at a final pH 2.2 with HCl without dilution and was taken to 1L distilled water. As a control, artificial gastric juice was adjusted at a final pH 6.6 with 5N NaOH. Sterilization was performed by filtration (filter membrane 0.22 µm).

Method

The Lactobacilli under study were grown in MRS broth at 37^{0}C for 16 h and the cells were harvested by centrifugation at 5000g for 10 min. The pellets were washed twice, obtained with 0.1 M phosphate buffer at pH 7. The cells were resuspended to the original volume with the buffer by vortex. Then the artificial gastric juice having pH 2.22 and pH 6.6 were inoculated with the 2% (v/v) bacterial suspension. Both the media were incubated at 37^{0}C and samples were taken out at 0, 1, 2, 3, and 4h and after 24h for cell viability measurement by reading optical densities at 620nm and streaking on to MRS agar plate for ensuring their viability.

Antimicrobial Assay

Agar well diffusion method was modified (Schillinger and Lucke, 1989; Toba *et al.*, 1991) and used to detect antimicrobial activities of "Cell Free Supernatant" (CFS) produced from *Lactobacilli and Bifidobacterium* strains. These assays were performed in duplicate. The Petridishes were filled with 20 ml nutrient agar media (MHA, Difco) and four uniform rectangular shaped holes were created by taking off the agar media in each petridishes using a sterile tip and 15 µl, 20 µl and 25 µl of the CFSs were placed into three different holes and MRS broth was placed in one hole as negative control. The pathogenic strains were adjusted to 10^9 cfu/ml by adding sterile water and spreaded on the surface of nutrient agar. The petridishes were pre-incubated at 4^{0}C for diffusion and incubated aerobically overnight at 37°C. The petridishes were examined for detecting and measuring zones of inhibition.

Bile Salt Hydrolase Activity Test: Plate Assay

Agar plates assay was developed to detect Bile salt hydrolase activity in lactobacilli. Agar plates MRS medium were prepared with 0.5% (w/v) of the taurodeoxycholic acid (TDCA). After boiling and steam sterilization, the plates were poured into sterile plastic Petri dishes (60 by 15 mm). Once solidified, the plates were inverted and placed in the anaerobic condition for at least 72 h before use. All plates were inoculated from an overnight culture by using a 10-µL loop. Plates were incubated in glass screw-cap jars at 37°C for 72 h. Precipitated bile acid could be seen around colonies representing highly active strains within 48 h., bile salt hydrolysis was

manifested at two intensities: (i) the formation of precipitate halos around colonies or (ii) the formation of opaque granular white colonies (Dashkevicz and Feighner, 1989).

Development of Yogurt

Preparation of Yogurt Culture

Nine isolates of lactic acid bacteria (three isolates of each *Lactobacillus. acidophilus, Lactobacillus delbrueckii ssp. bulgaricus* and *Bifidobacterium spp.* from three samples) were selected. The selection of cultures was based on the identification and characterization results of this study. They were sub-cultured in MRS broth in anaerobic condition for 24 hours at 37°C to obtain fresh culture. *Streptococcus thermophilus* was also collected from Food Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University,Khulna and cultured in ST agar at 37°. *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. Bulgaricus* was used as starter culture.

Preparation of Yogurt

In this study, yogurt was produced using the set method, as it is traditional, simple and because it favors the better survival of probiotic microorganisms. For preparation of yogurt, milk was collected from a regional farm and was heated at 95°C for 15 min, and then cooled to 43°C inoculated with a 5% (v/v) liquid culture of *isolated* LAB yoghurt culture. The inoculated mixes was poured into containers and incubated at 42°C until pH 4.6, cooled and stored at 4°C. The numbers of bacteria in freshly fermented yogurt were determined by established procedures to be approximately 10 CFU/ml (International Dairy Federation, 1988; Linn *et. al.*, 2008).

Analysis of Yogurt

pH **Determination**

The pH of produced yoghurt products was determined by using a Microprocessor pH meter of model pH 211(HANNA Instrument, Romania) on day 0.

Total Bacterial Count

Total bacterial count of lactic acid bacteria was done at MRS media using dilution method.

In Vivo Mice Trial

40 swiss (3-4 weeks) albino mice were purchased from ICDDR,B and housed in plastic cages with sow dust and paper bedding. Mice were allowed free access to water and maintained on a 12:12 h light:dark cycle. (Yeun *et al*, 2008). Rat pellet was also collected from ICDDR,B provided as 5 grams/mouse. Uneaten food was removed daily and replaced with fresh food. After 4 weeks of arrival mice were divided into four groups, each group comprises 10 mice. Group 1 was denoted as control; Group 2, group 3 and group 4 was denoted as treatment group 1, treatment group 2 and treatment group 3 respectively. For demarcation, individual mouse was marked using red, blue and green permanent marker in the tail.

Body Weight Gain and Blood Cholesterol Measuring Test

The control group (Group 1) was fed 5 grams rat pellet as basal diet plus additional 2 grams rat pellet/mouse/day (to equalize with at least one control group). The other three treatment groups (2, 3 and 4) received 5 grams rat pellet as basal diet plus additional 2, 4 and 6 grams of yoghurt/mouse/day as additional supplement, respectively. It could be mentioned that one gram set yoghurt contains 1.67 Kcal ME, whereas, one gram rat pellet contains 3.10 Kcal ME. Body weight was measured at day 0, day 7, day 14, day 21 and day 28 for determining body weight gain.

For the blood cholesterol lowering test, blood was collected from mice using tail nicking method on day 0 and day 28 and cholesterol level was measured using Easy Mate Blood Cholesterol meter.

Antibiotic Resistance Test

Twenty adult albino mice were selected and grouped in to four groups for antibiotic resistance test. Before starting of the experiment all mice were feed with 5 gm of basal diet and adequate water for 7 days. Then they were divided into 4 groups. Group 1 and Group 2 were selected as positive and negative control. Group 3 and 4 were selected as treatment groups. Group 1 was feed with 5gm probiotic yogurt with no antibiotic, Group 3 and Group 4 was feed with 2.5gm and 5 gm of probiotic yogurt along with antibiotic and group 2 was feed with only antibiotic. All the four groups were feed with normal basal diet (5 gm/per mouse). Amoxicillin was used

as antibiotic and 0.02 mg was administered in to mouse mixing with drinking water for per gram of mouse weight. Total experiment day was 14 days. Faces were collected from each group on day 0, day 7 and day 14 and cultured on MRS agar media for LAB colony count (Majhenic and Matijašic, 2001).

CHAPTER: FOUR

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Results

Morphological and Biochemical Test

Nine isolates of probiotic lactic acid forming bacteria (LAB) were isolated from three sample of yogurt and identified presumptively as three isolates of *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus and Bifidocterium spp each,* primarily by observing their colony morphology, physiological characteristics and finally performing the biochemical tests. The *Lactobacillus acidophilus* did grow well on MRS agar media supplemented with 0.5% salicin. It was observed that 0.05% cysteine enhanced the growth of *Lactobacillus delbrueckii ssp. bulgaricus and Bifidocterium spp.* Neomycin sulphate, paramomycin sulphate, nalidixic acid and lithium chloride containing MRS media was used as one of the selective media for growth of *Bifidocterium spp.* Morphological, physiological *and* biochemical characteristics of *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus* and *Bifidocterium spp* have shown in Table 4.1 and Table 4.2 sequentially. Microscopically the isolated LAB was Gram positive, catalase negative and absence of Endospore. Figure 4.1 to 4.18 shows Gram staining and Endospore tests, respectively. The isolates showed tolerance to MRS broth containing inhibitory substances such as 0.1-0.4% bacteriostatic phenol and 1-10% NaCl. According to their characteristics, the isolates were identified as *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus* and *Bifidocterium spp.*

Table 4.1 : Morphological characteristics of isolated lactic acid bacteria (LAB)

Identified isolates	Colony morphology
Lactobacillus acidophilus	Very small, round shaped and non transparent.
Lactobacillus delbrueckii ssp *bulgaricus.*	Medium size, irregular, smooth and deep white.
Bifidocterium spp.	Small, triangular, watery circle with deep white middle.

Table 4.2: Biochemical tests for *L. acidophilus* and *L. delbrueckii ssp. Bulgaricus* and *Bifidocterium spp.*

Identified bacteria	Catalase test	Gram staining test	Endospore test
L. acidophilus	-	+	-
L. delb.ssp. bulgaricus	-	+	-
Bifidocterium spp.	-	+	-

Gram Staining Test: Microscopic observation of isolates after gram staining test results from figure 4.1 to 4.9

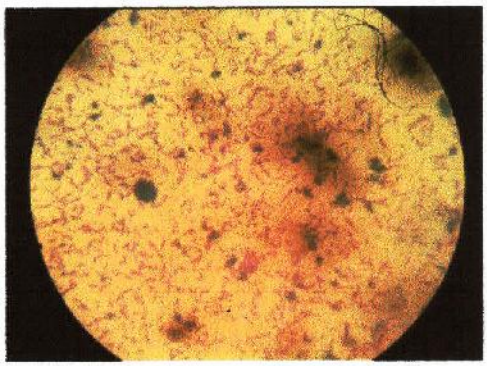

Figure 4.1: *L. acidophilus* from sample 1

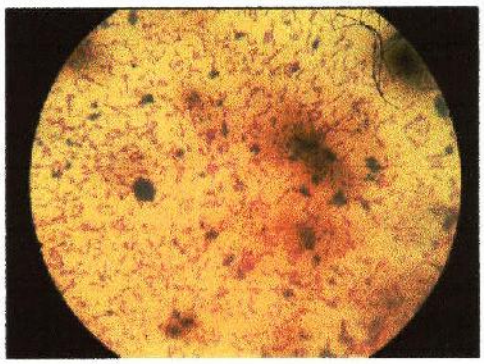

Figure 4.2: *L. acidophilus* from sample 2

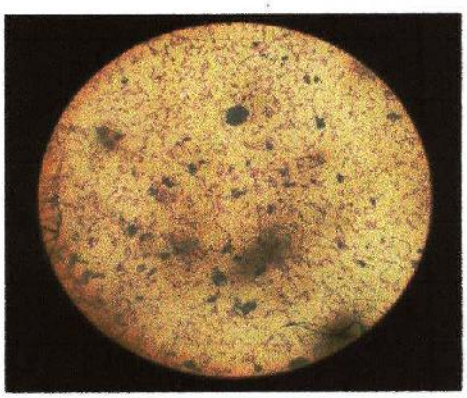

Figure 4.3: *L. acidophilus* from sample 3

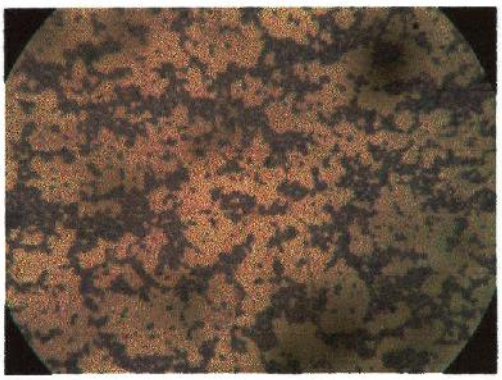

Figure 4.4: *L. delbrueckii ssp. Bulgaricus* from sample 1

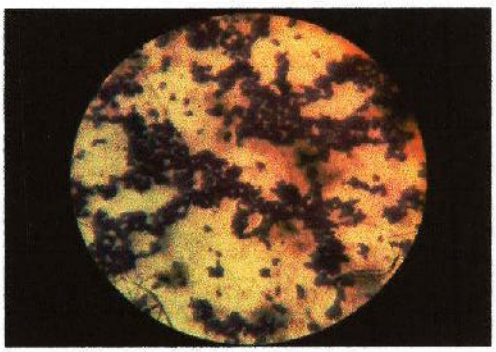

Figure 4.1: *L. delbrueckii ssp. Bulgaricus* from sample 2

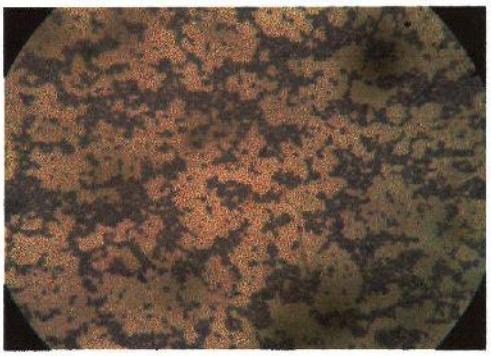

Figure 4.2: *L. delbrueckii ssp. Bulgaricus* from sample 3

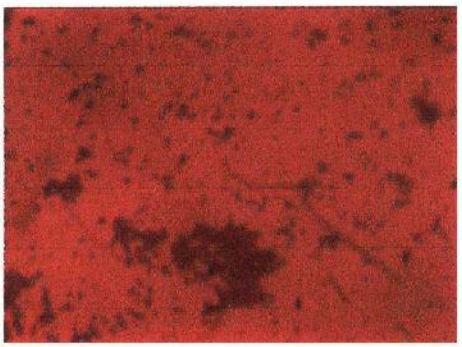

Figure 4.7: *Bifidocterium spp.* from sample 1

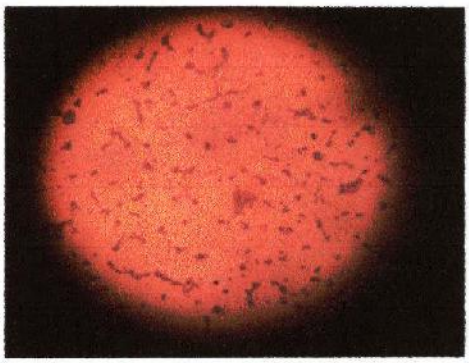

Figure 4.8: *Bifidocterium spp.* from sample 2

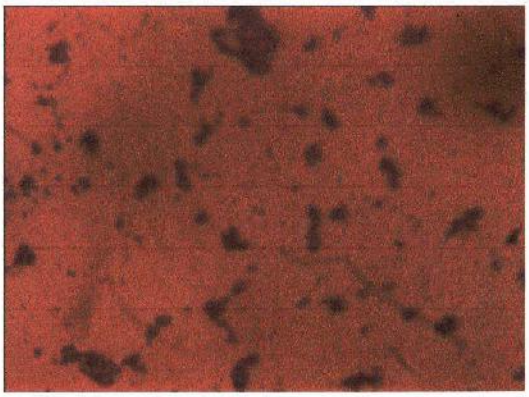

Figure 4.3: *Bifidocterium spp.* from sample 3

Endospore Test:

Endospore Test of probiotic bacteria (Fig. 4.10 to 4.18)

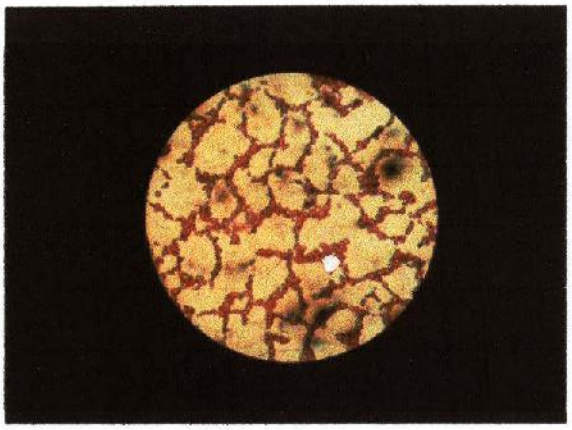

Figure 4.4: *L. acidophilus* from sample 1

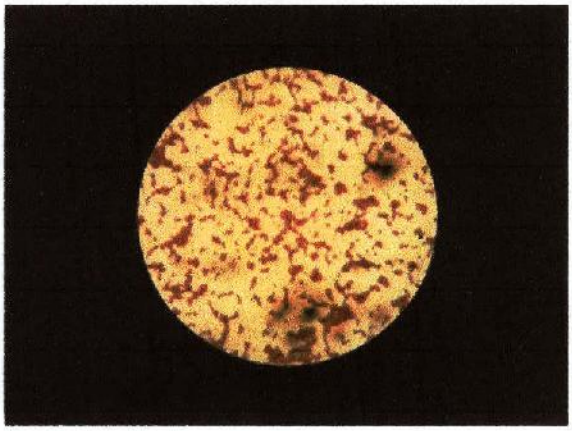

Figure 4.5: *L. acidophilus* from sample 2

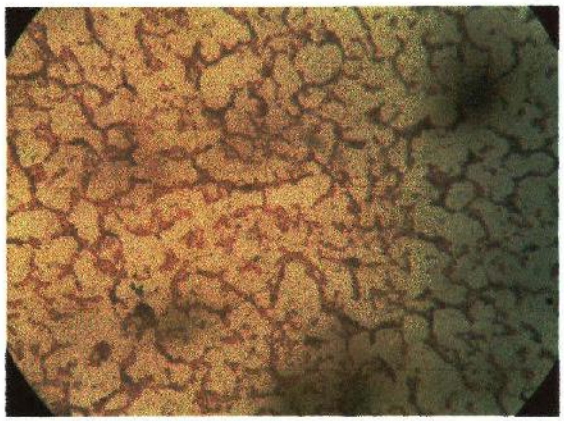

Figure 4.6: *Lactobacillus acidophilus* from sample 3

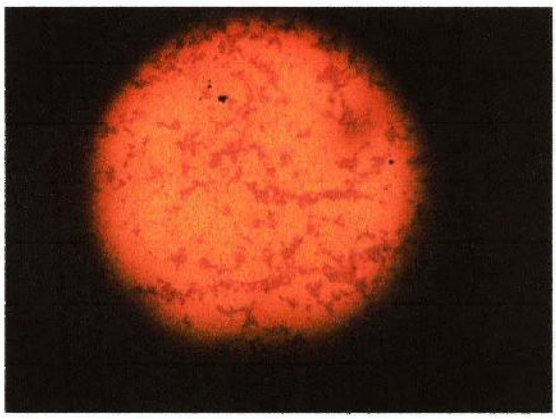

Figure 4.7:*Lactobacillus delbrueckii ssp. Bulgaricus* from sample 1

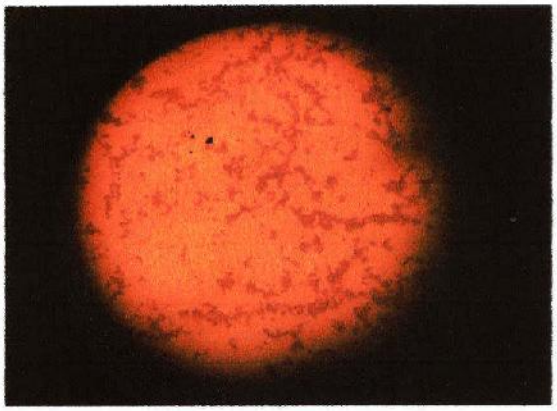

Figure 4.9: *L. delbrueckii ssp. Bulgaricus* from sample2

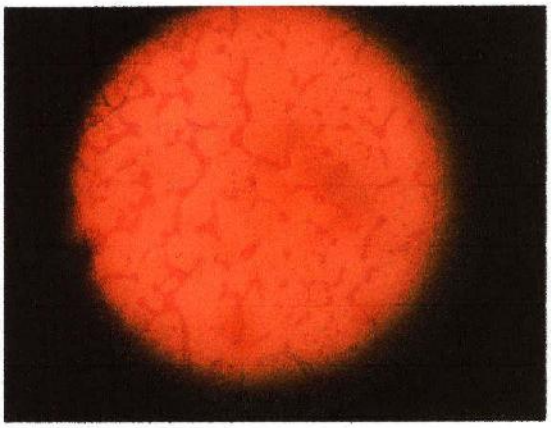

Figure 4.8: *L. delbrueckii ssp. Bulgaricus* from sample 3

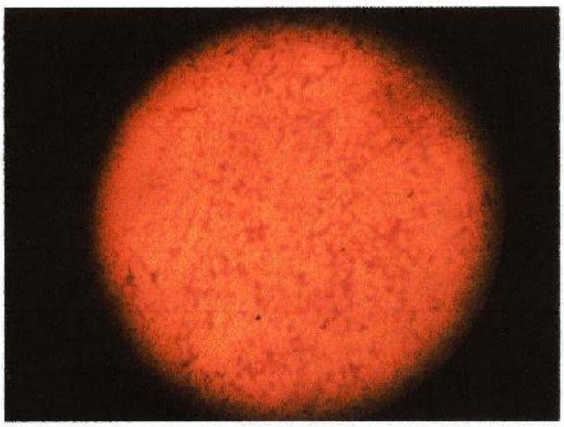

Figure 4.11: *Bifidocterium spp.* from sample 1

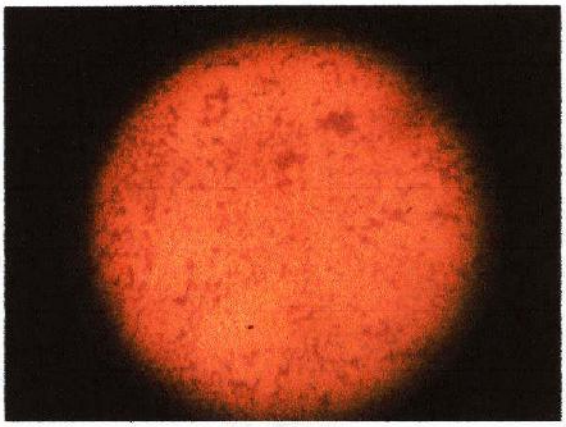

Figure 4.10: *Bifidocterium spp.* from sample 2

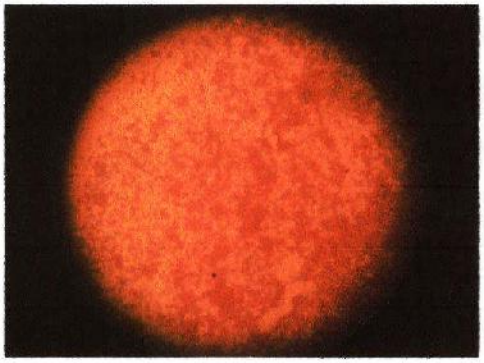

Figure 4.12: *Bifidocterium spp.* from sample 3

Sugar Fermentation Pattern

Isolated bacteria were identified up to species level on the basis of their growth at different temperatures and sugar fermentation tests as recommended by Harrigan and McCance (1976). Nine isolates, three from each (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. Bulgaricus* and *Bifidocterium spp*) were selected and subjected to sugar fermentation test. In the sugar fermentation patterns, mainly acid and gas production was observed. The isolates *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus* and *Bifidocterium spp* did not produce gas from glucose and other sugars. They also showed variation in their sugar fermentation patterns. *Lactobacillus acidophilus* fermented all the sugars used, except sorbitol. *Lactobacillus delbrueckii ssp. bulgaricus* fermented only three sugars (ribose, fructose, lactose) among eight sugars. On the other hand *Bifidocterium spp*fermented all eight sugars. The acid and gas production results are presented in Table-4.3.

Table 4.3: Sugar fermentation patterns of *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus* and *Bifidocterium spp.*

Sample no.	Name of the bacteria	Rib	Sor	Manni	Sucr	Fruct	Cello	Sal	Lact	Gas Production
1	*L. acidophilus*	+	-	+	+	+	+	+	+	no
	L. delb. ssp. Bulgaricus	+	-	-	-	+	-	-	+	no
	Bifidocterium spp	+	+	+	+	+	+	+	+	

Sample no.	Name of the bacteria	Rib	Sor	Manni	Sucr	Fruct	Cello	Sal	Lact	Gas Production
2	*L. acidophilus*	+	-	+	+	+	+	+	+	no
	L. delb. ssp. Bulgaricus	+	-	-	-	+	-	-	+	no
	Bifidocterium spp	+	+	+	+	+	+	+	+	no
3	*L. acidophilus*	+	-	+	+	+	+	+	+	no
	L. delb. ssp. Bulgaricus	+	-	+	+	+	+	+	+	no
	Bifidocterium spp	+	+	+	+	+	+	+	+	no

N.B. Rib=Ribose, Sor=Sorbitol, Manni=Mannitol, Sucr=Sucrose, Fruct=Fructose, Cello=Cellobiose, Sal=Salicin, Lact=Lactose, (+) means good fermentation, (-) means no fermentation

NaCl Tolerance Test

The isolated lactic acid forming bacteria (LAB) (*Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp)* from yogurt are able to tolerate 1-10% NaCl. In the present experiment, *Lactobacillus acidophilus* was able to tolerate 1-9% NaCl concentration. *Lactobacillus delbrueckii ssp. Bulgaricus* and *Bifidocterium spp*were able to tolerate 1-7% and 1-9% NaCl concentration respectively. Maximum growth were indicated as triple positive sign (+++), moderate growth as double positive sign (++), low growth as single positive sign (+) and no growth were indicated as negative sign (-). This results indicate that identified LAB were able to tolerate high concentration of NaCl like inhibitory substance. The results have been shown in Table-4.4, Table 4.5 and Table 4.6.

Table 4.4: NaCl tolerance tests of *Lactobacillus acidophilus* isolated from sample-1, and sample-2 and sample-3

Concentration of NaCl (%)	Sample-1	Sample-2	Sample-3
1	+++	+++	+++
2	+++	+++	+++
3	+++	+++	+++
4	+++	+++	+++
5	++	+++	++
6	++	++	++
7	++	++	+
8	++	++	+
9	+	+	+
10	-	-	-

N.B. (+ + +) indicates maximum growth, (+ +) indicates good growth, (+) indicates normal growth, (-) indicates no growth.

Table 4.5: NaCl tolerance tests of *Lactobacillus delbrueckii ssp. bulgaricus* isolated from sample-1 and sample-2 and sample-3

Concentration of NaCl (%)	Sample-1	Sample-2	Sample-3
1	+++	+++	+++
2	++	++	++
3	++	++	++
4	++	++	++
5	++	++	++
6	+	+	+
7	+	+	+
8	-	-	-
9	-	-	-
10	-	-	-

N.B. (+ + +) indicates maximum growth, (+ +) indicates good growth, (+) indicates normal growth, (-) indicates no growth.

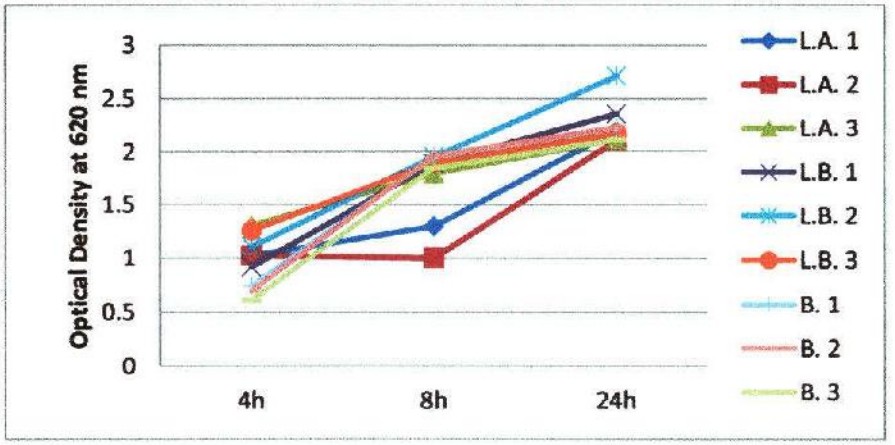

Figure 4.13: Survival and multiplication of isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* at 0.05 concentrations of bile salt in MRS broth (measured at optical density 620 nm)

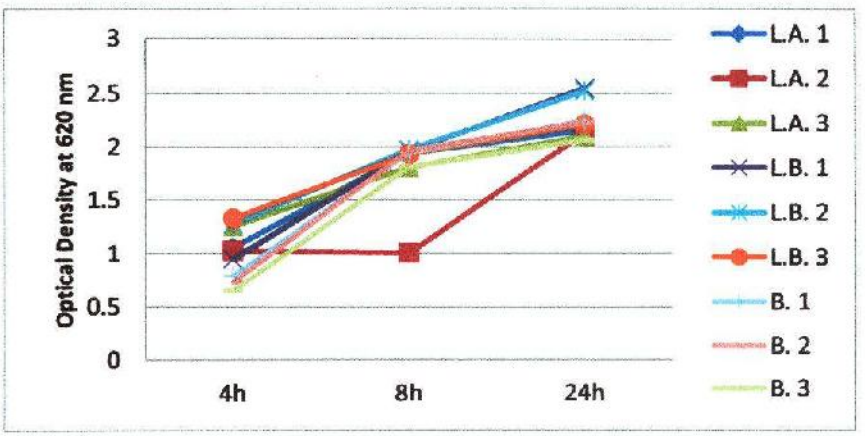

Figure 4.14: Survival and multiplication of isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp* at 0.1 concentrations of bile salt in MRS broth (measured at optical density 620 nm)

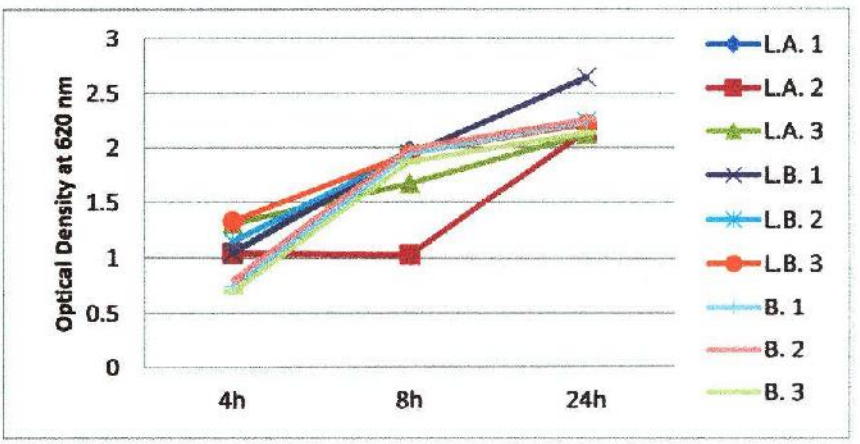

Figure 4.15: Survival and multiplication of isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* at 0.15 concentrations of bile salt in MRS broth (measured at optical density 620 nm)

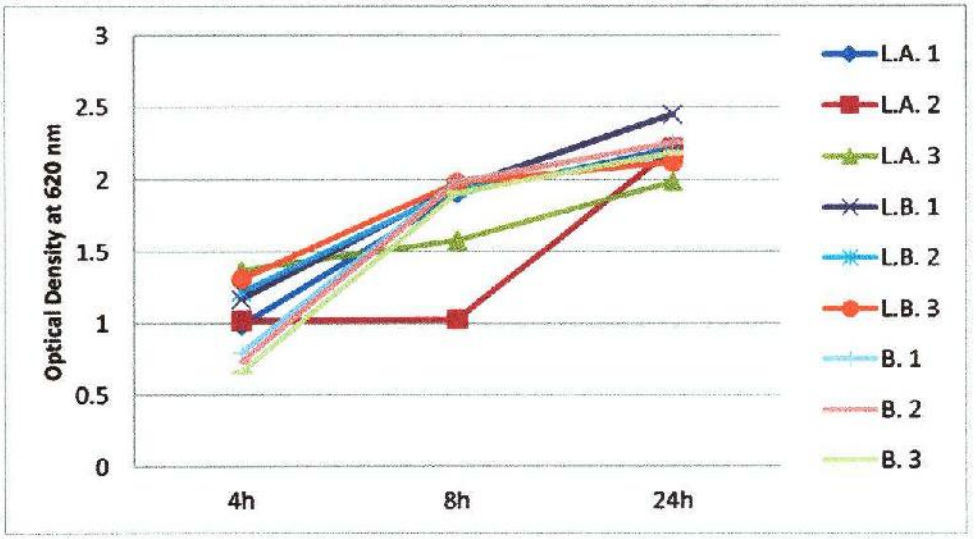

Figure 4.16: Survival and multiplication of isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* at 0.2 concentrations of bile salt in MRS broth (measured at optical density 620 nm)

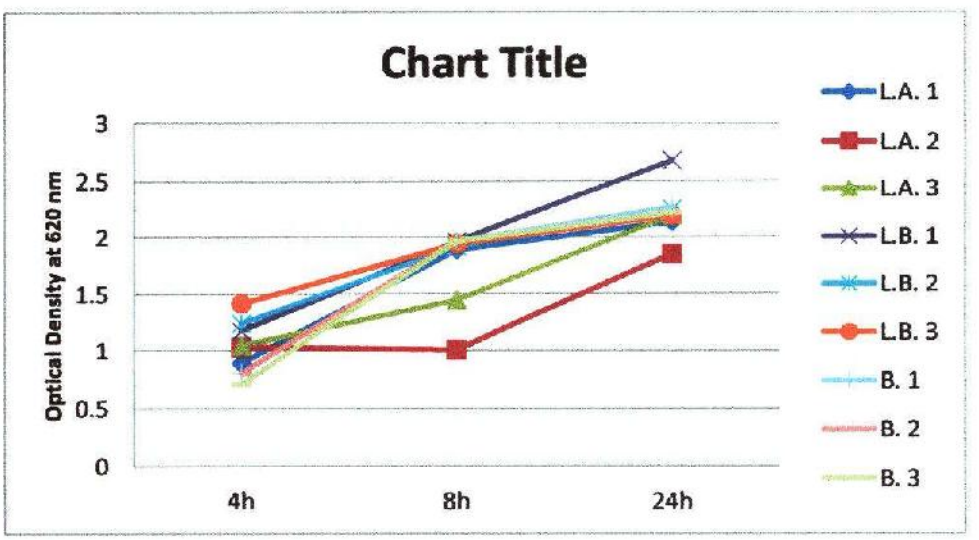

Figure 4.17: Survival and multiplication of isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* at 0.3 concentrations of bile salt in MRS broth (measured at optical density 620 nm)

Antimicrobial Activity of Metabolites Produced by Lactic Acid Forming Bacteria (LAB)

Isolated LAB did show antimicrobial activity against various disease causing bacteria (Table 4.19 to 4.27 and Fig. 4.28 to 4.42). *Lactobacillus acidophilus* showed antimicrobial activity against *Shigella flaxeri, Shigella dysenteriae, Vibrio cholerae and Salmonella typhi. Lactobacillus delbrueckii ssp. bulgaricus* also showed antimicrobial activity against *Staphylococcus epidermis, Shigella flexeri, Shigella dysenteriae, Vibrio cholerae, Salmonella typhi, Pseudomonas spp.* On the other hand, *Bifidocterium spp*did show antimicrobial activity against *Staphylococcus epidermis, E. coli, Shigella flexeri, Shigella dysenteriae, Vibrio cholerae, Salmonella typhi* and *Pseudomonas spp.* Diameter of zones of inhibition ranged from 10 mm to 20 mm, 8 mm to 23 mm and 10 mm to 19 mm for *Lactobacillus acidophilus, lactobacillus delbrueckii ssp. Bulgaricus* and *Bifidocterium spp*respectively.

Table 4.10: vitro antibacterial activity of *Lactobacillus acidophilus* from sample 1

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1.*Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	Nil		Nil	Nil
3. *E. coli*	Nil		Nil	Nil
4. *Shigella flexeri*	13		15	18
5.*Shigella dysenteriae*	10		16	17
6. *Vibrio cholerae*	13		18	19
7.*Enterococcus faecalis*	Nil		Nil	Nil
8.*Salmonella typhi*	13		17	20
9.*Pseudomonas spp.*	Nil		Nil	Nil

Table 4.11: In vitro antibacterial activity of *Lactobacillus acidophilus* from sample 2

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	Nil		Nil	Nil
3. *E. coli*	Nil		Nil	Nil
4. *Shigella flexeri*	12		15	19
5.*Shigella dysenteriae*	10		17	18
6. *Vibrio cholerae*	13		18	19
7. *Enterococcus faecalis*	Nil		Nil	Nil
8. *Salmonella typhi*	11		18	20
9. *Pseudomonas spp.*	Nil		Nil	Nil

Table 4.12: In vitro antibacterial activity of *Lactobacillus acidophilus* from sample3

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	Nil		Nil	Nil
3. *E. coli*	Nil		Nil	Nil
4. *Shigella flexeri*	12		16	19
5.*Shigella dysenteriae*	10		15	17
6. *Vibrio cholerae*	12		18	20
8.*Salmonella typhi*	14		18	20
9. *Pseudomonas spp.*	Nil		Nil	Nil

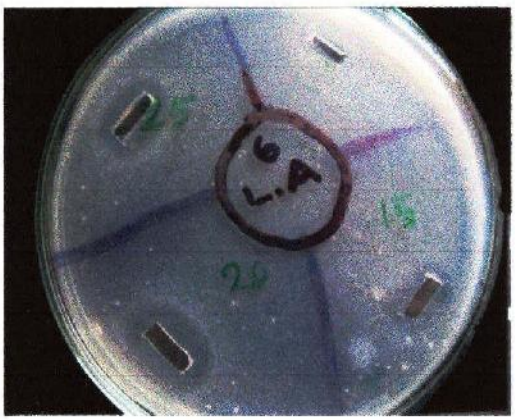

Figure 4.24: Antimicrobial activity of *Lactobacillus acidophilus* against *Vibrio cholerae*

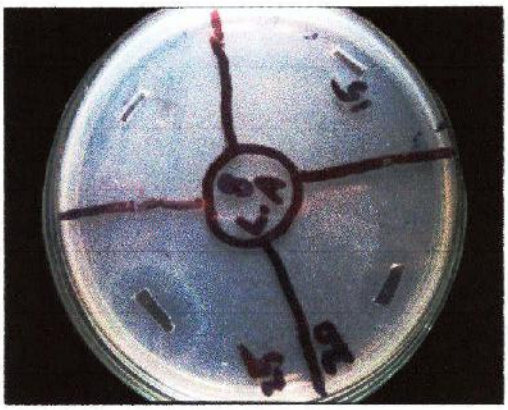

Figure 4.18: Antimicrobial activity of *Lactobacillus acidophilus* against *Salmonella typhae.*

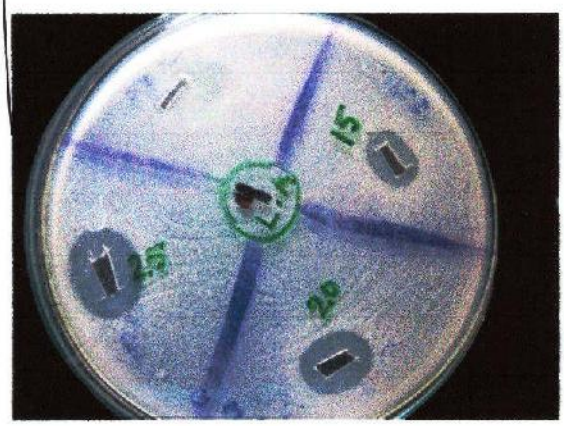

Figure 4.20: Antimicrobial activity of *Lactobacillus acidophilus* against *ShigellaFlaxeri*

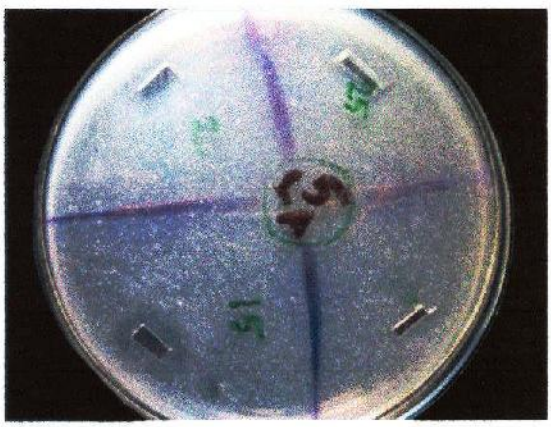

Figure 4.19: Antimicrobial activity of *Lactobacillus acidophilus* against *Shigella dysenteri*

Table 4.13: In vitro antibacterial activity of *Lactobacillus delbrueckii ssp. Bulgaricus*, sample 1.

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	9		16	17
3. *E. coli*	Nil		Nil	Nil

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl/ well	20µl / well	25µl / well
4. *Shigella flexeri*		15	17	19
5. *Shigella dysenteriae*		14	17	22
6. *Vibrio cholerae*		13	17	20
7.*Enterococcus faecalis*		Nil	Nil	Nil
8. *Salmonella typhi*		7	13	15
9. *Pseudomonas spp.*		15	16	9

Table 4.14: In vitro antibacterial activity of *Lactobacillus delbrueckii ssp. Bulgaricus*, sample 2

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl/ well	20µl / well	25µl / well
1.*Staphylococcus aureus*		Nil	Nil	Nil
2. *Staphylococcus epidermis*		10	15	18
3. *E. coli*		Nil	Nil	Nil
4. *Shigella flexeri*		15	17	20
5.*Shigella dysenteriae*		14	18	23
6. *Vibrio cholerae*		12	18	20
7.*Enterococcus faecalis*		Nil	Nil	Nil
8.*Salmonella typhi*		8	14	16
9.*Pseudomonas spp.*		16	16	20

Table 4.15: In vitro antibacterial activity of *Lactobacillus delbrueckii ssp. Bulgaricus*, from sample 3

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	9		15	17
3. *E. coli*	Nil		Nil	Nil
4. *Shigella flexeri*	15		16	19
5.*Shigella dysenteriae*	13		19	22
6. *Vibrio cholerae*	13		19	22
7.*Enterococcus faecalis*	Nil		Nil	Nil
8.*Salmonella typhi*	7		12	15
9.*Pseudomonas spp.*	14		16	20

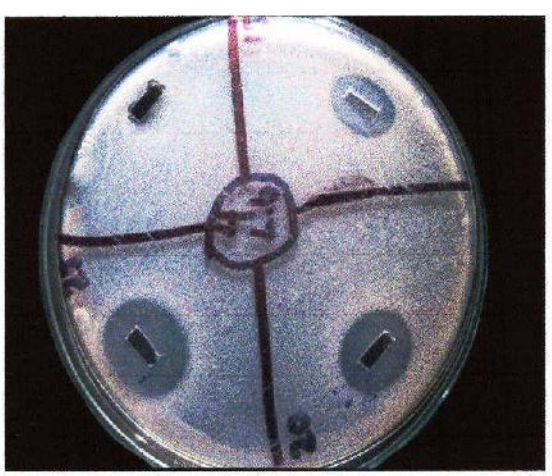

Figure 4.22: Antimicrobial activity of *Lactobacillus delbrueckii ssp. bulgaricus* against *Shigella flaxeri*

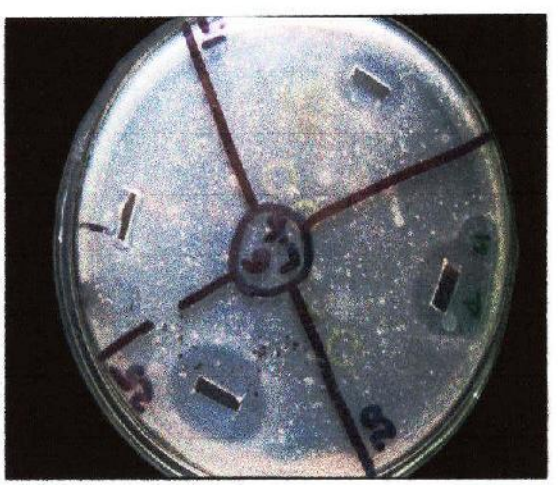

Figure 4.21: Antimicrobial activity of *Lactobacillus delbrueckii ssp. bulgaricus* against *Vibrio colerae*

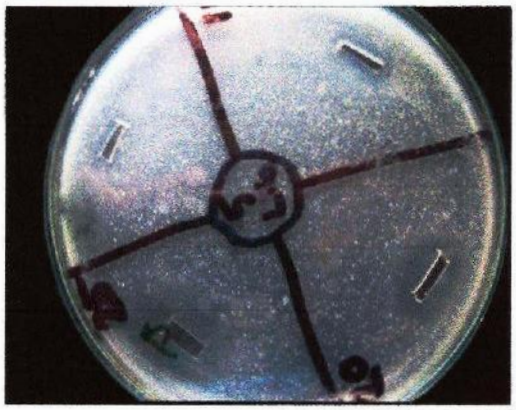

Figure 4.24: Antimicrobial activity of *Lactobacillus delbrueckii ssp. bulgaricus* against *Shigella dysenteri*

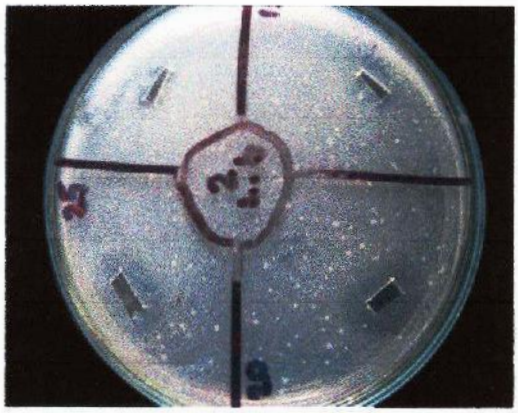

Figure 4.23: Antimicrobial activity of *Lactobacillus delbrueckii ssp. bulgaricus* against *S. epidermis.*

Table 4.16: In vitro antibacterial activity of *Bifidocterium spp.* from sample 1

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	12		16	16
3. *E. coli*	10		12	14
4. *Shigella flexeri*	15		17	18
5. *Shigella dysenteriae*	11		12	15
6. *Vibrio cholerae*	13		14	16
7. *Enterococcus faecalis*	Nil		Nil	Nil
8. *Salmonella typhi*	10		12	15
9. *Pseudomonas spp.*	12		15	15

Table 4.17: In vitro antibacterial activity of *Bifidocterium spp.* from sample 2

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	10		16	16
3. *E. coli*	10		12	14
4. *Shigella flexeri*	15		18	19
5. *Shigella dysenteriae*	10		14	17
6. *Vibrio cholerae*	12		12	15
7. *Enterococcus faecalis*	Nil		Nil	Nil
8. *Salmonella typhi*	10		15	16
9. *Pseudomonas spp.*	13		15	15

Table 4.18: In vitro antibacterial activity of *Bifidocterium spp.* from sample 3

Bacterial strains	Diameter of zone of inhibition in mm			
	Vol. →	15µl / well	20µl / well	25µl / well
1. *Staphylococcus aureus*	Nil		Nil	Nil
2. *Staphylococcus epidermis*	12		16	16
3. *E. coli*	10		12	14
4. *Shigella flexeri*	15		17	18
5.*Shigella dysenteriae*	11		12	15
6. *Vibrio cholerae*	13		14	16
7.*Enterococcus faecalis*	Nil		Nil	Nil
8. *Salmonella typhi*	9		13	15
9. *Pseudomonas spp.*	12		15	16

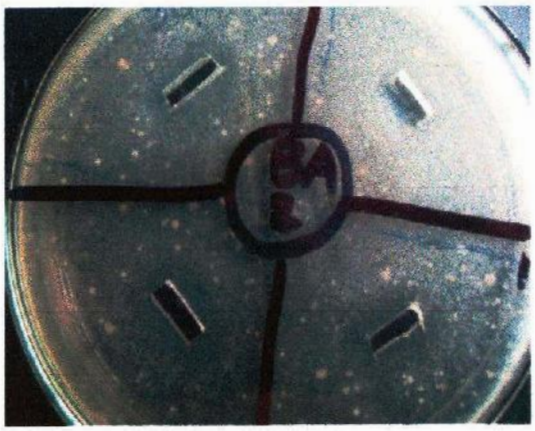

Figure 4. 25: Antimicrobial activity of *Bifidocterium spp.* against *Staphylococcus epidermis*

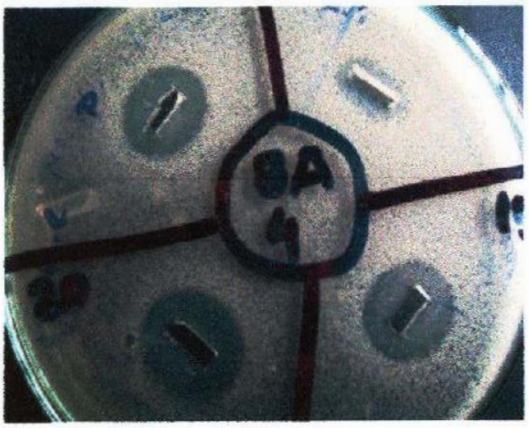

Figure 4. 26: Antimicrobial activity of *Bifidocterium spp.* against *Shigella flaxeri*

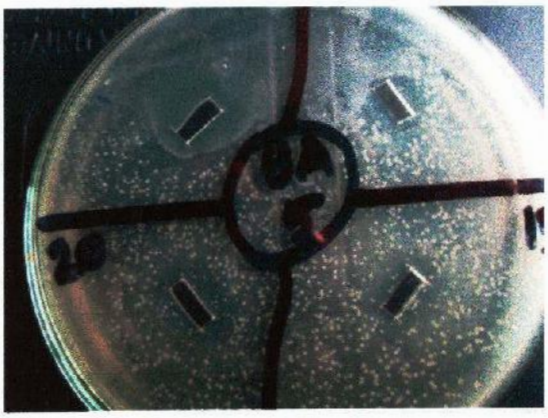

Figure 4. 27: Antimicrobial activity of *Bifidocterium spp. against Shigella dysenteri*

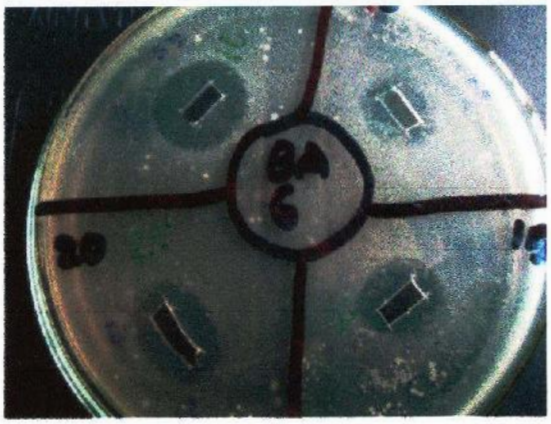

Figure 4. 28: Antimicrobial activity of *Bifidocterium spp.* against *Vibrio colerae*

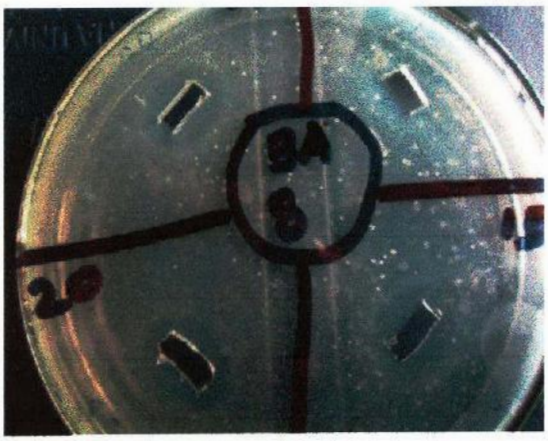

Figure 4.30: Antimicrobial activity of *Bifidocterium spp.* against *Salmonella typhae*

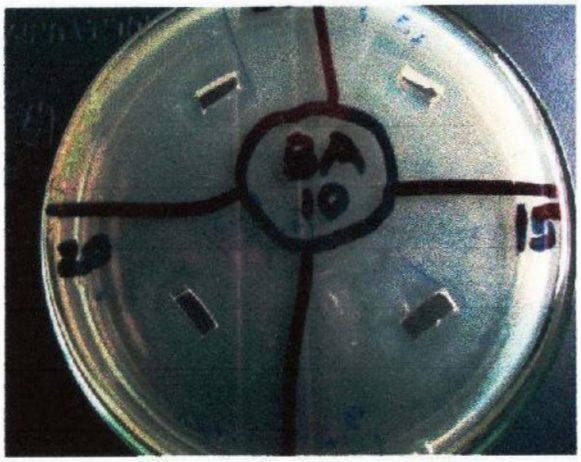

Figure 4.29: Antimicrobial activity of Bifidocterium spp against Pseudomonas spp.

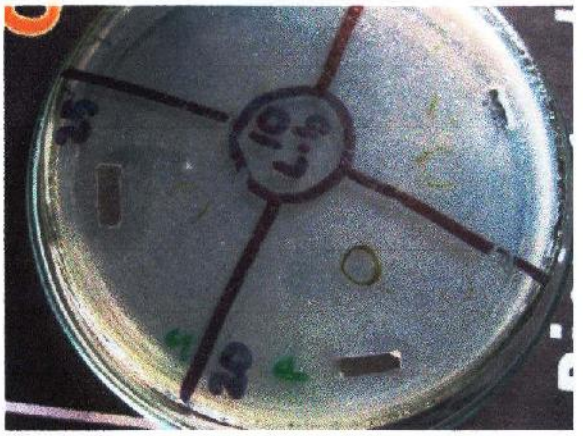

Figure 4.31: Antimicrobial activity of *Bifidocterium spp.* against *E. coli*

Gastric Juice Tolerance

Isolated *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp bulgaricus* and *Bifidocterium spp.* were subjected to gastric juice tolerance at pH 2.2 and also at pH 6.6 as control. Optical density was measured at 620 nm after 0, 1, 2, 3, 4 and 24 hours. The results indicate that all the isolates survived in artificial gastric juice at pH 2.2. But they didn't show rapid multiplication as there was no growth media for their growth and multiplication. On the other hand, isolates showed an optimum variation which indicates that cells were able to survive and could have little multiplication abilities in artificial gastric juice at pH 6.6. Survivability was ensured streaking on MRS agar plate after 1, 2, 3, 4 and 24 hours of incubation. From this experiment the results indicate that in GIT environment, the gastric juice will have minor or possibly no adverse effects on our isolated probiotic bacteria.

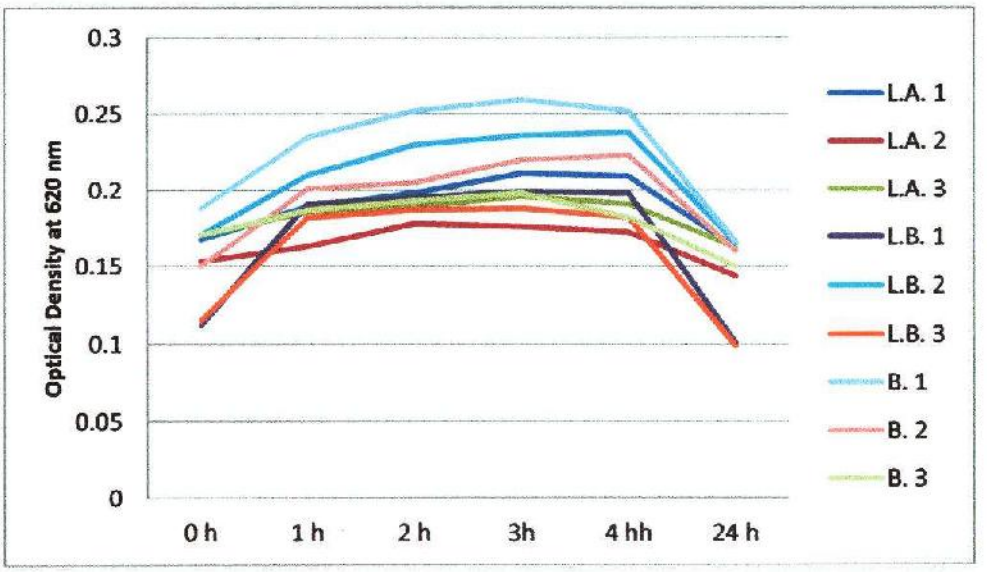

Figure 4.32 Survival and multiplication of isolated *Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* in artificial gastric juice at pH 2.2 (measured at optical density 620 nm)

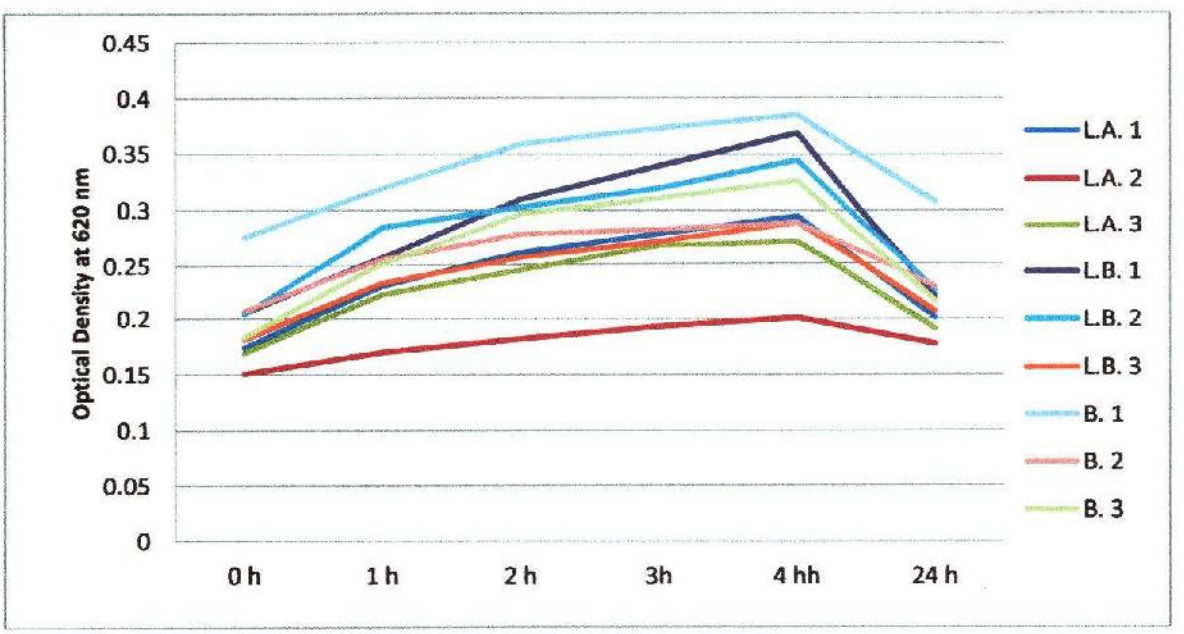

Figure 4.40: Survival and multiplication of isolated *Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. Bulgaricus and Bifidocterium spp.* in artificial gastric juice at pH 6.6 (measured at optical density 620 nm)

pH of Produced Yogurt:

Four sample of yogurt was produced on day 0, day 7, day 14 and day 21 and their pH and total bacterial count was measured. Test result of total bacterial count ensured yogurt's probiotic doses.

Table 4.19: pH and total bacterial count of produced yogurt

Day	pH	Total bacterial count
Day 0	4.78	2.6×10^9
Day 7	4.58	1.5×10^9
Day 14	4.49	2.3×10^9
Day 21	4.81	2×10^9

***In Vivo* Mice Trial:**

Mice feeding trial by laboratory based manufactured Probiotic yogurt was designed for 28 days. Forty mice were divided in to four groups, Group 1 was denoted as control; Group 2, group 3 and group 4 was denoted as treatment group 1, treatment group 2 and treatment group 3 respectively. Each group was comprised of 10 mice. The control group (Group 1) was fed 5 grams rat pellet as basal diet plus additional 2 grams rat pellet/mouse/day (to equalize with at least one control group). The other three treatment groups (2, 3 and 4) received 5 grams rat pellet as basal diet plus additional 2, 4 and 6 grams of yoghurt/mouse/day as additional supplement, respectively. It could be mentioned that one gram set yoghurt contains 1.67 Kcal ME, whereas, one gram rat pellet contains 3.10 Kcal ME. During 28 day visual observation, it was observed that mice of all Treatment Groups except Control Group were strong enough, active and were tolerant during high & sudden fluctuation of temperatures. On the other hand, mice of Control Group did not show such criteria. They were weak, less active and less heat tolerant. Besides, mice of all Treatment Groups had white and attractive far in comparison to the mice of Control Group.

Figure 4.41: Housing of mouse

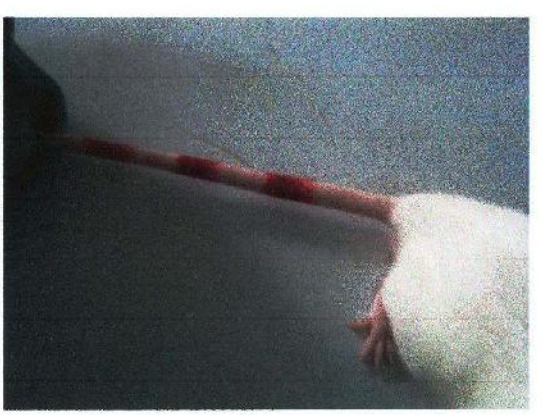

Figure 4.42: Tagging of mouse

Cholesterol Measuring:

Cholesterol level was measured using Easy Mate blood cholesterol meter on day 0 and day 28. The entire Treatment Groups showed significant lowering of cholesterol level after 28 day trial. But the control group didn't show any significant test result of cholesterol lowering level and didn't show any significant variation among collected data.

Figure 4.43: Cutting of tail for blood sample

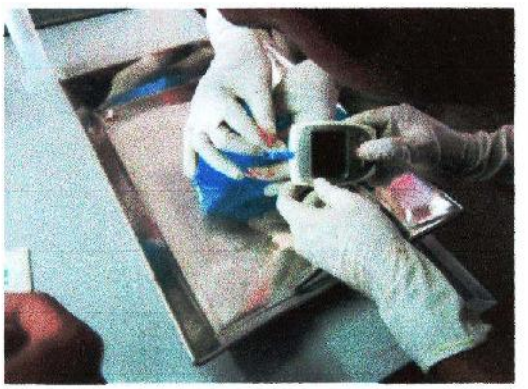

Figure 4.44: Taking reading of blood cholesterol level

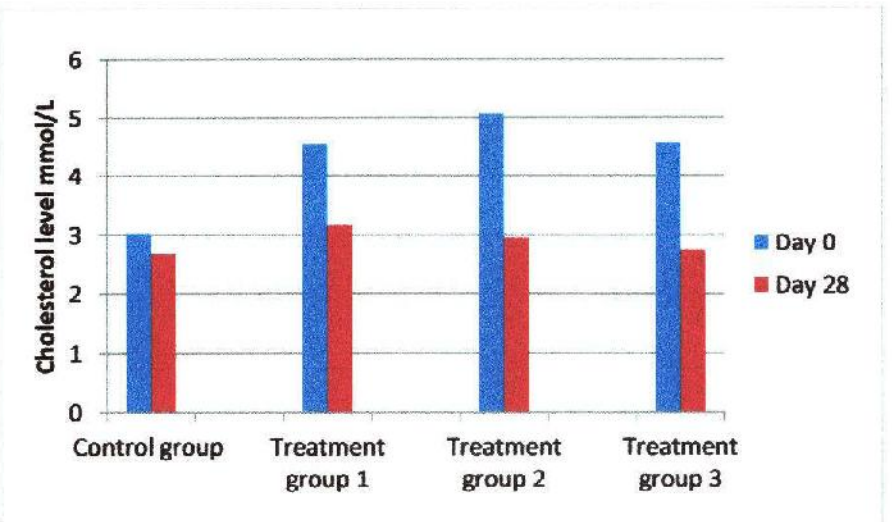

Figure 4.33: Comparison of measured average cholesterol level of different group on day 0 and day 28

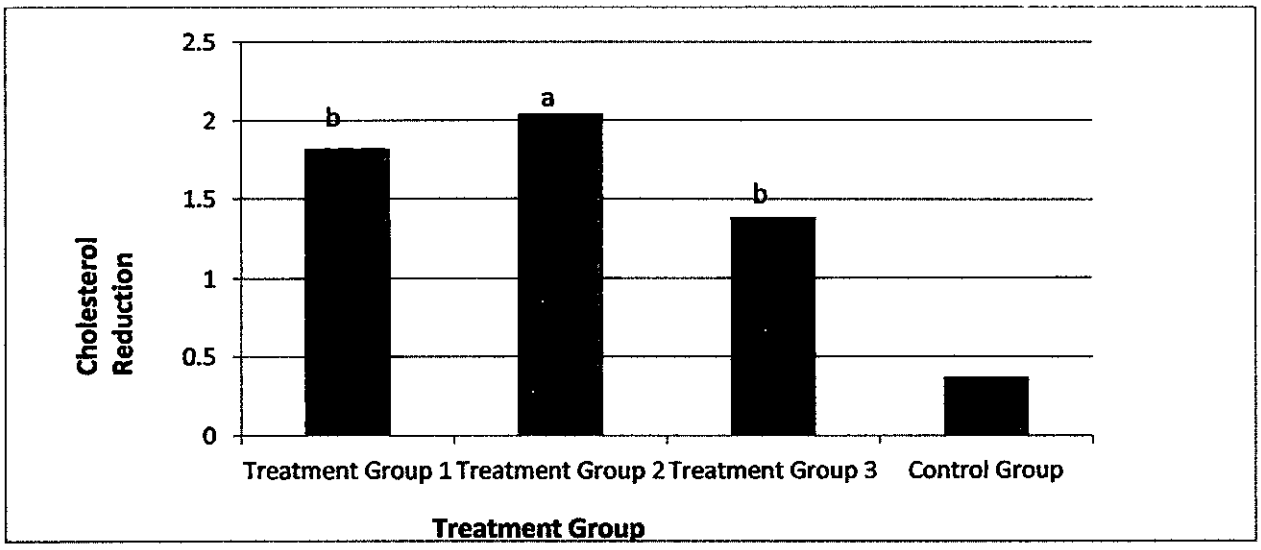

Figure 4.46: Blood Cholesterol level of Probiotic treatment and control group (b= statistically significant, a= statistically non significant)

Figure 54 represents the average blood cholesterol reduction level among the control and treatment groups. The average cholesterol reduction level of the control group was 0.37 mM/L over twenty eight days treatment period. Treatment Group 2 showed highest average cholesterol reduction level (2.04 mM/L)) and Treatment Group 1 and Treatment Group 3 showed 1.82 mM/L and 1.39 mM/L cholesterol reduction levels, respectively. The value of the F test in this experiment is 3.739 at 39 degree of freedom and the P value of this test is 0.019. So the overall result is significant at 0.05 level of significance ($P<\alpha$). The mean difference of treatment group 2 (P=0.02) with control group is significant at 0.05 level of significance.

Body Weight Gain:

Individual body weight of mice were measured at day 0, day 7, day 14, day 21 and day 28. The control group (Group 1) was fed 5 grams rat pellet as basal diet plus additional 2 grams rat pellet/mouse/day (to equalize with at least one control group). The other three treatment groups (2, 3 and 4) received 5 grams rat pellet as basal diet plus additional 2, 4 and 6 grams of yoghurt/mouse/day as additional supplement for 28 days, respectively

Mean Body Weight Gain:

Control Group: 1.85 gm
Treatment Group 1: 4.82 gm
Treatment Group 2: 6.03 gm
Treatment Group 3: 4.06 gm

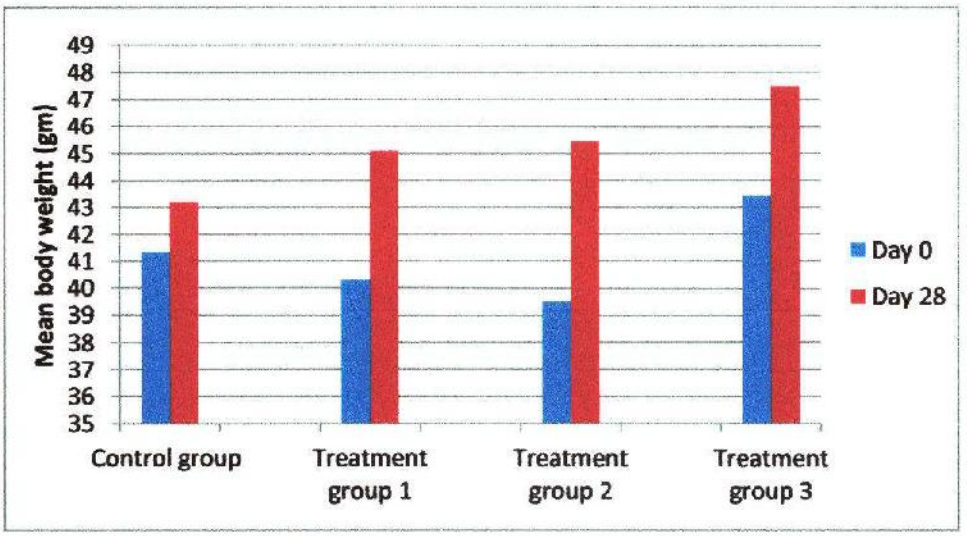

Figure 4.34 Comparison of measured average body weight of different group on day 0 and day 28

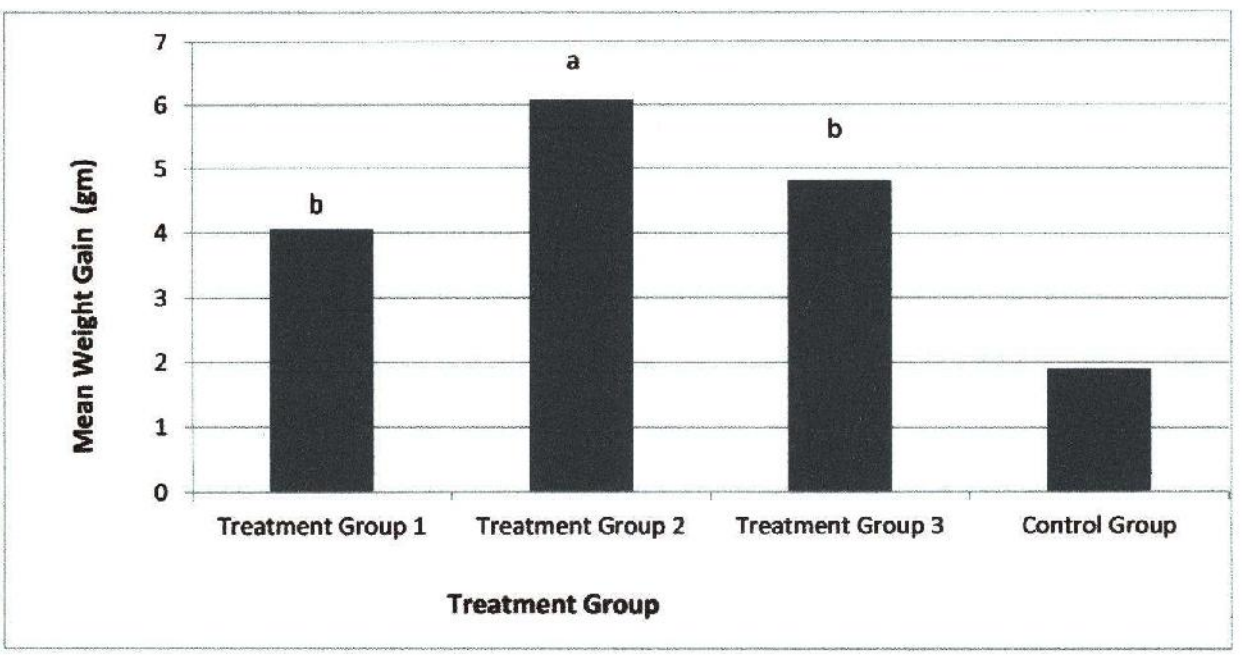

Figure 4.48: Mean weight gain of Treatment and Control Groups of Mice (b= statistically significant, a= statistically non significant)

Figure 56 represents the average body weight gain (gm) among the control and treatment groups of mice. The average weight gain of the control group was nearly 2 grams over twenty eight days treatment period. The other three treatment groups showed higher body weight gain than the control group. Treatment group 2 showed highest average weight gain (6 grams). The value of F Test in this experiment is 2.761 at 39 degree of freedom and the P value of this test is 0.056. Therefore, the overall result is insignificant at 0.05 level of significance (P>α). The mean difference of treatment group 2 (P=0.039) is significant with the control group at 0.05 level of significance whereas the mean difference of treatment group 1and 3 are not significant with the control group at this level.

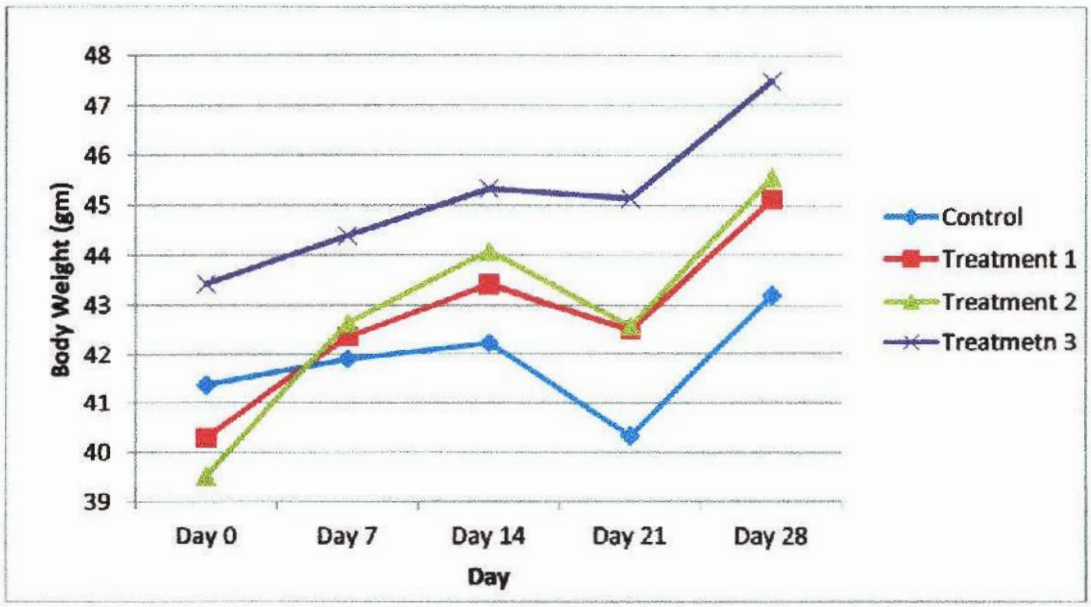

Figure 4.35: Body weight gain chart.

Due to sudden fluctuation of temperature and weather, weights of the mice showed variation time to time. But after the trial all the treatment group showed significant weight gain compared to control group.

Antibiotic Resistance Test:

Twenty mice were grouped in to 4 groups where group 1 and 2 were positive and negative control respectively and group 3 and 4 were treatment groups. In the 14 days of

experimental trial the log cfu/gm of faeces was calculated on basis of colony count method and compared with 0, 7 and 14 days count (Table-44 and Figure-58).

Table 4.20: Log CFu of per gm of faeces

Day	Group-1	Group-2	Group-3	Group-4
DAY 0	5.43	6.41	6.32	5.32
DAY 7	5.34	5.36	6.49	5.42
DAY 14	6.49	5.38	6.56	6.64

In this study Positive control and group 4 showed almost same growth. That means there was no so such adverse effect of antibiotic on group 4 that was feed with 5gm of probiotic yogurt along with amoxicillin. Group 3 shows significant rising of colony count despite of antibiotic treatment where Negative control shows sudden decrease of colony count in first 7days but showed steady colony count on day 14. All that result showed that feeding of probiotic yogurt confer intestinal growth of probiotic bacteria that have significant resistance for amoxicillin.

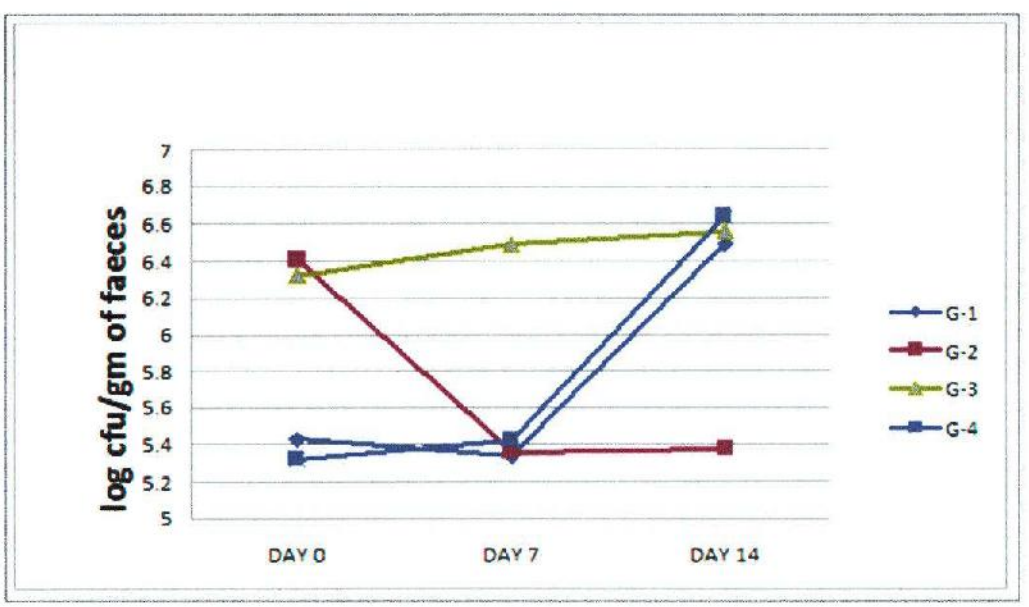

Figure 4.50: Comparison of log cfu/mg of faeces.

Discussion

Isolation and Identification of Lactic Acid Bacteria

Three isolates of of *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium spp.* were isolated from three different yoghurt samples of Khulna City Corporation area, Khulna, Bangladesh. *L. acidophilus, Lactobacillus delbrueckii ssp bulgaricus* and *Bifidobacterium* spp. were isolated using MRS agar medium with some modifications. Pure cultures were developed in MRS broth medium using streak plate method (Leboffe and Pierce, 2011). Some of the components of MRS culture media such as sodium acetate, magnesium sulfate, manganese sulfate and tween-80 are known to act as special growth factors for the growth of lactic acid bacteria as well as rich nutrient sources. Among them, Tween-80 is a surfactant which facilitates the uptake of nutrients and sodium acetate, ammonium citrate act as selective agents (de Man et al., 1960). On the other hand, addition of 0.05% L-cysteine in MRS media favored the specificity for the isolation of *Lactobacillus delbrueckii ssp bulgaricus.* (Lankaputhra et al.,1995; Hartemink et al., 1997; Shah, 2000). Addition of nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate in MRS confirm the selectivity of *Bifidobacterium spp* (Moriya et al, 2006). In the present study, the cultures were subjected to seven subcultures at pH 6.5 and incubated at the anaerobic condition with specific temperature, many fastidious organisms were removed and excellent growth of lactobacilli and *bifidobacteria* was favored. From their colony morphologies, physiological characteristic and finally by observing the biochemical characteristics (gram positive, endospore negative, catalase negative, non-motile, specific sugar fermentation profiles, resistance to inhibitory substances such as gastric acid pH 2.2, 0.05-0.3% bile acid, 0.1-0.4% phenol, 1-9% NaCl) all the nine isolates were identified presuptively as *L. acidophilus, Lactobacillus delbrueckii ssp bulgaricus* and *Bifidobacterium* spp. from three local Ghosh Dairy Shops viz. 1.Tala Adi Ghosh Dairy, 2. Satkhira Ghosh Dairy and 3. New Satkhira Ghosh Dairy of Khulna City Corporation area.

Characterization of Isolated Probiotic Bacteria

NaCl Tolerance

The identified nine isolates of LAB were tested for different concentration of NaCl (1-10%) in the MRS culture broth and the growth was observed according to turbidity after 24 hours. All the isolates had excellent tolerance against 1-5% NaCl, moderate in 6-8% and in 9%-10% NaCl, low or no growth were observed. Elizete and Carlos (2005) isolated lactobacilli from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Schillinger and Lucke (1987) found the growth of lactobacilli in the presence of 7.5% NaCl isolated from meat and meat products and the result is almost similar to the findings of present study. The NaCl test result of the present study were also found similar to Hoque et al (2010) who isolated *Lactobacillus* spp. from yoghurt and tested at different concentrations of NaCl (1-10%) and found 1-9% NaCl tolerance of their *Lactobacillus* spp.

Phenol Resistance

After 12 hours of incubation, nine isolates belonging to three species viz. *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp *bulgaricus and Bifidocterium* spp. showed good tolerance against 0.1 and 0.2% phenol and as the concentration increased to 0.3% and 0.4% their tolerance were decreased greatly. After 24 hours of incubation, better resistance and multiplication ability were observed against 0.1 and 0.2% phenol and as the concentration increased to 0.3% their tolerance were decreased and at 0.4% concentration of phenol their tolerance were lowest. The study of Hoque et al. (2010) was similar to this test result of this study. Ambalam et al. (2009) also shows lactobacillus grows in 0.4% and also remains viable at 0.5% phenol. Some authors suggest that a 0.4% concentration of phenol causes a bacteriostatic action in some microorganisms (Xanthopoulos, *et al.*, 2000).

Bile Resistance

Bile tolerance is also one of the most important attributes of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow and to perform their beneficial action in the gastrointestinal tract (Walker and Gilliland, 1983). The strains resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad, et al. 1998). The resistance capabilities of all the nine isolates showed more or less nearly equal resistance against 0.05%, 0.1%, 0.15% and 0.3% artificial bile acid concentrations after 1 , 2 , 3 and 4 hours of incubation respectively. After 16 hours of incubation, all the isolates started multiplication. Among the three species *Bifidobacterium spp.* showed stronger resistant than *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp *bulgaricus.* Klayraung et al,2008 studied bile tolerance of *lactobacilli* where concentrations of 0.3, 0.5 and 1.0% bile salts were used. All strains showed they could survive more than 60% in 0.3 and 0.5% bile salt solutions. The bile resistance test results of the FSA project by Gibson et al (2005) provide evidence of the bile tolerance nature of some of the *Lactobacillu* species. Elizete and Carlos (2005) stated that bile tolerance is an essential characteristic for better survival, not necessary for multiplication.

Gastric Juice Resistance

Gastric juice resistance ability is one of the most important characteristics of probiotic lactic acid bacteria to survive, to multiply and to exert their beneficial action in the gastrointestinal tract (GIT) (Kilara, 1982). LAB must survive the acidic environment of the stomach in order to reach the gut and modulate the microbiota. In the present study, pepsin was added in the experimental medium to stimulate the activity of gastric acid environment and the isolated lactic acid bacterial species were able to survive and to some extent showed multiplication in artificial gastric acid environment at pH 2.2. This was towards gastrointestinal condition at pH 6.6 after 1, 2, 3 and 4 hours of incubation respectively which was observed from the absorbance data of bacterial cell concentration in artificial gastric acid medium at 620 nm. However, after 24 hours of incubation the

survival as well as multiplication abilities were greatly reduced in both pH 2.2 and pH 6.6 of gastric juice.

All the three isolate of *Lactobacillus acidophilus* showed good multiplication and survivability at pH 2.2 specially in first hour. Sample no. 3 of *Lactobacillus acidophilus* showed better multiplication at the first 2 hours while the other two samples also showed satisfactory multiplication. But after 4 hours their multiplication was reduced and remains almost same after 24 hours. On the other hand three isolates of *Lactobacillus delbrueckii ssp bulgaricus* and *Bifidobacterium spp.* showed very good multiplication at the first 4 hours and showed survivability after 24 hours. In comparison to the tow species *Lactobacillus acidophilus* and *Bifidobacterium spp., the Lactobacillus delbrueckii ssp bulgaricus* showed better resistance and survivability to gastric juice and also showed multiplication after 24 hours incubation.

In addition, at pH 6.6 after 1, 2, 3 and 4 hours, all the nine isolates showed more or less equal survival and multiplication abilities. But *Lactobacillus delbrueckii ssp bulgaricus* and *Bifidobacterium* spp. showed very good multiplication after 24 hours where as *Lactobacillus acidophilus* showed steady survibabily. Overall, pH 6.6 served as control and showed greater survival and multiplication abilities than pH 2.2. The gastric juice resistance test results of the present study were found nearly similar with the results obtained by Gibson et al. (2005), where eight strains of lactic acid bacteria showed more than 50% survival abilities at pH 2 and 3. Shahidi et al., (2008) also showed that some species of *Bifidobacteria* is adapted with low pH though their growth become lower at pH 3. Linn et al., (2008) showed that all strains of *Lactobacillus* survived at pH 2 for a longer period although considerable variation among strains was observed.

Antimicrobial Activity

Isolated three species showed antimicrobial activity against *Shigella flexeri, Shigella dysenteriae, Vibrio cholerae* and *Salmonella typhi. Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp. also showed antimicrobial activity against *Staphylococcus epidermis, Pseudomonas spp. and E. coli.* Sample no. 3 of *Lactobacillus acidhphilus* showed strong antimicrobial activity in contrast of other two isolates. Sample no. 2 of *Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp. also sowed

better antimicrobial activity rather than other isolates. In terms of species, *Lactobacillus delbrueckii* ssp *bulgaricus* showed better activity in contrast to *Lactobacillus acidophilus* and *Bifidobacterium* spp. The antimicrobial activity of *Lactobacillus acidophilus* was found similar with the study of Coconier et al (1997). Other study of Gilliland and Speck (1977), Gilliland (1979) Lim, Huh,and Baek, (1993), Rasic and Kurmann (1983) and Sandine (1979) also stated the antimicrobial activity of *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp.

Product Development

For the production of yogurt, milk was collected from local source. All the nine isolates were used as inoculums. *Lactobacillus delbrueckii* ssp *bulgaricus* was used as starter culture which was used in the study by Haddadin et al, 2004. After production of yogurt pH and total bacterial count was done during day 0, 7, 14 and 21 for ensuring the perfect pH and survivability of bacteria and the probiotic dose. Davidson et al. 1999 showed the survivability of LAB in frozen state in -20 and the total bacterial count was much more good than the study showed by Maragkoudakis et al 2004.

Mice Trial

Body weight and Blood Cholesterol Level:

The comparative study of starter cultures showed that yoghurt inoculums containing *L. acidophilus*, *L. brevis, L. bulgaricus* and *B. adolescentis* together produced good quality yoghurt as compared to other combination of lactic acid forming bacteria. The efficacy, safety and beneficial effects of prepared bio-yoghurt was studied through laboratory mouse trail in order to know the acute toxicity, body weight gain, blood cholesterol level.

Forty albino mice were divided into 4 groups (one control and three treatment groups) comprising ten mice in each group. Group 1 was denoted as Control, and Group 2, Group 3 & Group 4 was denoted as Treatment Group 1, Treatment Group 2 and Treatment Group 3, respectively. The control group (Group 1) was fed 5 grams rat pellet as basal diet plus additional 2 grams rat pellet/mouse/day (to equalize with at least one control

group); Group 2, Group 3 and Group 4 mice were fed with rat pellet (5grams/mouse/day) along with 2 grams, 4 grams and 6 grams of Probiotic yoghurt, respectively. The mice trial was continued up to 28 days. After 28 days of treatment, no death and good body weight gain of mouse was observed and it may be stated that the prepared yoghurt sample was safe for consumption.

After 28 days trial, all the mice of three treatment groups showed significant body weight gain in contrast of the control group. Among the three treatment groups, treatment group 2 showed better body weight gain in comparison to group 1 and group 3. During the trial from day 14 to day 21, due to sudden rising of temperature cause lower food intake and sudden fall of body weight (Stanier, 1977). Control group showed too much fall in body weight in contrast of treatment group 2 and 3. Treatment group 1 showed steady body weight during this time.

Cholesterol level was measured on day 0 and day 28. Though the body weight of the mice of treatment group increased impressively but in the cholesterol lowering test they showed satisfactory decrease in cholesterol measurement. The entire treatment group showed significant lowering level of cholesterol after 28 day trial. But the control group didn't show any significant test result of low cholesterol level and didn't show any significant variation among collected data. Treatment group 1 and 2 showed better decrease in cholesterol lowering rather than group 3. On the other hand though the control group also showed slight decrease in cholesterol lowering but at the end they also showed their decrease in body weight. So decrease of the body weight may be the cause of cholesterol lowering for control group. The finding of the study is almost similar to the study of Ali et al (2011). Ghanem et al (2005) showed the significant body weight gain of mice where as Mahrous et al. (2011) showed significant reduction in serum blood cholesterol level in probiotic yoghurt feeding mice.

Antibiotic Resistance Test

Antibiotic resistance test was done with 20 albino mice grouped in to four groups where one positive and negative control and two other were treatment group. Experimental results showed that feeding of Probiotic yoghurt confers intestinal growth of Probiotic

4. The isolates showed very good tolerance against 0.1% and 0.3% phenol and moderate or low tolerance against at 0.4% phenol after 24 hours of incubation at 37^0 C, respectively.
5. All the three isolated species were bile salt tolerant at 0.05%, 0.1%, 0.15%, 0.2% and 0.3% during 4, 8 and 24 hours of incubation.
6. All the three isolated species were resistant to artificial gastric juice environment at pH 2.2 and 6.6 but their resistance capacity decreased after 24 hours of incubation at 37^0 C.
7. Isolated three species showed antimicrobial activity against *Shigella flexeri, Shigella dysenteriae, Vibrio cholerae* and *Salmonella typhi. Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp. They also showed *antimicrobial activity against Staphylococcus epidermis, Pseudomonas spp. and E. coli.*
8. After confirming Probiotic properties of isolates, yogurt was developed using all the nine isolates from three species as inoculums.
9. Twenty eight days mouse trial for body weight gain showed no death and good body weight gain of mouse for the three treatment group in comparison to the control group and it may be stated that the prepared yoghurt sample was safe for consumption
10. The entire treatment group showed significant lowering level of cholesterol after 28 day trial. But the control group didn't show any significant test results.
11. Fourteen days mouse trial showed that feeding of Probiotic yoghurt confers intestinal growth of Probiotic bacteria that have significant resistance for amoxicillin.
12. According to World Health Organization (WHO) and Food and Agriculture Organization (FAO, 2002) working group guidelines, the Probiotic organisms should possess the characteristics of resistance to gastric acid and bile with other attributed criteria. The present experimental isolated Probiotic lactic acid bacteria have shown almost similar characteristics defined by WHO and FAO standard.

Limitations:

i. Yoghurt samples were collected from Khulna District of Bangladesh which could limit the possibility of isolation of more efficient Probiotic bacteria from other regions.

ii. In the present study, only eight sugars were used in fermentation profile test for Probiotic bacterial species identification, which was a weakness of the study. More sugars tests would be worthy to investigate in future.

iii. There was a limitation of financial resources and time frame of the study to conduct such a huge experimental works and therefore, human trial was not conducted.

iv. The experimental results have shown that there were variations in Probiotic properties of the nine isolates belonging to three species viz. *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp *bulgaricus* and *Bifidobacterium* spp. Molecular identification is needed to study for isolation of best Probiotic lactic acid bacteria for technological interest and Probiotic product development for human.

Recommendations:

Further study needs to consider more yoghurt samples from different regions of Bangladesh. The study also needs to include molecular techniques like PCR-RFLP or SDS-PAGE and 16S rRNA sequencing for confirming the identification of Probiotic bacterial species. For better safety, evaluation trial on human is needed which may open the possibility to engineer new combinations of pre and Probiotic food products viz. ice-cream, fermented milk drinks and sausages for human consumption (from infant to adolescent) for the continental people.

REFERENCES

Ali, A. R. A., Metwally, A. M. M., Mahmoud, A. H., Attia, H. F., (2011). Effect of Feeding Probiotics on Rats' Immunityand Health Conditions during Pregnancy Food and Nutrition Sciences, 2, 96-104.

Ambalam P. S., Prajapati J. B., Dave J. M., Nair B. M., Ljungh A. & Vyas B. R. M. (2009), Isolation and characterization of antimicrobial proteins produced by a potential probiotic strain of human Lactobacillus rhamnosus 231 and its effect on selected human pathogens and food spoilage organisms. Microbial Ecology in Health and Disease.; 21: 211–220

Association of Official Analytical Chemists (AOAC), (1990). Official Methods of Analysis, 15th Edition .Association of Official Analytical Chemists Inc., Arlington, Virginia. USA.

Boudraa, G., M. Touhami, P. Pochart, R. Soltana, J.Y. Mary and J.F. Desjeux, (1990). Effect of feeding yogurt versus milk in children with persistent diarrhea. J. Paed. Gastroenterol. Nutr., 11: 509-512.

Bourlioux, P., Koletzko B., Guarner F. and Braesco, V. (2003).The intestine and its microflora are partners for the protection of the host: Report in Danone symposium the intelligent intestine. Am. J. Clin. Nutr., 78: 675-683.

Braat, H; Van Den Brande, J; Van Tol, E; Hommes, D; Peppelenbosch, M; Van Deventer, S (2004). "Lactobacillus rhamnosus induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function.". *The American journal of clinical nutrition* 80 (6): 1618–25.

Bulut, Ç. (2003). Isolation and molecular characterization of lactic acid bacteria fromcheese. *IYTE Thesis of Ms.*

Çakır, İ. (2003). Determination of some probiotic properties on Lactobacilli and Bifidobacteria. Ankara University Thesis of Ph.D.

Cardinal, M.J., Meghrous, J., Lacroix, C., Simard, R.E., (1997). Isolation of *Lactococcus lactis* strain producing inhibitory activity against *Listeria. Food Biotechnology* 11:129.

Catanzaro, J. and Green, L. (1997). Microbial ecology and probiotics in human medicine (Part-2). Altern Med Rev. 2:296-305

Chou, L.S and Weimer, B. (1999). Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus. Journal of Dairy Science* 82:23-31.

Chuayana, Jr. E.L., V. Ponce, C., Rivera, M.R.B., Cabrera, E.C. (2003). Antimicrobial activity of probiotics from milk products. *Phil J. Microbiol. Infect. Dis.* 32(2):71-74.

Coconnier M H, Liévin V, Camard M F B, Hudault S and Servin A L American Society of Microbiology (1997), Antibacterial effect of the adhering human Lactobacillus acidophilus strain LB. vol. 41 no. 5 1046-1052

Dashkevicz, M. and Feighner, S D., (1989). Development of a differential medium for bile salt Hydrolase-active lactobacillus spp. Applied and environmental microbiology, jan. vol. 55, no. 1 p. 11-16.

Davidson RH, Duncan S E, Hackney C R, Eigel W N and Boling J W (1999), Probiotic Culture Survival and Implications in Fermented Frozen Yogurt Characteristics, Blacksburg 24061-0418

De Man, J. C., Rogosa, M., and Sharpe, M.E. (1960). A medium for cultivation of lactobacilli, J. Appl. Bacteriol. vol. 23, pp. 130–135.

Donnet-Hughes A, Rochat F, Serrant P, Aeschlimann JM, Schiffrin EJ (1999): Modulation of nonspecific mechanisms of defense by lactic acid bacteria: Effective dose. J Dairy Sci, 82: 863-9.

Dugas, B., Mercenier, A., Lenoir-Wijnkoop, I., Arnaud, C., Dugas, N., and Postaire, E. (1999). Immunity and probiotics. Trends Immunology Today 20(9):387-390.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., C O'Sullivan, G., Shanahan, F., and Collins J.K., (2001). In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. Am. J. Clin. Nutr. 73:386S-392S.

Effect of Feeding Probiotics on Rats' Immunity and Health Conditions during Pregnancy (2011)

Elizete, D.F.R.P., and Carlos, S.R. (2005). Biochemical characterization and identification of probiotic lactobacillus for swine. B.CEPPA. Curitiba. Vol. 23, pp. 299-310.

Famularo G, De Simone C, Pandey V, Sahu AR, Minisola G. (2005). "Probiotic lactobacilli: an innovative tool to correct the malabsorption syndrome of vegetarians?". Med. Hypotheses 65 (6): 1132–5.

Fooks, L.J., Fuller, R., Gibson, G.R. (1999). Prebiotics, probiotics and human gut microbiology. *International Dairy Journal* 9:53-61.

Fuller , R. (1989) Probiotics in man and animals. J. Applied Bacteriology, vol. 66, pp.365-378.

Ghanem K Z, Salam A M A, Magharby A S (2005), Immunoprophylactic Effect Of Probiotic Yoghurt Feeding On Schistosoma Mansoni-Infected Mice, Polish Journal Of Food And Nutrition Sciences, 2005, Vol. 14/55, No 2, Pp. 123–126.

Gibson, G R., G. Rouzaud., J. Brostoff and N. Rayment.(2005) An evaluation of probiotic effects in the human gut: microbial aspects. Final Technical report for FSA project. University of Reading and King's College, London. pp. 1-9.

Gill HS, Cross ML, Rutherfurd KJ, Gopal PK (2001): Dietary probiotic supplementation to enhance cellular immunity in the elderly. Br J Biomed Sci, 57(2): 94-96.

Gilliland SE, Staley TE , Bush LJ (1984). Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J Dairy Sci.;67(12):3045-51.

Gilliland, S. E. (1979). Beneficial inter-relationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. Journal of Food Protection, 42, 164–167.

Gilliland, S. E., Speck, M. L. (1977a). Antagonistic action of Lactobacillus acidophilus toward intestinal and food-borne pathogens in associative cultures. Journal of Food Protection, 40(12),820–823.

Gilliland, S. E., a Speck, M. L. (1977b). Deconjugation of bile acids by intestinal lactobacilli. Applied Environmental Microbiology, 33(1), 15–18.

Gilliland, S. E., Nelson, C. R., a Maxwell, C. (1985). Assimilation of cholesterol by Lactobacillus acidophilus. Applied and Environmental Microbiology, 49(49), 377–385.

Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M (2000): Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: A double-blind, placebo-controlled trial. Gastroenterol, 119: 305-9.

Gismondo, M.R., Drago, L., Lombardi, A. (1999). Review of probiotics available to modify gastrointestinal flora. *International Journal of Antimicrobial Agents.* 12:287-292.

Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B. and Morelli, L.(2005). Should yoghurt cultures be considered probiotic?. *British Journal of Nutrition.* 93:783-786.

Haddadin M.S.Y., Awaisheh S.S. and Robinson R.K.(2004),The Production of Yoghurt with Probiotic Bacteria Isolated from Infants in Jordan, Pakistan Journal of Nutrition 3 (5): 290-293.

Harrigan W.F. and McCance, M.E. (1976). Laboratory Methods in Food and Dairy Microbiology. P. 19-20. Academic Press, London, UK.

Hartemink R., Domenech, V.R., and Rombouts, F.M. (1997). LAMVAB – A new selective medium for the isolation of lactobacilli from faeces, J. Microbiol. Meth. Vol. 29, pp. 77–84.edical J. vol. 4, pp. 1-11.

Hattingh A L, Viljoen B C (2001) Yogurt as probiotic carrier food, International Dairy Journal 11 (2001) 1–17

Heyman, M. (2000). Effect of lactic acid bacteria on diarrheal diseases. J. Am. Coll. Nutr., 19: 137S-146S.

Hitti, Miranda (2006). "Probiotics May Help Stressed Gut". WebMD. Retrieved. 10-24.

Hoda, M., (2011) Probiotics Bacteria from Egyptian Infants Cause Cholesterol Removal in Media and Survive in Yoghurt Food and Nutrition Sciences, 2, 150-155.

Hoque M.Z, Akter F, Hossain K M, Rahman M S M, Billah M M and Islam K M D (2010), Isolation, Identification and Analysis of Probiotic Properties of Lactobacillus Spp. From Selective Regional Yoghurts, World Journal of Dairy & Food Sciences 5 (1): 39-46,

IDF (International Dairy Federation), (1988). Fermented milks: Science and technology. Bulletin of the IDF No. 227, Brussels.

International Diary Federation (IDF), (1998). Yoghurt:Enumeration of characteristic microorganisms. Colony count technique at 370C . International Dairy Federation Bulletin 117A, Brussels, Belgium:1-5

Ishibashi, N., a Shimamura, S. (1993). Bifidobacteria: Research an development in Japan. Food Technology, 47(6), 126, 129–134.

Kailasapathy, K., a Rybka, S. (1997). L. acidophilus and Bifidobac-terium spp.Ftheir therapeutic potential and survival in yogurt. The Australian Journal of Dairy Technology, 52, 28–35.

Kim, H. S., a Gilliland, S. (1983). Lactobacillus acidophilus as a dietary adjunct for milk to aid lactose digestion in humans. Journal of Dairy Science, 66, 959–966.

Klayraung S., Viernstein H., Sirithunyalug J., Okonogi S. (2008), Probiotic Properties of Lactobacilli Isolated from Thai Traditional Food, Sci Pharm.; 76: 485–503.

Lankaputhra W.E.V., and Shah, N.P. (1995). Survival of *Lactobacillus* and *Bifidobacterium* spp. in the presence of acid and bile salts. Cult. Dairy Prod. J. vol. 30, pp. 2–7.

Leboffe J.M. and Pierce B.E. (2011), Section 2 in A Photographic Atlas for the Microbiology Laboratory, 4th edition, Morton Publishing

Lim, K. S., Huh, C. S., a Baek, Y. J. (1993). Antimicrobial susceptibility of Bifidobacteria. Journal of Dairy Science, 76(8), 2168–2174.

Lin, M., Savaiano, D., and Harlender, S. (1991). Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. J. of Dairy Sci. 74: 87-95

Linn M.Z., Aye K.N. and Yu S.S.(2008). Processing of Probiotic Yogurt, GMSARN International Conference on Sustainable Development: Issues and Prospects for the GMS 12-14.

Ljungh A, Wadstrom T (editors) (2009). *Lactobacillus Molecular Biology: From Genomics to Probiotics*. Caister Academic Press. ISBN 978-1-904455-41-7.

Mahrous H., Shaalan U.F. and Ibrahim A. M. (2011)The role of some probiotic lactic acid bacteria in the reduction of cholesterol on mice, International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 2(7) pp. 242-248.

Majhenic A.A., Matijašic B.B. (2001). Antibiotics influence on lactic acid bacteria inhabiting gastrointestinal tract. Mljekarstvo 51 (2) 119-134.

Mangin I., Suau A., Gotteland M., Brunser O., Pochart P. (2010). Molecular biology, genetics and biotechnology Amoxicillin treatment modifies the composition of Bifidobacterium species in infant intestinal microbiota, Anaerobe 16, 433e438.

Maragkoudakis PA, Miaris C, Rojez P, Manalis N, Magkanari F, Kalantzopoulos G, Tsakalidoua E. (2004), Production of traditional Greek yoghurt using Lactobacillus strains with probiotic potential as starter adjuncts International Dairy Journal 16 (2006) 52–60

Marshall, V. M. (1996). Bioyogurt: How healthy? Dairy Industries International, 61(1), 28–29.

Martini, M.C., Kukielka, D. and Savalano, D.A. (1991). Lactose digestion from yogurt: influence of a meal and additional lactose. Am J Clin Nutr 53, 1253–1258.

Mazza,G., 1998. Functional Food, Biochemical and ProcessingAspects. pp: 357-374.

Metchnikoff, E. 1908. The Prolongation of Life. G. P. Putnams & Sons, New York, NY.

Mian, M. F., Pek, E. A., Chenoweth, M. J. and A Ashkar, A. A. (2011). Humanized mice are susceptible to *Salmonella typhi* infection. Cellular & Molecular Immunology. 8, 83–87

Moriya J, Fachin L,Lourdes A,Gândara N, Viotto W H (2006),Evaluation Of Culture Media For Counts Of Bifidobacterium Animalis In The Presence Of Yoghurt Bacteria,Brazilian Journal Of Microbiology, 37:516-520

Patil MM, Pal A, Anand T and Ramana KV. Isolation and characterization of lactic acid bacteria from curd and cucumber. *Indian Journal of Biotechnology*. 2010; Vol: 9(April) pp166-172.

Pelczar, M.J., Chan, E.C.S. and Krieg, N.R. 1986. Microbiology.pp: 635-639. Mc Graw-Hill, Inc. New York.

Prasad J, Gill H, Smart J, Gopal PK (1998). Selection and characterization of Lactobacillus and Bifidobacterium strains for use as probiotics. Int Dairy J. 8: 993-1002.

Rasic, J. L., a Kurmann, J. A. (1983). Bifidobacteria and their (pp. 12,13, 21, 42–50, 102–133, 144–158). Basel: Birkhauser.

Reid, G, Jass J, Sebulsky M.T., McCormick, J.K. (2003). "Potential uses of probiotics in clinical practice". *Clin. Microbiol. Rev.* 16 (4): 658–72. doi:10.1128/CMR.16.4.658-672.2003

Reid, G. (2001). "Probiotic agents to protect the urogenital tract against infection". Am. J. Clin. Nutr. 73 (2 Suppl): 437S–443S.

Rolfe, R.D. 2000. The Role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 130:396S-402S

Ruis, W; Fric, P; Pokrotnieks, J; Lukás, M; Fixa, B; Kascák, M; Kamm, MA; Weismueller, J *et al.* (November, 2004). "Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine". *Gut* **53** (11): 1617–23. doi:10.1136/gut.2003.037747

Sanders ME (2000). "Considerations for use of probiotic bacteria to modulate human health". *The Journal of Nutrition* **130** (2S Suppl): 384S–390S. PMID 10721912. http://jn.nutrition.org/cgi/pmidlookup?view=long&pmid=10721912.

Sanders, M.E.(2003). Probiotics: Considerations for human health. *Nutr. Rev.* 61:91-99.

Sandine, W. E. (1979). Role of Lactobacillus in the intestinal tract. Journal of Food Protection, 42, 295.

Schillinger, U., and Lucke, F.K. (1987). Identification of lactobacilli from meat and meat products. Food Microbiology. Vol. 4, pp. 199-208

Schrezenmeir, J. and Vrese, M. (2001). Probiotics, prebiotics, and synbiotics – approaching a definition. Am. J. Clin. Nutr. 73:361S-364S.

Shah, N.P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci., vol. 83 pp. 894–907.

Shahidi, F, Mendosa A F, Boylstone T and Mohebbi M, A (2008), Perception to Survival of Bifidobacterium Spp.In Bioyoghurt, Simulated Gastric Juice and Bile Solution, World Applied Sciences Journal 3 (1): 40-44.

Soccol C.R., Vandenberghe L.P.D.S., Spier M.R., Medeiros A.B.P., Yamaguishi C.T., Lindner J.D.D. , Pandey A. and Soccol V.T, (2010), The Potential of Probiotics, Food Technol. Biotechnol. 48(4) 413–434

Stanier MW. (1977),Effect of environmental temperature and food intake on the distribution of fat in growing hairless mice. Br J Nutr. Mar;37(2):279-84.

Stanton, C., Ross, R. P., Fitzgerald, G. F., and Sinderen, D. V. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. Current Opinion in Biotechnology.16:198-203.

Tannock G (2004). A special fondness for lactobacilli. Appl. Environ. Microbiol. 70:3189-3194.Tolerance of Lactobacillus acidophilus used as dietary adjunct.J. Dairy Sci. 67: 3045-3051.

Van't Veer, P., J.M. Dekker, J.W.J. Lamers, F.J. Kok, E.G. Schouten, H.A.M. Brants, F. Sturmans and R.J.Jhermus, (1989). Consumption of fermented milk products and breast cancer: a case-control study in the Netherlands. Cancer Res., 49: 4020-4023.

Vesa, T.H., Marteau P. and Korpela, R. (2000). Lactose intolerance. J. Am. Coll. Nutr., 19: 165S-175S.

Yeun, K. J., Choi, Y. O. and Ji, G E. (2008). Effect of Oral Probiotics (Bifidobacterium lactis AD011 and Lactobacillus acidophilus AD031) Administration on Ovalbumin-Induced Food Allergy Mouse Model. J. Microbiol. Biotechnol. 18(8), 1393–1400.

APPENDIX

Bile Salt Tolerance (0.05%) Test Data (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
4h	1.02	1.022	1.306	0.916	1.104	1.259	0.74	0.695	0.61
8h	1.297	1	1.788	1.886	1.95	1.89	1.947	1.959	1.842
24h	2.196	2.101	2.114	2.358	2.711	2.172	2.222	2.23	2.111

Bile Salt Tolerance (0.1%) Test Data (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
4h	1.055	1.026	1.253	0.948	1.276	1.329	0.788	0.732	0.652
8h	1.94	1.005	1.805	1.961	1.974	1.933	1.945	1.962	1.809
24h	2.156	2.131	2.099	2.545	2.521	2.213	2.245	2.233	2.064

Bile Salt Tolerance (0.15%) Test Data (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
4h	1.044	1.043	1.304	1.045	1.144	1.328	0.744	0.803	0.698
8h	1.974	1.032	1.67	1.935	1.948	1.943	1.943	1.987	1.868
24h	2.214	2.147	2.12	2.644	2.24	2.215	2.237	2.261	2.133

Bile Salt Tolerance (0.2%) Test Data (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
4h	0.987	1.018	1.373	1.168	1.22	1.31	0.798	0.736	0.664
8h	1.901	1.03	1.58	1.948	1.933	1.978	1.989	1.978	1.906
24h	2.214	2.216	1.984	2.448	2.18	2.122	2.256	2.26	2.188

Bile Salt Tolerance (0.3%) Test Data (measured at optical density 620 nm)

	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
4h	0.894	1.031	1.045	1.172	1.238	1.415	0.806	0.804	0.71
8h	1.887	1.006	1.442	1.96	1.916	1.94	1.965	1.964	1.964
24h	2.135	1.851	2.195	2.685	2.246	2.193	2.262	2.211	2.219

N.B. L.A.1= *L. acidophilus* sample 1, L.A.2= *L. acidophilus* sample 2, L.A. 3= *L. acidophilus* sample 3, L.B.1= *L. delbrueckii ssp. Bulgaricus* sample 1, L.B.2= *L. delbrueckii ssp. Bulgaricus* sample 2, , L.B.3= *L. delbrueckii ssp. Bulgaricus* sample 3, B.1= *Bifidocterium spp.* sample 1, B.2= *Bifidocterium spp.* sample 2, B.3= *Bifidocterium spp.* sample 3.

Gastric Juice Tolerance Test in artificial gastric juice at pH 2.2 (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
0 h	0.168	0.153	0.171	0.112	0.171	0.115	0.188	0.15	0.171
1 h	0.189	0.163	0.186	0.191	0.21	0.182	0.235	0.201	0.187
2 h	0.198	0.178	0.189	0.195	0.23	0.187	0.252	0.205	0.193
3h	0.211	0.176	0.196	0.199	0.236	0.188	0.259	0.22	0.198
4 hh	0.209	0.172	0.191	0.198	0.238	0.182	0.252	0.223	0.182
24 h	0.162	0.144	0.162	0.101	0.165	0.099	0.166	0.16	0.15

Gastric Juice Tolerance Test in artificial gastric juice at pH 6.6 (measured at optical density 620 nm)

Hour	L.A. 1	L.A. 2	L.A. 3	L.B. 1	L.B. 2	L.B. 3	B. 1	B. 2	B. 3
0 h	0.174	0.151	0.169	0.205	0.204	0.181	0.276	0.207	0.183
1 h	0.23	0.17	0.222	0.258	0.285	0.233	0.32	0.256	0.252
2 h	0.262	0.182	0.245	0.31	0.303	0.257	0.359	0.279	0.297
3h	0.279	0.193	0.268	0.34	0.32	0.272	0.373	0.283	0.311
4 hh	0.295	0.201	0.271	0.369	0.345	0.289	0.385	0.289	0.327
24 h	0.201	0.178	0.191	0.22	0.226	0.207	0.308	0.229	0.216

N.B. L.A.1= *L. acidophilus* sample 1, L.A.2= *L. acidophilus* sample 2, L.A. 3= *L. acidophilus* sample 3, L.B.1= *L. delbrueckii ssp. Bulgaricus* sample 1, L.B.2= *L. delbrueckii ssp. Bulgaricus* sample 2, , L.B.3= *L. delbrueckii ssp. Bulgaricus* sample 3, B.1= *Bifidocterium spp.* sample 1, B.2= *Bifidocterium spp.* sample 2, B.3= *Bifidocterium spp.* sample 3

Measured blood cholesterol level (mmol/litre): on day 0

Mouse	Control group (No yogurt)	Treatment group 1(2gm of yogurt)	Treatment group 2 (4gm of yogurt)	Treatment group 3 (6gm of yogurt)
R1	2.8	4.1	3.9	3.6
R2	3.1	4.1	6.5	5.4
R3	3.1	4.0	6.3	8
B1	3.1	4.1	6.0	3.1
B2	2.6	3.4	6.0	2.9
B3	3.9	4.0	3.9	3.6
G1	3.4	3.6	3.1	6.7
G2	2.6	6.8	6.0	5.4
G3	2.6	4.5	5.4	3.2
Unmarked	3.1	7.1	3.6	3.6
Average:	3.03	4.57	5.07	4.55

Measured blood cholesterol level (mmol/litre): on day 28

Mouse	Control group	Treatment group 1	Treatment group 2	Treatment group
R1	2.6	2.6	3.4	3.1
R2	2.6	2.8	2.9	3.1
R3	2.9	2.6	3.1	3.9
B1	2.8	2.7	2.7	2.8
B2	2.8	2.9	3.0	2.7
B3	2.6	3.2	2.9	3.2
G1	2.6	2.6	2.7	3.4
G2	2.6	2.7	3.1	3.2
G3	2.6	2.6	2.6	3.1
Unmarked	2.7	2.8	3.1	3.2
Average:	2.68	2.75	2.95	3.17

Measured body weight on day 0 at gm

Mouse	Control group	Treatment group 1	Treatment group 2	Treatment group 3
R1	46.3	49	40	39.8
R2	36.6	44	42	44.5
R3	41-5	41.5	35.5	41.2
B1	40.3	42.5	39.5	39.6
B2	42.5	42.3	38.3	40.5
B3	39.3	44.5	41.4	38.4
G1	44.5	46.4	38.7	38.4
G2	39.1	37	40.2	39.3
G3	41.8	43	40.2	39.6
Unmarked	41.8	44	39.5	41.7
Average	41.36	43.42	39.53	40.3

Measured body weight on day 7 at gm

Mouse	Control group	Treatment group 1	Treatment group 2	Treatment group 3
R1	45.7	45.5	42	44.2
R2	36.9	45.3	45.3	45.8
R3	42.5	43.9	40	44.9
B1	42.9	46.9	41.5	42.3
B2	42.9	43.5	41.9	42.8
B3	41	43.2	45.3	41.7
G1	45.3	46.9	41.6	39.6
G2	38.9	37.7	45.5	40.1
G3	40.5	44.1	41.2	39.7
Unmarked	42.4	47.1	42.2	42.6
Average	41.9	44.41	42.65	42.37

Measured body weight on day 14at gm

Mouse	Control group	Treatment group 1	Treatment group 2	Treatment group 3
R1	45.4	45.9	43.5	45.3
R2	36.1	44.5	46.4	47.3
R3	44.4	44.10	42	44.9
B1	43.6	44.4	41.5	42.2
B2	44	44.6	42.8	44.7
B3	41.8	49.2	47.6	42.9
G1	43.9	47.4	44.9	41.2
G2	40	40	48.6	41.2
G3	41	44.8	41.3	41.3
Unmarked	42.1	48.4	42.2	43.2
Average	42.23	45.33	44.08	43.42

Measured body weight on day 21 at gm

Mouse	Control group	Treatment group 1	Treatment group 2	Treatment group 3
R1	45	47	38.4	45
R2	35.4	44.6	41.6	47
R3	43	43.2	44.58	43.6
B1	41.8	48.5	40.4	45
B2	40	45.1	40.5	40
B3	41.6	45.8	47	42.3
G1	38.6	47.4	44	41.2
G2	37.8	48	48.3	38
G3	39.3	43.7	41	39
Unmarked	39.7	48.1	40	43.9
Average	40.33	45.14	42.58	42.5