ANALYSIS OF PROBIOTIC PROPERTIES OF ISOLATED BACTERIA FROM SELECTED YOGURTS AND POULTRY FECES

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Submitted By

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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 The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.

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This research work is

Dedicated
To
My beloved Parents

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At first I thanks for the blessings of ALLAH, the Almighty to enable me to complete my work in time.

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ABSTRACT

Lactic acid bacteria (LAB) are beneficial probiotic organisms that provide to improved microbial balance, nutrition, and immune-enhancement of the intestinal tract, as well as lower cholesterol. In the present study four different yogurts and two poultry feces were used. The phenotypic and biochemical analyses gave a diversity of four presumptive species viz. *Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus brevis, and Bifidocterium* spp from yogurt sample and three presumptive species viz. *Lactobacillus acidophilus, Lactobacillus brevis, and Bifidocterium spp.* were found from two poultry feces sample respectively. The result was identified by their morphological, physiological and biochemical assay. It was observed that all the four species were gram positive, endospore negative, catalase negative and non-motile which are the characteristic to typical probiotic bacteria. Carbohydrate/sugar fermentation profiles experiment using sixteen important sugars ensured the presumptive identification of isolated species. The results showed that, the all selected species were resistant to artificial gastric juice environment at pH 2.2 and 6.6 but their resistance capacity decreased after 24 hours of incubation at 37^0C. They showed excellent tolerant ability to phenol and bile salt concentration. The NaCl tolerance test results of the isolated probiotic bacteria have shown that all the isolates have excellent tolerance against 1-6% NaCl, moderate in 7% to 9% and in 10% NaCl, no growth was observed after 24 hours of incubation at 37^0C. Moreover, all the isolates showed bile salt hydrolase activity using bile salt plate on solid MRS medium containing 0.5% taurodeoxycholic acid by forming opaque granular white colonies characteristic to probiotic lactic acid bacteria. Isolated bacteria from both yogurt and poultry feces were able to coagulate milk when 1% (v/v) culture of probiotic bacteria was inoculated into sterilized skim milk and incubated for overnight. *In vivo* culture experiments were performed by using Seven weeks old Swiss Albino mouse. Twenty four mice were used to investigate the effects on cholesterol level, antibiotic resistance ability, and inhibiting growth of pathogenic bacteria. For *in vivo* experiments, 12gm of yogurt made by selected bacterial cultures were orally administered to rats every day up to 4 weeks which showed reduced cholesterol levels significantly ($p < 0.05$), and antagonistic activity against potential pathogenic bacteria, the treatment group receiving probiotic bacteria showed significant resistance to the antibiotic which indicates that the isolated strains are potential for probiotic use.

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CHAPTER ONE

INTRODUCTION

To spend a healthy life style diet play a major role in preventing diseases and promoting health. Therefore there is an increasing trend for foods containing probiotic cultures. Growing human population urges the immense need to exploit the existing livestock resources to meet the animal protein requirements (Bilal, 2009). The concept of useful microbes is hundred years old when the people were in habit of consuming fermented milk. Lilly and Stillwell (1965), for the first time, used the word "probiotic" for this kind of microbes and described that probiotics are substances secreted by one microorganism that stimulate the growth of others. Fuller (1989) defined it as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. The application of probiotics in poultry has gained considerable interest during the last few years because antibiotic growth promoters (AGPs), added to animal feed to increase growth and decrease the incidence of diseases, are leaving harmful residues in meat and eggs. A wide range of microorganisms have been used as probiotics. The most commonly used organisms in probiotic preparations are lactic acid producing bacteria such as lactobacilli, streptococci, Bifidobacteria, Bacillus spp. and fungi like *Sacharomyces cerevisiae, Sacharomyces boulardii* and *Aspergillus oryzae* (Fuller, 1992; Medina *et al.*, 2001).

Probiotics are microorganisms introduced orally in the gastrointestinal tract (GIT) that are able to contribute positively to the activity of intestinal microflora and therefore, to the health of its host. Most probiotic bacteria belong to the group of lactic acid bacteria (LAB) and among them lactobacilli and bifidobacteria reportedly play a significant role in maintaining the intestinal ecosystem and in stimulating the immune system of the host (Saarela *et al.*, 2002). Many *in vitro* properties, such as adhesion, resistance to pH, etc., are usually investigated to determine if a specific selected strain would be suitable as a probiotic (Collins *et al.*, 1998).

The term 'probiotic' firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word 'probiotic' was used in different meaning according to its mechanism and the effects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined 'probiotic' as

'substances and organisms which contribute to intestinal microbial balance. The probiotics are living-health promoting microorganisms which will be incorporated into various kinds of foods. Lactic acid bacteria widely distributed in the nature and occurring indigenous microflora in raw milk that play an important role in many foods and feed fermentations with increased shelf life. These Lactobacilli and Bifidobacteria live along with 600 species of microorganisms in the mouth and 400 species in the small and large intestines. Fermented milks have been a part of the human diet since ancient times. Their efficacy in alleviating gastrointestinal disorders has been exploited in systems of traditional medicines world over. Lactic acid bacteria play an important role in preventing bacterial and fungal infections of the skin and gastrourinary tract. Lactobacilli have a protective role against vaginal infections. Improper function of the immune system that is related to decreased tolerance to indigenous microflora can lead to immunopathogenesis in chronic inflammatory bowel disease (Merger and Croitorou, 1998). Disruptions in intestinal permeability barriers can lead to translocation of bacteria into the blood stream (Wells *et al.*, 1988).

The works of Metchnikoff and Tissier were the first to make scientific suggestions about the probiotic use of bacteria, even if the word "probiotic" was not coined until 1960, to name substances produced by microorganisms which promoted the growth of other microorganisms (Lilly and Stillwell, 1965). Fuller (1989), in order to point out the microbial nature of probiotics, redefined the word as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". A quite similar definition was proposed by Havenaar and Huis in 't Veld (1992) "a viable mono or mixed culture of bacteria which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora". A more recent, but probably not the last definition is "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" (Guarner and Schaafsma, 1998).The observations of Metchnikoff and Tissier were so appealing that commercial exploitation immediately followed their scientific works. Unfortunately, results were not always positive and most of these observations were anecdotal. The probiotic concept was therefore regarded as scientifically unproven and it received minor interest for decades, with some research involving animal feeding, in order to find healthy substitutes for growth promoting agents. In the last 20 years, however, research in the probiotic area has progressed considerably

and significant advances have been made in the selection and characterisation of specific probiotic cultures and substantiation of health claims relating to their consumption. Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used, but not exclusively, as probiotic microorganisms and a growing number of probiotic foods are available to the consumer. Some ecological considerations on the gut flora are necessary to understand the relevance, for human health, of the probiotic food concept. Bacteria are normal inhabitants of humans (as well as the bodies of upper animals and insects) including the gastrointestinal tract, where more than 400 bacterial species are present (Tannock, 1999): half of the wet weight of colonic material is due to bacterial cells whose numbers exceed by 10 fold the number of tissue cells forming the human body. Normally the stomach contains few bacteria, whereas the bacterial concentration increases throughout the gut resulting in a final concentration in the colon of 10^{12} bacteria/g. Bacterial colonisation of the gut begins at birth, as new-borns are maintained in a sterile status until the delivery begins, and continues throughout life, with notable age-specific changes (Mitsuoka, 1992). Bacteria, forming the so-called resident intestinal microflora, do not normally have any acute adverse effects and some of them have been shown to be necessary for maintaining the well-being of their host. As an example of the beneficial role of intestinal microflora, it is possible to cite what has been referred to as "colonization resistance" or "barrier effect" (van der Waaij *et al.,* 1971; Vollaard and Clasener, 1994) meaning the mechanism used by bacteria already present in the gut to maintain their presence in this environment and to avoid colonization of the same intestinal sites The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian born Nobel Prize recipient working at the Pasteur Institute at the beginning of the last century, who suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907). At this time Henry Tissier, a French paediatrician, observed that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar, Y shaped morphology. These "bifid" bacteria were, on the contrary, abundant in healthy children (Tissier, 1906). He suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora.by freshly ingested microorganisms. Therefore, it could be assumed that dietary manipulation of gut microflora, in order to increase the relative numbers of "beneficial bacteria" could contribute to the well being of the host. This was also the original assumption of

Metchnikoff who however, cautioned that "Systematic investigations should be made on the relation of gut microbes to precocious

old age, and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body." Yogurt is one of the best-known foods that contain "probiotics" which is a living microorganism, upon ingestion in sufficient amount, exerts beneficial effects on the normal microbial population of the gastrointestinal tract (Bourlioux *et al.*, 2003). Different microbial species are considered as probiotics. The major strains *Lactobacillus acidophilus, L. casei.* and various *Bifidobacterium* species , *B. longum, B. bifidum* are the most dominant species in human small and large intestine, that can inhibit the growth of pathogenic organisms, through production of organic acids and bacteriocins (Mazza, 1998). Among the most important food products, supplemented with variable probiotic strains, like *Lactobacilli* and *Bifidobacterium* strains is yogurt. Yogurt is a coagulated milk product, that results from the fermentation of lactic acid in milk, by LAB bacteria *Lactobacillus bulgaricus* and *Strepetococcus thermophilus* (Pelczar *et al.,* 1986). It is traditionally manufactured by fortifying whole or skimmed milk by evaporation, or addition of skim milk powder, heating to 85-95°C for 10-30 min and inoculating with LAB bacteria and then incubating at 42-45° C. Bifidus yogurt prepared, by the addition of *B.bifidum* and *B.longum*, to the yogurt culture and incubating at 42°C for 3-4 h. *L. acidophilus* can also incorporate with yogurt culture to create acidophilus yogurt (Mazza, 1998).

The biological activity of yogurt bacteria used in the production of cultured diary product and their metabolites, the processing steps such as pasteurization, microfiltration and product formulation, modify the physiological activity of the final product (Mazza, 1998). National Yogurt Association rules, define the active culture yogurt as a final product that contains live yogurt bacteria in amount > 10^8 cells/g at the end time of the manufacture. Up to date, many research studies focused on the role of probiotic bacteria in the yogurt for the enhancement of the gastrointestinal function through increasing minerals absorption, reduction in lactose intolerance (lactase deficiency) and through consumption of *L. acidophilus* culture yogurt (Vesa *et al.*, 2000, Martini *et al.*, 1991). LAB bacteria enables the inhibition of the pathogen infection through production of acetic acid, lactic acid and bacteriocins (Bianchi-Salvadori, 1986) as well as stabilizing the intestinal microflora after long term antibiotics uses (Brown *et al.*, 2005).

Suppression of the harmful carcinogens associated with colon cancer (Tavan *et al.*, 2002; Wollowski *et al.*, 1999) and increasing the immune response by production of secretory immunoglobulin were achieved (Perdigon, 1995, 2003). Relief of constipation, reduction of serum cholesterol (Jones, 2002), allergy in young adults (Piaia *et al.*, 2003) and control of diarrhea were established through the consumption of yogurt (Heyman, 2000).

The quality of yoghurt, or any food product, can be defined against a wide range of criteria, including for example, the chemical, physical, microbiological and nutritional characteristics. Food or dairy manufacturers aim to ensure that the safety and quality of their products will satisfy the highest expectations of the consumers. On the other hand, the consumers expect to a great extent, unconditionally, that the manufacturer has ensured that the product:

- is safe for human consumption with respect to both chemical and microbial contamination;
- conforms to any regulations enshrined in law, or statutory requirements laid down by health or local authorities;
- is capable of achieving a specified shelf-life without spoilage; and
- has the highest possible organoleptic standard that can be achieved within the existing constraints of manufacture or marketing (Tamime and Robinson, 1999).

In the recent past, there has been an explosion of probiotic-based health products mostly in the form of fermented dairy products as well as dietary supplements. The markets for probiotic products and supplements are increasing worldwide (Playne, 1997). Today there are more than 70 "Bifidus" and "Acidophilus" containing products worldwide, including several fermented dairy products (Shah, 2001). Probiotic survival in products is affected by a range of factors including pH, post-acidification (during storage) in fermented products, hydrogen peroxide production, oxygen toxicity (oxygen permeation through packaging), storage temperatures, stability in dried or frozen form, poor growth in milk, lack of proteases to break down milk protein to simpler nitrogenous substances and compatibility with traditional starter culture during fermentation. Oxygen plays a major role in the poor survival of probiotic bacteria (Brunner *et al.*, 1993 Dave and Shah, 1997a, b, c; Kailasapathy and Rybka, 1997; Shah, 2000).

According to Gilliland (1990), *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are used for manufacturing of yogurt. *Lactobacillus acidophilus, Lactobacillus casei,* and

Streptococcus sp. are used for the production of ripened cheese, cultured milk, cream and ripened butter and *L. acidophilus* and *L. casei* are widely used as probiotic bacteria in human and animal health. *Lactobacillus brevis, L. plantarum, L. delbrueckii,* are used for the production of soda crackers. Saccaro *et al.*, (2011) described a number of health benefits have been claimed for many products containing one or more groups of probiotic organisms. *Acidophilus-bifidobacteria* products or yogurts containing *Bifidobacterium bifidum* and or *Lactobacillus acidophilus* are widely available in the world market. Currently, there is an increasing commercial interest to add probiotic bacteria to fermented dairy products due to the recent discoveries in several aspects of bioscience, which support the hypothesis that, beyond nutrition, the diet may modulate various functions in the body. Due to the fact that the yogurt starter cultures (*Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) grow fast in milk during the fermentation stage (*ca.* 6-8 h), as the probiotic micro-organisms. Lim *et al.*, 1995; Sanders *et al.*, 1999 observed that the viability of probiotic bacteria in yogurt depends of many factors, such as the strains used, interaction between species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism, final acidity of the product, and the concentrations of lactic and acetic acids. However, the main factors for loss of viability of probiotic organisms have been attributed to the decrease in the pH of the medium, and accumulation of organic acids as a result of bacterial growth during the fermentation of the milk. In practice, the differential enumeration of lactic acid bacteria is often difficult to achieve due to the presence of multiple (closely related) species, and the unstable phenotypic trait. Isolation media with truly selective properties are preferred for selective enumeration on a routine basis. Several studies reported selective media for enumeration of probiotic micro-organisms in mixed cultures. Vasiljevic and Shah, 2008 described a variety of interactions can occur between the lactic acid bacteria (LAB) during the manufacture of dairy products. Bellengier *et al.*, 1997 observed that microbial interactions, either beneficial (protoco-operation) or unfavourable (antagonism) among lactic acid bacteria may generate undesirable changes in the composition of the bacterial microbiota during the manufacture and cold storage of fermented dairy products. Therefore, for optimising the technological performance of probiotic cultures during development of 'functional' foods, possible interactions amongst probiotic cultures and LAB must be assessed such *delbrueckii* ssp. *bulgaricus* LB340, *L. acidophilus* LAC, *Bifidobacterium*

animalis subsp. *lactis* BLO4 and *L. rhamnosus* LBA were used as culture by Danisco Brasil Ltda, Cotia, Brazil. The cultures were kept in the cold store until they were required.

Holzapfel, 2001 concluded, lactic acid bacteria are among the most important probiotic microorganisms typically associated with the human gastrointestinal tract. Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, e.g., morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and fermentation of various carbohydrates. Studies based on comparative 16S ribosomal RNA sequencing analysis, however, showed that some taxa generated on the basis of phenotypic features do not correspond with the suggested phylogenetic relations. Thus, some species are not readily distinguishable by phenotypic characteristics. This is especially true for the so-called *Lactobacillus acidophilus* group, the *Lactobacillus casei* and *Lactobacillus paracasei* group, and some bifidobacteria, strains of which have been introduced in many probiotic foods, e.g., the novel yogurt-like commodities. Consequently, modern molecular techniques, including polymerase chain reaction-based and other genotyping methods, have become increasingly important for species identification or for the differentiation of probiotic strains. Probiotic strains are selected for potential application on the basis of particular physiologic and functional properties, some of which may be determined *in vitro*. The classification and identification of a probiotic strain may give a strong indication of its typical habitat and origin. The species, or even genus name, may also indicate the strain's safety and technical applicability for use in probiotic products. Molecular typing methods such as pulsed-field gel electrophoresis, repetitive polymerase chain reaction, and restriction fragment length polymorphism are extremely valuable for specific characterization and detection of such strains selected for application as probiotics.
However, lactic acid bacteria (LAB) have attained major attention for probiotic activity and have generally been considered as good probiotic organisms (Saavedra, 2001; Sullivan *et al.*, 1992). Among lactic acid bacteria, lactobacilli are the most important (Tannock, 2004). The crop and ileum flora are mainly composed of lactobacilli in poultry (Fuller, 1984). Many lactobacillus strains isolated from various sources are being used as probiotic agents and it is unlikely that each species/strain possesses all of the desired characters that will make it a suitable probiotic. The functional properties of the strains (Gibson and Fuller, 2000; Holzapfel *et al.*, 2001) should be well studied and documented. Lactic acid bacteria (LAB) are present in the microbiota of

mammals and birds (Fuller, 1989), and those originating in the intestine have undergone intensive study for their potential probiotic properties and their rapid establishment as bacterial communities for the prevention of colonization by pathogenic bacteria. Different studies aimed to identify the microbiota of the gastrointestinal tract of poultry pointed out the predominance of lactobacilli such as *Lactobacillus crispatus,* which was isolated from chicken crops and intestine (Beasley *et al.*, 2004); *Lactobacillus rhamnosus* TB1, from the intestinal tract of chicken and exhibiting good adherence and *in vivo* colonization (Bouzaine *et al.*, 2005); *Lactobacillus salivarius* with antagonism against *Escherichia coli* and *Salmonella* Enteritidis were found in gastrointestinal tracts of chicks (Garriga *et al.,* 1998); and, finally, strains of *Lactobacillus thermotolerans* G12, G22, G35T, G43, and G44 were isolated from chicken feces (Niamsup et al., 2003). *Lactobacillus* isolates from chicken origin are good sources of antimicrobial peptides, bacteriocins, or both (Lima *et al.,* 2007), whereas *L. salivarius* UCC118, a recently sequenced and genetically tractable probiotic strain of human origin, produces a bacteriocin *in vivo* that can significantly protect mice against infection with the invasive foodborne pathogen, *Listeria monocytogenes* (Corr et al., 2007). Recent studies revealed the role of bacteriocins from *Bacillus circulans, Paenibacillus polymyxa* (Stern *et al.,* 2005), and *L. salivarius* (Stern *et al.*, 2006) in reducing fecal *Campylobacter* colonizationin broiler chickens infected with *C. jejuni.*

The recent study emphasis that, analysis of probiotic properties of isolated bacteria from selected yogurt and poultry feces of the particular region of Bangladesh has need to be explored Therefore, the present research work was undertaken with the following objectives:

a. Isolation and identification of probiotic bacteria from selected yogurt and poultry feces
b. Analysis of probiotic properties of presumptively identified probiotic bacteria.
c. To focus on probiotic efficiency level of identified probiotic bacteria through *in vivo* mice trial

CHAPTER TWO

Review of Literature

Foods are no longer considered by consumers only in terms of taste and immediate nutritional needs, but also in terms of their ability to provide specific health benefits beyond their basic nutritional value. Currently, the largest segment of the functional food market is provided by the foods targeted towards improving the balance and activity of the intestinal microflora (Saarela *et al.*, 2002). *Lactobacillus* and *Bifidobacterium*, both of which have been extensively studied and established as valuable native inhabitants of the GIT (Fuller, 1989; Salminen *et al.*, 1998, Capela *et al.*, 2006). Various microorganisms, particularly species of *Lactobacillus* and *Streptococcus*, have traditionally been used in fermented dairy products to promote human health as well as food functionality and flavor.

2.1 Probiotics

The word probiotic is derived from the Greek meaning "for life". Probiotics were first defined by Kollath in 1953 to denote all organic and inorganic food complexes in contrast to harmful antibiotics. Lilly and Stillwell (1965) defined probiotics as "microorganisms promoting the growth of other microorganisms". Although numerous definitions have been proposed since then, most have failed to be completely satisfactory because they lack statements such as "stabilization of the gut flora" (Goktepe *et al.*, 2006). Havenaar and Veld (1992) have defined probiotics as "mono- or mixed cultures of live microorganisms which, when applied to animal or human, beneficially affect the host by improving the properties of the indigenous microflora". When these probiotic bacteria are present in yogurt and other fermented foods, they may beneficially alter the normal gut flora (Metchnikoff, 1907). Probiotics have also been defined by the European Union (EU) Expert Group on Functional Foods in Europe (FUFOSE) to be "viable preparations in foods or dietary supplements to improve the health of humans and animals" (FUFOSE working group, 1999). More recently, probiotics have been referred to as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

2.2 Historical perspective of probiotics

Escherich described the microbiota of the infant GIT and suggested benefits of their colonization indigestion. Around the same time, Doderlein postulated the beneficial association of vaginal bacteria in inhibiting the growth of pathogenic bacteria by producing lactic acid (Goktepe *et al.,* 2006). Studies by Moro in 1900 and by Beijerinck in 1901 reported the beneficial association of LAB with human host (Goktepe *et al.,* 2006). The longevity of Caucasians was related to the high intake of fermented milk products (Metschnikoff, 1907), as elucidated in his bestselling book The Prolongation of Life. LAB belong to a group of Grampositive, non-sporulating, non-respiring cocci or rods, which produce lactic acid as a major metabolic end product during the fermentation of carbohydrates (Salminen *et al.,* 1998). Although phylogenetically different, bifidobacteria are another group of lactic acid producing bacteria which are commonly accepted as LAB. Bifidobacteria were found to be typically associated with the feces of breast-fed infants and a lower incidence of intestinal upset was observed for breast-fed infants, when compared with formula-fed infants (Goktepe *et al.,* 2006).

2.3 Probiotic bacteria

Strains of LAB, such as *Lactobacillus, Bifidobacterium, Eubacterium* and *Streptococcus*, have traditionally been used in the manufacture of fermented dairy products and are generally regarded as safe (GRAS) (O'Sullivan *et al.,* 1992). In addition, these bacteria are desirable members of the intestinal microflora (Berg, 1998). Table 2.1 shows a list of microorganisms including both LAB and non-lactics which are generally considered as probiotics. Lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens (Collins *et al.,* 1998; Ouwehand *et al.,* 2002) are some of the general criteria that have been used for the selection of probiotics. *L. casei* strain "Shirota" has been reported to have the longest history of safe use as a probiotic in food with proven health benefits (Goktepe *et al.,* 2006). *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were used to make mildly acidified yogurts called "bio-yogurts" in Germany during late 1960s (Goktepe *et al.,* 2006). Viable probiotic strains with beneficial functional properties are supplied in the market as fermented food products, mainly "yogurt"-type, or in lyophilized form, both as food supplements and as pharmaceutical preparations. For many years, pharmaceutical preparations containing live microorganisms in capsules, also

known as "biotherapeutics", were used for the restoration of the GIT population, e.g., after or during antibiotic treatment (Goktepe *et al.,* 2006).

The prevalence of lactobacillus and bifidobacterial spp. in the intestinal tract of humans is not known accurately. *Lactobacillus crispatus*, *L. gasseri*, *L. salivarius*, and *L. reuteri* have been reported as the major species of the *Lactobacillus* microflora (Mitsuoka *et al.*, 1990). Whereas, *Lactobacillus johnsonii*, *Lactobacillus ruminis*, *Lactobacillus casei*, and *Lactobacillus brevis* have been detected occasionally. *Bifidobacterium longum* has been found predomonently in adult human GIT, while *Bifidobacterium bifidum* was detected occasionally. In contrast, *Bifidobacterium infantis* and *Bifidobacterium breve* were detected predominantly in infant feces, while *B. longum* and *B. bifidum* detected occasionally (Biavati *et al.,* 1984).

Table 2.1 Microorganisms considered as probiotics (Adapted from Holzapfel *et al.,* 2001)

Lactobacillus spp.	*Bifidobacterium spp.*	Other LAB	"Non-lactics" a
L. acidophilus	*Bifidobacterium adolescentis*	*Enterococcus faecalis* a	*Bacillus cereus* ("toyoi") a,c
L. amylovorus			
L. casei		*Ent. Faecium*	*Escherichia coli*a
L. crispatus	*B. longum*	*Sporolactobacillus inulinusc*	*Propionibacterium freudenreichii* a,c
L. delbrueckii subsp. bulgaricus a	*B. breve*		
	B. animalis		
	B. bifidum		*Saccharomyces cerevisiae* ("boulardii") a
L. gallinarum c	*B. infantis*		
L. gasseri	*B. lactis* b		
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

a Mainly in pharmaceutical preparations; b Synonym of *B. animalis;* c Mainly for animals

On the basis of their morphologic and phenotypic features, the LAB are subdivided (Table 2.2) into the genera *Betabacterium, Thermobacterium, Streptobacterium, Streptococcus, Betacoccus, Tetracoccus,* and *Microbacterium* (Holzapfel *et al.,* 2001). *Enterococcus, Lactococcus*, and *Vagococcus* have been separated from the original genus, *Streptococcus* (Holzapfel *et al.*, 2001). The genus *Streptococcus* represents mainly pathogenic bacteria, except *Streptococcus thermophilus*; whereas, some strains of *Enterococcus* spp. may be involved in opportunistic infections, and some are considered to play role in food fermentations, and are also found as commensals in the GIT. *Lactococcus* spp. are generally considered to be nonpathogenic and safe.

2.4 The gastrointestinal ecosystem

The GIT of the human body is a complex ecosystem with a diverse and concentrated microbial population that mediates numerous interactions with the chemical environment, such as digestion, adhesion and colonization in the GIT. The mucosal surface area increases by: circular folding which contributes to about a 3-fold increase, through the production of villi, for a 7to 10 fold increase, and by the formation of intestinal microvilli, which results in a 15 to 40 fold increase (Holzapfel *et al.,* 1998). Varying numbers of bacteria are found throughout the GIT, ranging from 10^1-10^3 cfu/mL or g in the stomach contents; 10^7 cfu/mL in the jejunum, up to 10^9 cfu/g in the terminal ileum and approximately 5×10^{11} cfu/g in the distal colon contents (Goktepe *et al.,* 2006). The bacteria detected in feces reflect the bacteria present in the distal colon, thus studies of the human GIT microflora usually involve analysis of fecal samples (Moore *et al.,* 1978). Traditional culture methods have been used to analyze and characterise microbial communities in a natural ecosystem to obtain a complete diversity picture. In contrast to the microaerophillic lactobacilli, the study of anaerobic bifidobacteria and eubacteria was made possible by thedevelopment of anaerobic techniques in the early 1970s (Goktepe *et al.*, 2006). However, cultivation techniques have major limitations, as many microbes in different ecosystems cannot be cultivated by standard culture methods (Ward *et al.*, 1990). Classical culture-independent techniques include direct microscopic analysis and monitoring specific enzymes or metabolites, and have provided valuable insight into the real numbers of microflora in faecal samples. Microscopic analysis of faecal samples by Langendijk *et al.* (1995) revealed approximately 10^{11} to 10^{12} organisms per g of wet feces. It is noteworthy that these techniques are very limited in their ability to give any in-depth characterisation of specific organisms

present or community diversity. Fluorescence microscopy, confocal laser scanning microscopy and flow cytometry have been used to detect viable populations through the use of fluorescent probes (Lipski *et al.*, 2001). If epifluorescence microscopy and/or confocal laser scanning microscopy are applied, the method is usually referred to as fluorescence *in situ* hybridization (FISH). FISH has been used to study the composition of GIT microbial system (Tannock *et al.*, 2000). Various studies reported that the microscopic technique itself is not perfect and may significantly under-report the true numbers (Ward *et al.*, 1990).

Various short chain fatty acids (SCFA), such as acetate, propionate and butyrate, are end products of anaerobic bacterial fermentation. Thus, measurement of these acids in feces can be correlated with specific bacterial metabolism in the intestine (Rowland, 1989). For example, *Lactobacillus casei* GG fed to children with an intestinal infection significantly increased the total SCFA concentration (Siigur *et al.*, 1996). Increases or decreases in specific enzymes for example, β-glucuronidase and β-galactosidase, in feces can also point to the metabolic activities of certain groups of bacteria. Reduction in β-glucuronidase levels was reported in humans during ingestion of *L. casei* GG (Ling *et al.* 1994). Also, a significant correlation has been observed between the levels of faecal β-galactosidase and numbers of bifidobacteria (Favier *et al.*, 1997). While many faecal enzymes, such as azoreductase and nitroreductase are mainly produced by the species *Bacteroides, Eubacterium* and *Clostridium,* more studies are needed to accurately correlate specific faecal enzymes with specific groups of bacteria (Rowland, 1989). Cell viability can also be inferred from enzymatic activities such as esterase conversion of carboxyfluorescein diacetate (cFDA). The reduction of tetrazolium salts, or dyes such as propidium iodide, TOTO-1, SYTO 9, carboxyfluorescein and oxonol, have been used as viability indicators (Goktepe *et al.*, 2006). Bunthof *et al.* (2001) have combined culture plating technique with dyes, and reported that cFDA labels the culturable subpopulation; whereas, TOTO-1 lables the non-culturable population. Determination of percentages of guanine+cytosine (G+C) content is one of the few methods depicting the total bacterial community of the GIT without any previous knowledge of component bacteria or their DNA sequences (Apajalahti *et al.*, 1998). This approach has been applied by Apajalahti *et al.* (2003) to determine the total bifidobacteria community in human feces.

2.5 Gastrointestinal strains of human origin

In spite of increased research on gut microbial ecology, only a small number of approximately 400 species of different genera have been cultivated and studied with regard to their physiology, metabolic interactions, and taxonomy (Goktepe *et al.*, 2006). The large intestine is densely populated by *Bacteriodes* and the Gram-positive, anaerobic genera *Eubacterium* and *Bifidobacterium.* Lactobacilli are the predominant species in the vagina and are also normally present in the oral cavity (10^3-10^4 cfu/g), the ileum (10^3-10^7 cfu/g), and colon (10^4-10^8 cfu/g), where they play an important role in maintenance of a stable gut mucosa (Lidbeck *et al.,* 1993). Long and Swenson (1977) showed that bifidobacteria and lactobacilli are the dominant bacterial species found to be present in the feces of breast-fed infants. There is a lack of research evidence that has demonstrated a single dominant species in the human GIT. However, *L. acidophilus* (commonly referred as simply "*acidophilus*") has been recovered in relatively high numbers from the GIT (Molin *et al.,* 1993). Strains of *acidophilus* have been isolated from the intestinal tract of humans as well as animals such as rodents and birds.

LABs are gram-positive, non-spore forming, catalase-negative organisms that are devoid of cytochromes and anaerobic but aerotolerant. They are fastidious, acid-tolerant, and strictly fermentative (either homo- or hetero); lactic acid is the major end product of sugar fermentation (Axelsson, 1998). However, some species can form catalase or cytochromes on media containing hematin or related compounds and some lactobacilli can also produce non-heme catalase, called pseudocatalase, which cause confusion for LAB identification (Holzapfel *et al.*, 2001).

Table 2.2 LAB associated with the human gastrointestinal tract (Goktepe *et al.*, 2006)

Lactobacilli	Other Lactic acid Bacteria
Intestinal Bacteria:	*Bifidobacterium adolescentis*
Lactobacillus acidophilus group	*B. angulatum*
L. acidophilus senso strictu	*B. bifidum*
L. delrueckii	*B. breve*
L. brevis	*B. cantenulatum*
L. paracasei	*B. infantis*
L. animalis	*B. longum*
L. buchneri	*B. pseudocantenulatum*
L.crispatus	*Enterococcus fecalis*
L. curvatus	*E. faecium*
L. fermentum	*Leuconostoc Mesenteroides*
L. gasseri	*Pedicoccus pentosaceus*
L. johnsonii	*Weissella confusa*
L. reuteri	
L. rhamnosus	
L. ruminis	
L. sakei	
L. plantarum	

2.6 Mechanism of action of Probiotics microorganism

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel, *et al.* 1998). There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly (Rolfe 2000, Çakır 2003, Salminen, *et al.* 1999, Castagliuola, *et al.* 1999).

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

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

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

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

5. Degradation of toxin receptor: Because of the degredation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* protects the host against *C. difficile* intestinal disease. Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks, *et al.* 1999).

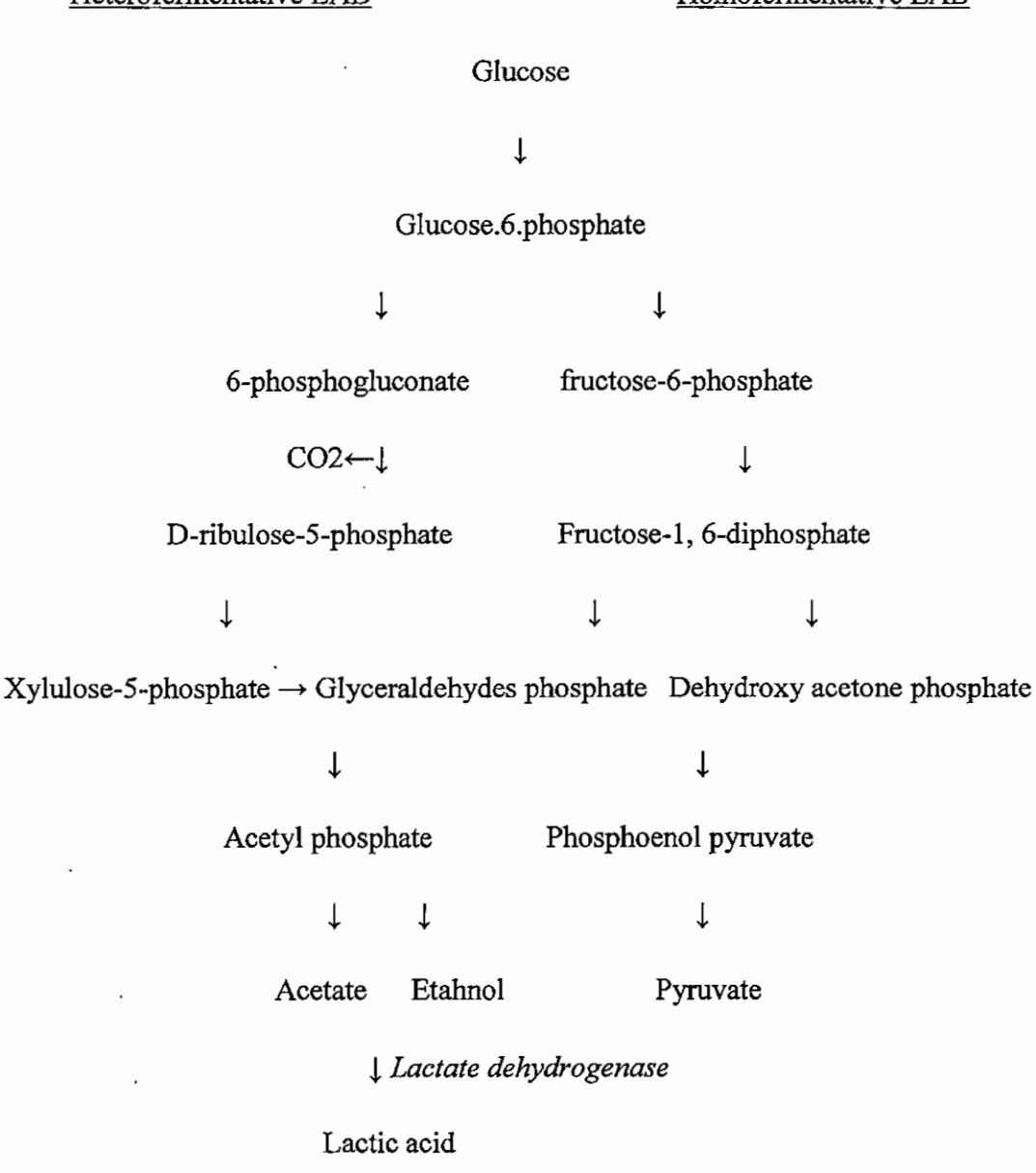
LAB are widespread in nature, their nutritional requirements are very complex. Hence, they predominate habitats that rich in carbohydrates, protein breakdown products, vitamins and environments with low oxygen. This confirms the prevalence in dairy products (Stiles and Holzapfel, 1997). Generally, lactic acid bacteria (LAB) can be defined as Gram positive, non-spore forming, catalase negative, devoid of cytochromes, acid tolerant, and facultative anaerobe group that produce lactic acid as the major end-product during fermentation of carbohydrates. According to carbohydrate metabolism, they can be divided into two main groups:

1. Homofermentative LAB (produce mainly lactic acid).

2. Heterofermentative LAB (produce lactic acid, carbon dioxide, ethanol and/or acetic acid).

Dairy microflora is further divided into two groups. Primary group includes starter flora which refer to stater LAB and secondary group includes non starter lactic acid bacteria (NSLAB), propionic acid bacteria (PAB), smear bacteria, moulds and yeasts (Beresford *et al.*, 2001).

Table 2.3 Metabolic pathways of glucose utilization by LAB (Garvie, 1984)



This classification is originated from metabolic routes that organisms used and resulting end product. While homofermentives use glycolysis (Embden- Meyerhof Pathway), heterofermentives use the 6-phosphogluconate/phosphoketolase Pathway (Garvie, 1984).

2.7 The Effect of Probiotics on Health

There are lots of studies on searching the health benefits of fermented foods and probiotics. However, in most of these studies researchers did not use sufficient test subjects or they use microorganisms were not identified definitely (Çakır 2003). So, while a number of reported effects have been only partially established, some can be regarded as well established and clinically well documented for specific strains. These health-related effects can be considered as in the below (Çakır 2003, Scherezenmeir and De Vrese 2001, Dunne, *et al.* 2001, Dugas, *et al.* 1999).

-Managing lactose intolerance.

- Improving immune system.
- Prevention of colon cancer.
- 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.
- Suppression of pathogenic microorganisms (antimicrobial effect).
- Prevention of osteoporosis.
- Prevention of urogenital infections.

2.7.1 Lactose Intolerance

Most of human commonly non-Caucasians become lactose intolerant after weaning. These lactose intolerant people cannot metabolize lactose due to the lack of essential enzyme β-galactosidase. When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence, cramping and diarrhoea ensue. If lactose passes through from the small intestine, it is converted to gas and acid in the large intestine by the colonic microflora. Also the presence of breath hydrogen is a signal for lactose maldigestion. The studies provide that the addition ofcertain starter cultures to milk products, allows the lactose intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms (Fooks, *et al.* 1999, Scheinbach 1998, Quewand and Salminen 1998, Lin, *et al.* 1991).

The beneficial effects of probiotics on lactose intolerance are explained by two ways. One of them is lower lactose concentration in the fermented foods due to the high lactase activity of bacterial preparations used in the production. The other one is; increased lactase active lactase enzyme enters the small intestine with the fermented product or with the viable probiotic bacteria (Salminen, *et al.* 2004).

When the yogurt is compared with milk, cause the lactose is converted to lactic acid and the yogurt consist of bacterial β-galactosidase enzyme; it is suitable end beneficial to consume by lactose intolerants. Furthermore, the LAB which is used to produce yogurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, are not resistant to gastric acidity. Hence, the products with probiotic bacteria are more efficient for lactose intolerant human.

2.7.2 Immune System and Probiotics

The effects of immune system are promising. However, the mechanism is not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts (Mombelli and Gismondo 2000). Several reserchers have studied on the effects of probiotics on immune system stimulation. Some in vitro and in vivo searchs have been carried out in mice and some with human. Data indicate that oral bacteriotherapy and living bacteria feeding in fermented milks supported the immune system against some pathogens (Scheinbach 1998, Dugas, *et al.* 1999). Probiotics affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA concentrations (Çakır 2003, Scheinbach 1998, Dugas, *et al.* 1999). Some of these effects are related to adhesion while some of them are not (Quwehand, *et al.* 1999).

Link-Amster *et al.* (1994) examined whether eating fermented milk containing *Lactobacillus acidophilus* La1 and bifidobacteria could modulate the immune response in human. They give volunteers the test fermented milk over a period of three weeks during which attenuated *Salmonella typhi* Ty21a was administered to mimic an enteropathogenic infection. After three weeks, the specific serum IgA titre rise to *S. typhi* Ty21a in the test group was >4-fold and significantly higher ($p=0.04$) than in the control group which did not ate fermented foods but received *S. typhi* Ty21a. The total serum IgA increased. These results showed that LAB which Cn survive in the gastrointestinal tract can act as adjuvants to the humoral immune response (Lime- Amster, *et al.* 1994, Quwehand, *et al.* 1999).

Perdigon *et al.* (1986) feed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations (Scheinbach 1998). Also in a human trial Halpern *et al.* (1991) feed human with 450 g of yogurt per day for 4 months and at the end a significant increase is observed in the production of γ-interferon (Fooks, *et al.* 1999). Mattilla-Sandholm and Kauppila (1998) showed that *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 derived extracts suppress lymphocyte proliferation in vitro. Further evidence for immunomodulation by these two strains a children trial with severe atopic eczema resulting from food allergy. Children fed with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 showed improvement in clinical symptoms compared to the placebo group (Saarela, *et al.* 2000).

2.7.3 Diarrhea

Diarrhea is many causes and many types so it is difficult to evaluate the effects of probiotics on diarrhea. But there are lots of searchs and evidence that probiotics have beneficial effects on some types of dierrhea. Diarrhea is a severe reason of children death in the worldwide and rotavirus is its common cause (Scheinbach 1998). In the treatment of rotavirus dierrhea, *Lactobacillus* GG is reported really effective. The best documented probiotic effect is shortened duration of rotavirus diarrhea using *Lactobacillus* GG. It has been given proof in several studies around the world by some researchers like Guandalini *et al.* (2000), Pant *et al.* (1996). Also *Lactobacillus acidophilus* LB1, *Bifidobacterium lactis* and *Lactobacillus reuterii* are reported to have beneficial effects on shortening the diarrhea (Salminen, *et al.* 2004). One of types of dierrhea is traveller's diarrhea (TD) which affects the healthy travellers not only in developing countries but also in Europe. Probiotics have beneficial effects in preventing some forms of TD. Oksanen *et al.* (1990) evaluated the efficacy of *Lactobacillus* GG in preventing diarrhea in 820 people travelling from Finland to Turkey. In a double-blind study by Black *et al.* (1989) lyophylised bacteria (*L.acidophilus, B.bifidum, L.bulgaricus, S.thermophilus*) were given to 56 Danish tourists on a 2-week trip to Egypt. The occurence of diarrhea in the group receiving the lactic acid bacteria was 43% while it was 71% in the placebo group (Gismondo, *et al.* 1999).

Antibiotic therapy causes mild and severe outbreaks of diarrhea. The normal microflora may be suppresed during the microbial therapy and resulting with filling with pathogenic strains. The changes of microflora may also ancourage the resistant strains at least *Clostridium difficile* which is the reasan of antibiotic associated diarrhea (ADD). Several clinical trials (Surewiez, et al.,

Adam, *et al.,* Mcfarland, *et al.*, etc.) have used *Saccharomyces boulardii, Lactobacillus* spp. and *Bifidobacterium* spp. in ADD. Probiotics which are able to restore and replace the normal flora should be used. Also they shoukd be used in high risk patients such as old, hospitalised or immunocompromised. Studies with *Saccharomyces boulardii* proved that *Clostridium difficile* concentraiton is decreased in the presence of *Saccharomyces boulardii* (Gismondo, *et al.* 1999).

2.7.4 Cancer

Epidemiological studies point out that if the consumption of saturated fats increases in the diet, the occurrence of colon cancer increases in Western World. Bacterial enzymes (β-glucornidase, nitroreductase and azoreductase) convert precarcinogens to active carcinogens in the colon. It is thought that probiotics could reduce the risk of cancer by decreasing the bacterial enzymes activity. Although the exact mechanism for the anti tumor action is not known, some suggestions have been proposed by McIntosh as follows (Fooks, *et al.* 1999, Scheinbach 1998):

1. Carcinogen/procarcinogen are suppressed by binding, blocking or removal.
2. Suppressing the growth of bacteria with enzyme activities that may convert the procarcinogens to carcinogens.
3. Changing the intestinal pH thus altering microflora activity and bile solubility.
4. Altering colonic transit time to remove fecal mutagens more efficiently.
5. Stimulating the immune system.

There are in vitro and in vivo evidences not only from animal studies but also from human studies that probiotics have beneficial effects on suppression of cancer. Oral administration of lactic acid bacteria has been shown to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa in rats. The consumption of lactobacilli by healthy volunteers has been demonstrated to reduce the mutagenicity of urine and feaces associated with the ingestion of carcinogens in cooked meat. When it comes to epidemiological studies, they show an association between fermented dairy products and colorectal cancer. The consumption of a large quantity of dairy products especially fermented foods like yogurt and fermented milk with containing *Lactobacillus* or *Bifidobacterium* may be related to a lower occurrence of colon cancer (Rafter 2003, Hirayama and Rafter 2000). A number of studies have shown that predisposing factors (increases in enzyme activity 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).

2.7.5 Cholesterol Reduction

Lots of researchers proposed that probiotics have cholesterol reduction effects. However, the mechanism of this effect could not been explained definitely. There are two hypotheses trying to explain the mechanism. One of them is that bacteria may bind or incorporate cholesterol directly into the cell membrane. The other one is, bile salt hydrolysis enzymes deconjugate the bile salts which are more likely to be exerted resulting in increased cholesterol breakdown (Çakır 2003, Scheinbach 1998, Prakash and Jones 2004).

A study on the reduction of cholesterol was showed that *Lactobacillus reuteri* CRL 1098 decreased total cholesterol by 38% when it is given to mice for 7 days in the rate of 104 cells/day. This dose of *Lactobacillus reuteri* caused a 40% reduction in triglycerides and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen and liver (Kaur, *et al.* 2002).

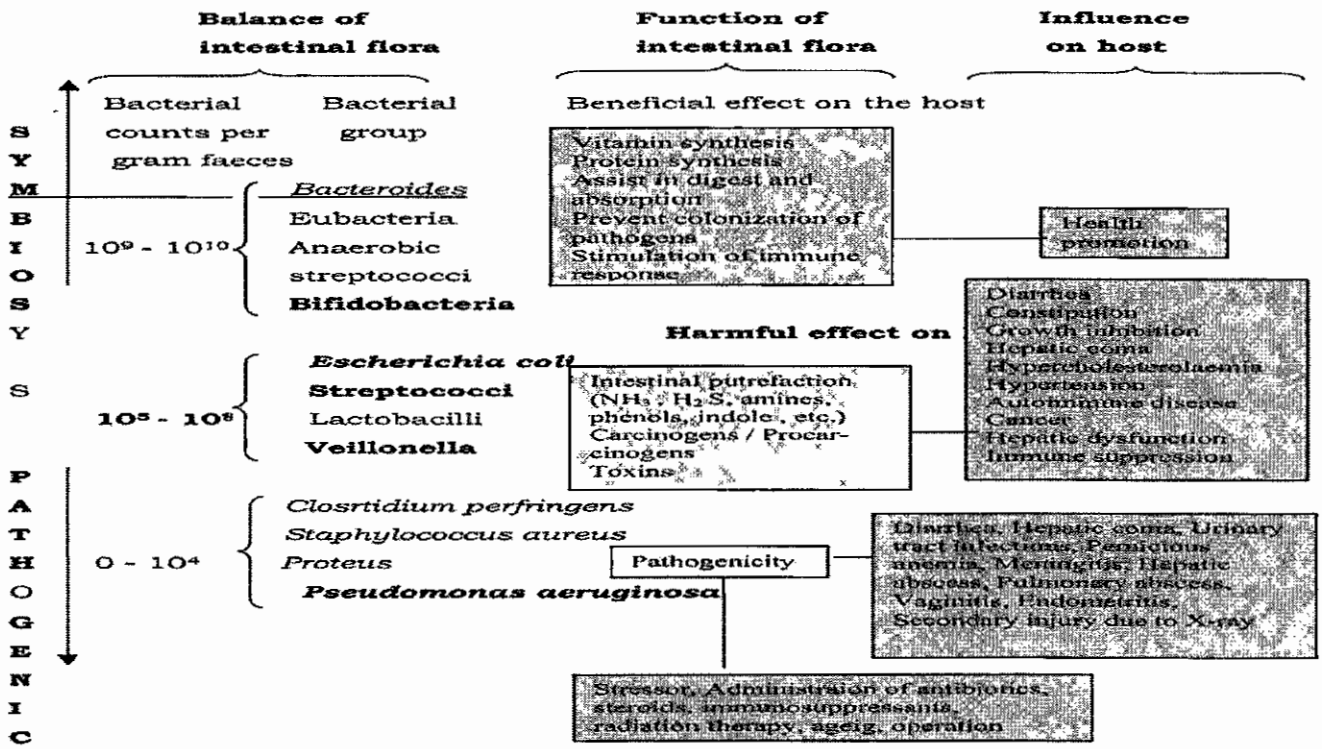


Fig. 2.1 the interrelationship between intestinal bacteria and human health as proposed by Mitsuoka (Ishibashi a Shimamura, 1993).

2.8 Yogurt as probiotic carrier food

Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotic microorganisms by which consumers can take in large amounts of probiotic cells for the therapeutic effect. Yogurt has long been recognized as a product with many desirable effects for consumers, and it is also important that most consumers consider yogurt to be 'healthy'. In recent years, there has been a significant increase in the popularity of yogurt (HamannaMarth, 1983) as a food product, accentuating the relevance of incorporating L. acidophilus and B. bifidum into yogurt to add extra nutritional-physiological value. The conventional yogurt starter bacteria, L. bulgaricus and Streptococcus thermophilus, lack the ability to survive passage through the intestinal tract and consequently do not play a role in the human gut (Gilliland, 1979).

2.8.1 Background of yogurt

Yoghurt is an acidified, coagulated product obtained from milk by fermentation with lactic acid producing bacteria. Of all cultured milk products, yoghurt is the most well known and most popular worldwide (Early, 1998). The culinary art of yoghurt making originated thousands of years ago. It is likely, however, that the origin of yoghurt was the Middle East, and the evolution of this fermented product through the ages can be attributed to the culinary skills of the nomadic people living in that part of the world. Although modern large-scale production is designed to handle thousands of litres per day, using highly sophisticated technology with mechanization and automatisation, the basic principles underlying the manufacturing process, have altered little with time (Tamime and Robinson, 1999). In recent years it became increasingly important, for various reasons, to manage and control all the elements of food manufacturing processes. One approach in this regard, that is well established and implemented world-wide is the HACCP system (Tamime *et al.*, 2002).

Flavour, texture and aroma of yoghurt vary dependent upon country of origin (as well as other factors including raw material formulation and manufacture process). In some areas, yoghurt is produced in the form of a highly viscous liquid, whereas in other countries it takes the form of a softer gel. Yoghurt is also produced in a drinking form and can be frozen or blended with other ingredients to create, for example, mousse type products, sorbet, yoghurt ice-cream or other forms of dairy dessert (Early, 1998).

The initial popularity of yoghurt in Western Europe owed much to the work of the Russian bacteriologist and 1908 Nobel Prize Laureate, E. Metchnikoff, who at the turn of the century studied the bacteria used to produce yoghurt. In his book The Prolongation of Life, written in 1907, he attributed the good health and longevity of Balkan peasants to the effects of certain bacteria in the yoghurt they consumed. He postulated the theory that prolongation of life would follow ingestion of a lactic acid bacterium named as Bulgarian bacillus. The presence of this organism in yoghurt was supposed to inhibit the growth of putrefactive organisms in the intestine.The Bulgarian bacillus is, in fact, Thermobacterium bulgaricum, later designated as Lactobacillus bulgaricus (currently known as Lactobacillus delbrueckii subsp. bulgaricus) (Tamime and Robinson, 1999).

2.8.2 Production of Yogurt

Yoghurt is a very nutritious food and its continued consumption in the Western World owes much to the development of its health food image (Early, 1990). Consumption of yoghurt is highest in countries around the Mediterranean, in Asia and in Central Europe (Bylund, 1995). The methods of production of yoghurt have, in essence, changed little over the years and although there have been some refinements, especially in relation to lactic acid bacteria, that bring about fermentation, the essential steps in the process are still the same, namely:

- raising the level of total solids in the process milk to around 14 – 16 g / 100 g;
- heating the milk, ideally by some method that allows the milk to be held at high
- temperature for a period of 5 – 30 min; the precise time will depend on the temperature selected;
- inoculating the milk with a bacterial culture in which Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus are the dominant organisms;
- incubating the inoculated milk, in bulk or retail units, under conditions that promote the
- formation of a smooth viscous coagulum and the desired aromatic flavour/aroma;
- cooling and, if desired, further processing, e.g. the admixture of fruit and other ingredients,
- pasteurisation or concentration; and
- packaging for distribution to the customer under chilled conditions.

2.9 Types of Yogart

At present there are many different types of yoghurt produced worldwide, and (Tamime and Deeth, 1980) have proposed a scheme of classification that separates all types of yoghurt into four categories based on the physical characteristic of the product. However, these products and in particular yoghurt are subdivided into different groupings based on the following aspects (Tamime and Robinson, 1999):

- legal standards (i.e. existing or proposed) to classify the product on the basis of chemical composition or fat content (full, semi-skimmed/medium or skimmed/low fat);
- physical nature of the product, i.e. set, stirred or fluid/drinking; the latter is considered stirred yogurt of low viscosity;
- flavours (plain/natural, fruit or flavoured; the latter two types are normally sweetened); and
- Post-fermentation processing (vitamin addition or heat treatment).

2.10 Fermentation products of yogurt

During the production of yogurt, changes to the milk constituents are attributed to fermentation, and the ingredients added during manufacturing. Changes induced during fermentation, include the fermentative action of the inoculated starter cultures, the secretion of nutritional and chemical substances by the microorganisms, as well as the presence of the microorganisms and their associated enzymes (Gurr, 1987). The primary role of lactic acid bacteria is to utilize lactose as a substrate and convert it into lactic acid during fermentation of milk. Lactose is taken up as the yogurt does not differ substantially from milk but the free amino acid content is higher due to proteolytic activity of microorganisms (Rasic a Kurmann, 1983). The microbial inoculum has a substantial influence on the vitamin content of yogurt. While some bacteria require B vitamins for growth, several others synthesise certain vitamins. Fermentation has little effect on the mineral content of milk and therefore the total mineral content remains unaltered in the yogurt (Gurr, 1987). In summary, the concentrations of lactic acid, galactose, free amino acids and fatty acids increase as a result of fermentation while lactose concentration decreases. Addition of ingredients mainly increases the protein and sugar content.

2.11 Bio-yogurt

In recent years some yogurt products have been reformulated to include live strains of L. acidophilus and species of Bifidobacterium (known as AB-cultures) in addition to the conventional yogurt organisms, S. thermophilus and L. bulgaricus. Therefore, bio-yogurt is yogurt that contains live probiotic microorganisms, the presence of which may give rise to claimed beneficial health effects.

2.12 Production of AB-yogurt

For the production of AB-yogurt, similar processing procedures to traditional yogurt are applied with the exception of the incorporation of live probiotic starter cultures. Heat treated, homogenised milk with an increased protein content (3.6–3.8%) is inoculated with the conventional starter culture at 451C or 371C and incubated for 3.5 and 9 h, respectively (Anon., 1994). The probiotic culture can be added prior to fermentation simultaneously with the conventional yogurt cultures or after fermentation to the cooled (41C) product before packaging.

2.13 starter cultures in a bio-yogurt

Bio-yogurt, containing L. acidophilus and B. bifidum (AB-yogurt), is a potential vehicle by which consumers can take in probiotic cells. The number of probiotic bacteria required to produce a beneficial effect, has not been established. Kurmann and Rasic (1991) suggested to achieve optimal potential therapeutic effects, the number of probiotic organisms in a probiotic product should meet a suggested minimum of >106 cfumL−1. These numbers required, however, may vary from species to species, and even among strains within a species. Other authors stipulate >107 and 108 cfu mL_1 as satisfactory levels (Davis et al., 1971; Kailasapathya and the addition of bifidogenic factors to achieve the desired levels of growth (von Hunger, 1986; Modler, 1994; Klaver et al., 1990).The survival of probiotic bacteria in fermented dairy bio-products depends on such varied factors as the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (e.g. carbohydrate source), final acidity, milk solids content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for Bifidobacterium sp.), level of inoculation, incubation temperature, fermentation time and storage temperature (Hamman aMarth, 1983; YoungaNelson, 1978; Kneifel, Jaros, a Erhard, 1993).

2.14 Health benefits of yogurt

Yogurt is a nutrient-dense food that meets a wide variety of nutritional needs at for everyone. Yogurt is a good source of protein-an average 8-ounce serving contains between 8 and 10 grams of protein, or 16 to 20 percent of the Daily Recommended Value (DRV). Because yogurt is cultured the amount of protein often succeeds liquid milk. Yogurt is also an excellent source of calcium. Yogurt may contain up to 35 percent of the Recommended Daily Intake (RDI) for calcium. Yogurt is low in fat and high in certain minerals and essentials vitamins, including riboflavin B2, Vitamin B12, phosphorus and potassium.

- May help reduce osteoporosis risk
- Yogurt can be eaten by people who are lactose intolerant
- Diets rich in calcium may help reduce hypertension
- May enhance the immune system of certain individuals
- Versatile and convenient –use as a substitute for mayonnaise, sour cream and cream cheese to lower calories
- May reduce the risk of colon cancer
- Excellent source of calcium
- Yogurt is considered a meat alternative because of high protein content
- Large variety of flavors and styles that can be used to reduce calories (**http://aboutyogurt.com/index.asp**)

2.15 The market for functional and probiotic food

The most active area within the functional foods market in Europe has been probiotic dairy products, in particular, probiotic yogurts and milks. In 1997 these products accounted for 65% of the European functional foods market, valued at US$889 million, followed by spreads, valued at US$320 million and accounting for 23% of the market (Hilliam M, *et al.*, 1998). In a recent study undertaken by Leatherhead Food RA, the market for functional foods in the United Kingdom, France, Germany, Spain, Belgium, Netherlands, Denmark, Finland, and Sweden was reviewed. The results of the study showed that the probiotic yogurt market in these 9 countries totalled > 250 million kg in 1997 (Hilliam M, *et al.*, 1998), with France representing the largest

market, having sales of ~90 million kg, valued at US$219 million. The German market for probiotic yogurts is growing rapidly; for example, during 1996–1997, it increased by 150%, whereas the UK market grew by a more modest 26% during the same period. On average, probiotic yogurts accounted for ~10% of all yogurts sold in the 9 countries studied, with Denmark having the highest proportion (20%) of probiotic yogurts, followed by Germany and the United Kingdom (both at 13%) and then France (11%). On the lower end of the scale were the Netherlands and Belgium (both at 6%) and then Finland and Sweden (both at 5%) (11). Seen as crucial to market expansion in Europe is further clarity on the use of health claims. The market for functional foods in Europe could ultimately account for ~5% of total food expenditure in Europe, which, based on current prices, would equate to ~US$30 billion (5)

Consumers are continually bombarded with both direct and indirect messages to focus on healthful foods and nutrition (Shrewsbury, *et al.*, 1997). As a result of the continued repetition of this message, diet and health have been inextricably linked. Consumer knowledge of nutrition, although not yet perfect, is improving and today's consumers find greater meaning in nutritional messages. Consequently, the marketing of functional products requires a 2-fold strategy. First, it is imperative that these new products fit the consumers' lifestyles and beliefs for future health goals (Shrewsbury, *et al.*, 1997). Second, the most appropriate market segment must be targeted.

2.16 Safety Aspects of Probiotics

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

1. Strains for human use are preferred to be human origin.
2. They are isolated from healthy human gastrointestinal tract.
3. They have to be non-pathogenic.
4. They have to no history of relationship with diseases like, infective endocarditis or gastrointestinal tract disorders.
5. They do not deconjugate bile salts.
6. They should not carry transmissible antibiotic resistance genes (Saarela, *et al.* 2000).

Probiotics are generally considered safe. As evidenced by epidemiologic studies, bacteremia or sepsis from lactobacilli is extremely rare. Numerous probiotics have a long history of safe use and no health concerns have been observed (Salminen S *et al.,* 1998) there are, however, isolated reports of fungemia with Saccharomyces following its use as a probiotic especially in immunocompromised or ICU patients (Pletincx M *et al.,* 1995; Rijnders BJ *et al.*, 2000). Thus although administration of probiotics generally can be considered safe, each strain of probiotic has specific properties that should be considered before its use in any patient. Novel microbes, including probiotics and genetically modified probiotics need to be assessed for their safety on a strain- by-strain basis. The safety of probiotics in conditions where the mucosal integrity of gastrointestinal tract is compromised requires more studies before sweeping safety recommendations can be formulated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling

Different sources of yogurt and poultry feces were collected for the isolation of probiotic bacteria and study of their probiotic properties. The samples were stored carefully at low temperature refrigerator (- 4°C) to protect it from contamination and deterioration. The names of the shops from which the samples were collected were:

Sample no. 07: Govt.Poultry Farm, Sylhet

Sample no. 08: Regional Govt. Poultry Farm, Rajabarihat, Rajshahi.

Sample no. 21: Mohonlal Sweets and Food industries Ltd, Humayan Rashid chattar, Sylhet.

Sample no. 22: Fullkolly Sobhanighat Bishwaroad, Sylhet.

Sample no. 23: Fiza and Company, Uposohor Market, Mendibag, Sylhet.

Sample no.24: Modhubon Ovijat Misti Biponi Rozview Complex, Sobhanighat, Bishwaroad, Sylhet.

3.2 Site of the experiment

The present study was conducted in Food Biotechnology laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh.

3.3 Media Preparation

MRS media were used for isolating Lactic acid bacteria from selected yogurt (De Man *et al.*, 1960). L-cysteine (0.05%) was added to MRS media to improve the specificity of this medium for isolation of *Bifidobacterium* spp. by creating an anaerobic condition (Zinedine and Faid, 2007). ST agar medium was used for the isolation *Steptococcus thermophilus* (Saccaro *et al.*, 2011). Only MRS agar medium was used for the isolation of *L. acidophilus* and *L. brevis* present in the yoghurt samples. The P^H of the medium for isolation of *L. acidophilus and L. brevis* was adjusted at 6.5 ± 2 and for isolation of *Bifidobacterium* spp. P^H was adjusted to 5.2. However, ST agar medium was adjusted to pH 6.8 (Saccaro *et al.*, 2011) GYP agar medium was used for the isolation of lactic acid bacteria from poultry feces sample at specific P^H 6.8.

3.4 Isolation of probiotic bacteria from collected yoghurt and poultry feces

Chemicals required for Isolation of *Lactobacillus spp*

MRS medium (peptone, meat extract, yeast extract, D- glucose, tween-80, K_2HPO_4, $MgSO_4$, $MnSO_4$, $(NH_4)_2C_6H_6O_7$, sodium citrate, agar), 0.15% peptone for peptone water preparation.

GYP medium (Peptone, yeast extract, D- Glucose, Tween-80, K, $MgSO_4$, $MnSO_4$, $FeSO_4$, NaCl sodium citrate, Agar).

Equipments required for Isolation of Lactic acid bacteria pH meter, test tubes, petri plates, autoclave, deionizer, vortex mixture, laminar airflow cabinet, pipette, micropipette, micropipette tips, anaerobic chamber, incubator, and refrigerator.

Protocol

One gram of yoghurt was taken aseptically and dissolved in 9 ml of peptone water solution (0.15% peptone) and serially diluted up to ten logarithmic (10^{-10}) fold. The diluted sample was then inoculated into the MRS (oxoid) agar acidified with glacial acetic acid to pH 5.7 and incubated anaerobically for 24 hours, 48 hours, 72 hours at 24°C, 48°C, 72°C, respectively as required based on target species. During the tests, the cultures were kept in MRS agar stabs at refrigeration temperature (Erdourul *et al.*, 2006). Most of the fastidious organisms are removed in the presence of acetate, citrate and Tween 80, because of anaerobic condition. The growth of lactic acid bacteria were ensured by observing the colony morphology and biochemical tests. Finally, the single colony of *Lactoacillus acidophilus, Bifidobacterium* spp, *Lactoacillus brevis and Steptococcus thermophilus* were isolated. The isolated culture was maintained in MRS broth as a pure culture at P^H 6.5 (International Dairy Federation, 1998).

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 30^0C 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 30^0C for 24 hr. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain, cell morphology, and catalase reaction before further sugar fermentation and characterization test. During the test the cultures were kept in MRS agar stabs at refrigeration temperature (Bayane *et al.*, 2005).

3.5: Bacterial Identification/Characterization

3.5.1 Colony Morphology

Isolates were further purified by subculture on MRS agar plates, and the colony morphologies were examined

3.5.2 Gram Staining

Materials required for gram staining: activated broth culture, crystal violet, Gram's iodine mordant, alcohol, safranine.

Protocol: Single colony was taken aseptically, smeared on to a clean dry slide and heat-fixed. Then heat-fixed smear was flooded with crystal violet solution for 30 sec and rinsed with water for 5 sec. Then grams iodine solution was used to cover over the slide for 1minute and rinsed with tap water for 5 sec. The slide was then decolorized with 95% ethanol for 15 to 30 sec and rinsed with 5 sec. Finally safranin was used as counter stain for 60-80 sec and rinsed with water. Then under light microscope gram positive bacteria were determined because the gram positive organism stain blue to purple and gram negative organisms stain pink to red color.

3.5.3. Endospore test

For endospore staining Schaeffer-Fulton and Wirtz-ConklinSch method described by Harley and Prescott (2002) in Laboratory Exercise in microbiology was used.

Materials required for endospore staining: Malachite green stain (0.5% (wt/vol) aqueous solution), 0.5 g of malachite green, 100 ml of distilled water, decolorizing agent: tap water, safranin counterstain.

Stock solution: (2.5% (wt/vol) alcoholic solution)

Safranin (2.5 grams) and 100 ml of 95% ethanol.

Working solution: 10 ml of stock solution and 90 ml of distilled water.

Protocol: Schaeffer-Fulton (1933) method was used for staining endospores of isolated bacteria. LAB is non spore forming, and by using loop single colony of isolated probiotic bacteria was aseptically transferred into a slide air dried and heat fixed. The slide was covered with filter paper and then soaked with malachite green and then heated to steaming the stain for 5 to 6 minutes. Paper towel was removed and allowed to cool the slide and rinsed with water for 30 sec. Finally, counter stained with safranin for 60 to 80 sec and rinsed with water for 30 sec

and then examined under light microscope. Vegetative cells stained as red and both endospore and free spore stained as green.

3.5.4. Catalase Test

Materials Required: Cultures: 24-48 hour broth cultures of isolated bacteria.

Reagent: 3% hydrogen peroxide.

Equipments: Bunsen burner, inoculating loop, test tubes, test tube rack, microscopic slides

Protocol: The test was done by Holt *et al*, (1994) method. A clean glass slide was divided into two sections with grease pencil. One should be labeled as test and the other as control. A small drop of normal saline on each area was placed with a sterilized and cooled inoculating loop; a small amount of the culture from the nutrient agar slant or Petri plate was picked up. One or two colonies were emulsified on each drop to make a smooth suspension. One drop of hydrogen peroxide was given over the test smear. The other drop serves as control. The fluid over the smears was observed for the appearance of gas bubbles.

Catalase enzyme breaks down hydrogen peroxide into oxygen and water molecules and oxygen production is observed by the generation of O_2 bubbles.

$$H_2O_2 \xrightarrow{\text{Catalase enzyme}} 2H_2O + O_2$$

The generation of gas bubbles indicates the presence of the enzyme, hence the catalase positive nature of the bacterium. *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are both catalase negative and no O_2 production (gas bubbles) were observed when 3% H_2O_2 solution was dropped on top of the colonies grown overnight on agar medium.

3.5.5 Determination of Carbohydrate/Sugar Fermentation Profiles

Carbohydrate/Sugar fermentation profiles were performed according to Erkus (2007).

Materials for Carbohydrate /Sugar Fermentation: Sixteen different sugars: 5% Glucose, 5% Sucrose, 2% Cellubiose, 2% Fructose, 5% Maltose, 5% Lactose, 1% salicin, 5% mannitol, 2% Ribose, 2% Xylose, 2% Arabinose, 5% Galactose, 2% Raffinose, 5% D-sorbitol, 2% Mannose, 2% Rhamnose.

Equipment required: Test tube, centrifuge, 2 ml centrifuge tubes, micropipette, micropipette tips etc.

Protocol: Lactic acid bacteria were screened for their ability to ferment 16 different carbohydrates, and all the reactions were performed three times using test tube. The procedure included two steps. The first step was the preparation of active cells free from sugar residues and the preparation of sterile sugar solutions.

Preparation of active cell culture free from sugar:

1) Overnight activation of isolates in 10 ml MRS and at 42°C.
2) Centrifugation was done for 10 min at 10000 rpm.
3) Resuspending the pelleted cells in 5 ml MRS without glucose containing bromecresol purple.
4) Centrifugation for 10 min at 10000 rpm.
5) Resuspending the pelleted cells in actual volume of 10 ml MRS without glucose containing bromecresol purple.

Preparation of Test Sugar Solution:

- Each sugar was dissolved in deionized water at a final concentration of 2-10% (w/v).
- Filter sterilization of sugar solutions was done by filters with 0.22 μm pore diameter.

Combination of sugar solutions and active cell culture without sugar:

- One ml of sugar solution was pipetted into the test tube
- Two hundred microliters of active cell solution without sugar was added to the sugar solution pipetted tubes.
- One negative controls was used for the indication of any contamination coming from basal media or any activity problem with culture. Negative Control: 5 ml of active cell solution without sugar
- All the reactions were performed three times.
- After overnight incubation at 42°C, the turbidity and the color change from purple to yellow with respect to negative and positive controls was recorded as positive fermentation result.

3.5.6. Motility Test

Purpose: Motility test medium was used to determine the motility of microorganisms.

Recipes: **Motility-Indole-Lysine (MIL) Medium**

Peptone	10.0 g
Tryptone	10.0 g
Yeast Extract	3.0 g
L-lysine Hydrochloride	10.0 g
Dextrose	1.0 g
Ferric Ammonium Citrate	0.5g
Bromcresol Purple	0.02g
Agar	2.0 g

Protocol

1) MRS broth medium was prepared and dispensed in 5 ml aliquots in screw-top test tubes.
2) The medium was autoclaved at 121°C under 15 psi pressures for 15 minutes.
3) To test for motility, using a sterile needle, a well-isolated colony was picked and stabbed the medium within 1 cm of the bottom of the tube. The needle was kept in the same line as it entered and as it was removed. Incubation was done at 35°C for 18 hours or until growth was evident. A positive motility test indicated by a diffuse cloud of growth away from the line of inoculation. The MIL medium is a multi-test medium used to test for motility while simultaneously determining other metabolic characteristics (ASM Microbe Library Protocol).

3.6: Study of Probiotic Properties of Lactic Acid Bacteria

3.6.1. Assay for determination of gastric juice resistance

Gastric juice resistances of isolated lactic acid bacteria from selected yoghurts and poultry feces were assayed using the protocol by Graciela and Maria (2001) with some modification in which 620 nm filter absorbance of cell concentration in gastric juice medium was taken.

3.6.1.1 Media for determination of gastric juice resistance

Artificial gastric juice: NaCl (2 gram), pepsine (3.2 gram) (Sigma, UK), were adjusted at a final pH 2.2 with HCl without dilution and was taken to 1 litre with sterile distilled water. As a control, artificial gastric juice was adjusted at a final pH 6.6 with 5 N NaOH. Sterilization was done by filtration with filter membrane of 0.22μm.

3.6.1.2 Materials for gastric juice resistance

1. An overnight culture in MRS broth.
2. Artificial gastric juice, pH 2.2 and pH 6.6.

3.6.1.3 Methods

The identified lactic acid bacteria were grown in MRS broth at 37°C for 24 hours. The artificial gastric juice having pH 2.2 and pH 6.6 were inoculated with the 1% (v/v) bacterial suspension. After that both the media were incubated at 37°C and then samples were taken at 0, 2, and 4 hours and after 24 hours. 620 nm filter absorbance of cell concentration in gastric juice medium was taken for detecting the cell resistance and multiplication by spectrophotometer. Uninoculared gastric medium at pH 2.2 and pH 6.6 served as control.

3.6.2. Bile salt tolerance test

3.6.2.1. Assay for determination of Bile salt tolerance test: Bile salt tolerance test of isolated lactic acid bacteria from selected yoghurts and poultry sample were assayed using the protocol by Graciela and Maria (2001) with some modification and according to Zinedine and Faid (2007) to some extent where it was necessary.

3.6.2.2 Materials for Bile salt tolerance test

1. An overnight culture in MRS broth
2. MRSO (MRS-Oxgall) broth medium
3. Sterile 0.1% peptone water
4. MRS agar medium

3.6.2.3 Methods

MRS broths with different concentration (0.05%, 0.1%, 0.3% and 0.6 %) of bile oxgall (Sigma Laboratories, UK) were used to evaluate the tolerance and growth rate of isolated LAB species. Final P^H of the medium was adjusted to 6.5 and autoclaved at 121°C for 15 psi for sterilization. Then 1% overnight culture of isolated LAB were inoculated into the MRSO (MRS-Oxgall) broth medium and incubated at 37°C for 1, 4, 12 and 24 hours under anaerobic condition for the detection of survival rates of the isolates were measured by taking absorbance at 620 nm filter for detecting the cell viability and multiplication by spectrophotometer. Uninoculared MRS broth served as control.

3.6.3. Phenol tolerance test

MRS broth was modified with 0.1%-0.4% phenol to determine the phenol tolerance of identified lactic acid bacteria. The P^H of the MRS broth was adjusted at 6.5. After sterilization of the MRS broth, 1% (V/V) of 24 hour active culture was inoculated and incubated at 37°C for 12 and 24hours. After incubation tolerance was determined by measuring absorbance at 620 nm filter of cell concentration for detecting the cell viability and growth by spectrophotometer. Uninoculared phenol solution served as control (Hoque et al., 2010).

3.6.4. NaCl tolerance test

LAB species shows different levels of NaCl tolerance. Different concentration (1%-10%) of NaCl was inoculated into MRS broth. After sterilization of each test tube containing MRS broth medium, 1% (V/V) fresh overnight culture of isolated LAB species was inoculated and incubated at 37°C for 24 h. After 24 h of incubation growth was determined by observing turbidity. Maximum growth rate based on turbidity indicated as double plus sign (++) and normal growth indicated as negative sign (+) and no growth indicated as negative sign (-) (Hoque *et al.*, 2010)

3.6.5. Bile salt hydrolase activity test: Plate assay

Materials: MRS medium components, granulated agar (Difco), sodium salt of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA) or taurodeoxycholic acid

Equipments: Autoclave, Anaerobic chamber, CO_2 incubator, glass screw-cap jars,

Protocol:

The isolated cultures were subcultured at least 3 times in anaerobic MRS broth (Difco Laboratories, Detroit, Mich.). The MRS broth was dispensed into screw-cap tubes, autoclaved, and placed, while still hot, into an anaerobic condition. All transfers and manipulations were performed anaerobically.

Agar plates were prepared by adding 1.7% (wt/vol) granulated agar (Difco) to broth medium and poured into sterile plastic petri dishes (60 by 15 mm). Once solidified, the plates were inverted and placed in the anaerobic condition for at least 48 hours. All plates were inoculated from an overnight culture using a sterile loop. Plates were incubated in glass screw-cap jars at 37°C in the anaerobic condition for 72 h. Precipitated bile acid could be seen around colonies representing highly active strains within 48 h.

Bile salt plates: Test plates were prepared with 0.5% (wt/ vol) of the sodium salt of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), or taurodeoxycholic acid (TDCA). After

boiling and steam sterilization, the plates were handled as described by Dashkevicz and Feighner, 1989.

3.7 Maintenance of Lactic acid bacteria

Material requirements: MRS medium, eppendorf tubes etc.

Protocol: Subculture will be done for maintenance of lactic acid bacteria for daily or weekly use. For long term preservation the purified bacteria with homogeneous cell morphology were stored in a deep freeze in MRS broth medium for preservation. (Bulut, 2003).

3.8 Development of bio-yogurt

3.8.1 Preparation of Yogurt culture

Starter culture Ten isolates of lactic acid bacteria (four isolates of each *Lactobacillus. acidophilus,* three of each *Lactobacillus brevis,* two *streptococcus thurmophilus, and Bifidobacterium spp.* from four selected samples) were selected. The selection of cultures was based on the identification and characterization results of this study used as starter culture.

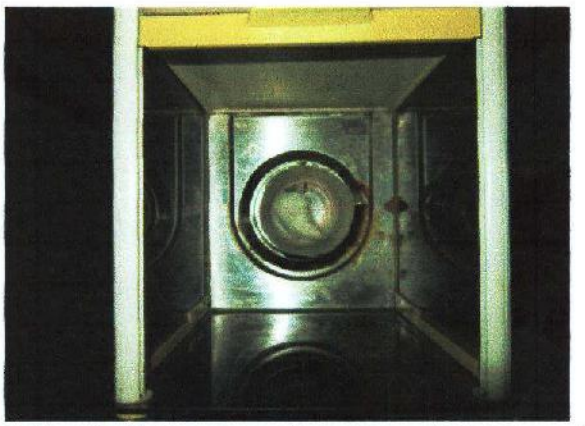
Protocol

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

3.9 Trial on mice

Material requirements Twenty four Swiss albino mice (3-4 weeks) were purchased from ICDDR,B and housed in plastic cages with sow dust and paper bedding. **Protocol:** Mice were allowed open access to water and maintained on a 12:12 h light: dark cycle. (Yeun *et al*, 2008). Rat pellet was also collected from ICDDR,B and was provided 8 grams/mouse/day. Leftover food was removed daily and was replaced with fresh food. After 4 weeks of arrival mice were divided into two groups, each group comprised 12 mice. Group 1 was denoted as treatment group and group 2 as control. For differentiation, individual mouse was marked different colours viz. red, blue, black, yellow, green, white, red black, blue black, yellow black, blue yellow, red blue and red yellow by using permanent marker in the tail.

A. Housing and feeding of mouse



B. Measuring body weight of mouse by using electric balance

C. Cutting of tail for blood sample collection

D. Taking reading of cholesterol level by using Easy Touch GC

Figure 3.1: Mice trial A. Housing and feeding of mouse B. Measuring body weight of mice C. blood sample collection D. cholesterol level measurement.

3.9.1 Body weight gain and blood cholesterol measuring test

Materials: Easy Touch GC blood cholesterol measuring device, cholesterol measuring strips and sharp edge.

Protocol

Group 1 was feed with rat pellet (5grams/mouse) along with 8 grams of probiotic yoghurt per day up to 28 days. Group 2 mice were feed with only rat pellet (5grams/mouse); 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 for measuring blood cholesterol level in mmol/l by using, on day 0 and day 28 and cholesterol level was measured by using Easy Touch GC blood cholesterol measuring strip.

3.9.2 Antibiotic Resistance Test

Materials requirements: Amoxicillin

Protocol

Twenty four adult albino mice were selected and grouped into two groups for antibiotic resistance test. Before starting of the experiment all mice were feed with 5 gm of basal diet and adequate water for 7 days. Then they were divided into 2 groups. Groups 1 were selected as treatment group and group 2 as control. Group 1 was feed with basal diet (5 gm/per mouse) along with 8 gm probiotic yogurt per day with antibiotic while Group 2 were feed with basal diet and probiotic without antibiotic. Amoxicillin was used as antibiotic and 0.02 mg was administered in to mouse mixing with drinking water for per gram of mouse weight. Total experiment day was 14 days. Feces were collected from each group on day 0, day 7 and day 14 and cultured on MRS agar media for LAB colony count (Majhenic and Matijašic, 2001)

3.9.3 Inhibition of the growth of pathogenic microorganisms *in vivo*:

Requirements: Pathogenic bacteria, MacConkey broth,

Protocol

Escherichia coli was grown in MacConkey broth (P^H 7.1±0.2) at 37° C for 24 h. and mice were inoculated with *E. coli* by 0.1 ml of the bacterial suspension. A single dose of 0.1 ml sample yogurt containing about 10^9 colony forming units (cfu) was fed to mice before 5 day of testing with the pathogenic bacteria (Silva *et. al,* 1999). The feces of control infected and yoghurt supplemented infected mice were collected individually after day 7 of post infection. The feces

were homogenized with a tissue grinder and suspended to produce 10 ml sample. Then the samples were diluted 10 fold serially and 0.2 ml amount was plated onto MacConkey agar (p^H 7.1±0.2) for *E. coli* respectively. The petridishes were cultured at 37° C for 24h and subsequently colonies were observed. Purple colonies of *E. coli* were easily distinguishable from other normal resident enterobacteria. The no. of pathogenic bacteria/ml in samples was counted by the following equation.

$$No.of\ bacteria/ml\ in\ sample = \frac{No.of\ colonies\ (cfu)}{Dilution\ no. \times Amount\ of\ sample\ plated}$$

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Isolation of Lactic acid bacteria from yogurt sample

Probiotic Lactic acid forming bacteria (LAB) were isolated and identified presumptively as *Lactobacillus acidophilus* from sample number 21, 22 and 23, *Streptococcus thermophilus* from sample number 21 and 24. *Lactobacillus brevis* from sample number 23, 24 and *Bifidocterium spp.* from sample number 23 primarily by observing their colony morphology, physiological characteristic and finally by observing the biochemical characteristics. *Lactobacillus acidophilus* did grow well on MRS agar media supplemented with 0.5% salicin and 0.05% cysteine in MRS enhances the growth of *Bifidocterium spp. Streptococcus thermophilus* grows well on ST agar medium.*Lactobacillus brevis*grows on MRS agar medium. Morphological, physiological *and* biochemical characteristics of *Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus brevis,* and *Bifidobacterium*spp. have shown in Table 4.1, 4.2 and 4.3 sequentially. Microscopically the isolated LAB were Gram positive (Figure 2.1, 2.2, 2.3 and 2.4), absence of endospore (Figure 3.1, 3.2, 3.3, 3.4), non-motile (Figure 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 5.10) and catalase negative (Table 4.3). Consequently, sugar fermentation profiles were performed for identification. The isolates have the ability to show tolerance to MRS broth containing inhibitory substances such as bacteriostatic phenol, gastric acid, NaCl and bile salt. According to their characteristics, the isolates were identified as *Lactobacillus acidophilus Streptococcus thermophilus, Lactobacillus brevis,* and *Bifidocterium*spp.

4.2. Identification

4.2.1. Colony Morphology

Colony morphologies of the identified isolates are given in Table 4.1 from which it was observed that the colony morphology of *Lactobacillus acidophilus* Very small, round shaped and non transparent from sample no. 21, 22 and 23. Moreover, Medium, circular, low convex, irregular creamy white. were observed in *Streptococcus thermophilus* from sample no. 21. Small, white rod shaped and non-transparent colonies were observed in *Lactobacillus brevis* from sample no. 23, 24. Small, triangular, watery circle with deep white middle were observed in *Bifidocterium* spp. from sample no. 23. Colony morphology of the above mentioned isolated probiotic bacterial species were found characteristic to the corresponding typical species.

Table 4.1: Colony morphology of isolated probiotic bacteria from yogurt sample.

Identified isolates	Sample No.	Colony morphology
Lactobacillus acidophilus	Sample no. 21, 22 and 23	Very small, round shaped and non transparent
Streptococcus thermophilus	Sample no. 21 and 24	Medium, circular, low convex, irregular creamy white.
Lactobacillus brevis	Sample no. 23, 24	Small, white rod shaped and non-transparent.
Bifidocterium spp.	Sample no. 23	Small, triangular, watery circle with deep white middle.

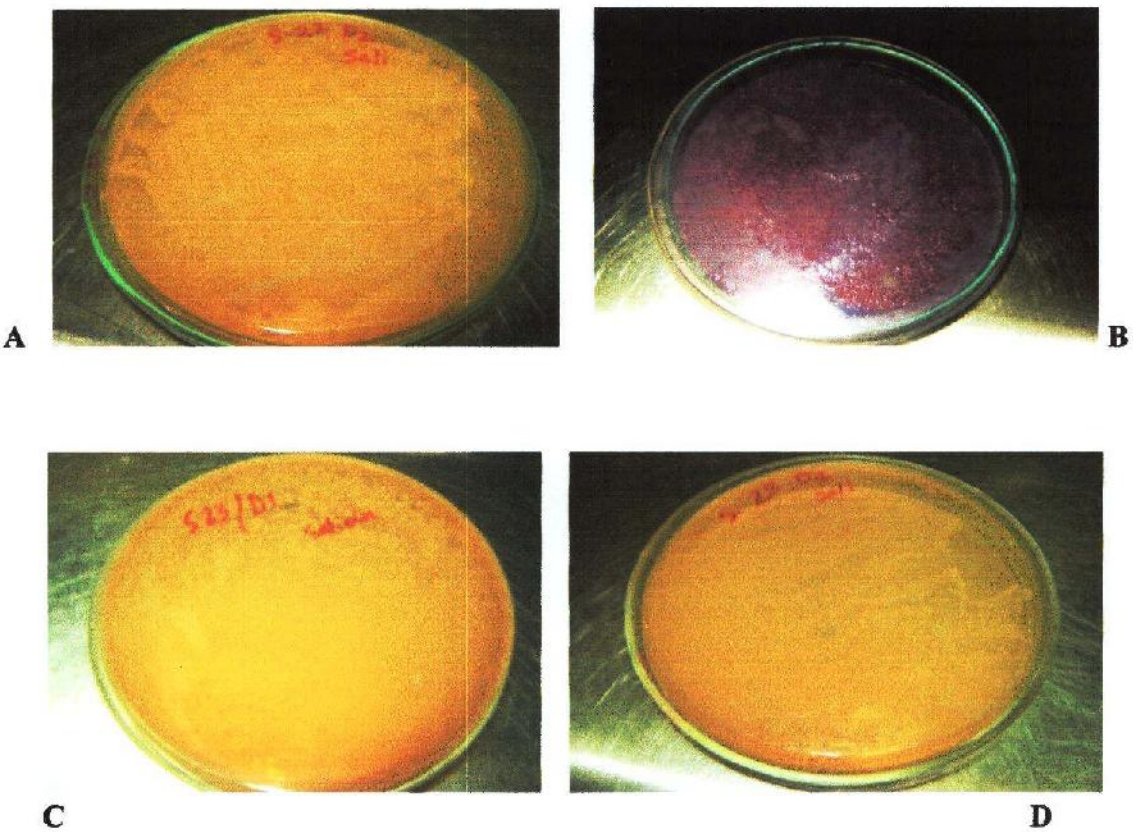


Figure 4.1 (A to D): Colony morphology of presumptive isolated bacteria: Isolate A (*lactobacillus acidophilus*, sample-22); B (*Streptococcus thermophilus*, sample-21); C (*lactobacillus brevis*, sample-23); D (*Bifidocterium* spp. sample- 23) grown on selective MRS agar and ST media.

4.2.2. Gram staining test

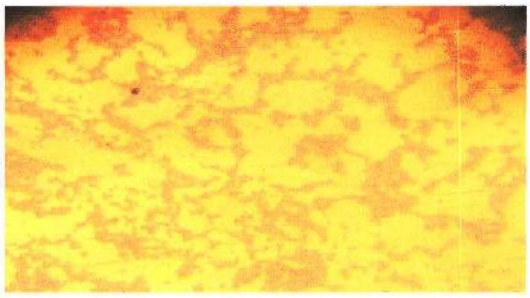
All the isolated probiotic bacteria were found gram positive due to retain violet blue color which are given as positive sign in Table 4.3. *Lactobacillus acidophilus* were found Rod shaped, regular in long chains from sample no. 21, 22 and 23. In addition, Coccoid shape, spherical or oval, occur in chains were observed in *Streptococcus thermophilus* from sample no. 21. Rod shape, regular, occur singly/chain were observed in *Lactobacillus brevis* from sample no. 23 and 24. Tiny rod, branched, v and y shape in chains were observed in *Bifidocterium* spp. from sample no. 23. Cell shape of the above mentioned isolated probiotic bacterial species after Gram staining were the characteristics to the corresponding typical species (Figure 4.1, 4.2, 4.3, 4.4).

Table 4.2. Cell shape of isolated probiotic bacteria after Gram staining

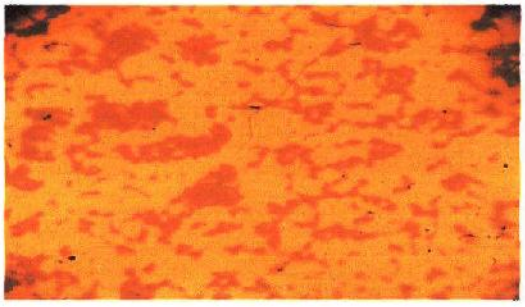
Identified LAB	Sample No.	Cell shape
Lactobacillus acidophilus	Sample no. 21, 22 and 23	Rod shaped, regular in long chains
Streptococcus thermophilus	Sample no. 21 and 24	Coccoid shape, spherical or oval, occur in chains
Lactobacillus brevis	Sample no. 23 and 24	Rod shape, regular, occur singly/chain
Bifidocterium spp.	Sample no. 23	Tiny rod, branched, v and y shape in chains

Table 4.3. Physiological and biochemical characteristics of isolated probiotic bacteria

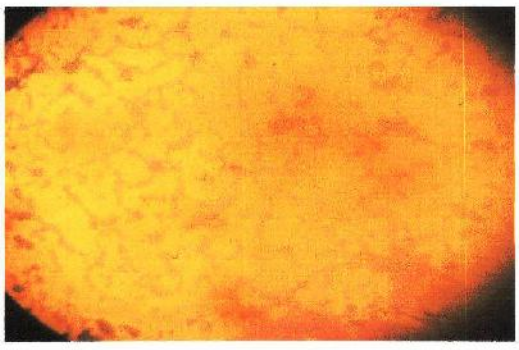
Physiological and biochemical characteristics	*Lactobacillus acidophilus* Sample no. 21, 22 and 23	*Streptococcus thermophilus* Sample no. 21, 24	*Lactobacillus brevis* Sample no. 23 and 24	*Bifidocterium* spp. Sample no. 23
Gram stain	+	+	+	+
Catalase test	-	-	-	-
Endospore test	-	-	-	-
Motility test	-	-	-	-



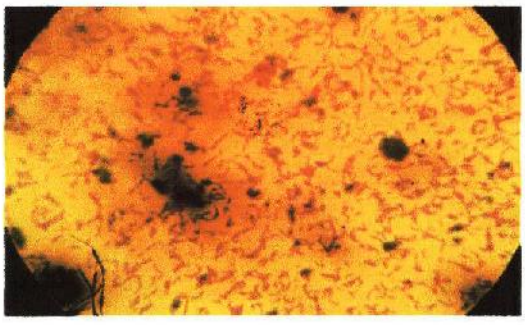
Lactobacillus acidophilus (sample no. 21), Isolate no. 01



Lactobacillus acidophilus (sample no. 22), Isolate no. 03

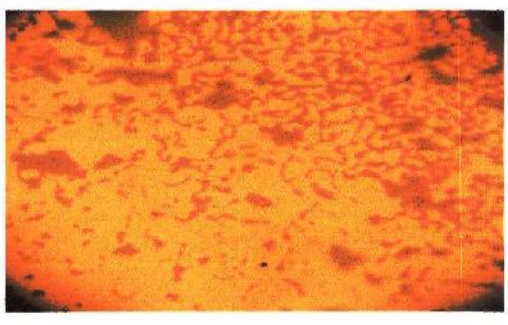


Lactobacillus acidophilus (sample no. 23), Isolate no. 08

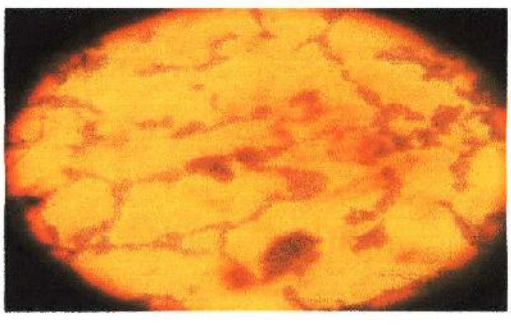


Lactobacillus acidophilus (sample no. 22) Isolate no. 05

Figure 4.2.1: Microscopic observation (40X) of *L. acidophilus* after Gram staining.

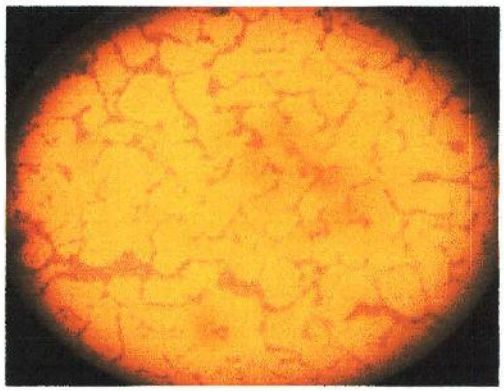


S. thermophilus (sample no. 21) Isolate no. 02



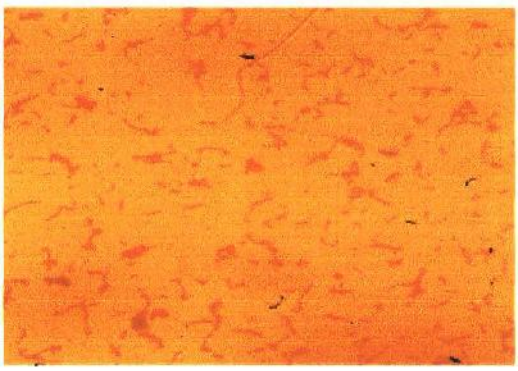
S. thermophilus (sample no. 24) Isolate no. 06

Figure 4.2.2: Microscopic observation (40X) of *S. thermophilus* after Gram staining.



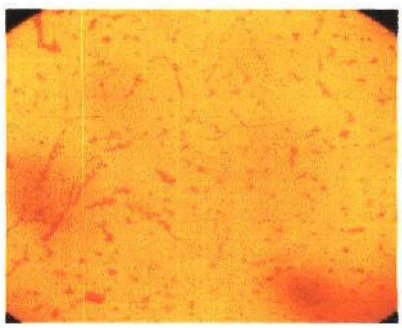
Lactobacillus brevis (sample no. 23)

Isolate no. 04



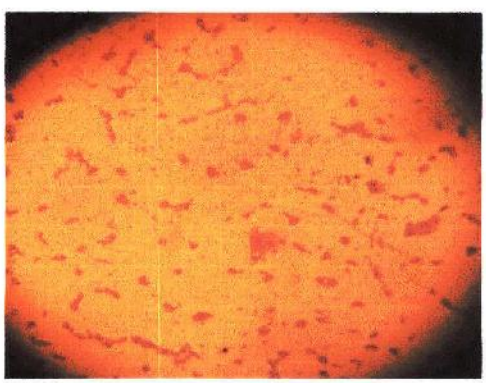
Lactobacillus brevis (sample no. 24)

Isolate no. 07



Lactobacillus brevis (sample no. 24) Isolate no. 10

Figure 4.2.3: Microscopic observation (40X) of *Lactobacillus brevis* and after Gram staining.



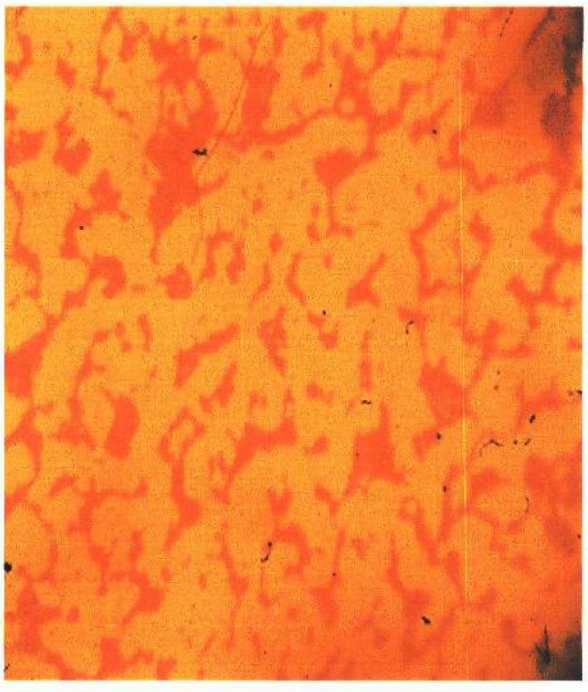
Bifidobacterium spp. (sample no. 23) Isolate no. 09

Figure 4.2.4: Microscopic observation (40X) of *Bifidobacterium spp.* after Gram staining.

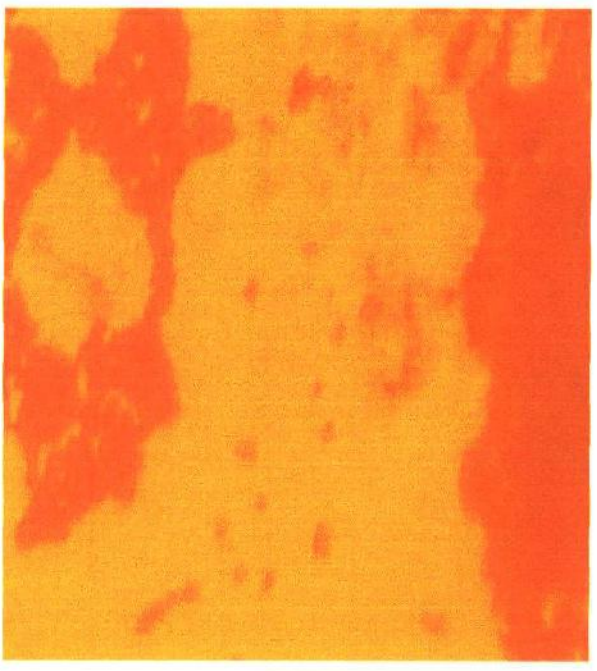
4.2.3. Endospore test:

The Endospore test of the isolates was performed by Schaeffer-Fulton method described by Harley and Prescott (2002). using loop, single colony of isolated probiotic bacteria was aseptically transferred on a slide which was air dried and heat fixed. The slide was covered with filter paper and then soaked with malachite green and then heated to steaming the stain for 5 to 6 minutes. Paper towel was removed and allowed to cool the slide and was rinsed with water for 30 sec. Finally, counter stained with safranin for 60 to 80 sec and rinsed with water for 30 sec and then examined under light microscope.

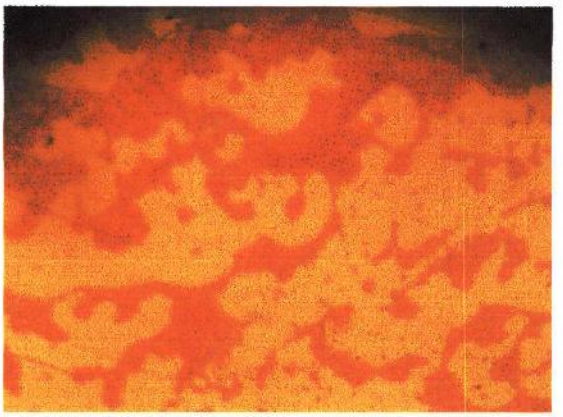
Due to Microscopic examination all the isolated LAB was endospore-negative [(Figure 6(A to C), Figure 7 (A to C), Figure 8 (A to D)]. Under light microscope, vegetative cells stained show as red and both endospore and free spore stained as green and from microscopic observation green color was not observed and this result indicate absence of endospores.

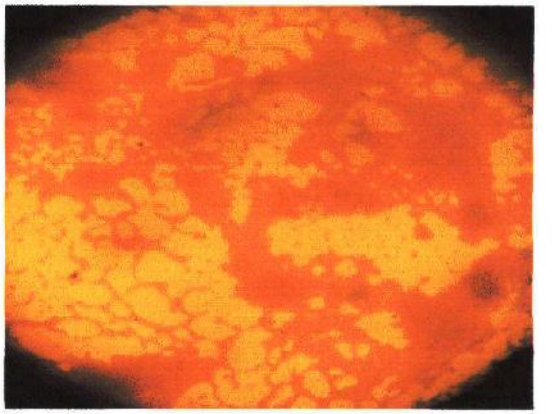


Lactobacillus acidophilus (sample no. 21)
Isolate no. 01



Lactobacillus acidophilus (sample no. 22)
isolate no. 03





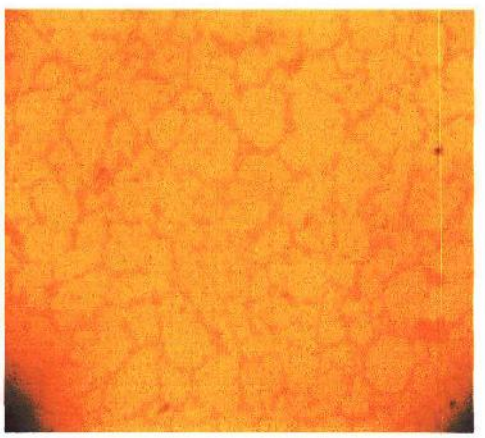
Lactobacillus acidophilus (sample no. 23)

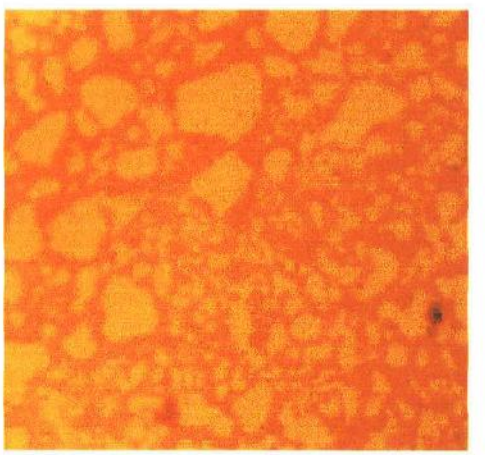
Isolate no. 08

Lactobacillus acidophilus (sample no. 22)

Isolate no.05

Figure 4.3.1: Endospore test of *L. acidophilus* of yogurt sample





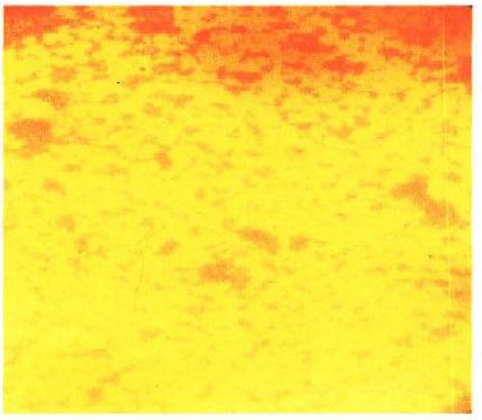
Steptococcus thermophilus (sample no. 21)

Isolate no. 02

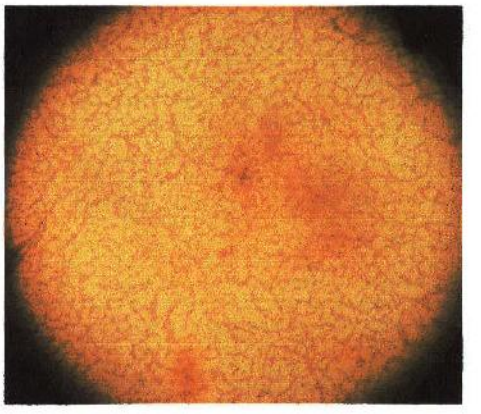
Steptococcus thermophilus (sample no. 24)

Isolate no. 06

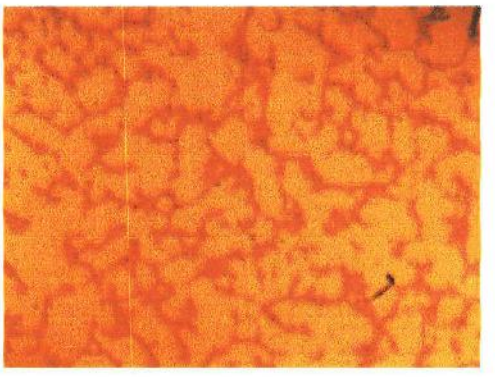
Figure 4.3.2: Endospore test of *Steptococcus thermophilus* of yogurt sample



L. brevis (sample 23), Isolate no. 04

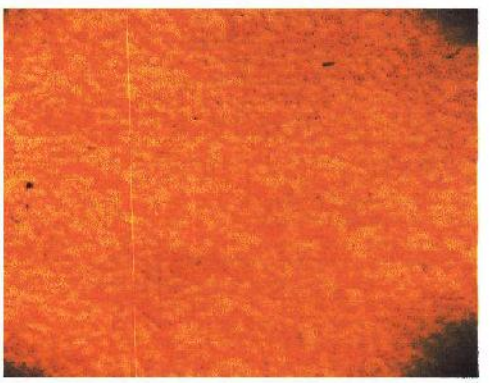


L. brevis (sample 24), Isolate no. 07



L. brevis (sample 24), Isolate no. 10

Figure 4.3.3: Endospore test of *L. brevis* of yogurt sample



Bifidobacterium spp. (Sample no. 23)

Isolate no. 09

Figure 4.3.4: Endospore test of *Bifidobacterium* spp. of yogurt sample

4.2.4. Catalase test

All the isolated and presumptively identified probiotic bacteria (*Lactobacillus acidophilus* from sample no. 21, 22 and 23, *Streptococcus thermophilus* from sample no. 21, 24 and *Lactobacillus brevis* from sample no. 23, 24 and *Bifidocterium* spp. from sample no. 23) were found catalase negative which are produce negative reaction due to no bubble production during addition of 3% H_2O_2 (Table 4.3).

Fig. 4.4: Catalase test of yogurt sample showing no bubble formation

4.2.5. Motility test

Non-motile formation were found for all of the isolated and presumptively identified probiotic bacteria which is also characteristic properties of typical probiotic bacteria like (*Lactobacillus acidophilus* from sample no. 21, 22 and 23, *Streptococcus thermophilus* from sample no. 21, 24 and *Lactobacillus brevis* from sample no. 23, 24 and *Bifidocterium* spp. from sample no. 23) given as negative sign in Table 4.3.

Figure 4.5.1: Motility test, Sample no. 21

Non-motile (Isolate no. 01)

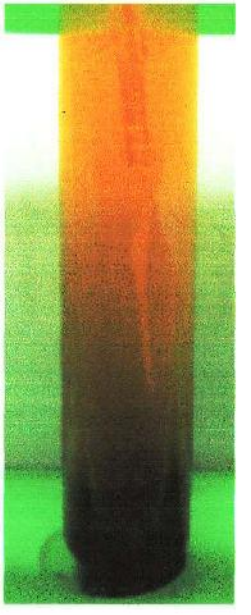


Figure 4.5.2: Motility test, Sample no. 22

Non-motile (Isolate no. 03)

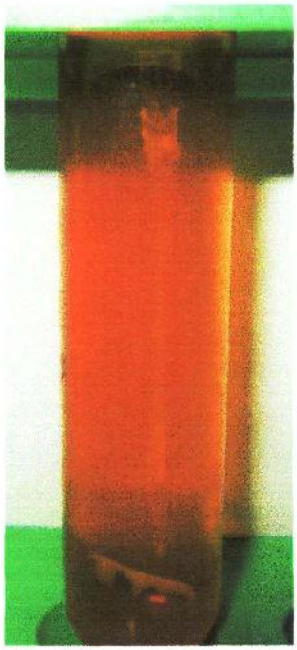


Figure 4.5.3: Motility test, Sample no. 23

Non-motile (Isolate no. 08)

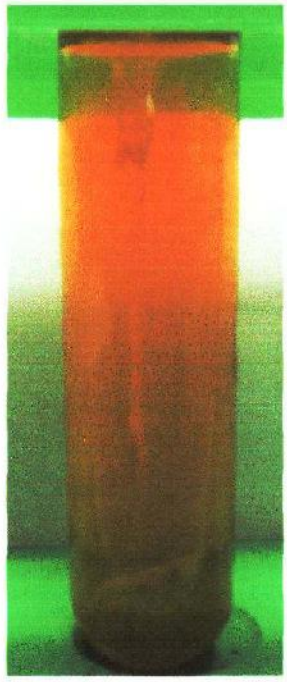


Figure 4.5.4: Motility test, Sample no. 22

Non-motile (Isolate no. 05)

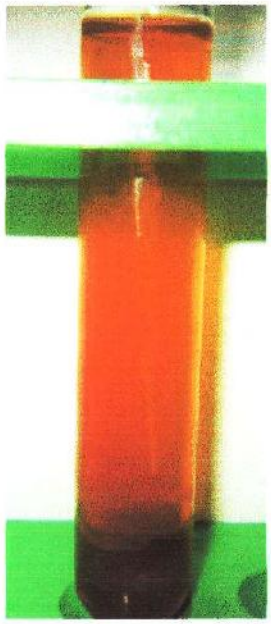


Figure 4.5.5: Motility test, Sample no. 21

Non-motile (Isolate no. 02)

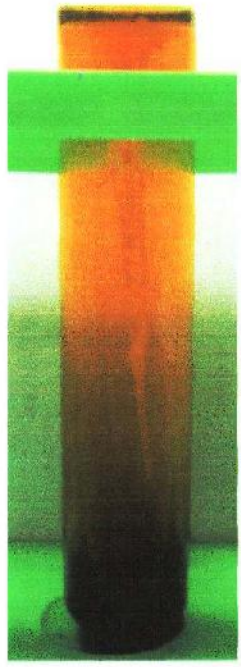


Figure 4.5.6: Motility test, Sample no. 24

Non-motile (Isolate no. 06)

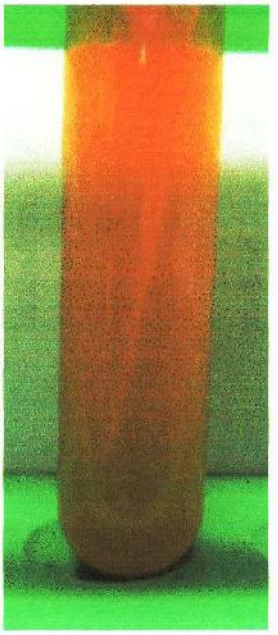


Figure 4.5.7: Motility test, Sample no. 23

Non-motile (Isolate no. 04)

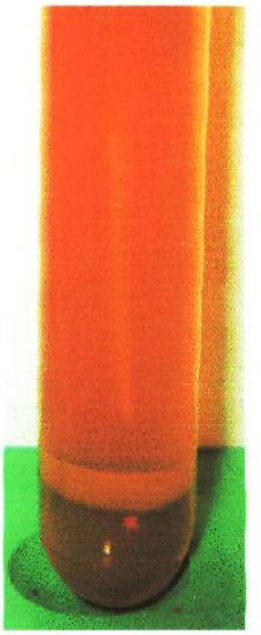


Figure 4.5.8: Motility test, Sample no. 24

Non-motile (Isolate no. 07)

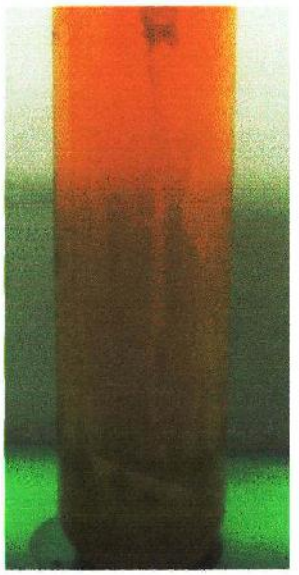


Figure 4.5.9: Motility test, Sample no. 24

Non-motile (Isolate no. 10)

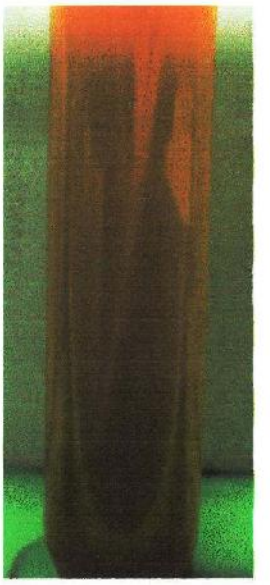


Figure 4.5.10: Motility test, Sample no. 23

Non-motile (Isolate no. 09)

4.2. Sugar fermentation test

Isolated bacteria were identified on the basis of their fermentation or sugar metabolism pattern which was determined by color change of the broth medium due to acid production pattern as well as for bacterial identification. In Table 4.4 and Figure 6.1 to 6.16 it was observed that every probiotic bacterium has specific sugar fermentation pattern of its own. LAB produces lactic acid as the major metabolic end-product of sugar fermentation. Besides exerting its activity through lowering the pH and through its undissociated form, lactic acid is also known to function as a permeabilizer of the Gram-negative bacterial outer membrane (Alakomi *et al.*, 2000), allowing other compounds to act synergistically with lactic acid (Niku-Paavola *et al.*, 1999).

Figure 4.6.1: Sugar (Lactose) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 04, 05, 07, and 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar.

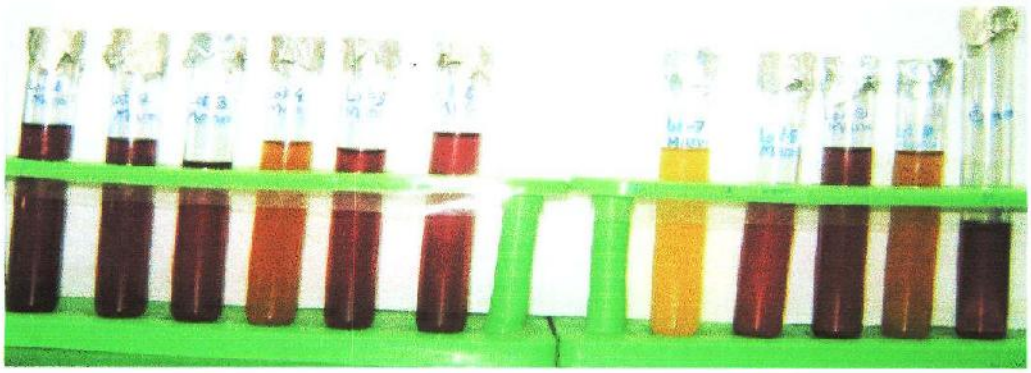


Figure 4.6.2: Sugar (Mannitol) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 04, 07, and 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar.

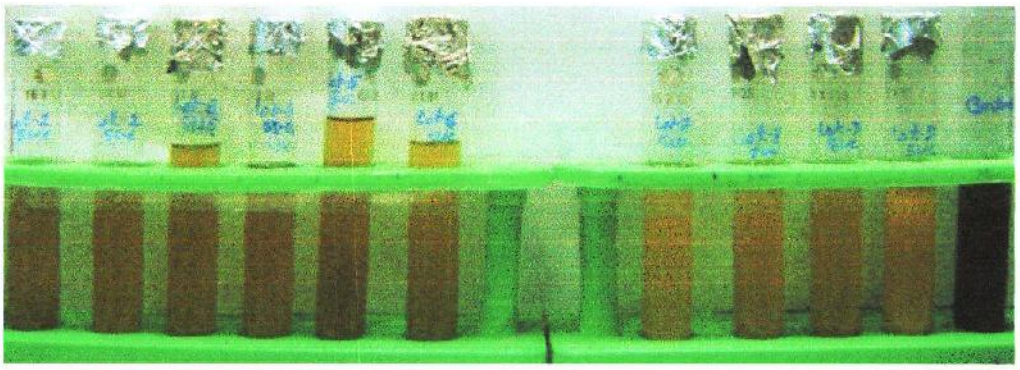


Figure 4.6.3: Sugar (Sucrose) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar.

Figure 4.6.4: Sugar (Fructose) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

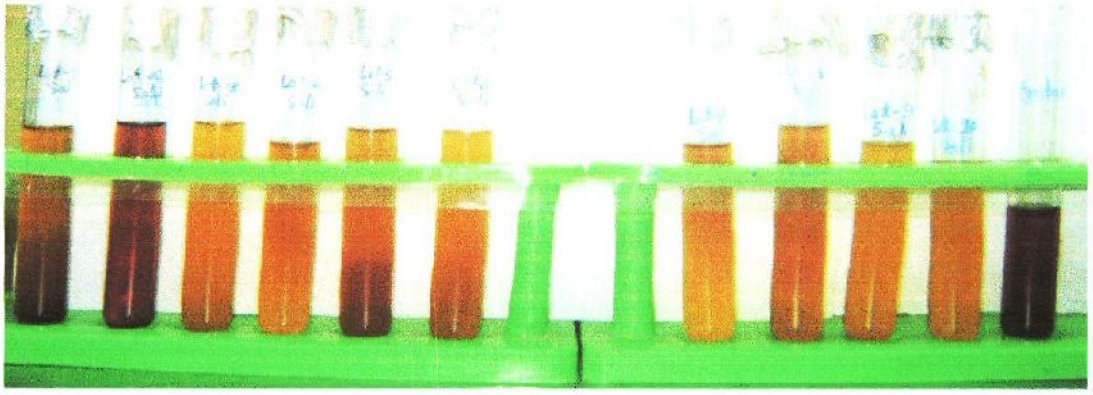


Figure 4.6.5: Sugar (Salicin) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 01 and 03 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

Figure 4.6.6: Sugar (Ribose) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 01 to 06 and 08 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

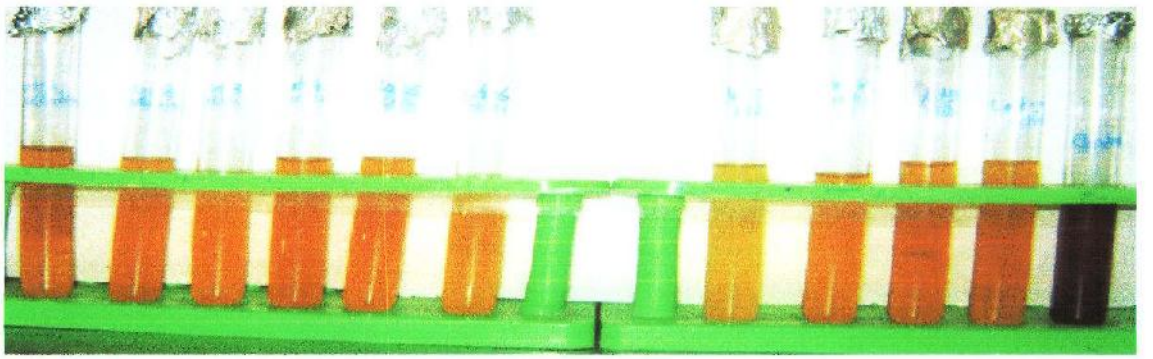


Figure 4.6.7: Sugar (Cellubiose) fermentation patterns of isolate no. 01 to 10. Yellow colour indicates fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

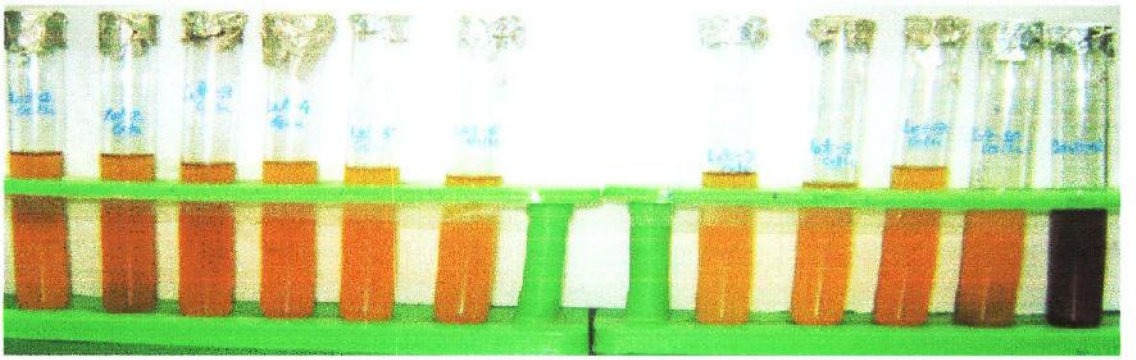


Figure 4.6.8: Sugar (Glucose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

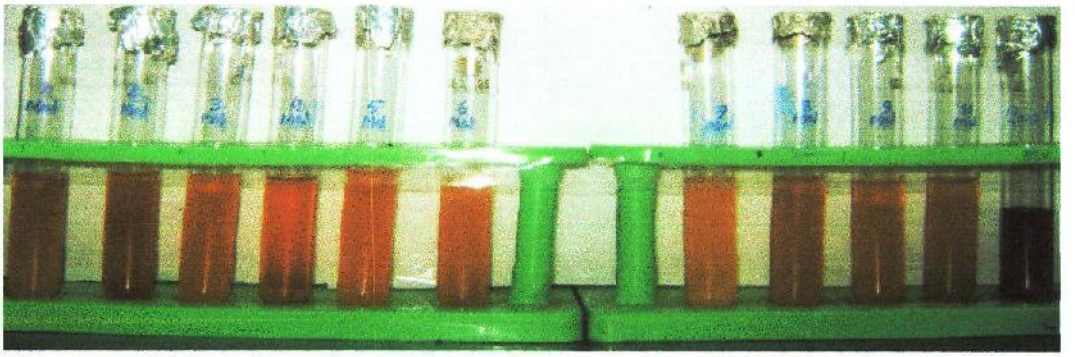


Figure 4.6.9: Sugar (Maltose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

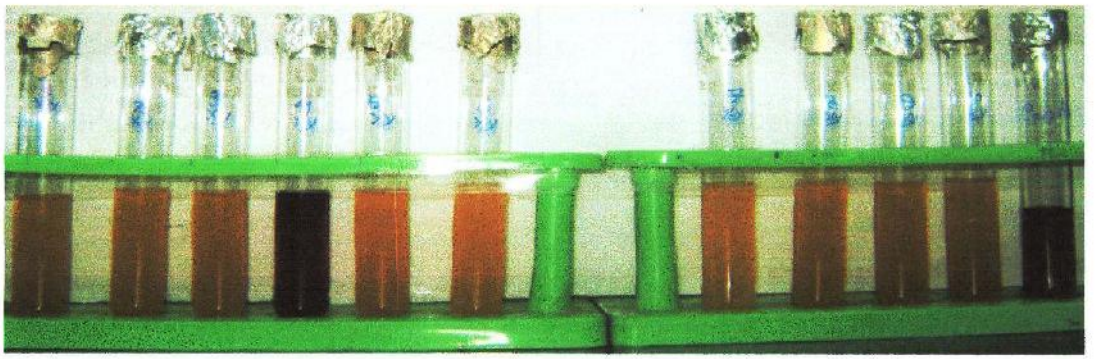


Figure 4.6.10: Sugar (Xylose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 10 except 04 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

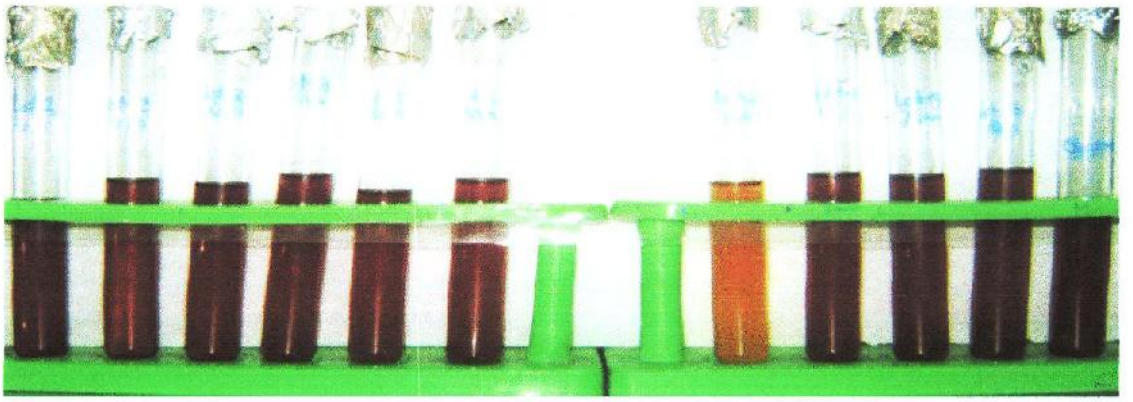


Figure 4.6.11: Sugar (Rhamnose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 07 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

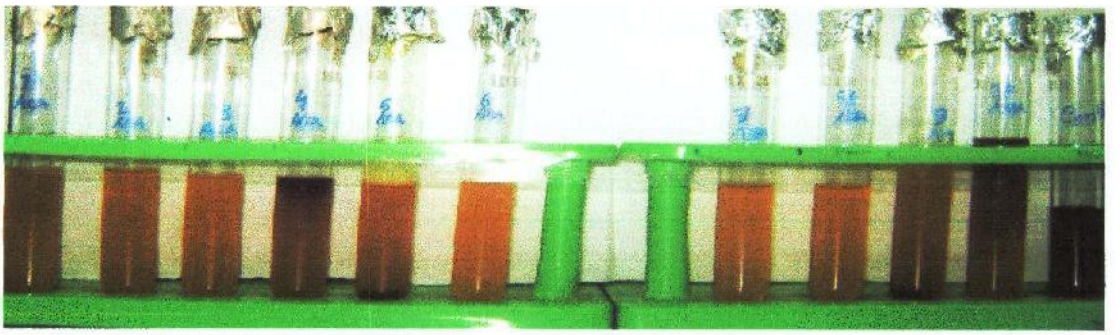


Figure 4.6.12: Sugar (L-Arabinose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

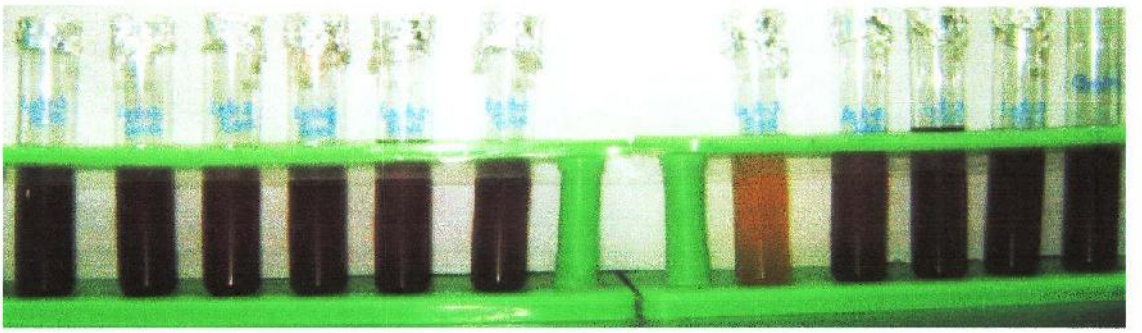


Figure 4.6.13: Sugar (D-Sorbitol) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 07 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

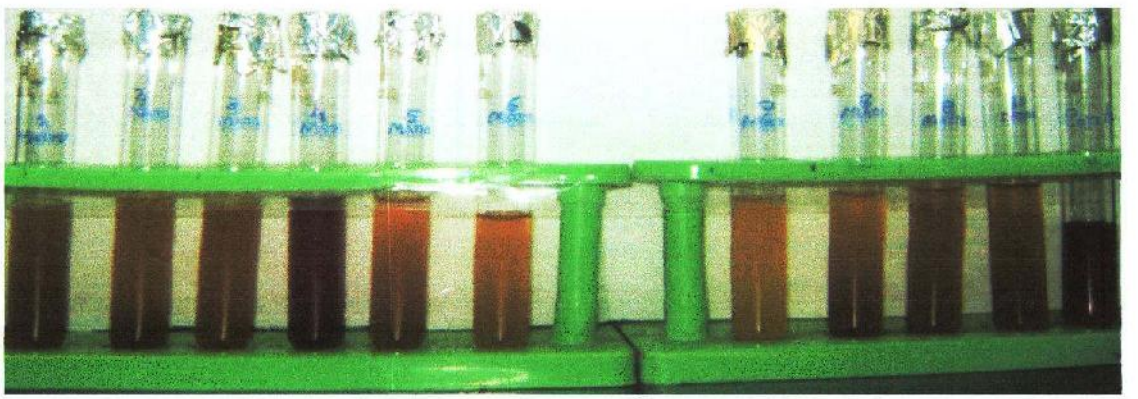


Figure 4.6.14: Sugar (D-Mannose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 03 and 05 to 10 except 04 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

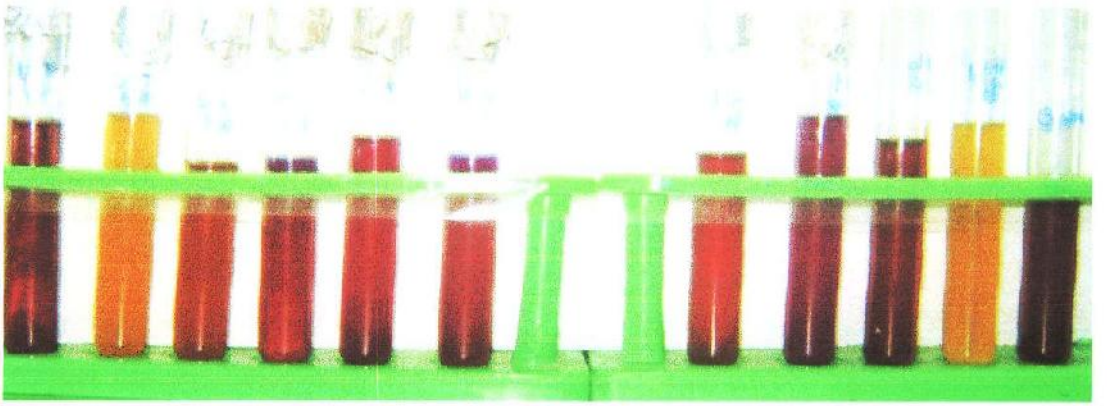


Figure 4.6.15: Sugar (Raffinose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 02 and 10 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

Figure 4.6.16: Sugar (Galactose) fermentation patterns of isolate no. 01 to10 .Yellow colour indicate fermentation done by isolate no. 01 to 08 and 10 except 09 (From left to right). The 11th test tube as negative control having only bacterial culture without any sugar

Table 4.4: Sugar fermentation patterns of isolated LAB from yogurt sample

Isolate number	Lactose	Mannitol	Sucrose	Fructose	Salicin	Ribose	Cellubiose	Glucose	Maltose	Xylose	Rhamnose	L-Arabinose	D-Sorbitol	D-Mannose	Raffinose	Galactose
Isolate no.01	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.02	-	-	+	+	-	+	+	+	+	+	-	+	-	+	+	+
Isolate no.03	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.04	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+
Isolate no.05	+	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.06	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.07	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
Isolate no.08	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.09	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	-
Isolate no.10	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+

(+) means fermentated;(-) means non fermentated.

Adapted from Scardovi (1986); Sgorbati *et al.*, (1995); Ghanbari *et al.*, (2009) and Karna *et al.*, (2007).

4.3. Determination of Probiotic Properties of Identified Probiotic Bacteria

4.3.1 Phenol tolerance test

At 0 hour, 1ml of lactic acid bacterial culture were inoculated into MRS culture medium containing 0.1%, 0.2%, 0.3%, 0.4% phenol. After 12 and 24 hours the ten isolates of the species *Lactobacillus acidophilus, Streptococcus thermophilus Lactobacillus brevis, and Bifidocterium spp* showed good tolerance against 0.1%, 0.2% and 0.3% phenol and moderate tolerance against 0.4% phenol. The absorbances were taken at 620 nm wavelength to measure bacterial isolates cell concentration by spectrophotometer.

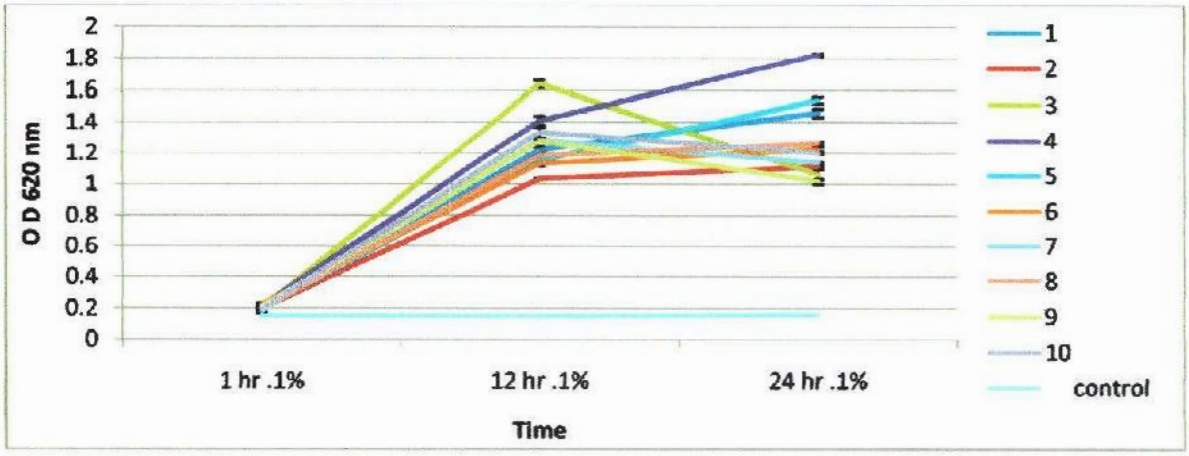


Figure 4.7.1: Phenol tolerance of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) at 0.1% concentrations after 12 hours and 24 hours of incubation at 37^0C. The rising lines indicate more tolerance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

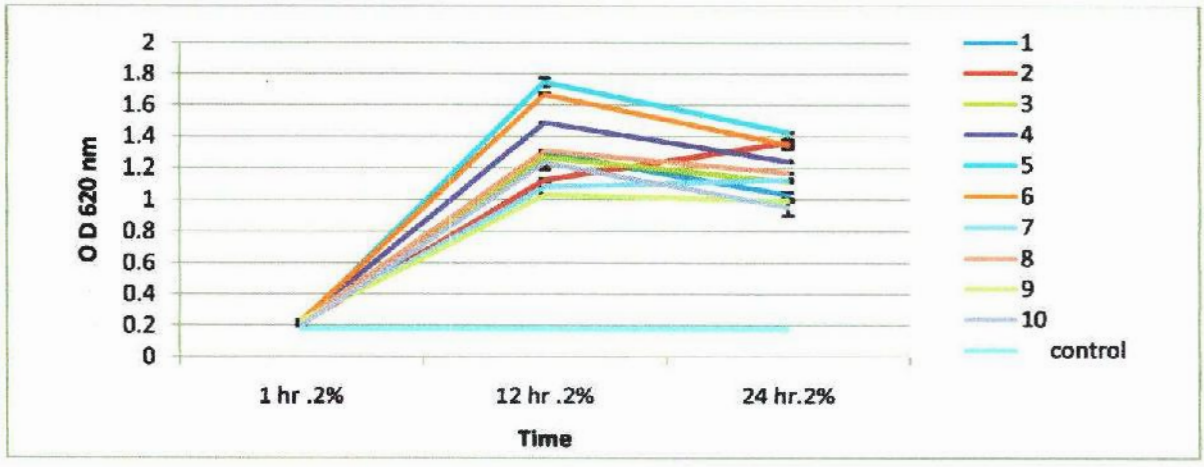


Figure 4.7.2: Phenol tolerance of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) at 0.2% concentrations after 12 hours and 24 hours of incubation at 37^0C. The rising line indicates more tolerance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

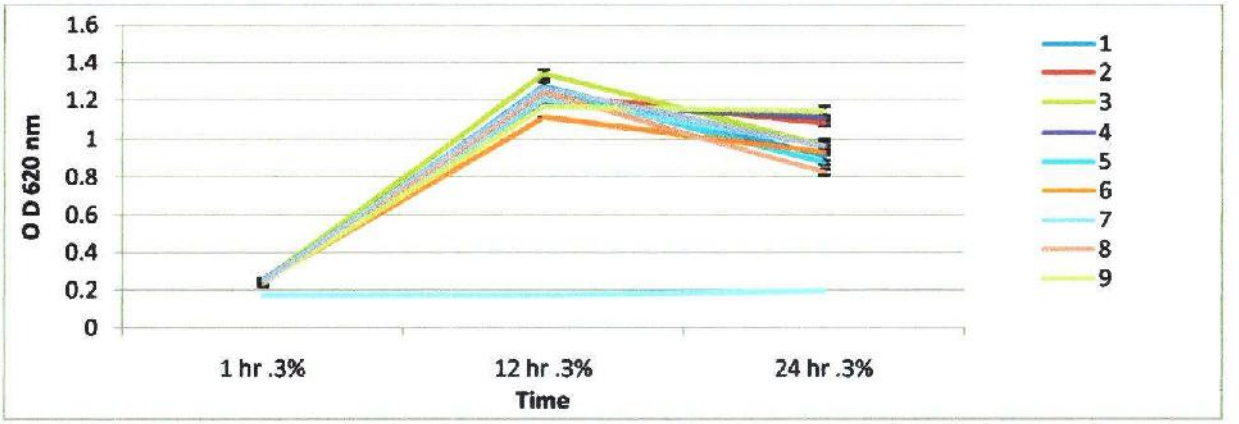


Figure 4.7.3: Phenol tolerance of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) at 0.3% concentrations after 12 hours and 24 hours of incubation at 37^0C. The rising line indicates more tolerance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

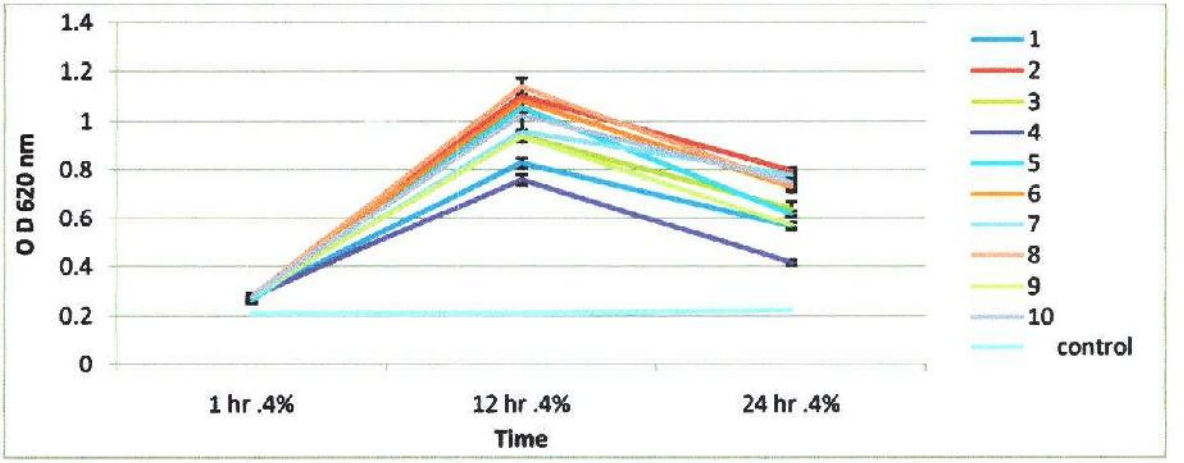


Figure 4.7.4: Phenol tolerance of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) at 0.4% concentrations after 12 hours and 24 hours of incubation at 37^0C. The rising line indicates more tolerance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) was presented.

4.3.2. Gastric Juice Resistance

At 0 hour, 1ml of bacterial culture were inoculated into MRS culture medium containing gastric juice at pH 2.2 and 6.6, into a single well and the same case was followed for all the isolates The identified ten isolates of probiotic bacteria had the ability to survive in artificial gastric acid environment at low pH (pH 2.2) but their survival ability decreased after 24 hours of incubation at 37^0C. The absorbance of identified ten isolates of probiotic bacterial culture in gastric juice environment at 620 nm indicated the nature of survival and multiplication abilities

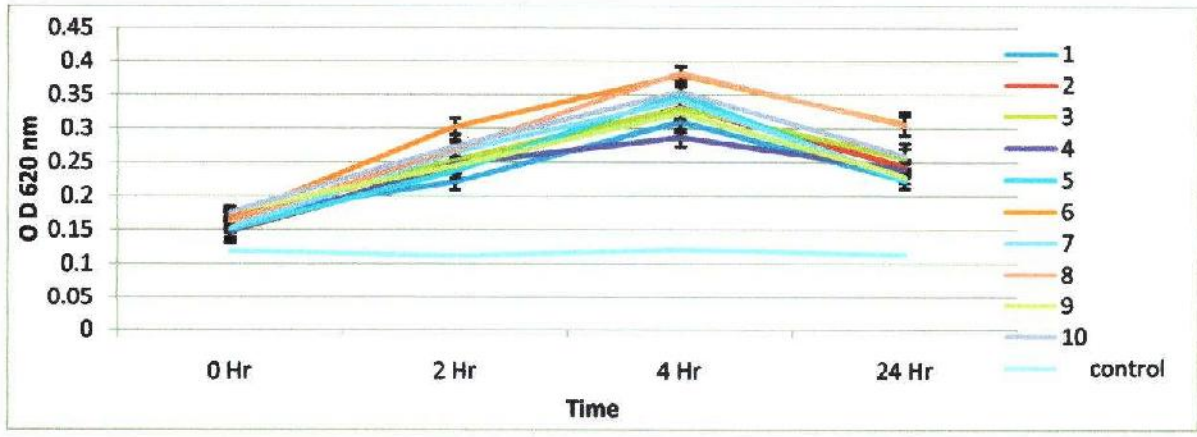


Figure 4.8.1: Survival and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial gastric juice at pH 2.2. The upward movement of the line diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) is presented.

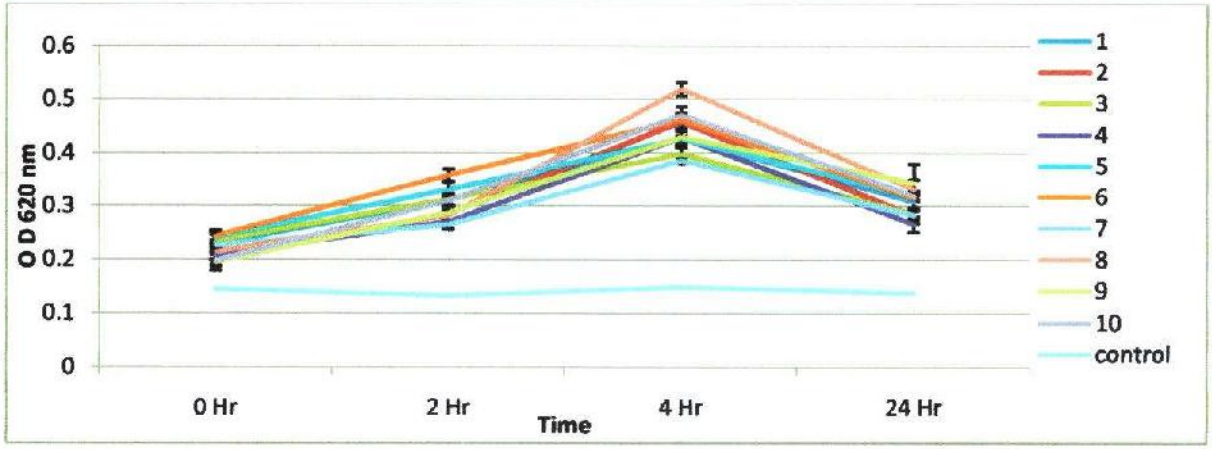


Figure 4.8.2: Survival and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial gastric juice at pH 6.6. The upward movement of the line diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3).

4.3.3. Bile Salt Tolerance:

At 0 hour, 1ml of probiotic bacterial culture were inoculated with MRS culture medium containing 0.05%, 0.1%, 0.3% and 0.6% inhibitory substance bile, were poured into a single well and the same case was followed for all the isolates. Concerning bile salt tolerance, ten isolates of the probiotic bacteria were able to survive in 0.05%, 0.1%, 0.3% and 0.6% inhibitory substance, bile salt. The isolates were also able to multiply in the above mentioned concentrations of bile acid after 4, 12 and 24 hours of incubation at 37^0C. In the figure 4.34, 4.35, 4.36 and 4.37 the optical densities values 620 nm against time and concentration were plotted in line diagram representing tolerance to artificial bile salt.

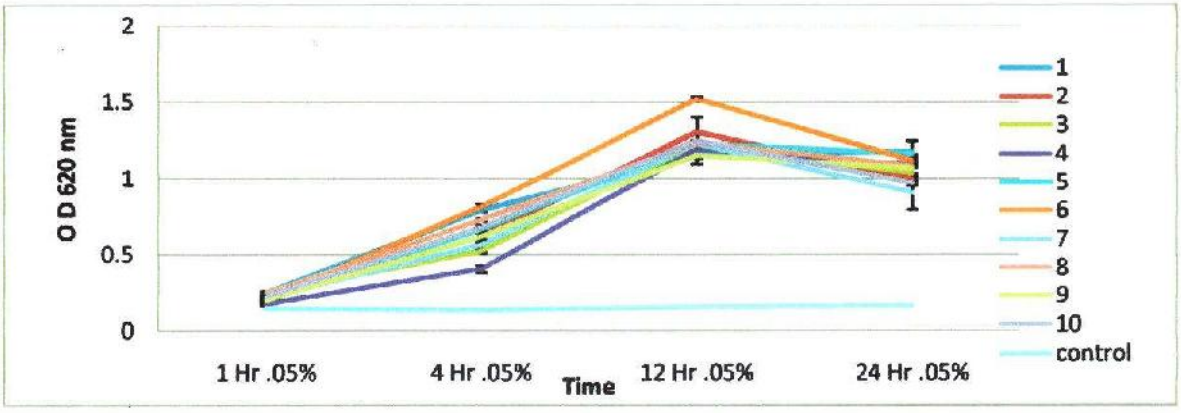


Figure 4.9.1: Tolerance and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial bile salt at .05% concentration. The upward movement of the diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) is presented.

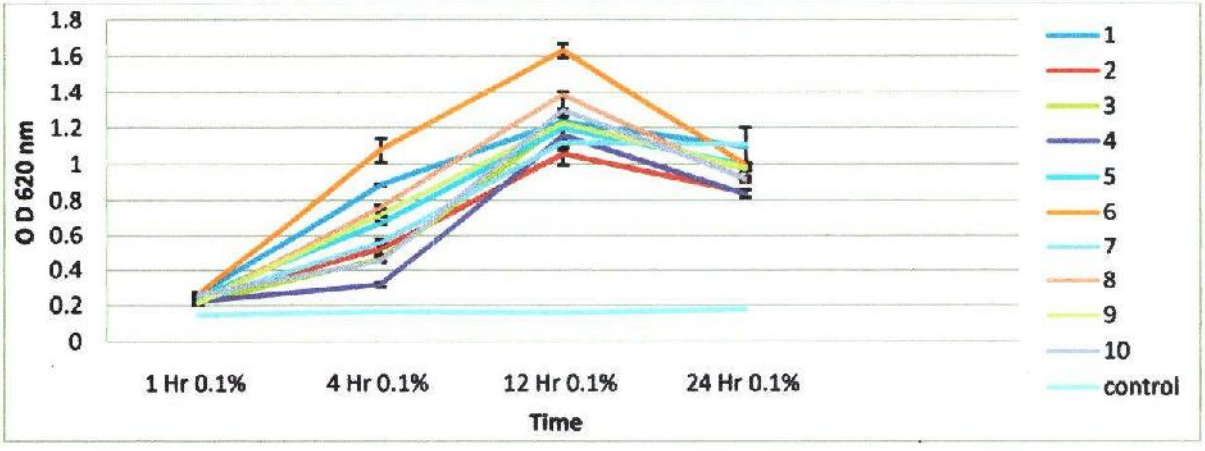


Figure 4.9.2: Tolerance and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial bile salt at 0.1% concentration. The upward movement of the diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) is presented.

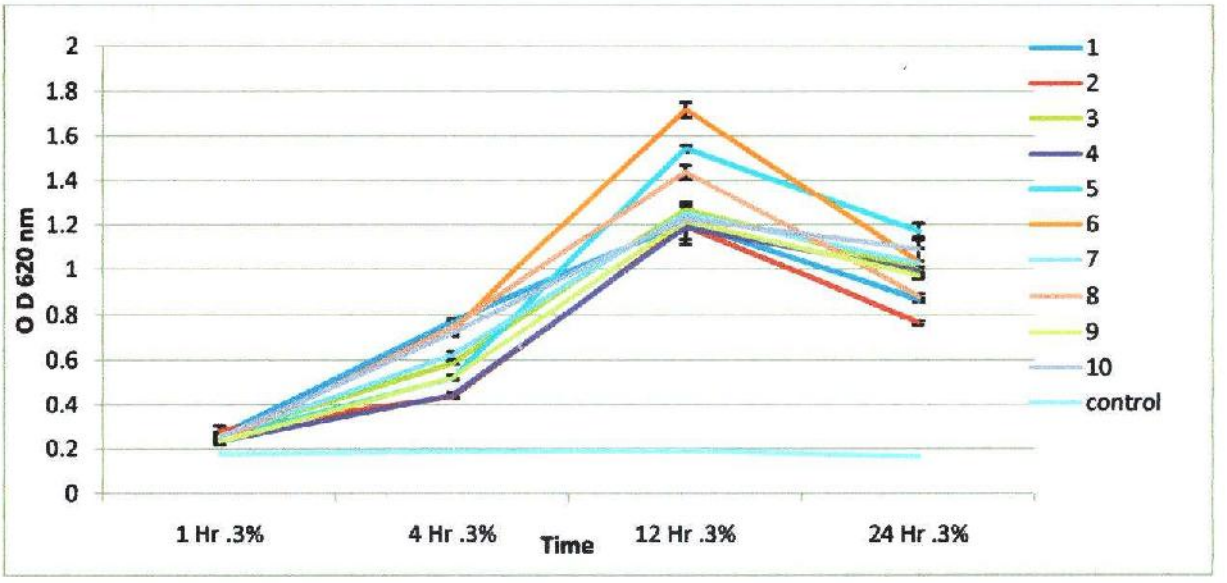


Figure 4.9.3: Tolerance and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial bile salt at 0.3% concentration. The upward movement of the diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) is presented.

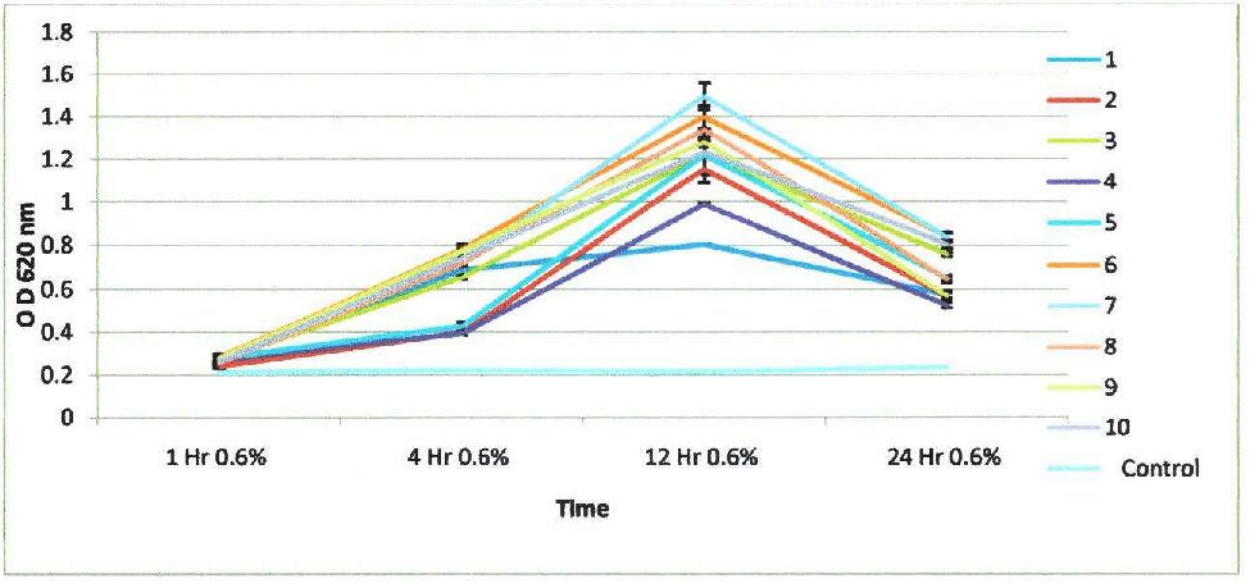


Figure 4.9.4: Tolerance and multiplication abilities of identified probiotic bacteria (isolate 1-10 from sample no. 21, 22, 23 and 24) in artificial bile salt at 0.6% concentration. The upward movement of the diagram indicates the resistance ability of the ten isolates. Data were expressed as mean and one standard deviation (n=3) is presented.

4.4. NaCl tolerance test from yogurt sample

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. The present results showed that 10 isolated probiotic bacteria (from sample no. 21, 22, 23 and 24) have excellent tolerance activity against 1-6% NaCl and in 7% to 9% NaCl they showed moderate level of growth except isolate no. 01 sample no.21 and isolate no.03 sample no.22 by observing turbidity after 24 hours of incubation at 37^0C (Table 4.5). Moreover, in 10% of NaCl, no growth was observed. NaCl tolerances of ten isolates at 1-10% are shown in Table 4.5

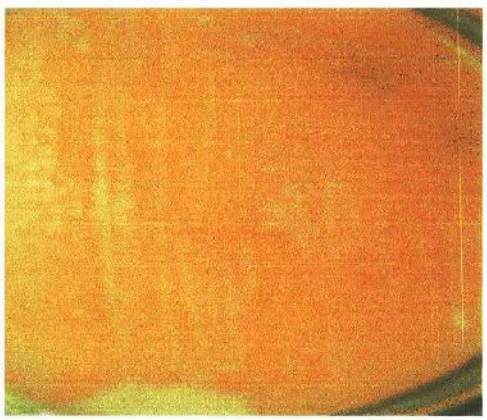
Table 4.5. NaCl tolerance test of isolated probiotic bacteria from yogurt sample

NaCl concentration (%)	Isolate no. 1 (Sample no. 21)	Isolate no. 2 (Sample no.21)	Isolate no.3 (Sample no. 22)	Isolate no.4 (Sample no. 23)	Isolate no.5 (Sample no. 22)	Isolate no. 6 (Sample no. 24)	Isolate no.7 (Sample no. 24)	Isolate no. 8 (Sample no. 23)	Isolate no.9 (Sample no. 23)	Isolate no. 10 (Sample no. 24)
1	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++
7	+	++	+	++	++	++	++	++	++	++
8	-	+	+	+	++	+	+	+	+	+
9	-	+	-	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	-

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

4.5 Bile salt hydrolase activity test: Plate assay

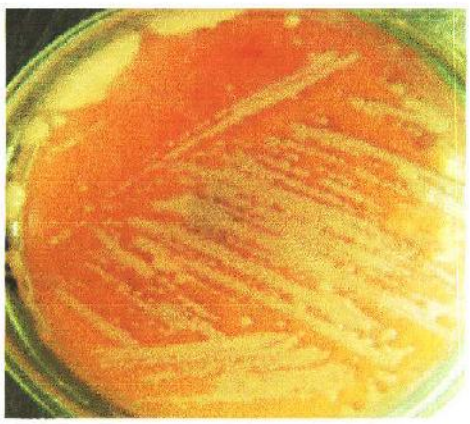
When bile salt hydrolase-producing isolated and identified lactic acid bacteria were streaked out on MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA), the taurine-conjugated bile acid was deconjugated, producing deoxycholic acid. This deconjugation activity of isolates colonies were turn into opaque granular white colonies or precipitate halos around colonies indicating the bile salt hydrolase- active lactic acid bacteria. From the pictures, it is observed that copius amount of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium or producing precipitate halos (Figure 4.10.1 to 4.10.10).



L. acidophilus, Sample no. 21 (isolate no. 01)
MRS agar medium with 0.5% TDCA

L. acidophilus, Sample no. 21 (isolate no. 01)
Control

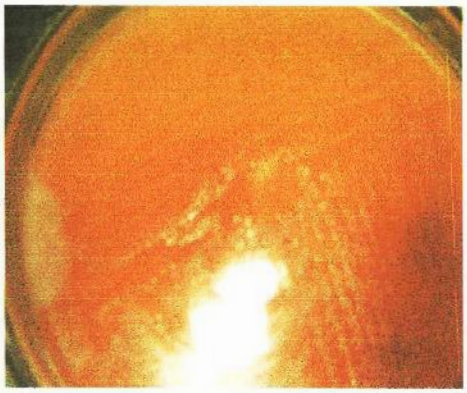
Figure: 4.10.1: Bile salt hydrolise activity test



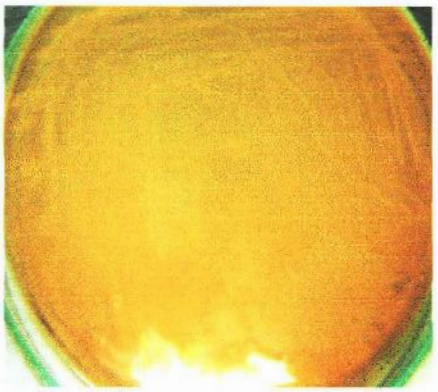
S. thermophilus Sample no. 21 (isolate no. 02)
MRS agar medium with 0.5% TDCA

S. thermophilus Sample no. 21 (isolate no. 02)
Control

Figure: 4.10.2: Bile salt hydrolise activity test

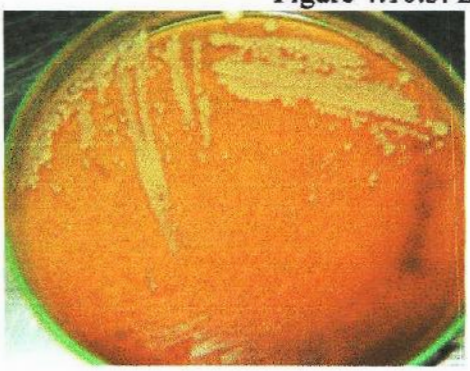


L. acidophilus, Sample no. 22 (isolate no. 03)
MRS agar medium with 0.5% TDCA

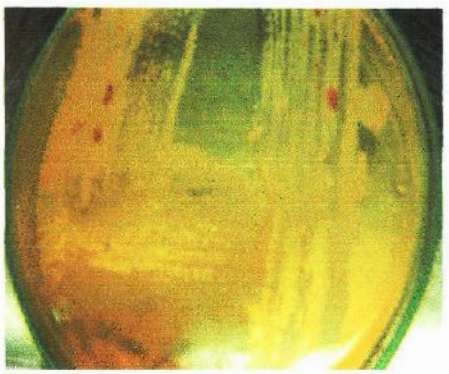


L. acidophilus, Sample no. 22 (isolate no. 03)
Control

Figure 4.10.3: Bile salt hydrolise activity test

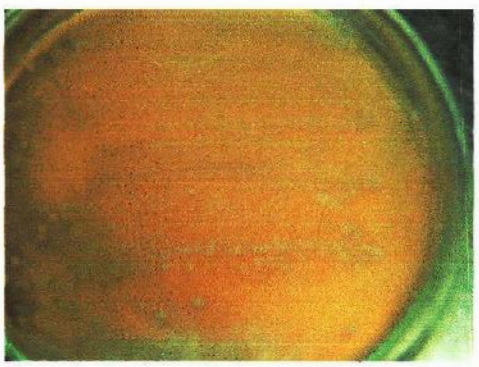


Lactobacillus brevis sample no. 23 (isolate no. 04)
MRS agar medium with 0.5% TDCA

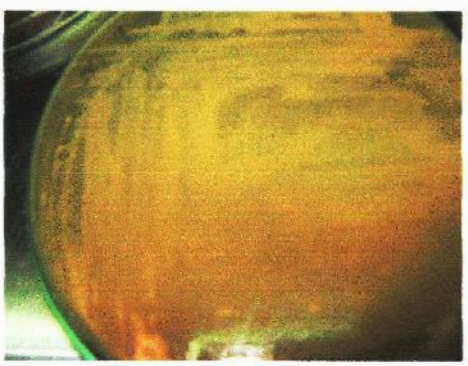


Lactobacillus brevis sample no. 23 (isolate no. 04)
Control

Figure 4.10.4: Bile salt hydrolise activity test

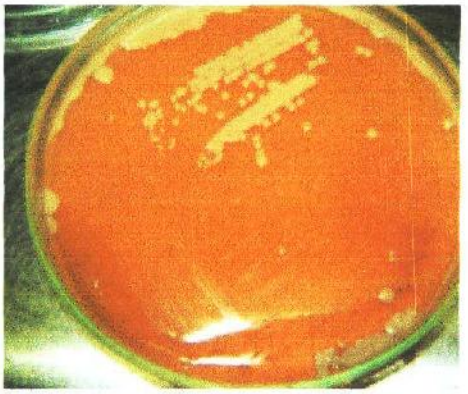


L. acidophilus, Sample no. 22 (isolate no.05)
MRS agar medium with 0.5% TDCA



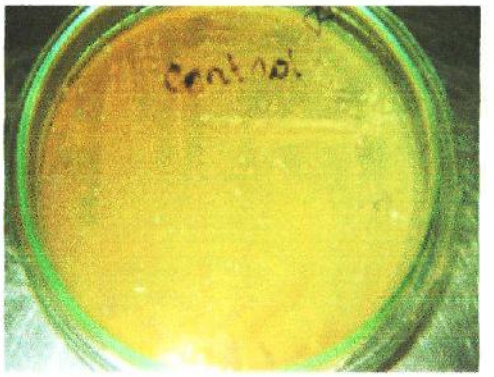
L. acidophilus, Sample no. 22 (isolate no. 05)
Control

Figure 4.10.5



S. thermophilus Sample no. 24 (isolate no. 06)

MRS agar medium with 0.5% TDCA



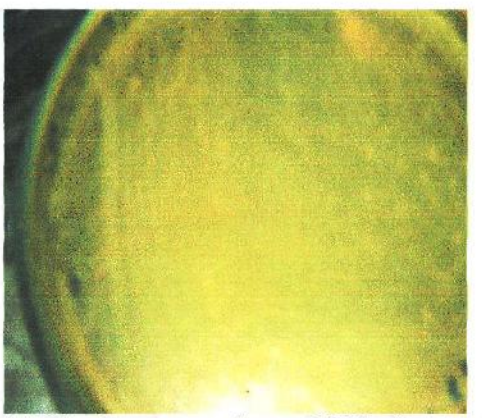
S. thermophilus Sample no. 24 (isolate no. 06)

Control

Figure 4.10.6: Bile salt hydrolise activity test

Lactobacillus brevis sample no. 24 (isolate no. 07)

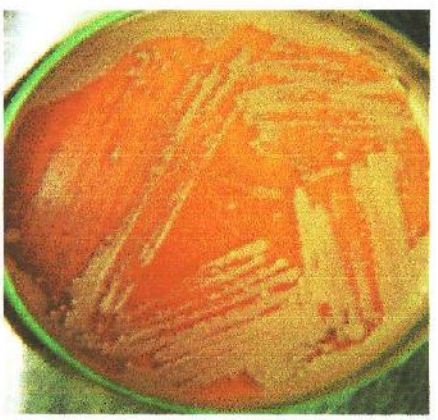
MRS agar medium with 0.5% TDCA



Lactobacillus brevis sample no. 24 (isolate no. 07)

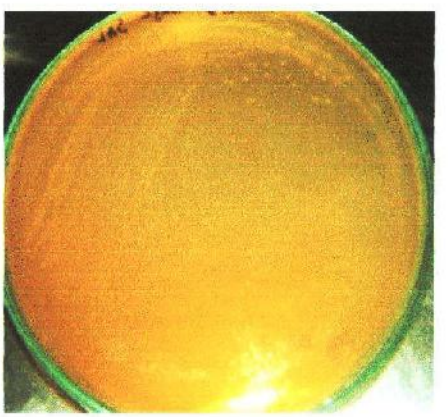
Control

Figure 4. 10.7



L. acidophilus, Sample no. 23 (isolate no.08)

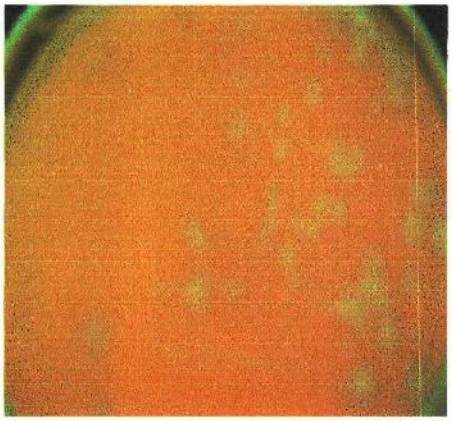
MRS agar medium with 0.5% TDCA

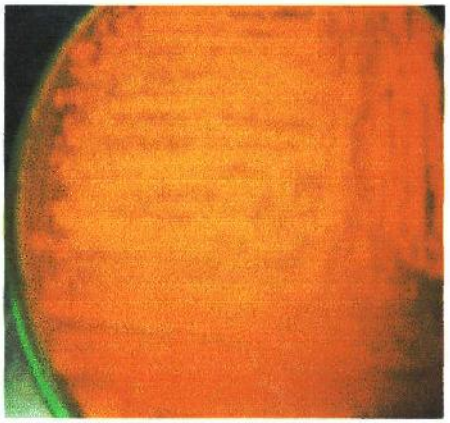


L. acidophilus, Sample no. 23 (isolate no.08)

Control

Figure 4.10.8

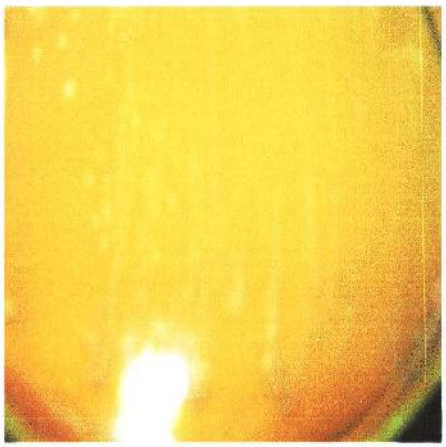


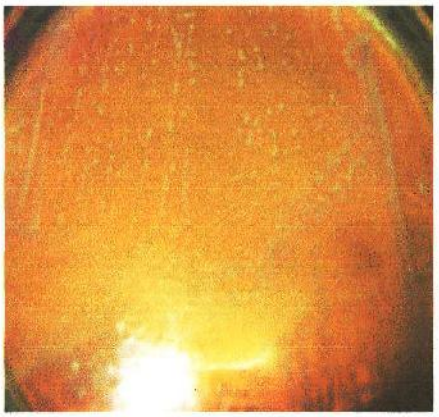


Bifidobacterium spp. Sample no. 23 MRS agar medium with 0.5% TDCA

Bifidobacterium spp. Sample no.23 (isolate no. 09) Control

Figure 4.10.9: Bile salt hydrolise activity test





Lactobacillus brevis sample no. 24 (isolate no. 10) MRS agar medium with 0.5% TDCA

Lactobacillus brevis sample no. 24 (isolate no. 10) Control

Figure 4.10.10: Bile salt hydrolise activity test

Figure 4.10.1 to 4.10.10 is Bile salt hydrolise activity test

Manifestation of bile salt hydrolase activity through plate assay using bile salt plate on solid MRS medium containing 0.5% taurodeoxycholic acid (TDCA) by isolated and identified *L. acidophilus S. thermophilus*, *L. brevis*, and *Bifidobacterium* spp. TDCA containing MRS agar media forming opaque granular white colonies or precipitate halos around colonies indicating the bile salt hydrolase-active lactic acid bacteria while only MRS medium (control, right sight) does not develop white colonies or precipitate halos around colonies.

Table 4.6: Bile salt hydrolase activity in isolated probiotic bacteria from yogurt sample

Isolated probiotic bacteria	Plate assay
Lactobacillus acidophilus, Sample no. 21, 22 and 23 (isolate no. 01 , 03, 05, 08)	+
Streptococcus thermophilus, Sample no. 21 and 24 (isolate no. 02, 06)	+
Lactobacillus brevis, Sample no. 23 and 24 (isolate no. 04, 07, 10)	+
Bifidocterium spp, Sample no. 23 (isolate no. 09)	+

A plus (+) sign indicates white granular opaque colonies.

Four isolates of *Lactobacillus acidophilus*, two isolates of *Streptococcus thermophilus*, three isolates of *Lactobacillus brevis* and one isolate of *Bifidobacterium* spp. showed excellent bile salt hydrolase activity in plate assay (Table 4.6).

4.6 Trail on mice

4.6.1 Body weight gain

Individual body weight of mice were measured at day 0, day 7, day 14, day 21 and day 28. Due to sudden fluctuation of environmental condition (temperature, weather etc), weights of the mice showed variation in various time. However, after the trail all the treatment groups showed significant weight gain compared to control group.

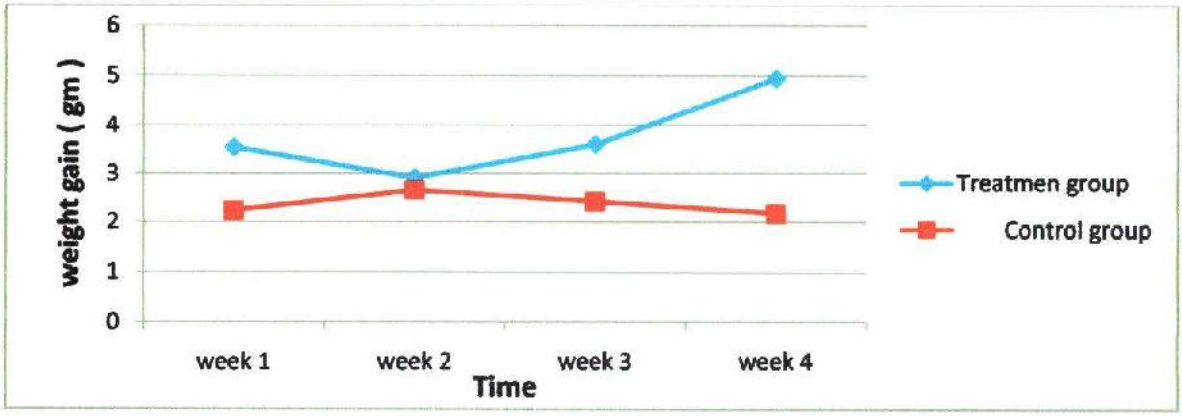


Figure 4.11.1: Weekly body weight gain of the treatment and control group.

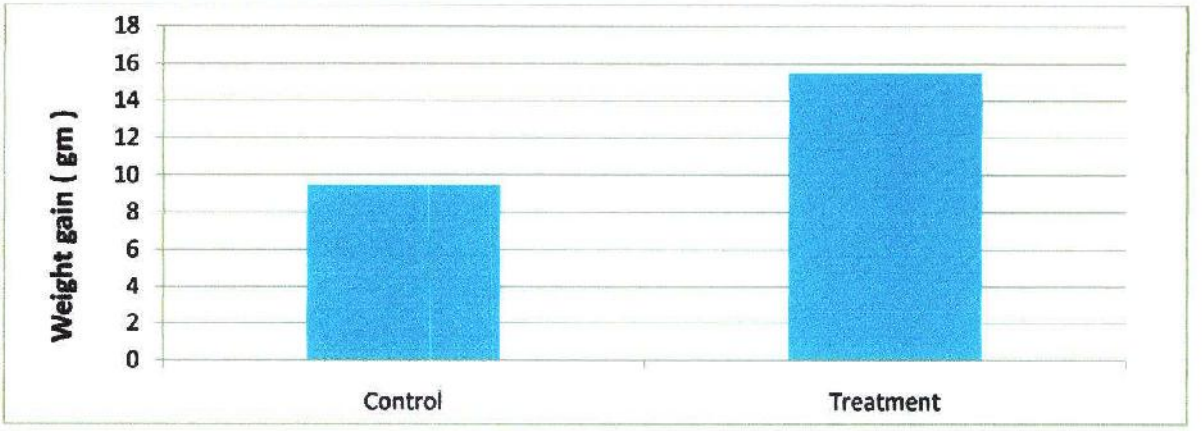


Figure 4.11.2 Total body weight gain of treatment and control group of mice

Figure 4.11.2 represents the average body weight gain among the control and treatment group. The average weight gain of the control group is nearly 9 grams over twenty eight days treatment period and the treatment group shows higher body weight gain then the control group. Treatment group show average weight gain 15.5 grams here the value of t test is 18.124 at 23 degree of freedom and the value of significance is 0.016. Therefore, the overall results are significant at 0.05 level of significance.

4.6.2 Cholesterol Reduction

Cholesterol level was measured on day 0 and day 28. The treatment group showed significant lowering of cholesterol level after 28-day trail. However, the control group did not show any significant test result of cholesterol level lowering and did not show any significant variation among collected data.

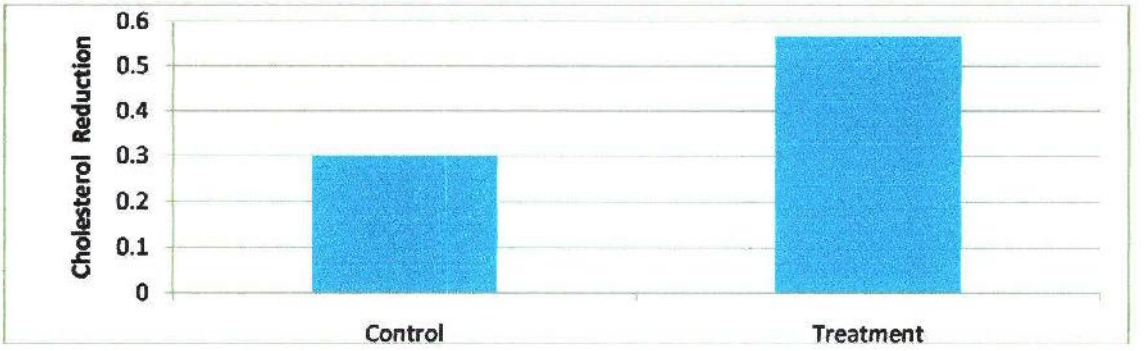


Figure 4.12: Blood cholesterol level of yogurt treated and control group of mice.

Figure 4.12: represents the average blood cholesterol level among the control and treatment groups. The average cholesterol reduction of the control group is 0.3milimole per liter (mM/L) over twenty eight days treatment period and the treatment group shows higher cholesterol reduction then the control group. Treatment group show average cholesterol reductions 0.566(mM/L).Here the value of t test is 11.984 at 23 degree of freedom and the value of significance is 0.009. Therefore, the overall results are significant at 0.01 and 0.05 level of significance.

4.7. Antibiotic Resistance Test

Feces were collected from each group on day 0, day 7 and day 14 and cultured on MRS agar media for LAB colony count.

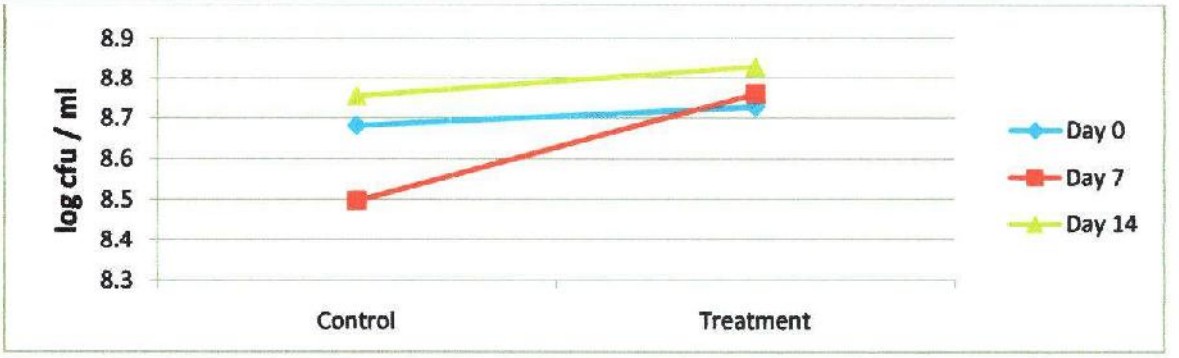


Figure 4.13: Number of lactic acid bacteria in mice over the antibiotic resistance test.

Figure 4.13 represents the antibiotic resistance activity of lactic acid bacteria. Here control group and the numbers of bacteria in this group decrease slightly throughout the treatment period. Treatment group shows increased amount of bacteria over time. This indicates the antibiotic resistance activity of the treatment groups.

4.8. Inhibition of growth of pathogenic microorganism *in vivo*

All the treatment groups were observed the Inhibition of growth of *E. coli* while control group didn't show much Inhibition of growth of *E. coli*.

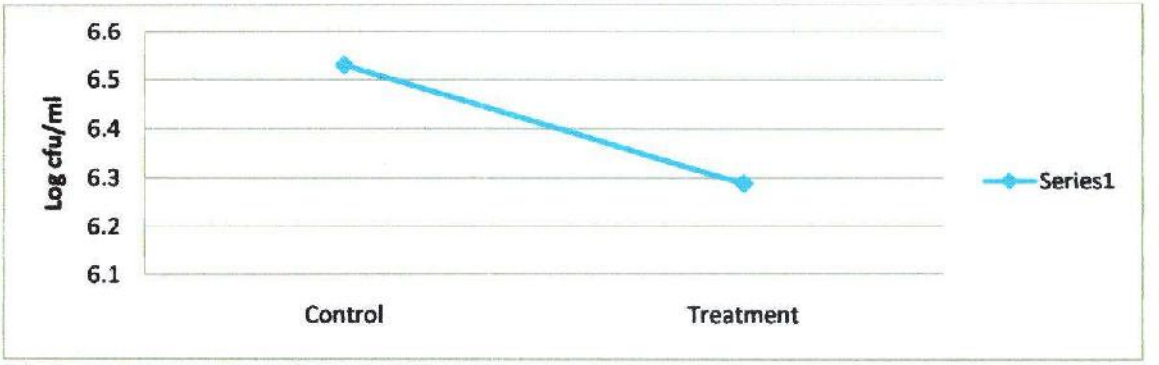


Figure 4.14: Inhibition of growth of pathogenic microorganism of treatment and control group.

Figure 4.14 represents the inhibition of growth of pathogenic microorganism (*E.coli*) among the control and treatment groups. The number of pathogenic bacteria is 6.28 million (cfu/ml) in control group and the treatment group show higher inhibition of growth of pathogenic microorganism then the control group.

4.7. Identification of lactic acid bacteria from selected poultry feces

Two varieties of poultry feces sample were collected from different poultry farm among Sylhet and Rajshahi district. Probiotic Lactic acid forming bacteria (LAB) were isolated and identified as *Lactobacillus brevis, Lactobacillus acidophilus*, and *Bifidobacterium spp* primarily by observing their colony morphology, physiological characteristic and finally by observing the biochemical characteristics.

4.7.1. Colony Morphology

Colony morphologies of the isolates are given in Table 4.8 from which it was observed that the colony morphology of Isolate no. 3, 5 and 9 small to medium sized, irregular, smooth and deep white. Small, triangular, watery circle with deep white middle were observed in isolates 2 and 8. Colony morphology of the above mentioned bacterial species were found in different isolates.

Table 4.7: Colony morphology of isolated bacteria from poultry feces

Identified isolates	Isolate No.	Colony morphology
Lactobacillus brevis	Isolate no.02 (sample no.08) and isolate no.04 (sample no.07)	Small, white rod shaped and non-transparent.
Lactobacillus acidophilus	Isolate no. 01 (sample no. 07)	Very small, round shaped and non transparent
Bifidocterium spp.	isolate no. 03 (sample no. 08)	Small, triangular, watery circle with deep white middle.

4.7.2. Gram staining test

All the isolated bacteria were found gram positive due to retain violet blue color which are evaluated given as positive. *Lactobacillus* spp. were found rod shaped, regular, occured singly/chain. Tiny rod, branched, v and y arrangement in chains were observed in *Bifidocterium* spp. Cell shape of the isolated bacterial species after Gram staining were the characteristics to the corresponding typical species.

Table 4.8: Cell morphology of isolated bacteria of poultry feces after Gram staining.

Identified bacteria	Cell shape
Lactobacillus brevis	Rod shape, regular, occur single or chain
Lactobacillus acidophilus	Rod shaped, regular in long chains
Bifidocterium spp.	Tiny rod, branched, v and y shape in chains

4.7.3. Endospore test

All the isolated bacteria from poultry sample were found non-endospore forming due to stain red characteristic to typical *Lactobacillus* spp. and *Bifidocterium* spp.

4.7.4. Catalase test

All the isolated and presumptively identified bacteria from poultry sample were found catalase negative which is given as negative sign due to no bubble production during addition of 3% H_2O_2. This test results are the characteristic to typical bacteria like *Lactobacillus* spp. *and Bifidocterium* spp. The isolates have the ability to tolerate MRS broth containing inhibitory substances such as 0.1-0.4% bacteriostatic phenol and 1-10% NaCl. Physiological and biochemical characteristics of *Lactobacillus* spp. and *Bifidobacterium* spp. from poultry sample are shown in Table 4.9

Table 4.9: Physiological and biochemical characteristics of isolated Lactic acid Bacteria

Physiological and biochemical characteristics	*Bifidobacterium* spp.	*Lactobacillus* spp.
Gram stain	+	+
Endospore test	–	–
Catalase test	–	–
1-8 % NaCl tolerence	+	+

+: Positive, -: Negative.

4.8. Sugar/carbohydrate fermentation profiles test

Isolated lactic acid bacteria were presumptively identified based on their carbohydrate/sugar metabolism or fermentation pattern which was determined by color change of the broth medium due to acid production. It was observed that every probiotic bacterium has specific carbohydrate/ 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 probiotic bacteria (due to lactic acid production) of that particular carbohydrate/sugar. Sixteen carbohydrates/sugars were used in the present study. These were: lactose, mannitol, sucrose, fructose, salicin, ribose, cellubiose, glucose, maltose, xylose, rhamnose, L-arabinose, D-sorbitol, D-mannose, raffinose and galactose.

Table 4.10: Sugar fermentation patterns of isolated probiotic bacteria from poultry feces.

Isolate number	Lactose	Mannitol	Sucrose	Fructose	Salicin	Ribose	Cellubiose	Glucose	Maltose	Xylose	Rhamnose	L-Arabinose	D-Sorbitol	D-Mannose	Raffinose	Galactose
Isolate no.02, 04	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+
	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+
Isolate no.01	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+
Isolate no.03	-	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-
	-	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-

4.9. NaCl tolerance test of poultry feces

The NaCl tolerance test results of four isolated probiotic bacteria collected from poultry sample (isolate 2, 3, 5, and 7) have shown that all the isolates have excellent tolerance activity against 1-6% NaCl, in 7% they showed moderate growth rate and 9% NaCl they showed low level of growth by observing turbidity after 24 hours of incubation at 37^0C (Table 4.11). Moreover, in 10% of NaCl, no growth was observed. NaCl tolerances of ten isolates at 1-10% are shown in Table 4.11.

Table 4.11 NaCl tolerance test of isolated probiotic bacteria collected from poultry feces

NaCl concentration (%)	Isolate no. 1	Isolate no. 2	Isolate no.3	Isolate no.4
1	++	++	++	++
2	++	++	++	++
3	++	++	++	++
4	++	++	++	++
5	++	++	++	++
6	++	++	++	++
7	+	+	++	+
8	-	+	+	+
9	-	-	-	+
10	-	-	-	-

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

4.10. Determination of Probiotic Properties of Identified Probiotic Bacteria

4.10.1. Gastric Juice Resistance

The identified four isolates of probiotic bacteria from poultry sample had the ability to survive in artificial gastric acid environment at low pH (pH 2.2) but their survival ability decreased after 24 hours of incubation at 37^0C. The absorbance of identified four isolates of probiotic bacterial culture in gastric juice environment at 620 nm indicated the nature of survival and multiplication abilities (Figure 12.1, 12.2). At 0 hour, 100 µl of bacterial culture containing approximately 10^7 isolated bacteria along with MRS culture medium containing gastric juice at pH 2.2 and 6.6, were poured into a single well and the same method was followed for all the isolates.

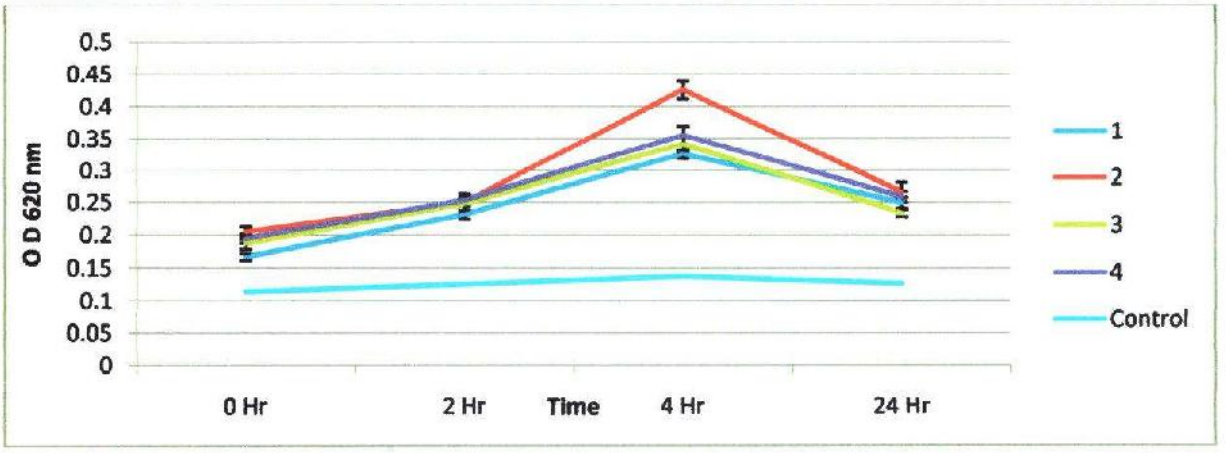


Figure 4.15: Survival and multiplication abilities of identified probiotic bacteria (isolate 1, 2, 3, 4) from poultry sample in artificial gastric juice at pH 2.2. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

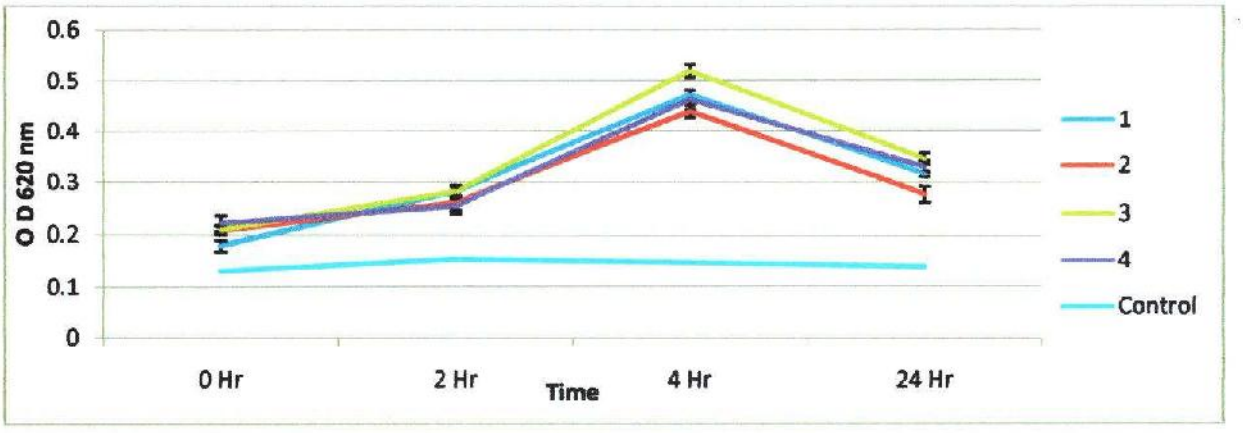


Figure 4.16: Survival and multiplication abilities of identified probiotic bacteria (isolate 1, 2, 3 and 4) from poultry sample in artificial gastric juice at pH 6.6. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

4.10.2. Bile Salt Tolerance

Regarding bile salt tolerance, four isolates of the probiotic bacteria collected from poultry sample were able to survive in 0.05%, 0.1%, 0.3% and 0.6% inhibitory substance, bile salt. The isolates were also able to multiply in the above mentioned concentrations of bile acid after 1, 4, 12 and 24 hours of incubation at 37^0C (Figure 12.3 to 12.6). In the figure the optical densities values 620 nm against time and concentration were plotted in a line diagram representing tolerance to artificial bile salt. At 0 hour, 100 µl of bacterial culture containing approximately 10^7 isolated bacteria along with MRS culture medium containing 0.05%, 0.1%, 0.3% and 0.6% inhibitory substance bile, were poured into a single well and the same method was followed for all the isolates collected from poultry sample.

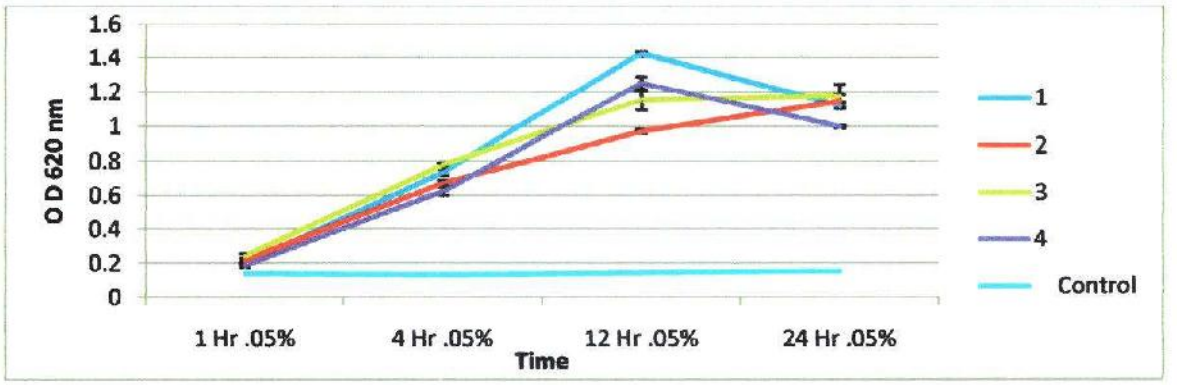


Figure 4.17: Survival and multiplication abilities of identified probiotic bacteria (isolate 1, 2, 3, and 4) collected from poultry sample in 0.05% concentration of bile salt. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

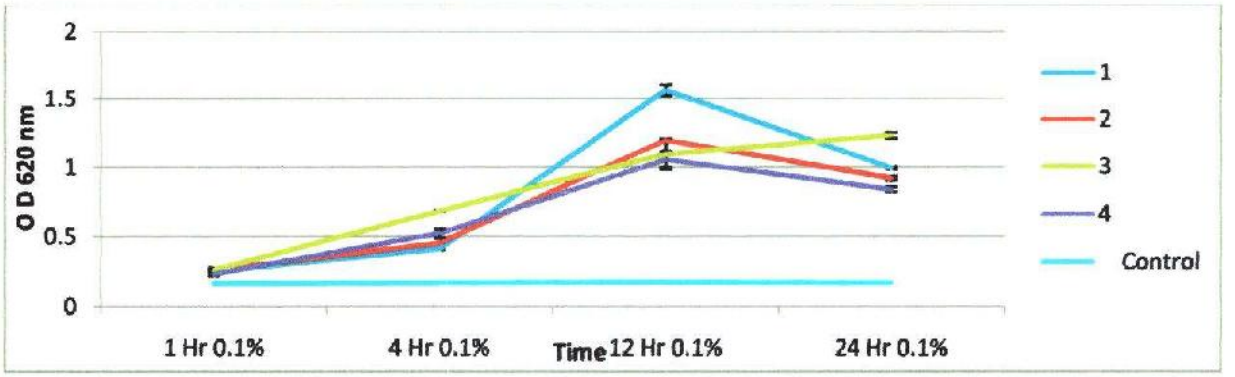


Figure 4.18: Survival and multiplication abilities of identified probiotic bacteria collected from poultry sample (isolate 1, 2, 3, and 4) in 0.1% concentration of bile salt. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

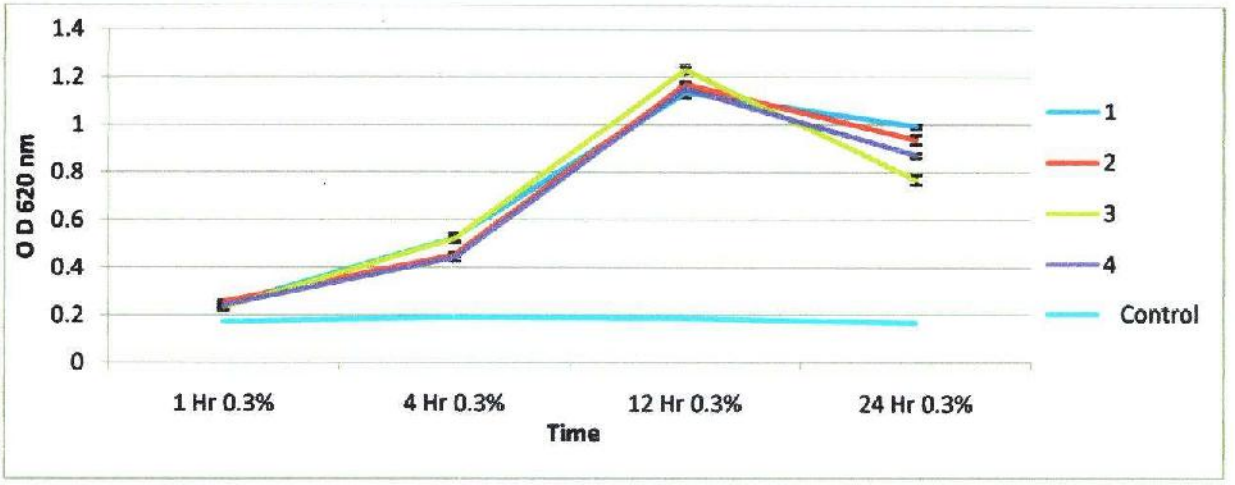


Figure 4.19: Survival and multiplication abilities of identified probiotic bacteria collected from poultry sample (isolate 1, 2, 3, and 4) in 0.3% concentration of bile salt. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

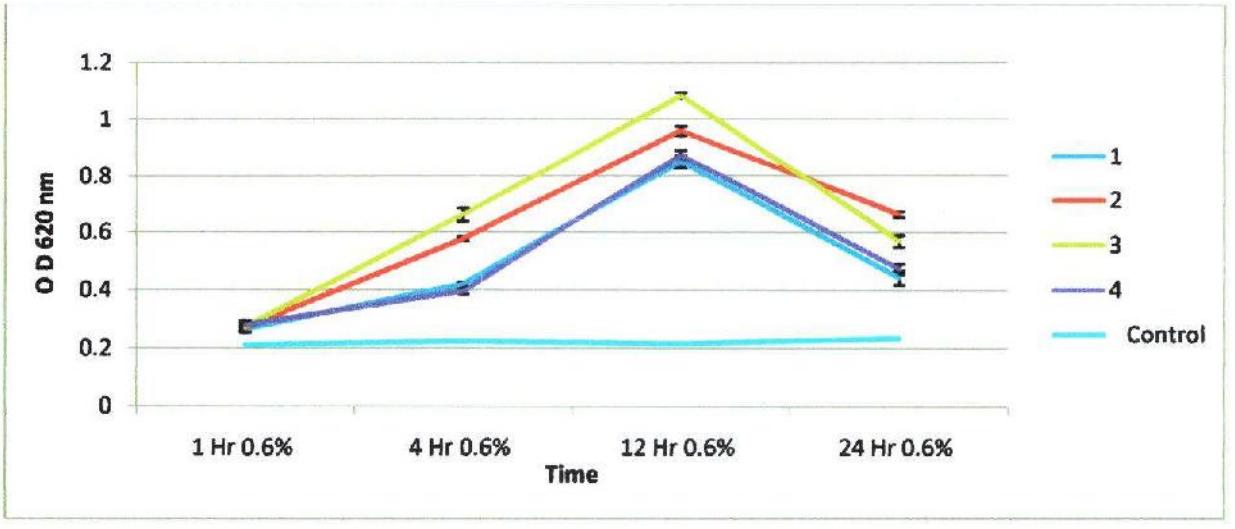


Figure 4.20: Survival and multiplication abilities of identified probiotic bacteria collected from poultry sample (isolate 2, 3, 5, and 7) in 0.6% concentration of bile salt. The upward movement of the line diagram indicates the resistance ability of the five isolates. Data are expressed as mean and one standard deviation (n=3).

4.10.3. Phenol tolerance test

The four isolates of the *Lactobacillus* spp. *and Bifidocterium* spp collected from poultry sample showed good tolerance against 0.1 - 0.2% phenol and moderate tolerance against 0.3% and 0.4% phenol. The absorbances were taken at 620 nm wavelength to measure bacterial islolates cell concentration. Observation of turbidity after 24 hours showed excellent tolerance and growth at 0.1%, 0.2% and 0.3% of phenol and moderate growth at 0.4% phenol. At 0 hour, 1 ml of lactic acid bacterial culture containing approximately 10^8 lactic acid bacteria were inoculated into MRS culture medium containing 0.1%, 0.2%, 0.3%, 0.4% phenol at 0 hour.

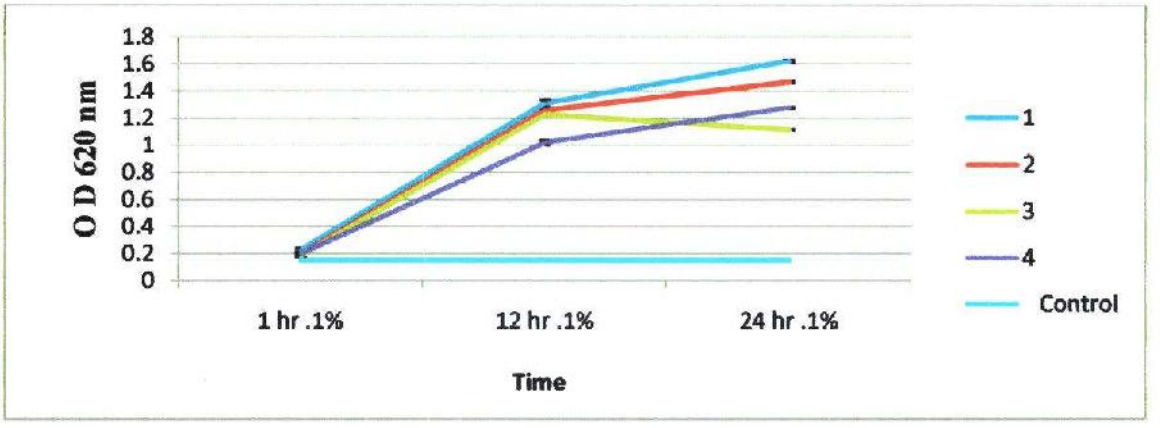


Figure 4.21: Phenol tolerance of identified probiotic bacteria collected from poultry sample (isolate 1, 2, 3 and 4) at 0.1% concentrations after 1 hour 12 hours and 24 hours of incubation at 37^0C. The upward lines indicate more tolerance ability of the four isolates. Data were expressed as mean and one standard deviation (n=3).

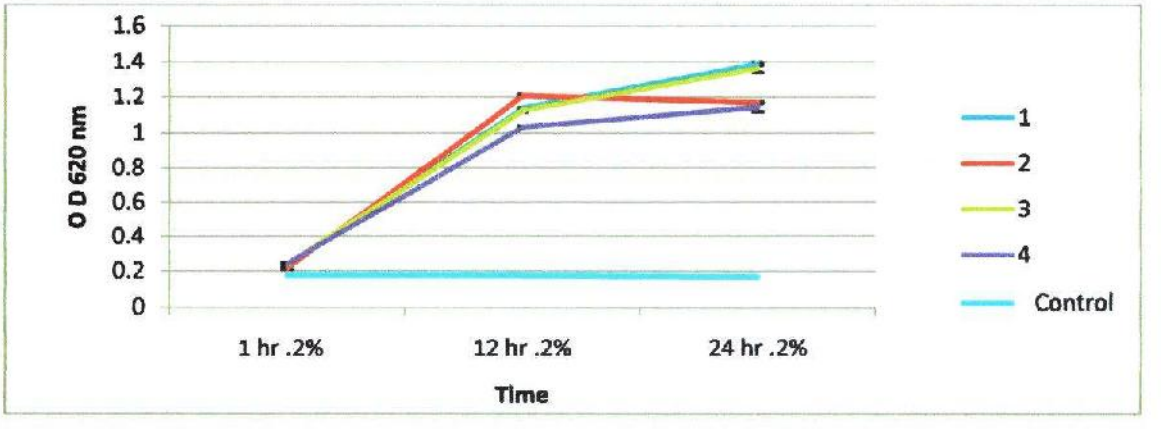


Figure 4.22: Phenol tolerance of identified probiotic bacteria (isolate 1, 2, 3, and 4) at 0.2% concentration after 1 hour 12 hours and 24 hours of incubation at 37^0C. The upward lines indicate more tolerance ability of the four isolates. Data are expressed as mean and one standard deviation (n=3).

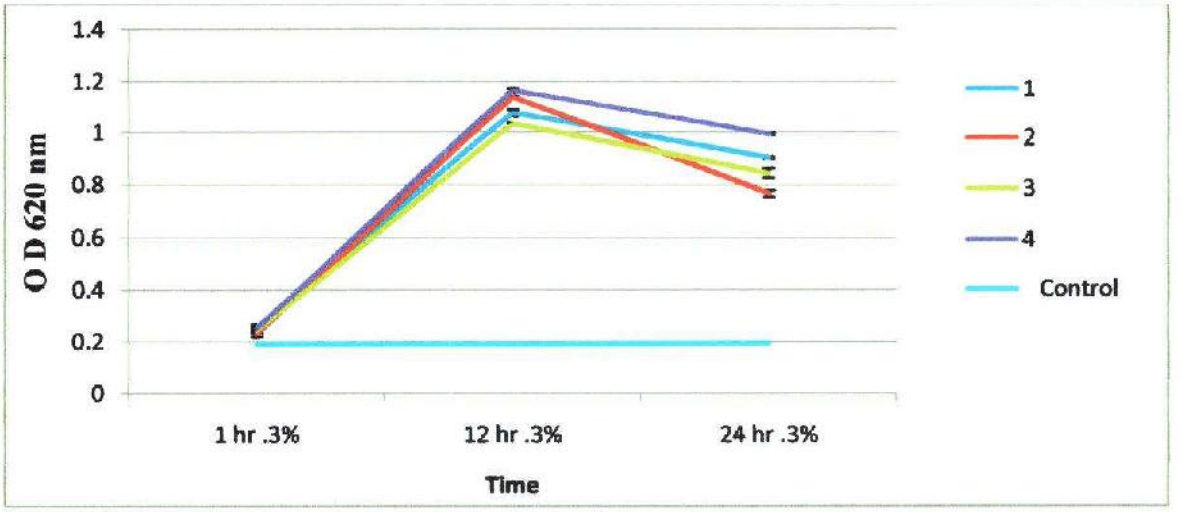


Figure 4.23: Phenol tolerance of identified probiotic bacteria (isolate 1, 2, 3, and 4) at 0.3% concentration after 1 hour 12 hours and 24 hours of incubation at 37^0C. The upward lines indicate more tolerance ability of the four isolates. Data are expressed as mean and one standard deviation (n=3).

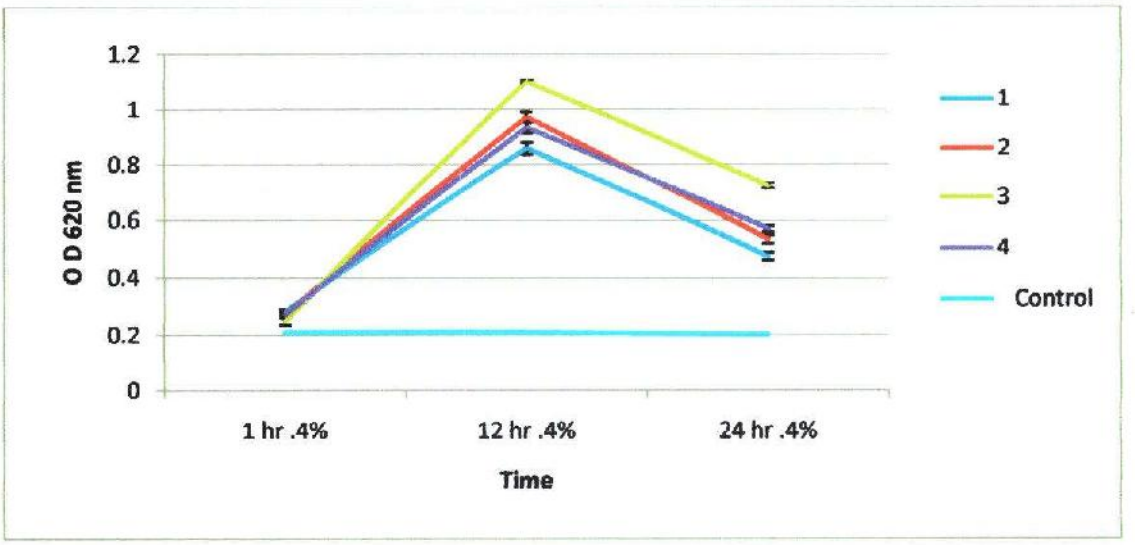


Figure 4.24: Phenol tolerance of identified probiotic bacteria (isolate 1, 2, 3, and 4) at 0.4% concentration after 1 hour 12 hours and 24 hours of incubation at 37^0C. The upward lines indicate more tolerance ability of the four isolates. Data are expressed as mean and one standard deviation (n=3).

DISCUSSION

Four yogurt samples and two poultry feces were used in the present study. Two different samples were first grown on agar plates containing MRS (pH6.2-6.8) and GYP (pH6.2-6.8) media respectively. Variation of probiotic bacteria were found from different geographical locations and also sample difference of yogurt and poultry feces. Three isolates of *Lactobacillus acidophilus*, two isolates of *Streptococcus thermophilus*, two isolates of *Lactobacillus brevis* and one isolate of *Bifidobacterium* spp. were isolated from four yoghurt samples from Sylhet district and two isolates of *Lactobacillus brevis,* one isolate of *Lactobacillus acidophilus*, one isolate of *Bifidobacterium* spp. were isolated from two different poultry feces from Sylhet and Rajshahi district of Bangladesh. Optimum growth of probiotic bacteria as well as others organism depends on optimum temperature, pH and media nutrient. In the present study *Lactobacillus acidophilus, Lactobacillus brevis* and *Bifidobacterium* spp. from yogurt samples were isolated using MRS agar medium with some modifications. *Streptococcus thermophilus* were isolated on ST agar medium containing 0.5 g/100 ml of bromocresol purple. Pure cultures were developed in MRS broth medium from the streaking plate. *Lactobacillus spp and Bifidobacterium* spp. from poultry feces were isolated using GYP agar medium. In the present study the Lactobacillus isolated from yogurt and poultry did not produce H_2O_2 which was similar to the findings of Yuksekdag *et al.,* 2004. Lactic acid forming bacteria (LAB) were isolated and identified primarily by observing their colony morphology, physiological characteristic and the results are presented in table 4.3. Theis results found almost similar to Hoque et al (2010). Finally all the isolated probiotic bacteria were presumptively confirmed by biochemical characteristics and the results are presented in Table 4.4. These results indicate that *Lactobacillus acidophilus* showed positive reactions for sucrose, fructose, ribose, glucose, maltose, galactose and arabinose, xylose, except mannitol and raffinose. The results for *Steptococcus thermophilus* indicate that all of these bacteria gave positive reactions with raffinose, fructose, sucrose, ribose, while gave negative reactions with lactose, mannitol, and rhamnose. *L. brevis* gave positive reactions with lactose, glucose, mannitol, sucrose and fructose, ribose and negative reactions with xylose, arabinose, and mannose. *Bifidobacterium* spp gave positive reaction with lactose, mannitol, sucrose and fructose, but negative reaction with rhamnose and sorbitol. These results are almost similar to the Bergey's manual of determinative bacteriology (Anonymous, 1974). Similar results of sugar test for *L. acidophilus* were reported by Abu-Tarboush (1994).

Gastric Juice Resistance

Most bacteria do not survive well at low pH values. Thus, it has been suggested that microbial culture to be used as growth promotants should be screened for their resistance to acidity (Conway *et al*). During the present study, pepsin was added in the experimental medium to stimulate the activity of gastric acid environment and the isolated lactic acid bacterial species were able to survive and to some extent multiply in artificial gastric acid environment at pH 2.2. This was towards gastrointestinal condition at pH 6.6 after 1, 2, 3 and 4 hours of incubation respectively which was observed from the absorbance data of bacterial cell concentration in artificial gastric acid medium at 620 nm. However, after 24 hours of incubation the survival as well as multiplication abilities of both yogurt and poultry isolates were reduced in both pH 2.2 and pH 6.6 of gastric juice. *L. acidophilus* from isolate no.08 (sample no. 23) showed better survival ability than *L. acidophilus* from isolate no. 01, 03 and 05 (sample no. 21, 22) at both pH 2.2 and pH 6.6. *streptococcus thermophilus* from isolate no. 02 and 06 (sample no. 21 and 24) showed moderate survival ability compared at pH 2.2.When considered only *L. brevis* from isolate no. 04 and 07 (sample 23 and 24), showed poor survival ability compared to *L. acidophilus* from isolate no. 08 (sample 23).In case of *streptococcus thermophilus species*, after 4 hour of incubation at pH 2.2, from isolate no. 02 (sample no.21) showed good resistance ability but *Bifidobacterium* spp. isolate no. 10 (sample no. 23) showed lowest multiplication rate compared to other nine bacteria at pH 2.2. Moreover almost bacteria show best survival and multiplication abilities at 4 hours . But after 24 hours, at pH 2.2, all the isolates show lowest survival ability compared to earlier hours. In addition, at pH 6.6 after 1, 2, 3 and 4 hours, all the ten isolates showed more or less equal survival and multiplication abilities except *L. acidophilus* from isolate no.08 (sample no. 23) which showed highest multiplication rate at 4 hours but moderate survival ability after 24 hours. The gastric juice resistance test results of the present study were found nearly similar with the results obtained by Jin *et al.* Overall, pH 6.6 served as control and showed greater survival and multiplication abilities than pH 2.2. In case of poultry, *L. acidophilus* showed excellent multiplication rate at 4 hours incubation period at low pH 2.2 but they were poorly survive like as other isolated bacteria after 24 hours incubation period but in pH 6.6 they were able to multiply and survived as well. In case of pH 2.2 after 4 hours, the multiplication ability was highest, as the same result found in case of pH 6.6. According to Goldin, 1992 gastric juice resistance ability is one of the most important characteristics of

probiotic lactic acid bacteria to survive, to multiply and to exert their beneficial action in the gastrointestinal tract.

Bile Salt Tolerance

Resistance to bile is an important characteristic that enables lactobacillus to survive and grow in the intestinal tract Gililand *et al.* (1984) reported that supplementation of the diet with the more bile resistant strain of *Lactobacillus acidophilus* increased the number of the strain in the upper small intestine. The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. In the present experiments, the resistance capabilities of all the ten isolates of yogurt and four isolates of poultry showed more or less nearly equal resistance against 0.05%, 0.1%, 0.6% and 0.3% artificial bile acid concentrations after 4 hours all the isolates moderately multiplication, all the isolates showed highest multiplication rate after 12 hours incubation period.

After 12 hours of incubation, all the isolates of yogurt started multiplication and among them *streptococcus thermophilus* species, isolate no.06 (sample no. 24) exhibited highest bile acid resistance against 0.05%, 0.1%, 0.3%, artificial bile acid concentrations. Moreover, after 12 hours of incubation among the *L. brevis* species, isolate no. 07 (sample no. 24) showed the highest resistance against 0.3% artificial bile acid concentrations. In addition, *Lactobacillus acidophilus* from isolate no. 01 (sample no. 21) showed slightly better resistance than *Lactobacillus acidophilus* from isolate no. 03, and 08 (sample no. 22 and 23) and *Bifidobacterium* spp. from isolate no. 10 (sample no. 24) showed moderate bile acid resistance compared to other nine isolates after 12 hours of incubation against 0.05%, 0.1%, 0.3%, 0.6% artificial bile acid concentrations. In addition after 12 hours and 24 hours of incubation period four isolated bacteria of poultry sample showed highest multiplication and survival rate at 0.05%, 0.1%, among them *Lactobacillus acidophilus* isolate no. 01 (sample no.07) showed highest multiplication rate but the multiplication rate decreased at 0.3%, 0.6% of bile acid concentration. After 24 hours of incubation period of *Bifidobacterium* spp. Isolate no.03 (sample no.08) showed highest survival rate but after 12 hours later same bacteria showed better multiplication rate at 0.3%, 0.6% but lower at 24 hours later compared to *Lactobacillus brevis* isolate no. 02 (sample no. 08) although other isolated bacteria showed nearly good survival rate.

Elizete and Carlos (2005) stated that bile tolerance is an essential characteristic for better survival, not necessary for multiplication.

Phenol resistance

According to the present study after 12 hours of incubation, ten isolates belonging to four species viz. *Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus brevis,* and *Bifidocterium* spp.showed good tolerance against 0.1% and 0.2% phenol as the concentration increased to 0.3% and 0.4% their tolerance decreased greatly. After 12 hours of incubation period the survival rate of *Streptococcus thermophilus* isolate no. 06 (sample no. 24) comparatively low among others isolated bacteria at 0.1% phenol concentration. In poultry samples, the three isolates of the species *Lactobacillus acidophilus, Lactobacillus brevis,* and *Bifidocterium spp.* showed good tolerance against 0.1 - 0.2% phenol and moderate tolerance against 0.3% and 0.4% phenol. In both cases of 0.1 and 0.2% phenol concentrations after 24 hours incubation the result was highest than other concentrations.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, better resistance and multiplication ability were observed against 0.1 and 0.2% phenol and when the concentration increased to 0.3%, their tolerance were moderate and at 0.4% concentration of phenol, their tolerance were lowest, same as Xanthopoulos, *et al.,* 2000.

Sodium Chloride (NaCl) tolerance:

Sodium chloride is one of the most important factors for bacterial multiplication. NaCl may inhibit the growth of harmful bacteria and enhance the desirable fermentation (Masui *et al.* 1979). In the present study identified isolated probiotic bacteria from yogurt of samples were performed for different concentration of NaCl (1-10%) in the MRS culture broth and the growth was observed according to turbidity after 24 hours. All the isolates had excellent tolerance against 1-6% NaCl. At 7% to 9% NaCl concentrations they showed moderate level of bacterial growth and in 10% of NaCl, no growth was observed. In addition of poultry samples NaCl tolerance test results is finally identified four isolates of probiotic bacteria (from sample no. 07 and 08) have shown that all the isolates had excellent tolerance activity against 1-6% NaCl concentration and moderate survival rate in 7% to 8% as well as very low multiplication rate in

9% of NaCl concentration and in 10% of NaCl, no growth was observed. They showed low level of growth by observing turbidity after 24 hours of incubation at 37^0C. Elizete and Carlos (2005) isolated lactobacilli from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Schillinger and Lucke (1987) found the growth of lactobacilli in the presence of 7.5% NaCl isolated from meat and meat products and the result is almost similar to the findings of present study. Shockey and Borger (1991) speculated that salt concentration inhibits the growth and proteolytic activity of harmful bacteria but not the growth of lactic acid bacteria.

Bile salt hydrolase activity test: Plate assay

Isolated and identified lactic acid bacteria were streaked out on MRS agar plates containing 0.5% taurodeoxycholic acid (TDCA) and all the ten isolates of yogurt showed colonies like opaque granular white indicating the bile salt hydrolase-active lactic acid bacteria. The bile salt hydrolase activity test results of the present study were similar to Ahn, Kim, Lim, Baek, and Kim (2003) found precipitated halo and opaque granular white material on agar plugs around L. acidophilus colonies, which were confirmed to bacterial species exhibited different bile salt hydrolysis activity

Mice Trial

All selected probiotic bacteria of yogurt were tested for *in vivo* experiments. Seven weeks old Swiss Albino mouse was used *in vivo* experiments. *Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus brevis,* and *Bifidocterium* spp were used to prepare yoghurt. In case of mouse trial, blood cholesterol level, body weight , antibiotic resistance, inhibition of growth of pathogenic microorganism *in vivo* tests were performed by feeding yoghurt and compared with control group (without yoghurt) which containing all those species were similar to Modler, 1994; Klaver et al., 1990).

Body weight measurement

After 28 days of treatment period all the treatment group showed significant weight gain results compared to control group in some case variation occurs of weight gain due to environmental factors. Inadequate housing provision is also responsible for variation. Comfortable requirements are also very essential to get maximum significant results. Other researchers also have found a significant increase in body weight for enriched housed mice. They also found mice from super

enriched housing conditions consumed more food, which is consistent with the higher body weights (Chapillon, Manneché, Belzung, & Caston, 1999; Dahlborn, Van Gils, Van de Weerd, Van Dijk, & Baumans, 1996; Van de Weerd, Baumans, Koolhaas, & Van Zutphen, 1994). This has major implications for all contexts in which mice are maintained and cared for in the confined settings, because any compromising of the factors in the animals' environment that can influence their behavior may lead to less variability in the experimental results and can thereby reduce the number of animals needed for experimental procedures. Standardization of environmental enrichment, meeting the animal's species appropriate needs, without increasing the variability in experimental results although the environment of the animal can never be standardized entirely (Crabbe et al. 1999, Nevison et al. 1999, Olsson & Dahlborn 2002). Concerning body weight, treatment group showed significant results due to feed yogurt containing probiotic bacteria which may increase feed intake and digestion as well as altering metabolism by increasing digestive enzyme activity. 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*.

Blood cholesterol measurement

In the present study, the treatment group showed significant level of cholesterol reduction that was feed with yogurt compared with control group which group was feed without yogurt. In other studies (de Rodas, B.Z., S.E. Gilliland & C.V. Maxwell. 1996), with diet induced hypercholesterolemia were observed that for animals receiving the milk supplemented with lactobacilli the cholesterol level declined sooner and reached the significantly lower level.

Antibiotic resistance test

In regard to antibiotics acting on cell wall, *lactobacilli* are usually sensitive to penicillin and β lactamase inhibitors, but more resistant to cephalosporins. Many *Lactobacillus* species show a high level of resistance to vancomycin. Most *Bifidobacterium* species are resistant to aminoglycosides, metronidazole and Gram-negative spectrum antibiotics. They are also intrinsically resistant to mupirocin, an antibiotic that is being used in the selective isolation of this genus. In contrast, *bifidobacteria* are very susceptible to macrolides, vancomycin, rifampicin, spectinomycin, chloramphenicol, and β-lactams. (Dimitris et al., 2009; Hummel et al., 2007; Zhou et al., 2005; Klare et al., 2007; Perreten et al., 1997). During the study period, the

treatment group showed significant resistance to the antibiotic compared to the control group. In addition of control group showed trivial decreased level of lactic acid bacteria when the treatment group showed increase level of lactic acid bacteria.

Inhibition of Growth of Pathogenic Microorganism *in vivo*

In the present experiment the treatment group mice which fed with yogurt showed lowest amount of pathogenic *Escherichia coli* bacteria where as compared with control group without yogurt and exhibited highest amount of pathogenic bacteria that could happen due to lack of antimicrobial activity by yogurt. In previous studies findings to Verschuere, 2000; Ishibashi and Yamazaki, 2001; Maragkoukako *et al.*, 2006; Heller, 2001; Ray, 2004 showed that lactic acid bacteria can neutralize harmful products from foods in the gastrointestinal tract due to their antagonistic potential against pathogen such as *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* which is almost similar with the present experimental results.

Conclusion

Analysis of probiotic properties of isolated bacteria from selected yogurt and poultry feces were the main focus of this study. In order to achieve this aim, colony morphology, gram staining, endospore test, catalase test, motility test, carbohydrate fermentation test, gastric juice resistance test, bile tolerance test, phenol tolerance test, NaCl tolerance test, and bile salt hydrolase activity tests were performed. *In vivo* tests by mice trial viz. body weights, blood cholesterol measurements, antibiotic resistance and inhibition of growth of pathogenic microorganisms were also conducted. The concluding results of the study are:

1. A total of ten isolates were identified as probiotic bacteria from selected yogurt and four isolates from poultry.
2. Four species viz. *Lactobacillus acidophilus Streptococcus thermophilus*, *Lactobacillus brevis,* and *Bifidocterium* spp from yogurt and three species of *Lactobacillus brevis, Lactobacillus acidophilus,* and *Bifidocterium* spp were presumptively identified as probiotic bacteria.
3. The isolated bacteria were found gram positive, endospore negative, catalase negative, non-motile, had the abilities to ferment different biochemical test and shown characteristic to specific probiotic lactic acid bacterial species, resistance to inhibitory

substances such as 0.1-0.4% phenol, gastric acid at pH 2.2 and 6.6, 0.05-0.3% bile, 1-9% NaCl and also showed bile salt hydrolase activity on plate assay.

4. Isolated bacteria from yoghurt and poultry samples were able to coagulate milk to produce curd at specific temperature and culture conditions.
5. Blood cholesterol level is lowering; antibiotic resistance, antimicrobial activities and body weight gain properties tests were done by *in vivo* using mice models. In all test significant results were found, so it was understandable that all isolates have probiotic properties.

According to World Health Organization (WHO) and Food and Agriculture Organization (FAO, 2002) working group guidelines, the probiotic organisms should possess the characteristics of resistance to gastric acid and bile with other attributed criteria. The present experimental isolated probiotic lactic acid bacteria have shown almost similar characteristics defined by WHO and FAO standard.

Recommendation

The study needs to Use of at least fifty carbohydrates/sugars or use of API-50 kit in carbohydrate/sugar fermentation profiles experiment can strengthen the lactic acid bacterial species identification. The study also needs to include molecular techniques like SDS-PAGE or PCR-RFLP and 16S rRNA sequencing for accurate identification of probiotic bacterial species and multiplex RAPD-PCR technique could be used to reveal the complete metabolic potential of each of the probiotic strain which may open the possibility to engineer new combinations of pre- and probiotic food products viz. fermented milk drinks, yoghurt, ice-cream and sausages for human consumption whereas liquid and lyophilized form of probiotic products for poultry uses.

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APPENDICES

APPENDIX-A

RECIPIES FOR CULTURE MEDIA

A1. MRS BROTH

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
D-glucose	20
K_2HPO_4	2
Sodium acetate	5
Triammonium citrate	2
$MgSO_4$, $7H_2O$	0.2
$MnSO_4$, $7H_2O$	0.05
Tween-80	1 ml
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

A2. MRS AGAR

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
D-glucose	20
K_2HPO_4	2
Sodium acetate	5
Triammonium citrate	2
$MgSO_4$, $7H_2O$	0.2
$MnSO_4$, $7H_2O$	0.05
Tween-80	1 ml
Deionized water	1000 ml
Agar	15

All ingredients were dissolved in deionized water and pH was adjusted to 6.3-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

A3. MODIFIED MRS FOR CARBOHYDRATE FERMENTATIONS

MRS broth	Gram/litre
Peptone	10
Meat extract	10
Yeast extract	5
K_2HPO_4	2
Sodium acetate	5
Triammonium citrate	2
$MgSO_4$, $7H_2O$	0.2
$MnSO_4$, $7H_2O$	0.05
Tween-80	1 ml
Bromocresol purple	0.04
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.2-6.6. Medium was sterilized by autoclaving at 121°C for 15 minutes.

A4. ST (*Streptococcus thermophilus*) AGAR

MRS broth	Gram/litre
Tryptone	10
Sucrose	1
Yeast extract	5
K_2HPO_4	2
Agar	12

All ingredients were dissolved in 1 litre of distilled (deionized) water and pH was adjusted to 6.8. 6 ml of 0.5 gram/100 ml of bromocresol purple was added to the rehydrated ingredients. The medium was sterilized by autoclaving at 121°C for 15 minutes.

A5. GYP Broth

GYP broth	Gram/litre
Peptone	5
Yeast Extract	10
Dextrose	10
Manganese Sulfate	0.2
Magnesium Sulfate	0.4
Ferrous Sulfate	0.2
Sodium Acetate	2
Nacl	0.2
Tween 80	1 ml

All ingredients were dissolved in deionizer water and pH was adjusted to 6.8 and medium was sterilized by autoclaving at 121^0 C for 15 minutes. In case of GYP agar media 16gm/l agar is added with above composition and autoclaving at 121^0C for 15 minutes.

Body Weight Gain Data

	Day 0		Day 07	
Mice	**Control Group (gm)**	**Treatment group**	**Control Group (gm)**	**Treatment Group**
Red	13.3	14.7	15.2	17.3
Blue	15.2	14.1	18.4	16.0
Black	11.8	16.1	14.1	18.9
Yellow	11.3	15.8	12.8	19.8
Green	12.9	17.0	14.5	22.5
White	12.2	19.1	14.8	24.6
Red Black	13.7	18.8	16.3	23.5
Blue Black	14.1	20.4	15.7	24.8
Yellow Black	14.6	12.5	17.9	15.1
Blue Yellow	10.8	12.9	13.1	16.3
Red Blue	18.4	16.8	21.6	18.3
Red Yellow	15.4	19.5	17.4	21.8
	Day 14		**Day 21**	
Red	17.1	19.5	18.3	23.5
Blue	21.6	18.3	24.6	20.8
Black	16.9	23.7	19.5	27.3
Yellow	14.6	21.9	17.1	25.7
Green	18.1	25.8	19.8	30.9
White	19.2	26.3	21.0	29.8
Red-Black	18.5	27.4	21.5	30.6
Blue-Black	17.1	26.8	18.6	32.2
Yellow-Black	20.6	17.3	23.7	21.2
Blue-Yellow	15.7	19.2	18.3	23.6
Red-Blue	25.4	21.2	28.7	26.3
Red-Yellow	19.7	24.3	22.9	29.9

Body Weight Gain Data

Day 28		
Mice	**Control Group (gm)**	**Treatment group**
Red	19.7	27.8
Blue	29.1	22.1
Black	20.1	31.2
Yellow	19.1	29.3
Green	22.4	35.8
White	23.1	36.7
Red Black	24.6	34.9
Blue Black	21.9	38.8
Yellow Black	28.3	25.3
Blue Yellow	22.6	27.8
Red Blue	31.1	29.9
Red Yellow	24.3	35.4

Cholesterol Measuring Data

	Day 0		Day 28	
Mice	**Control Group (Mm)**	**Treatment Group (Mm)**	**Control Group (Mm)**	**Treatment Group (Mm)**
Red	3.4	3.5	3.6	3.0
Blue	3.3	4.0	3.9	3.1
Black	3.5	3.4	3.8	2.9
Yellow	3.4	4.2	3.8	3.3
Green	4.2	3.8	3.6	3.2
White	3.4	4.2	3.0	3.1
Red Black	4.2	3.8	3.7	3.2
Blue Black	3.1	4.9	3.5	3.5
Yellow Black	4.3	3.7	3.9	3.0
Blue Yellow	3.4	3.6	3.3	2.8
Red Blue	3.6	3.1	3.5	2.6
Red Yellow	4.1	4.3	4.0	3.3

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

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Control
0 Hr	0.182	0.169	0.189	0.209	0.215	0.2	0.211	0.217	0.202	0.238	0.215	0.211	0.132
2 Hr	0.269	0.285	0.291	0.265	0.244	0.271	0.284	0.290	0.271	0.254	0.239	0.268	0.155
4 Hr	0.461	0.479	0.475	0.427	0.439	0.451	0.533	0.515	0.509	0.443	0.462	0.481	0.148
24 Hr	0.316	0.310	0.321	0.278	0.261	0.291	0.336	0.342	0.357	0.319	0.328	0.342	0.139

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

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Control
0 Hr	0.172	0.168	0.162	0.205	0.214	0.199	0.185	0.197	0.181	0.188	0.197	0.201	0.114
2 Hr	0.239	0.225	0.231	0.255	0.234	0.261	0.245	0.258	0.241	0.245	0.255	0.261	0.126
4 Hr	0.319	0.332	0.325	0.418	0.42	0.442	0.341	0.33	0.353	0.352	0.371	0.342	0.138
24 Hr	0.242	0.249	0.259	0.268	0.251	0.281	0.235	0.228	0.239	0.259	0.268	0.251	0.127

Phenol Tolerance Test Data for Poultry Sample (OD 620 nm)

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Control
1 Hr 0.1%	0.212	0.229	0.241	0.195	0.186	0.209	0.185	0.191	0.179	0.189	0.199	0.203	0.15
1 Hr 0.2%	0.225	0.237	0.241	0.21	0.215	0.211	0.231	0.244	0.229	0.23	0.251	0.239	0.173
1 Hr 0.3%	0.259	0.246	0.264	0.241	0.219	0.233	0.235	0.242	0.256	0.249	0.268	0.237	0.19
1 Hr 0.4%	0.279	0.285	0.293	0.262	0.278	0.287	0.251	0.239	0.267	0.261	0.286	0.277	0.21

Phenol Tolerance Test Data for Poultry Sample (OD 620 nm)

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Control
12 Hr 0.1%	1.341	1.285	1.309	1.259	1.275	1.243	1.233	1.221	1.23	1.01	1.011	1.04	0.15
12 Hr 0.2%	1.139	1.142	1.137	1.209	1.215	1.202	1.115	1.128	1.132	1.031	1.038	1.025	0.173
12 Hr 0.3%	1.065	1.079	1.089	1.138	1.144	1.135	1.054	1.037	1.039	1.167	1.175	1.158	0.19
12 Hr 0.4%	0.882	0.857	0.839	0.951	0.971	0.991	1.102	1.1	1.11	0.914	0.939	0.951	0.21

Phenol Tolerance Test Data for Poultry Sample (OD 620 nm)

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Control
24 Hr 0.1%	1.611	1.623	1.629	1.461	1.469	1.472	1.112	1.118	1.114	1.271	1.28	1.284	0.15
24 Hr 0.2%	1.387	1.38	1.391	1.17	1.173	1.165	1.341	1.384	1.358	1.17	1.12	1.144	0.168
24 Hr 0.3%	0.901	0.885	0.893	0.783	0.759	0.764	0.827	0.849	0.861	0.995	0.978	0.982	0.196
24 Hr 0.4%	0.463	0.478	0.489	0.531	0.552	0.524	0.716	0.723	0.735	0.561	0.574	0.583	0.203

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

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Contro l
1 Hr 0.05%	0.187	0.195	0.21	0.225	0.212	0.215	0.234	0.241	0.259	0.18	0.195	0.185	0.134
4 Hr 0.05%	0.733	0.756	0.717	0.691	0.67	0.663	0.79	0.771	0.782	0.639	0.641	0.597	0.144
12 Hr 0.05%	1.439	1.413	1.421	0.983	0.959	0.972	1.1	1.21	1.15	1.21	1.262	1.281	0.147
24 Hr 0.05%	1.109	1.11	1.139	1.191	1.111	1.135	1.186	1.24	1.11	1.008	0.991	0.998	0.156
1 Hr 0.1%,	0.243	0.253	0.264	0.259	0.248	0.267	0.25	0.265	0.276	0.22	0.243	0.231	0.165
4 Hr 0.1%,	0.406	0.413	0.421	0.453	0.459	0.446	0.687	0.681	0.693	0.551	0.536	0.487	0.169
12 Hr 0.1%,	1.575	1.52	1.598	1.212	1.198	1.189	0.997	1.12	1.19	1.07	0.991	1.11	0.176
24 Hr 0.1%,	1.007	0.989	0.995	0.937	0.91	0.919	1.213	1.235	1.257	0.821	0.849	0.858	0.17
1 Hr 0.3%,	0.248	0.228	0.253	0.265	0.253	0.259	0.235	0.223	0.245	0.248	0.231	0.249	0.174
4 Hr 0.3%,	0.517	0.511	0.545	0.446	0.455	0.463	0.511	0.531	0.524	0.428	0.442	0.457	0.189
12 Hr 0.3%,	1.116	1.155	1.137	1.182	1.169	1.161	1.217	1.227	1.246	1.126	1.171	1.159	0.185
24 Hr 0.3%,	1.001	0.987	0.994	0.914	0.941	0.957	0.789	0.769	0.751	0.881	0.869	0.862	0.167
1 Hr 0.6%,	0.276	0.267	0.256	0.273	0.261	0.279	0.264	0.275	0.286	0.263	0.284	0.295	0.213
4 Hr 0.6%,	0.428	0.416	0.422	0.583	0.571	0.579	0.669	0.681	0.637	0.389	0.396	0.407	0.224
12 Hr 0.6%,	0.831	0.873	0.847	0.962	0.942	0.974	1.094	1.072	1.083	0.853	0.872	0.891	0.217
24 Hr 0.6%,	0.457	0.411	0.459	0.651	0.664	0.672	0.549	0.579	0.587	0.489	0.456	0.487	0.235

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

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Isolate 05		
0 Hr	0.268	0.252	0.279	0.193	0.2	0.215	0.223	0.234	0.247	0.208	0.217	0.199	0.297	0.279	0.288
2 Hr	0.324	0.319	0.31	0.296	0.288	0.271	0.301	0.315	0.321	0.275	0.254	0.281	0.325	0.349	0.317
4 Hr	0.413	0.422	0.431	0.441	0.473	0.461	0.388	0.396	0.411	0.417	0.429	0.441	0.423	0.432	0.426
24 Hr	0.306	0.312	0.297	0.285	0.267	0.293	0.277	0.291	0.285	0.268	0.251	0.281	0.313	0.319	0.325

	Isolate 06			Isolate 07			Isolate 08			Isolate 09			Isolate 10			Control
0 Hr	0.282	0.269	0.289	0.225	0.238	0.216	0.218	0.227	0.2	0.194	0.181	0.21	0.198	0.21	0.187	0.145
2 Hr	0.359	0.345	0.369	0.271	0.263	0.258	0.284	0.29	0.271	0.286	0.275	0.3	0.311	0.318	0.3	0.132
4 Hr	0.451	0.469	0.469	0.391	0.379	0.388	0.533	0.515	0.509	0.425	0.437	0.419	0.468	0.458	0.488	0.149
24 Hr	0.316	0.31	0.321	0.295	0.273	0.279	0.311	0.338	0.346	0.284	0.261	0.297	0.319	0.31	0.33	0.138

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

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Isolate 05		
0 Hr	0.174	0.182	0.169	0.186	0.175	0.162	0.197	0.177	0.184	0.188	0.208	0.197	0.185	0.194	0.197
2 Hr	0.221	0.234	0.218	0.241	0.229	0.231	0.265	0.251	0.244	0.244	0.251	0.237	0.228	0.234	0.249
4 Hr	0.309	0.299	0.313	0.319	0.332	0.325	0.331	0.319	0.342	0.288	0.298	0.273	0.354	0.337	0.346
24 Hr	0.237	0.213	0.222	0.232	0.249	0.251	0.235	0.241	0.252	0.239	0.249	0.229	0.225	0.219	0.231

	Isolate 06			Isolate 07			Isolate 08			Isolate 09			Isolate 10			Control
0 Hr	0.226	0.238	0.219	0.189	0.199	0.182	0.194	0.189	0.21	0.195	0.179	0.188	0.188	0.197	0.201	0.112
2 Hr	0.309	0.31	0.317	0.285	0.28	0.271	0.267	0.283	0.257	0.254	0.242	0.238	0.275	0.255	0.291	0.125
4 Hr	0.373	0.356	0.38	0.341	0.33	0.353	0.371	0.393	0.384	0.321	0.315	0.332	0.352	0.371	0.342	0.128
24 Hr	0.308	0.314	0.289	0.231	0.223	0.219	0.311	0.298	0.315	0.239	0.227	0.221	0.259	0.268	0.251	0.141

Phenol Tolerance Test Data (OD 620 nm)

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Isolate 05		
1 Hr 0.1%	0.197	0.173	0.187	0.187	0.195	0.204	0.197	0.182	0.206	0.213	0.219	0.215	0.183	0.197	0.204
1 Hr 0.2%	0.21	0.215	0.219	0.218	0.205	0.211	0.225	0.214	0.21	0.235	0.217	0.227	0.221	0.214	0.218
1 Hr 0.3%	0.231	0.254	0.241	0.261	0.236	0.247	0.245	0.261	0.249	0.256	0.249	0.266	0.239	0.257	0.245
1 Hr 0.4%	0.271	0.289	0.267	0.279	0.291	0.275	0.293	0.274	0.289	0.288	0.271	0.276	0.253	0.265	0.269
12 Hr 0.1%	1.23	1.2	1.24	1.034	1.037	1.035	1.618	1.649	1.663	1.441	1.385	1.409	1.153	1.15	1.157
12 Hr 0.2%	1.3	1.28	1.31	1.115	1.128	1.132	1.237	1.32	1.241	1.239	1.242	1.237	1.428	1.422	1.425
12 Hr 0.3%	1.23	1.29	1.3	1.233	1.221	1.23	1.314	1.348	1.359	1.185	1.179	1.188	1.26	1.263	1.265
12 Hr 0.4%	0.85	0.809	0.827	1.102	1.1	1.11	0.942	0.921	0.957	0.782	0.757	0.739	1.073	1.058	1.039
24 Hr 0.1%	1.435	1.487	1.461	1.112	1.118	1.114	1.036	1.103	1.059	1.811	1.823	1.829	1.526	1.565	1.531
24 Hr 0.2%	1.01	1.05	1.03	1.341	1.348	1.358	1.115	1.119	1.112	1.487	1.48	1.491	1.718	1.751	1.769
24 Hr 0.3%	0.96	0.86	0.93	1.074	1.1	1.08	0.952	0.988	0.971	1.101	1.115	1.121	0.891	0.876	0.87
24 Hr 0.4%	0.55	0.58	0.57	0.786	0.809	0.795	0.614	o.639	0.671	0.413	0.428	0.409	0.616	0.632	0.61

	Isolate 06			Isolate 07			Isolate 08			Isolate 09			Isolate 10			Control
1 Hr 0.1%	0.21	0.226	0.235	0.169	0.196	0.181	0194	0.187	0.207	0.211	0.209	0.199	0.184	0.194	0.202	0.15
1 Hr 0.2%	0.228	0.231	0.254	0.218	0.236	0.224	0.216	0.221	0.227	0.224	0.236	0.231	0.198	0.201	0.209	0.178
1 Hr 0.3%	0.249	0.236	0.254	0.256	0.241	0.262	0.245	0.225	0.233	0.247	0.231	0.243	0.241	0.253	0.238	0.173
1 Hr 0.4%	0.278	0.284	0.292	0.259	0.293	0.277	0.265	0.281	0.292	0.287	0.271	0.292	0.271	0.296	0.287	0.21
12 Hr 0.1%	1.155	1.119	1.129	1.288	1.28	1.285	1.189	1.175	1.193	1.271	1.28	1.284	1.203	1.209	1.2	0.15
12 Hr 0.2%	1.668	1.66	1.677	1.084	1.08	1.076	1.309	1.315	1.302	1.034	1.038	1.025	1.24	1.271	1.192	0.178
12 Hr 0.3%	1.11	1.117	1.113	1.205	1.209	1.202	1.238	1.244	1.235	1.167	1.175	1.158	1.278	1.221	1.291	0.173
12 Hr 0.4%	1.083	1.076	1.088	0.957	0.951	0.963	1.1	1.15	1.17	0.914	0.939	0.951	1.095	0.992	0.989	0.21
24 Hr 0.1%	1.11	1.225	1.247	1.139	1.136	1.143	1.261	1.269	1.272	1.01	1.011	1.04	1.321	1.38	1.311	0.16
24 Hr 0.2%	1.327	1.34	1.367	1.116	1.35	1.129	1.17	1.173	1.165	1.17	1.12	1.144	0.895	0.988	0.976	0.176
24 Hr 0.3%	0.921	0.948	0.934	0.982	0.997	0.93	0.843	0.829	0.814	0.995	0.998	0.992	0.987	0.927	0.979	0.196
24 Hr 0.4%	0.721	0.749	0.717	0.781	0.765	0.792	0.731	o.752	0.724	0.561	0.574	0.583	0.746	0.771	0.739	0.225

Bile salt Test Data (OD 620 nm)

	Isolate 01			Isolate 02			Isolate 03			Isolate 04			Isolate 05			Contro l
1 Hr 0.05%	0.232	0.245	0.257	0.184	0.215	0.195	0.24	0.245	0.259	0.176	0.162	0.186	0.218	0.209	0.212	0.145
4 Hr 0.05%	0.799	0.781	0.792	0.229	0.241	0.217	0.525	0.537	0.513	0.214	0.225	0.211	0.669	0.66	0.673	0.134
12 Hr 0.05%	1.1	1.21	1.15	1.2	1.32	1.39	1.234	1.212	1.229	1.171	1.211	1.197	1.206	1.233	1.247	0.156
24 Hr 0.05%	1.186	1.24	1.11	1.008	0.991	0.998	1.014	1.101	0.999	1.025	1.1	1.15	1.116	1.25	1.107	0.163
1 Hr 0.1%	0.25	0.265	0.276	0.22	0.243	0.231	0.213	0.223	0.209	0.235	0.215	0.223	0.238	0.249	0.257	0.148
4Hr 0.1%	0.887	0.881	0.893	0.251	0.236	0.247	0.469	0.452	0.473	0.307	0.314	0.331	0.683	0.661	0.675	0.165
12 Hr 0.1%	1.213	1.235	1.257	1.07	0.991	1.11	1.219	1.227	1.234	1.088	1.21	1.18	1.145	1.18	1.27	0.16
24 Hr 0.1%	0.977	1.12	1.19	0.921	0.929	0.938	0.978	0.959	0.988	0.857	0.816	0.839	0.996	0.979	1.01	0.176
1 Hr 0.3%	0.258	0.237	0.263	0.193	0.174	0.181	0.253	0.241	0.259	0.219	0.227	0.235	0.239	0.224	0.247	0.174
4 Hr 0.3%	0.778	0.761	0.784	0.428	0.439	0.447	0.593	0.581	0.589	0.446	0.431	0.457	0.52	0.512	0.529	0.185
12 Hr 0.3%	1.12	1.19	1.31	1.16	1.151	1.169	1.282	1.269	1.261	1.181	1.187	1.195	1.658	1.631	1.643	0.189
24 Hr 0.3%	0.877	0.861	0.869	0.781	0.769	0.762	1.o14	0.991	0.997	1.013	0.997	0.991	1.064	1.1	1.13	0.163
1 Hr 0.6%	0.265	0.25	0.276	0.233	0.221	0.239	0.263	0.251	0.269	0.25	0.261	0.269	0.259	0.265	0.269	0.213
4 Hr 0.6%	0.687	0.681	0.693	0.389	0.396	0.407	0.646	0.655	0.663	0.387	0.396	0.398	0.665	0.679	0.683	0.224
12 Hr 0.6%	0.786	0.81	0.806	1.233	1.2	1.34	1.12	1.24	1.29	0.981	0.987	0.995	1.15	1.19	1.31	0.217
24 Hr 0.6%	0.576	0.563	0.585	0.879	0.866	0.858	0.751	0.764	0.772	0.527	0.539	0.515	0.663	0.641	0.655	0.235

	Isolate 06			Isolate 07			Isolate 08			Isolate 09			Isolate 10			Control
1 Hr 0.05%	0.207	0.197	0.211	0.221	0.191	0.213	0.245	0.227	0.257	0.211	0.189	0.196	0.235	0.22	0.215	0.145
4 Hr 0.05%	0.833	0.796	0.817	0.559	0.579	0.567	0.72	0.729	0.738	0.641	0.597	0.625	0.694	0.675	0.669	0.134
12 Hr 0.05%	1.539	1.513	1.51	1.248	1.209	1.231	1.224	1.249	1.237	1.172	1.131	1.154	1.296	1.216	1.233	0.156
24 Hr 0.05%	1.109	1.11	1.139	0.998	0.954	0.778	1.03	1.09	1.13	1.016	1.1	1.09	0.983	0.959	0.972	0.163
1 Hr 0.1%	0.249	0.263	0.274	0.21	0.227	0.235	0.208	0.212	0.218	0.201	0.227	0.214	0.259	0.248	0.267	0.148
4Hr 0.1%	1.006	1.13	1.1	0.563	0.543	0.581	0.772	0.751	0.769	0.737	0.712	0.721	0.453	0.459	0.446	0.165
12 Hr 0.1%	1.675	1.62	1.598	1.116	1.119	1.127	1.405	1.385	1.371	1.11	1.39	1.18	1.312	1.298	1.289	0.16
24 Hr 0.1%	1.007	0.989	0.995	1.009	1.1	1.21	0.902	0.914	0.921	0.979	0.971	0.989	0.937	0.91	0.919	0.176
1 Hr 0.3%	0.229	0.249	0.253	0.258	0.238	0.263	0.228	0.224	0.241	0.233	0.219	0.248	0.24	0.257	0.261	0.174
4 Hr 0.3%	0.72	0.734	0.747	0.617	0.611	0.645	0.725	0.779	0.743	0.513	0.535	0.524	0.71	0.736	0.724	0.185
12 Hr 0.3%	1.757	1.691	1.708	1.216	1.295	1.237	1.401	1.45	1.461	1.207	1.227	1.216	1.205	1.45	1.229	0.189
24 Hr 0.3%	1.103	0.999	0.997	1.1	0.997	0.981	0.871	0.884	0.897	0.985	0.964	0.971	1.11	1.03	1.13	0.163
1 Hr 0.6%	0.289	0.297	0.261	0.285	0.257	0.266	0.247	0.273	0.254	0.266	0.276	0.287	0.287	0.245	0.229	0.213
4 Hr 0.6%	0.789	0.755	0.803	0.71	0.716	0.722	0.68	0.691	0.785	0.789	0.761	0.77	0.755	0.732	0.745	0.224
12 Hr 0.6%	1.34	1.416	1.44	1.421	1.533	1.527	1.321	1.399	1.3	1.294	1.272	1.283	1.206	1.26	1.23	0.217
24 Hr 0.6%	0.817	0.841	0.863	0.857	0.816	0.839	0.645	0.634	0.661	0.549	0.579	0.587	0.823	0.786	0.815	0.235

Antibiotic Resistance Test

	Control				Treatment				Control				Treatment			
	Day 0								Day 07							
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	Col-ony	Dilu-tion	No of Cfu/ml	Log cfu/ml
Red	86	10^{-6}	4.30×10^{8}	8.634	119	10^{-6}	5.95×10^{8}	8.775	79	10^{-6}	3.95×10^{8}	8.597	102	10^{-6}	5.10×10^{8}	8.708
Blue	78	10^{-6}	3.90×10^{8}	8.592	92	10^{-6}	4.60×10^{8}	8.663	71	10^{-6}	3.55×10^{8}	8.551	109	10^{-6}	5.45×10^{8}	8.737
Black	69	10^{-6}	3.45×10^{8}	8.538	64	10^{-6}	3.20×10^{8}	8.506	69	10^{-6}	3.45×10^{8}	8.538	125	10^{-6}	6.25×10^{8}	8.796
Yellow	75	10^{-6}	3.75×10^{8}	8.575	92	10^{-6}	4.60×10^{8}	8.663	83	10^{-6}	4.15×10^{8}	8.619	112	10^{-6}	5.60×10^{8}	8.749
Green	64	10^{-6}	3.2×10^{8}	8.506	75	10^{-6}	3.75×10^{8}	8.575	77	10^{-6}	3.80×10^{8}	8.586	79	10^{-6}	3.95×10^{8}	8.597
White	75	10^{-6}	3.75×10^{8}	8.575	75	10^{-6}	3.75×10^{8}	8.575	66	10^{-6}	3.30×10^{8}	8.519	69	10^{-6}	3.45×10^{8}	8.538
Red-Black	88	10^{-6}	4.4×10^{8}	8.644	79	10^{-6}	3.95×10^{8}	8.596	74	10^{-6}	3.70×10^{8}	8.569	97	10^{-6}	4.85×10^{8}	8.686
Blue-Black	78	10^{-6}	3.90×10^{8}	8.592	73	10^{-6}	3.65×10^{8}	8.563	78	10^{-6}	3.90×10^{8}	8.592	71	10^{-6}	3.55×10^{8}	8.551
Yellow -Black	81	10^{-6}	4.05×10^{8}	8.608	97	10^{-6}	4.85×10^{8}	8.686	94	10^{-6}	4.70×10^{8}	8.673	84	10^{-6}	4.20×10^{8}	8.623
Blue-Yellow	91	10^{-6}	4.50×10^{8}	8.653	101	10^{-6}	5.05×10^{8}	8.703	58	10^{-6}	2.90×10^{8}	8.463	83	10^{-6}	4.45×10^{8}	8.618
Red-Blue	71	10^{-6}	3.55×10^{8}	8.551	84	10^{-6}	4.20×10^{8}	8.623	64	10^{-6}	3.20×10^{8}	8.506	91	10^{-6}	4.55×10^{8}	8.659
Red-Yellow	79	10^{-6}	3.95×10^{8}	8.597	86	10^{-6}	4.30×10^{8}	8.634	71	10^{-6}	3.55×10^{8}	8.551	97	10^{-6}	8.85×10^{8}	8.686

Antibiotic Resistance Test

	Control				Treatment				Control				Treatment			
	Day 0								Day 07							
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	Col-ony	Dilu-tion	No of Cfu/ml	Log cfu/ml
Red	15	10^{-7}	7.50×10^{8}	8.875	16	10^{-7}	8.00×10^{8}	8.904	12	10^{-7}	6.00×10^{8}	8.779	21	10^{-7}	1.05×10^{8}	9.022
Blue	13	10^{-7}	6.50×10^{8}	8.813	14	10^{-7}	7.00×10^{8}	8.846	14	10^{-7}	7.00×10^{8}	8.846	16	10^{-7}	8.00×10^{8}	8.904
Black	14	10^{-7}	7.00×10^{8}	8.846	11	10^{-7}	5.50×10^{8}	8.741	12	10^{-7}	6.00×10^{8}	8.779	15	10^{-7}	7.50×10^{8}	8.875
Yellow	14	10^{-7}	7.00×10^{8}	8.846	17	10^{-7}	8.50×10^{8}	8.929	11	10^{-7}	5.50×10^{8}	8.741	10	10^{-7}	5.00×10^{8}	8.699
Green	13	10^{-7}	6.50×10^{8}	8.813	10	10^{-7}	5.00×10^{8}	8.699	10	10^{-7}	5.00×10^{8}	8.699	12	10^{-7}	6.00×10^{8}	8.779
White	10	10^{-7}	5.00×10^{8}	8.699	11	10^{-7}	5.50×10^{8}	8.741	11	10^{-7}	5.50×10^{8}	8.741	12	10^{-7}	6.00×10^{8}	8.779
Red-Black	11	10^{-7}	5.50×10^{8}	8.741	15	10^{-7}	7.50×10^{8}	8.876	13	10^{-7}	6.50×10^{8}	8.813	17	10^{-7}	8.50×10^{8}	8.929
Blue-Black	10	10^{-7}	5.00×10^{8}	8.699	16	10^{-7}	8.00×10^{8}	8.904	15	10^{-7}	7.50×10^{8}	8.875	14	10^{-7}	7.0×10^{8}	8.845
Yellow -Black	14	10^{-7}	7.00×10^{8}	8.846	15	10^{-7}	7.50×10^{8}	8.876	11	10^{-7}	5.50×10^{8}	8.741	16	10^{-7}	8.00×10^{8}	8.904
Blue-Yellow	11	10^{-7}	5.50×10^{8}	8.741	12	10^{-7}	6.00×10^{8}	8.779	13	10^{-7}	6.50×10^{8}	8.813	21	10^{-7}	1.05×10^{8}	9.022
Red-Blue	12	10^{-7}	6.00×10^{8}	8.779	13	10^{-7}	6.50×10^{8}	8.813	8	10^{-7}	4.00×10^{8}	8.603	11	10^{-7}	5.50×10^{8}	8.741
Red-Yellow	15	10^{-7}	7.50×10^{8}	8.876	12	10^{-7}	6.00×10^{8}	8.779	9	10^{-7}	4.50×10^{8}	8.654	12	10^{-7}	6.00×10^{8}	8.779

Antibiotic Resistance Test

	Control				Treatment				Control				Treatment			
	Day 14 (Dilution-10^{-6})								Day 14 (Dilution-10^{-7})							
Mice	Col-ony	Dilut ion	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	Col-ony	Dilu-tion	No of Cfu/ml	Log cfu/ml
Red	91	10^{-6}	4.55×10^{8}	8.659	136	10^{-6}	6.80×10^{8}	8.833	16	10^{-7}	8.00×10^{8}	8.904	17	10^{-7}	8.50×10^{8}	8.929
Blue	75	10^{-6}	3.75×10^{8}	8.575	140	10^{-6}	7.00×10^{8}	8.846	19	10^{-7}	9.50×10^{8}	8.978	18	10^{-7}	9.00×10^{8}	8.954
Black	87	10^{-6}	4.35×10^{8}	8.639	121	10^{-6}	6.05×10^{8}	8.781	17	10^{-7}	8.50×10^{8}	8.929	11	10^{-7}	5.50×10^{8}	8.740
Yellow	71	10^{-6}	3.55×10^{8}	8.551	112	10^{-6}	5.60×10^{8}	8.749	19	10^{-7}	9.50×10^{8}	8.978	17	10^{-7}	8.50×10^{8}	8.929
Green	76	10^{-6}	3.80×10^{8}	8.579	152	10^{-6}	7.60×10^{8}	8.881	21	10^{-7}	1.05×10^{8}	9.022	19	10^{-7}	9.50×10^{8}	8.987
White	87	10^{-6}	4.35×10^{8}	8.639	109	10^{-6}	5.45×10^{8}	8.737	19	10^{-7}	9.50×10^{8}	8.978	17	10^{-7}	8.50×10^{8}	8.929
Red-Black	96	10^{-6}	4.80×10^{8}	8.682	148	10^{-6}	7.40×10^{8}	8.869	17	10^{-7}	8.50×10^{8}	8.929	16	10^{-7}	8.00×10^{8}	8.904
Blue-Black	77	10^{-6}	3.85×10^{8}	8.585	57	10^{-6}	2.85×10^{8}	8.455	15	10^{-7}	7.50×10^{8}	8.875	14	10^{-7}	7.00×10^{8}	8.846
Yellow -Black	73	10^{-6}	3.65×10^{8}	8.563	75	10^{-6}	7.50×10^{8}	8.876	18	10^{-7}	9.00×10^{8}	8.955	15	10^{-7}	7.50×10^{8}	8.876
Blue-Yellow	63	10^{-6}	3.15×10^{8}	8.499	81	10^{-6}	4.05×10^{8}	8.608	16	10^{-7}	8.00×10^{8}	8.904	18	10^{-7}	9.00×10^{8}	8.955
Red-Blue	57	10^{-6}	2.85×10^{8}	8.455	71	10^{-6}	3.55×10^{8}	8.551	13	10^{-7}	6.50×10^{8}	8.813	19	10^{-7}	9.50×10^{8}	8.978
Red-Yellow	67	10^{-6}	3.35×10^{8}	8.526	96	10^{-6}	4.80×10^{8}	8.682	15	10^{-7}	7.50×10^{8}	8.876	18	10^{-7}	9.00×10^{8}	8.955