Lactobacillus sp. as probiotics for human health with special emphasis on colorectal cancer

Gayathri D¹, Asha¹ and T. N. Devaraja²

¹Department of Studies in Microbiology, Davangere University, Shivangangothri, Davanagere- 577002, India. ²Taralabalu Krishivigyan Kendra, Davanagere- 577005, India

gayathridevaraja@gmail.com

Abstract

Probiotics are live microorganisms when administered in adequate amount confer health benefit to the host. Extensive usage of probiotics in dairy as a starter in preparation of cheese and other dairy products has become popular in the recent past. The representatives of probiotics mainly include Lactic Acid bacteria such as Lactobacillus acidophilus, L. plantarum, L. johnsonii, L. gasseri, L. casei, L. rhamnosus and Bifidobacterium longum, B. breve, B. infantis, B. thermophilum, B. pseudolongum and others. A number of animal studies conducted by various research groups indicated that the probiotics possess potential anticancer effects. Colorectal cancer (CRC) is one of the most devastating diseases causing high morbidity and mortality among human in most of the urban, eastern and western parts of the world. Oral intake of large number of substances among human perhaps act as mutagenic compounds resulting in change of native microbiota of intestine and could induce cancer. In the present review, the beneficial effects of probiotics in reducing the risk of colorectal cancer (CRC) have been discussed drawing observations from an ongoing related research work in our laboratory. The present article also focuses to describe the beneficial applications of Lactobacillus spp. particularly in human.

Keywords: Probiotics, Lactic acid bacteria, Lactobacillus, Colorectal cancer.

Introduction

Probiotics are dietary supplements containing potentially beneficial bacteria needed for the betterment of gastrointestinal tract (FAO, 2002). Probiotics can also be defined as the preparations or product containing viable, defined microorganisms in sufficient numbers, which alter the microflora by implantation or by colonization in the host and exert beneficial health implications on their host. While, prebiotics are the non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Prebiotics are also known as colonic foods and mainly are the symbiotic make up of fructooligosaccharide molecules and short-chain sugar molecules containing fructose molecules. Prebiotics derived from insoluble fiber and fructosaccharide sugars, are often found in fruit and honey (Thomas & Greer 2010).

Probiotics convert sugars and other carbohydrates into lactic acid and produce a characteristic sour taste to fermented dairy foods and in fortified foods. Some of the probiotic bacteria act as preservative by lowering the pH of the medium and reduce opportunities for spoilage organisms to grow. Hanish & Varghese (2006) reported that the fermented dairy products and freeze-dried bacteria are most popular vehicles for delivering these organisms to the gastrointestinal tract of human and animal system. Further, they have also been used in preparation of feed for poultry, dairy and fisheries to maintain better health in recent times.

Probiotic bacteria

Gastrointestinal tract of human have been inhabited by a variety of microorganisms. Physiological balance of this microbiota is greatly influenced by intestinal environment. Among the numerous intestinal bacteria that beneficially affect the host intestine, some could be recognized as probiotics (Ishibashi & Yamazaki 2001).

More than eighty species of microorganisms have been recorded as probiotics from various sources. Among them, the most used Lactobacillus sp. are present in raw milk, dairy products as well as infant, children and adult faeces (Coeuret et al., 2003). The representative species of probiotics viz., Lactobacillus and Bifidobacterium include Lactobacillus acidophilus, L. plantarum, L. johnsonii, L. gasseri, L. casei, L. rhamnosus, Bifidobacterium longum, B. breve, B. infantis, B. thermophilum, B. infantis, B. pseudolongum and others. In addition to these, dairy product comprising L. bulgaricus, Streptococcus thermophilus and Leuconostoc could be used as probiotics.

However, Lactococcus which is not in the group of probiotics help in intestinal microbial balance (Ishibashi & Yamazaki, 2001). The extensive usage of Lactobacillus, Leuconostoc and Pediococcus in food processing has been recorded in the history of mankind. However, safety of consumption of these probiotics have not been questioned until now, although, absence of pathogenicity and infectivity are the prerequisites in them. In addition to this, factors to be addressed for evaluation of probiotics include virulence as well as intrinsic properties. Epidemiological studies or post marketing surveillance,
are the criteria that are recommended to assess the safety of probiotics. In addition, efficacy and health promoting effects of probiotics have been considered for specific strains (Salminen, 2001). Mateau (2001) had proposed studies on intrinsic factors, pharmacokinetics and interaction between hosts and probiotics to assess the safety of probiotics.

Probiotic bacteria are found to be potentially useful in managing lactose intolerance, lowering of cholesterol and blood pressure, Helicobacter pylori infection, antibiotic associated diarrhea, inflammation, irritable bowel syndrome, colitis, gastro-enteritis, rotavirus gastro-enteritis in children, ulcerative colitis, Crohn's disease, pouchitis, intestinal cancer, mammary gland cancers and in colorectal cancer (Harish & Varghese, 2006).

Isolation of probiotic lactobacilli

Coeuret et al. (2003) reported a number of culture media for Lactobacilli which include resuspension medium enriched with phosphate, cysteine, antifoaming agents and sugar before plating on de Man, Rogosa, Sharpe (MRS) medium, MRS medium has been routinely employed for the isolation and enumeration of lactic acid bacteria (LAB) in most food (fermented) products (Shah, 2000; Mirdohi et al., 2008). Coeuret et al. (2003) reviewed the application of MRS with vancomycin and bromocresol green, a new anaerobic media for the enumeration of Lactobacilli from faeces and various dairy products. Furthermore, they described the X-glu agar being selective for L. acidophilus from yogurt and related milk products. Presently, widely used MRS medium is considered to be an ideal one for the cultivation of lactic acid bacteria.

Phenotypic identification of lactobacilli

Lactobacillus is a non-motile, non-spore forming, Gram-positive bacteria and the cell morphology varies widely from long, straight or slightly crescent shaped bacilli to coryneform coccobacilli. Classical phenotypic tests for identification of Lactobacilli are based on physiological characteristics such as respiratory type, motility, growth, temperature sensitivity as well as growth in NaCl. Lactobacilli are typically chemo-organotrophic and ferment carbohydrates producing lactic acid as a major end product (Kandler & Weiss 1986). Protein analysis such as protein finger printing or multi-locus enzyme electrophoresis are advanced phenotypic methods used in identification of Lactobacillus spp (Coeuret et al., 2003).

Molecular methods for identification of lactobacilli

A number of probiotic research groups have described molecular methods for the identification of species through taxonomic analysis by DNA/DNA hybridization, sequencing, polymerase chain reaction (PCR), ribotyping. Polymorphism analysis permit strain differentiation by the use of techniques such as, restriction enzyme assay (REA), randomly amplified polymorphic DNA (RAPD), repeated sequence extragenic palindromic PCR (REP-PCR), amplified fragment length polymorphism (AFLP), plasmid profiling and pulsed field gel electrophoresis (PFGE) (Gilliland et al., 1975; Collins et al., 1987; Williams et al., 1990; Janssen et al., 1996; Brandt et al., 2000; De-Angelis et al., 2001; Coeuret et al., 2003).

Furthermore, Pot et al. (1993) described the partial sequence analysis of 23S rRNA genes of L. acidophilus, L. johnsonii, L. gasseri and showed that the DNA probe Lgb was proven to be specific for the strains belonging to L. johnsonii. The combined use of differential plating and molecular strain typing methodologies provided food and medical microbiologists a best tool for the enumeration and identification of Lactobacillus groups.

Ribotyping proved to be more effective, although some strains appeared to have identical ribotype and chemotype patterns. Therefore, Zhong et al. (1998) reported that more specific identification methods such as the use of repetitive sequence PCR probes, are most preferred. The taxonomy and physiology of probiotic LAB can only be understood by using polyphasic taxonomy combining morphological, biochemical and physiological characteristics with molecular based phenotype and genotype techniques (Klein et al., 1998). Screening new primers in RAPD analysis and using other restriction enzymes in ribotyping could possibly increase specificity for strain typing (Tynkkynen et al., 1999). In addition, Torrani et al. (2001) differentiated a L. plantarum through phylogenetic analysis of partial rRNA gene sequence.

Dimitrov (2009) identified Lactobacillus and Bifidobacterium at species level by the combination of ARDRA (amplified ribosomal DNA restriction analysis) (by using enzymes Hae I, Msp I and Eco RI) and SDS-PAGE. Stoyancheva et al., (2009) performed ARDRA analysis for the identification of L. delbrueckii, L. helveticus and L. acidophilus and other thirteen isolates from dairy foods.

Culture independent method of identification involves the extraction of nucleic acid DNA or RNA from raw samples by using probes for hybridization and primers for denaturing gradient gel electrophoresis (DGGE), temperature gradient gel and by linear temperature gradient, and a single strand conformation polymorphism (SSCP) which detect the variable region of the 16S r-RNA gene and used neutral non-denaturing polyacrylamide gels (Coeuret et al., 2003). They further reported that the analysis of Lactobacilli in dairy product is very complicated and use of molecular based phenotypic or genotypic technique would give more accurate results, indicating the diversity of Lactobacillus sp.

Probiotics for human health

Various investigations have projected numerous therapeutics and control measures for gastrointestinal disorders, asthma in addition to several types of cancers...
using probiotics. Native microorganisms from yogurt would prevent infections of gastrointestinal tract by influencing the microbial ecosystem. The inhibitory mechanisms of LAB against pathogenic bacteria are primarily due to the production of organic acid and bacteriocins (Gorbach et al., 1987; Kim 1988). Kaila et al. (1992) have recorded that, in children using oral microbial feeding with LAB has prevented acute rotavirus associated diarrhea and antibiotic induced gastrointestinal disorders. Further, they reported that LAB augmented the local immune defense by increasing the number of immunoglobin secreting cells and perhaps improve the gastrointestinal system of children with acute rotavirus associated diarrhea. Harish & Varghese (2006) also reported that probiotics have been proposed to be a novel approach in the management of allergic diseases especially in infants.

In addition, oral intake of *Bifidobacterium longum* in yogurt with erythromycin have been observed to reduce the frequency of gastrointestinal disorders. Thus, consumption of LAB would reduce antibiotic induced changes of the intestinal ecosystem (Kaila et al., 1992). Audin et al., (2008) suggested that the daily consumption of Acimei, a probiotic fermented dairy product significantly improved seroprotection against H1N1 strain observed in elderly women although the mechanism of protection is yet to be resolved. Several animal and studies in human have shown beneficial effects of LAB in yogurt consumption as it aids in building resistance to gastrointestinal pathogens. Recent report reveals that probiotics play precise role in preventing all types of diarrhea, gastroenteritis (Thomas & Greer, 2010), inflammatory bowel diseases, ulcerative colitis, Crohn's disease, pouchitis, irritable bowel syndrome (IBS), lactose intolerance, colon cancer, constipation (Ajmal & Ahmed, 2008), infection and allergy (Rijikers et al., 2010), peptic ulcer, stimulation of immune system, antifungal actions and preservation of food (Masood et al., 2010).

**Immunological aspects of probiotics**

It has been found that phagocytic activity of macrophages was significantly higher in mice fed with milk containing *B. bifidum, L. acidophilus, L. casei, L. helveticus* than in control mice fed with unfermented milk (Perdigon et al., 1988). Furthermore, they showed that feeding milk fermented with *L. casei* and *L. acidophilus* increased in vitro and in vivo phagocytic activity of peritodal macrophages against sheep RBC in Swiss mice. In addition, *L. acidophilus* induced the production of interferon (INF-α) and INF-β in murine peritoneal macrophage cell culture (Kitazawa et al., 1992). Also, dose dependent orally administered LAB and yogurt increased secretory IgA producing cells in the small intestine in an experimental animal (Perdigon et al., 1988).

Later, Puri et al., (1996) reported that serum IgA concentration in yogurt fed mice were significantly higher than the milk fed mice after *Salmonella* challenge. Earlier, Takahashi et al., (1993) reported that specific IgG and IgA production in mice administered with LAB when compared to control non administered group. These studies perhaps indicated that the IgA secreted by the intestinal B cell enter the circulation and raise the serum IgA concentration. The influence of a yogurt-supplemented diet on the immunocompetence and survival of animals subsequently infected with *Salmonella typhimurium* suggested that live LAB would enhance local and systemic immune response (De Simone et al., 1988). Additionally, increased phagocytic activity of human blood cells particularly granulocytes after the injection of fermented milk with *L. acidophilus* and *B. bifidum* have also been reported (Schiffin et al., 1997 & Schiffin et al., 1995).

An interesting report has been made on innate acquired immune influence and observed the expression of innate immune response of CV206, TLR2, IgA*, CD4*, CD8* cells as well as cytokines which increased upon *L. casei* treatment in the small intestine of mice. In addition, the consumption of yogurt containing viable LAB was shown to increase INF-β, IL(interleukin)-6, IL-10, INF-γ and TNF (Tumor necrosis factor)-α production (Halpem et al., 1991; Miettinen et al., 1996; Aattouri & Lemonnier, 1997). Human and animal studies indicated that oral intake of LAB yogurt would stimulate the immune response with the higher production of cytokine, macrophage activity leading to better cell mediated immune response. Further, Crittenden et al., (2005) reviewed the intestinal microbial ecosystem and interaction between gut bacteria, diet and health of the human host.

Tao et al., (2006) investigated that the probiotic *Lactobacilli* induced heat shock in intestinal epithelial cell in a time and concentration dependent manner in conditioned media. They observed that the effects were mediated by low molecular weight peptide which was an acid and heat stable. This induced the expression of cytoprotective proteins in gut epithelial cells and activated signal transduction pathway, thus, inducing beneficial clinical effects.

Very recently, it has been found that, curd enriched with *L. bulgaricus* and *S. thermophilus* increased the levels of cytokine (INF-α, INF-λ, IL-10, IL-4) there by increased secondary immune response (Dewan et al., 2009), perhaps a boon for malnourished children. A probiotic *Bacillus subtilis* natto increased serum IgG and IFN-λ levels and increased general performance by improving the average daily weight gain in Holstein male calves (Sun et al., 2010). van Hemert et al., (2010) isolated *L. plantarum* from human source and identified the genes responsible for anti inflammatory cytokine IL-10 and secretion of pro-inflammatory cytokine IL-12. Also, *L. fermentum* was found to induce immunological response by reducing pro-inflammatory cytokine interleukin-10 in the liver without typhoid nodule in mice.
challenged with *S. typhimurium* (Truuusalu et al., 2010). Probiotic curd containing *L. acidophilus* and *L. casei* suppressed ovalbumin specific IgE and lymphocyte in ovalbumin induced allergy in mice (Jain et al., 2010) indicating antiallergic potential of *Lactobacillus* spp.

**Probiotics in controlling cancer**

Rats challenged with carcinogen DMH fed with *L. acidophilus* developed fewer tumors than animals treated with other LAB (Mcintosh et al. 1999). In addition, *Lactobacillus* strains were found to protect against chemically induced tumors in rats and reduce the formation of superficial bladder tumors in humans (Aso & Akazan 1992; Yamazaki et al., 2000).

Seow et al. (2002) investigated the high growth inhibition in human bladder cancer cell lines using *Lactobacillus* and compared with the effect of *Mycobacterium bovis* (BCG) on human bladder cell lines. They reported that *Lactobacillus* spp induced cytotoxic effects in bladder cancer cell and stated the use of *L. rhamnosus* and *L. casei* bacterial species in treating bladder cancer was encouraging.

*Enterococcus faecium, E. durans, E. avium* and *E. reuteri* isolated from human feces had the capacity to transfer 2- amino 1- methyl 1-6 phenylimidazo(4,5-b)pyridine (PhIP) a dietary carcinogen into its non toxic form (Vanhaecke et al., 2008). The symbiotic combination of *L. rhamnosus* GG and *B. lactis* as a probiotic, oligofructose enriched inulin as a prebiotic had the capacity of fecal water to induce necrosis in colonic cells and showed improved epithelial barrier function in polypectomized patients (Liang, 2008).

**Colon cancer risk in India and in the world**

Colon cancer rates in India are lower than those seen in western countries. Colon cancer rates vary from 4.7 in male and 3.2 in female per 1,00,000 population in India to 40.6 in male, 30.70 in female in United States (Sinha et al., 2003). Colorectal cancer (CRC) is the fourth most common cancer in men and third most common cancer in women worldwide. IARC (International Agency for Research on Cancer) found that in Slovenia, CRC incidence increased 70% among men and 28% among women. In Miyagi, Japan rates are 92% among men and 2% among women. In Miyagi, Japan rates are 92% among men and 28% among women. Substantial regional variations of CRC incidence have been observed in the countries such as, Japan, Israel and Singapore. Recent reports states that, the United States was the only country where CRC incidence rates declined in both in males and females (Centere et al., 2009; Umar & Greenwald, 2009).

**Probiotics in controlling colorectal cancer**

Colorectal cancer is one of the most devastating diseases causing high morbidity and mortality among human in most of the urban, eastern and western countries. Change of life style, food would perhaps attribute to change of native microbiota of intestine and act as mutagen in inducing cancer.

Development of colorectal cancer (CRC) involves a sequence of events although, partially being understood. During initiation step, mutagen causes alteration in the native DNA, followed by metabolic activation of a precursor to produce the carcinogen. Next step is involved with changes in signal transduction pathway followed by overgrowth in colonic crypts or aberrant crypt. These aberrant crypts were considered to be pre-neoplastic structures. They are enlarged and elevated than normal crypts and show cell lines growth patterns. Aberrant crypt would occur singly or in groups and certain fractions of these aberrant crypts would progress to polyps to become tumors. Brady et al. (2000) suggested that pre-biotics with or without pre-biotics have an inhibitory effect on the development of aberrant crypts and reported that it is difficult to analyze the efficacy of probiotics to control tumor development at initiation stage or post initiation stage.

Rafter et al. (2007) investigated on dietary symbiotics that would reduce cancer risk factors in polycystomised and colon cancer patients. Animal studies suggested that pre and probiotics exert protective effects against tumor development in the colon. The probiotic concept as induced by a symbiotic preparation - oligofructose enriched inulin and *L. rhamnosus, Bifidobacterium lactis* to reduce the risk of colon cancer in human. It was found that symbiotic intervention resulted in significant changes in faecal microbiota that is, *Bifidobacterium* and *Lactobacillus* increased while *Clostridium perfringens* decreased. This intervention reduced colorectal cancer proliferation and the ability of faecal water to induce necrosis in colonic cells and improved epithelial barrier function in polypectomised patients and increased production of INF-α in cancer patients. They further reported that several colorectal biomarkers can be altered by symbiotic intervention.

Oligofructose and inulin, selective fermentable chicory fructans, have been shown to stimulate the growth of *Bifidobacteria* (Reddy, 1999) and evaluated inulin and oligofructose for their potential inhibitory properties against the development of colonic aberrant crypt foci in rats. Their results indicated that dietary administration of oligofructose and inulin inhibited the development of ACF in the colon and showed antitumor activities. Further, the degree of ACF inhibition was more pronounced in animals given inulin than those fed oligofructose. And, reasoned that probiotics selectively stimulated the growth of bifidobacteria, ornithine decarboxylase activities, ras-p21 oncoprotein expressions and tumor inhibitory activity of typhosphilized cultures of *Bifidobacteria longum* against chemically induced colon and mammary carcinogenesis and against colonic tumor cell proliferation. However, human studies in this line perhaps broaden the application of pre and probiotics.

Further, Reddy & Rivenson (1993) evaluated the inhibitory effect of *B. longum* on colon, mammary and...
liver carcinogenesis induced by 2-amino-3-methylimidazol [4,5-f] quinoline (IQ), a food mutagen in rats. Their results indicated that dietary B. longum significantly inhibited the IQ induced incidence of tumors in colon, and liver and in female rats mammary carcinogenesis was suppressed to 50%. Thus usage of B. longum significantly reduced the risk of colon cancer and considerably the mammary cancer in rats.

O’Keefe et al., (2007) reported that the incidence of CRC was dramatically higher in African Americans than in native Africans. They conducted a study to analyze the difference in incidence by examining interaction between diet and colonic bacterial biota using healthy middle aged group of humans. Diet was measured by 3-d recall and colonic metabolism by breath hydrogen and methane responses to oral lactulose. On culturing faecal samples, 7-α dehydroxylating bacteria and L. plantarum were recovered. Further, colonoscopic mucosal biopsies were used to measure the proliferation rates. They found that when compared to native Africans, African American consume meat, saturated fat and cholesterol along with more calcium, vitamin A and C with fiber intake. Breath hydrogen was higher and methane was lower in African American and faecal colony counts of 7-α dehydroxylating bacteria were higher and while Lactobacilli were lower. It was also observed that colonic crypt cell proliferation rates were dramatically higher in African Americans and proposed that higher CRC risk in African Americans than native Africans. Thus they hypothesized that CRC risk was determined by interaction between external- dietary and internal- bacterial environments. In addition to this, most recently Saraf et al., (2010) reported that the beneficial uses of probiotics in reducing HIV infection and cancers including colorectal carcinomas.

Therefore, the consumption of probiotic and prebiotic would decrease the risk of colon cancer particularly in producing aberrant crypt in tumor development. The results of these could be beneficial in reducing the risk of CRC in animal models that can be extrapolated to human system. Majority of evidence for anticancer effects of probiotics are gathered from animal studies, while the evidence from human studies is scarce. In addition, human clinical trials would record significant observations on the beneficial effects of probiotics. Further, characterization of highly potential strains of probiotics having specific anti-cancerous effects and their mechanisms need to be addressed.

References
Indian Journal of Science and Technology

Vol. 4 No. 8 (Aug 2011) ISSN: 0974-6846

44. Schiffen AM, Brassart D, Servin AL, Rochat F and Donnet-Hughes A (1997) Immune modulation of...


60. Williams JGK, Kubelik AR, Kenneth JL, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res.18(22), 6531-6535.


I send you my best wishes and respect.

We fell pleasure to inform you that your paper [Molecular characterization and variation of Lactobacillus sp. of remote malnad regions of Kamataka, India.] has been reviewed by expert referees and accepted for publication in Advances in Environmental Biology (ISI Journal: SJR* = 0.026). Our reviewers have made some corrections during the evaluation process. Additional questions concerning your paper will be sent along with the galley proof, if needed also, revised article will be checked by the editors, go through language editing and be sent to the publisher. Your article has been scheduled to appear in the fourth coming issue of Advances in Environmental Biology (October 2011).

Please pay the printing cost of your article (180USA $) to go to the next step of publication. Please make sure that your payment is timely received in order to avoid delay in publication of your article. Kindly note that this paper will not be published until the publication fees are paid before the deadline (10/11/2011).

Payment Options
1) Western Union:
   Receiver Name: Ali Mohammad Al-Tawaha
   Address: Amman P.O. Box 1388, Jordan
   After transferring money, provide us the following information via
   E-mail.
   MTCN (Money Transfer Control Number) C Sender's First and Last Name,
   Address
   Amount C Secret Answer (If any)
   Article No.

   Important Note:
   1) By submitting the processing fee, it is understood that the author has agreed to our terms and
      conditions which may change from time to time without any notice.
   2) Author will take the responsibility what so ever if any copyright infringement or any other violation of
      any law is done by publishing the research work by the author
   3) Before publishing, author must check whether this journal is accepted by his employer, or any authority
      he intends to submit his research work. we will not be responsible in this matter.
   4) If at any time, due to any legal reason, if the journal stops accepting manuscripts or could not publish
      already accepted manuscripts, we will have the right to cancel all or any one of the manuscripts without
      any compensation or returning back any kind of processing cost.
   5) The cost covered in this invoice is only for online publication of a single manuscript.

   * The SJR indicator measures the scientific influence of the average article in a journal; it expresses how central
   to the global scientific discussion an average article of the journal is. Cites per Doc. (2y) measures the
   scientific impact of an average article published in the journal, it is computed using the same formula
   that journal impact factor ™ (Thomson Reuters).
BEST REGARDS
Dr. Abdel Rahman Mohammad Said Al-Tawaha (Ph.D McGill University)
Founder President of American-Eurasian Network for Scientific Information
http://www.aensionline.com/
Department of Biological Sciences
Al Hussein Bin Talal University
Maan, P.O. Box 20, Jordan
E-mail: abdel.al-tawaha@mall.mcgill.ca
         abdeltawaha@yahoo.com
Office phone: +962-3-2179000 ext 7560 Home phone: 962-2-7305196 Mobile 962-776693869
Molecular characterization and variation of *Lactobacillus* sp. of remote malnad regions of Karnataka, India.

Asha¹, Gayathri D¹* and Batish V²
¹Department of Studies in Microbiology, Davangere University, Shivagangothri, Davanagere- 577002, India,
²Department of Biotechnology and molecular biology, NDRI, Karnal, Haryana-132001, India.

Abstract

A total of 150 strains of *Lactobacillus* isolated from traditional curd samples from remote regions of malnad districts of Karnataka, India were characterized. All the isolates were identified by phenotypic, biochemical and molecular methods. The phenotypic characterization was carried out by morphological, fermentation patterns and various biochemical parameters and identified as *Lactobacillus* was distinct from available data by characteristic heterofermentation products. Out of 150 isolates 10 isolates were selected (L1-L10) to repeated PCR analysis and confirmed as the genus *Lactobacillus* and five (L1-L5) isolates were subjected to species level characterization and identified as *L. fermentum* (L3) and *L. plantarum* (L5). A large number of diversity in fermentative reactions was observed among 150 isolates of *Lactobacillus*. In addition, L3 was the only isolate producing xylose in addition to fructose, trehalose, arabinose, maltose, mannose and galactose. The sample collection sites have special emphasis that the individuals of the region maintained the curd since a century or more and the consumers showed high disease resistance in general specifically to gastrointestinal disorder and longevity. Among the various isolates studies, L3 was distinct producing characteristic aroma. Therefore, L3 isolate perhaps could use as health adjuvant or immune boosters for human and identified as *L. fermentum*.

Key words: *Lactobacillus fermentum*, *L. plantarum*, PCR, heterofermenting

*corresponding author: gayathridevaraja@gmail.com

Introduction

*Lactobacillus* species constitute a significant proportion of probiotic cultures used in developed countries in microbial adjuvant nutrition [1]. Members of the genus *Lactobacillus* play an important role in human and animal gastrointestinal tract as well as in production and spoilage of many foods, feeds and beverages. *Lactobacillus* represents commensal mammalian gastrointestinal microbiota and is useful as probiotics, functional foods and dairy products [2]. Because of the potential health benefits, these organisms are increasingly incorporated in dairy foods [3] in recent times.

During the last fifteen years, the *Lactobacillus* genus has evolved and contains more than 80 species [2]. Due to the growing interest in using bacteria as probiotics or starters in dairy products, the identification of these microorganisms at species level is becoming more and more required [4] particularly from traditional dairy samples.

The genus *Lactobacillus* encompasses a diverse assemblage of Gram positive, catalase negative, non-sporing rod shaped organisms [5]. Conventionally, *Lactobacillus* sp have been identified on the basis of cell morphology, analysis of fermentation products-ability to utilize various carbohydrate substrates [6]. The precise identification of these bacteria to the species level is relatively difficult. *Lactobacillus* and other lactic acid bacteria (LAB) and several other groups of bacteria are found in the same habitats during ecological studies of microbiota succession. Methodological procedures to handle such a high number of isolates should be brief and precise to avoid inconclusive result. 16s rRNA gene sequence have been used in the identification of many of the *Lactobacillus* sp through the derivation of specific oligonucleotide probes or polymerase chain reaction based techniques [6]. In addition, the diversity of *Lactobacillus* sp in different
regions of the world have been documented [7, 8, 9, 10]. However, Indigenous Lactobacillus sp distributed in
certain regions of India have become health secret of some of the conservative traditional families. Therefore,
the curd samples were collected from very remote regions of malnad districts of Karnataka, where they
maintained curd samples since centuries and were regular consumers. In addition, they showed high disease
resistance and longevity. In this context, the present study was undertaken to determine the most dominant
strains of Lactobacillus and further could be used as biotechnological tool as starter or food adjuvant or health
boosters.

Material and Methods

Sample: Curd sample

Isolation of Lactobacillus sp

The curd samples were collected from remote regions of malnad districts (Shivamogga and
Chikkamagulure) of Karnataka, India (Table 1). The samples were serially diluted and pour plated on MRS
(de Man, Rogosa, Shrape) selective media incubated at 37°C for 24 hours. Minute oval shaped colonies
beneath the culture plates have been selected and streaked on MRS plates and incubated at 37°C for 24 to 48
h. The colonies were subcultured and stored in MRS slants at 4°C [11].

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date of collection</th>
<th>Place of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curd sample</td>
<td>17/06/08</td>
<td>Nichidi, Hosanagar, Shivamogga, India 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S1 – S5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6 – S9</td>
<td>19/06/08</td>
<td>Tyagarthi, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S10 – S12</td>
<td>19/06/08</td>
<td>Balegundi, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S13 – S14</td>
<td>19/06/08</td>
<td>Keladi, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S15</td>
<td>20/06/08</td>
<td>Shiraguppa, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S16 – S17</td>
<td>20/06/08</td>
<td>Beleuru, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S18 – S19</td>
<td>20/06/08</td>
<td>Keladipura, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S20 – S21</td>
<td>20/06/08</td>
<td>Ulluru, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S22 – S24</td>
<td>25/06/08</td>
<td>Bimaneri, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S25 – S29</td>
<td>14/07/08</td>
<td>Sunakallu, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S30 – S32</td>
<td></td>
<td>Kamblikoppa, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S33 – S35</td>
<td></td>
<td>Humcha, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S36</td>
<td></td>
<td>Malla, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S37</td>
<td></td>
<td>Hunakallu, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S38 – S40</td>
<td>24/07/08</td>
<td>Sullalae, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S41 – S44</td>
<td>25/07/08</td>
<td>Hadiagallu, Hosanagar, Shivamogga, 13.92°N 75.07°E 585m</td>
</tr>
<tr>
<td>S45 – S48</td>
<td>01/08/08</td>
<td>Kigga, Sringeri, Chikkamagalure, 12.54°N 74.04°E 1037m</td>
</tr>
<tr>
<td>S49 – S52</td>
<td>06/08/08</td>
<td>Guddemanee, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S53 – S55</td>
<td>22/08/08</td>
<td>Malavalli, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S56 – S57</td>
<td>02/10/08</td>
<td>Kalsi, Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S58 – S59</td>
<td>02/10/08</td>
<td>Hecche Sagar, Shivamogga, 14.17°N 75.03°E 580m</td>
</tr>
<tr>
<td>S60 – S65</td>
<td>10/06/09</td>
<td>Honnebne, Tirthahalli, Shivamogga, 13.7°N 75.23°E 591m</td>
</tr>
</tbody>
</table>

Table 1: Sample collection sites
Phenotypic and Biochemical characterization

*Lactobacillus* sp. have been identified on the basis of cell morphology, analysis of fermentation products, enzymatic activities and ability to utilize various carbohydrates. Further, the isolates were subjected to Gram staining, KOH test, catalase test, oxidase test, MR-VP test, nitrogen reduction test and gelatin hydrolysis tests [5, 12, 13]. The biochemical identification of the curd isolates at genus/species level was performed according to established phenotypic criteria. The isolates were tested for gas production from glucose, arginine hydrolysis and fermentation of L-arabinose, cellobiose, lactose, mannitol, melibiose, raffinose, ribose, sucrose, sorbitol, trehalose and xylose according Samells et al.[14] with modifications.

PCR

Out of 150 isolates, only ten were selected for identification up to genetic level using PCR. Out of ten, only five isolates were selected for characterization species and were incubated for 16 h. 0.5 ml of this culture was then subcultured overnight in MRS broth, centrifuged (1000 rpm/20 at 5°C) and washed once in SET buffer (containing 10mg/ml lysozyme) followed by incubation for 60 min at 37°C, after the addition of 10% sodium dodecyl sulfate (SDS) and 200 μl of 5 M NaCl. Deproteinizations have been performed by extracting twice with equal volume of phenol and the phenol was removed using chloroform. The aqueous phase was transferred to a clean tube containing isoamyl alcohol. Aqueous phase was then added to equal volume of isopropanol after centrifugation (4500 rpm/15'). Further, DNA was precipitated by adding 1/10 th vol of 3-M sodium acetate (pH 5.2) followed by two volumes of ice cold ethanol. After DNA precipitation condensed into a small clump, it has been hooked out and washed in 90 and 70% ethanol, dried and resuspended in TAE (Tris base, acetic acid and EDTA) buffer [15].

Restriction digestion and agarose gel electrophoresis

Tagsments were separated by gel electrophoresis according to Zong et al [16] using 1.5% submerged horizontal agarose slab gels prepared in TAE buffer. Gels have been run at a constant voltage of 6 V/ml for one hour. DNA fragments have been visualized with uv transilluminator and photographed.

PCR amplification

PCR mixture consisted of NF buffer, Taq buffer (buffer containing Taq DNA polymerase), 10X dNTPs, primer F, primer R and TAQ enzyme. In addition 20 mM TRIS HCl, 50 mM KCl (pH 8.4) dNTPs, 1 μM Taq DNA in final volume of 10μl. PCR was performed by denaturation at 94°C for 30 s. DNA extraction at 72°C for 30 s and final extension at 72°C for 5 of total 35 cycles were performed. PCR products were electrophoresed in 1.4% - 2% agarose gel and visualized by uv transillumination after ethidium bromide staining (5μg/ml) [4, 17].

REP-PCR analysis for species identification

PCR was repeated with other primers specific for the DNA of *Lactobacillus fermentum* and *L. plantarum*. Species specific PCR with primers for 5 isolates of *Lactobacillus* were performed. Products were analyzed in uv transilluminator system.
Results

A total of 150 isolates of *Lactobacillus* was isolated on MRS selective media which were analyzed by phenotypic and biochemical characterization. Out of these 150 isolates, only 10 isolates were selected for the molecular characterization. The stains were Gram positive, non spore forming single cells, pair or short chains. The isolates were facultative anaerobic and catalase and oxidase negative (Table-2). Biochemically the strains were relatively homogenous and produced acids, but no gas from glucose. None of the isolates exhibited alkaline phospho amidase activity and urease activity or hydrolyse gelatin. All were Voges-proskauer negative and failed to reduce nitrite. In carbohydrate fermentation 65% of isolates reduced arabinose, maltose and mannose, while, mannitol was not been fermented. In addition, 50% of isolates reduced galactose, sorbitol and trehlole. Further, 35% of isolates reduced salcin and only 15% of isolates reduced xylose. However, highest number of isolates (75%) could able to ferment fructose (Graph 1). Among the ten selected isolates, 60% reduced arabinose, maltose and mannose. 50% were able to reduce galactose and trehlole. Further, 30% of the isolates were able to reduce sorbitol, 40% salcin and highest 80% were able to reduce fructose. Only 10% of the isolate (L3) reduced the xylose while none of the isolates reduced the mannitol (Table 3). The primer tested in PCR had given a thick band with the ten isolates represented the genus *Lactobacillus* (Fig 1 and 2). Among them five isolates have been given thick bands observed with the primer specific for *L. fermentum* and *L. plantarum* (Fig 3 and 4), and identified as *L. fermentum* and *L. plantarum*.

Table 2: Physiological test results for *Lactobacillus* isolates

<table>
<thead>
<tr>
<th>Gram’s stainig</th>
<th>Catalase Test</th>
<th>Oxidase test</th>
<th>KOH test</th>
<th>Nitrate Reduction test</th>
<th>MR test</th>
<th>VP test</th>
<th>Indole Production Test</th>
<th>Gelatin Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI-L10</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table3: Fermentation results for ten potential *Lactobacillus* isolates

<table>
<thead>
<tr>
<th><em>Lactobacillus</em> isolates</th>
<th>Arabinose</th>
<th>Maltose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Sorbitol</th>
<th>Trehlole</th>
<th>Salcin</th>
<th>Xylose</th>
<th>Mannitol</th>
<th>fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lp1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lp2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Graph 1: Fermentation test results of Lactobacillus species with sugars

Fermentation positive test results of Lactobacillus sp. in percentage in the graph indicated the different fermentation patterns with different sugars such as, 55% arabinose, maltose and mannose positive. Mannitol was not reduced. 45% of isolates reduced galactose, sorbitol and trehlose. 35% of isolates reduced salicin and only 8% of isolates reduced xylose while, 75% reduced the fructose.

Fig 1 and 2: Genus identification of Lactobacillus isolates showed thick band with LbLM primer. L1,L2,L3,L4 and L5 are Lactobacillus isolates where, P1 is the test organism

Fig 3 and 4: Species identification with species specific primer
LFP is the species specific primer for L. fermentum. L1, L2 and L3 are showing thick band with the primer LFP. L4 and L5 no band formation with the primer LFP. LPP is the species specific primer to L. plantarum. L4 and L5 are producing thick band with the primer LPP.
Discussion

A total of 150 isolates of *Lactobacillus* sp. were isolated from very remote regions of malnad districts (12.54° N - 14.17° N to 75.03° E - 76.21° E) of India. The collection area had the tradition of maintaining the curd sample since a century or more, who showed high degree of disease resistance particularly to gastrointestinal diseases and longevity (lived 90 to 100 years, observed usually). Curd samples were collected from these families to isolate the most potential *Lactobacillus* isolates property. All the isolates were uniformly positive with the Gram reaction, catalase, oxidase, KOH, MR-VP, indole test in addition to nitrate reduction test and gelatin hydrolysis (In fact, a high degree of variation among *Lactobacillus* isolates were observed in fermentation test using varieties of sugars (Graph 1). Arabinose, maltose and mannose were 55% positive, while mannitol was not being fermented. In addition, galactose, sorbitol and trehalose were 45% positive. Further, salicin was 35% and the least fermenting xylose (5%) by only (one) isolate. Highest ability to ferment fructose by majority of isolates was recorded (75%) except two isolates. A number of physiological and biochemical properties have been used to differentiate the *Lactobacillus* sp have been recorded by other research groups [18]. They found a variation in biochemical as well as fermentative reactions particularly with maltose and millibiose. However, distinct type of biomolecule being produced by them and perhaps in the present study, distinct variation was observed with xylose and other sugars. This would clearly indicate that heterofermentative nature of *Lactobacillus* having specific phenotypic line.

Of the 150 isolates, 10 isolates were chosen for molecular characterization. Ten isolates were further characterized by PCR using LbLM primer to identify up to generic level. A characteristic banding pattern compare with molecular marker determined as the genera *Lactobacillus*. Further, 5 most significant isolates were characterized up to species by using species specific primer LFP and LPP using repeated PCR and it was identified as *L. fermentum* (L1, L2 and L3) and *L. plantarum* (L4 and L5). Although *L. fermentum* and *L. plantarum* were isolated and characterized by other workers [11, 15, 17, 18, 19]. The present isolates showed high heterofermentative activity exhibiting its diverse nutritive nature, particularly by L3, consequently indicating the unique taste and odour of the curd sample. Although, L3 is the only isolate ferment xylose in addition to arabinose, maltose, mannose and galactose, it perhaps contributed distinct aroma and taste to the curd sample. This may indicate that, the presence of xylose fermented products and other perhaps acts as health boosters.

The present study provided important evidence that this phenotypic group is practically well differentiated from curd sample isolates. Further, typical kind of byproduct produced on fermentation perhaps contributes to flavor and enhancing the beneficial gastrointestinal system consequently better health was observed. Although the isolates were identified as *L. fermentum* and *L. plantarum*, is distinct from convectional bacterial sp in that they are derived from remote areas of Karnataka state, India, the people of this region were highly conservative showed high longevity. Therefore, these isolates perhaps have a potential as health boosters for human.

References