

**A STUDY ON PROBIOTIC PROPERTIES OF BACTERIA
ISOLATED FROM SELECTED YOGHURT (COMILLA) AND
POULTRY FAECES (COMILLA AND PABNA)**

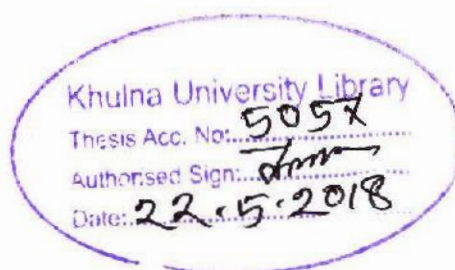


A Thesis Submitted To

Biotechnology and Genetic Engineering Discipline, Life Science School,
Khulna University, Khulna, Bangladesh, in partial fulfillment of the
requirements for the degree of Master of Science (MS) in Biotechnology and
Genetic Engineering

Submitted By

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Examination Roll No.: MS 110701
Session: 2011-2012



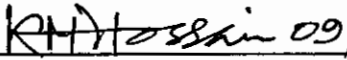
**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH
AUGUST, 2014**

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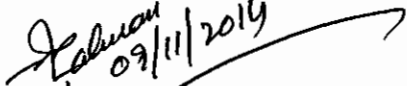
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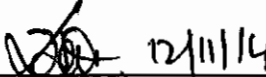
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DECLARATION

This thesis is the original research work of the author, except as otherwise stated. The material presented has not 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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.



K.M. Mehedi Hasan

Date: 09.11.14

The Research Work is
Dedicated to
My beloved twin sisters

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The Author

ABSTRACT

Probiotic bacteria are a heterogeneous group found widely in nature. They colonize in gastrointestinal and urogenital tracts of humans and animals and are present in foods. The objectives of the present research work were isolation, identification, study of probiotic properties and *in vivo* mice trial of isolated bacteria. Three isolates of *Lactobacillus acidophilus*, two isolates of *Lactobacillus brevis*, one isolate of *Lactobacillus delbrueckii* and two isolates of *Streptococcus thermophilus* were isolated from four yoghurt samples. Two *Streptococcus spp.*, one *Lactobacillus spp.* and one *Bifidobacterium spp.* were isolated from poultry samples. All isolated bacteria were gram positive, endospore negative, catalase negative, non-motile and showed bile salt hydrolase activity. In this study sixteen carbohydrates were used to construct carbohydrate fermentation profile of the isolated bacteria. Isolated probiotic bacteria showed NaCl tolerance activity against 1-8% NaCl and few showed 9% NaCl tolerance. They also showed artificial gastric acid resistance at low pH (pH 2.2) and favorable pH (pH 6.6) after 24 hours of incubation at 37°C. Isolated bacteria were capable to survive in 0.05%, 0.1%, 0.3% and 0.6% bile salt at 2, 4 and 24 hours of incubation at 37°C respectively. The Isolates showed excellent phenol tolerance activities and were capable to survive in 0.1%, 0.2% and 0.3% of phenol and moderate growth at 0.4% phenol after 24 hours of incubation at 37°C. In mouse trial, the treatment group with maximum quantity of yoghurt supplement showed as significant body weight gain, decreased blood cholesterol level, antibiotic resistance and pathogenic organisms inhibition activities.

TABLE OF CONTENTS

Topics	Page No.
ACKNOWLEDGEMENT	i
ABSTRACT	ii
TABLE OF CONTENTS	iii-iv
LIST OF TABLES	v
LIST OF FIGURES	vi-vii
LIST OF ABBREVIATIONS	viii-ix
CHAPTER 1 INTRODUCTION	01-04
CHAPTER 02 REVIEW OF LITERATURE	05-22
2.1. The Background of Probiotics	07
2.2. Beneficial Effects and Application of Probiotic Bacteria in Human	07-11
2.3. Mechanism of Probiotics	12
2.4. Selection Criteria for Probiotic Strain	13
2.5. Safety Aspects of Probiotics	14
2.6. Yoghurt as Carrier for Probiotic Microorganisms	14
2.7. Isolation of Probiotic Microorganisms	14-18
2.8. Bile Salt Hydrolases Activity	18
2.9. Gastric Juice Tolerance	19
2.10. Bile and Phenol Tolerance Resistance	19
2.11. Body Weight Gain and Blood Cholesterol Reduction	20
2.12. Antibiotic Resistance	21
2.13. Pathogenic Microorganism Inhibition	21-22
CHAPTER 3 MATERIALS AND METHODS	23-38
3.1. Isolation of Probiotic Bacteria from Yoghurt and Poultry Feces	23-25
3.2. Identification	25-29
3.2.1. Colony Morphology	25
3.2.2. Gram Staining	26
3.2.3. Endospore Test	27
3.2.4. Motility Test	27-28
3.3.5. Catalase Test	28
3.3.6. Carbohydrate(Sugar) Fermentation Test	29
3.3. Probiotic Properties Study	30-32
3.3.1. NaCl Tolerance Test	30
3.3.2. Bile Salt Hydrolase Activity Test: Plate Assay	30
3.3.3. Gastric Juice Tolerance Test	31
3.3.4. Bile Tolerance Test	31
3.3.5. Phenol Tolerance Test	32
3.4. Trial on Mice	33-36
3.4.1. Preparation of Yogurt in the Laboratory	34
3.4.2. Body Weight Gain and Blood Cholesterol Reduction Test	35
3.4.3. Antibiotic Resistance Test	35
3.4.4. <i>In vivo</i> Inhibition of Pathogenic Microorganism	36

3.5. Isolation of Probiotic Bacteria from Poultry Sample	37
3.6. Identification and Probiotic Properties Study	37
3.7. Preservation of Lactic Acid Bacteria	38
3.7.1. Long Term	38
3.7.2. Short Term	38
CHAPTER 04 RESULTS AND DISCUSSION	39-77
4.1. Isolation of Probiotic Bacteria	39
4.2. Identification	39-53
4.2.1. Colony Morphology	39-41
4.2.2. Gram Staining Test	42-44
4.2.3. Endospore Test	45-46
4.2.4. Motility Test	47
4.2.5. Catalase Test	48
4.2.6. Carbohydrate(Sugar) Fermentation Test	48-53
4.3. Determination of Probiotic Properties of Identified Probiotic Bacteria	53-57
4.3.1. Sodium Chloride (NaCl) Tolerance Test	53
4.3.2. Bile Salt Hydrolase Activity Test: Plate assay	54
4.3.3. Gastric Juice Resistance Test	55
4.3.4. Bile Salt Tolerance Test	56
4.3.5. Phenol Tolerance Test	57
4.4. Trial on Mice	58-61
4.4.1. Body Weight Gain Test	58
4.4.2. Blood Cholesterol Reduction Test	59
4.4.3. Antibiotic Resistance Test	60
4.4.4. <i>In vivo</i> Inhibition of Pathogenic Microorganism	61
4.5. Isolation of Probiotic Bacteria from Poultry Sample	62-68
4.5.1. Colony Morphology	62
4.5.2. Gram Staining Test	62-63
4.5.3. Endospore Test	64
4.5.4. Motility Test	65
4.5.5. Catalase Test	65
4.5.6. Carbohydrate(Sugar) Fermentation Test	66-68
4.6. Determination of Probiotic Properties of Identified Probiotic Bacteria from Poultry	68-72
4.6.1. Sodium Chloride (NaCl) Tolerance Test	68
4.6.2. Bile Salt Hydrolase Activity Test: Plate Assay	69
4.6.3. Gastric Juice Resistance Test	70
4.6.4. Bile salt tolerance Test	71
4.6.5. Phenol tolerance test	72
4.7. Discussion	73-77
CONCLUSION	78-79
REFERENCES	80-89
APPENDIX	90-107

LIST OF TABLES		
Table No.	Title of the Tables	Page No.
2.1.	<i>Bifidobacterium</i> species detected in human feces	15
2.2.	<i>Lactobacillus</i> species detected in the intestinal tract and used in pr obiotic products	17
3.1.1.	Collected youghurt samples and its sources	23
3.1.2.	Collected poultry samples and its sources	23
3.2.	Optimum conditions for growth of LAB isolates	25
4.1.	Colony morphology of isolated probiotic bacteria from yoghurt samples	40
4.2.	Cell morphology of isolated probiotic bacteria after gram staining from yoghurt samples	42
4.3.	Carbohydrate(Sugar) fermentation of the Isolated bacteria from yoghurt samples	49
4.4.	Isolated presumptively identified bacteria from yoghurt samples	53
4.5.	Sodium Chloride (NaCl) tolerance of isolated probiotic bacteria from yoghurt samples	53
4.6.	Bile salt hydrolase activity of isolated probiotic bacteria from yoghurt samples	54
4.7.	Colony morphology of isolated probiotic bacteria from poultry samples	62
4.8.	Cell morphology of isolated bacteria (poultry samples) after gram staining	62
4.9.	Carbohydrate(sugar) fermentation test of isolated bacteria from poultry samples	66
4.10.	Isolated presumptively identified bacteria from poultry samples	68
4.11.	Sodium Chloride (NaCl) tolerance of isolated probiotic bacteria from poultry samples	68
4.12.	Bile salt hydrolase activity of isolated probiotic bacteria from poultry samples.	69

LIST OF FIGURES		
Figure No.	Title of the Figures	Page No.
2.1	Schematic representation of the concept of probiotics	06
2.2	Beneficial Effects in the Intestine	09
3.1.1	Feeding of Mouse (Swiss Albino)	33
3.1.2	Taking Blood Sample from tail	33
3.1.3	Blood Cholesterol Measuring	33
4.1.1	Colonies of different isolated bacteria from yoghurt samples	40
4.1.2	Colonies of different isolated bacteria from yoghurt samples	41
4.2.1	Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X	42
4.2.2	Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X	43
4.2.3	Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X	44
4.3.1	Endospore test of isolated bacteria from yoghurt samples	45
4.3.2	Endospore test of isolated bacteria from yoghurt samples	46
4.4	Motility test of isolated bacteria from yoghurt samples	47
4.5.1	Sugar fermentation of the isolates (yoghurt samples) in glucose; L-arabinose; ribose; xylose; fructose and D-mannose	50
4.5.2	Sugar fermentation of the isolates (yoghurt samples) in D-sorbitol; rhamnose; salicine; mannitol and maltose	51
4.5.3	Sugar fermentation of the isolates (yoghurt samples) in lactose; galactose; cellubiose; sucrose and raffinose	52
4.6	Bile salt hydrolase activity test	54
4.7	Survival and growth abilities of isolated probiotic bacteria in artificial gastric juice at pH 2.2 and 6.6	55
4.8	Bile salt tolerance test of isolated probiotic bacteria from yoghurt samples at 0.05%, 0.1% , 0.3% and 0.6% concentrations after 0, 2, 4 and 24 hours of incubation at 37°C	56
4.9	Phenol tolerance of isolated bacteria from yoghurt samples at 0.1%,0.2%, 0.3% and 0.4% concentrations after 1 hours, 12 hours and 24 hours of incubation at 37°C	57
4.10	Body weight gain of treatment and control groups of mice after 28 days treatment with laboratory made yoghurt	58
4.11	Blood cholesterol Reduction level of treatment and control groups of mice after 28 days treatment with laboratory made yoghurt	59
4.12	The antibiotic resistance activity of lactic acid bacteria in experimental mice	60
4.13	Inhibition of pathogenic microorganism (<i>E. coli</i>) by lactic acid bacteria in experimental mice	61
4.14	Microscopic observation of isolated bacteria after gram staining from poultry samples at 40X zoom	63
4.15	Endospore test of isolated bacteria from poultry samples	64

4.16	Motility test of of isolated bacteria from poultry samples	65
4.17	Sugar fermentation of the isolates (poultry samples) in glucose; L-arabinose; ribose; xylose; fructose; D-mannose; D-sorbitol; rhamnose; salicine; mannitol; maltose; lactose; galactose; cellubiose; sucrose and raffinose	67
4.18	Survival and growth abilities of isolated probiotic bacteria in artificial gastric juice at pH 2.2 and 6.6	70
4.19	Bile salt tolerance test of isolated probiotic bacteria from yoghurt samples at 0.05%, 0.1% , 0.3% and 0.6% concentrations after 0, 2, 4 and 24 hours of incubation at 37 ⁰ C	71
4.20	Phenol tolerance of isolated bacteria from yoghurt samples at 0.1%,0.2%, 0.3% and 0.4% concentrations after 1 hours, 12 hours and 24 hours of incubation at 37 ⁰ C	72

LIST OF ABBREVIATIONS

β-ga	β-galactosidase
B	B- Lymphocytes
BPW	Buffered Peptone Water
BSCIC	Bangladesh Small & Cottage Industry Corporation
BSH	BSH bile salt hydrolase
CFU	Colony forming unit
CLA	Conjugated linoleic acids
DNA	Deoxyribonucleic Acid
DSS	Dextrin Sulfate Sodium
EPS	Exo polysaccharides
EU	European Union
EAE	Experimental Autoimmune Encephalomyelitis
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FUFOSE	Functional Foods in Europe
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
GALT	Gut-Associated Lymphoid Tissue
GYP	Glucose Yeast Peptone
H	Hour
ICDDR,B	International Centre for Diarrhoeal Diseases Research, Bangladesh
IgA	Immunoglobulin A
IFN-γ	Interferon-Gamma
IL	Interleukin
LAB	Lactic Acid Bacteria
<i>L. or Lb</i>	<i>Lactobacillus</i>
MHC	Major Histocompatibility Complex
M	Mast cells
Mm	Millimetre
mM/L	Milimole per liter
MIL	Motility-Indole-Lysine
MRS	de Man, Rogosa and Sharp
NIH	National Institute Of Health
OD	Optical Density
NK	Natural Killer
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
rDNA	Ribosomal DNA
ST	<i>Streptococcus thermophilus</i>
TDCA	Taurodeoxycholic acid
ELISA	Enzyme Linked Immune sorbent Assay
NaOH	Sodium Hydro-oxide
V/V	Volume/Volume
TDCA	Taurodeoxycholic Acid
wt/vol	Weight/Volume

TNF- α

Th

TGF- β

WHO

μ g

Tumor Necrosis Factor alpha

T-helper cell

Transforming Growth Factor- Beta

World Health Organization

Microgram

CHAPTER 1

INTRODUCTION

Probiotics are live microorganisms which give health benefit to their host and a number of *Lactobacillus sp.*, *Enterococcus sp.*, *Bifidobacterium sp.* and *Bacillus sp.* have been reported to be used as probiotics in human and animal (Klein et al., 1998; Chukeatirote, 2003). Most probiotic foods are milk based and yoghurt is one of the popular probiotic dairy food products produced from the spontaneous lactic acid fermentation of milk by probiotic microorganisms. Lactic acid produced from the fermentation of lactose acts on milk protein to give yoghurt its most characteristic texture and flavor (Ashrab and Shah, 2011). Besides, probiotic microorganisms produce small organic compounds that give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999). In yoghurt, lactic acid is produced by the probiotic microorganisms to prevent the growth of undesirable microorganisms in non-sterile raw materials and these culture bacteria help to make the products shelf-stable (Yali, 1996). Most probiotic bacteria belong to the group of lactic acid bacteria (LAB) and most LAB naturally present in food and the strains used as starter culture are also present in the gastrointestinal tract (GIT) of human and animal (Molin, 2001; Saarela et al., 2002). LAB were first isolated from milk (Carr et al., 2002) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Caplice and Fitzgerald, 1999; Jay, 2000; Liu, 2003; O'Sullivan et al., 2002). LAB occur naturally in fermented food (Caplice and Fitzgerald, 1999) and have been detected in soil, water, manure and sewage (Holzapfel et al., 2001). However, after prolonged treatment, accumulation of antibiotics in meat products occurs and becomes a serious problem. Most of antibiotics used are tetracycline, penicillin G, bacitracin, oxytetracycline and chloramphenicol. Some intestinal microflora have also developed resistance to these antibiotics (Pascual, 1999). Moreover, antibiotics can be harmful to beneficial microorganisms in GIT. Since antibiotics using harmful as feed additives, other natural biological control agents including probiotic bacteria have been used as an alternative to antibiotics (Klein, 1998; Chukeatirote, 2003).

Studies on probiotics report that probiotics have many beneficial effects, such as inhibitory effects on pathogens, aid in the management or prevention of chronic diseases and support to the immune system. Research showed that daily consumption of probiotic helps to booster immunity in elderly people (Ibrahim et al., 2010). *L. salivarius* and *B. infantis* reduce the secretion of IL-8 and the pro-inflammatory responses generated by pathogenic bacteria, and stimulate production of IL-10 (Interleukin-10) and TNF- α (Tumor Necrosis Factor alpha) by the dendritic cells (O'Hara et al., 2006). Kailasapathy and Rybka (1997) reported on several animal studies confirming that the intake of yoghurt and fermented milks containing probiotic bacteria inhibited tumor initiation and proliferation. The antitumor action of probiotics is attributed to the prevention of carcinogens, prevention of bacteria that convert procarcinogens to carcinogens (Gilliland, 1989; Gorbach et al., 1987). Probiotic microbes inhibit harmful bacterial growth by competing invading strain for essential nutrients or adhesion sites or by making the surroundings adverse for the growth of the invader microbes by producing antibacterial substances (Sandine, 1979; Gurr, 1987). Studies indicated that *lactobacilli* and *bifidobacteria* involve in production of inhibitory substances such as organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids; their acting as competitive antagonists, i.e. competition for adhesion sites and nutrients, inhibit harmful bacterial colonization of a habitat by competing more effectively for essential nutrients or adhesion sites or by making the local environment unfavorable for the growth of the invader (Sandine, 1979). The consumption of probiotic products is helpful in maintaining good health, restoring body vigor, and in combating intestinal and other disease (Mital and Garg, 1992). Consumption of probiotic food that produces linoleic acid can control weight gain caused by a lipid rich diet (Lee et al., 2006). Certain people incapable of digestion of lactose adequately due to the absence of β -D-galactosidase in the human intestine and this leads to various degrees of abdominal discomfort (Kim and Gilliland, 1983). Kim and Gilliland (1983) investigated the effect of *L. acidophilus* in milk aid lactose digestion in humans. Some lactic acid bacteria used as starter cultures in milk fermentation and these probiotic bacteria such as *L. acidophilus* and *B. bifidum* produce β -D-galactosidase. This enzyme hydrolyses lactose, which results in increased tolerance for dairy products.

LAB consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are recognized as LAB (Ercolini et al., 2001; Jay, 2000; Holzapfel et al., 2001; Stiles and Holzapfel, 1997). Some lactic acid producing gram-positive bacteria belong to the phylum Actinobacteria such as genera *Aerococcus*, *Microbacterium*, *Propionibacterium* (Sneath and Holt, 2001) as well as *Bifidobacterium* (Gibson and Fuller, 2000; Holzapfel et al., 2001). Members of LAB share the property of being Gram-positive bacteria (Fooks et al., 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Study conducted using human gastric juice (Goldin et al., 1992) showed that *L. rhamnosus* was unable to survive when challenged at pH 1 but there was no loss of viability at pH 3 or higher. Strains of lactobacilli which have a documented ability to survive and reproduce in the human gut scored poorly when challenged *in vitro* for 4 hours at pH 2.5. (Conway et al., 1987; Charteris et al., 1998). Bile salt hydrolases activity has been detected in *Lactobacillus* (De-Smet et al., 1995), *Bifidobacterium* (Kim et al., 2004), *Enterococcus* in chicken Knarreborg et al., 2002). *Lactobacilli* and *Bifidobacteria* are routinely used as probiotic strains. In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999). Strains of *Lactobacillus*, *Bifidobacterium*, *Eubacterium* and *Streptococcus*, have traditionally been used in the manufacture of fermented dairy products and are generally regarded as safe (O'Sullivan et al., 1992).

Yoghurt is one of the most common fermented milk products known for many years and has taken much attention in few years with the introduction of probiotics. Preservation of the different food products in order to achieve longer and more stable shelf life, antimicrobials become a crucial alternative to overcome many spoilage possibilities caused by pathogenic or non-pathogenic microorganisms in the product. Due to the fact that the yoghurt starter cultures grow fast in milk during the fermentation stage (6-8 h), whilst the probiotic microorganisms tend to grow slowly (18-24 h and up to 5 days depending on the used microbiota) and, as a consequence, the normal commercial practice is to blend the yoghurt bacteria with the probiotic cultures in order to achieve a shorter production time. It is a well-established fact that the yoghurt bacteria do not survive the gastrointestinal conditions or colonize the human gut (Shah, 2000) and for this reason, the probiotic microorganisms are added to the yoghurt starter cultures to achieve some

functional health benefits to humans when they consume fermented milk products. In the present research work probiotic bacteria were isolated from both regional yoghurt and poultry feces and characterized so that more potent strain can be identified and could be used in further research for the development of more potential probiotic food products for human and poultry. Therefore, the present research work was undertaken with the following objectives:

- i. Isolation, identification and study of properties of presumptively identified probiotic bacteria from regional yoghurt and poultry faeces.
- ii. *In vivo* laboratory mice trials for study of blood cholesterol level, body weight gain, antibiotic resistance test and pathogenic microorganism inhibition test.

CHAPTER 02

REVIEW OF LITERATURE

Greek word “Probiotic” originated from Latin word “pro” and Greek word “biotic” that means “for life”. Probiotics are living microorganisms when applied to human or animal, beneficially affect the health of the host by influencing the activity of microflora of the gastrointestinal tract or indigenous microbial balance. (Fuller, 1989). Probiotics are viable non-pathogenic microorganisms that colonize in intestine, modify the intestinal microflora and their metabolic activities positively affect the health of the host (Weizman et al., 2006). FAO/WHO defines Probiotics as viable non-pathogenic that are administered orally in the gastrointestinal tract and confer a health benefit to the host’s health when introduced in adequate amounts.

To be considered as potential probiotics, the bacteria must be able to tolerate extreme gastrointestinal conditions (acid, bile, enzymes, low levels of oxygen), ability to adhere to gastrointestinal mucosa and competitive exclusion of pathogens. There are certain requirements for these organisms as they must be alive, not have gone through pasteurization, and must be present as viable cells in large numbers (Gorbach, 2002) and it should be capable of exerting beneficial effects on the host animal, (Figure 2.1) nonpathogenic and non-toxic, capable of surviving in gastrointestinal conditions (low pH, organic acids and bile) (Singh et al., 2011).

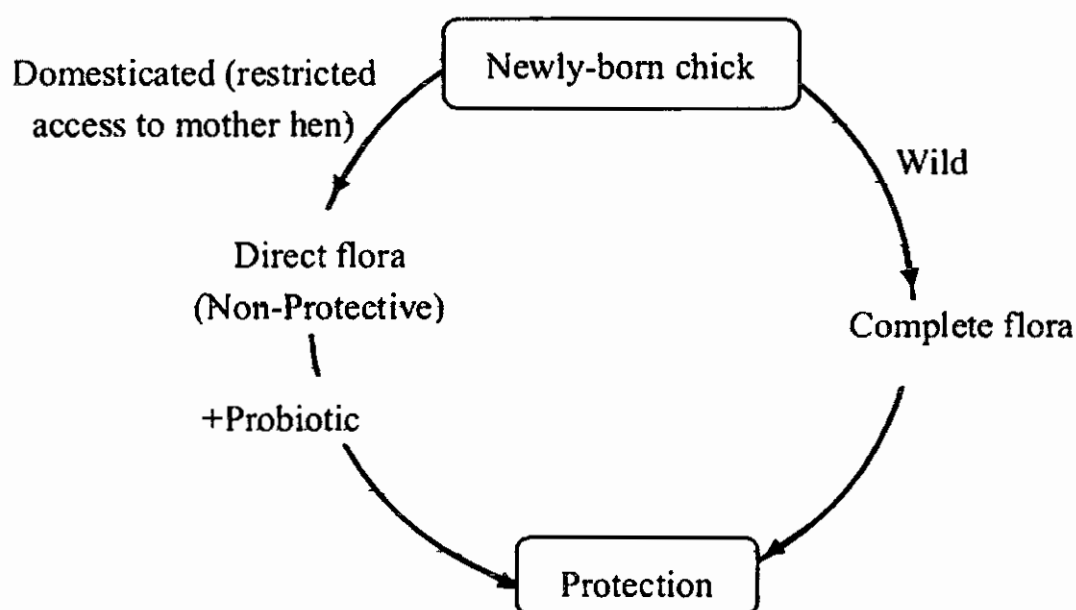


Figure 2.1: Schematic representation of the concept of probiotics (Fuller, 2001).

The most commonly known probiotic bacteria are the lactic acid bacteria, the most popular ones. Some of these are *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bifidobacterium* species can be listed as the most common ones (Singh et al., 2011).

Probiotics are produced directly by fermentation of food such as yoghurt. Although the term "Prebiotics" is also used often in such cases, the two terms should not be confused, since "prebiotics" actually refers to non-digestible food ingredients that confer benefits on the host by stimulating probiotic microorganisms in the gastrointestinal tract. Prebiotics are commonly found in, or extracted from, plant material including fruits cereal, and vegetables, but are also present in human milk and colostrums. The best characterized prebiotics include inulin, fructo-oligosaccharide, galacto-oligosaccharide, xylo-oligosaccharide, isomalto-oligosaccharide, beta-glucans, and lactulose.

2.1. The Background of Probiotics

The beneficial properties of live microbial food for example fermented milk get back many centuries ago. Their use in treatment has been mentioned in early ages, fermented milk considered not only a food product but a medicine as well. Sour milks were prescribed for prolong life, medication of disorders of the stomach and intestines (Oberman, 1985, Hughes and Hoover, 1991). Around 1908, Russian bacteriologist Eli Metchnikoff presented a scientific reason for the advantageous effects of lactic acid bacteria present in fermented milk (Hughes and Hoover, 1991; O'Sullivan et al., 1992). He attributed the good health and longevity of the Bulgarians for their consumption of large amounts of fermented milk, yoghurt. His 'longevity-without-aging' theory suggested that lactic acid bacteria resulted in the displacement of toxin forming bacteria usually present in the intestine resulting in prolonged life. In 1899, *Bifidobacteria* were isolated from the feces of breast fed infants and found that *Bifidobacteria* were a predominant element of the humans intestinal microbial flora (Ishibashi and Shimamura, 1993) and the introduction of *bifidobacteria* to infants suffering from diarrhea prevent bacteria responsible for diarrhea by re-establishing themselves as the dominant intestinal microbial flora (O'Sullivan et al., 1992). Now a days probiotic bacteria have increasingly been incorporated into foods results in several benefits.

2.2. Beneficial Effects and Application of Probiotic Bacteria in Human

- Alleviation lactose intolerance.
- Modulation host immune system
- Prevention of colon cancer.
- Anti-mutagenic properties
- Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence).
- Lowering blood pressure.
- Reducing inflammation.
- Reduction of allergic symptoms.
- Beneficial effects on mineral metabolism, particularly bone density and stability.
- Reduction of *Helicobacter pylori* infection.
- Prevent antibiotic associated diarrhoea
- Prevent acute common childhood diarrhoea
- Prevent travellers' diarrhoea
- Suppression of pathogenic microorganisms (antimicrobial effect).

- Prevention of osteoporosis.
- Prevention of urogenital infections.
- Prevent autoimmunity
- Prevent enterocolitis and sepsis in very low birth weight infants
- Prevent irritable Bowel Syndrome
- Prevent ulcerative colitis in adults.
- Alleviation antibody production in response to vaccination
- Prevention of dental caries
- Improved nutritional value of foods
- Alleviation of constipation
- Protection against colon/bladder cancer

(Source: Canducci et al., 2000; Armuzzi, 2001; Kailasapathy and Chin, 2000; Jones, 2009; Mikelsaar, 2009; Manisha, 2012; Fuller, 1989).

Alleviation Lactose Intolerance

A generally accepted health benefit of yoghurt is improved digestion of lactose in lactose-intolerant people (Ouwehand et al., 1998). This may be due to the presence of the enzyme β -galactosidase (β -gal) produced by probiotics and starter cultures that break down lactose or the physical characteristics of yoghurt (Vesa et al., 2000). β -gal extensively hydrolyses lactose into glucose and galactose (Mahoney, 1998). *Lactobacillus delbrueckii* sp. *Bulgaricus* is a promising microorganism for production of β -gal (Vasiljevic et al., 2005). It has been found that improved digestion of lactose was not caused by hydrolysis of the lactose prior to consumption, indicating that the beneficial effect must have occurred in the digestive tract after consumption of milk containing *L. acidophilus*. The continued utilization of lactose within the gastrointestinal tract depends on the survival of the *lactobacilli* in that environment. Lactose can be hydrolyzed with β -gal to avoid lactose crystallization in frozen concentrated deserts and milk consumption by lactose-intolerant individuals can be improved (Shah, 1993; Shah et al., 1993; Kim and Rajagopal, 2000). In addition to this, lactose acts as a galactosyl donor and an acceptor to form di-, tri-, or higher galacto-oligosaccharides (Wallenfels and Weil, 1972; Prenosil et al., 1987). *Bifidobacterium* and *Lactobacillus* have been used for their probiotic properties (Shah, 2007). *Bifidobacterium* and *Lactobacillus* have become organisms of interest for commercial production of β -gal (Shah and Jelen, 1990).

Modulation Host Immune System

Researchers have analyzed the immune responses caused *in vitro* in human gut epithelial cells by endogenic bacteria (*Bifidobacterium infantis* and *Lactobacillus salivarius*) and by a pathogenic bacterium *Salmonella typhimurium* increases the expression of 36 of the 847 genes involved in immunity including NF- κ B and IL-8 whereas neither of the commensal bacteria damage the expression of any of these genes. However, *L. salivarius* and *B. infantis* reduce the secretion of IL-8 and the pro-inflammatory responses generated by pathogenic bacteria, and stimulate production (Figure 2.2) of IL-10 and TNF- α by the dendritic cells. It therefore appears that even if the gut epithelium remains quiescent in contact with *L. salivarius* and *B. infantis*, these bacteria exert an effect on the intestinal immune cells (O'Hara et al., 2006).

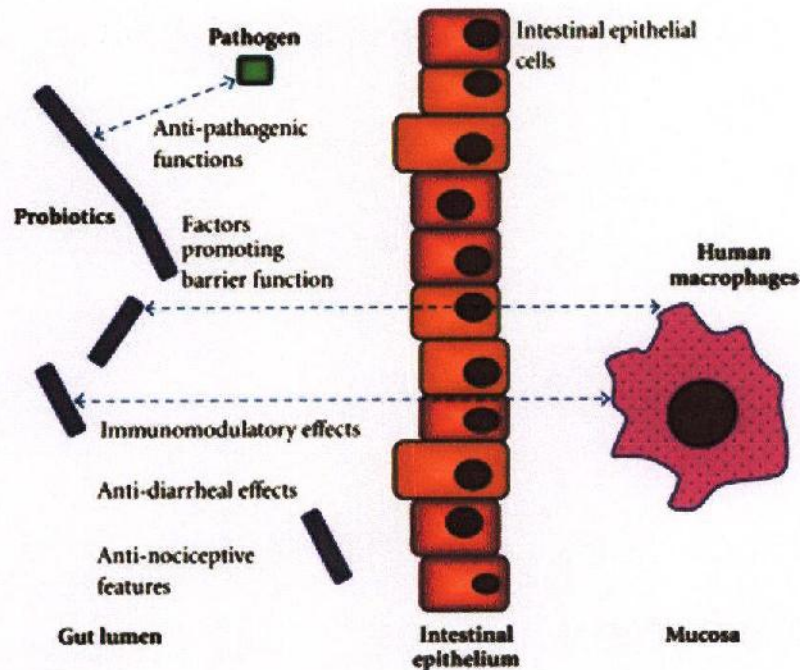


Figure 2.2: Beneficial Effects in the Intestine. Depiction of the interactions between beneficial bacteria (left side), their secreted factors, pathogens, and the intestinal mucosa (right side). Only two host cell types are shown, intestinal epithelial cells and macrophages. The arrows indicate the release and possible distribution of secreted factors derived from probiotics (Britton, 2008).

Following Activities have been Increased by Probiotics in Immune Function

Phagocytosis: The process by which phagocytic cells (for example, neutrophils and monocytes) engulf foreign cells and debris; this can lead to presentation of antigens on the phagocyte surface and facilitation a cell-mediated immune response.

Natural killer (NK) cell activity: NK cells are involved in killing infected or tumor cells by causing apoptosis or tumor cells by causing apoptosis or necrosis.

Cytokine production by T lymphocytes: Cytokines, such as Inteferon, Tumor Necrosis Factor alpha, Interleukins, etc. modulate the function of various cell types.

Antibody production in response to vaccination: Antibodies are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses.

Effect of probiotics on infections, diseases, allergies and inflammatory condition:

Enterocolitis and sepsis in very low birth weight infants: *L. acidophilis* reduced the incidence of necrotizing enterocolitis, sepsis and death.

Antibiotic-Associated Diarrhoea: Some Lactobacilli have been shown to reduce risk of developing the diarrhoea in infants and children.

Helicobacter pylori infection: Although there was no significant effect on *H. pylori* eradication, severity and frequency of overall symptoms were reduced.

Acute Common Childhood Diarrhoea: Probiotic supplementation was reported to reduce the duration of non-rotavirus diarrhoea. *L. casei*, *L. acidophilus*, *L. helveticus*, and *S. thermophilus* reduce incidence of diarrhoea in healthy children.

Travellers' Diarrhoea: A mostly beneficial effect has been observed regarding incidence, risk of frequency of the diarrhoea.

Irritable Bowel Syndrome (IBS): Supplementation with *Lactobacillus GG* decreased abdominal distention, but other gastrointestinal symptoms and abdominal pain were not altered.

Ulcerative Colitis in Adults: *L. acidophilus* and *L. reuteri* alleviated symptoms and decreased exacerbations. Other non-LAB probiotics also had some beneficial effects.

Allergies (nasal and food allergies, atopic dermatitis): Neutral and some beneficial results were achieved in various clinical studies in adults and children (Lomax and Calder, 2009; Jones, 2009; Mikelsaar, 2009).

Cancer Prevention

A number of studies have shown that predisposing factors (increases in enzyme stimulation that activate carcinogens, increase procarcinogenic chemicals within the colon or alter population of certain bacterial genera and species) are altered positively by consumption of certain probiotics (Brady et al., 2000). Kailasapathy and Rybka (1997) reported on several animal studies confirming that the intake of yoghurt and fermented milks containing probiotic bacteria inhibited tumor initiation and proliferation. The antitumor action of probiotics is attributed to the prevention of carcinogens and/or procarcinogens, prevention of bacteria that convert procarcinogens to carcinogens (Gilliland, 1989; Gorbach et al., 1987), stimulation of the host's immune system (Rasic, 1983) and/or lessening of the intestinal pH to decrease microbial function.

Anti-obesity Effect

Conjugated linoleic acids (CLA) are a major focus of scientific research since numerous health benefits would appear to be linked to their consumption such as anti-carcinogenic and anti-atherogenic effects and the reduction of body fat mass. A study on mice, explore the role of probiotic in weight management. Consumption of a probiotic that produces linoleic acid can control weight gain caused by a lipid rich diet (Lee et al., 2006). However, another study shows that fat loss via the CLA can lead in mice to a lipodystrophia associated with severe side effects (hyperinsulinemia, insulino-resistance, hepatic steatosis). Consequently, it isn't time to make extrapolations to humans (Poirier, 2005).

2.3. Mechanism of Probiotics

- Increased competition with pathogenic bacteria for nutrients and adhesion sites.
- Production of antimicrobial substances such as bacteriocins.
- Production of short-chain fatty acids by fermentation of carbohydrates, which may:
 - Reduce pH of the colon which is detrimental to pathogenic bacteria;
 - Provide nutrition to colonocytes.
- Reduction of intestinal permeability.
- Modulation of immune function of gastrointestinal mucosa.

Probiotic bacteria have been observed to have inhibitory effect against food borne pathogens (Lim et al., 1993; Rasic and Kurmann, 1983). Several studies indicated the preventative mechanisms for the inhibition of pathogens described to *lactobacilli* and *bifidobacteria* include production of inhibitory/antimicrobial substances such as: organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids; their acting as competitive antagonists, i.e. competition for adhesion sites and nutrients, inhibit harmful bacterial colonization of a habitat by competing more effectively than an invading strain for essential nutrients or adhesion sites or by making the local environment unfavorable for the growth of the invader. Stimulation of the immune system by the production of organic acids by the probiotics lowers the pH and alters the oxidation-reduction potential in the intestine, resulting in antimicrobial action (Sandine, 1979). They have also been a subject of interest with respect to the production of growth inhibition compounds, including bacteriocins (Klaenhammer, 1993). Bacteriocins are antimicrobial proteinaceous compounds that are generally inhibitory towards related bacterial strains (Tagg, 1976; Nes, 1996). The use of bacteriocins has attracted increased attention as potential bio-preservatives and as a possible substitute for chemical preservation (Abee, 1995).

2.4. Selection Criteria for Probiotic Strain

- Human source for human Probiotics
- Extreme gastrointestinal conditions (acid, bile, enzymes, low levels of oxygen) tolerance
- Gastrointestinal Mucosal surface adhesion
- Ability to competitive exclusion of pathogens
- Must be alive, not have gone through pasteurization, and must be present as viable cells in large numbers
- Capable of exerting beneficial effects on the host animal
- Non-pathogenic, non-toxic and safe for health and medical use
- Favourable technological characteristics

(Source: Gorbach, 2002; Singh et al., 2011; Quwehand et al., 1999).

The probiotic strains must be a normal inhabitant of the human intestinal tract, survives passage through the upper digestive tract in large numbers, be capable of filling an ecological niche, and have beneficial effects when in the intestine (Gilliland, 1989). In order to survive, the strain must be resistant to bile salts present in the lower intestine, gastric conditions (pH 1–4), enzymes present in the intestine (lysozyme) and toxic metabolites produced during digestion (Hoier, 1992). The bacteria used in traditional yoghurt fermentation, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, do not belong to the indigenous intestinal flora, are not bile acid resistant and do not survive passage through the gut (Gilliland, 1979). These traditional yoghurt bacteria may, nevertheless, have positive effects as a result of fermentation metabolites, either by an inhibitory action towards pathogens or improvement of lactose digestion (Hoier, 1992). Since criteria in literature generally states that not less than a million viable cells/mL probiotic product have to be present for transfer of the probiotic effect to consumers, it is desirable that the probiotic culture multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage. The selected strains must be able to ferment milk relatively quickly, either alone or in combination with other strains (Rybka and Kailasapathy, 1995).

2.5. Safety Aspects of Probiotics

Potential probiotic strains present in breast milk contains which stimulate the growth of the beneficial bacteria neonate gut (Martin et al., 2004). During their pregnancy and for the six months following childbirth, the women were given the *Lactobacillus rhamnosus* probiotic. the administration of *L. rhamnosus* during the first months of life, while being well tolerated by the babies, did not affect the composition of their gut microflora (Rinne, 2006).

2.6. Yoghurt as Carrier for Probiotic Microorganisms

yoghurt is an important food and the high concentration of probiotic microorganisms in yoghurt play important biological role for health benefits. Studies on this biological food indicate that yoghurt is a challenging source for potential probiotic microorganisms. Probiotic organisms isolated by Priya et al. (2011) from different commercially prepared yoghurt and the isolates were identified using standard morphological, cultural and biochemical reactions (Howells, 1992). The count of probiotic bacteria in the fermented milk $\geq 10^6$ colony forming units (cfu)/g at the end of the shelf-life of the product (Sanders and Veld, 1999). Yoghurt contains prebiotic substances which stimulate the growth of the beneficial bacteria. In a general view human milk contains fat, protein, carbohydrate, minerals and bacteria.

2.7. Isolation of Probiotic Microorganisms

Bifidobacterium one of the most important probiotic bacteria used in the dairy industry. They are gram-positive, non-motile, rod-shaped, and often branched anaerobic bacteria. They were first isolated from a breast-fed infant by Henry Tissier from the Pasteur Institute. *Bifidobacteria* have a positive effect on the immune system and help to control intestinal pH. In addition, *Bifidobacteria* produce bacteriocins and bacteriocin-like inhibitory compounds which inhibit the growth of other bacteria. *Bifidobacteria* possesses many glycosylases able to degrade various plant or milk-derived oligosaccharides. Several such enzymes were identified on the *Bifidobacterium* genome. Also diverse glycosyl hydrolase, ABC transporter and the fos gene cluster that is involved in the processing of health promoting fructooligosaccharides, called bifidogenic factors, can be found on the genome (Kim et al., 2009). Obviously *Bifidobacteria* are able to utilize a broad range of substrates as energy sources, such as plant polymers, glycoproteins and glycoconjugates, as well as having specialized proteins for the catabolism of oligosaccharides. *Bifidobacteria* also have a unique

hexose metabolism called the bifid shunt. The key enzyme, fructose-6-phosphate phosphoketolase is not found in any other gram-positive intestinal bacteria and therefore provides an ideal target for a diagnostic test. It was found that live *B. lactis* bacteria can directly counteract the harmful effects of coeliac-toxic gliadin and this may prove to be a future potential treatment of coeliac disease (Lindfors et al., 2008).

Only 3-6% of the fecal flora of adult intestines is composed of *Bifidobacteria* (Table 2.1). With rising age, the number of *Bifidobacteria* reduced (Bundesamt für Gesundheit, 2004).

Table 2.1. *Bifidobacterium* species detected in human faces.

<i>Bifidobacterium</i> species	
<i>B. adolescentis</i>	<i>B. gallicum</i>
<i>B. angulatum</i>	<i>B. globosum</i>
<i>B. bifidum</i>	<i>B. infantis</i>
<i>B. breve</i>	<i>B. longum</i>
<i>B. catenulatum</i>	<i>B. pseudocatenulatum</i>
<i>B. dentium</i>	

(Source: Sgorbati et al., 1995)

Individual with lower numbers of *Bifidobacteria* have a higher threat for diarrhea and allergies. That's why, *Bifidobacteria* are used as a probiotic infant supplement as drinks, yoghurts and other products. Fluka has developed *Bifidobacteria* Selective Media (BSM) which allows easy and fast quality control of yoghurt made with *bifidus*. *Bifidobacterium* colonies raise within 24-48 hours and purple brown colonies are formed. Therefore it is easier to differentiate *Bifidobacteria* from other organisms. A study by Swiss governmental on enumeration of *bifidobacteria* in sour milk products showed that using BSM Agar, the *bifidobacteria* forms purple brown colonies which allows easy and fast enumeration (Siegrist, 2011).

Probiotic microorganisms isolated from different commercially prepared yoghurt (Priya et al., 2011). The isolate was initially tested for colony morphology phase, using phase contrast microscopy, motility, gram staining and catalase reaction (with 10% H₂O₂). Gas production from glucose was determined in MRS broth supplemented with 1% glucose containing inverted Durham tubes at 30°C for 48 h. Arginine dihydrolase was determined in MRS broth supplemented with 0.3% arginine, which was incubated for 3 days (Jeppesen and Huss, 1993). In addition, the LAB isolate was tested for the ability to grow in MRS-broth containing 2, 5, 7, 10 and 15% NaCl (w/v) at 30°C for 3 days. The ability of LAB strain to grow at different pH (2, 3, 4 and 6.5) and temperature values (15, 37, 40 and 45°C) was also checked using MRS broth and carbohydrate fermentation profiles of the selected isolate were investigated using API 50 CH strips (Ndaw, 2008). Enumeration of the yoghurt species, *L. bulgaricus* and *S. thermophilus*, were performed using MRS and M17 agar, respectively (Hattingh, 2001). Agar media allowing the simultaneous enumeration of *S. thermophilus* and *L. bulgaricus* are Lactic acidbacteria (LAB) agar (Davis and McCaskill, 1971). *S. thermophilus* were only evident in M17 and ST agar media. *L. delbrueckii* ssp. *bulgaricus* did not grow in MRS-vancomycin media and reinforced clostridium agar (RCA) media. In addition, *L. rhamnosus* and *B. animalis* ssp. *lactis* grew only in MRS-vancomycin and RCA agar media respectively. The MRS (pH 5.4) agar was suitable for counting *L. delbrueckii* ssp. *Bulgaricus* selectively (Saccaro et al., 2011).

Lactobacilli are straight rod-shaped, gram-positive, fermentative, facultative anaerobic or microaerophilic organotrophs. Under certain circumstances spiral or coccobacillary forms have been observed. As a general, they appear as chains of varying length. Lactobacilli belong to the lactic acid bacteria group. They produce lactic acid and obtain energy from the fermentation of lactose, glucose and other sugars to lactate through homofermentative metabolism. Conversely, there are some heterofermentative lactobacilli that produce alcohol as well lactic acid from sugars. This acid forming inhibits growth of other organisms and supports the growth of lactobacilli that flourish in low pH. During the process, non-oxidative substrate level phosphorylation ATP is formed. Some strains produce bacteriocin like substance that inhibit a broad range of pathogens (Ljungh and Wadström, 2009). Lactobacilli also produce adhesins, a proteins, that play a critical role in specific host component recognition. It is very important for the bacterial adhesion and colonization at host extracellular matrix surfaces (Ljungh and Wadström, 2009). Several new *Lactobacillus* species (Table 2.2) have been introduced as probiotics, including *Lactobacillus rhamnosus*,

Members of the genus *Lactobacillus* can be selected on solid culture media that have an acidic pH (e.g., Rogosa SL agar) while many *Lactobacillus* strains used in the dairy industry can be cultured under microaerophilic, or even aerobic conditions, intestinal isolates proliferate best under anaerobic conditions. *Lactobacilli* are gram-positive, non-spore-forming rods (ranging from coccobacilli to long, slender bacilli). Confirmation that the isolates belong to the genus *Lactobacillus* can be accomplished by testing for the absence of catalase activity, and the presence of lactic acid as the major acid produced from the fermentation of glucose. Acetic, succinic and formic acids may be detected in minor quantities in some cultures. Task of *lactobacillus* isolates to homofermentative or heterofermentative categories is also a useful phenotypic test by way of a Durham tube, whether gas is produced from glucose (Tannock, 1999). In broiler, probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have a beneficial effect on broiler performance (Jin, 1998) modulation of intestinal microflora and pathogen inhibition (Jin, 1998; Kabir, 2005), intestinal histological changes, immunomodulation (Samanya, 2002; Chichlowski, 2009), certain haemato-biochemical parameters (Jin, 1998), improving sensory characteristics of dressed broiler meat (Pelicano, 2003) and promoting microbiological meat quality of broilers (Kabir, 2005).

2.8. Bile Salt Hydrolases Activity

The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and a number of bile salt hydrolases (BSHs) have been identified and characterized. BSH activity has been detected in *Lactobacillus* (Dashkevicz and Feighner, 1989; De-Smet et al., 1995), *Bifidobacterium* (Kim et al, 2004), *Enterococcus* in chicken (Knarreborg et al., 2002). *Lactobacilli* and *Bifidobacteria* are routinely used as probiotic strains. BSH activity had not been detected in bacteria isolated from environments from which bile salts are absent (e.g., *Lactococcus lactis* or *Streptococcus thermophilus*) (Moser and Savage, 2001).

2.9. Gastric Juice Tolerance

More than two liters of gastric juice is secreted each day, with a pH as low as 1.5 providing a barrier to the entrance into the gut of bacteria (Conway et al., 1987). Study conducted using human gastric juice (Goldin et al., 1992) showed that *L. rhamnosus* was unable to survive when challenged at pH 1 but there was no loss of viability at pH 3 or higher. Strains of lactobacilli which have a documented ability to survive and reproduce in the human gut scored poorly when challenged *in vitro* for 4 hours at pH 2.5. (Conway et al., 1987). *In vitro* results (Hood and Zottola, 1988; Charteris et al., 1998) showed that enteric lactobacilli could tolerate exposure to pH 2, while higher pH slightly affected their viable counts and pH 1 was destructive for all of the lactobacilli that were tested.

2.10. Bile and Phenol Tolerance Resistance

Probiotic strains are being resistant to acid and bile. Bile acids are synthesized in the liver from cholesterol and sent to the gallbladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli* subspecies, *Klebsiella* spp., and *Enterococcus* spp. *in vitro*. The deconjugated acid forms are more effective on gram positive bacteria (Dunne et al., 1999, Çakır, 2003). Many studies have reported the ability of *Lactobacillus acidophilus* (Usman and Hosono, 1999) and *bifidobacteria* (Dambekodi and Gilliland, 1998) to assimilate cholesterol from laboratory media. Thus, both types of bacteria may have the potential to reduce serum cholesterol in humans. One proposed mechanism is the deconjugation of bile salts by bacteria producing bile salt hydrolase. Most conjugated bile salts are recirculated through the enterohepatic circulation, while deconjugated bile salts are less soluble and excreted in the feces. The bile salts that are excreted must be replaced by new bile salts, which are formed from cholesterol in the body. Thus, the more bile salts excreted, the more cholesterol is removed from the body. Furthermore, deconjugated bile salts do not stimulate the absorption of cholesterol and other lipids from the small intestine as well as do conjugated bile salts. Walker and Gilliland (1993) reported that some strains of *Lactobacillus* can deconjugate bile salts. Raja et al. (2009) reported that *Lactobacillus fermentum* strain from chicken tolerates 0.3% and 0.4% phenol.

2.11. Body Weight Gain and Blood Cholesterol Reduction

The consumption of probiotic products is helpful in maintaining good health, restoring body vigor, and in combating intestinal and other disease (Mital and Garg, 1992). Goldin and Gordbach (1992) reported that the activities of nitroreductase, azoreductase and β -glucuronidase in the gut of mice could be reduced by feeding supplement of *Lactobacillus acidophilus*. The same results were found in case of human consumption (Goldin and Gordbach, 1984). Nahanshon *et al.* (1992) determined that consumption of maize/soybean supplemented with *Lactobacillus* cultures stimulated appetite and increased fat, nitrogen, calcium, phosphorus, copper, and manganese retention layers. In case of body weight gain, all these factors play important roles and highest amount of probiotic intake results highest body weight gain. However, several evidences show that probiotic bacteria may have a beneficial effect on blood lipid levels (Taranto *et al.*, 1998). *In vitro* experiments also showed that cholesterol assimilation was influenced by the presence of bile salts. Uptake of cholesterol was particularly higher in a medium containing higher concentration of bile salts (Pereira and Gibson, 2002). Cholesterol removal may be attributed to binding to the bacterial cell wall or incorporating into bacterial cellular membrane (Usman, 2000). It has been postulated that cholesterol was not closely associated with the membrane, but merely attached to the cell surface (Dambekodi and Gilliland, 1998). The fact that cholesterol may be incorporated into cellular membrane was supported by changes in membrane properties of *L. acidophilus* that were grown in the presence of cholesterol. Cells that were grown in the presence of cholesterol micelles showed greater resistance to lysis by sonication than did cells grown in control broth (Noh *et al.*, 1997). A study on the reduction of cholesterol was showed that *Lactobacillus reuteri* decreased total cholesterol by 38% when it is given to mice for 7 days (Kaur *et al.*, 2002).

2.12. Antibiotic Resistance

After prolonged treatment, accumulation of antibiotics in meat products occurs and becomes a serious problem. Most of antibiotics used are tetracycline, penicillin G, bacitracin, oxytetracycline and chloramphenicol. Some intestinal microflora have also developed resistance to these antibiotics (Pascual, 1999). Several species of *Lactobacillus* including *L. rhamnosus* and *L. casei* are intrinsically resistant to vancomycin. Charteris et al. (2001) found vancomycin resistance strains of *Lactobacillus* strain, rapidly growing, facultatively anaerobic, potentially probiotic *L. rhamnosus* strains. In the study of Toomey et al. (2010), strains of *Lactobacillus* spp. including *L. paracasei*, *L. reuteri* and *L. curvatus*, except *L. plantarum* were resistant to erythromycin. Tetracycline resistance was demonstrated by only *L. plantarum* and was also intrinsically resistant to vancomycin. Intrinsic streptomycin resistance was observed in *lactobacilli* besides *streptococci* species (Ouoba et al., 2008).

2.13. Pathogenic Microorganism Inhibition

A bacterial strain is resistant when it stay alive and reproduce in the presence of an antimicrobial agent that would normally inhibit or kill this particular kind of bacterial strain (Michigan State University, 2011). Probiotic microbes inhibit harmful bacterial growth by competing invading strain for essential nutrients or adhesion sites or by making the surroundings adverse for the growth of the invader microbes by producing antibacterial substances (Sandine, 1979; Gurr, 1987). Antimicrobial resistance is just one of the many adaptation characteristics that bacterial populations able to compete and survive amongst their microbial neighbors in whatever environment they are (Michigan State University 2011). Studies indicated that *lactobacilli* and *bifidobacteria* involve in production of inhibitory substances such as organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids; their acting as competitive antagonists, i.e. competition for adhesion sites and nutrients, inhibit harmful bacterial colonization of a habitat by competing more effectively for essential nutrients or adhesion sites or by making the local environment unfavorable for the growth of the invader (Sandine, 1979). *Lactobacillus reuterii*, 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; *Enterococcus faecalis* produces bacteriocin cytolysin; *Lactobacillus plantarum* produces bacteriocin plantaricin S; *Lactobacillus acidophilus* produces bacteriocin acidophilucin A

(Quweh and Vesterlund, 2004). The resistance of probiotic bacteria opens the possibility to develop probiotic food products that will behave adequately in the presence of antimicrobial agents without negatively affecting the survival of the probiotic bacteria and thus ensure an aggregated protection to these products against pathogenic bacteria, and extending the products shelf life (Klaenhammer Kullen, 1999). Tcanpaisan et al. (2010) reported that oral *Lactobacillus* strains showed a strong inhibitory effect against *Strep. mutans* and *Streptococcus sobrinus*, as well as, gram-negative periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Administration of the human *L. fermentum* strain in the vaginal tract of the mice indicated that the probiotic *L. fermentum* produced both preventative and curative effects on *E. coli* growth (Pascual et al., 2010). *Lactobacillus* exerts an antagonistic action against *streptococci* by inhibiting cell invasion (Principalli et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1. Isolation of Probiotic Bacteria from Yoghurt and Poultry Feces

This research work was conducted on yoghurt samples (Table 3.1) of four different commercial sources collected from Comilla District of Bangladesh. Collected samples were stored carefully at 4°C in refrigerator for prevention of contamination and deterioration of the samples.

Table 3.1.1. Collected yoghurt samples and its sources.

Sample no.	Yoghurt Sample source
17	Vogoboti, Pora Vander, Comilla.
18	Matri Vander, Monohorpur, Comilla.
19	Misti Mela, Jela School Road, Comilla.
20	Porabari Sweets, Station Road, Kandir Para, Comilla.

Table 3.1.2. Collected poultry samples and its sources.

Sample no.	Poultry Sample source
01	Government Poultry Farm, Comilla.
02	Government Poultry Farm, BSCIC, Pabna.

This research was carried out in Food Biotechnology and Molecular Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh.

Reagents

0.15% peptone, distilled water, L-cysteine, MRS medium (Peptone, Meat extract, yeast extract, D- Glucose, Tween-80, K₂HPO₄, MgSO₄, MnSO₄, (NH₄)₂C₆H₆O₇, sodium citrate, Agar), ST agar media.

Apparatus

Anaerobic chamber, autoclave, deionizer, freezer, incubator, laminar airflow cabinet, micropipette tips, micropipette, petri plates, pH meter, pipette, test tubes and vortex.

Procedure

For the isolation of *L. acidophilus* and *L. brevis* present (Saccaro et al., 2011) in the yoghurt samples MRS agar medium was used. The P^H of the medium for isolation of *L. acidophilus* and *L. brevis* was adjusted at 6.5 ± 2 . Using MRS media (De Man et al., 1960) probiotic bacteria were isolated and L-cysteine (0.05%) was added to the media to develop the specificity of the medium for isolation of *Bifidobacterium* spp from the collected yoghurt. This modification creates an anaerobic condition (Zinedine and Faid, 2007) in the media. On other hand, for the isolation *Streptococcus thermophilus* (Saccaro et al., 2011) ST agar medium was used. For isolation of *Bifidobacterium* spp. P^H was adjusted to 5.2 and ST agar medium was adjusted to pH6.8 (Saccaro et al., 2011). During media preparation, all the ingredients were dissolved in distilled water and medium was sterilized by autoclaving at 121⁰ C for 15 min. One gram of sample was suspended in 9 ml of peptone water solution (0.15% peptone) and serially diluted up to ten logarithmic (10⁻¹⁰) fold. Then the diluted samples were inoculated in the MRS agar plates, the incubation temperature and incubation time are shown in Table 3.2.

Table 3.2. Optimum conditions for growth of LAB isolates.

probiotic bacterial	pH	Incubation temperature (°C)	Incubation time (hour)
<i>Bifidobacterium</i> spp.	5.2	45	72
<i>Lactococcus</i> <i>acidophilus</i> , <i>L. brevis</i> and other <i>Lactococcus</i>	6.5	37	24
<i>Streptococcus</i> <i>thermophilus</i>	6.8	37	48

Source: Linn et al., 2008 and Saccaro et al., 2011.

3.2. Identification

After the incubation, the plates with contamination free and separate colonies were observed and colonies with typical characteristics were randomly selected for further tests.

3.2.1. Colony Morphology

Selected isolates were further examined after streaking. On MRS agar plates the colonies of the isolates were observe by repeated streaking. After incubation the colonies were examined morphologically by eye according to appearance, color, shape, size and nature.

3.2.2. Gram Staining

Isolates were examined morphologically under the light microscope using staining (Erkus 2007) method.

Apparatus and chemicals

Bunsen burner, distilled water, droppers, glass slides, activated broth culture, alcohol, crystal violet, Gram's iodine mordant and safranine.

Procedure

- ⇒ On the center of the glass slides, one drop of distilled water was transferred.
- ⇒ Using inoculating loop a colony was singled out loop and smear was prepared.
- ⇒ Crystal violet staining for 1 min
- ⇒ The excess stain is removed by washing under tap water
- ⇒ Stained with Gram's iodine mordant for 1 min
- ⇒ Washed gently under tap water
- ⇒ Fixation with 95% alcohol for 15 seconds
- ⇒ Stained with Safranine for 30 seconds
- ⇒ Washed gently under tap water
- ⇒ Drying

Gram positive bacteria give blue-purple and Gram negative bacteria give pink-red colour after Gram staining.

3.2.3. Endospore Test

Cells from fresh cultures have to be used for Gram staining. Cultures were grown in appropriate mediums at 37 °C for 24 h under anaerobic conditions and endospores test performed (Schaeffer-Fulton 1933) as follow. Endospores are bright green and vegetative cells with brownish red to pink.

Apparatus and reagents

Blotting paper, Bunsen burner, droppers, glass slides, inoculation loop, activated broth culture, distilled water, Malachite green stain (0.5% (w/v)), Safranin (2.5% (w/v) alcoholic solution) and tap water.

Procedure

- ⇒ On the center of the glass slides, one drop of distilled water was transferred. Using inoculating loop a colony was singled out loop.
- ⇒ Air dried and heat fixed covered with a square of blotting paper.
- ⇒ Then the blotting papers were flooded by malachite green stain solution keeping the paper moist and adding more dye as required.
- ⇒ Washed in tap water.
- ⇒ Stained with safranin for 30 seconds.
- ⇒ Washed with tap water.
- ⇒ Dried by blotting paper
- ⇒ Then the slides were observed under microscope for the existence of endospores.

3.2.4. Motility Test

Motility test medium was used to For determination of the motility of microorganisms motility test (ASM Microbe Library Protocol) is performed.

Apparatus and reagents

Sterile needles, test tub, Motility-Indole-Lysine (MIL) medium (Peptone, tryptone, yeast extract, L-lysine hydrochloride, dextrose, ferric ammonium citrate, bromcresol purple and agar).

⇒ Incubation at 37°C for 72 h in the anaerobic chamber.

3.3.3. Gastric Juice Tolerance Test

Apparatus and Reagents

Filter membrane of 0.22µm, aluminum foil, micropipette tips, micropipette, test tub, active culture, MRS broth, NaCl, distilled water, HCl, NaOH and , pepsine.

Procedure

- ⇒ Gastric juice (pH 2.2 and pH 6.6) was prepared artificially by 2g NaCl, 3.2g pepsine, pH 2.2 was adjusted at with HCl without dilution and was get n to 1 liter with sterile distilled water.
- ⇒ Another artificial gastric juice was adjusted at a pH 6.6 using 5 N NaOH.
- ⇒ Gastric juice was sterilized by filter membrane.
- ⇒ Next gastric juice of pH 2.2 and pH 6.6 were inoculated in test tube with the 1% (v/v) bacterial culture.
- ⇒ After incubation at 37°C and then samples were taken at 0, 2, 4 and 24 hours absorbance of cell concentration in gastric juice medium was measured by spectrophotometer at 620 λ.

3.3.4. Bile Tolerance Test

Apparatus and Reagents

Aluminum foil, micropipette tips, micropipette, test tub, active culture (overnight), 0.1% peptone water and MRSO (MRS-Oxgall) broth medium.

Procedure

- ⇒ MRS broths were prepared with different concentration of 0.05%, 0.1%, 0.3% and 0.6 % of bile oxgall, P^H of the media was 6.5.
- ⇒ Sterilization of media by autoclave.
- ⇒ After that 1% overnight culture of isolates were inoculated into the media.
- ⇒ Incubation at 37°C for 24h under anaerobic condition.
- ⇒ Survival rates of the organism were measured by spectrophotometer at 620 λ.

⇒ After 0, 2, 4h and after 24 h for cell viability was measured.

3.3.5. Phenol Tolerance Test

Apparatus and Reagents

Aluminum foil, micropipette tips, micropipette, test tub, active culture, MRS broth, NaCl, distilled water, phenol.

Procedure

- ⇒ MRS broth was adjusted with different concentration (0.1%, 0.2%, 0.3% and 0.4%) of phenol.
- ⇒ The P^H of the MRS broth was adjusted at 6.5. MRS broth with phenol was inoculated in test tube with the 1% (v/v) bacterial culture.
- ⇒ After incubation at 37°C samples were taken at 12 and 24 hours.
- ⇒ Absorbances of cell concentration of the media were measured by spectrophotometer at 620 λ .

3.4.1. Preparation of Yoghurt in the Laboratory

Apparatus and reagents:

Micropipette tips, micropipette, active culture, milk etc.

Procedure:

Well blended whole milk heated at 95-100°C for 15 min and then

↓
Cooled to 40-45°C

↓
Then 5% (v/v) liquid culture of isolated LAB culture added

↓
Then the milk mixer were poured into containers and incubated at 37°C for 8-12
hr.
↓

After coagulation yoghurt was preserved in 4°C.

3.4.2. Body Weight Gain and Blood Cholesterol Reduction Test

Materials:

Easy Mate blood cholesterol meter, mice, yoghurt etc.

Procedure:

Control group mice were fed with equivalent feed without yoghurt; group 2 and group 3 were fed with 5grams/mouse feed along with 5grams, 10 grams of probiotic yoghurt respectively for 28 days. Body weight was measured at day 0, day 7, day 14, day 21 and day 28 for determining body weight gain. Blood was collected from mice using tail nicking method on day 0 and day 28 for the blood cholesterol lowering test and using Easy Mate blood cholesterol meter cholesterol level was measured. For Blood cholesterol measurement EasyMate^R cholesterol measuring stripes were used. EasyMate^R cholesterol measuring stripes were previously adjusted with digital EasyTouch^R cholesterol measuring device. At least 1.5 µl blood was needed to activate EasyTouch^R cholesterol measuring device.

3.4.3. Antibiotic Resistance Test

Materials:

Micropipette tips, micropipette, test tub, mice, yoghurt, amoxicillin, MRS agar media etc.

Procedure:

All mice were feed with 5 gm of basal diet and adequate water for 7 days. Then they were divided into 3 groups. Group 1 and Group 2 were selected as positive and negative control respectively whereas Group 3 was selected as treatment groups. Group 1 was designed as no yoghurt + no antibiotic and group 2 was feed with only antibiotic but no yoghurt. Group 3 feed with 10 gm of probiotic yoghurt along with antibiotic, respectively. Antibiotic 0.02 mg amoxicillin mg was administered into mouse mixing with drinking water for per gram of mouse weight. Total experiment carried out for 14 days. Faces (Majhenic and Matijašić, 2001) were collected and cultured on MRS agar media for LAB colony count.

3.5. Isolation of Probiotic Bacteria from Poultry Sample

Reagents:

GYP medium (Peptone, yeast extract, D- Glucose, Tween-80, K, MgSO₄, MnSO₄, FeSO₄, NaCl sodium citrate, Agar).

Apparatus:

pH meter, test tubes, petri plates, autoclave, deionizer, vortex, laminar airflow cabinet, pipette, micropipette, micropipette tips, anaerobic chamber, incubator, and freezer.

Procedure:

All ingredients were dissolved in distill water and pH was adjusted to 6.8 and medium was sterilized by autoclaving at 121⁰ C for 15 min. Microbes were isolated from regional poultry feces by using GYP agar medium at specific P^H. Five grams of sample were mixed with 100 ml of GYP broth medium (P^h = 6.8) and the sample suspension was incubated anaerobically at 30⁰C for 24 hr. Then 100 µl of the turbid broth was diluted up to ten logarithmic (10⁻¹⁰) fold and spread onto GYP agar medium after dilution, the culture was incubated aerobically at 30⁰C for 24 hr.

3.6. Identification and Probiotic Properties Study

Colony morphology, gram staining, catalase test, endospore test, motility test, determination of sugar /carbohydrate fermentation test were accomplished as following the same procedure of yoghurt samples. Probiotic properties assay, NaCl tolerance test, phenol tolerance test, gastric juice resistance test , bile salt tolerance test, bile salt hydrolase activity test were performed as yoghurt sample.

4.2.2. Gram Staining Test

Isolated bacteria were further test for gram staining. In gram staining, the isolates retained the gram positive color. The isolated bacteria found as gram positive with different morphology (Table 4.2).

Table 4.2. Cell morphology of isolated bacteria after gram staining from yoghurt samples.

Isolate no.	Microscopic observation
I ₀₉ , I ₁₀ , I ₁₃	Rod shaped, chains
I ₁₀	Spherical/oval, chains
I ₀₂ , I ₀₄	Rod shaped, single/chain
I ₀₅	Long rod shaped, single/chain
I ₁₈ , I ₁₉	Cocccoid shaped, chain

Isolate I₀₉ and I₁₃ observed as rod shaped and occurred as chains form. Isolate I₁₀ showed spherical, some oval shaped and chained appearance. Bacteria of I₀₂ and I₀₄ isolates are rod shaped, appeared in single and chain form. Isolate I₀₅ bacteria long rod shaped, appeared in single and chain form. I₁₈ and I₁₉ isolates observed as cocccoid shaped in chain form. Microscopic observation at 40X zoom of the isolated bacteria.



Isolate no. I₀₉



Isolate no. I₁₃

Figure 4.2.1: Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X.

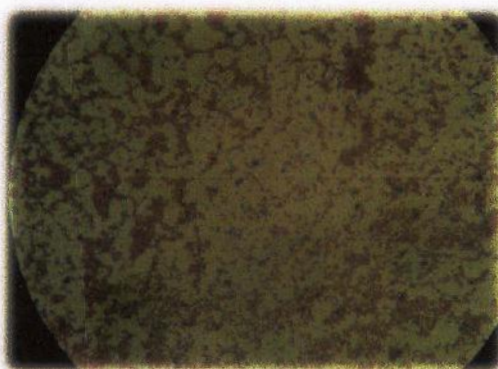
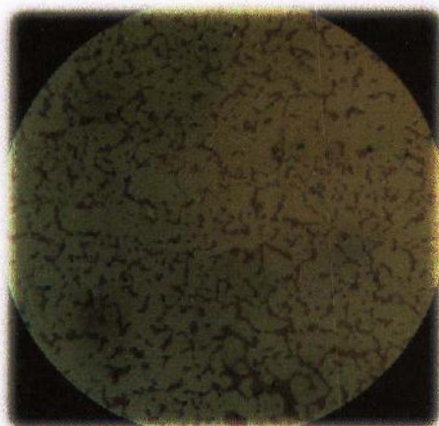
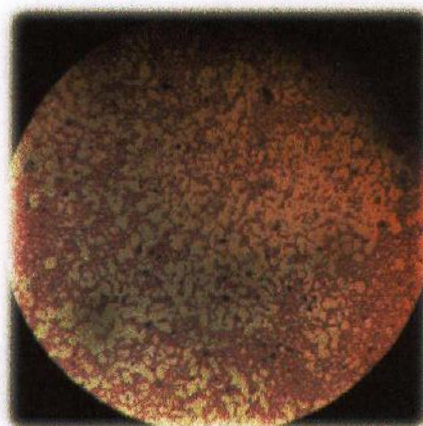
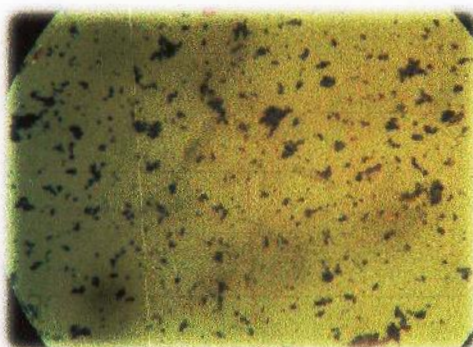
Isolate no. I₁₀Isolate no. I₀₂Isolate no. I₀₄Isolate no. I₀₅

Figure 4.2.2: Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X zoom.

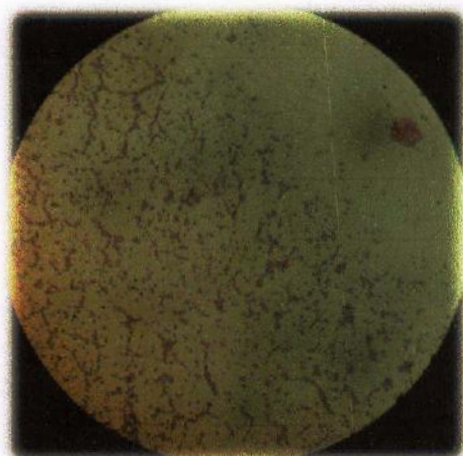
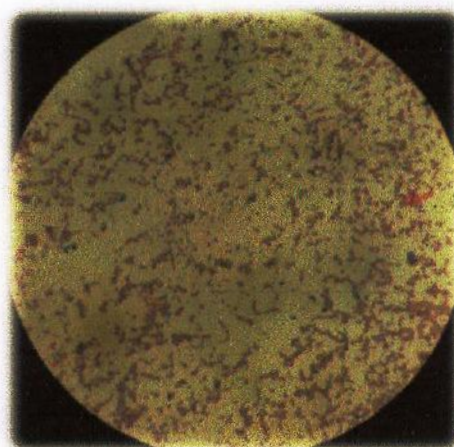
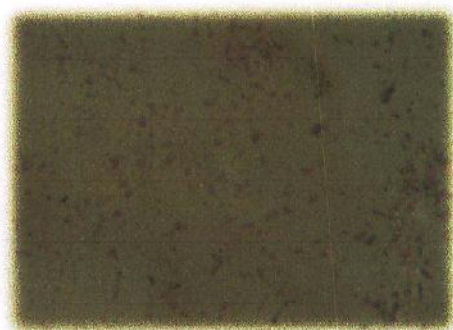
Isolate no. I₁₈Isolate no. I₁₉

Figure 4.2.3: Microscopic observation of isolated bacteria after gram staining from yoghurt samples at 40X zoom.

4.2.3. Endospore Test

Endospore test performed for the isolated bacteria. All the isolates showed negative result. In this test, no spore observed on slide prepared from the isolates under microscope. The isolated bacteria found as non-spore forming bacteria (Figure 4.3.1-4.3.2).



Isolate no. I₀₉

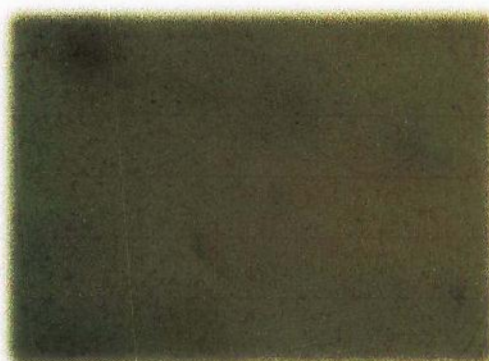
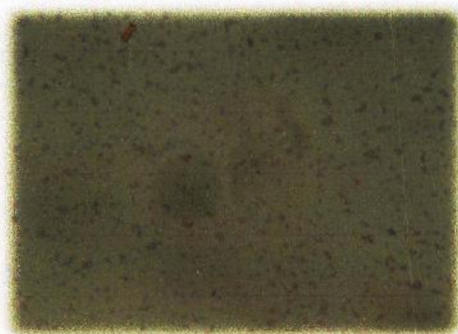


Isolate no. I₁₀



Isolate no. I₁₃

Figure 4.3.1: Endospore test of isolated bacteria from yoghurt samples.

Isolate no. I₀₂Isolate no. I₀₄Isolate no. I₀₅Isolate no. I₁₈Isolate no. I₁₉**Figure 4.3.2:** Endospore test of of isolated bacteria from yoghurt samples.

4.2.4. Motility Test

Motility test of the isolated bacteria shows that the bacteria are found non-motile. Figures shows (Figure 4.4) growth initiation along the inoculation line. These lines indicate non-motility nature characteristic of the isolated bacteria.

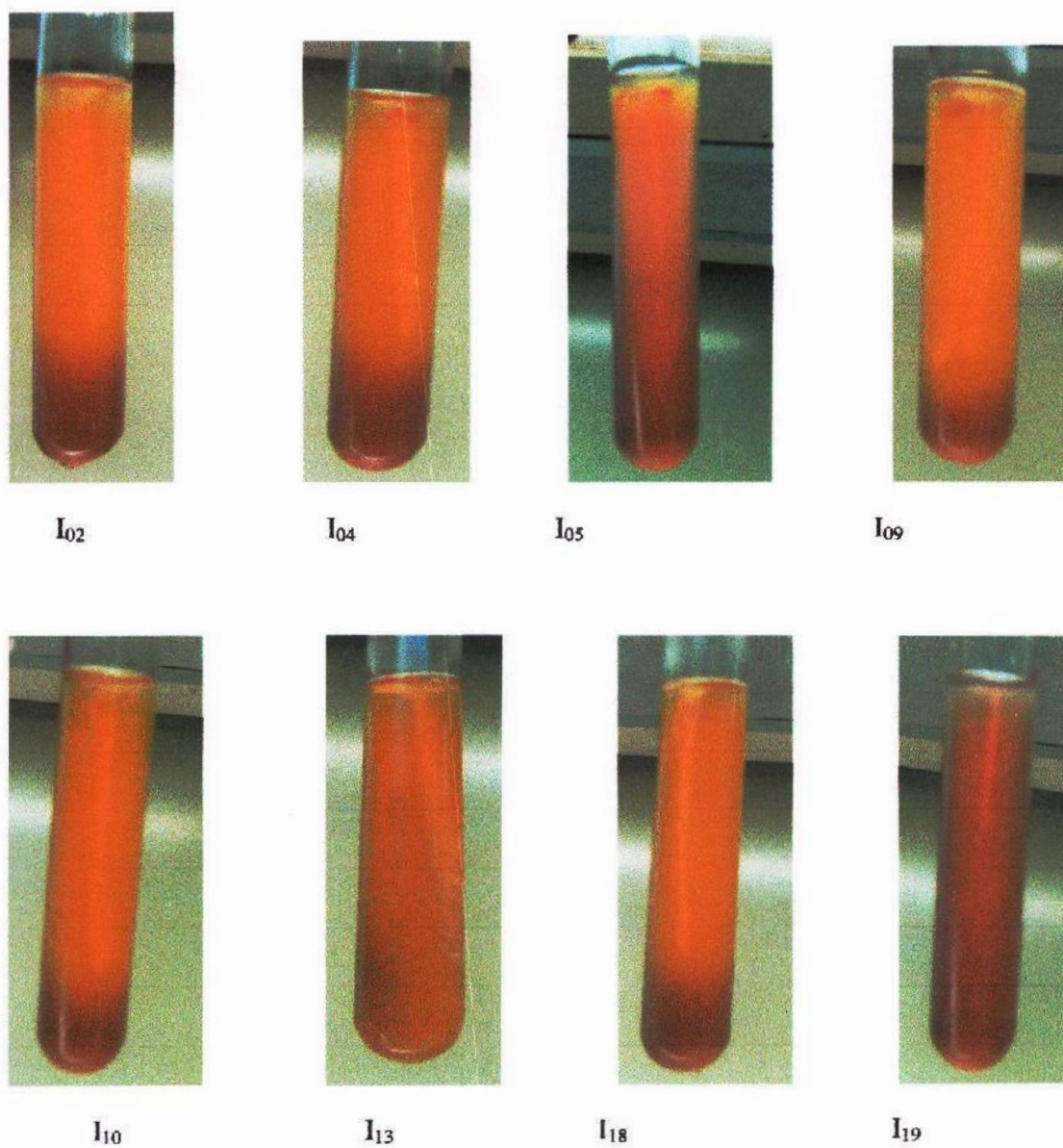


Figure 4.4: Motility test of isolated bacteria from yoghurt samples.

Table 4.1. Colony morphology of isolated bacteria from yoghurt samples.

Isolates no.	Colony morphology
I ₀₉ , I ₁₀ , I ₁₃	Very small size, circular and white
I ₀₂ , I ₀₄	Small, Circular, white and creamy
I ₀₅	Medium size, irregular and white
I ₁₈ , I ₁₉	Medium size, Circular, convex, semi-transparent

Isolate no. I₀₉Isolate no. I₁₀Isolate no. I₁₃**Figure 4.1.1:** Colonies of different isolated bacteria from yoghurt samples.

4.2.5. Catalase Test

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules. Oxygen formation is examined by the production of Oxygen bubbles. The production of gas bubbles shows the presence of the enzyme (the catalase positive nature of the bacterium). Probiotic bacteria are catalase negative. In catalase test, the isolated bacteria produced no gas bubbles, that means the bacteria exhibited catalase negative activity. All the isolated bacteria are found catalase negative due to no bubble production by addition of 3% H_2O_2 .

4.2.6. Carbohydrate (Sugar) Fermentation Test

Probiotic bacteria are anaerobic and they utilize glucose to lactic in anaerobic fermentation. These lactic acid decrease the pH in media. To detect the fermentation as well as decreased pH, a pH indicator is used. pH indicator changes color along pH change. Each types of bacteria cannot ferment each type of sugars. Each types of bacteria has a specific carbohydrate fermentation profile. In this research sixteen carbohydrate were used to construct carbohydrate fermentation profile of the isolated bacteria. Bromocresol purple used as pH indicator which purple color change to yellow indicate the fermentation of carbohydrate. The isolated bacteria presumply identified as probiotic bacteria based on their carbohydrate fermentation profile (Table 4.3.).

Table 4.3. Carbohydrate (Sugar) fermentation of the Isolated bacteria from yoghurt samples.

Isolate no. Carbo- hydrate	I ₀₂	I ₀₄	I ₀₅	I ₀₉	I ₁₀	I ₁₃	I ₁₈	I ₁₉
Glucose	+	+	+	+	+	+	+	+
L-Arabinose	+	+	-	-	+	-	-	+
Ribose	+	+	-	+	+	+	-	+
Xylose	+	+	-	+	-	+	+	+
Fructose	+	+	+	+	+	+	+	+
D-Mannose	+	+	-	+	+	+	-	-
D-Sorbitol	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Salicin	+	-	-	-	+	-	+	-
Mannitol	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+
Lactose	-	-	+	-	-	-	+	-
Galactose	+	+	-	-	+	-	-	-
Cellubiose	+	+	-	+	+	+	-	-
Sucrose	+	-	+	+	+	-	+	+
Raffinose	-	-	+	+	-	-	-	-

+ means fermentated; - means not fermentated.



Figure 4.5.2: Sugar fermentation of the isolates (yoghurt samples) in D-sorbitol; rhamnose; salicine; mannitol and maltose.



Figure 4.5.3: Sugar fermentation of the isolates (yoghurt samples) in lactose; galactose; cellulose; sucrose and raffinose.

Isolated bacteria had specific sugar fermentation pattern of its own. The change of purple colour of the broth medium to yellow colour is the indication of fermentation performed by the isolated bacteria (Fig 4.5.1-4.5.3). The sugar fermentation pattern of isolated bacteria (Table 4.4.) correlated with the study of Ghanbari et al., (2009), Karna et al., (2007), Shah et al., (2009) and Azizpour et al., (2009).

Table 4.4. Isolated presumptively identified bacteria from yoghurt samples.

Isolated bacteria	Isolates no.	Sample no.
<i>Lactobacillus acidophilus</i>	I ₀₉ , I ₁₀ , I ₁₃	S ₁₉ , S ₂₀ , S ₁₇
<i>Lactobacillus brevis</i>	I ₀₂ , I ₀₄	S ₂₀ , S ₁₇
<i>Lactobacillus delbrueckii</i>	I ₀₅	S ₁₉
<i>Streptococcus thermophilus</i>	I ₁₈ , I ₁₉	S ₁₉ , S ₁₇

4.3. Determination of Probiotic Properties of Identified Probiotic Bacteria

4.3.1. Sodium Chloride (NaCl) Tolerance Test

Isolated of probiotic bacteria show NaCl tolerance activity against 1-8% NaCl and few show against 9% NaCl. After 24 hours of incubation at 37°C turbidity indicate % NaCl tolerance substance which inhibit other types of bacteria. Isolates showed good growth at 1-7% of NaCl. In case of 8 and 9 % of NaCl each isolates showed moderate growth. Finally in 10% of NaCl, no growth was observed. NaCl tolerances of eight isolates at 1-10% have shown in Table 4.5.

Table 4.5. Sodium Chloride (NaCl) tolerance of isolated probiotic bacteria from yoghurt samples.

NaCl	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)
I ₀₂	++	++	++	++	++	++	++	+	-	-
I ₀₄	++	++	++	++	++	++	++	+	-	-
I ₀₅	++	++	++	++	++	++	++	+	-	-
I ₀₉	++	++	++	++	++	++	++	+	-	-
I ₁₀	++	++	++	++	++	++	++	+	-	-
I ₁₃	++	++	++	++	++	++	++	+	-	-
I ₁₈	++	++	++	++	++	++	++	+	+	-
I ₁₉	++	++	++	++	++	++	++	+	+	-

N.B. (++) means excellent and normal growth; (+) means low level of growth; (-) means no growth.

4.3.2. Bile Salt Hydrolase Activity Test: Plate assay

Lactic acid bacteria produce bile salt hydrolase. Due to this activity when isolated bacteria streaked out on MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA), the taurine-conjugated bile acid that was deconjugated, producing deoxycholic acid, colonies were turn into opaque granular white colonies, precipitated halos around colonies (Table 4.6).

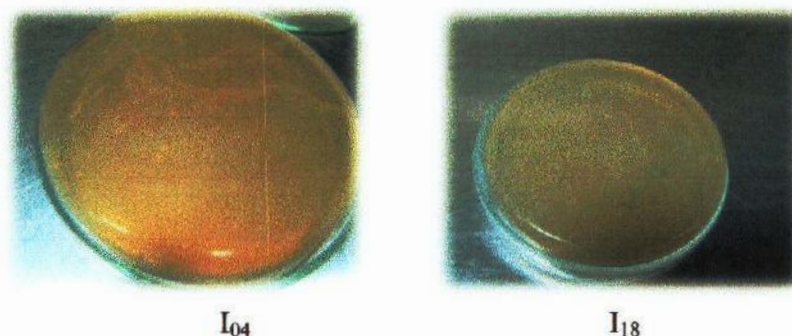


Figure 4.6: Bile salt hydrolase activity test.

Table 4.6. Bile salt hydrolase activity of isolated probiotic bacteria from yoghurt samples.

Isolated bacteria	Isolates no.	Plate assay
<i>Lactobacillus acidophilus</i>	I ₀₉ , I ₁₀ , I ₁₃	+
<i>Lactobacillus brevis</i>	I ₀₂ , I ₀₄	+
<i>Lactobacillus delbrueckii</i>	I ₀₅	+
<i>Streptococcus thermophilus</i>	I ₁₈ , I ₁₉	+

4.3.3. Gastric Juice Resistance Test

Presumptively identified probiotic bacteria had the capability to stay alive in artificial gastric acid surroundings at low pH. (pH 2.2) and favorable pH (pH 6.6). Their survival capability decreased towards 24 hours of incubation at 37°C. The character of survival and multiplication abilities indicated by (Figure 4.7) the absorbance of the isolates bacterial culture in gastric juice environment at 620 nm. The rising lines indicate more tolerance capability of. Data were expressed as average value. The incubation periods were 0 hr, 2 hr, 4 hr, and 24 hr.

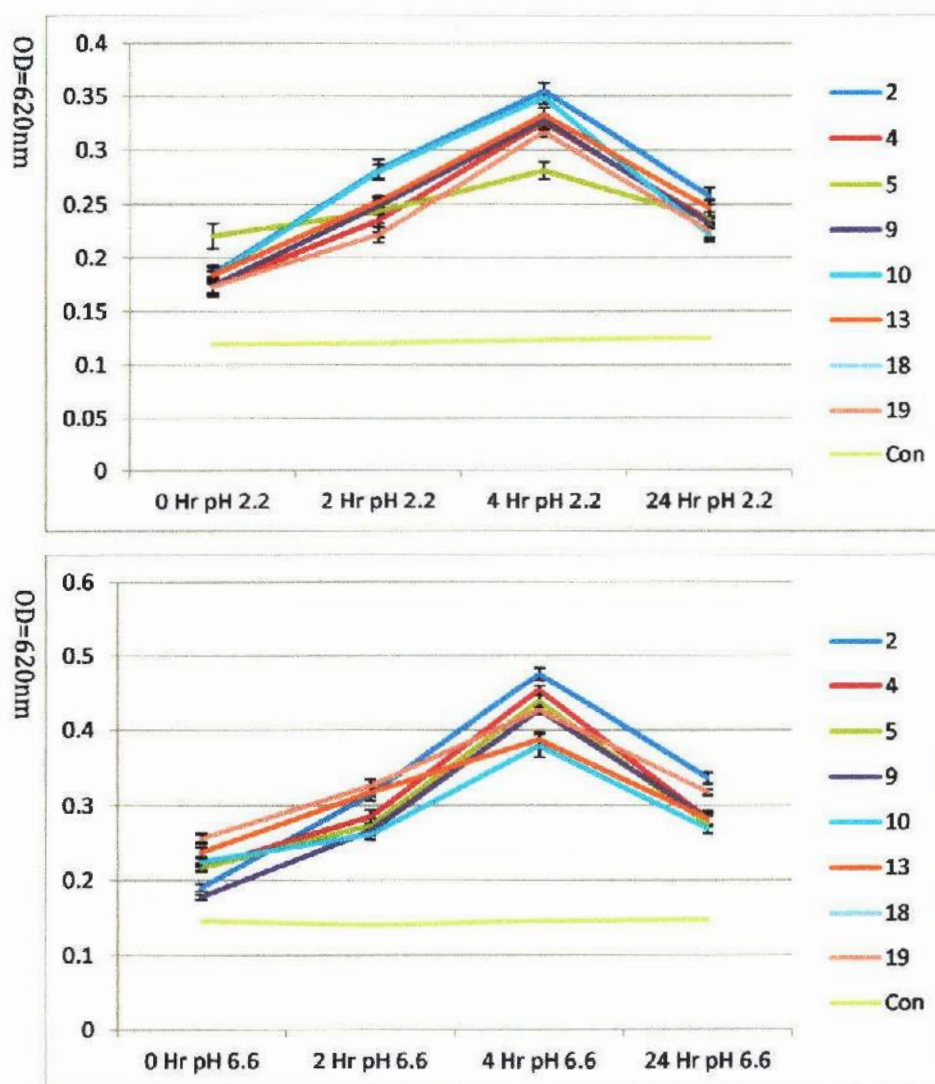


Figure 4.7: Survival and growth abilities of isolated probiotic bacteria in artificial gastric juice at pH 2.2 and 6.6. The rising lines indicate the resistance ability of the isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.3.4. Bile Salt Tolerance Test

Survival of probiotic culture through the stomach and intestine examined by bile salt tolerance test. The broth of bacterial culture containing different concentrations of bile salt was determined by observing turbidity. The turbidity of the culture decreases with the increase of bile salt concentration and with the time of incubation. Isolated bacteria are capable to survive in 0.05%, 0.1%, 0.3% and 0.6% bile salt after 24 hours of incubation at 37°C. In the optical densities values 620 nm against time and concentration were plotted in a line diagram (Figures 4.8) representing tolerance to artificial bile salt. Data were expressed as average value of isolates at different concentrations (0.05%, 0.1%, 0.3% and 0.6%) after 0 hour, 2 hours, 4 hours and 24 hours of incubation at 37°C.

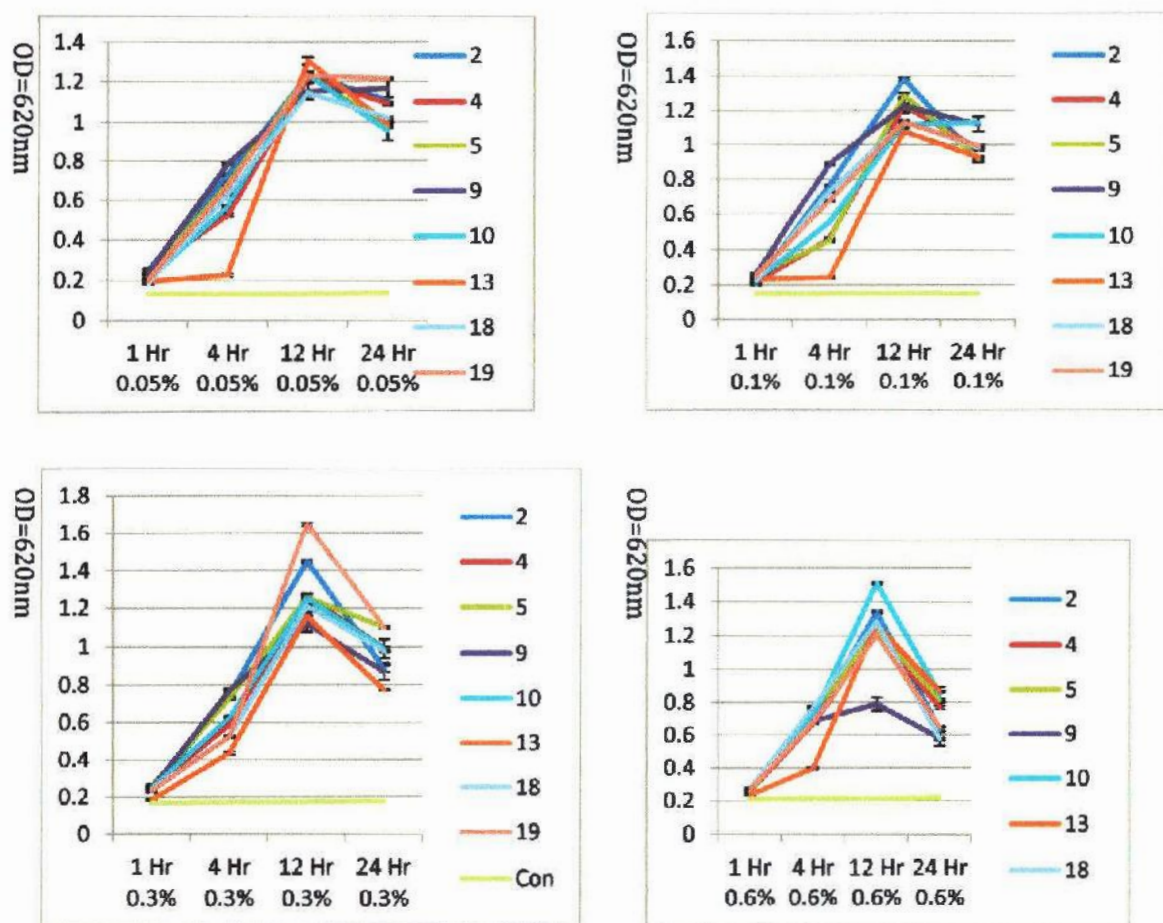


Figure 4.8: Bile salt tolerance test of isolated probiotic bacteria from yoghurt samples at 0.05%, 0.1% , 0.3% and 0.6% concentrations after 0, 2, 4 and 24 hours of incubation at 37°C. The rising lines indicate more tolerance capability of the isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.3.5. Phenol Tolerance Test

The Isolates have excellent tolerance Phenol. The turbidity of the culture decreases with the increase of concentration. Isolated bacteria are capable to survive in 0.1%, 0.2% and 0.3% of phenol and moderate growth at 0.4% phenol after 24 hours of incubation at 37°C. In the optical densities values 620 nm against time and concentration were plotted in a line diagram (Figures 4.9) representing tolerance. Data were expressed as average value of isolates at different concentrations 0.1%, 0.2%, 0.3%, 0.4% after 1 hour, 12 hours 24 hours of incubation at 37°C.

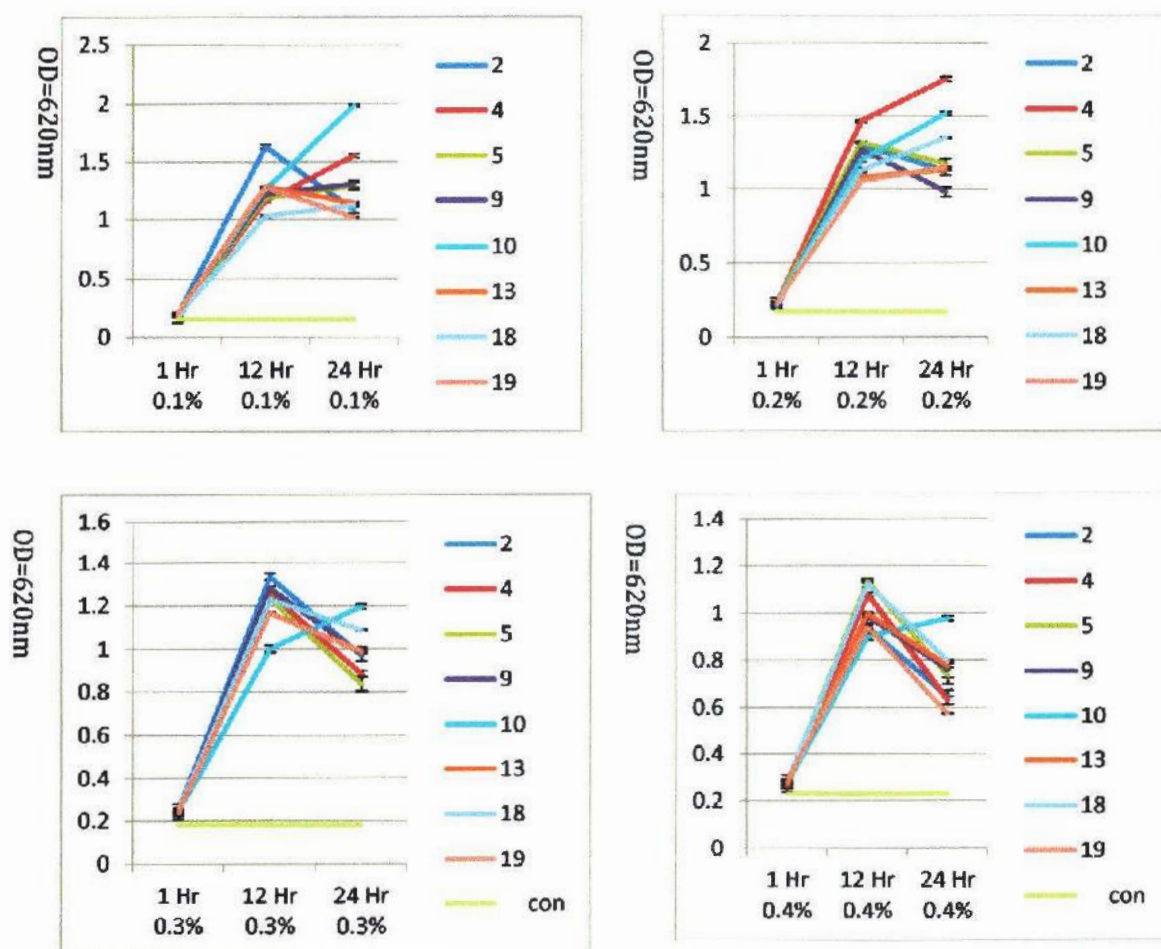


Figure 4.9: Phenol tolerance of isolated bacteria from yoghurt samples at 0.1%, 0.2%, 0.3% and 0.4% concentrations after 1 hours, 12 hours and 24 hours of incubation at 37°C. The rising lines indicate more tolerance ability of the eight isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.4. Trial on Mice

4.4.1. Body Weight Gain Test

Due to sudden fluctuation of temperature and weather, weights of the mice showed variation. But after the trial all the treatment group showed significant weight gain while control group did not show similar weight increment. That means yoghurt consumption help to gain more body weight than normal. Individual body weight of mice was measured at every week. The average body weight gain of control group is nearly 7.2 gm over 28 day's treatment period. Treatment group 2 showed highest average body weight gain (11.78 gm) and treatment group 1 showed nearly 9 gm weight gain. Here T2 was statistically significant but T1 was non-significant. Therefore, the overall results are significant at 0.05 level of significance. The mean difference of treatment group 2 was significant with the control group at 0.05 level of significance ($F=4.237$), whereas the mean difference of treatment group 1 is not significance with the control group at this level.

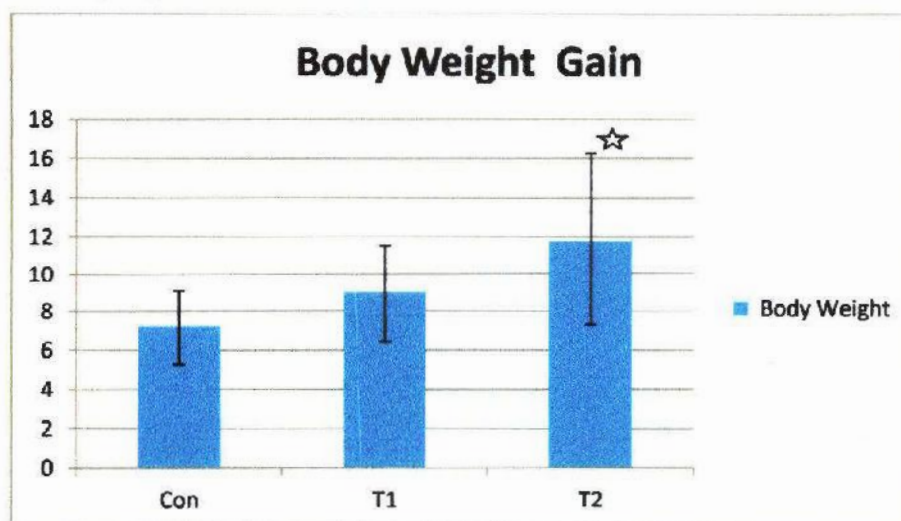


Figure 4.10: Body weight gain of treatment and control groups of mice after 28 days treatment with laboratory made yoghurt.

4.4.2. Blood Cholesterol Reduction Test

Cholesterol level was measured on day 0 and day 28. However the control group didn't show any significant test result of cholesterol level reduction and didn't show any significant variation among collected data. The average cholesterol reduction of the control group was 0.412 milimole per liter (mM/L) over 28 day's treatment period. Treatment group 2 showed highest average cholesterol reductions 0.95mM/L and treatment group 1 showed 0.82 mM/L cholesterol reduction. Here T2 was statistically significant but T1 was non-significant. So, the overall result is significant at 0.05 level of significance. The mean difference of treatment group 2 was significant with the control group at 0.05 level of significance ($F= 4.097$), whereas the mean difference of treatment group 1 is not significance with the control group at this level.

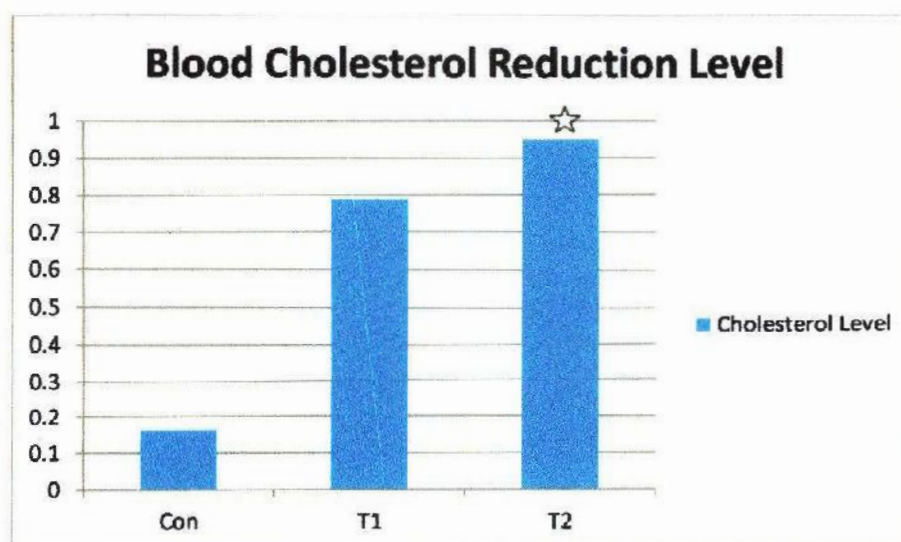


Figure 4.11: Blood cholesterol reduction level of treatment and control groups of mice after 28 days treatment with laboratory made yoghurt.

4.4.3. Antibiotic Resistance Test

Twenty four adult albino mice were selected and grouped in to four groups for antibiotic resistance test. Group 1 and Group 2 were selected as positive and negative control. Group 3 was selected as treatment groups. Total experimental day was 14. Faces were collected from each group on day 0, day 7 and day 14 and cultured on MRS agar media for LAB colony count. The logarithm of colony forming unit per ml was described in. The upward lines of T1 and T2 indicated the ability of survival and multiplication of LAB even under antibiotic treatment.

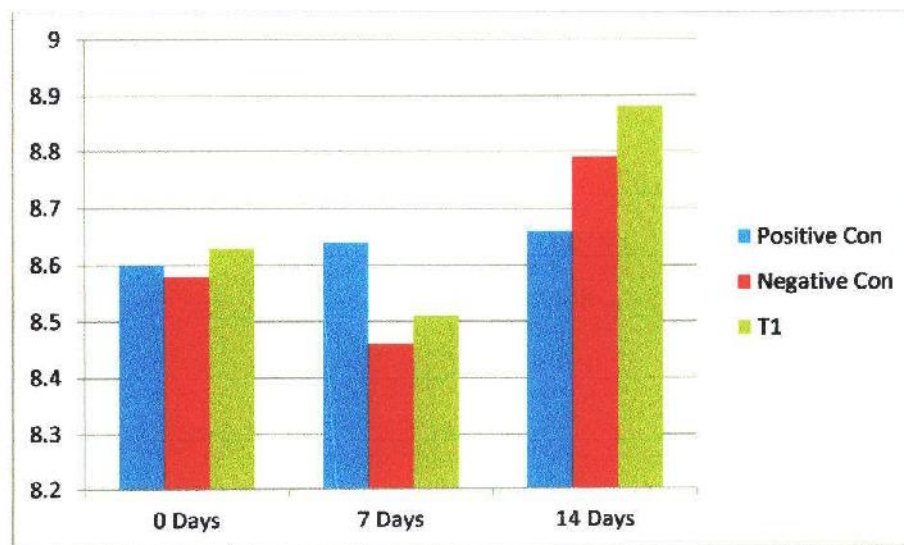


Figure 4.12: The antibiotic resistance activity of lactic acid bacteria in experimental mice.

4.4.4. *In vivo* Inhibition of Pathogenic Microorganism

Inhibition of growth of pathogenic microorganism was determined after day 7 of post infection. The yoghurt sample was fed to mice before 5 day of challenging with the pathogenic bacteria and then mice were inoculated with *E. coli* with 0.1 ml of the bacterial suspension. The no. of pathogenic bacteria/ml in samples was counted by the standard equation in logarithm of colony forming unit per ml. Treatment group 2 showed highest inhibition of growth of pathogenic bacteria and treatment group 1 showed significant inhibition of growth of pathogenic microbes than the control group of mice.

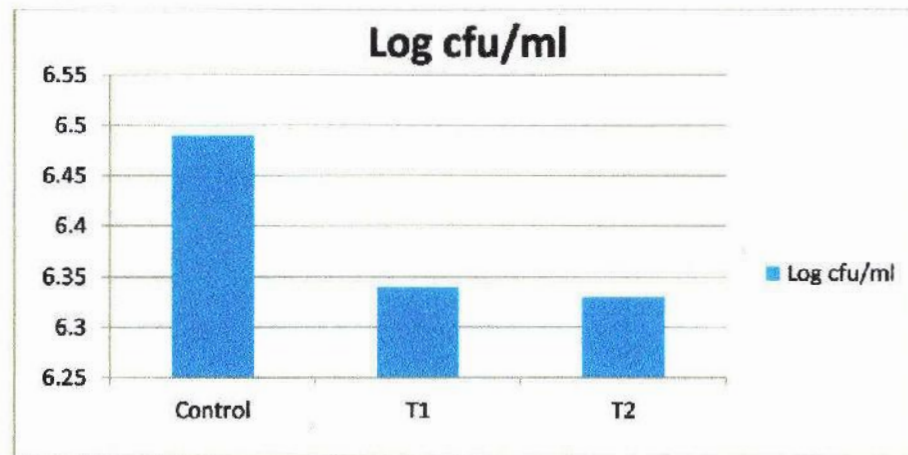


Figure 4.13: *In vivo* Inhibition of pathogenic microorganism (*E. coli*) by lactic acid bacteria in experimental mice.

4.5. Isolation of Probiotic Bacteria from Poultry Sample

4.5.1. Colony Morphology

Different types of bacteria form different types colonies. Thus colony morphology is preliminary observing criteria. Colony morphologies of the isolated bacteria (Table 4.7) are observed as colonies of isolates no. I₂₀ and I₂₁ are medium, circular. Isolate no. I₂₅ form small, circular, white and creamy colonies. Isolate no I₀₅ form medium, irregular and white colony. The colonies of isolate no. I₂₀, I₂₁ are medium, circular and semi-transparent.

Table 4.7. Colony morphology of isolated bacteria from poultry samples.

Isolated bacteria	Isolates no.	Colony morphology
<i>Streptococcus spp.</i>	I ₂₀ , I ₂₁	Medium size, Circular, semi-transparent
<i>Lactobacillus spp.</i>	I ₂₅	Circular, Small, convex, creamy and white
<i>Bifidobacterium spp.</i>	I ₂₇	Medium size, irregular and white

4.5.2. Gram Staining Test

Isolated bacteria were further test for gram staining. In gram staining, the isolates retained the gram positive color. The isolated bacteria found as gram positive with different morphology (Table 4.8).

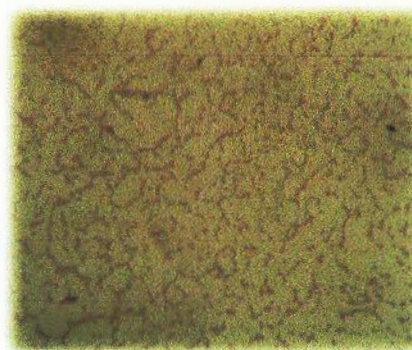
Table 4.8. Cell morphology of isolated bacteria (poultry samples) after gram staining.

Isolate no.	Microscopic observation
I ₂₀ , I ₂₁	Coccoid shaped, chain
I ₂₅	Rod shaped, single/chain
I ₂₇	Small rod or oval, single/chain,

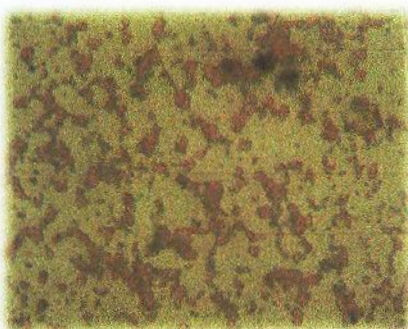
Microscopic observations at 40X zoom of the isolated bacteria are showed in figure (4.14).



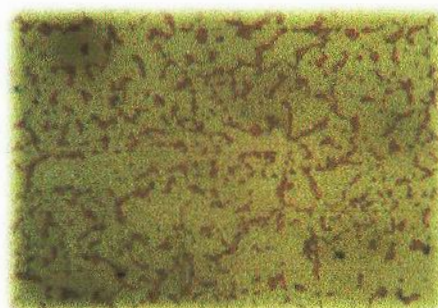
Isolate no. I₂₀



Isolate no. I₂₁



Isolate no. I₂₅



Isolate no. I₂₇

Figure 4.14: Microscopic observation of isolated bacteria after gram staining from poultry samples at 40X zoom.

4.5.3. Endospore Test

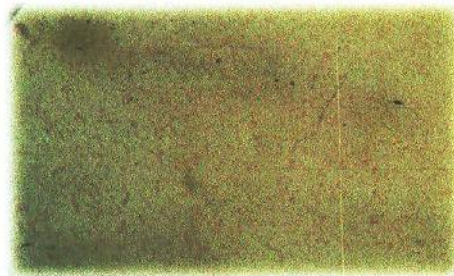
Endospore test performed for the isolated bacteria. All the isolates showed negative result. In this test, no spore observed on slide prepared from the isolates under microscope. The isolated bacteria found as non-spore forming bacteria (Figure 4.15).



Isolate no. I₂₀



Isolate no. I₂₁



Isolate no. I₂₅



Isolate no. I₂₇

Figure 4.15: Endospore test of isolated bacteria from poultry samples.

4.5.4. Motility Test

Motility test of the isolated bacteria shows that the bacteria are found non-motile. Figures shows (Figure 4.16) growth initiation along the inoculation line. These lines indicate non-motility nature characteristic of the isolated bacteria.

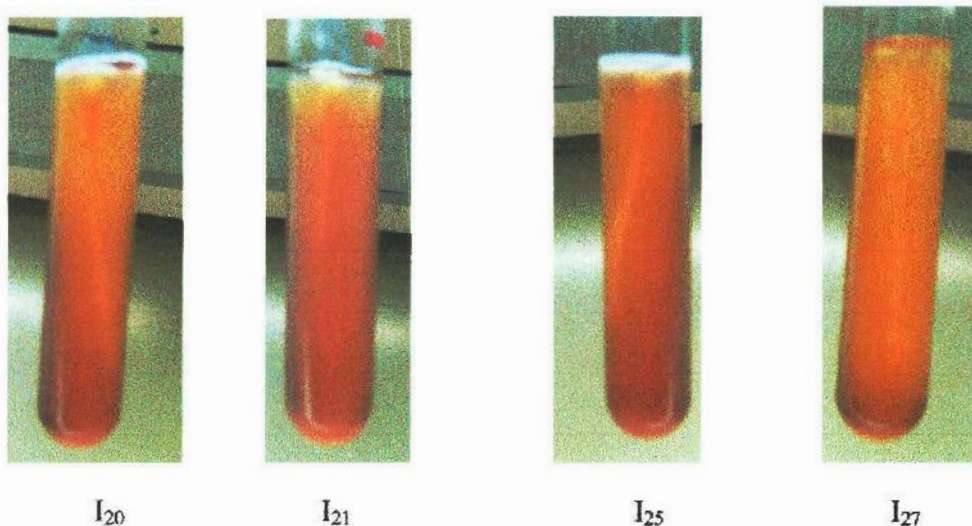


Figure 4.16: Motility test of isolated bacteria from poultry samples.

4.5.5. Catalase Test

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules. Oxygen formation is examined by the production of Oxygen bubbles. The production of gas bubbles shows the presence of the enzyme (the catalase positive nature of the bacterium). Probiotic bacteria are catalase negative. In catalase test, the isolated bacteria produced no gas bubbles, that mean the bacteria exhibited catalase negative activity. All the isolated bacteria are found catalase negative due to no bubble production by addition of 3% H_2O_2 .

4.5.6. Carbohydrate (Sugar) Fermentation Test

Probiotic bacteria are anaerobic and they utilize glucose to lactic in anaerobic fermentation. These lactic acids decrease the pH in media. To detect the fermentation as well as decreased pH, a pH indicator is used. pH indicator changes color along pH change. Each types of bacteria cannot ferment each type of sugar. Each type of bacteria has a specific carbohydrate fermentation profile. In this research sixteen carbohydrates were used to construct carbohydrate fermentation profile of the isolated bacteria. Bromocresol purple used as pH indicator which purple color change to yellow indicate the fermentation of carbohydrate. The isolated bacteria presumptly identified as probiotic bacteria based on their carbohydrate fermentation profile (Table 4.9).

Table 4.9. Carbohydrate(sugar) fermentation test of isolated bacteria from poultry samples.

Isolate no. Carbo- hydrate	I ₂₀	I ₂₁	I ₂₅	I ₂₇
Glucose	+	+	+	+
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
Xylose	-	-	-	+
Fructose	+	-	+	+
D-Mannose	+	+	+	+
D-Sorbitol	-	-	+	-
Rhamnose	-	-	-	-
Salicin	+	-	+	-
Mannitol	-	-	+	+
Maltose	+	+	-	-
Lactose	+	+	+	+
Galactose	+	-	+	+
Cellubiose	-	+	-	+
Sucrose	+	+	+	-
Raffinose	+	-	-	+

+ means fermented; - means not fermented.

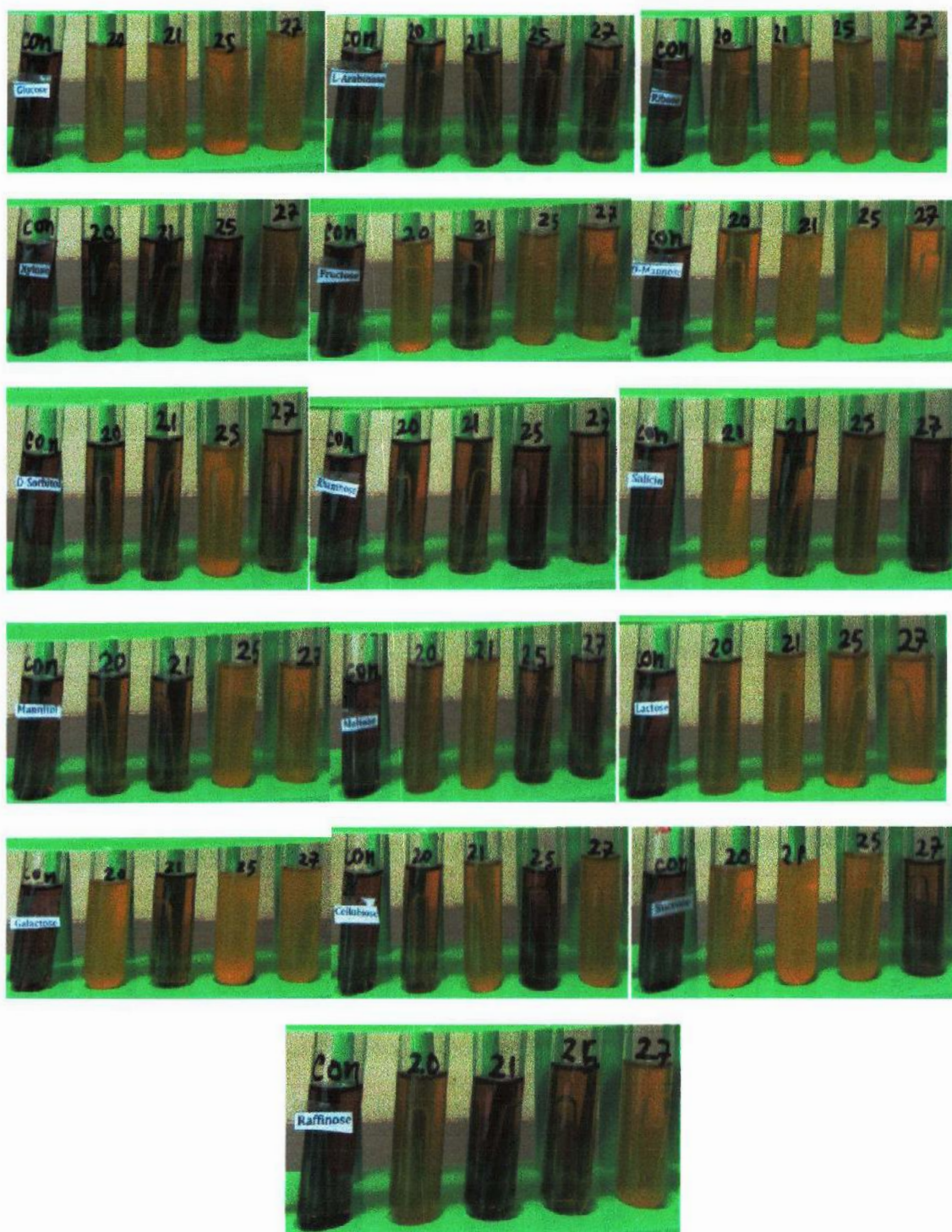


Figure 4.17: Sugar fermentation of the isolates (poultry samples) in glucose; L-arabinose; ribose; xylose; fructose; D-mannose; D-sorbitol; rhamnose; salicine; mannitol; maltose; lactose; galactose; cellubiose; sucrose and raffinose.

Isolated bacteria had specific sugar fermentation pattern of its own. The change of purple colour of the broth medium to yellow color is the indication of fermentation performed by the isolated bacteria (Fig 4.17). The sugar fermentation pattern of isolated bacteria (table 4.10) correlated with the study of Ghanbari *et al.*, (2009), Karna *et al.*, (2007), Shah *et al.*, (2009) and Azizpour *et al.*, (2009)

Table 4.10. Isolated presumptively identified bacteria from poultry samples.

Isolated presumptively identified bacteria	Isolate no.	Sample No
<i>Streptococcus spp.</i>	I ₂₀ , I ₂₁	S ₁ , S ₂
<i>Lactobacillus spp.</i>	I ₂₅	S ₁
<i>Bifidobacterium spp.</i>	I ₂₇	S ₂

4.6. Determination of Probiotic Properties of Identified Probiotic Bacteria from Poultry

4.6.1. Sodium Chloride (NaCl) Tolerance Test

Isolated of probiotic bacteria show NaCl tolerance activity against 1-9% NaCl. After 24 hours of incubation at 37°C turbidity indicate % NaCl tolerance substance which inhibit other types of bacteria. Isolates showed good growth at 1-7% of NaCl. In case of 8 and 9 % of NaCl each isolates showed moderate growth. Finally in 10% of NaCl, no growth was observed. NaCl tolerances of eight isolates at 1-10% have shown in Table 4.11.

Table 4.11. Sodium Chloride (NaCl) tolerance of isolated probiotic bacteria from poultry samples.

NaCl	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)
I ₂₀	++	++	++	++	++	++	++	+	+	-
I ₂₁	++	++	++	++	++	++	++	+	+	-
I ₂₅	++	++	++	++	++	++	++	+	-	-
I ₂₇	++	++	++	++	++	++	++	+	-	-

N.B. (++) means excellent and normal growth; (+) means low level of growth; (-) means no growth.

4.6.2. Bile Salt Hydrolase Activity Test: Plate Assay

Lactic acid bacteria produce bile salt hydrolase. Due to this activity when isolated bacteria streaked out on MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA), the taurine-conjugated bile acid was deconjugated, producing deoxycholic acid colonies were turn into opaque granular white colonies, precipitated halos around colonies (Table 4.12).

Table 4.12. Bile salt hydrolase activity of isolated probiotic bacteria from poultry samples.

Isolated presumptively identified bacteria	Isolates no.	Plate assay
<i>Streptococcus spp.</i>	I ₂₀ , I ₂₁	+
<i>Lactobacillus spp.</i>	I ₂₅	+
<i>Bifidobacterium spp.</i>	I ₂₇	+

4.6.3. Gastric Juice Resistance Test

Presumptively identified probiotic bacteria had the capability to stay alive in artificial gastric acid surroundings at low pH. (pH 2.2) and favorable pH (pH 6.6). Their survival capability decreased towards 24 hours of incubation at 37°C. The character of survival and multiplication abilities indicated by (Figure 4.18) the absorbance of the isolates bacterial culture in gastric juice environment at 620 nm. The rising lines indicate more tolerance capability of. Data were expressed as average value. The incubation periods were 0 hr, 2 hr, 4 hr, and 24 hr.

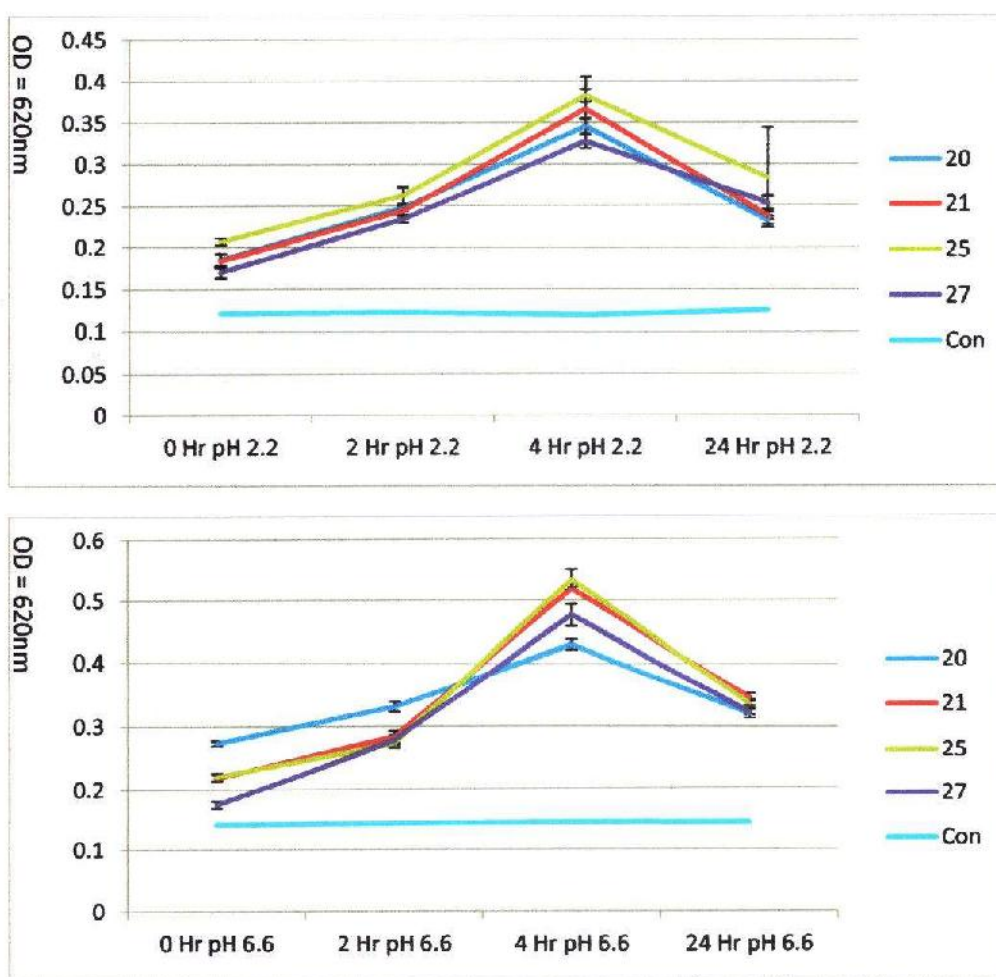


Figure 4.18: Survival and growth abilities of isolated probiotic bacteria in artificial gastric juice at pH 2.2 and 6.6. The rising lines indicate the resistance ability of the isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.6.5. Phenol Tolerance Test

The Isolates have excellent tolerance. The turbidity of the culture decreases with the increase of concentration. Isolated bacteria are capable to survive in 0.1%, 0.2% and 0.3% of phenol and moderate growth at 0.4% phenol after 24 hours of incubation at 37°C. In the The optical densities values 620 nm against time and concentration were plotted in a line diagram (Figures 4.20) representing tolerance. Data were expressed as average value of isolates at different concentrations 0.1%, 0.2%, 0.3%, 0.4% after 1 hour, 12 hours 24 hours of incubation at 37°C.

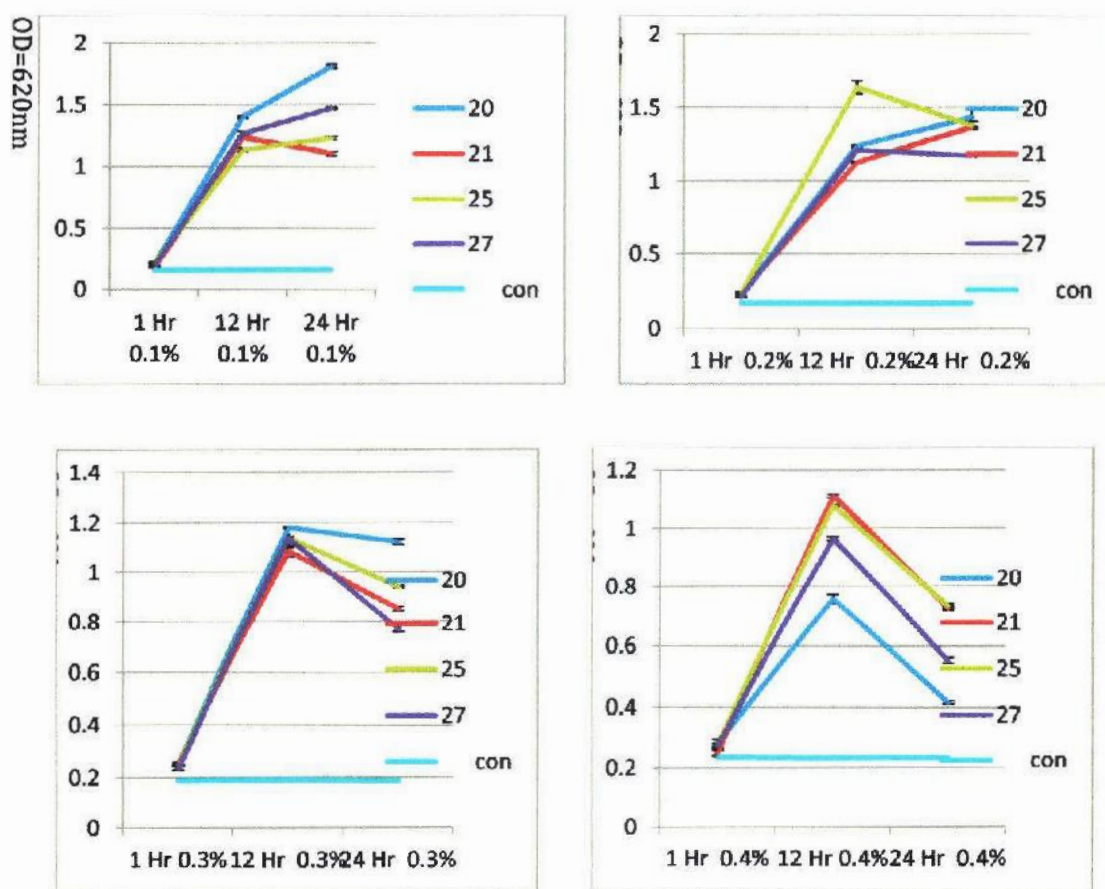


Figure 4.20: Phenol tolerance of isolated bacteria from poultry samples at 0.1%, 0.2%, 0.3% and 0.4% concentrations after 1 hour, 12 hours and 24 hours of incubation at 37°C. The rising lines indicate more tolerance ability of the isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.7. Discussion

Three isolates of *Lactobacillus acidophilus*, two isolates of *Lactobacillus brevis*, one isolate of *Lactobacillus delbrueckii* and two isolates of *Streptococcus thermophilus* were isolated from yoghurt samples of Comilla district of Bangladesh. Two *Streptococcus spp.*, one *Lactobacillus spp.* and one *Bifidobacterium spp.* were isolated from poultry feces samples of Pabna and Comilla districts. Different probiotic bacteria originated from different locations and the growth varies from different environmental conditions, temperature, availability of energy sources and pH radically affects the growth of specific strains. The identified isolates were tested for different concentration of NaCl (1-10%) in the MRS broth and the growth was observed according to turbidity after 24 hours of incubation at 37°C. All the isolates had excellent tolerance against 1-7% NaCl. At 8% and 9% NaCl concentrations they showed low level of growth and in 10% of NaCl, no growth was observed. The NaCl test results of the research were found similar to Hoque *et al* (2010) who isolated *Lactobacillus spp.* from yoghurt and tested at different concentrations of NaCl. Schillinger and Lucke (1987) found the growth of *lactobacilli* in the presence of 7.5% NaCl isolated from meat and meat products. In poultry samples, four isolates of probiotic bacteria showed excellent tolerance activity against 1-7% NaCl and isolate no. 20 and 21 tolerated 9% NaCl.

The bacteria survived in artificial gastric juice at low pH (pH 2.2) and favorable pH 6.6. Their survival capability decreased towards 24 hours of incubation at 37°C. The survival and multiplication abilities indicated by the absorbance of the isolates bacterial culture in gastric juice environment at 620 nm. 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 GIT (Kilara, 1982). Pepsin was added in the experimental medium to stimulate the activity of gastric acid environment. Towards the gastrointestinal condition (pH 6.), *L. acidophilus* from isolate no. 10 (sample no. 20) showed better survival ability than *L. acidophilus* from isolate no. 09, 13 (sample no. 19, 17) at pH 2.2. *Lactobacillus brevis* from isolate no. 02 (sample no. 20) showed better survival ability compared to other at pH 2.2. When considered only *Lactobacillus delbrueckii* from isolate no. 05 (sample 19), showed poor survival ability compared to overall, pH 6.6 showed greater survival and multiplication abilities than pH 2.2. In case of poultry, isolated *Lactobacillus spp.* (sample 1) showed good tolerance ability to survive in gastric environment at low pH 2.2 but *Bifidobacterium spp.*

(sample 2) showed poor tolerance. In pH 6.6 they were able to multiply and survived as well where *Lactobacillus spp.* (sample 1) showed better tolerance.

Isolated bacteria showed good tolerance against 0.1 and 0.2% phenol. At concentration increased to 0.3% and 0.4% their tolerance decreased greatly. Some authors suggest that a 0.4% concentration of phenol causes a bacteriostatic action in some microorganisms (Xanthopoulos, *et al.*, 2000). After 24 hours of incubation, improved resistance and multiplication capability were observed against 0.1 and 0.2% phenol and when the concentration increased to 0.3%, their tolerance were temperate and at 0.4% concentration of phenol, their tolerance were lowest. In poultry samples, the four isolates of the species *Lactobacillus spp.*, *Streptococcus spp.* and *Bifidoacterium spp.* proved good tolerance against 0.1 - 0.2% phenol and low tolerance against 0.3% and 0.4% phenol.

Bile concentration of the human GIT 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). Resistance capabilities of isolates showed more or less nearly equal resistance against 0.05%, 0.1%, 0.3% and 0.6% artificial bile acid concentrations after 0, 2, 4 and 24 hours of incubation respectively. All the isolates started multiplication and among the *L. acidophilus* species, isolate no. 13 (sample no.17) exhibited poor bile acid resistance. Moreover, after 4 hours of incubation among the *L. acidophilus* species, isolate no. 09 (sample no.19) exhibited good resistance against 0.05%, 0.1%, 0.3% and 0.6% artificial bile acid concentrations. In addition, *S. thermophilus* from isolate no. 19 (sample no. 17) showed slightly better resistance than *S. thermophilus* from isolate no. 18 (sample no. 19) However, after 24 hours of incubation, the resistance capabilities of all the eight isolates showed better at 0.05% and 0.1% than at 0.3% and 0.6%. Walker and Gilliland (1993) stated that turbidity of the culture decreased with the increase of bile salt concentration in the culture broth and the turbidity increased with the time of incubation in case of poultry. After 24 hours of incubation all the isolates at 0.05%, 0.1% concentrations of bile salt showed better result than at 0.3% and 0.6% . On MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA) streaking all the isolates from yoghurt and poultry developed colonies like opaque granular white that is similar to Dashkevich and Feighner (1989).

After biochemical assay, those selected strains were tested for *in vivo* experiments. For conducting *in vivo* experiments seven weeks-old Swiss Albino mouse (*Mus musculus*) were used. Yoghurt was prepared using all the isolates that containing *Lactobacillus acidophilus*,

Conclusion

In this Research work twelve isolates of probiotic bacteria were isolated and presumptively identified based on morphology, physiology and biochemical assay. Eight isolates were obtained from four yoghurt samples of Comilla district of Bangladesh and four isolates were obtained from two poultry feces samples of Pabna and Comilla districts. These isolates were further studied for probiotic properties.

- Three isolates of *Lactobacillus acidophilus*, two isolates of *Lactobacillus brevis*, one isolate of *Lactobacillus delbrueckii* and two isolates of *Streptococcus thermophilus* were obtained from four yoghurt. Two *Streptococcus spp.*, one *Lactobacillus spp.* and one *Bifidobacterium spp.* were obtained from two poultry feces.
- All isolates were found as gram positive, endospore negative, catalase negative and non-motile. For sugar fermentation profile of each isolates, sixteen sugars were used to construct sugar fermentation profile.
- The Isolates showed Bile salt hydrolase activity and NaCl tolerance activity at 1-8% NaCl and few showed tolerant 9% NaCl.
- Probiotic bacteria survived in artificial gastric juice at both low pH (pH 2.2) and favorable pH 6.6. Their survival capability decreased towards 24 hours of incubation at 37°C.
- Isolated bacteria were capable to survive in 0.05%, 0.1%, 0.3% and 0.6% bile salt for 24 hours of incubation at 37°C. The Isolates showed excellent phenol tolerance activities and were capable to survive in 0.1%, 0.2% and 0.3% of phenol and moderate growth at 0.4% phenol after 24 hours of incubation at 37°C.
- In mouse trail, the treatment group with maximum quantity of yoghurt supplement showed significant body weight gain, decreased blood cholesterol level, antibiotic resistance and pathogenic organisms inhibition activities.

Recommendation

In the present study cultural, morphological, physiological, biochemical assay, probiotic properties were studied but future research work needs for strain identification, viability in products, attachment ability of probiotic bacteria at intestine membrane. Human and poultry trials would be required for safe probiotic product development like fermented milk drinks, yoghurt, ice-cream, juice and sausages for human consumption whereas liquid and lyophilized form of probiotic products for poultry. Molecular characterization of the isolated bacteria should be conducted in order to ensure the species level identification of the isolates.

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APPENDIX-I

MRS BROTH

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
D-glucose	20
K ₂ HPO ₄	2
Sodium acetate	5
Triammonium citrate	2
MgSO ₄ , 7H ₂ O	0.2
MnSO ₄ , 7H ₂ O	0.05
Tween-80	1 ml
Deionized water	1000 ml
pH	6.3-6.6

MRS AGAR

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
D-glucose	20
K ₂ HPO ₄	2
Sodium acetate	5
Triammonium citrate	2
MgSO ₄ , 7H ₂ O	0.2
MnSO ₄ , 7H ₂ O	0.05
Tween-80	1 ml
Deionized water	1000 ml
Agar	15
pH	6.3-6.6

MODIFIED MRS FOR CARBOHYDRATE FERMENTATIONS

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
K ₂ HPO ₄	2
Sodium acetate	5
Triammonium citrate	2
MgSO ₄ , 7H ₂ O	0.2
MnSO ₄ , 7H ₂ O	0.05
Tween-80	1 ml
Bromocresol purple	0.04
Deionized water	1000 ml
pH	6.2-6.6

(*Streptococcus thermophilus*) AGAR

MRS broth	Gram/litre
Tryptone	10
Sucrose	1
Yeast extract	5
K ₂ HPO ₄	2
Agar	12
pH	6.8

The composition of GYP Broth media

Ingredients		g/L
Peptone		5
Yeast Extract		10
Dextrose/ D-Glucose		10
Sodium Acetate		2
Salt solution:		5
➤ Magnesium Sulfate + 7H ₂ O	40 mg	
➤ Manganese Sulfate + 4H ₂ O	2mg	
➤ Ferrous Sulfate + 7H ₂ O	2mg	
➤ Sodium Chloride	2mg	
➤ Deionized Water	1 ml	
Tween 80		10 ml
Deionized Water		1000 ml
pH		6.8

The composition of GYP Agar media

Ingredients		g/L
Peptone		5
Yeast Extract		10
Dextrose/ D-Glucose		10
Sodium Acetate		2
Salt solution:		5
➤ Magnesium Sulfate + 7H ₂ O	40 mg	
➤ Manganese Sulfate + 4H ₂ O	2mg	
➤ Ferrous Sulfate + 7H ₂ O	2mg	
➤ Sodium Chloride	2mg	
➤ Deionized Water	1 ml	
Tween 80		10 ml
Agar		16
Deionized Water		1000 ml
pH		6.8

APPENDIX-II

Body Weight Data

Day 0			
Mice	Control Group (gm)	T1 (gm)	T2 (gm)
Red	14	11.3	20.2
Yellow	15	16.8	19.9
Blue	16.3	17.8	13.5
Green	17.8	20.9	19.4
Black	17	21.3	21.2
Black-Blue	16.7	10	14.8
Black-Red	16	17.4	18.4
White	17.3	18.6	17
Day 7			
Red	16	13.8	24.9
Yellow	19.8	20.4	26
Blue	19.2	19.9	16.4
Green	18.8	25.7	20.7
Black	22.7	21.5	18.4
Black-Blue	17.7	14.5	20
Black-Red	17.8	19	21.9
White	21	20.1	20
Day 14			
Red	17.7	16.9	28.9
Yellow	21.9	21.6	30
Blue	22.8	22	18
Green	19.9	28.5	25
Black	14.9	26.7	23.7
Black-Blue	18.9	15	26.3
Black-Red	18	22	24.8
White	21.4	21	22.2
Day 21			
Red	17.3	19.6	32
Yellow	22.2	28	31.7
Blue	24.6	23.9	25
Green	21	27	31
Black	17	25	23.6
Black-Blue	20	23	28
Black-Red	19.7	24	25.4
White	23	24.6	26

Day 28			
Red	21	21	37
Yellow	25	23.6	35.5
Blue	23.2	30.4	30.9
Green	27	32.3	27.1
Black	24.8	32.7	30
Black-Blue	24	17	27.8
Black-Red	20	23.8	23.5
White	22.7	25.3	27.4

APPENDIX-III

Cholesterol Measuring Data

Day 0			
Mice	Control Group (gm)	T1 (gm)	T2 (gm)
Red	4.7	3.9	4.5
Yellow	4.1	4.5	4
Blue	4.7	4.3	4.3
Green	4.4	4.9	3.9
Black	3.9	4.1	4
Black-Blue	3.6	3.3	4.5
Black-Red	4.3	4.1	4
White	4	4.7	4.8
Day 28			
Red	4.3	3.1	3.5
Yellow	4.0	3.9	2.9
Blue	4.4	3.4	3.9
Green	3.3	3.9	3.2
Black	3.5	3	3.1
Black-Blue	4.5	2.9	3.9
Black-Red	4.3	3.2	3.1
White	4.1	4.1	2.8

Antibiotic Resistance Test Data

96

G1 (Positive control)					G2 (Negative control)					T1				
Day 14														
Mice	Col- ony	Dilu- tion	No of Cfu/ml	Log cfu/ml	Col- ony	Dilu- tion	No of Cfu/ml	Log cfu/ml	Col- ony	Dilu- tion	No of Cfu/ml	Log cfu/ml	No of Cfu/ml	Log cfu/ml
Red	89	10 ⁻⁶	4.4×10 ⁸	8.64	73	10 ⁻⁶	3.6×10 ⁸	8.55	155	10 ⁻⁶	7.7×10 ⁸	8.88		
Yellow	92	10 ⁻⁶	4.6×10 ⁸	8.66	61	10 ⁻⁶	3.0×10 ⁸	8.47	169	10 ⁻⁶	8.4×10 ⁸	8.92		
Blue	91	10 ⁻⁶	4.5×10 ⁸	8.65	65	10 ⁻⁶	3.2×10 ⁸	8.50	137	10 ⁻⁶	6.8×10 ⁸	8.83		
Green	79	10 ⁻⁶	3.9×10 ⁸	8.59	73	10 ⁻⁶	3.6×10 ⁸	8.55	149	10 ⁻⁶	7.4×10 ⁸	8.86		
Black	84	10 ⁻⁶	4.2×10 ⁸	8.62	69	10 ⁻⁶	3.4×10 ⁸	8.53	131	10 ⁻⁶	6.5×10 ⁸	8.81		
Black-Blue	98	10 ⁻⁶	4.9×10 ⁸	8.69	71	10 ⁻⁶	3.5×10 ⁸	8.54	179	10 ⁻⁶	8.9×10 ⁸	8.94		
Black-Red	87	10 ⁻⁶	4.3×10 ⁸	8.63	67	10 ⁻⁶	3.3×10 ⁸	8.51	157	10 ⁻⁶	7.8×10 ⁸	8.89		
White	83	10 ⁻⁶	4.1×10 ⁸	8.61	59	10 ⁻⁶	2.9×10 ⁸	8.46	167	10 ⁻⁶	8.3×10 ⁸	8.91		

APPENDIX-V

Data of Growth Inhibition of Pathogenic Microorganism

Mice	Control				T1				T2			
	Colony	Dilution	No of Cfu/ml	Log cfu/ml	Colony	Dilution	No of Cfu/ml	Log cfu/ml	Colony	Dilution	No of Cfu/ml	Log cfu/ml
Red	59	10^{-4}	2.9×10^6	6.46	51	10^{-4}	2.55×10^6	6.40	23	10^{-4}	1.15×10^6	6.06
Yellow	45	10^{-4}	2.2×10^6	6.34	65	10^{-4}	3.25×10^6	6.51	69	10^{-4}	3.45×10^6	6.53
Blue	73	10^{-4}	3.6×10^6	6.55	33	10^{-4}	1.65×10^6	6.21	38	10^{-4}	1.90×10^6	6.27
Green	89	10^{-4}	4.4×10^6	6.64	53	10^{-4}	2.65×10^6	6.42	49	10^{-4}	2.45×10^6	6.38
Black	43	10^{-4}	2.1×10^6	6.32	37	10^{-4}	1.85×10^6	6.26	41	10^{-4}	2.05×10^6	6.31
Black-Blue	79	10^{-4}	3.9×10^6	6.59	35	10^{-4}	1.75×10^6	6.24	52	10^{-4}	2.60×10^6	6.41
Black-Red	63	10^{-4}	3.1×10^6	6.49	49	10^{-4}	2.45×10^6	6.38	73	10^{-4}	3.65×10^6	6.56
White	69	10^{-4}	3.4×10^6	6.53	43	10^{-4}	2.15×10^6	6.33	29	10^{-4}	1.45×10^6	6.16

APPENDIX-VI

Phenol Tolerance Test Data (OD 620 nm) Yoghurt Sample

PHENOL: Isolate no.2			PHENOL: Isolate no.4			PHENOL: Isolate no.5			PHENOL: Isolate no.9		
1 Hr .1%	0.187	0.192	0.201	0.195	0.183	0.177	0.203	0.195	0.177	0.195	0.187
1 Hr .2%	0.215	0.234	0.22	0.211	0.229	0.212	0.223	0.227	0.218	0.205	0.211
1 Hr .3%	0.248	0.241	0.269	0.258	0.248	0.24	0.228	0.234	0.246	0.236	0.257
1 Hr .4%	0.273	0.294	0.285	0.272	0.266	0.251	0.283	0.263	0.289	0.286	0.291
12 Hr .1%	1.615	1.639	1.643	1.163	1.155	1.147	1.192	1.184	1.177	1.211	1.227
12 Hr .2%	1.232	1.42	1.231	1.528	1.432	1.441	1.313	1.318	1.32	1.256	1.267
12 Hr .3%	1.311	1.338	1.355	1.254	1.271	1.258	1.233	1.236	1.242	1.283	1.292
12 Hr .4%	0.922	0.941	0.955	1.106	1.075	1.062	1.114	1.146	1.153	0.995	0.988
24 Hr .1%	1.046	1.101	1.089	1.563	1.569	1.538	1.27	1.276	1.331	1.331	1.325
24 Hr .2%	1.125	1.119	1.12	1.752	1.731	1.762	1.163	1.168	1.173	0.993	0.979
24 Hr .3%	0.95	0.978	0.982	0.882	0.878	0.892	0.852	0.833	0.825	0.983	0.969
24 Hr .4%	0.643	0.628	0.662	0.624	0.643	0.629	0.723	0.741	0.734	0.758	0.753

PHENOL: Isolate no.10				PHENOL: Isolate no.13			PHENOL: Isolate no.18			PHENOL: Isolate no.19			Control
1 Hr .1%	0.12	0.171	0.188	0.172	0.189	0.18	0.185	0.188	0.202	0.192	0.213	0.205	0.157
1 Hr .2%	0.23	0.243	0.211	0.219	0.222	0.234	0.228	0.225	0.212	0.239	0.233	0.221	0.171
1 Hr .3%	0.235	0.244	0.251	0.245	0.263	0.254	0.26	0.233	0.257	0.235	0.241	0.249	0.181
1 Hr .4%	0.272	0.288	0.261	0.261	0.289	0.276	0.281	0.292	0.271	0.277	0.2782	0.293	0.231
12 Hr .1%	1.264	1.251	1.281	1.291	1.283	1.287	1.021	1.027	1.045	1.283	1.278	1.271	0.155
12 Hr .2%	1.191	1.178	1.212	1.077	1.073	1.082	1.113	1.138	1.122	1.073	1.035	1.047	0.172
12 Hr .3%	1.051	0.979	0.975	1.103	1.211	1.189	1.223	1.231	1.238	1.156	1.173	1.165	0.18
12 Hr .4%	0.917	0.898	0.884	1.023	1.014	0.957	1.112	1.14	1.121	0.943	0.931	0.947	0.227
24 Hr .1%	1.972	1.985	1.993	1.129	1.145	1.138	1.115	1.118	1.124	1.017	1.021	1.014	0.159
24 Hr .2%	1.515	1.528	1.519	1.124	1.128	1.135	1.351	1.345	1.348	1.147	1.152	1.142	0.17
24 Hr .3%	1.201	1.188	1.204	0.982	0.994	0.987	1.084	1.11	1.07	0.987	0.996	0.991	0.18
24 Hr .4%	0.989	0.98	0.965	0.778	0.783	0.769	0.796	0.808	0.785	0.582	0.567	0.569	0.229

Phenol Tolerance Test Data (OD 620 nm) Poultry Sample

	PHENOL: Isolate no.20			PHENOL: Isolate no.21			PHENOL: Isolate no.25			PHENOL: Isolate no.27			Control
1 Hr .1%	0.208	0.217	0.212	0.178	0.187	0.189	0.216	0.211	0.232	0.193	0.201	0.183	0.156
1 Hr .2%	0.224	0.215	0.236	0.243	0.235	0.221	0.221	0.238	0.246	0.213	0.209	0.217	0.174
1 Hr .3%	0.244	0.259	0.251	0.257	0.253	0.245	0.246	0.241	0.252	0.228	0.241	0.237	0.188
1 Hr .4%	0.297	0.283	0.269	0.24	0.253	0.258	0.281	0.276	0.272	0.273	0.282	0.265	0.236
12 Hr .1%	1.402	1.394	1.411	1.241	1.228	1.237	1.149	1.128	1.122	1.243	1.271	1.278	0.156
12 Hr .2%	1.248	1.234	1.235	1.133	1.119	1.125	1.657	1.586	1.671	1.211	1.203	1.215	0.172
12 Hr .3%	1.176	1.185	1.18	1.109	1.066	1.073	1.18	1.121	1.112	1.143	1.131	1.137	0.191
12 Hr .4%	0.752	0.773	0.741	1.112	1.107	1.118	1.073	1.081	1.078	0.97	0.957	0.962	0.233
24 Hr .1%	1.823	1.816	1.795	1.087	1.115	1.113	1.215	1.235	1.243	1.478	1.462	1.47	0.158
24 Hr .2%	1.385	1.418	1.501	1.362	1.373	1.367	1.41	1.374	1.358	1.172	1.181	1.163	0.173
24 Hr .3%	1.124	1.108	1.131	0.843	0.852	0.86	0.935	0.942	0.943	0.761	0.782	0.764	0.1187
24 Hr .4%	0.423	0.415	0.413	0.724	0.719	0.731	0.729	0.739	0.726	0.541	0.535	0.553	0.233

APPENDIX-VII

Bile Salt Tolerance Test Data (OD 620 nm) Yoghurt Sample

2				4			5			9		
1 Hr 0.05%	0.253	0.246	0.236	0.235	0.251	0.257	0.212	0.231	0.224	0.229	0.234	0.242
4 Hr 0.05%	0.732	0.726	0.733	0.518	0.526	0.532	0.678	0.691	0.687	0.782	0.778	0.791
12 Hr 0.05%	1.235	1.228	1.243	1.213	1.233	1.223	1.237	1.226	1.251	1.2	1.132	1.127
24 Hr 0.05%	1.125	1.113	1.092	1.084	1.095	1.103	0.975	0.981	0.967	1.145	1.218	1.138
1 Hr 0.1%	0.213	0.209	0.203	0.228	0.217	0.21	0.262	0.257	0.249	0.263	0.251	0.268
4 Hr 0.1%	0.768	0.773	0.762	0.471	0.468	0.453	0.442	0.454	0.461	0.891	0.895	0.882
12 Hr 0.1%	1.395	1.378	1.382	1.231	1.221	1.241	1.287	1.305	1.278	1.223	1.218	1.237
24 Hr 0.1%	0.919	0.908	0.914	0.967	0.973	0.983	0.921	0.918	0.914	1.086	1.132	1.138
1 Hr 0.3%	0.238	0.229	0.234	0.242	0.257	0.259	0.263	0.249	0.258	0.246	0.255	0.236
4 Hr 0.3%	0.738	0.747	0.753	0.582	0.595	0.587	0.727	0.721	0.735	0.763	0.772	0.781
12 Hr 0.3%	1.456	1.441	1.446	1.273	1.265	1.258	1.258	1.263	1.251	1.122	1.118	1.121
24 Hr 0.3%	0.893	0.876	0.887	0.993	0.988	0.995	1.085	1.108	1.115	0.872	0.867	0.875
1 Hr 0.6%	0.256	0.242	0.261	0.253	0.262	0.258	0.252	0.256	0.238	0.257	0.262	0.267
4 Hr 0.6%	0.693	0.687	0.703	0.668	0.671	0.678	0.743	0.752	0.737	0.679	0.692	0.685
12 Hr 0.6%	1.332	1.347	1.341	1.212	1.23	1.192	1.231	1.229	1.238	0.793	0.79	0.785
24 Hr 0.6%	0.627	0.641	0.634	0.762	0.774	0.769	0.815	0.823	0.807	0.587	0.572	0.578

10				13			18			19			Con	
1 Hr 0.05%	0.195	0.201	0.198	0.201	0.198	0.193	0.187	0.192	0.183	0.201	0.205	0.197	0.138	
4 Hr 0.05%	0.576	0.565	0.571	0.238	0.227	0.221	0.636	0.625	0.631	0.676	0.665	0.671	0.137	
12 Hr 0.05%	1.237	1.231	1.242	1.283	1.312	1.317	1.137	1.153	1.146	1.223	1.242	1.231	0.138	
24 Hr 0.05%	0.985	0.979	0.898	0.997	0.988	0.992	1.022	1.013	1.027	1.213	1.219	1.22	0.141	
1 Hr 0.1%	0.218	0.231	0.225	0.231	0.226	0.243	0.221	0.219	0.225	0.255	0.259	0.248	0.15	
4 Hr 0.1%	0.559	0.563	0.552	0.243	0.252	0.238	0.737	0.729	0.723	0.672	0.678	0.681	0.152	
12 Hr 0.1%	1.121	1.115	1.119	1.016	1.103	1.114	1.137	1.131	1.127	1.135	1.127	1.129	0.155	
24 Hr 0.1%	1.123	1.132	1.131	0.935	0.923	0.928	0.987	0.981	0.975	0.985	0.992	1.003	0.151	
1 Hr 0.3%	0.248	0.242	0.251	0.186	0.19	0.177	0.245	0.235	0.237	0.227	0.236	0.239	0.169	
4 Hr 0.3%	0.625	0.631	0.628	0.437	0.443	0.428	0.527	0.521	0.532	0.519	0.524	0.531	0.172	
12 Hr 0.3%	1.243	1.259	1.253	1.154	1.167	1.159	1.213	1.202	1.22	1.647	1.652	1.644	0.173	
24 Hr 0.3%	0.987	0.993	0.996	0.765	0.771	0.78	0.973	0.982	0.978	1.107	1.113	1.087	0.176	
1 Hr 0.6%	0.268	0.283	0.278	0.228	0.235	0.241	0.283	0.268	0.271	0.268	0.263	0.27	0.209	
4 Hr 0.6%	0.721	0.718	0.725	0.402	0.393	0.398	0.768	0.787	0.763	0.674	0.683	0.679	0.217	
12 Hr 0.6%	1.52	1.497	1.518	1.225	1.239	1.33	1.285	1.291	1.279	1.215	1.189	1.201	0.215	
24 Hr 0.6%	0.842	0.851	0.847	0.857	0.875	0.868	0.578	0.568	0.573	0.642	0.647	0.651	0.221	

Bile Salt Tolerance Test Data (OD 620 nm) Poultry Sample

20				21			25			27			Con		
1 Hr 0.05%	0.182	0.168	0.173	0.183	0.187	0.198	0.195	0.198	0.191	0.197	0.186	0.193	0.197	0.186	0.136
4 Hr 0.05%	0.219	0.213	0.223	0.643	0.637	0.634	0.821	0.81	0.815	0.741	0.726	0.735	0.741	0.726	0.138
12 Hr 0.05%	1.207	1.187	1.193	1.274	1.286	1.268	1.541	1.538	1.545	1.419	1.427	1.422	1.419	1.427	0.135
24 Hr 0.05%	1.183	1.201	1.195	0.987	0.982	0.995	1.114	1.121	1.123	1.119	1.123	1.128	1.119	1.123	0.141
1 Hr 0.1%	0.227	0.234	0.231	0.233	0.228	0.241	0.253	0.262	0.258	0.257	0.252	0.263	0.257	0.252	0.156
4 Hr 0.1%	0.327	0.319	0.33	0.549	0.556	0.461	1.108	1.112	1.123	0.423	0.432	0.419	0.423	0.432	0.154
12 Hr 0.1%	1.193	1.197	1.207	1.084	1.104	1.095	1.664	1.659	1.659	1.579	1.592	1.583	1.579	1.592	0.157
24 Hr 0.1%	0.845	0.861	0.858	0.854	0.857	0.848	0.998	1.006	1.014	0.993	1.013	1.018	0.993	1.013	0.159
1 Hr 0.3%	0.221	0.232	0.229	0.237	0.242	0.234	0.238	0.242	0.249	0.245	0.241	0.252	0.245	0.241	0.173
4 Hr 0.3%	0.439	0.445	0.454	0.453	0.452	0.436	0.728	0.743	0.738	0.527	0.541	0.531	0.527	0.541	0.173
12 Hr 0.3%	1.186	1.177	1.184	1.167	1.174	1.161	1.786	1.792	1.778	1.141	1.138	1.135	1.141	1.138	0.178
24 Hr 0.3%	1.014	1.021	1.029	0.863	0.879	0.873	1.093	1.105	1.112	0.993	0.986	0.984	0.993	0.986	0.177
1 Hr 0.6%	0.258	0.268	0.263	0.281	0.292	0.285	0.278	0.283	0.291	0.269	0.273	0.285	0.269	0.273	0.22
4 Hr 0.6%	0.391	0.389	0.395	0.395	0.387	0.392	0.792	0.788	0.781	0.423	0.414	0.421	0.423	0.414	0.223
12 Hr 0.6%	0.982	0.977	0.984	0.864	0.885	0.875	1.436	1.412	1.385	0.838	0.856	0.841	0.838	0.856	0.226
24 Hr 0.6%	0.516	0.523	0.528	0.467	0.461	0.472	0.846	0.838	0.852	0.449	0.455	0.453	0.449	0.455	0.225

APPENDIX-VIII

Gastric Juice Resistance (pH 2.2) Test Data (OD 620 nm) Yoghurt Sample

	2			4			5			9					
	0 Hr pH 2.2	2 Hr pH 2.2	4 Hr pH 2.2	0.179	0.183	0.194	0.165	0.183	0.172	0.192	0.183	0.187	0.182	0.168	0.173
0 Hr pH 2.2	0.179	0.183	0.194	0.179	0.183	0.194	0.165	0.183	0.172	0.192	0.183	0.187	0.182	0.168	0.173
2 Hr pH 2.2	0.283	0.291	0.272	0.283	0.291	0.272	0.229	0.236	0.241	0.232	0.242	0.256	0.241	0.257	0.248
4 Hr pH 2.2	0.346	0.357	0.361	0.346	0.357	0.361	0.319	0.332	0.325	0.271	0.282	0.292	0.332	0.324	0.326
24 Hr pH 2.2	0.252	0.254	0.267	0.252	0.254	0.267	0.243	0.234	0.229	0.228	0.237	0.243	0.228	0.237	0.2311

	10				13			18			19			Con
	0 Hr pH 2.2	0.179	0.183	0.188	0.183	0.192	0.176	0.167	0.173	0.179	0.235	0.226	0.22	
0 Hr pH 2.2		0.273	0.283	0.285	0.247	0.255	0.257	0.228	0.223	0.214	0.318	0.313	0.315	0.119
2 Hr pH 2.2		0.355	0.343	0.347	0.339	0.326	0.332	0.315	0.321	0.313	0.364	0.368	0.372	0.12
4 Hr pH 2.2		0.222	0.215	0.227	0.253	0.238	0.249	0.231	0.225	0.219	0.283	0.295	0.298	0.123
24 Hr pH 2.2														0.125

Gastric Juice Resistance (pH 6.6) Test Data (OD 620 nm) Yoghurt Sample

2				4				5		9		
0 Hr pH 6.6	0.188	0.196	0.187	0.211	0.217	0.223	0.213	0.22	0.217	0.178	0.174	0.181
2 Hr pH 6.6	0.308	0.313	0.323	0.276	0.293	0.287	0.282	0.276	0.263	0.259	0.271	0.268
4 Hr pH 6.6	0.484	0.467	0.473	0.453	0.447	0.459	0.439	0.431	0.445	0.423	0.431	0.426
24 Hr pH 6.6	0.336	0.344	0.329	0.289	0.283	0.274	0.273	0.264	0.286	0.293	0.281	0.274

10				13			18			19			Con
0 Hr pH 6.6	0.219	0.223	0.233	0.245	0.231	0.236	0.262	0.256	0.251	0.273	0.265	0.279	0.146
2 Hr pH 6.6	0.256	0.269	0.259	0.313	0.323	0.317	0.318	0.334	0.326	0.368	0.358	0.375	0.141
4 Hr pH 6.6	0.381	0.393	0.363	0.398	0.379	0.385	0.432	0.427	0.42	0.467	0.463	0.471	0.146
24 Hr pH 6.6	0.263	0.268	0.275	0.286	0.271	0.29	0.318	0.314	0.321	0.327	0.323	0.319	0.148