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Immunomodulation of gastrointestinal tract by probiotics: an insight into the role of *Lactobacillus* sp. and *Bacillus* sp. on immunity

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ABSTRACT

The gastrointestinal tract (GIT) is an immunologically active ecosystem with a crucial role in activating or stimulating the immune system as well as a barrier against microbial antigens. About a hundred trillion microorganisms such as bacteria, viruses, fungi, and protozoa cohabit with the GIT. Gut microbes play a vital role in health and disease, leading to the emergence of 'probiotics' a novel therapeutic consumption of live, beneficial cultures. Probiotics exhibit an extensive mechanism of action which include destroying/blocking pathogens, production of antimicrobial compounds, and modulation of the immune system without affecting intestinal homeostasis. Although *Lactobacillus* sp. is most abundantly used as a probiotic, reports also show the emergence of *Bacillus* sp. in boosting the immune system and promoting good health. This review attempts to highlight the potential of Lactobacillus and Bacillus species as a probiotic in stimulating the immune system.

Key words: Probiotics, *Lactobacillus*, *Bacillus*, immune system, gastrointestinal tract

Introduction

Probiotic which is 'for life' in Greek has numerous definitions. Probiotics are live microorganisms that help to maintain normal microflora of the body including the gastrointestinal tract and thereby benefit the host. Research findings indicating the importance of probiotics in modulating host immune responses, thereby affording protection from arthritis and intestinal inflammation, as well as resistance to diseases, diabetes etc., also point to their increased use over the past few years in treating diseases. Some of the definitions given to probiotics are listed in Table 1. In U.S.A. as dietary supplements, they are not permitted to claim cure or treat any disease. Mixtures of *Lactobacillus* and *Streptococcus* species are being used in fermented milk and milk products to promote human health since the middle ages (Berg, 1998). Microorganisms that meet the criteria of probiotics include lactic acid bacteria (LAB), most commonly *Lactobacillus* and *Bifidobacterium* sp., yeast, *Lactococcus*, *Streptococcus*, non-pathogenic strains of *Escherichia coli* and *Enterococcus* species. Similarly, other studies have contributed new insights into the immune-mediated mechanisms in infections and metabolic diseases (Borchers et al., 2009). Vertebrate gut with its antigenically diverse microflora has a definite role in immune modulations. The main purpose of this review is to compare the health benefits of probiotics *Lactobacillus* sp. and *Bacillus* sp. on the immunity of various organisms.

Table 1. *Definitions of probiotics cited over years.*

Year	Author	Definition	Reference
1965	Lilly & Stillwell	"Substances secreted by microorganism which stimulate the growth of the other"	Lilly & Stillwell, 1965
1971	Sperti	"Tissue extracts which enhanced the growth of microorganisms"	Fuller, 1992
1974	Parker	"Organisms and substances which contribute to intestinal microbial balance"	Parker, 1974
1989	Fuller	"A live microbial feed supplement which beneficially affects the host animal by improving its microbial balance"	Fuller, 1992
1992	Havenaar	"A viable mono or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous 'microflora'"	Havenaar et al., 1992
2002	WHO	"Live microorganisms which when administered in adequate amounts confer a health benefit on the host"	FAO & WHO, 2002

Table 2. *Properties of probiotics.*

Probiotic properties	Remarks
Acid and Bile stability	To survive in the intestine, retaining cell integrity and metabolic activity
Mucosal adhesion	Modulation of immunity by competitive exclusion of pathogens, preventing adherence of pathogen and its colonisation in the intestine
Safe for consumption	Intestinal mucus is not degraded.
Production of antimicrobial substances	Inactivation of pathogens which are harmful for normal microflora
Increase bowel movement	Relieves constipation and reduces diarrhoea
Absence of pathogenic characteristics	lack of enterotoxins, cytotoxins, hemolysis, presence of antibiotic resistant genes

In a normal state, the intestinal immune system is homeostatic, tolerating most intestinal microbes that provide vitamins, short-chain fatty acids, and amino acids and protecting from pathogen invasion by competitive exclusion. Besides, intestinal microbes produce antimicrobial compounds to create a hostile environment for pathogens. In turn, the microbe provided with a nutrient-rich, stable environment has a mutualistic relationship with the host. The positive health effects of intestinal bacteria like cancer prevention, reduction in blood cholesterol, medication for gastrointestinal disorders, vaginal infections, urinary infections, an inhibitor of dental caries, pulmonary infections, enhanced growth, etc. have led researchers to reinforce these effects through the supply of beneficial probiotic bacteria. There is increased evidence that probiotic strains exhibit similar activities as commensal bacteria including immunomodulation (Quijano, 2014). Microorganisms need to meet several criteria to qualify as probiotics, which is summarized in Table 2.

1.1. Probiotics and Immune system

The role of probiotics in immunity can be better understood by using animal models which are ideal prototypes. Various animals used include fish (adult and larvae), poultry, mouse, pig, cow, monkeys, and humans. *In vitro* systems are also extensively used to look into biological mechanisms like signalling pathways (Patel et al., 2015). The immune defense mechanism of the gut comprises three parts; the intestinal epithelial barrier, the lamina propria, and the gut-associated lymphoid tissue (GALT). GALT is composed of organised Peyer patches, isolated lymphoid follicles and mesenteric lymph nodes (Ahluwalia et al., 2017). Foreign antigens are prevented from entering the body from the lumen of mucosal tissues by a variety of mechanisms. Some of these are site-specific. Being an accessible organ the gut immune system has been the most widely studied to date because tissue samples are often removed during a number of clinical procedures. It is thought that there is more lymphoid tissue along the length of the gut than anywhere else in the body. This is likely to be the consequence of harboring such a large amount of commensal microorganisms, the vast majority of which are composed of hundreds of different species of bacteria.

Commensal microorganisms are ubiquitously present on epithelial surfaces throughout the human body. These microbes have co-evolved with their hosts, ensuring a symbiotic relationship that is often beneficial to both the commensal and the host species. The vast majority of commensal microorganisms are Gram-negative bacteria found in the distal parts of the intestines. Although under normal circumstances commensal bacteria are not pathogenic, they still possess similar pathogen-associated microbial peptides (PAMPs) as pathogenic bacteria. PAMPs recognized by TLRs (Toll-like receptors) and NOD (nucleotide oligomerization domain-containing) proteins are shared between both pathogenic and commensal microorganisms. The mechanism by which the immune system distinguishes between the two types of PAMPs is not fully understood, although several hypotheses have been proposed. There is some evidence to suggest that certain commensal bacteria possess altered PAMPs that prevent or antagonize TLR recognition, thereby preventing the activation of inflammatory pathways. However, this is not the case for all commensals, while certain pathogenic species employ the same strategy to avoid immune recognition. Evidence also exists demonstrating that commensal species actively down-regulate inflammatory pathways or promote immunoregulatory responses. For example, *Lactobacillus* sp. reduced the expression of the proinflammatory transcription factor NF-κB and IL-10 (Rocha-Ramírez et al., 2017) while commensal *Helicobacter* sp. enhance Foxp3+ CD4+ Treg cell expression (Gorman, 2016). Probiotics stimulate, modulate and regulate host immune response by initiating the activation of specific genes of localized host cells. They modulate gastrointestinal hormone release and regulate brain behaviour through bidirectional neuronal signalling, as part of the gut-brain axis (Hsu et al., 2007).

Mechanisms, by which probiotics work on intestinal microbial communities, are suppression of pathogens, modulation of the immune system, stimulation of the proliferation of epithelial cells, and by differentiating and strengthening the intestinal barrier (Thomas & Versalovic, 2010). Antimicrobial compounds produced by probiotics suppress the growth of other microorganisms (Spinler et al., 2008) and compete with other microbes in the intestine for receptors and binding sites on intestinal mucosa (Collado et al., 2007). *Lactobacillus* strains augment the integrity of the intestinal barrier, preserve immune system tolerance, decrease

the transfer of bacteria across the intestinal mucosa and reduce gastrointestinal infections like IBS and IBD (Lee & Bak, 2011). Probiotics alter the receptiveness of epithelial cells and immune cells to microbes in the intestinal lumen which results in the modulation of intestinal immunity (Strähle et al., 2012; Bron et al., 2017). The mechanism of probiotic lactobacilli on the immune system is strain specific. The immune system being multi-compartmental, every probiotic strain interacts differently by, inducing a visible and quantifiable effect (Kemgang et al., 2014).

The decade saw an array of research on probiotics. *Lactobacillus* sp. is the most commonly studied to date. Besides the genus *Bacillus* sp., consisting of spore-forming bacteria, carry a number of probiotic attributes. The benefits of LAB and its role in the maintenance of health in humans and animals have been established both scientifically and commercially. However, in comparison to LAB, bacterial spore formers have not scored much acceptance in probiotic research. In the present review, to get a general perception about the research articles published in the area of probiotics, CSVs were collected using the key words '*Lactobacillus*+probiotics' and '*Bacillus*+probiotics' in the search field 'PubMed'. Research papers published over the last 10 years i.e., from 2009 to 2018 for both *Lactobacillus* sp. and *Bacillus* sp. were compared as shown in Figure 1. The figure illustrates the fact that majority of research in the area of probiotics was done using *Lactobacillus* sp. when compared to *Bacillus* sp. Even though the number of articles published related to *Bacillus* sp. is fewer when compared to *Lactobacillus* sp., there has been sustained research activity in *Bacillus* sp. since 2009. This showed that considerable effort is invested in studying the in-depth molecular mechanisms of probiotics. The quest for understanding the mechanism of action of probiotics has brought about a large interest in understanding the molecular processes critical in host-microbe interactions.

A number of studies have examined the potential of *Bacillus* sp. as a probiotic in *in vitro* and *in vivo* models. Besides meeting all the qualities of a probiotic, *Bacillus* sp. shows tolerance to high acidic conditions and enhanced stability during heat processing and storage at low temperatures (Bader et al., 2012) They also eliminate pathogens and exhibit anti-microbial, anti-oxidant, immunomodulatory (Lefevre et al., 2015; Shobharani et al, 2015; Ripert et al., 2016), and food fermentation (Terlabie et al., 2006) capabilities. The addition of *lactobacilli / bifidobacterium* to fermented milk products (Colombel et al., 1987) claims to maintain the balance of intestinal flora, where exogenous bacteria reach the large intestine in a viable form. The mechanism by which probiotic organisms modulate the immune system is not entirely known. But it is considered to be a result of competition for nutrients in the colon, for colonization, for binding on epithelial cells in the gut, for bacteriocin production, lowering the pH of the colon, and by nonspecifically stimulating the immune system (Liu et al., 2007). By activating the host's nonspecific resistance probiotic organisms eliminate microbial pathogens. The gut flora is stabilized by the prevention of inflammation and intestinal permeability. In addition, they promote the immunologic barrier by enhancing intestinal immunoglobulin A (IgA) as well as inflammatory responses, and non-immunologic gut defense barrier by controlling increased intestinal permeability and altered gut microflora (Isolauri et al., 2001).

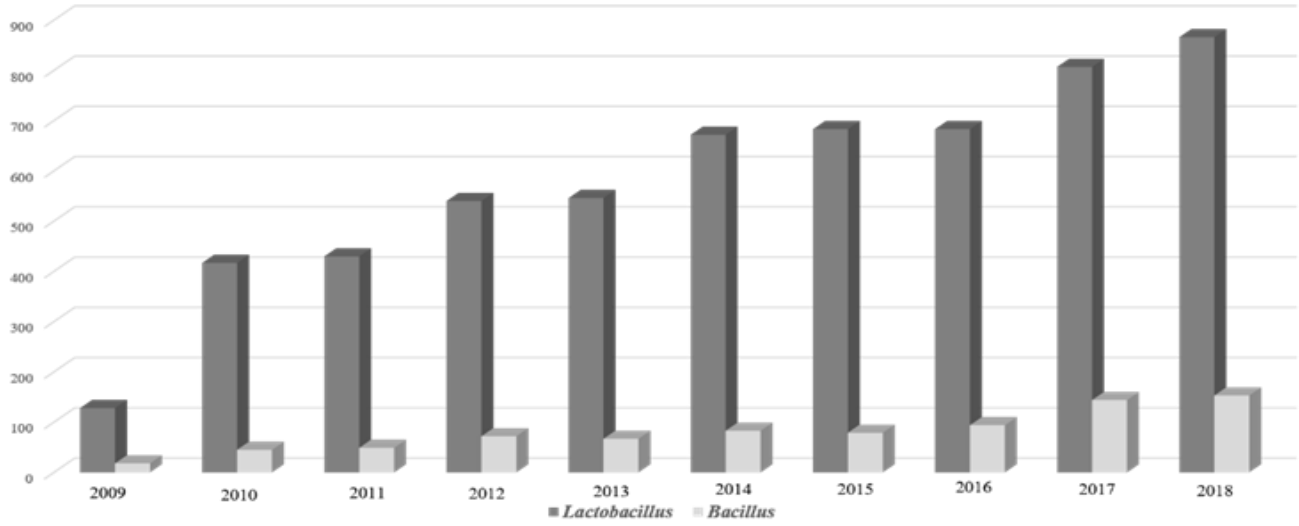

Figure 1. *Shows the trend in research articles published in the area related to probiotics Lactobacillus sp. and Bacillus sp. over the past 10 years (Source: Generated from CSVs obtained from PubMed using keywords 'Lactobacillus+probiotics' and 'Bacillus+probiotics' from January 2009 to December 2018).*

1.2. General mechanism of action of probiotics

The body prevents the entry of foreign antigens by a wide array of mechanisms. The immune system comprises biological structures that are capable of detecting pathogens and protecting against diseases. Apart from that it also has the ability to distinguish pathogens from the host's own healthy tissue. The mucosa-associated lymphoid tissue (MALT) is the largest immune organ in the body. The mucosal layer of the gastrointestinal tract, respiratory tract, and genitourinary tract together constitute a total area of 400m^2. The host intestinal gut microbiome associated with GIT is present in the large intestine plays a role in host defense (Isolauri et al., 2001). The intestinal gut microbiome is involved in the fermentation of exogenous carbon and energy sources and also produces short-chain fatty acids (SCFAs) beneficial to the host. It also provides protection against invading pathogens by a phenomenon called 'colony resistance'. The intestinal microbiome competes for the same attachment sites and nutrients as the pathogens. They also produce antimicrobial compounds that inhibit the growth of pathogens and also provide an important stimulus for the maturation of the immune system.

The mucosa-associated lymphoid tissue initiates an immune response to pathogens along mucosal linings in the body. Gut-associated lymphoid tissue a component of MALT relies on the large population of antibody-producing plasma cells. MALT is composed of two regions; a diffused lymphoid tissue scattered in lamina propria and an organised lymphoid tissue found in the submucosa. GALT eliminates antigens by non-specific and specific defense mechanisms. The specialised epithelial cells called M cells in the enterocytes are involved in the transport of antigens. The reduced brush border, glycocalyx, and amount of hydrolytic enzymes on the apical membrane allow interaction between antigens and M cells (Collins et al., 2012). Conserved pathogen-associated microbial peptides (PAMPs) in microorganisms are recognized by pattern recognition receptors (PRRs) which play a key role in the innate immune response. Pathogen and pathogen-derived products are recognized by toll-like receptor (TLR) family, which in turn, initiates an innate immune response. TLRs are pattern recognition receptor family conserved from insects to mammals. PAMPs recognised by TLRs include lipids, lipoproteins, proteins, and nucleic acid derived from microorganisms like bacteria viruses, parasites, and fungi (Jault et al., 2004).

TLRs are of two types, the protostome-type (P-type) and the vertebrate-type (V-type) (Schnare et al., 2001). P-type TLRs and V-type TLRs are mostly found in invertebrates and vertebrates respectively (Leulier & Lemaitre, 2008). TLRs are divided into six major subfamilies, namely, the TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11 subfamilies. TLR1 subfamily recognizes lipoproteins and consists of TLR1, 2, 6, 10, 14, 15, 16, 18, 25, 27 & 28. dsRNA, LPS, and bacterial flagellin are recognized by TLR3, 4, and 5 subfamilies respectively. Nucleic acid motifs are recognized by TLR7 subfamily which include TLR7, 8, and 9. Proteins and nucleic acid motifs are recognized by the TLR11 subfamily which consists of TLR11, 12, 13, 19-23, and 26. Depending on their cellular localisation and PAMP ligands TLRs are also divided into two groups. TLR (1, 2, 4, and 6) which are expressed on cell membranes and stimulated by microbial membrane components, and TLR (3, 7, 8, and 9) which are mainly stimulated by nucleic acid derived from microbes, particularly viruses. TLR7 and TLR9 are mainly sequestered in the endoplasmic reticulum in unstimulated cells and endolysosomes (Nie et al., 2018).

Depending on TIR domain-containing adaptor-inducing IFN-β (TRIF), TLR signalling pathways are classified into two specific types of pathways; myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and MyD88-independent pathway (Akira & Takeda, 2004). All TLRs except TLR3 depend on MyD88-dependent pathway. Dimerization of TLR occurs after ligand recognition and MyD88 binds to the TIR domain of TLR with the help of homotypic/heterotypic interactions. A Myddosome complex is formed by the association of IL-1 receptor-associated kinase 4 (IRAK4) and the death domain of MyD88, which leads to auto-phosphorylation of IL-1 receptor-associated kinase 1 (IRAK1) (Lin et al., 2010). Protein tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is activated which activates the TAK1/TGF-β-activated kinase (TAB) complex by K-63-linked poly ubiquitination of TAK1 and TRAF6. IκB kinase (IKK)-mediates the phosphorylation and degradation of I kappa B alpha (IκBα). This leads to the nuclear translocation of the transcription factor NF-κB which induces the activation of inflammatory genes like TNF-α, IL-1, IL-6, and IL-8 (Wang et al., 2001). TLR2 is the PRR that recognises bacterial lipoproteins and peptidoglycans in gram-positive bacteria and TLR5 is the PRR for flagellin of bacterial flagella. TLR2 forms heterodimers with TLR1 and TLR6. The TLR1/TLR2 and TLR6/TLR2 complex recognises bacterial lipopeptides and bacterial diacylated lipopeptides, respectively. TLR7 binds to single-stranded RNA (ssRNA) from viruses such as influenza and human immunodeficiency virus I (HIV-I) and play no role in bacteria and mice. TLR3 is expressed in dendritic cells and epithelial cells of the respiratory system, cervix, intestine, uterus, cornea, and brain. Bacterial DNA is sensed by TLR9 (Testro & Visvanathan, 2009). The mechanism of action of probiotics and the role of Toll-like receptors in enhancing immunity has been schematically shown in Figure 2.

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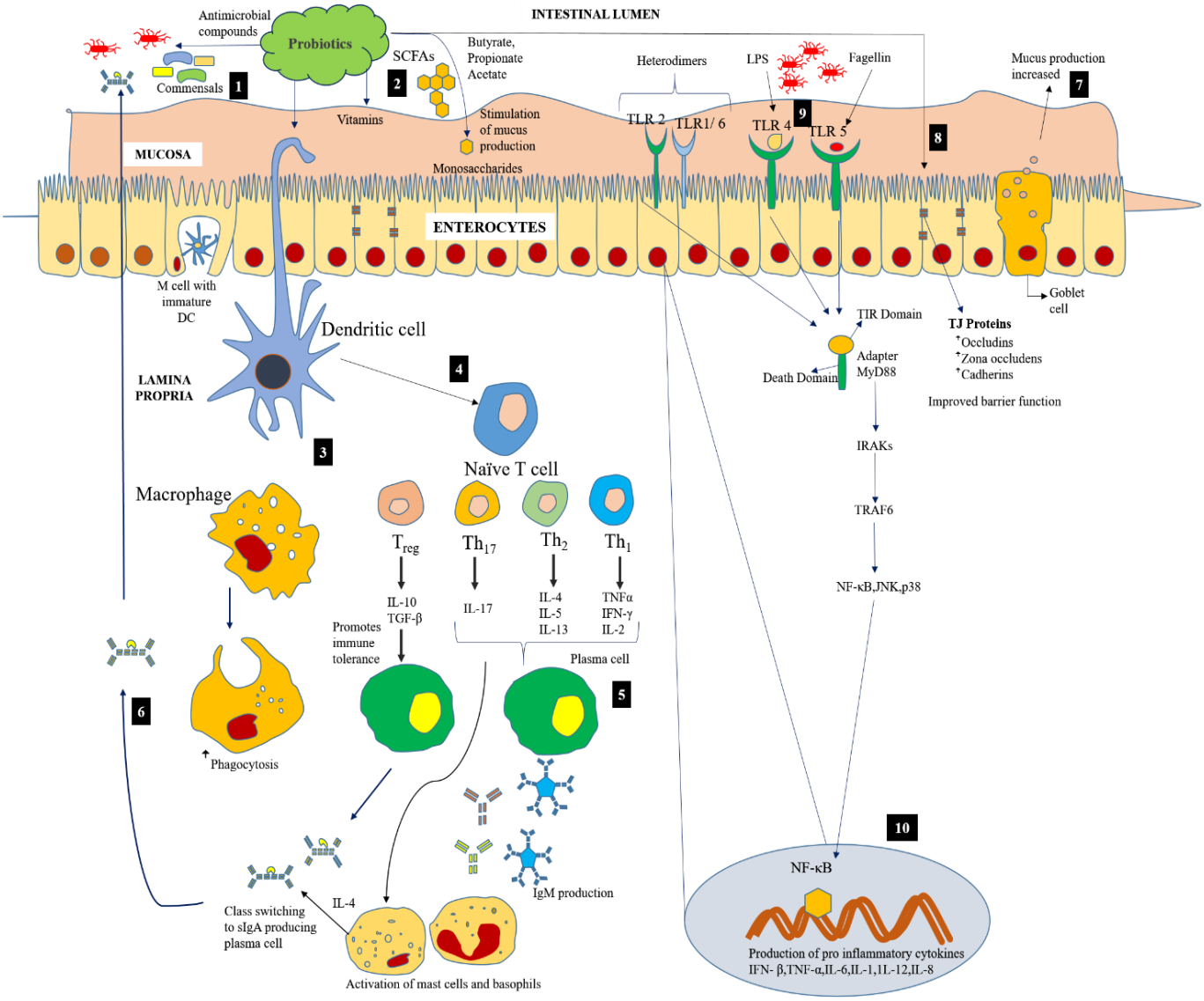

Figure 2. *Diagrammatic representation of the role of probiotics in immune response in host gastrointestinal system.*
1. Probiotics compete with microbial pathogens for nutrients and adherence to the epithelium. Probiotics secrete anti-microbial compounds like bacteriocins that kill pathogens 2. Probiotics also secrete SCFAs (acetate, butyrate, and propionate), vitamins and folate. Butyrate stimulates mucosal restitution and inhibits inflammation. SCFAs increase mucus secretion and production. Immature dendritic cells phagocytose pathogens and present protein fragments using MHC molecules. Dendritic cells endocytose bacterial products either by extending into the enteric lumen throughout epithelial cells.3. Along with dendritic cells, macrophages present antigens, and also produce chemical substances like enzymes, complement proteins, and regulatory factors such as interleukin-1 and also aid in phagocytosis.4. Dendritic cells interact with naive T cells by migrating to the T cell zones of the secondary lymphoid organs. Helper T cells on exposure to IL-12, IL-4, and IL-6 become Th1, Th2, and Th-17 type cells. Th1 cells secrete the cytokines IFN-γ, IL-2, and TNF-β and secretes IL-17, IL-17F, IL-6, IL-22, and TNF-α. Treg cells maintain homeostasis and secrete IL-10 and transforming growth factor (TGF)-β 5.Th2 cells secrete interleukin IL-4, -5, -10 and -13 and activate B cells. Mast cells produce the cytokine IL-4, IL-6 and TGF-β.6.sIgA eliminate antigens and pathogens.7.Goblet cells in the gut produce a protective mucus blanket by secretion of glycoproteins known as mucins.8.Probiotics improve intestinal integrity by up-regulation of tight junction (TJ) proteins like claudin, occludin and zonula occludens-1 (ZO-1).9. Pathogens are recognised by pattern recognition receptors (PRR) which bind to pathogen-associated molecular patterns (PAMPs). The mechanism of action has been explained earlier.10 NF-κB leads to the transcription of effector genes TNF-α and IL-8.

2. Lactobacillus sp. as probiotics

Lactobacilli are Gram-positive, non-spore-forming and non-flagellated rods or coccobacilli (Hammes & Vogel, 1995). *Lactobacilli* are found in various ecological niches like the gastrointestinal and genital tracts. They form a vital part of the indigenous microflora of humans and higher animals. Environmental factors that affect the distribution of lactobacilli include oxygen availability, pH, the presence of specific substrates, and bacterial interactions. They are not correlated with a gastrointestinal or intestinal infection. They are generally safe and are regarded as non-pathogenic microorganisms (Salminen, 1996).

Lactobacilli the most commonly used probiotic microorganisms include strains of *L. plantarum, L. delbreuckii ssp. bulgaricus, L. casei, L. brevis, L. acidophilus, L. reuteri*, *L. fermentum*, and *L. lactis*. The genera *Lactobacillus* and Pediococcus belong to the family *Lactobacillaceae* which also includes *ParaLactobacillus* and *Sharpea*. They are all included in the trivial expression "lactobacilli" (Molin, 2001). Beneficial effects of *Lactobacilli* include:

a. Decreased risk of colon cancer (Chang et al., 2012; Zhu et al., 2014; Zhang et al., 2015)

b. Treatment of Hypercholesterolemia (Taranto et al., 2000)

c. Enzyme production and vitamin synthesis (Yao et al., 2018)

d. Enhanced cell mediated immunity (Wagner et al., 2000)

e. Enhance innate immunity by production of antimicrobial substances like bacteriocins (Zacharof & Lovitt, 2012)

f. Prevention of antibiotic associated diarrhoea (Merenstein et al., 2009)

g. Balances intestinal microflora (Nogacka et al., 2019)

h. Treatment of vaginal and urinary tract infections (Stapleton, 2016)

i. Improves quality of food / feed (Giraffa et al., 2010)

j. Improve Symptoms of Irritable Bowel Syndrome (Herías et al., 1999)

2.1. Lactobacillus and Immune system

Studies on *Lactobacillus helveticus*, *L. casei*, *L. rhamnosus*, and *L. rhamnosus* induced early pro-inflammatory cytokines such as IL-8, TNF-α, IL-12, and IL-6. NF-κB and TLR2-dependent signalling were increased by treatment with these probiotics, which further shows its immunostimulatory effects (Rocha-Ramírez. Et al., 2017). DNA isolated from the probiotic mixture containing various lyophilized lactic acid bacterial strains including various species of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* evoked non-inflammatory responses in immune cells. Experiments in rat and mouse models showed an increased proportion of T cells in the lamina propria (Nogacka et al., 2019) and a decrease in T-cell reactivity (Kirjavainen et al., 1999; Mike et al., 1999). The ingestion of L. rhamnosus was associated with an increase in mitogen-induced IL-10 from peripheral blood mononuclear cells which translated into elevated serum concentrations of IL-10 (Pessi, et al., 2000; Pessi et al., 1999) and was proved unprotective against IBD in humans (Prantera et al., 2002). In vitro studies have recommended that lactobacilli may have anti-proliferative effects on T cells along with suppression of cytokine secretion by T cells (Pessi et al., 1999). *Lactobacillus* and *Bifidobacterium* strains have been shown to influence cytokine production. They also influence the activity of regulatory T cells by dendritic cells microorganisms including probiotics (Christensen et al., 2002; Hart et al., 2004).

In HT-29 cells stimulated by *Salmonella typhi*, the probiotic *Lactobacillus plantarum* inhibited IL-8 production and prevented pathogen adhesion to epithelial cells. They modulated TNF-α, IL-1β, and IL-17 secretion by J774 macrophages. *L. plantarum* inhibited inflammatory stimulation in epithelial cells and activated a tolerogenic profile in mononuclear cells of healthy donors (Ferreira et al., 2016). Oral administration of strain pPG- α/*L. casei* 393 in BALB/c mice effectively evoked mucosal, humoral, and cellular immunity (Gao et al., 2019). Supplementation of *Lactobacillus plantarum* in broilers on production, immunity, antioxidant property, and intestinal microflora was studied and an increase serum total IgG and IL-6 concentration were observed. It also increased catalase activities in serum and liver and also increased intestinal diversity (Shen et al., 2014). Similar work has also been pursued by others (Mohammadian et al., 2017) with *L. delbrueckii* ssp. *bulguricus* and *Lactobacillus plantarum* isolated from the intestine of *Barbus grypus* showed disease resistance to *Aeromonas hydrophila*, with enhanced serum lysozyme, complement, and respiratory burst activity suggesting the use of *Lactobacillus* sp. in augmenting fish immune response. Skin mucus studies in goldfish (*Carassius auratus*), with *Lactobacillus acidophilus* showed an influence on immune and appetite-related genes (Hosseini. Et al., 2016). *Lactobacillus plantarum* HM218749.1 fermented supernatant of aloe showed the strong scavenging capacities of α, α-diphenyl-β-picrylhydrazyl (DPPH), $O2^{\cdot-}$, ·OH, and $Fe2+$ chelation. Inhibition zones for *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella flexneri*, *Escherichia coli*, *Listeria monocytogenes*, *S. dysenteriae*, *Staphylococcus aureus*, and *Propionibacterium* were also observed (Jiang et al., 2016). The effect of *Lactobacillus casei* (Ya et al., 2008) and *Lactobacillus crispatus* (Tobita et al., 2010) on IgA secretion in the intestinal fluid has been proved by previous studies. They were shown to improve resistance to infections in gastrointestinal mucosa by the increase in the concentration of sIgA in intestinal fluid. Studies on both viable and heat-killed *Lactobacillus* GG in an epithelial cell model showed a decrease in IkB degradation which led to the translocation of NF-kB into the nucleus,

which resulted in decreased TNF induced IL-8 production (Zhang et al., 2005).

A related study of epithelial cells pretreated with *Lactobacillus casei* DN114001 decreased *Shigella flexneri* induced NF-kB activation as a result of inhibition of IkB degradation. *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 challenged with virulent human rotavirus were studied in gnotobiotic pigs (Vlasova et al., 2013). *Lactobacillus* colonization increased frequencies of CD4, and MHC II expressing MNCs isolated from ileum, duodenum and blood of probiotic-treated pigs. A decrease in the frequency of toll-like receptors TLR2 and TLR4 expressing MNCs and an increased frequency of TLR3 expressing MNCs in pigs post-challenge, suggested the anti-inflammatory effect of the probiotics mediated by TLR signalling. Oral administration of *Lactobacillus* casei in BALB/c mice showed activation of immune cells related to innate immune response without affecting the number of T cells (Galdeano & Perdigo, 2014). Immunomodulation of *Lactobacillus helveticus* NS8 isolated from koumiss, a traditionally fermented mare's milk evaluated in TNBS-induced colitis mice models and LPS treated macrophage cell line RAW264.7 resulted in diminished proinflammatory effects of lipopolysaccharide (LPS) in mouse macrophage cell line by elevating the levels of IL-10 (Rong et al., 2015) Intranasal or oral administration of *Lactobacillus plantarum* DK119 isolated from fermented Korean cabbage food was used to study the antiviral effects on the influenza virus in the mouse model. The treatment elevated the levels of cytokines IL-12 and IFN-γ in bronchoalveolar lavage fluids with a low degree of inflammation in influenza-virus infected mouse models (Park et al., 2013). *Lactobacillus rhamnosus* IMC 501 supplementation in zebrafish (*Danio rerio*) showed an increase in intestinal innate immunity and hepatic stress with a decrease in oxidative stress levels and decreased DNA damage (Gioacchini et al., 2014).

Studies on *Lactobacillus salivarius*, *L. gasseri*, *L. rhamnosus*, and *L. acidophilus* F-1 strains isolated from samples of feces, breast milk, and vagina from healthy donors from Taiwan *in vitro* in different cultures of human immune cells showed elevation in the secretion of cytokines like IL- 10, IL-12p70, IFN-γ, and TNF-α (Hsieh et al., 2013) which supported the use of a combination of probiotic strains to yield desirable probiotic benefits in different human epithelial cells. *Bifidobacterium longum* and *Lactobacillus helveticus* supplementation improved immunity by regulation of naive and memory T cells, Treg cells, and natural killer activity and modulation of gut Treg cells and γδT cells in elderly humans and aged mice (Finamore et al., 2019). Immunomodulatory functions were significantly augmented with an increase in Treg cells in new-born suckling rats treated with probiotic *Lactobacillus reuteri* DSM 17938 (Hoang et al., 2019). The immunomodulatory effect of *L. reuteri* in pigs showed enhanced T-cell differentiation and cytokine expression (Wang et al., 2009). In a similar study *L. reuteri* supplementation improved serum-specific anti-OVA IgG levels (Yu et al., 2008).. Conceptually similar work has also been carried out in neonatal piglets in which *L. reuteri* decreased mRNA expression of IL-1β in the ileum, whereas its combination with *L. acidophilus* resulted in immunological homeostasis in pigs infected with human rotavirus (Liu et al., 2014).

Effect of *Bifidobacterium animalis* subsp. *lactis* IPLA 20020 and *Lactobacillus gasseri* on intestinal microbiota and cytokine production in adult BALB/c mice showed that the expression of cytokines like TNF-α, IFN-γ, IL-12 and IL-10, IL-6 in small and large intestine respectively. In a study where were co-cultured with Caco-2 cells co-cultured with *Lactobacillus salivarius* and *L. plantarum*, increased expression of mRNAs of T helper, cytokines, surface receptors, TLRs (2, 4, and 9), phagocytosis and macrophage energy metabolism. A decrease in IFN-γ secretion and sIgA secretion levels along with inhibition of NF-κB inflammation signal pathway and down regulation of mRNA expression of interleukins were noted (Ren et al., 2019). Table 3 summarizes the commonly studied probiotic strains of *Lactobacillus* and the work related to immune response in various models worked on so far.

3. Bacillus sp. as probiotics

Bacillus spp. are generally aerobic to facultative aerobic (Hoffmann et al., 2002). To be considered a probiotic, the *Bacillus* strain must possess the primary requirements of GIT, stress tolerance, good adherence capability, and biotherapeutic properties (Panwar et al., 2016). For the safe transit of probiotics and localization in the gut it must survive the stress conditions prevailing in the GIT. *Bacilli* are mainly soil organisms and also include *B. subtilis* have also been found in samples of feces and small intestine of humans (Elshaghabee et al., 2017). The ability to form a biofilm, anaerobic sporulation, and antimicrobial production have helped *B. subtilis* to adapt in the human gut which make them considered gut commensals (Hong et al., 2008). Members of the genus *Bacillus* are most widespread in nature (Garbeva et al., 2003), and were also isolated frequently from water and air (Andersson et al., 1999). Being universal in soil, air, and water they easily find their way into food products. *Bacillus* counts in wheat, grain, and whole meal, were found to be 10^6 CFU/g (Rogers, 1978; Pepe et al., 2003). The ability of bacilli spores to resist high heat, enables their survival in the baking process and hence they are found in bread and bakery products (Sorokulova et al., 2003). *Bacillus* microflora was detected in milk, even after pasteurisation which makes them predominant in pasteurised milk products (Pendurkar & Kulkarni, 1990).

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Table 3. *Works related to immune system using Lactobacillus sp. as probiotics.*

Sl No	Species	Mechanism of action	Reference
1.	*Lactobacillus rhamnosus*	IFN-γ stimulated T_H1/T_H2 response	(Ghadimi et al., 2008)
		Increased level of interleukin IL-12 when co-cultured with macrophages enhanced and IL-12 and IFN-γ when co-cultured with mouse spleen cells, induction of T_H1 cytokine	(Takeda et al., 2013)
		IgG, IgA, and IgM secreting cells increased with enhanced nonspecific humoral response in acute rotavirus diarrhea.	(Kaila et al., 1992)
		Treatment of several gastrointestinal conditions like Crohn's disease	(Gupta et al., 2000)
		Increased IFN-γ and IL-10, activation of dendritic cells with generation of (+) Th1 cells during respiratory syncytial virus (RSV) challenge.	(Chiba et al., 2013)
2.	*Lactobacillus gasseri*	The levels of reactive oxygen and nitrogen species produced by peritoneal macrophages were increased. Innate immunity was increased	(Kato-mori et al., 2010)
		Anti-atherogenesis action	(Ding et al., 2017)
3.	*Lactobacillus crispatus*	Modulation of toll-like receptor (TLR) expression in HeLa cells	(Rizzo et al., 2013)
4.	*Lactobacillus plantarum*	Elevated increase in serum immunoglobulin IgM concentration	(Lee et al., 2017)
		Lysozyme and alternative complement pathway activities of serum, phagocytosis, and respiratory burst activity with intensified immunity and growth in *L. rohita*.	(Giri et al., 2013)
		Elevated levels of cytokines IL-12 and IFN-γ in broncho alveolar fluids and protection against influenza.	(Park et al., 2013)
		Up regulation of dendritic cells in Peyer's Patches.	(Smelt et al., 2013)
		Treatment of IBS	(Niedzielin et al., 2013)
5.	*Lactobacillus acidophilus*	Decreased TNF-α and IL-10 expression in allergic children	(Taylor et al., 2006)
		Use in systemic candidiasis	(Wagner et al., 1997)
		Increase in serum lysozyme activity	(Talpur et al., 2014)
		Increase in phagocytosis increase of endogenous interferon production	(Mokrozub et al., 2012)
		Increased IgA antibody with enhanced mucosal and systemic IgA responses	(Tejada-Simon et al., 1999)
		Produces antimicrobial compounds and used in treatment of pediatric diarrhoea	(Sanders & Klaenhammer, 2008)
		Increased delayed-type hypersensitivity, improved intestinal health and humoral and cell-mediated immunity.	(Wang et al., 2012)
		Increased IgG levels.	(Paineau et al., 2008)
		Induction of TNF-α, Interleukins (10,6,and 12) and TNF-α production	(Morita et al., 2002)
		Regulation of nonspecific immune response	(Pelto et al., 1998)
		Reduced incidence of diarrhoea	(Gill, 2003)
6.	*Lactobacillus delbrueckii*	Concentration of the pro-inflammatory cytokine IL-8 decreased	(Moro-Garcia et al., 2013)
		Increased enzyme activities of superoxide dismutase, catalase and glutathione peroxidase in *Cyprinus carpio*	(Zhang et al., 2017)
		Induced TNF-α and IL-1β production	(Hong et al., 2015)
7.	*Lactobacillus paracasei*	Protection against atopic eczema, conjunctivitis, and asthma in children	(Bertelsen et al., 2014)
		Up regulated expression of genes related to fecundity with boost in oocyte maturation	(Qin et al., 2013)
8.	*Lactococcus lactis*	Antibacterial activity against *Streptococcus and Enterococcus* with increased IL-12, IFN-γ and lysosomal activities.	(Kim et al., 2013)
		Induction of CD4 and CD8 T cell activation in intestine.	(Smelt et al., 2013)

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Table 3. *Works related to immune system using Lactobacillus sp. as probiotics (continued).*

Sl No	Species	Mechanism of action	Reference
9.	*Lactobacillus caseis*	Increase in IgAs and IL-6producing cells involved in innate immune response.	(Galdeano & Perdigo, 2014)
		Activation of immune response against this breast tumour	(Aragón et al., 2014)
		Anti-tumour and anti-allergy immunotherapy	(Cross, 2002)
		Enhanced macrophage activation	(Mike et al., 1999
		Secretory IgA response increased and protected from microorganisms *Salmonella typhimurium* and *Escherichia* to prevent intestinal infections.	(Taranto et al., 2000)
10.	*Lactobacillus salivarius*	Modulate immune responses and intestinal barrier dysfunction in inflammatory bowel disease (IBD).Enhanced growth performance, gut health and reduced incidence of diarrhoea.	(Sayan et al., 2018)
11.	*Lactobacillus johnsonii* (formerly known as *Lactobacillus acidophilus* La1)	Serum IgA concentrations of total and specific immunoglobulin and proteins were increased.	(Marteau et al., 1997)
		Phagocytic activity of peripheral blood leukocytes was increased	(Donnet-Hughes et al., 1999)

The activity of *Bacillus* strains on the host includes antimicrobial, anti-oxidative, and immunomodulatory effects. The features contributing to the probiotic characteristics of *Bacillus* sp. were analysed in various studies. Probiotic properties of *Bacillus* include their ability to produce antimicrobial peptides (AMPs) and extracellular effector molecules. Their capability to interact with the host with the help of adhesion and attachment components (Khochamit al., 2015). The major microflora involved in the fermentation of soya beans for preparation of soya food products and condiments are *Bacillus* sp. (Ray et al., 2000; Inatsu et al., 2006) with *Bacillus subtilis* and *B. licheniformis* as prevalent species at 10^8CFU/g (Omafuvbe et al., 1999).Immune responses were modified by oral administration of probiotics (Bermudez-Brito et al., 2012). Probiotics showed the ability to prevent or attenuate allergic conditions by regulation of T helper (Th1) responses. Probiotic supplementation stimulates cytokine production, and induction of interferons like IFN-γ, interleukin IL-12, IL-6, TNF-α, and IL-2 from Th1/Th17 cells (Boirivant & Strober, 2007). Administration of *Bacillus coagulans* together with other microorganisms was used in treating antibiotic-associated diarrhoea (Hempel et al., 2012; Doron et al., 2008). *Bacillus subtilis* spores were used as probiotics for animal consumption (Larsen et al., 2014; Zokaeifar et al., 2014) and are recommended for treating diarrhoea and elimination of *Helicobacter pylori* in humans (Tompkins et al., 2010).

Bacillus amyloliquefaciens H57 helped in weight gain and nitrogen retention in dairy calves. It also reduced the risk of diarrhoea and improved the growth of calves as they transitioned through weaning to the paddock (Le et al., 2017) *Bacillus amyloliquefaciens* S1 isolated from the cecum of goose showed cellulose activity and its supplementation in goose feed improved fertilization and hatching rate of goose eggs significantly (Ye et al., 2017). Supplementation with the probiotic *Bacillus subtilis* in the diet improved eggshell quality along with lower levels of plasma cholesterol and triglyceride without affecting internal egg quality. A significant increase in IgM immunoglobulin concentration was observed (Fathi et al., 2018). *Bacillus subtilis* isolated from the intestinal tract of grass carp provided protection to grass carp against oxidative stress damage induced by the pathogen *Aeromonas hydrophila* (Tang et al., 2018). Co-supplementation of probiotic *Bacillus subtilis* L10 and G1 strains in juvenile white shrimp (*Litopenaeus vannamei*) showed increased growth performance, digestive enzyme activity, immune gene expression, and resistance to disease. It also enhanced immune response by stimulating the expression of immune-related genes (Zokaeifar et al., 2012). Studies have shown that a diet supplemented with *Bacillus subtilis* in cherry valley ducks has improved growth performance, innate immune response, and resistance against *E. coli* and reovirus infection. The major pro-inflammatory factors like IL- 1β, IL-6, IL-8, and IL-10 and antiviral proteins were up-regulated in ducks fed with probiotics (Guo et al., 2017). Increased immune stimulation and resistance to infectious disease episodes were observed in healthy elderly humans on the consumption of *Bacillus subtilis*. It was substantiated by an increase in levels of serum interferon, intestinal and salivary sIgA in elderly individuals (Lefevre et al., 2015). *Bacillus subtilis* was shown to induce β defensins which strengthen innate defense mechanisms. Studies in mice showed that exopolysaccharide from *Bacillus subtilis* protected them from acute colitis induced by *Citrobacter rodentium*. Probiotics stimulated the development of anti-inflammatory macrophages and inhibited T cell activation. The study also suggests the use of *B.subtilis*

exopolysaccharide to control T cell-mediated immune response (Paynich et al., 2017).

Probiotics have also been used in aquaculture as a preventive measure against diseases (Magnadottir, 2010) as well as in animal models. Extensive studies have shown that fish models share homology with some vertebrates like humans with the complex immune system. Fish is widely used as laboratory animals in toxicology and other fields because of their small size and availability in large numbers of many species such as the Japanese medaka (*Oryzias latipes*), common guppy (*Poecilia reticulata*), goldfish (*Carassius auratus*), zebrafish (*Danio rerio*) and swordtails (*Xiphophorus*). Studies done in yellow perch have shown the use of mixed *Bacillus* sp. as a promising candidate for aquaculture. The supplementation improved the early innate responses, reduced cortisol, and elevated Igf1 levels. *In vivo* assessments of *Bacillus indicus* and *B. subtilis* administration in guinea pigs and rabbits was found to be safe for oral use (Hong et al., 2008). Supplementation of *Bacillus velezensis* in *Carassius auratus* showed an upregulation of cytokines like IL-2, IL-4, and IL-12 (Yi et al., 2018). The effects of probiotic *Bacillus amyloliquefaciens* SC06 in piglets to check immune function, tight junction, and goblet-cell number proteins of intestine showed downregulation of gene expression of intestinal mucosal defensin-1, human mucin-1, claudin3 and claudin4 were observed. A decrease in serum IFN-α, IFN-γ, IL-1β, and IL-4 levels along with an increase in TNF-α and IL-6 secretion were observed. In a similar study, *Bacillus amyloliquefaciens* have shown to decrease cytokines TNF-α, IFN-γ, IL-1β, and IL-4 levels in the liver, with an increase in IFN-α (Du et al., 2018). Intranasal administration of GFP labelled *Bacillus subtilis* in piglets showed the presence of probiotics in the lamina propria of the nasopharyngeal tonsils, nasal mucosa, and soft palate tonsils. *B. subtilis* administration increased the expression of TLR-2 and TLR-9 in the tonsils of piglets and the study also supports the use of *Bacillus subtilis* to enhance the immunity to respiratory diseases (Yang et al., 2018).

Administration of inactivated *Bacillus coagulans* in healthy adults showed changes in cytokines related to immune regulation which included IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSF, and TNF-α (Kalman et al., 2018). In another study, *Bacillus coagulans* evaluated in healthy school children showed a decrease in the incidence of upper respiratory tract infection symptoms which include nasal congestion, bloody nasal mucus, and itchy nose. Immunomodulation of serum TNF-α, CD163, G-CSF, ICAM-1, IL-6, IL-8 was also noticed which supports its use as a probiotic in humans (Anaya-Loyola et al., 2019). *Bacillus subtilis* (BS1 and BS2) and *Bacillus velezensis* (BV1) isolated from Tibetan yaks showed anti-inflammatory, growth-promoting, and antioxidant effects in mice models. Serum IgG, IgM, and IgA were enhanced, and pro-inflammatory factor TNF-α, IL-6, and IL-8 were down-regulated while anti-inflammatory factor IL-10 expression was up-regulated (Li et al., 2019).

Bacillus subtilis administration in grass carp (*Ctenopharyngodon idella*) showed stimulation of cytokine-related pathways. It significantly induces expression of both pro-inflammatory and anti-inflammatory cytokines like IL-1β, IL-6, IL-8, IL-12, TNF-α and IL-4, IL-10, TGF-β, respectively (Zhou et al., 2019). *Bacillus subtilis* supplementation on Pengze crucian carp enhanced the immunity and antioxidant capacity by stimulating alkaline phosphatase, acid phosphatase, total superoxide dismutase, catalase, and glutathione peroxidase activities (Cao et al., 2019). In laying hens dietary supplementation of *Bacillus amyloliquefaciens* BLCC1-0238 showed a decrease in the expression of IL-1β, IL-6, and TNF-α. The increase in serum IL-4 levels suggested its role in maintaining intestinal mucosal immune system integrity and increasing nutrient absorption by reducing the levels of pro-inflammatory cytokines and increasing the levels of anti-inflammatory cytokines (Zhou et al., 2019). *Bacillus coagulans* MTCC 5856 probiotic was used efficiently in irritable bowel syndrome (IBS) patients suffering from depression (Majeed et al., 2018). Table 4 lists works related to probiotic *Bacillus* sp. on immune response in various models worked so far.

Conclusion

Studying interactions between microbial flora and the host immune system has been of special interest to researchers all over the world. As there are different strains of microorganisms with distinct health benefits the present knowledge of microorganisms with probiotic properties is not complete. Besides *Lactobacillus* sp. the use of *Bacillus* sp. as a probiotic is increasing rapidly. Studies reviewed demonstrated their role in immune stimulation, antimicrobial production, and pathogen exclusion. Probiotics have GRAS (generally regarded as safe) status given to probiotics leads to their consumption without much safety concern. Studies reviewed pinpoint the immunomodulatory effects of probiotics. Although there is supporting evidence that probiotic microorganisms play a crucial role in immune stimulation, there is clearly an increasing need for further research to confirm the role of probiotic-mediated immunostimulation on various diseases in the host. The mechanism of action and maintenance of intestinal homeostasis is obvious from *in vitro* and *in vivo* studies.

The review of major studies in this area makes it clearly obvious that more studies and clinical trials in humans can generate a vast majority of probiotics commercially.

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Table 4. *Works related to immune system using Bacillus sp. as probiotics.*

Sl No	*Bacillus* sp.	Mechanism of action	Reference
1.	*Bacillus coagulans*	Increased expression of immune-related genes like myeloid differentiation factor (MyD) 88, interleukin (IL)-1β, tumor necrosis factor (TNF)-2, and IL-1β in groupers, and TLR- 4, TNF-α, TRAM 1, NF-κB in zebrafish. Enhanced immune response in zebrafish challenged with vibrios.	(Pan et al., 2013)
		Treatment of patients with acute diarrhoea.	(Sudha & Bhonagiri, 2012)
		Used in treatment of IBS.	(Urgesi et al., 2014)
		Lessen exercise-induced muscle damage enhances recovery.	(Jäger et al., 2016)
2.	*Bacillus subtilis*	Expression levels of occludin, cloudin2 and IgA-positive cells significantly increased.	(Qin et al., 2018)
		Increase in folate metabolism and nitric oxide synthesis.	(Clark & Hodgkin, 2014)
		Anti-bacterial activity	(Ramachandran et al., 2014)
		Decreased frequency of respiratory infections.	(Lefevre et al., 2015)
3.	*Bacillus pumilus*	Increased immunity and disease resistance *in Oreochromis niloticus* Improved growth performance.	(Aly et al., 2008)
4.	*Bacillus licheniformis*	Improved systemic immunity	(Wang et al., 2017)
		Disease resistance improved with increase in Innate immunity and antioxidant capability	(Zhang et al., 2013)
5.	*Bacillus circulans*	Disease resistance and non-specific immunity levels were increased in catla-catla	(Bandyopadhyay & Das Mohapatra, 2009)
6.	*Bacillus amyloliquiefaciens*	Induction of autophagy. Antibacterial activity against *E.coli* increased by modulating immunity	(Wu et al., 2017)
		Enhanced expression of innate immune-related genes like IL-1β, IL-6, IL-21, and TNF-α, and TLR-1, -3, and -4.	(Lin et al., 2019)
		The serum IgG, IgA and IgM were improved in broiler chickens.	(Luan et al., 2019)
		Higher expression of immune relevant genes IL-1β, TNF-α, C3 and iNOS in head-kidney tissues of catla- catla	(Singh et al., 2017)
		Induction of DC maturation. Increased CD80, CD86, CD40 and MHCII expression and cytokine secretion. The frequencies memory T cells were increased in spleens.	(Huang et al., 2016)
		Decreased inflammatory response regulation of microbiota in the small intestines.	(Li et al., 2018)

Since we are in an era where we can modify our health through foods, substantial care should be given to future research on probiotics. The adverse effects of the consumption of antibiotics and other drugs on the microbiota of GIT must not be ignored. Although permanent modulation of intestinal microbiota is not possible by probiotic consumption, it is well-studied that probiotics help in the restoration of homeostasis to some extent in acute infections or diseases. One of the most important advantages of probiotics is that they can be easily incorporated into everyday foods. So it is important that future studies and clinically controlled trials be carefully done for their approval as a suitable drug instead of antibiotics.

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