SCREENING OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM FRESH WATER FISH INTESTINE

Thesis submitted to

National Institute of Technology, Rourkela for
the partial fulfilment of the Master degree in
Life Science

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2012

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CERTIFICATE

This is to certify that the thesis entitled "**SCREENING OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM FRESH WATER FISH INTESTINE**" submitted to National Institute of Technology, Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by SWATI CHAUHAN under my supervisions and guidance.

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DECLARATION

I hereby declare that the thesis entitled **"Screening Of Potential Probiotic Lactic Acid Bacteria From Fresh Water Fish Intestine"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Bismita Nayak, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: SWATI CHAUHAN

Place:

ACKNOWLEDGEMENT

If words are considerable as symbols of approval and brought as acknowledgement then let the words play a heralding role in expressing my gratitude.

I would like to express my extreme sense of gratitude to **Dr. Bismita Nayak**, Asst. Professor, Dept. of Life Science, NIT Rourkela for her guidance throughout the work and her encouragement, positive support and wishes extended to me during the course of investigation.

I would like to thank the other faculties of Life Science, Dr. Samir Kumar Patra (HOD Life Science), Dr. Surajit Das, Dr. Sujit Kumar Bhutia Dr. Rasu Jayabalan, Dr. Bibekanand Mallik and Dr.Suman jha for their constant support and guidance.

I express my sinceare gratitude to Mr. Pradipta Ranjan Rauta for their inspiring conversation and the good spirit of scientific work.

I express special thanks to all my friends for being there whenever I needed them.

It is my pleasure to be indebted to various people, who directly or indirectly contributed in the development of this work and who influenced my thinking, behaviour, and acts during course of study.

Finally, I am forever indebted to my parents and sister for their understanding and encouragement when it was most required.

Swati Chauhan

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ABSTRACT

Probiotic, Lactic Acid Bacteria are associated with normal microbial ecosystem of gastrointestinal tract of human and fish and plays a vital role during *in-vivo* interactions occurring in Gastro Intestinal Tract (GI) of human beings, hence exert health benefits beyond inherent basic nutrition. In the recent study, some potential probiotic lactic acid bacteria were screened from fresh water fish intestine. Sixteen strains have been selected on the basis of growth in *Lactobacillus* selective media (MRS) and subjected to Gram staining, Catalase activity and Oxidase test. Out of these, 6 strains have been selected for further studies on the basis of morphology, *Lactobacillus* specific biochemical test and amplification of *Lactobacillus* genus specific gene. Five strains named (KTIT, KT2W, KT1B, KA2, FS) have been identified as *Lactobacillus casei. S*ixth strain has been identified and named as KT1, *Lactobacillus delbrueckii* on the basis of bergey's manual of systematic bacteriology. Out of these six strains KT1 has proved its probiotic credibility more by producing antimicrobial Bacteriocin-like compound, which were having inhibitory property against potential pathogenic strains. Strain KT1 was found to be more potential probiotic lactic acid bacteria after further identification by biochemical analysis and molecular identification (16s rDNA Sequencing) was done. The study is extremely promising, that underscores the important role of *Lactobacillus* strains, having probiotic effects, which may play an important role in food industry as starter-culture, co-culture and bio protective cultures to improve quality and safety of preserved food and beverages.

Key words: Probiotics, Lactic acid bacteria, Fish intestine, Bacteriocin, Molecular analysis.

1. INTRODUCTION

Probiotics are defined as “living micro-organisms, which on ingestion in specific numbers exerts health advantages beyond inherent basic nutrition” (Guarner and Schaafsma, 1998; Tannock, 2002) still interest in this area was initiated by Metschnikov 100 years ago (Metschnikoff, 1907). Traditionally, the lactic acid bacteria (LAB) are defined by formation of lactic acid as a sole or main end-product from carbohydrate metabolism. They are associated with normal microbial ecosystem of gastrointestinal tract of human and fish. Lactic acid bacteria comprise a diverse group of non-spore forming, Gram-positive bacteria. They occur as cocci or rods and are generally lacking catalase, although pseudo-catalase can be found in rare cases. They are chemoorganotrophic and grow only in complex media. Fermentable carbohydrates are consumed as energy source. Hexoses are degraded primarily to lactate (homofermentatives) or to lactate and additional products such as acetate, ethanol, CO2, formate or succinate (heterofermentatives). Lactic acid bacteria are present in foods (dairy products, sour dough, fermented meat, fermented vegetables, silage, beverages), in sewage, on plants, as well as within the genital, intestinal and respiratory tracts of humans and animals (Schleifer and Ludwig, 1995; Hammes *et al*., 1991). Lactic acid bacteria (LAB) produce a variety of low molecular mass compounds together with acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide and different metabolites. Several of those metabolites have a broad activity spectrum against different species, and their production is basically affected by the food matrix itself.

Lactic acid bacteria produce a variety of antagonistic factors that consists of end products which are produced metabolically, bactericidal proteins and antibiotic-like substances termed bacteriocins. The rate of inhibitory activity by bacteriocins of lactic acid bacteria can be either narrow inhibiting only those strains that are closely related to the producer organism, inhibiting a diverse group of Gram-positive microorganisms. Bacteriocins of LAB are considered as safe natural preservatives or biopreservatives, because it is assumed that they're degraded by the proteases in gastrointestinal tract (Cleveland *et al*., 2001). Bacteriocins are extracellularly released peptides or protein molecules, with a bacteriostatic mode of action against closely related species. The inhibitory spectrum of several bacteriocins also includes food spoilage and/or food-borne pathogenic microorganisms (Schillinger *et al*., 1996). The discovery of nisin, the first bacteriocin utilized on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocins of LAB has expanded

in the last two decades, looking for novel bacteriocin producing strains from dairy, meat, plant products, and traditional fermented products. Various bacteriocins have been isolated and characterized (Cleveland *et al*., 2001). Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum *Firmicutes*. The genera *Carnobacterium, Lactobacillus, Lactococcus, Lactosphaera, Melissococcus, Pediococcus, Streptococcus, Tetragenococcus* and *Weissella* are recognized as LAB (Stiles and Holzapfel, 1997).

Due to emergence of multi-drug resistant bacteria as serious problem over the past decades, major analysis efforts are aimed toward finding effective drugs. Under such conditions Lactic acid bacteria and their metabolites are good alternatives as a source of antimicrobial agents. Primarily *Lactobacillus* and *Bifidobacterium* species are found in several dairy foods, normal inhabitants of the human gut and supplements and are currently attracting keen interest as health supplements from both consumers and researchers due to heightened awareness of the beneficial connections among health, diet and nourishment.

The properties expected from potential probiotic strains of lactic acid bacteria, compiled by many authors (Kullen and Klaenhammer, 1999; Veld and Shortt, 1996; Havenaar *et al*., 1992; Salminen *et al*., 1996; Tannock 1997) are:

a) Accurate taxonomic identification.
b) Normal inhabitant of the species targeted human origin for human probiotics.
c) Nontoxic and non-pathogenic.
d) Genetically stable.
e) Capable of survival, proliferation, and metabolic activity at the target area.
f) Adherance and colonization potential preferred.
g) Stability of desired characteristics during culture preparation, storage, and delocalisation.
h) Viability at high populations preferred at $10^6 – 10^8$
i) Production of antimicrobial substances, as well as bacteriocins, hydrogen peroxide and organic acids.
j) Antagonistic for pathogenic/cariogenic bacteria.
k) Able to compete with the normal microflora, as well as the same or closely related species; potentially resistant to acid, bacteriocins and other antimicrobials produced by residing microflora.
l) Resistant to bile.

m) Resistant to acid.
n) Immunostimulatory.
o) Able to exert one or more clinically documented health benefits.
p) Amenable to production processing adequate growth, concentration, recovery, freezing, dehydration, storage and distribution.
q) Provision of suitable organoleptic qualities (or no undesirable qualities) when included in fermented products.

Microorganisms used as probiotics:

- *Lactobacillus acidophilus*
- *L.plantarum*
- *L. casei*
- *L. casei subsp. Rhamnosus*
- *L. delbreuckii subsp. Bulgaricus*
- *L. fermentum*
- *L.reuteri*
- *Lactococcuslactis subsp. Lactis*
- *L. lactis subsp. Cremoris*
- *Bifidobacteriumbifidum*
- *B. infantis*
- *B.adolescentis*
- *B. longum*
- *B. breve*
- *Streptococcus salivarius subsp. Thermophiles*
- *Enterococcus faecalis*
- *E.faecium*
- *Saccharomyces boulardii*

The immune system of mammals includes a complex array of cells and molecules, which interact to produce protection from challenge by pathogenic microbes (bacteria, viruses, parasites). Antigens are substances that induce an immune response, are usually parts of invading microbes. Varied organs participate during this immune response. As an example, the central lymphoid organs (bone marrow, thymus) contribute towards the ontogenesis of the

different immune cells, whereas the peripheral lymphoid organs (spleen, lymph nodes, mucosal lymphoid tissue) orchestrate the immune response. Most of the antigens penetrate into the body through the mucosa and the mucosal immune system of the host plays a key role in the defense response to pathogens. The intestinal microbiota is that the largest supply of microbial stimulation that exerts both harmful and beneficial effects on human health. Therefore, it acts as a primary agent because it participates in the development of the postnatal immune system as well as oral tolerance and immunity. It's possible that the microbiota acquired during and immediately after birth is necessary for the newborn's systemic and mucosal immunity, and it should also be responsible for controlling inflammatory responses in allergic and inflammatory bowel diseases. If so, probiotics may impede these inflammatory processes by stabilizing the intestinal microbial environment and intestinal permeability barrier by fostering the degradation of enteric antigens and altering their immunogenicity. The leading proposed explanations for the action mechanisms of probiotics against bacterial pathogens are immunostimulation and immunomodulation. Lots of various immunologic studies have been made in the probiotic field using different strains and different models. The objective of this review was to create the point about the immunological potential of probiotic and to highlight what is already acquired and what is the direction to take to better define an immunologic profile of a probiotic strain. An excellent review (Cummings *et al*., 2004) gave excellent explanations regarding the normal function of the gut and the immune system in healthy person with a detailed description of the systemic and mucosal immunity. Our review is split into two parts. The first, discussing the work of (Cummings *et al*., 2004), briefly described the key actors and answers of the innate and adaptive immune system (1) and the mucosal immune system (2). The second half is concentrated on the interactions between probiotics and intestinal epithelium and their impact in innate immunity (3), adaptive immunity (4) and particularly on the Th1/Th2 balance (5). The *in vitro* tests show the cytokine profile of probiotic strains are described (6) and clinical studies evaluating the effects of probiotics in the treatment of several chronic inflammatory diseases and allergies are reported (7).

Probiotic cultures are associated traditionally with cultured of milks and dairy product, from which there's substantial proof for positive effects on human health and general well-being (Kaenhammer, 2000; Reuter, 2001). Many *in vitro* and *in vivo* experiments on antagonism of various *Lactobacillus* strains against *Helicobacter pylori* and *Clostridium difficile, Campylobacter jejuni*, *E. coli* was done. All tested human *Lactobacillus* strains are able to

inhibit the expansion of all strains of anaerobic human gastrointestinal pathogens. Additionally, bacteriocins have properties like antitumour and anticholestrol activity. Chemical reactions are related to reduction of nitrate, improvement in immunological status and adsorption of vitamins B group. The transity lactic acid bacteria among the GI tract are capable of delivering enzymes and different substances into the intestine that probably facilitate to regulate the intestinal flora (Collins *et al*., 1999). Also, the antioxidative activity of lactic acid bacteria is reported. Due to inhibitory impact, selected probiotic *lactobacilli* could also be used as biological preservative, so, the objective of this study was to provide information on isolation, growth antimicrobial activity, impact of pH, heat, and sensitivity to proteolytic enzymes of *lactobacillus* as probiotic bacteria.

Growing human population urges the immense need to exploit the existing livestock resources to meet the animal protein requirements. The concept of helpful microbes is hundred years old when the people were in habit of consuming fermented milk. (Lilly and Stillwell, 1965), 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, that beneficial for the host animal by improving the 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. The most commonly used organisms in probiotic preparations are lactic acid producing bacteria such as *lactobacilli, streptococci, Bifidobacteria* and fungi such as *Sacharomyces cerevisiae, Sacharomyces boulardii* and *Aspergillus oryzae* (Medina *et al*., 2001). 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. 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 should be well studied and documented. The present study was aimed at isolating and characterizing *lactobacilli* from avian microbiota and fermented milk products and to study probiotic properties of these isolated *lactobacillus* species for their use in chicken.

The modern term ‘probiotic’ was first used by Fuller (Fuller, 1989), a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance. After that it was demonstrated as heat-inactivated bacteria or fragments of bacterial DNA have positive effects as well. (Marteau *et al*., 2002) defined probiotics as ‘microbial cell preparations or components of microbial cells that have a beneficial effect on the health and wellbeing’. The mechanisms by which probiotics beneficially affect the host are multiple. Probiotics can prevent or ameliorate diarrhoea and inflammation through their local effects and/or their effect on the immune system. In the gut, probiotic bacteria may occupy binding sites on the gut mucosa, preventing pathogenic bacteria binding to the mucosa. *Lactobacilli* produce proteinaceous compounds, particularly bacteriocins, that act as local antibiotics against more pathogenic organisms and decrease production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-12. Probiotics stimulate IgA production (Kaila *et al*., 1992). *Lactobacilli* give acetic and lactic acid and inhibit the growth of bacterial pathogens. It has been postulated that probiotics compete with pathogens for nutrients and modify toxins produced by pathogens or toxin receptors found in the gut wall. (Saavedra, 1995) have shown that specific DNA repeats isolated from probiotics can attenuate experimental colitis in various animal models. This is true even with inactivated bacteria. By using toll-like receptor 9 (TLR-9) deficient mice, they have showed that TLR9 signaling was essential in mediating the anti-inflammatory effect of probiotics. Prerequisites for probiotics are to be effective and safe. The properties of an good probiotic defined by Saavedra (Saavedra, 1995) are resistance to digestion by enteric or pancreatic enzymes, gastric acid and bile, ability to stop the adherence, establishment and replication of pathogens in the gastrointestinal tract (GI Tract). The examples of probiotic bacteria are members of the *Lactobacilli* family such as *Lactobacillus Rhamnosus* GG, *Bifidobacteria* and the yeast *Saccharomyces Boulardii*. There are many bacteria which can be qualified as probiotics, but different bacteria have unlike actions in different disease states, taking into explanation that some disease are better treated with a combination of bacteria and that there is an issue of dosing and viable vs. non-viable elements of the bacteria. Treatment with probiotics can safe, but it was not risk free. Probiotics are potentially pathogenic. A recent report describes 3 patients with fungemia in whom the probiotic origin was proven by DNA fingerprinting (Munoz *et al*., 2005). Reports of infections of probiotic origin emphasize the fact that these patients are usually immunosupressed with multiple ports of entry, like venous and urinary catheter.

Probiotic *Lactobacillus* is known to confirm various health promoting activities on their host after either parenteral or oral administration in rats. Some of their beneficial effects include prevention of intestinal infection (Tannock, 1983), control of serum cholesterol, enhancement of immunity in human and rats, and growth enhancement of poultry and pigs. The mechanisms by which these probiotics affect their host and improve gut barrier can be due to: competition for adhesion site, production of inhibitory compounds, and rebalancing of interrupted gastrointestinal (GI) microbial composition and metabolism. *Lactobacilli* are not regular inhabitants of the digestive tract in rabbits and poorly adhere to epithelial cells; therefore, their usefulness is doubtful in such species. Studies on different clinical approaches in pet rabbits (Fann *et al*., 2001) showed that *Lactobacillus* can be successfully used in therapies instituted for antibiotic-associated enteritis, and suggested two possible mechanisms of action. The first is that *Lactobacillus* has been shown to have an inhibitory effect on pathogenic *E. coli* and so it would be useful in the event of *E. coli* population. The second theory is that, also in rabbit, *Lactobacillus* is a normal gut inhabitant (Das *et al*., 1997) that may be eradicated with inappropriate antibiotic administration. Another condition is that, being living microrganisms, the application of probiotics to a large number of animals as under commercial conditions must be effective, should be administered early in life as possible, and should understate uncontrolled variables such as water quality and proportioner/ medicator function and consistency. These cosequences can be addressed and minimized if the probiotic were administered by spray application, as observed in poultry.

Interest in the role of probiotics for human health goes back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life. It is only recently, however, that the interrelationship between intestinal microorganisms and the health benefits deriving from it are beginning to be understood. Presently it is generally recognised that an optimum 'balance' in microbial population in our digestive tract is associated with good nutrition and health. The microorganisms primarily related to this balance are *Lactobacilli* and *Bifidobacteria*. Factors that negatively effects the interaction between intestinal microorganisms, like stress and diet, tends to detrimental effects in health. Increasing evidence indicates the consumption of 'probiotic' microorganisms can help maintain such a favourable microbial profile and results in several therapeutic benefits. Recently probiotic bacteria have increasingly been incorporated into foods as dietary adjuncts. One of the most famous dairy products for the delivery of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cells is bio-yogurt. Sufficient number of viable cells, such as the 'therapeutic minimum' needs to be consumed regularly for

transfer of the 'probiotic' effect to consumers. *Lactobacillus acidophilus* non-pathogenic and a member of the normal intestinal microflora is widely used in fermented dairy products and is of considerable industrial and medical interest because it has been reported to aid in the reduction of the levels of harmful bacteria and yeasts in the small intestine and to produce lactase, an enzyme which is important for the digestion of milk (Deraz et al., 2007). So, *L. acidophilus* group of lactic acid bacteria (LAB) is added as dietary adjuncts to commercial fermented milk products and the intake of these bacteria may have beneficial effects on human health. The properties of *L. acidophilus* have been investigated in order to establish its specific role in the complex microbial intestinal equilibrium, both of man and higher animals. The *L. acidophilus* has been considered to be the predominant *lactobacillus* in the intestinal tract of healthy humans (Ray, 1996). *L. acidophilus* strains have been widely utilized as a dairy starter culture for their therapeutic activities associated with an intestinal microbial balance, and has been used in fermented foods, and as an probiotic in dietary supplements. Recent in vitro studies showed that *L. acidophilus* is a strong Th1 cytokine (IL-12, IFN-γ) inducer. *L. acidophilus* significantly up-regulated surface markers on dendritic cells (DCs), including HLA-DR, CD40, CD86 and CD83. There is an increasing interest in the research of antimicrobial peptides (bacteriocins and bacteriocin-like compounds) produced by lactic acid bacteria (LAB) because of their potential use as antimicrobial agents for improving the safety of food products. Among Lactobacilli, strains belonging to species of the *L. acidophilus* complexes are most frequently used as probiotics. Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (Klaenhammer and Kullen, 1999). It may contribute to the colonisation resistance of the host and its protection against gastrointestinal pathogens. As the bacteriocin producing strain *L. acidophilus* IBB 801, a dairy isolate, shows antibacterial activity against *Escherichia coli* and *Salmonella*, the authors told that it may have potential as a probiotic. Numerous reports have proved the power of L. acidophilus strains to produce bacteriocins. *L. acidophilus* strains exhibiting antagonistic activity towards certain types of psychrotrophic microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium sp*. were especially important as these microorganisms even at low levels in food pose a significant spoilage and public health threat. Bacteriocins are lethal to closely related bacteriocin associated species, food-borne pathogens and spoilage bacteria (Klaenhammer, 1993). Among the Lactobacillus species, *L. acidophilus* strains have been extensively utilized as probiotic cultures in dairy and pharmaceutical products and numerous reports have proved its ability to produce bacteriocins, yet no review has been published summarizing its bacteriocin production. Since

(Metchnikoff, 1908) proposed a role for *Lactobacilli* in suppressing undesirable intestinal microflora, numerous researchers have investigated the antimicrobial activities of *L. acidophilus*. Broad spectrum inhibition has been clearly demonstrated for organic acids and hydro-gen peroxide produced by *L. acidophilus*. In addition, a number of reports suggest that antimicrobial proteins, or bacteriocins, either mediate or facilitate antagonism by *L. acidophilus*. (Vincent *et al*., 1959) first described a bacteriocin-type inhibitor produced in aged liver veal agar cultures of *L. acidophilus*. Crude "lactocidin" was non-volatile, non-dializable, insensitive to catalyze, active at neutral pH and displayed inhibitory activity against numerous genera, including *Proteus, Salmonella*, *Escherichia*, *Staphylococcus*, *Bacillus*, *Streptococcus*, and *L. actobacillus*. No further studies on lactocidin, or similar broad-spectrum bacteriocins produced by *L. acidophilus* has been reported. However previous studies of antagonism by *L. acidophilus* do not specifically address, identify, or confirm the involvement of bacteriocins.

The objectives of the current work are given below:

1) Isolation of LAB from fish intestine by growing on *Lactobacillus* selective media (MRS) in anaerobic Condition.
2) Selection of LAB by amplification of *Lactobacillus* specific gene.
3) Inhibitory action of isolated LAB strains against Bacillus sp., Klebsiella sp., *Proteous sp*., *E. coli*, and *Pseudomonas sp*.
4) Identification of potential probiotic LAB by biochemical as well as molecular characteristics.

2. REVIEW OF LITERATURE

2.1. PROBIOTICS

The original observation of the positive role played by bound bacteria was 1st introduced by Russian scientist and Nobel laureate Elie Metchnikoff, who within the starting of the twentieth century urged that it'd be doable to switch the gut flora and to exchange harmful microbes with helpful microbes (Metchnikoff, 1907). Metchnikoff, proposed the hypothesis that the aging method results from the activity of putrefactive (proteolytic) microbes manufacturing toxic substances within the giant bowel. Proteolytic bacteria like *Clostridia*, that are a part of the traditional gut flora, manufacture toxic substances together with phenols, indols and ammonia from the digestion of proteins. According to Metchnikoff these compounds were liable for what he referred to as "intestinal autointoxication" that caused the physical changes related to adulthood. It absolutely was at that point known that milk fermented with lactic-acid bacteria inhibits the expansion of proteolytic bacteria thanks to the low pH created by the fermentation of lactose. Metchnikoff had conjointly observed that bound rural populations in Europe, for instance in Bulgaria and therefore the Russian steppes that lived largely on milk fermented by lactic-acid bacteria were exceptionally long lived. Primarily based on these facts, Metchnikoff proposed that consumption of fermented milk would "seed" the intestine with harmless lactic-acid bacteria and reduce the intestinal pH which this is able to suppress the expansion of proteolytic bacteria. Metchnikoff himself introduced in his diet bitter milk fermented with the bacteria he referred to as "*Bulgarian Bacillus*" and located his health benefited. *Bifidobacteria* were 1st isolated from a breast-fed infant by Henry Tissier. The isolated bacterium named *Bacillus bifidus communis* (Tissier, 1900) was later renamed to the genus *Bifidobacterium*. Tissier found that *bifidobacteria* are dominant within the gut flora of breast-fed babies and he observed clinical advantages from treating diarrhoea in infants with *bifidobacteria*. The claimed result was *bifidobacterial* displacement of proteolytic bacteria inflicting the disease. Throughout a pestilence of shigellosis in 1917, German professor Alfred Nissle isolated a strain of *Escherichia coli* from the faeces of a soldier who wasn't suffering from the disease. Ways of treating infectious diseases were required at that point when antibiotics weren't nevertheless obtainable, and Nissle used the Escherichia coli strain in acute gastrointestinal infectious salmonellosis and shigellosis. In 1920, Rettger demonstrated that Metchnikoff's "*Bulgarian Bacillus*", later

referred to as *Lactobacillus delbrueckii subsp. bulgaricus*, couldn't live within the human intestine, (Cheplin and Rettger, 1920) and therefore the fermented food phenomenon diminished. Metchnikoff's theory was disputable, and folks doubted his theory of longevity. Once Metchnikoff's death in 1916, the centre of activity moved to the U.S. It absolutely was reasoned that bacteria originating from the gut were a lot of seemingly to provide the required result within the gut, and in 1935 bound strains of *Lactobacillus acidophilus* were found to be terribly active when implanted within the human digestive tract. Trials were administrated using this organism, and inspiring results were obtained particularly within the relief of chronic Constipation. The term "probiotics" was 1st introduced in 1953 by Werner Kollath (Hamilton-Miller *et al*. 2003). Contrasting antibiotics, probiotics were outlined as microbially derived factors that stimulate the expansion of different microorganisms. In 1989, Roy Fuller urged a definition of probiotics that has been widely used: "A live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance". (Fuller, 1989) Fuller's definition emphasizes the necessity of viability for probiotics and introduces the facet of a helpful result on the host. Within the following decades, intestinal lactic acid bacterial species with alleged health helpful properties are introduced as probiotics, together with *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus johnsonii* (Tannock, 2003).

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* and fungi like *Sacharomyces cerevisiae* and *Aspergillus oryzae* (Fuller, 1992; Medina *et al*., 2001). 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 should be well studied and documented. The present study was aimed at isolating

and characterizing *Lactobacilli* from avian microbiota and fermented milk products and to study probiotic properties of these isolated *Lactobacillus* species for their use in chicken.

2.1.1 EARLY HISTORY OF THE USE OF MICROORGANISMS FOR HUMAN BENEFIT

There is proof from wall carvings that cultured milk merchandise was created a minimum of 4500 years ago. Written proof for fermented milks seems in Genesis 18: eight, "He then brought some curds and milk that had been ready and set these before them". The assembly of wine is cited in Genesis 9: twenty, "Noah a person of the soil, proceeded to plant a vineyard, where he drank a number of its wine, he became drunk and lay uncovered within his tent". In Exodus 12: thirty-nine the utilization of microorganisms to arrange bread is cited: "They baked the dough that that they had brought out of Egypt into cakes of unleavened bread. The exodus from Egypt is believed to own occurred approximately in 1440 B.C. Homer within the Iliad, written between 900 and 800 B.C. makes various references to wine and cheese. In book eleven of the Iliad there's the subsequent passage: "Pours an oversized portion of Pramnian wine; with goats milk cheese a flavourous style bestows, and last with flour the smiling surface stows". The traditional production of wine, cheese and bread served variety of helpful functions. It altered the flavour and texture of the natural foods and within the case of milk merchandise extended the time of edible use by preventing speedy spoilage by random bacterial or fungal growth. Within the case of wine, additionally to its pleasurable mind-altering properties, wine was used as an anesthetic. During a 10th-century Persian work, the Shahnameh, the utilization of wine was described for performing Caesarean sections. In India wine was used as an anesthetic by the surgeon Sushruta around 600 B.C. so a protracted history exists for the utilization of microorganisms to learn the human condition. In additional recent times an early reference to the utilization of microorganisms for a particular medical condition was proposed by (Doderlein, 1892), during which year he proposed to treat vaginal infections with *Lactobacilli*. In 1900 Henry Tissier at the Pasteur Institute isolated a *Bifidobacterium* from a breast-fed infant (Tissier, 1905). This bacterium is currently designated *Bifidobacterium bifidus*. Tissier additionally showed that *Bifidobacteria* are the predominant organism found in breast-fed infant faeces and counseled administering this organism to infants with diarrhoea. In 1907 the utilization of a particular category of microorganisms to learn human health was introduced to the overall public by the

Nobel Prize winner Elie Metchnikoff. In his book The Prolongation of Life (1907), Metchnikoff stated his belief that bacteria within the colon were liable for adverse health in adults which consuming bitter milk or yogurt would counteract these harmful bacteria. He proposed that the strain "*Bulgaricus Bacillus*", later named *Lactobacillus bulgaricus,* was the strain liable for conferring higher health and longer life in humans. In 1911 Douglas revealed The Bacillus of Long Life that supported the concept of human longevity and also the consumption of fermented milk. In 1917 Alfred Nissle isolated an *Escherichia coli* that he used to treat acute intestinal diseases like salmonellosis and shigellosis, with a big success rate. This organism is currently designated *E. coli* Nissle and remains used as a probiotic and is an example of a non-lactic acid bacteria probiotic. In 1935, Retteger at Yale University proposed that *Lactobacillus acidophilus* would be an acceptable species to use for human clinical trials (Retteger et al., 1935). This approach was followed by a study demonstrating positive results for patients with chronic constipation. The utilization of specific bacteria for human disorders dates to the Twenties however the term "probiotic" wasn't employed in this context till 1974. (Parker, 1974) described probiotics as "organisms and substances, that contribute to intestinal microbial balance". In 2002, a EEC knowledgeable Committee outlined probiotics as "living microorganisms, that upon ingestion in adequate amounts exert health advantages beyond inherent general nutrition".

2.1.3 OVERVIEW OF PROBIOTIC STUDIES AND RESULTS FOR THE PAST 35 YEARS

Based on the definitions for a probiotic expressed in 1974 and changed in 2002, a major variety of microorganisms are isolated and identified as probiotics. A number of these probiotics are fed to humans and animals to check, treat or forestall varied diseases, disorders and syndromes. The approximate variety of various bacterial strains in every genera that are attributed as probiotics are as follows: *Lactobacillus*, 23; *Bifidobacterium*, 5; *E. coli*, 2; and one strain every of *Bacillus, Streptococcus, Enterococcus* and *Lactococcus*. Additionally there's solely yeast, specifically *Saccharomyces boulardii* that has probiotic attributes (Sanders, 2007). With the corresponding isolation and identification of probiotic microorganisms there has been an increasing variety of basic researches, clinical analysis, clinical trial and intervention studies printed. Year to year, since the mid-1980s, the quantity of papers has increased exponentially. It'll not be doable in a very chapter or a book to hide

all the studies in print and thus the subsequent sections describe the highlights of the findings on health advantages.

2.1.4 CURRENT EVIDENCE FOR PROBIOTIC HEALTH BENEFITS

2.1.4.1 Lactose Intolerance

Worldwide several scores of individuals expertise lactose malabsorption. The frequency of the disorder will increase with age. The cause for this disorder may be a decline within the activity of the enzyme lactase within the intestinal brush border mucosa. This decline in activity ends up in lactose malabsorption. This incomplete absorption causes flatus, bloating, abdominal cramps, and moderate to severe diarrhoea. A serious consequence of this sequence of events may be a severe limitation in consumption of dairy merchandise, that is especially pronounced within the elderly. Many studies have demonstrated that in the fermentation of milk to create yogurt lactase is made and on consumption of yogurt this lactase is active within the intestinal tract (Kim and Gilliland, 1983; Kolars *et al*., 1984). The organisms used for the assembly of yogurt are *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*. (Kim and Gilliland, 1983) found that feeding yogurt to participants who were lactose-intolerant caused a major reduction within the levels of hydrogen found within the breath compared with feeding milk to subjects with constant condition. the extent of hydrogen within the breath reflects the intestinal microflora metabolism of lactose not absorbed within the little intestine and therefore gift within the colon, where the microflora are gift in high concentrations. (Kolars *et al*., 1984) found that subjects who ingested eighteen g of lactose in yogurt had sixty seven less hydrogen in their breath compared with constant lactose dose delivered in milk. An analysis of intestinal duodenal aspirates obtained from the themes consuming yogurt indicated that there have been vital levels of lactase within the duodenum. A scientific review of the printed literature in 2005 analysing studies of probiotic treatment of adult lactose intolerance concluded that the proof doesn't support the effectiveness of probiotics for treatment of this disorder (Levri *et al*., 2005). However, the authors conclude that this could result from the variation within the nature or variety of probiotic employed in the particular study. For instance, *lactobacilli* that have low levels of lactase may be potential confounder. The strains selected for yogurt production have high lactase levels, needed for the economical preparation of yogurt.

2.1.4.2. Inflammatory bowel disease

Inflammatory bowel disease (IBD) may be a major medical drawback. IBD may be a general term used for intestinal inflammation, and also the specific diseases and disorders that fall into the IBD class embrace Crohn's disease, ulcerative colitis, and irritable bowel syndrome. One amongst the necessary potential medical applications for probiotics is that the treatment and prevention of IBD relapses. There are a restricted range of reports of the useful effects of probiotics in treating or assuaging IBD symptoms. It's been shown that *E. coli* Nissle is useful in maintaining the remission part for patients with Crohn's disease. Administration of *Lactobacillus salivarius* in milk to interleukin (IL)-10 knockout mice considerably reduced inflammation within the cecum and colon compared with identical knockouts fed milk alone. IL-10 is an anti-inflammatory cytokine that causes progressive colonic inflammation when levels are low or absent, as is that the case for these knockout mice. These results counsel that probiotics, alone or by interaction with the present intestinal flora, will influence the colonic immune system and counteract low IL-10 levels. IL-10 is often expressed in T cells within the lamina propria of the colon. In another murine model study, IL-10 knockout mice treated with a mixture of *L. salivarius* and *Bifidobacterium longum subsp. infantis* in an exceedingly dairy product resulted in an exceedingly decrease in disease severity. The severity of disease was evaluated by weight loss, colon pathology and general look over a 6-week amount (McCarthy *et al*., 2003). Management animals fed solely dairy product exhibited a chronic wasting disease throughout identical time amount. A study involving thirty two patients with Crohn's disease in clinical remission and given mesalamine or mesalamine and *S. boulardii* showed that thirty seventh of patients given the drug alone relapsed in half-dozen months whereas half-dozen.5% of patients receiving drug and *S. boulardii* relapsed. The knowledge counsel that *S. boulardii* may well be a helpful adjuvant for preventing symptomatic relapse in patients with Crohn's disease. The total of the present human and animal probiotic IBD literature is preliminary and equivocal; but, it will counsel that specific probiotics may well be helpful in preventing symptomatic relapse for patients with ulcerative colitis and/or Crohn's disease.

2.1.4.3 Treatment of gastroenteritis

The most in depth probiotic medical literature is within the space of diarrheal diseases (gastroenteritis).The treatment and prevention may be more categorized by etiologic agent or by the sort of disease.

2.1.4.3a. Antibiotic-associated diarrhoea

There are various studies investigating the efficacy of probiotics for preventing or reducing the frequency and severity of diarrhoea related to the clinical use of antibiotics (Arvola *et al*., 1995; Vanderhoof *et al*., 1999; Armuzzi *et al*., 2001a, b). When finding out 119 youngsters who received antibiotics for respiratory infections and concomitant *Lactobacillus rhamnosus* GG (LGG) or placebo throughout the antibiotic treatment amount, investigators found a seventieth reduction in diarrheal symptoms for the cluster administered LGG compared with a placebo arm (Arvola *et al*., 1995). In an exceedingly larger study involving 202 youngsters treated with oral antibiotics, V-E Day of the youngsters who got LGG concurrently with antibiotic experienced diarrheal symptoms compared with twenty sixth of the placebo cluster (Vanderhoof *et al*., 1999). In 2 studies with sixty and one hundred twenty adult patients respectively receiving antibiotic treatment to eliminate a *Helicobacter pylori* infection, investigators found that a considerably lower variety of patients who received concurrent LGG experienced nausea and diarrhoea compared with a gaggle given placebo (Armuzzi et al., 2001a,b). *Helicobacter pylori* are identified as an etiologic agent for gastric ulcers. *Saccharomyces boulardii* has conjointly been shown to cut back antibiotic associated diarrhoea (Marchand and Vandenplas, 2000). A meta-analysis of the impact of probiotic administration on antibiotic-associated diarrhoea comprising twenty two placebo-controlled studies found a combined relative risk of 0.39 for diarrhoea among the probiotic-treated cohorts. The investigators concluded that a robust profit exists for probiotic administration for antibiotic-associated diarrhoea, though they cautioned that the proof isn't however definitive and a lot of studies are needed.

2.1.4.3b. Acute diarrhoea

Numerous studies have reported the employment of probiotics to forestall or treat acute diarrhoea (Cetina-Savri and Sierra, 1994; Pant *et al*., 1996; Shornikova *et al*., 1997a, b; Mastretta *et al*., 2002; Allen *et al*., 2003). The bulk of the studies concerned infants or kids and therefore the etiologic agent was rotavirus or of unknown cause. Probiotics that are shown to be effective for the treatment of acute gastroenteritis embrace LGG, *Lactobacillus reuteri* and *S. boulardii* (Pant *et al*., 1996; Shornikova *et al*., 1997a, b; Mastretta *et al*., 2002; Allen *et al*., 2003). A multicenter European based mostly trial with 287 kids aged 1–36 months from ten countries is one amongst the foremost intensive trials investigating probiotic treatment for acute diarrhoea reported. The youngsters were experiencing moderate to severe diarrhoea. The patients were randomized to be given placebo or LGG together with oral

rehydration answer. The youngsters receiving LGG had a shorter period and decreased severity of disease together with a shorter hospital keep. Another necessary finding was that on follow-up the probiotic treated kids had a decreased probability of persistent diarrheal illness. There are alternative samples of findings the same as those described on top of in kids with diarrheal disease (Pant *et al*., 1996; Shornikova *et al*., 1997b). A review of the double-blind randomized literature for probiotic biotherapeutic agents found that LGG and *S. boulardii* had the foremost favorable impact for treatment of acute diarrhoea in kids and adults (Marchand and Vandenplas, 2000).

2.1.4.3c. Traveler's diarrhoea

Visitors from temperate climate countries when move to areas with tropical or subtropical climates, they experience a high incidence of diarrhoea. The incidence rate typically approaches five hundredth. There are many printed studies that have investigated the efficacy of probiotic treatment for lowering the diarrheal incidence rate. A study that tracked Finnish travellers to Turkey showed that in one among 2 resorts oral ingestion of LGG conferred a big protection rate of thirty.5% and 27.9% in weeks one and a couple of the study respectively. The travelers were given LGG or a placebo before their trip and LGG afforded a protection rate of forty seventh (Hilton *et al*., 1997). (McFarland, 2007) performed a meta-analysis of studies designed to analyze probiotics for the prevention of traveler's diarrhoea. The analysis included twelve studies that met the inclusion and exclusion criteria. The results of the analysis showed that the pooled relative risk was zero.85 ($P < 0.001$) which probiotics considerably stop traveler's diarrhoea. The meta-analysis investigator conjointly concluded that *S. boulardii* and a combination of *L. acidophilus* and *Bifidbacterium bifidum* had important treatment efficacy.

2.1.4.3d. Treatment of relapsing gastroenteritis caused by *Clostridium difficile* toxin

Often as a result of antibiotic treatment, the conventional intestinal microflora is altered. The disturbance to the microflora may end up in *C. difficile* growth from living spores, with the concomitant production of toxin within the intestinal tract. Many studies have shown that treatment with LGG prevents relapse of gastroenteritis (i.e. recurrent *C. difficile* associated disease, RCDAD) formerly use of antibiotics. Clinical observations have indicated an hour relapse rate once therapy with metronidazole or vancomycin. Solely 16 PF which have received LGG had a relapse and once a second course of treatment with LGG, there was a ninety four overall cure rate. There are many recent studies that have solid doubt

on these earlier findings. No profit was found for a yogurt/LGG formulation for patients with RCDAD (Pochapin, 2000). In an exceedingly tiny study using capsules containing lyophilized LGG there once more was no profit noted with the probiotic, though the study had too few subjects to produce statistical power. It's so not clear if probiotics are useful for patients with RCDAD.

2.1.4.4. Cholesterol lowering

There is some proof based mostly on human studies that probiotics could lower total serum cholesterol and/or low-density lipoprotein (LDL) cholesterol. The results don't seem to be definitive and sometimes conflicting. The lowering of LDL cholesterol would have vital implications for decreasing the danger of coronary artery disease and for fatal myocardial infarction. The human studies that have shown a bearing for fermented milk merchandise on plasma cholesterol levels found a lowering of total cholesterol between five.4 and 23.2% and for LDL between nine and nine.8%. A recent study of fourteen subjects in an exceedingly randomized crossover trial involving normal yogurt or yogurt and *L. acidophilus* and *B. animalis subsp. lactis* for 6-week feeding amounts and a 4-week washout period found a big decline in serum total cholesterol when comparing the yogurt and probiotics to the yogurt alone. The cholesterol studies have had little numbers of subjects and were restricted in length, typically in weeks. Based mostly on in vitro and animal studies, many mechanisms for the probiotic lowering of serum cholesterol are proposed. These involve absorption or assimilation of cholesterol by probiotics. There has been a study showing optimal removal of cholesterol from growth media within the presence of *L. casei* and a prebiotic (Liong & Shah, 2005). A separate mechanism that has been proposed for probiotic-induced cholesterol lowering is that the ability of *bifidobacteria* and *lactobacilli* to deconjugate bile acids. The deconjugation would cause additional fast excretion of bile acids within the faeces and since cholesterol may be a precursor for bile acid synthesis, the lower bile acid concentration would act as a positive feedback for increasing synthesis from cholesterol to bile acids.

2.1.4.5. Treatment for urogenital infections

Vaginal infections are caused by such agents as *Candida, Trichomonas*, or bacterial organisms like *Gardnerella vaginalis* and *Mycoplasma hominis*. Urinary tract infections are much common in girls and are typically caused by *E. coli, Chlamydia* and *Candida*. There are approximately three hundred million urogenital infections reported per year. Traditional healthy girls have approximately fifty completely different species of microorganisms within

the vaginal flora. (Reid *et al*,. 1995) accounted that weekly intravaginal instillation of lactobacilli in ten premenopausal girls reduced urinary tract infections from 6.3 per patient per year before treatment to 1.3 per patient per year throughout treatment. (Hilton *et al*,. 1992) found that yogurt containing *L. acidophilus* lowers the *Candida*-caused vaginitis by threefold in an exceedingly crossover-designed trial. The results of studies using probiotics for treatment or prevention of urogenital infections are terribly restricted, though there are investigators trying to style specific probiotics to be administered orally to stop or cut back the incidence of urogenital infections.

2.1.4.6. Treatment of allergic reactions

The most in depth studies directed at probiotic modulation of the immune response to food allergens are done with LGG for preventing and treating atopic eczema. In an exceedingly study of 159 pregnant girls with a family history of atopic disease the themes got either LGG or placebo for 2–4 weeks before their expected delivery. Girls who breast-fed their infants received LGG or placebo for six months and ladies who bottle-fed their new-borns fed them LGG or placebo for six months. A five hundredth reduction within the incidence of atopic eczema was noted within the 1st a pair of years of the child's life for the cluster receiving LGG compared with the placebo cluster. In an exceedingly follow-up to the current study, when four years the youngsters given LGG had a considerably lower incidence of atopic eczema compared with the placebo cluster (Kalliomaki *et al*., 2003). In another study twenty seven infants with atopic eczema were randomized into 3 teams and given LGG, *Bifidobacterium animalis subsp. lactis* (Isolauri *et al*., 2000). When a pair of months the clinical score for the severity and extent of the eczema indicated a big improvement within the skin condition of the infants fed the probiotics (P = zero.002). an identical study within which thirty one infants with atopic eczema had their exposure to cows' milk terminated and were treated with LGG showed a big improvement compared with a gaggle who weren't fed cows' milk and were fed placebo. *Bifidobacterium animalis* has additionally been shown to scale back the severity of atopic eczema in young kids.

2.1.4.7 Prevention of dental caries

After oral ingestion, probiotics will be isolated from the oral cavity. Thus it'd be logical to review their efficacy in preventing dental caries. Additionally, LGG has been shown to possess antimicrobial activity against the Streptococcus spp., an organism concerned in inflicting tooth decay (Silva *et al*., 1987). Kids during a multicenter daycare trial got LGG-

containing milk or non-supplemented milk and examined before and when the 7-month intervention study. The kids receiving the probiotic had a lower rate of clinical development of dental caries, that was most pronounced within the cluster aged 3–4 years (Nase *et al*., 2001). Lot of studies are required to check if this observation will be repeated and if alternative probiotics can have identical useful result.

2.1.4.8 Treatment and prevention of cancer by probiotics

By virtue of their metabolic activity, probiotics will influence the etiology of colon cancer and presumably tumors at different sites. Probiotics are shown to cut back intestinal bacterial enzymes concerned within the activation of procarcinogens (Hosoda *et al*., 1996). Probiotics can also manufacture short-chain fatty acids which will even be protecting within the colon. Animal studies in rats have shown that probiotics will inhibit the formation of aberrant crypt foci within the colon. Acombination of inulin and *B. longum* reduced chemically induced aberrant crypt foci by seventy four (Rowland *et al*., 1998). Inulin alone lowers the aberrant crypts by twenty first. Rats fed a combination of oligofructose, inulin, LGG and *B. animalis subsp. lactis* had considerably lower azoxymethane-induced colon tumors (Marotta *et al*., 2003). Mice genetically bred to be at risk of colitis and colon cancer had a tenth incidence rate of adenocarcinoma when fed *L. salivarius* compared with the five hundred rate for management animals (O'Mahoney *et al*., 2001). Rats injected with DMH and fed LGG had a considerably lower colon cancer incidence than animals receiving DMH alone. Human colon cancer trials haven't been conducted with probiotics, primarily owing to the problem of conducting a preventive intervention trial. There's one report within the literature of an individual's trial of patients with superficial bladder cancer. The patients receiving *L. casei* had an eightieth longer disease free amount, with a mean of 350 days compared with 195 days for the management cluster.

2.1.4.9. Additional health benefits attributed to probiotics

There are variety of alternative health edges that are observed for probiotic use over the past number of years. Some are included during this section. A study conducted in Italy with kids laid low with cystic fibrosis and given LGG for the chronic abdominal pain typically related to the disease indicated that the frequency and severity of abdominal issues were reduced which intestinal inflammation as judged by the faecal marker calprotectin and rectal nitric oxide was conjointly decreased. Rheumatoid arthritis may be a systemic inflammatory disease. Animal studies using experimental arthritis model in Lewis rats

showed that these rats improved when fed LGG compared with placebo. The findings of a preliminary study involving twenty one patients with rheumatoid arthritis receiving either placebo or LGG showed that the LGG cluster had a decreased variety of swollen joints and lower overall arthritic activity, though the distinction failed to reach statistical significance. (Nanji *et al.*, 2005) studied the flexibility of probiotics to stop alcohol-induced liver disease during a rat model. Rats were conditioned to drink ethanol and one cluster was administered LGG orally. The rats fed LGG had reduced liver disease and lower plasma endotoxin levels. During a connected study rats got carbon tetrachloride to induce chronic liver disease as a model to check the efficacy of probiotics in spontaneous bacterial peritonitis. LGG wasn't effective and failed to stop bacterial overgrowth or bacterial translocation from the colon into mesenteric lymph nodes or portal blood. The result of probiotics on radiation exposure has been studied during a mouse model (Dong *et al*., 1987). Mice were either fed LGG or maintained on a standard diet and then exposed to total body irradiation. The LGG-fed rats had a considerably lower mortality rate at forty eight hours when irradiation. Of the twenty one management mice ten had *Pseudomonas aeruginosa* bacteremia, compared with one of twenty one mice fed LGG. None of the LGG-fed mice had LGG bacteremia. There's a preliminary study from Japan employing a streptozotocin-induced diabetic mouse model that showed that feeding LGG lowered hemoglobin A1c blood levels and improved glucose tolerance compared with controls. Bone marrow transplantation patients will develop graft-versus-host disease (GVHD). Bacterial lipopolysaccharide (LPS) is believed to be concerned during this method. A mouse model of GVHD has been developed where the disease is induced by using a significant histocompatibility mismatch (Gerbitz *et al*., 2004). The animals show serious injury to the bowel mucosa and high levels of serum LPS and inflammatory cytokines. The animals were divided into 3 teams, receiving in their drinking water LGG, ciprofloxacin or no additive for seven days before transplantation. Treatment with LGG reduced mortality that was most distinguished within the early post-transplantation amount and was mirrored during a lower GVHD score compared with the opposite teams. Mesenteric lymph nodes of LGG treated animals had a lower concentration of translocated intestinal organisms.

2.1.4.10. Conclusions based on past and present use of probiotics for health applications

This section has outlined the current knowledge regarding the application of probiotics for preventing and treating medical diseases and disorders. Table 1 (Barry., 2011)

lists the medical applications for probiotics that have been studied in the past and which are currently under investigation.

TABLE 1: Past and Current Applications for Probiotics

Medical condition	Example of probiotic used or studied
Antibiotic-associated diarrhea	*Lactobacillus, Saccharomyces boulardii*
Lactose malabsorption	*Lactobacillus, Streptococcus thermophilus, Streptococcus salivarius*
Acute diarrhea	*Lactobacillus, Bifidobacterium, S. boulardii*
Traveler's diarrhea	*Lactobacillus*
Vaccine adjuvant	*Lactobacillus*
Vaginitis	*Lactobacillus*
Dental caries	*Lactobacillus*
Relapsing *C. difficile* colitis	*Lactobacillus*
Inflammatory bowel disease	*Lactobacillus, Bifidobacterium, S. boulardii*
Rheumatoid arthritis	*Lactobacillus*
Cirrhosis of the liver	*Lactobacillus*
Cystic fibrosis abdominal side effects	*Lactobacillus*
Food allergies	*Lactobacillus*
Diabetes	*Lactobacillus*
Graft-versus-host disease	*Lactobacillus*
Cancer	*Bifidobacterium, Lactobacillus*
Nasal pathogen colonization	*Lactobacillus*
Radiation side effects	*Lactobacillus*
Hypercholesterolemia	*Bifidobacterium, Lactobacillus*

2.2 LACTIC ACID BACTERIA

2.2.1. Historical background of lactic acid bacteria

Lactic acid-manufacturing fermentation is a recent invention. Varied cultures in various elements of the planet have used fermentation to enhance the storage qualities and nutritive worth of perishable foods like milk, vegetables, meat fish and cereals. The organisms that manufacture this kind of fermentation, lactic acid bacteria, have had a very important role in preserving foods. In developed world, lactic acid bacteria are mainly related to fermented dairy product like cheese, buttermilk, and yogurt. The utilization of dairy starter cultures has become an trade throughout this century.

The concept of the cluster name 'lactic acid bacteria' was created for bacteria inflicting fermentation and coagulation of milk, and defines as those that manufacture lactic acid from lactose. The family name *Lactobacteriaceae* was applied by (Orla-Jensen, 1919) to a physiological cluster of bacteria manufacturing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide. Today, lactic acid bacteria are considered synonymous by and huge with the family *Lactobacteriaceae* (Breed et al., 1957).

Since the times of Russian scientist Metchnikoff, lactic acid bacteria have conjointly been related to helpful health effects. Today, an increasing range of health food and so-called purposeful foods similarly as pharmaceutical preparation are promoted with health claims based mostly on the characteristics of sure strains of lactic acid bacteria. Most of those strains, however, haven't been completely studied, and consequently the claims aren't well substantiated. Moreover, health edges are judged mainly using subjective criteria. Additionally, the particular bacterial strains employed in the studies are typically poorly identified. Most data regarding the health effects of lactic acid bacteria is therefore anecdotal. There's clear want for important study of the result on health of strain choice and therefore the quality of fermented foods and their ingredients.

Lactic acid bacteria are a gaggle of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They're non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative. Usually they're non-motile and don't scale back nitrite. They're subdivided into four genera *Streptococcus, Leuconstoc, Pediococcus*, and *Lactobacillus*. Recent taxonomic revisions counsel that lactic acid bacteria cluster can be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus*. Originally, *Bifidobacteria* were included within the genus *Lactobacillus* and therefore the organism was spoken as *Lactobacillus bifidus*. Though the classification of lactic acid bacteria into completely different genera is principally based mostly on the characteristics utilized by (Orla-Jensen, 1919); but, confusion was still prevalent when the monograph of (Orla-Jensen, 1919) appeared. This work has had an oversized impact on the systematic of lactic acid bacteria, and, though revised to some extent, it's still valid and therefore the basis of classification remarkably unchanged. The classification of lactic acid bacteria into completely different genera is basically based mostly on morphology, mode of glucose fermentation, growth at completely different temperatures, and configuration of the lactic acid created, ability to grow at high salt concentrations, and acid or alkaline tolerance. Even a number of the newly described genera of lactic acid bacteria, extra characteristics like fatty acid composition and motility are used because the basis of classification. The term lactic acid bacteria were used synonymously with "milk souring organisms." Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic-acid producing bacteria of other habitats was recognized (Axelsson, 1993). Lactic acid

bacteria are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals. The genera that, in most respects, fit the general description of the typical lactic acid bacteria are (as they appear in the latest Bergey's Manual from 1986) *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus*. The genera *Lactobacillus, Leuconostoc*, and *Pediococcus* have largely remained unchanged, but some rod-shaped lactic acid bacteria, previously included in *Lactobacillus*, is now forming the genus *Carnobacterium* (Collins *et al*., 1987).

2.2.2. Classification at genus level

The basis for the classification of lactic acid bacteria in different genera has essentially remained unchanged since the work of (Orla-Jensen, 1919). Although their morphology is regarded as questionable as a key character in bacterial taxonomy (Woese, 1987), it is still very important in the current descriptions of the lactic acid bacteria genera. Thus lactic acid bacteria can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). An important characteristic used in the differentiation of the lactic acid bacteria genera is the mode of glucose fermentation under standard conditions, i.e., non-limiting concentrations of glucose and growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability. Under these conditions, lactic acid bacteria can be divided into two groups: homofermentative, which convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO_2. In practice, a test for gas production from glucose will distinguish between the groups. *Leuconostocs* and a subgroup of *Lactobacillus* are heterofermentative; all other lactic acid bacteria are homofermentative.

Growth at certain temperatures is mainly used to distinguish between some of the cocci. *Enterococci* grow at both 10^0C and 45^0C, *Lactococci* and *Vagococci* at 10^0C, but not at 45^0C. Streptococci do not grow at 10^0C, while growth at 45^0C in dependent on the species (Axelsson, 1993). Salt tolerance (6.5% NaCl) may also be used to distinguish among *Enterococci, Lactococci/Vagococci*, and *streptococci*, although variable reactions can be found among *Streptococci*. Extreme salt tolerance (18% NaCl) is confined to genus *Tetragenococcus*. Tolerances to acid and/or alkaline conditions are also useful characteristics.

Enterococci are characterised by growth at both high and low pH. The formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to

distinguish between *Leuconostoc* and most heterofermentative lactobacilli, as the former produce only D-lactic acid and the latter a racemate (DL-lactic acid).

2.2.3 Metabolism of lactic acid bacteria

The essential feature of lactic acid bacteria metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated ATP is subsequently used for biosynthesis purposes. Lactic acid bacteria as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is of course, lactic acid (> 50% of sugar carbon). It is clear however, that lactic acid bacteria adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end-product patterns.

2.2.4 Grouping of Lactobacillus

The primary interest of (Orla-Jensen's, 1919) early description of the lactic acid bacteria was directed to identify these bacteria useful in the dairy industry, with the particular interest in the study of those bacteria occurring in Danish 'dairy cheese'. Orla-Jensen recognized 10 species in his time. This number increased only slowly to 15 and 25 species, in the 7^{th} and 8^{th} editions of Bergey's Manual respectively. Finally 44 species have been recognized in the latest 9^{th} edition of Bergey's Manual. The numbers of species are still increasing due to emerging of new taxonomic methods, which allow a more precise identification of strains isolated some time ago and, to some extent, from the continued investigation of habitats. The latest grouping of *lactobacilli* by (Kandler and Weiss, 1986) relies on biochemical-physiological criteria and neglects classical criteria of Orla-Jensen such as morphology and growth temperature since many of recently described species did not fit into the traditional classification scheme. Unfortunately, the description of new species usually does not include the analysis of the end products derived from the fermentation of pentoses, and therefore, the enzymes of the pentose phosphate pathway may be present permitting a homofermentative metabolism of pentose in lactobacilli. Nevertheless, maintaining the traditional terms is justified with regards to hexose utilization. However, at low substrate concentration and under strictly anaerobic conditions, some facultatively heterofermentative species may produce acetate, ethanol and formate instead of lactate from pyruvate. Thus, the definitions have to be used in awareness of their limitations.

When glucose is used as a carbon source, lactobacilli could be homofermentative or heterofermentative. When homofermentative, they could produce more than 85% lactic acid, whereas the heterofermentative strains produce lactic acid, carbon dioxide, ethanol or acetic acid. In the presence of oxygen or other oxidants, increased amounts of acetate may be produced at the expense of lactate or ethanol. A total of 56 species of lactobacilli have been divided into three metabolic groups (Hammes and Vogel, 1995).

- **Group A**: Obligatory homofermentative lactobacilli: Hexoses are fermented to lactic acid by EMP pathway. Pentose or gluconate are not fermented.
- **Group B**: Facultatively heterofermentavie lactobacilli: Hexoses are fermented to lactic acid by EMP pathway. The organisms possess aldolase and phosphoketolase and therefore, not only ferment hexose but alsopentoses (and often gluconate). In the presence of glucose, the enzymes of the phosphogluconate pathway and may be fermented.
- **Group C**: Obligatory heterofermentavie lactobacilli: the phospogluconate pathway ferments hexoses, yielding lactate, acetic acid (ethanol) and CO_2 in equimolar amounts. Pentose enters in this pathway and may be fermented.

Within these three groups the species are arranged according to their phylogenetic relationship. The letter indicates the affiliation of the *L. delbrueckii* group, b to the *L. casei-Pediococcus* group. Thus, the combination of the letter Aa defines a species as belonging to the obligatory homofermentative lactobacilli affiliated in the *L. delbrueckii* group, whereas Cb means that the species is obligatory heterofermentative phylogenetically belonging to the *L. casei-Pediococcus* group, etc. The *L. casei* species belonging to the group facultatively a heterofermentavie organism comes under group B. Two species, *L. acetotolerans* and *L. hamsteri* constitute group Ba, meaning that phylogenetically these organisms fall into the *L. delbrueckii* group. The presence of the Lys-Dasp type peptidoglycan is consistent with this grouping.

Group Bb contains 15 species, 12 of which contain Lys-Dasp and three DAP in their peptidoglycan. In contrast to (Kandler and Weiss, 1986), (Hammes and Vogel, 1995) have included into group Bb *L. bifermentans* since, in agreement with the group definition; this organism possesses key enzymes, aldolase and phosphoketolase. *L. bifermentans* is characterized by fermenting glucose homofermentatively. However, dependent on the pH,

lactate can be metabolized to ethanol, acetic acid and CO_2 and H_2. The utilization of lactate (and/or pyruvate) is rather common for group Bb-organisms. It can be foreseen that changes will occur for the species *L. casei* and *L. paracasei*. With ample evidence, (Dellaglio *et al*,.1991) presented a request for an opinion. They showed that the type strain of *L. casei* (ATCC 393) is not genetically closely related to several subspecies of *L. casei* as they were described by (Kandler and Weiss, 1986). This had led (Collins *et al*,. 1989) to describe *L. paracasei* sp. Nov. that included these types of strains. It appears, however, that the species *L. paracasei* should be rejected and changed for *L. casei*. The type strain of *L. casei* would have to be allotted to a new species that includes also one strain of *L. rhamnosus* and *L. zeae*.

2.2.5 Taxonomic diversity of Lactobacillus

The human gastrointestinal tract contains hundreds of different bacterial species. (Tannock, 1995). Members of the genus *Lactobacillus* are commonly present as members of microbial communities and have received considerable attention with respect to their putative health conferring properties as probiotics. *Lactobacillus* has worldwide industrial use as starters in the manufacturing of fermented milk products. Moreover, some of *Lactobacillus* strains have probiotic characteristics and are therefore included in fresh fermented products or used in capsular health products, such as freeze-dried powder. The use of some *Lactobacillus* strains as probiotics is based on studies that show that these species belong to the normal intestinal flora and that the strains have beneficial effects on human and animal health (Salminen *et al*., 1996). Major bacterial species isolated from human gastrointestinal tract fall generally into three distinct categories. These include: 1) Organisms almost always present in large number, and constituting the indigenous and resident flora, e.g. *Bacteroides*, *Bifidobacterium*; 2) Organisms normally present in small or moderate numbers, and part of the resident flora, e.g. *Enterobacteriaceae, Streptococcus* and *Lactobacillus*; and 3) Organisms present in small numbers, probably contaminants from other regions of the body e.g. *Staphylococcus*, *Haemophilus*, etc., or from the environment, e.g. *Bacillus*, *Corynebacterium*, which constitute transient flora. More specifically, organisms of the human gastrointestinal tract include diverse bacterial genera or families, and are divided into the following three groups: 1) Lactic acid bacteria in a broad sense, including *Bifidobacterium, Lactobacillus,* and *Streptococcus* (including *Enterococcus*); 2) Anaerobic group, including *Bacteriodaceae, Eubacterium, Peptococcaceae, Veillonella, Megasphera, Hemmiger, Clostridium* and *Treponema*; and 3) Aerobic group, including *Enterobacteriaceae, Staphylococcus, Bacillus, Corynebacterium, Pseudomonas* and *yeasts*.

3. OBJECTIVES

1) Isolation of LAB from fish intestine by growing on *Lactobacillus* selective media (MRS) in anaerobic Condition.
2) Selection of LAB by amplification of *Lactobacillus* specific gene.
3) Inhibitory action of isolated LAB strains against *Bacillus sp., Klebsiella sp., Proteous sp., E. coli,* and *Pseudomonas sp*.
4) Identification of potential probiotic LAB by biochemical as well as molecular characteristics.

4. MATERIALS AND METHODOLOGY

4.1 SAMPLE COLLECTION:

Fresh water fish intestine

Fresh water fishes such as Kau (*Anabas scandens)*, Singhi (*Heteroneuster fossilis)* were obtained from Pahad kata fish market, Rourkela, Odisha in living condition.

4.2 ISOLATION OF LACTIC ACID BACTERIA:

From fish intestine

The ventral surfaces of the fishes were sterilised using 70% ethanol and aseptically dissected to remove intestines. The intestines were opened by a longitudinal incision and thoroughly flushed with sterilised chilled normal saline solution (NSS) to remove feed materials, dirt and other impurities. Then the intestines were homogenised with normal saline solution (0.87% NaCl) in sterile mortar and pestle. The homogenised intestines were centrifuged at 5000 rpm for 5minute.The supernatants were serially diluted up to 10^{-6} dilutions. From 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions, 100 µl of samples were plated by spread plate method on prepared MRS agar plate adjusted with different pH i.e. 3.5, 4.5, 6.5, 7.5 and incubated anaerobically at 37^{0}C for 48 hrs.

4.3. IDENTIFICATION OF LACTIC ACID BACTERIA

Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics by following Bergey's Mannual of Systematic Bacteriology (Kandler and Weiss, 1986)

4.3.1 MORPHOLGICAL IDENTIFICATION

(i) Gram Staining Method

Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical, primarily the presence of high levels of peptidoglycan, and physical properties of their

cell walls . The Gram stain is almost always the first step in the identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming *Gram-variable* and *Gram-indeterminate* groups as well. A Gram positive results in a purple/blue colour while a Gram negative results in a pink/red colour.

- In Gram staining the single colony from the agar plate was taken & placed on the grease free slide.
- The smear was air-dried and then heat fixed.
- Then the slide was treated with crystal violet for 1 min followed by washing with running distilled water.
- Then Gram's iodine for 1 min followed by washing with running distilled water.
- After that the slide was treated with alcohol for 30 sec.
- Finally the slide was counterstained with safranin for 30 sec followed by washing with running distilled water.
- Then it was air dried & then examined under microscope to know the morphology of bacteria.

(ii) **Colony Morphology**

Cultures in the petriplate were examined to study colony morphology.

4.4 BIOCHEMICAL ANALYSIS

Biochemical identification of the isolated strains was done by using Biochemical identification kit (HI Bacillus identification kit, HIMEDIA) and some manual biochemical methods. Biochemical Identification test kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a colour change in the media that is either interpreted visually or after addition of a reagent. The biochemical test includes different sets of tests like as follows:

4.4.1 Catalase Test

The catalase test was performed to detect the presence of catalase enzyme, by inoculating a loopful of culture into tubes containing 3% hydrogen peroxide solution.

Positive reaction was indicated by formation of effervescence or appearance of bubbles, due to the breaking down of hydrogen peroxide to O_2 and H_2O.

4.4. Oxidase Test

This oxidase test was done with the help of a commercially available disc coated with a dye N-tetramethylparaphenylenediaminedihydrochloride (HI media), to detect the presence of cytochrome 'C' oxidase which is responsible for the oxidation of the dye. Rubbing a little quantity of the bacterial culture by means of a sterile toothpick on the disc causes formation of purple colour within 10-30 sec indicating positive reaction whereas no colour change indicates negative reaction.

4.4.4 Motility Test

The motility test was done to determine the motility of the organism. Bacterial cultures were stabbed into the motility test medium (HI media) and were incubated at 37ºC for 48h.Turbidity and observation of growth besides the stab line indicated a positive reaction whereas clear visibility with growth along the stab line indicated a negative reaction.

4.4.3 Endospore Formation Test

The endospore formation test was performed for all isolated strains to observe the spore formations. The purpose of this test is to differentiate between the microorganism which produce endospore and which are not. Spores have a durable outer coating that is composed of the protein keratin. This keratin coat resists staining so in order to stain a spore the primary stain, malchite green, must be heated to drive the stain into the spores. Vegetative cells are then decolorized with water and 0.5% safranin is used to counterstain. Thus endospores are stained green, while vegetative cells are stained red.

1. The smear of 4-5 days old bacterial culture was made on the cleaned glass slide.
2. The smears were heat fixed.
3. The smears were covered with a piece of absorbent paper cut to fit the slide and place the slide on wire gauze on a ring stand.
4. The paper is saturated with malachite green and holding the Bunsen burner in the hand the slide was heated until steam can be seen rising from the surface. Heat was removed and reheated the slide as needed to keep the slide steaming for about three minutes.

5. The paper was removed with forceps and rinsed the slide thoroughly with tap water.
6. The slide was drained and counterstained 45 seconds with 0.5% safranin.
7. Then the slide was washed, dried and examined.
8. Slides were observed under microscope. The vegetative cells will appear red and the spores will appear green.

4.5 CONFORMATION OF LACTIC ACID BACTERIA

4.5.1 Amplification of Lactobacillus genus specific gene

The primers used for the study taken from the published papers in heterologous species. The Lactobacillus genus specific primer pairs were used to amplify ~327 bp products from bacterial cell lysate. First the master mix was prepared, from which 23.75µl was mixed to 1.25µl of each bacterial cell lysate sample. Each PCR reaction consisted of 20.35µl dH_2O, 2.5µl 10X buffer (HIMEDIA), 0.25µl dNTPs (Chromus Biotech), 0.5µl of each forward and reverse primer, followed by 0.5µl Taq DNA polymerase (HIMEDIA). All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 52°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min. Then the PCR product was stored at 4^0C until further use. The generated PCR products (8µl) were then analysed by electrophoresis on 1% agarose gel.

Forward primer: 5' AGCAGTAGGGAATCTTCCA 3'
Reverse primer: 5' ATTYCACCGCTACACATG 3'

PCR condition 96ºC/5 min, (94ºC/15 sec, 52ºC/30 sec, 72ºC/1 min)$_{30\ cycle,}$ 72ºC/10 min, 4ºC/∞

4.6 BACTERIOCIN ASSAY BY WELL DIFUSION METHOD

- LAB strains were previously cultured in MRS broth was centrifuged at 5000 rpm for 10 minutes.
- Cell free supernatant was filtered through 0.22 µm syringe filter and collected in sterile vials.
- Pre-incubated MHA plates were punched with sterile 5 mm cork borer to make uniform wells. 4-5 wells were made in each MHA plate.

- All Pathogenic strains were mixed with NSS separately and density was adjusted by comparing with 0.5 Mc. Farlands standard as described above.
- Indicator organisms were streaked at 60^0 rotations in MHA plates with sterilised cotton swabs.
- 200µl of LAB supernatant filtrate was poured into the well.
- All the plates were labelled and incubated aerobically at 37^0 C for 24-48 hrs.
- After incubation inhibition zone size was measured in millimetre scale.

4.7 FURTHER IDENTIFICATION OF POTENTIAL LAB

4.7.1 Biochemical Analysis

4.7.1.1 Gas Prodution from Glucose

Gas production from glucose was assessed by inoculating the isolated strains in MRS broth containing glucose (1%) containing Durham tube in inverted condition and incubated at 37ºC for 48-72 hrs. The upward movement of inverted Durham tube indicates positive reaction (gas production).

4.7.1.2 Malonate Utilisation

Malonate utilisation test was performed to observe the utilisation of malonate present in the Malonate test medium (Himedia). Malonate test medium contains Bromothymol blue as indicator. Sodium malonate is the carbon source and ammonium sulphate is the nitrogen source. Organisms, which are able to utilise malonate, release sodium dioxide. The resulting alkaline conditions cause the indicator to change from light green to blue. Colour of the medium changes from light green to blue if the test is positive. Medium remains light green in colour if the test is negative.

4.7.1.3 Voges Proskauer's Test

Some organisms have the ability to produce a neutral end product acetoin from glucose utilisation. This can be detected by adding 1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B to the biochemical kit (after 48 hrs incubation). A positive test showing pinkish red colour within 2-5 min. No amendment in colour indicates negative result.

4.7.1.4 Citrate Utilisation Test

The citrate utilisation test was performed to test the ability of bacteria to convert citrate (an intermediate of the Kreb's cycle) into oxaloacetate (another intermediate of the Kreb's cycle). In this media, citrate is that the solely carbon source available to the bacteria. The change of medium colour to a bright blue as a result of an increase in the pH was considered as positive result.

4.7.1.5 Nitrate Reduction Test

This test was performed to test whether microorganisms could able to convert nitrate to nitrite or not by the addition of 1-2 drops of sulphanilic acid and 1-2 drop of N, N-Dimethyl-1-Napthylanine reagent to kit medium. Immediate development of pinkish red colour on addition of reagent indicated positive reaction. No change in colour indicated a negative reaction.

4.7.1.6 Arginine Decarboxylation Test

The arginine decarboxylation test was performed to detect arginine decarboxylation. The medium of this test contains Bromocresol purple as pH indicator. When carbohydrate present in the medium is utilised, pH was lowered due to acid production changing the colour of the medium to yellow. The acid produced stimulated decarboxylase enzyme which caused the formation of amine due to this reaction increased in pH of the medium, converting the colour of the indicator from olive green to light purple which is a positive result and no change in colour indicated negative result.

4.7.1.7 ONPG Test

Two enzymes, permease and galactosidase are required to lactose fermentation. True non-lactose fermenters are barren of both enzymes; however some organisms may lack permease but possess the enzyme galactosidase. O-nitro phenyl-D-galactopyranoside (ONPG) is structurally similar to lactose. Medium changed from colourless to yellow if the test was positive and the medium remained colourless if the test was negative.

4.7.1.8 Esculine Hydrolysis

Esculine is substituted glucoside that can be hydrolysed by bacteria to yield glucose and esculetin. The latter combines with ferric ions in the medium to form black coloured complex. Colour of the medium changed from cream to black if the test was positive and the medium remained cream in colour if the test was negative.

4.7.2 Molecular identification (16s rDNA Sequencing)

The 16S rDNA sequence was amplified using 16S universal primers (B27F and U1492R) of 1.53 kb size. First the master mix was prepared, from which 23.75µl was mixed to 1.25µl of each bacterial cell lysate sample. Every PCR reaction consisted of 20.35µl dH_2O, 2.5µl 10X buffer (HIMEDIA), 0.25µl dNTPs (Chromus Biotech), and 0.5µl of each forward and reverse primer, followed by 0.5µl Taq DNA polymerase (HIMEDIA). All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 49°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min. Then the PCR product was stored at 4^0C until further use. The generated PCR products (8µl) were then analysed by electrophoresis on 1% agarose gel. The PCR product was sent for sequencing.

Universal 16s Forward Primer (B27F): 5’ AGAGDDDGATCCPGGCTCAG 3’

Universal 16s Reverse Primer (U1492R): 5’ GGTTACATTGTTACGACTT 3’

5. RESULTS

5.1 GROWTH IN MEDIA

- After 2-3 days of incubation growth was observed on MRS agar plate 16 colonies have been isolated by observing colony morphology, pure cultured and stored in soft agar tube.
- Out of 16 colonies, 6 colonies were selected on the basis Lactobacillus specific biochemical tests and were grown in MRS broth and yellow precipitate was observed at the bottom of the tube.
- All the selected strains shown both aerobic and anaerobic growth.

5.2 MORPHOLOGICAL IDENTIFICATION

(A) Gram Staining

All the 6 isolated strains KT2W, KT1, KT1B, KT1T, KA2, FS were found to be Gram positive.

(B) Colony morphology

- All the 6 selected strains named KT2W, KT1, KT1B, KT1T, KA2, FS were found to be convex and flat, entire margin, brown, white and transparent, single rods and in chain.
- Based on colony morphology, cell shape, cell arrangement all the 6 strains were subjected to further study.

5.3. BIOCHEMICAL IDENTIFICATION

5.3.1 Catalase activity

All the isolates were subjected to catalase test showed no effervescence on reacting with H_2O_2 and thus were catalase negative.

5.3.2 Oxidase activity

All the isolates were oxidase negative as no colour change was observed on the oxidase disc rubbed with the broth culture of the isolates.

5.3.3 Endospore formation

All the isolates were non-endospore forming as no green coloured spore has been seen under microscope after staining with malachite green.

5.3.4 Motility test

This test was done to determine the motility of the organism and all the isolates were found to be non-motile as there was no turbidity and growth resumed only along stab line.

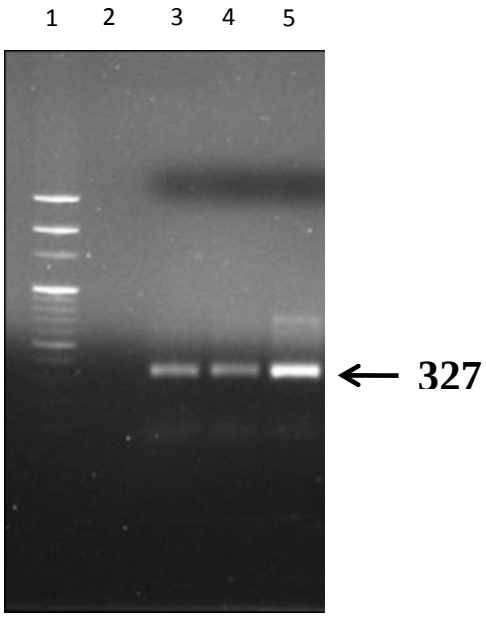

FIGURE 1: Lactobacillus genus specific gene amplification (1: Marker, 3, 4, 5: KT2W, KT1 and KT1B respectively)

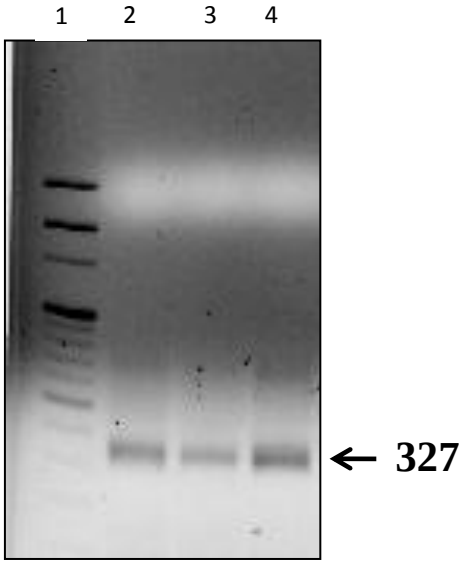

FIGURE 2: Lactobacillus genus specific gene amplification (1: Marker, 2, 3, 4: KT1T, KA2 and FS respectively)

TABLE 2. Bacteriocin Assay by Agar well diffusion method

Sl.No.	PATHOGEN	KT2W	KT1	KT1T	KTIB	KA2	FS
1	*E.Coli*	+++	+++	+++	++	++	++
2	*Pseudomonas sp.*	_	++	+	++	++	++
3	*Bacillus sp.*	+++	+++	+++	+++	++	++
4	*Proteus sp.*	+++	+++	++	++	++	+++
5	*Kleibsella sp.*	++	+++	++	++	++	+++

FIGURE 3: Bacteriocin Activity Test

TABLE 3. Biochemical characteristics of the isolated strains from kitchen waste and fresh water fish intestine (KT2W, KT1, KT1T, KT1B, KA2, FS)

Sl No.	Tests	KT1
1	**Gram stain**	+
2	**Catalase activity**	_
3	**Oxidase activity**	_
4	**Endospore formation**	_
5	**Motility**	_
6	**Gas production from Glucose**	_
7	**Malonate**	+
8	**V.P.**	_
9	**Citrate**	+
10	**Nitrate Reduction**	+
11	**Arginine**	_
12	**O.N.P.G.**	_
13	**Esculine Hydrolysis**	+

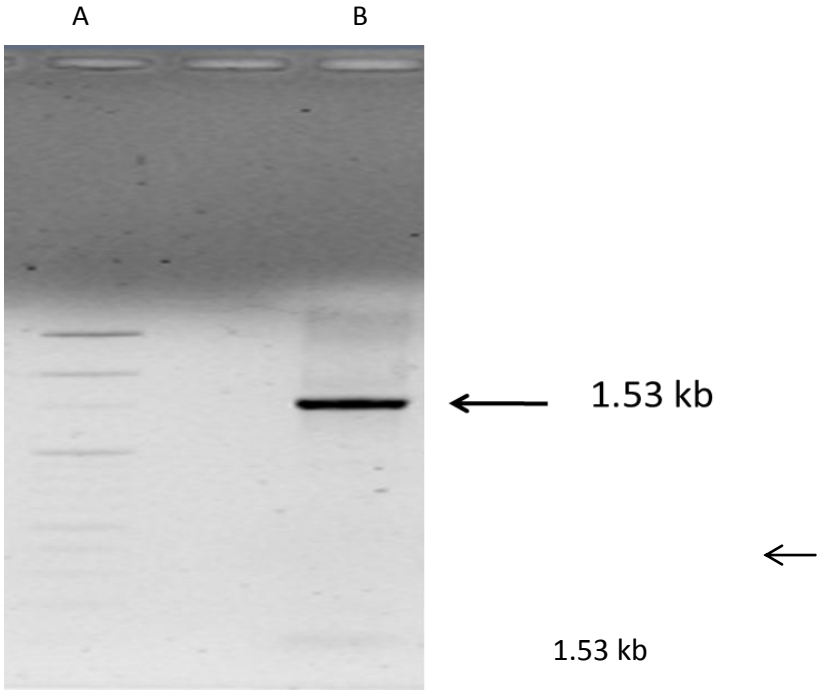

FIGURE 4: 16S rDNA amplification (A: Molecular weight marker, B: Amplified 16S rDNA sequence of KT1 strain)

6. DISCUSSION

A cursory review of literature indicates that strains of *L. casei* and *L. delbrueckii* have beneficial effects against watery diarrhea in infants, decreasing lactose intolerance in adults, has anti-cancerous properties, reducing cholesterol and leading to irritable bowel disease (IBD)(Anderson *et al*., 2001).

Isolation of *Lactobacillus* strains has been accomplished by using *Lactobacillus* selective medium MRS agar (HIMEDIA). Sixteen strains has been isolated and subjected to further study. Six Strains KT1, KT2W, KT1T, KT1B, KA2 and FS were selected as they were gram positive, oxidase negative, catalase negative bacilli. Similar result was found by (Sharp, 1979; Bukola, 2008; Ali, 2011). Above six strains were also confirmed as belong to LAB by amplifying the Lactobacillus genus specific primer designed from regions of identity within the 16S ribosomal DNA (rDNA) sequence (Byun *et al*., 2004).

All the strains were found to be facultative anaerobes, as the strains grown in anaerobic condition under candle jar condition in CO_2 incubator and growth also found in presence of oxygen. Similar results were found by Gibson *et al*., 2005, Markiewicz, 2005, Sawet *et al*., 2011.

The stages of the pre-identification based on morphological aspects showed that LAB strains were Gram positive rods found in chain, singly and in pairs. Their colonies were circular (KTI, KT2W, KT1B, KT1T, KA2), flat (FS), low convex with entire margin, as reported by (Markiewicz, 2005, Edna *et al*., 2006, Bukola, 2008; Baukhemis, 2009, Pati *et al*., 2010).

All the 6 isolates were subjected to different biochemical tests results. Isolates were subjected to test, gas production from Glucose by adding 1% glucose in MRS broth with inverted Durham tube. After 48 hrs of incubation gas was not produced by all the strains. Similar result was reported by (Edna *et al*., 2006; Baukhemis, 2009; Ali, 2011).

Four to seven days old cultures were subjected to endospore staining test, all the strains were found to be non-spore forming (Adesokan, 2009; Hoque *et al*., 2010).

Then non-spore forming strains were tested for motility in Manitol Nitrate Motility agar (Himedia). All the strains were found to be non-motile as turbidity was absent and growth found along stab line only.

The isolation of *Lactobacillus* species from fresh water fish intestine has been carried out. Strains KTIT, KT2W, KT1B, KA2 and FS have been identified as *Lactobacillus casei* and the strain KT1 as *Lactobacillus delbrueckii* according to the characteristics followed in Bergey's manual and in the prokaryotes (Hargrove *et al*., 1978; Hoizapfel *et al*., 1992). Presently, bacterial species identification using the 16S rDNA-based method is the most widely accepted, as large public domain sequence databases were are available in genebank for comparasion (Rauta *et al*., 2011; Maidak *et al*., 1996; Peer *et al*., 1997). In the current study, the 16S rDNA sequences of above discussed six strains were amplified and sequenced. But the sequences are yet to be analyzed for molecular confirmation.

As shown in Table 2, the antimicrobial spectra of LAB strains were assessed against *E.coli, Pseudomonas sp., Bacillus sp., Proteus sp*. and *Kleibsiella sp.* All srains showed maximum inhibition against *E.coli* and *Proteus sp*. All strains except KT2W showed inhibition zone against *Pseudomonas sp. L.casei* and *L.delbrueckii* is effective against genera *Listeria, Enterococcus* and *Salmonella* is reported by (Edna *et al*., 2006). (Adekosan *et al*., 2009) reported the antagonism against *Pseudomonas aeriginosa*, *Staphylococcus aureus*. Boukhemis in 2009 reported the antagonist activity against *E.coli*. LAB strains showing effective inhibition spectra can be used as probiotics to replace chemical antibiotics in animal and in fish –feed industry. Considering probiotic properties, all the isolated strains can be used as potential probiotics with further detailed studies.

7. CONCLUSION

The use of probiotics in enhancing the gut microflora of human, animals and in fishes shows signs of becoming a success which infers that the isolated strains can be used as probiotics both in human and fishes after proper certification. Six strains were screened under harsh conditions of acid by lowering the pH of the media. All the isolates showed good to excellent acid tolerance, which support the fact that the strains can be survive under acidic conditions in the GI. KT1 out of these different strains of *L. casei* and were assessed against five enteric pathogenic genera like *Kleibsiella sp*., *Proteus sp*., *Pseudomonas sp*., *E.coli*, *Bacillus sp*. Inhibition zone was observed due to production of bacteriocin-like compound. In this study Bacteriocin activity test results proved KT1 strain more potential probiotic Lactic Acid Bacteria. Hence this strain can be used against disease caused by the above mentioned enteric pathogens.

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