Romanian Biotechnological Letters *Vol. 20, No. 2, 2015*

REVIEW

Genetic diversity of lactic acid bacteria strains towards their potential probiotic application

Received for publication, October 20, 2014
Accepted, January 20, 2015

LUCIA YÉPEZ[1] AND GABRIELA N. TENEA[1,2,*]
[1]*Faculty of Engineering in Agricultural and Environmental Sciences, Universidad Tecnica del Norte, Av. 17 de Julio s-21 y Jose Maria Cordova. Barrio El Olivo, Ecuador.*
[2]*Centre of Molecular Microbiology, Laboratory of Molecular Biology, 59 Marasti, Bucharest, Romania*
[*] ***Corresponding author:*** gntenea@utn.edu.ec, gtenea@hotmail.com

Abstract

Lactic acid bacteria found as sub-dominant component of the human and animal microbial gut represents one of the most significant groups of probiotic organisms. During the last decade probiotics become an important and viable ingredient in the functional foods as well as the pharmaceutical industry. In this paper, we shall review the most important findings with regards to the in vitro screening of specie-specific probiotic strains, the molecular mechanism of probiotic action, and applications in functional food. Moreover, we shall punctuate alternative sources for the isolation of novel probiotic strains to potentially satisfy the market need in the development of new functional products containing probiotic cultures more active and with better probiotic characteristics than those already existed.

Keywords: lactic acid bacteria, probiotics, functional foods, *Lactobacillus ssp.*, immune stimulation

1. Introduction

The „probiotic" (originated from the Greek word „probios" meaning „for life") micro-organisms refers to mostly bacteria which when taken in adequate amounts confer a nutrition benefits (FAO/WHO, [1]).

Early studies of Eli Metchnikoff [1908] [2], a Nobel laureate immunologist, of Balkan peasants which lives longer, generated great interest for the research community in deciphering the role of gut microflora in health. Further studies have emphasized the benefit of probiotics and theirs commercially advantages in many different products in the world (LONGDET & al. [3]). Currently, the interest of studying the potential health benefits of numerous probiotic strains are under investigation, from improving the microbial balance in the intestine to enhancing immune system function.

The probiotics are non-pathogenic group microorganisms comprising a wide variety of species and subspecies, with defined characteristics, such as to adhere, colonize and modulate the human gastrointestinal system. Over decade, the benefic effect of probiotic products including different enzymes, vitamins, capsules or tablets and some fermented foods, on the health of host have been investigated.

The most known microorganisms *Lactobacillus* and *Bifidobacterium* are the main bacteria group studied for their probiotics potential (BEHNSEN & al. [4]). Recent studies have been shown that the identified probiotic strains exhibit anti-inflammatory, anti-allergic and positive therapeutic effect (CHARTERIS & al. [5], EZENDAM & VAN LOVEREN, [6], FLOCH & al. [7]), and play a protective role by directly competing with intestinal pathogens through the release of antibacterial substances such as bacteriocine (COTTER & al. [8]) or metabolites such as acetic acid and lactic acid (SERVIN, [9]). The protective effect on gut flora provides health benefits beyond basic nutrition and make this microorganisms to be considered foods besides nutrients. Therefore, new terminology has been developed, namely *functional foods,* referring to the presence of significant biologically active components.

Ongoing scientific research on the genomics, proteomics and genetic engineering of lactic acid bacteria is increasing our knowledge of their physiology and potential applications. However, despite the presence in the market of many probiotics comprising strains isolated from human gut, there is very little research addressing the exploiting of novel biological resources to identify of new probiotic candidates with enhanced potential benefits. Thus, is a need for improving useful properties of starter cultures by characterizing the genetic diversity of natural microbial population.

2. *In vitro* screening of potential probiotic strains

The basis for assessing probiotic efficacy in humans requires the understanding of the main characteristics of probiotic strains. Each strain has unique and different properties. Therefore, the isolation and characterization of probiotic bacteria is of special interest the criteria used for strain selection, physiological characteristics, tolerance to conditions of the digestive tract, multiplication and operating capacity in the intestine, the effect on the immune system, antibacterial factors, the ability to colonize, resistance to industrial processing, their efficacy and safety.

In general, lactic acid bacteria (LAB) are non-sporing, rods and cocci, aero-tolerant anaerobes that lack catalase and respiratory chain, with a DNA base composition of less than 53 mol% G+C (STILES & al. [10], JOHNSON-GREEN, [11]), and classified as homofermentative and heterofermentative groups in concordance to their mode of glucose fermentation. The homofermentative strains convert carbohydrates to lactic acid as the only or major end product, while the heterofermentative produce lactic acid and additional products such as ethanol, acetic acid and carbon dioxide (HALASZ, [12]). Thus, the main metabolism of LAB is the degradation of different carbohydrates and related compounds by producing primarily lactic acid and energy. To the most used probiotic strains members of the *Lactobacillus* and *Bifidobacterium* genera, other groups of *Enterococcus*, *Oenoccoccus*, *Propionibacterium*, *Bacillus*, *Escherichia coli*, *Clostridium butyricum*, and some yeast strains, such as *Saccharomyces boulardii,* have been also used as probiotics (STANTON & al. [13]). Table 1 shows the most known strains used up to date as probiotics.

Nowadays, the screening for potential probiotic strains is performed using *in vitro* tests, consisting of the selection of strain and further determine its rigorous taxonomic classifycation, related to their origin, habitat and physiology (MORELLI, [14]). A panel group of experts from FAO/WHO (2002) suggested that the specificity of probiotic action is more important to be considered than the source of provenience of the microorganism tested. This conclusion was drawn due to uncertainty of the origin of the human intestinal microflora since the infants are borne with virtually sterile intestine. However, the panel also underlined a need for improvement of *in vitro* tests to predict the performance of probiotics. While many probiotics meet criteria such as acid and bile resistance and survival during gastrointestinal transit, an ideal probiotic strain remains to be identified for any given indication.

Table 1. Microorganisms known as probiotics

Genus *Lactobacillus*	Genus *Bifidobacterium*	Other strains
L. acidophilus *L. casei* *L. crispatus* *L. delbrueckii* subsp. *Bulgaricus* *L. plantarum* *L. fermentum* *L. gasseri* *L.gallinarum* *L. helveticus* *L. johnsonii* *L.plantarum* *L. rhamnosus* *L. reuteri* *L. salivarus*	*B. animalis* *B. adolascentis* *B. bifidum* *B. breve* *B. infantis* *B. longum* *B. lactis*	*Enterococcus faecalis* *Enterococcus faecium* *Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* *Leuoconostoc mesenteroides* *Propionibacterium freudenreichii* *Pediococcus acidilactici Streptococcus salivarus* subsp. *thermophilus* *Saccharomyces boulardii*

The initial screening and selection of probiotic strains consisted of the evaluation of *phenotype and genotype stability, plasmid stability; carbohydrate and protein utilization patterns; acid and bile tolerance and survival and growth; intestinal epithelial adhesion properties; production of antimicrobial substances; antibiotic resistance profiles; antimicrobial properties, immunogenicity.* Secondly, the bacteria must arrive in the intestines in sufficient quantities, strain dependent, to have a potential benefic effect (TUOMOLA & al. [15]). Therefore, the crucial parameters to be considered in the screening of the probiotic strains are viability and ability to adhere to the intestinal mucosa. Viability (generally assessed as CFU/g) should be maintained at the level shown to deliver heath effects. The bacteria may need to adhere to the wall of the intestine (i.e. ”implant”) and colonize in order to have an effect. The lack of standardized procedure to be used in the analysis, as well as the *in vivo* and *in vitro* discrepancies open debate in the values of the parameters to be considered when identify a new probiotic strain. Therefore, it might be valuable approach to establish the strain properties and its target population of the species investigated.

One of the discoverers of *Lactobacillus* GG, dr. Sherwood Gorbach, states: „Our research over the previous 20 years had established beyond doubt that implantation in the gut was the critical feature that a strain must possess to influence the intestinal milieu...” (SILVA & al. [16]). However, proper characterization and identification of the strains is required in case of a probiotic product to be used in food industry or pharmacy (TEMMERMAN & al. [17]). Traditional phenotypic analysis such as microscopy and cultivation has been improved by the introduction of genotypic studies. Table 2 displayed the most important selection criteria for probiotics.

Table 2. Selection criteria for probiotics

Probiotic Strain Properties	Comments
Secure	Accurate identification and characterization of the strains.
Acid and bile tolerance	Survival through the intestine, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surface	Improve immune system, compete with pathogens, maintain metabolic activity, prevents pathogens to adhesion and colonization.
Valuable technological properties	Survival in products, phage resistance, strain stability, oxygen resistance, no negative effects on product flavor.
Clinically validated	Minimum effective dosage has to be known for each particular strain and in different products.

Recently, identification using molecular technologies such as PCR (polymerase chain reaction) with specific primers sequences for the analysis of ribosomal RNA with sequences containing hyper-variable subunits that are specific to each bacteria species has been performed (TANGANAUT & al. [18]). Currently, about 16,000 sequences 16S rRNA in the databases associated with new bioinformatics techniques has been described for the identification and characterization of probiotic bacteria strains. Other molecular approaches such as RAPD (random amplify polymorphic DNA), RFLP (restriction fragments length polymorphism), REP-PCR (repetitive sequenced based PCR) have been used in the process of the molecular identification of the bacterial strains. For example, in one study, the PCR-ARDRA technique was used to identify potential probiotic *Lactobacillus* species isolated from bovine vagina (OTERO & al. [19]). The 16S rRNA gene was amplified by PCR and the amplification products were further digested with different restriction enzymes. Based on this digestion profile, the bacteria isolates were classified as *Lactobacillus fermentum* (OTERO & al. [19]). In other studies, a novel multiplex PCR primer set containing of seven specific and two conserved primers, from the integrated sequences of 16S and 23S rRNA genes and their rRNA intergenic spacer region was used to identify seven probiotic *Lactobacillus* species with 93.6% accuracy. This study showed that the multiplex primer set is really efficient tool for simple, rapid and reliable identification of *Lactobacillus* species (KWON & al. [20]). The molecular identification of the bacillus species is important knowing that the probiotic characteristic is specie-specific.

3. The molecular functionality of the probiotics

During the past decade, scientists from the field of microbiology, immunology, and gastroenterology have actively studied the mechanism by which commensal bacteria improve mucosal defenses of the gastrointestinal tract by trying to explain how probiotics could protect the host from the intestinal disorders (O'HARA & SHANAHAN, [21]). The application of probiotic lactobacilli starts with the assumption that the mechanisms by which lactobacilli had health benefits are to one of the following categories: (i) pathogen inhibition and restoration of microbial homeostasis through microbe-microbe interactions, (ii) enhancement of epithelial barrier function, and (iii) modulation of immune responses (Table 3) (O'HARA & SHANAHAN, [21])

Table 3. Mechanisms of action of probiotics

Probiotics: mechanisms of action
(i) Pathogen inhibition and restoration of microbial homeostasis through microbe-microbe interactions
Decrease luminal pH
Secrete antimicrobial peptides
Inhibit bacterial invasion
Block bacterial adhesion to epithelial cells
(ii) Enhancement of barrier function
Increase mucus production
Enhance barrier integrity
(iii) Immunomodulation
Effects on epithelial cells
Effects on dendritic cells
Effects on monocytes/macrophage
Effects on lymphocytes
o *B lymphocytes*
o *NK cells*
o *T cells*

(1)Pathogen inhibition and restoration of microbial homeostasis through microbe-microbe interactions

The capacity of lactobacilli to inhibit pathogens is well known since they have been used for centuries in food preservation to prevent microbial spoilage. However, some important characteristics has been pointed out by the Council for Agricultural Science and Technology: a) the microbes colonizing different regions of the human body are multiple and numerous and differs according to their habitat. b) microbes have stable viability through time: colonizing *microbiota* can be impacted by antibiotics, diet, immunosuppression, intestinal cleansing, and other factors; c) the composition of the "normal intestinal *microbiota*" is not currently elucidate (FORSSTEN & al. [22]). In the same manner, the characteristics of the intestinal *microbiota* are not well understood. The nature of the end products of growth of these microbes may be as important as which specific microbes are present.

(2)Enhancement of epithelial barrier function

The intestinal barrier considered major defense mechanism used to maintain epithelial integrity and to protect the organism from the environment, is maintained by several interrelated systems including mucus secretion, chloride and water secretion, and binding together of epithelial cells (NG & al. [23]). The consumption of non-pathogenic bacteria could increase the intestinal barrier function, and the functionality of probiotic bacteria have been extensively studied for their contribution in the preservation of this barrier. It has been shown that the microorganisms that interact with the gastrointestinal mucosa can communicate with the primary epithelial and mucosal lymphoid elements and such interaction would stimulate host defenses in the gut (WALKER, [24]).

As the mechanisms by which probiotics enhance the intestinal barrier function are not fully understood, numerous studies underline that the enhancing the expression of genes involved in close-fitting of the junction signaling are linked with the strengthen of intestinal barrier integrity. For example, it has been suggested that toll-like receptors (TLRs) from the eukaryotic epithelial, endothelial, and lymphoid cells, could interact with molecular patterns on both pathogens and commensal bacteria (WEISS & al. [25], CASTILLO & al. [26]). It has been shown that after being anchored to the cell surface by a surface molecule, CD14, the TLR-4 interacts with LPS as an LPS-binding protein complex. As result of this interactions numerous signaling molecules are activated in the cell to release the transcription factor nuclear factor kB (NFkB) into the nucleus, which in turn transcribes inflammatory cytokines (IL-8 and IL-6) to supply the fundamental point for an acute innate inflammatory response to an invading pathogen (CASTILLO & al. [26]).

(3) Modulation of immune responses

Lactobacilli have been investigated for their capacities to employ immune-stimulatory and -regulatory effects. As probiotics are administered to the gastrointestinal tract via beverages, food, or capsules, their capacity to enhance the barrier function of the gut wall epithelium against pathogens and toxins is also gaining attention to the scientific community (ISOLAURI, [27]). It has been found significant improvement when a supplement containing two lactobacilli strains (*L. rhamnosus* or *B. lactis)* was administrated to children's who showed atopic eczema. Another study has shown that the consumption of probiotic *L. rhamnosus* GG by the pregnant woman reduced the rate of newborns having atopic dermatitis (KALLIOMAKI & al. [28]). Moreover, in another study involving hypercholesterolemic mice, the probiotic potential of *L. plantarum* PHO4 was established (NGUYGEN et al. [29]). It has been shown that the mice feed with 10^7 CFU/day over two weeks possess about 7 to 10% lesser serum cholesterol and triglycerides than the control one. More recently, a specific role of intestinal bacteria in the pathogenesis of various intestinal disorders, such as aberrant immune responses that contribute to the pathogenesis of inflammatory bowel disease (O'HARA & SHANAHAN, [21], HAKANSSON & MOLIN, [30]) has been recognized. For example, innate immune responses to indigenous bacteria prime the immune system and influence adaptive responses to exogenous antigens.

It is known that several probiotic strains are able to stimulate and regulate, several features of natural immune responses (EZENDAM & VAN LOVEREN [31]). These properties contribute to the improvement of human health and could influence infectious disease, response to vaccines, autoimmune disorders, and other inflammatory diseases. Some studied performed in animal demonstrated the different effect on the immune system, which depends mostly on the dose applied and immune status of the host (SAAVEDRA, [32]).

The probiotic efficacy of *L. casei* isolated from human breast milk in the prevention of shigellosis of albino rats infected with clinical strains of *Shigella dysenteriae* has been well investigated. The results have demonstrated that the experimental group of rats infected with *S. dysenteriae* but not treated suffer for the disease and the one which were treated with the L. casei showed no effect on the liver and do not develop the disease (LONGDET & al. [33]).

Probiotic microorganisms can stimulate the immune responses or down-regulate numerous inflammatory responses (CUMMINGS & al. [34]; ERICKSON & HUBBARD, [35]; GILL & GUARNER, [36]; GUARNER, [37]; MADSEN [38]), as well as the modulate the activity of the monocytes, macrophages and neutrophils cells, natural killer cells (NK-cells), the major class of cellular effectors of innate immunity (GILL & GUARNER [36]).

4. Probiotics and their use as functional food

In recent years, consumers have begun to look at food not only as a source of energy but also for health benefits beyond basic nutrition (ASHWELL & al. [39]). Functional foods are also known as nutraceuticals, pharma-foods or designer foods.

A functional food contains an added ingredient that makes the traditional food functional as known the added probiotic bacteria into traditional yogurt. However, a food can also be naturally functional such as *oatmeal* that naturally contains beta-glucans that has been proven to reduce blood cholesterol levels. Another product, such as cooked tomatoes containing phytochemical lycopene and yogurt and other fermented milk products, may reduce the risk of prostate and cervical cancer and respectively, enhance gastrointestinal system function (DE ROSS & al. [40], STANTON & al. [41]). Actually, foods containing living organisms form a special sub-group of functional foods namely: ***probiotic foods.***

Based on their health benefits the functional foods can be divided on gut, bone, heart and immune system foods. The dairy products including fermented milk products are known for their beneficial effect of gut microflora and are considered the most powerful in the area of functional foods (COLLADO & al. [42]).

The main ingredients of gut health include probiotics, prebiotics and synbiotics. Probiotics help to maintain the balance of beneficial and harmful bacteria whereas prebiotics are a natural food for probiotic bacteria supporting their growth. A symbiotic product contains both a probiotic and a prebiotic ingredient.

The products for heart health emphasis on the reducing risk factors for cardiovascular disease including elevated blood cholesterol levels and hypertension, includes soya, omega-3 fatty acids, phytostanols as well as phytosterols. All of these have shown to have cholesterol-lowering ability. Functional foods known to increase the immune system have mainly been fortified with vitamins or contain probiotic cultures. Some probiotics have an ability to boost the immune system and the antioxidant vitamins A, C and E can increase the resistance of the body to infection.

Nowadays, the new formulated food products that could improve the immune system contained in different food matrices consisted of probiotic yogurt drinks, various types of cheese, ice creams, milk-based desserts, powdered milk for new-born infants, butter, mayonnaise, powder products or capsules and fermented food of vegetable origin.

5. Conclusion remarks

With concomitant developments of the research, commercial, food industry and medical sectors, the field of probiotics is also growing rapidly with accumulation of many data about their health and nutrition benefits. The complete genome sequencing of the most known lactic acid bacteria as well as the identification of functional properties will contribute to the developing of most powerful products with improvement functional properties. As the mainly source of knowing probiotics is represented by the human microflora it would be more valuable to search for other sources of probiotic microorganisms.

According to the new territorial redistribution several zones of Ecuador, known as undeveloped natural areas, were included in the governmental policy as important resources to be considered. In this context, our current work relates to isolation, characterization and evaluation the probiotic capacity of lactic acid bacteria isolated from native un-exploited ecological niches originated from Ecuador. The preliminary results indicated the presence of a larger number of lactic acid bacteria to be tested for their probiotic potential (Tenea and col., un-publish data). The effort is carried out to rigorous characterization of these micro-organisms to be further tested in as a starter culture for the development of a **novel probiotic** product. In addition, wild strains might provide phenotypic and genetic variation resources for the improvement of current commercial probiotic strains.

6. Acknowledgements

G.N.T was sponsored by the Secretariat for Higher Education, Science, Technology and Innovation of the Republic of Ecuador under Prometeo Research project.

References

(1) FAO/WHO, Guidelines for the evaluation of probiotics in food – *Joint Food and Agricultural Organization of the United Nations and World Health Organization Working Group Meeting Report*, London Ontario, Canada, (2002).

(2) E. METCHNIKOFF, *The Prolongation of Life, 1st edition.* G.P. Putnam's Sons, NY, (1908).

(3) I.Y. LONGDET, R.J. KUTDHIK, I.G. NWOYEOCHA, The probiotic efficacy of *Lactobacillus casei* from human breast milk against Shigellosis in Albino rats, *Advances in Biotechnology & Chemical Processes* 1: 12-16, (2011).

(4) J. BEHNSEN, E. DERIU, M. SASSONE-CORSI, M. RAFFATELLU M, Probiotics: Properties, Examples, and Specific Applications, *Cold Spring Harb Perspect Med* 2013; doi:10.1101/ cshperspect.a010074, (2013).

(5) W.P. CHARTERIS, P.M. KELLY, L. MORELLI, J.K. COLLINS, Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract, *Journal of Applied Microbiology* 84: 759-768, (1998).

(6) J. EZENDAM, H. VAN LOVEREN, Probiotics: Immunomodulation and evaluation of safety and efficacy. *Nutrition Reviews*, 64(1): 1-14, (2008).

(7) M.H. FLOCH, W.A. WALKER, K. MADSEN, M.E. SANDERS, G.T. MAC-FARLANE, H.J. FLINT, L.A. DIELEMAN, Y. RINGEL, S. GUANDALINI, C.P. KELLY & al. Recommendations for probiotic use. *J Clin Gastroenterol* 45 (Suppl): S168 – S171, (2011).

(8) P.D. COTTER, C HILL, R.P. ROSS, Bacteriocins: Developing innate immunity for food. *Nat Rev Microbiol* 3: 777 – 788, (2005).

(9) A.L SERVIN, Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* 28: 405–440, (2004).

(10) M.E. STILES, W.H. HOLZAPFEL, Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microb*. 36: 1–29, (1997).

(11) P. JOHNSON-GREEN, *Introduction to food Biotechnology*. CRC Press, Boca Raton (2002).

(12) A. HALASZ, Lactic Acid Bacteria. In: Lasztity R, editor. *Food Quality and Standards* (Vol. 3). EOLSS Publishers Co Ltd, UK, (2009).

(13) C. STANTON, C. DESMOND, M. COAKLEY, J.K. COLLINS, G. FITZGERALD, R.P ROSS, Two challenges facing development of probiotic-containing functional foods, Farnworth ER (Ed.), *Handbookof fermented functional foods.* CRC Press, Boca Raton, FL, 27–58 (2003).

(14) L. MORELLI, In vitro assessment of probiotic bacteria: from survival to functionality. *Int Dairy J.* 17: 1278-128, (2007).

(15) E. TUOMOLA, R. CRITTENDEN, M. PLAYNE, E. ISOLAURI, S. SALMINEN, Quality assurance criteria for probiotic bacteria, *Am J Clin Nutr.* 73 (2): 393s-398s, (2001).

(16) M. SILVA, N.V. JACOBUS, C. DENEKE, S.L. GORBACH, Antimicrobial substance from a human *Lactobacillus* strain. *Antimicrob. Agents Chemother*. 31 (8): 1231–3 (1987).

(17) R. TEMMERMAN, B. POT, G. HUYS, J. SWINGS, Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol* 81(1): 1–10, (2003).

(18) W. TANGANURAT, B. QUINQUIS, V. LEELAWATCHARAMAS, A. BOLOTIN, Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables, *Journal of basic microbiology.* 49: 377 – 385, (2009).

(19) M.C. OTERO, L. MORELLI, M.E. NADER-MACIAS, Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. *Letters in applied microbiology* 43:91-97, (2006).

(20) H.S. KWON, E.H. YANG, S.W. YEON, B.H. KANG, T.Y KIM, Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16s rRNA through 23s rRNA. *FEMS Microbiology Letters* 239:267-275, (2004).

(21) A.M. O'HARA, F. SHANAHAN, Mechanisms of action of probiotics in intestinal diseases, *The Scientific World JOURNAL*. 7: 31–46 ISSN 1537-744X; DOI 10.1100/tsw.2007.26, (2007).

(22) S.D. FORSSTEN, S.J. LAHTINEN, A.C. OUWEHAND, J.J. MALAGO & al. (eds.) Chapter 2: *The Intestinal Microbiota and Probiotics 24 pp. In: Probiotic bacteria and enteric infections.* Springer Science Business Media B.V. (2011).

(23) S.C. NG, A.L. HART, M.A. KAMM, A.J. STAGG, S.C. KNIGHT, Mechanisms of Action of Probiotics: Recent Advances, *Inflamm. Bowel Dis*. 15(2), (2009).
(24) W.A. WALKER, Mechanism of action of probiotics, *Probiotic Mechanisms. CID.* 46 (Suppl 2). S87, (2008).
(25) D.S. WEISS, B. RAUPACH, K.TAKEDA, S. AKIRA, A. ZYCHLINSKY. Toll-like receptors are temporally involved in host defense. *J Immunol;* 172: 4463–4469, (2004).
(26) N.A. CASTILLO, G. PERDIGÓN, A. DE MORENO DE LE BLANC, Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar *typhimurium* infection in mice. *BMC Microbiol*.11: 177–189, (2011).
(27) E. ISOLAURI, Probiotics: Effects on immunity. *Am. J Clin Nutr.* 2001; 73 (suppl): 444S-450S, (2001).
(28) M. KALLIOMAKI, S. SALMINEN, H. ARVILOMMI, P. KERO, P. KOSKINEN, E. ISOLAURI, Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial, *Lancet* 357: 1076-1079, (2001).
(29) T.D.T. NGUYGEN, J.H. KANG, M.S. LEE, Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol lowering effects, International *Journal of Food Microbiology* 113(3): 358-361, (2007).
(30) A. HAKANSSON, G. MOLIN, Gut microbiota and inflammation. *Nutrients*. 3:637, (2011).
(31) J. EZENDAM, H. VAN LOVEREN, *Lactobacillus casei* Shirota administered during lactation increases the duration of autoimmunity in rats and enhances lung inflammation in mice. *Br J Nutr.* 99: 83-90, (2008).
(32) J. SAAVEDRA. Probiotics and infectious diarrhea, *American Journal of Gastroenterology* 95: 16-18, (2000).
(33) I.Y. LONGDET, R.J. KUTDHIK, I.G. NWOYEOCHA The probiotic efficacy of *Lactobacillus casei* from human breast milk against Shigellosis in Albino rats*, Advances in Biotechnology & Chemical Processes* 1: 12-16, (2011).
(34) J.H. CUMMINGS, J.M. ANTOINE, F. AZPIROZ, R. BOURDET-SICARD, P. BRANDTZAEG, P.C. CALDER, G.R. GIBSON, F. GUARNER, E. ISOLAURI, D. PANNEMANS, C. SHORTT, S. TUIJTELAARS, B. WATZL, PASSCLAIM—Gut health and immunity. *Eur J Nutr* 43 (Suppl. 2): II/118–173, (2004).
(35) K.L. ERICKSON, N.E. HUBBARD, Probiotic immunomodulation in health and disease. *J Nutr* 130: 403S–409S, (2000).
(36) H.S. GILL, F. GUARNER, Probiotics and human health: A clinical perspective. *Post-grad Med J* 80:516–526, (2004).
(37) F. GUARNER, Enteric flora in health and disease. *Digestion* 73 (Suppl 1): 5–12, (2006).
(38) K. MADSEN, Probiotics and the immune response. *J Clin Gastroenterol* 40:232–234, (2006).
(39) M. ASHWELL, Concepts of Functional Foods (ILSI Europe Concise Monograph Series Ed Walker, Available at: http://www.ilsina.org/file/ILSIFuncFoods.pdf, (2002).
(40) N.M. DE ROOS, M.B. KATAN, Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcino-genesis: A review of papers published between 1988 and 1998. *Am. J Clin Nutr.* 71: 405-411, (2000).
(41) C. STANTON, R.P. ROSS, G.F. FITZGERALD, D.V. SINDEREN, Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotechnol* 16:198–203, (2005).
(42) M.C. COLLADO, E. ISOLAURI, S. SALMINEN, Y. SANZ, The impact of probiotic on gut health. *Current Drug Metabolism,* 10: 68-78, (2009).