

## Role of probiotics, prebiotics, synbiotics and postbiotics in inhibition of pathogens

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A microbial pathogen is a potentially armed opportunist entity to colonize intestinal mucosa. They have phenomenal ability to evolve and adapt accordingly which further seizes signalling molecules and pathways of host to become pathogenic. In order to colonize mucosal layer, they need to cross the physicochemical barrier formed by intestinal epithelium. This gastrointestinal (GI) epithelium with inhabitant microbiota acts as synergistic partners in system of defense that protect the host from intrusion into GI tract. Resident microbiota ferment carbohydrates to lower the luminal pH by production of organic acids to attenuate gene expression of toxin genes in pathogens, they down regulate pro-inflammatory cytokine production induced by pathogens, displays competitive exclusion with pathogenic bacteria for attachment sites and nutrition and enhance the barrier integrity. Among them, *Lactobacillus* and *Bifidobacterium* species (members of Lactic acid bacteria) are found to be the most active inhabitant that provides better therapeutic benefits. Consumption of these live health beneficial microorganisms in adequate amounts constitutes probiotics. Colonial microbiota can be modified by some potential non digestible oligosaccharides known as prebiotics. These sugars transform gut microflora towards healthier composition. They are not only found to enhance anti-pathogenic ability and survival of probiotic microorganisms as synbiotic synergy but a few of them have an immense capability to mimic adhesion sites of pathogens thereby providing cytoprotection to intestine. Recent studies also demonstrate anti-pathogenic action of postbiotic metabolites obtained from these lactic acid bacteria. Antimicrobial potency can be further enhanced by creation of recombinant designer probiotics expressing oligosaccharide receptors for adhesins and toxins of pathogens on surface. This chapter illustrates anti-pathogenic mechanisms and actions of probiotic microorganisms alone as live or dead cells or in combination with prebiotics achieved through interaction with pathogenic bacteria.

**Keywords:** Pathogen; Probiotic; Lactobacillus; Postbiotics

### 1. Introduction

Human beings are always being a favorable host for a pathogen. As pathogens live at the expense of the host and human as host, provide them a perfect nutrient pool, excellent temperature and moist environment which constantly renew itself. This constantly changing host environment makes them more versatile and robust. Pathogen that colonizes in humans mainly comes from oral route, through our food or unhygienic habits. It is a tissue colonizing entity which provokes through cellular damage and finally displays symptoms of either morbidity or mortality. They replicate itself in the host by a continued destruction process of humoral or cellular breaching. Ordinarily, pathogens are classified on their transmission patterns in their hosts or vectors. Transmission of a pathogen could be zoonotic, geonotic or escalated from other human beings. In human body, the gastrointestinal (GI) tract represents the largest contact area between the body and the external environment [1]. Here, the pathogen adapts on ingestion, colonizes and translocates well to establish an infection. Gastrointestinal tract is the natural abode of trillions of microflora collectively known as gut microbiota. There exists a well established relationship between intestinal mucosa and gut microflora where gut microflora protect host from infection by foreign or unwanted pathogenic microorganisms and in return host mucosa provides plentiful nutrient for their prosperous growth. *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Escherichia*, *Eubacterium*, *Enterococcus*, *Streptococcus*, and *Lactobacillus* are considered as most common commensal groups of microorganisms found in intestine. Among them, *Lactobacillus* and *Bifidobacterium* are found as health-beneficial bacteria for the human host [2]. Among these abundant microbial communities, certain species of bacteria are known to offer different health benefits when administered in adequate amounts and they are labelled as “probiotics”.

Chemotherapy as inhibitory action was known to fastly eradicate infectious pathogen from last hundreds of years but the inclined incidence of development of antibiotic resistance among them and side effects leads to a precautionary thinking among peoples. The use of antibiotics also has been shown to increase colonization of enteric pathogens by altering the composition of gut microflora [3]. Now days, people are more concerned for green consumerism or food supplementation based on natural agents which can make their food functional with health benefits to restrict disease probability to lower limits [4]. In the same mode, applaudable research shown probiotics as naturally potential agents which can restrict pathogens and prevent dysbiosis by influencing gut microbiota in a manner which can intensify enteric or specific cytoprotection. Probiotics and other beneficial commensal initiate inhibition of pathogens by providing colonization resistance to them. The competition for similar nutrients and secretion of antimicrobial compounds, stimulation and development of innate and adaptive immunity directly or indirectly impede pathogen colonization.

In order to impart its benefits, the use of probiotics and prebiotics is emerging as a strategy for the reduction and prevention of GI infections. One of the main differences between probiotics and prebiotics is that probiotics are viable

food components whereas prebiotics are nonviable food component. Prebiotics are non-digestible oligosaccharides, remain intact through the digestive system and act as food for already established microflora. These sugars overcome many of the traditional limitations of introducing probiotic bacteria in to the GI tract. Therefore, using prebiotics is arguably a more practical and efficient way to manipulate the gut microflora [5]. Prebiotics are described as functional components of food which are metabolized by particular commensal bacteria in the gut conferring various health benefits to the host. To be classified as prebiotic, a food ingredient has to 1) resist the acidity of the gut, hydrolysis by mammalian enzymes and absorption in the GI tract, 2) be fermented by the intestinal microbiota, and 3) selectively stimulate the growth and/or activity of intestinal bacteria associated with health and wellbeing. Each of the above mentioned criteria is very important, the third is the most difficult to fulfill [6].

Prebiotics need to reach the large intestine with their chemical and structural properties unchanged. Prebiotics are able to escape the digestive processes in the upper part of the gut due to their molecular and structural composition, which makes them essentially resistant to our digestive enzymes. Prebiotics have been considered to be short-chain carbohydrates that have a degree of polymerization of two or more, and which are not susceptible to digestion by pancreatic and brush border enzymes. Most of the studies about prebiotics have been focused on fructans, such as inulin, FOS and galacto-oligosaccharides (GOS). They are also valuable functional ingredients for the food industry with the potential to improve the sensory properties of food. Others important prebiotics include lactulose, Xylo-oligosaccharides (XOS) and mannan-oligosaccharides (MOS).

Synbiotics refer to nutritional supplements combining probiotics and prebiotic food ingredients and in a form of synergism that improve the survival and implantation of live microbial dietary supplements in the tract, either by stimulating growth or by metabolically activating the health promoting bacteria [7]. The effect of whole synbiotic synergy primarily depends upon efficacy of probiotic strain used, while the prebiotic surges the strain to produce its maximum attributes. Synbiotics products offer the potential to develop prebiotics targeted at specific probiotic strains to optimize health benefits. Dose standardization of prebiotic sugar and knowledge of probiotic beneficial effects with their well studied safety aspects place foundation of active synbiotic combinations. This book chapter focuses on studies based on potent probiotic strains and their antimicrobial mechanisms in host. It also highlights the synergistic research outcomes in combination with prebiotics and postbiotics as substances.

## 2. Anti-Pathogenic Approach of Probiotics

Probiotic bacteria exhibit array of strategies in respect to mitigate infectious attempt of a pathogen. Probiotics works antagonistically in conjunction with gut epithelium lining and naturally inhabiting beneficial microbes [8]. Inhibition of pathogen and possible mechanism of action are mainly strain specific in nature and implementation of a new probiotic for its antimicrobial potential may also depend on the origin and its place of isolation too. Basically, screening of probiotics with anti-pathogenic activity is normally based on production of antimicrobial compounds confirmed from step by step initial *in vitro* trials and later *in vivo* experiments through animal models and final confirmatory validation through clinical trials seeking specific cytoprotection. The use of probiotics strains is based on the assumption that they modify the composition of gut microbiota and counteract pathogens at specific dose.

Based on elucidated molecular mechanisms behind the beneficial role of commensal and probiotic strains it becomes clear that they provide colonization resistance to pathogens by two major mechanisms.

- A. Direct antagonism by inhibitory metabolites, competitive exclusion for nutrients or niche establishment [9, 10].
- B. Host-mediated indirect antagonism effects such as improvement in gut barrier function and heightened immune response [11, 12].

## 3. Direct Antagonism

In direct antagonism, colonization resistance against pathogens is achieved through commensal and probiotic bacteria by directly competing for the same niche. They release array of metabolites that may be bacteriostatic or bactericidal to the pathogenic bacteria. These inhibitory compounds mainly include organic acids, bacteriocins and short chain fatty acids (SCFA) [13]. They may also elicit anti-pathogenic effects by capturing the adhering sites of the pathogenic strains by co-aggregating with the pathogens [14]. Nutrients based competition is mostly appeared in metabolically related bacteria. Below, we discuss some major aspects of direct antagonism between beneficial probiotics and pathogens.

## 4. Protection through production of antimicrobials

Probiotic bacteria produce a variety of anti-pathogenic substances that inhibit pathogenic bacteria by direct antagonism. The probiotics antimicrobial molecules can kill pathogens before they could adhere to the intestinal lining. In addition to organic acids, hydrogen peroxide, and bacteriocins as inhibitory compounds some probiotic lactic acid bacteria like *L. acidophilus* also produces Lactocidin, Acidolin, Acidophilin, Lactacium-B as inhibitory compounds. Similarly,

*Bifidobacterium* species produces Bifidolin and Bifilong which can inhibit several pathogenic bacteria [15]. However, among organic acids, lactic and acetic acids account for over 90% of their production which lowers pH at local sites with SCFA to primarily implement a bactericidal or bacteriostatic zonal effect. The effectivity of antimicrobial molecules by the probiotics needs to be ascertained in the presence of complex intestinal microbiota through *in vivo* studies. Furthermore, it may be possible that the inhibition action against enteric pathogens could be due to a combination of mechanisms that act through highly localized effects on intestinal epithelial cells. Many *in vitro* and *in vivo* studies depicts already known probiotic culture like *L. rhamnosus* GG, *L. casei* Shirota, *L. johnsonii* NCC 533, *L. reuteri* ATCC 55730 and *L. acidophilus* LB to show major anti-pathogenic effect on direct contact with culture or cell free spent culture supernatants (CFCs) against infectious target microorganisms leading to decrease of 3-4 log CFU/ml in population [16, 17]. Another *in vitro* study shown restricted adhesion and invasiveness of *E. coli* O157:H7 to human intestinal cell lines on pretreatment with concentrated probiotic CFCs. (*L. rhamnosus* DR20 and *B. lactis* DR10 strains) [18, 19] demonstrated *Lactobacillus* and *Bifidobacterium* strains are capable of inhibiting *Listeria monocytogenes* invasion to C2BBel epithelial cells through secretion of proteinaceous molecules through transwell chamber system having separation by a non-permeable membrane. The membrane prevented direct contact between the probiotic and the pathogen.

The presence of probiotic strain or their CFCs (*L. rhamnosus* GG, *L. casei* Shirota, *L. johnsonii* NCC 533, *L. acidophilus* LB) was found to cease entry of some invasive pathogens, such as Salmonella, who can reside inside cell cytoplasm vesicles for replication and found unaffected on antibiotic therapy [20, 21, 22, 23]. Some strains also found to have affinity to block the swimming motility of *S. typhimurium*, which plays a essential role in swimming into the intestinal contents and interact with the intestinal epithelial cells [24]. In preinfected Caco-2/TC7 cells with *S. typhimurium*, it was observed that CFCs obtained from *L. acidophilus* LB can irreversibly decrease intracellular level of *S. typhimurium* by 4 to 5 log CFU/ml (136). Earlier [25] observed restricted invasion of Caco-2 cells by *S. enterica* serovar *typhimurium* as pH-dependent mechanism in case of *L. rhamnosus* GG spent culture supernatant but later experiments using acidified culture medium of non-cultivated *Lactobacillus* to pH 4.5 demonstrated that the inhibitory activity of CFCs of *L. rhamnosus* GG, *L. johnsonii* NCC 533, *L. casei* Shirota, *L. acidophilus* LB, and *L. casei* DN-114 001 did not result solely from the acidic pH [26]. [27] examined the production of hydrogen peroxide by the human gut commensal *Lactobacillus johnsonii* NCC533 and found that the hydrogen peroxide was produced *in vitro* at levels that were inhibitory for *S. typhimurium*.

Action and ability of Bacteriocins produced by lactobacilli and bifidobacteria is well documented against pathogens. These are bactericidal proteinaceous molecules with wide variety of peptides and proteins in terms of their size, microbial targets, mechanisms of action and immunity. Bacteriocins plays vital role in gaining competitive advantage against unwanted pathogenic microbes. Strains isolated from human stool have been found to be capable of bacteriocins production [28, 29]. Recently, Thuricin CD is a two component bacteriocin (Trn- $\alpha$  and Trn- $\beta$ ) produced by a strain of *Bacillus thuringiensis* isolated from a human fecal sample, demonstrated antimicrobial activity against *C. difficile* in a mouse model of infection [30]. Some commensal Enterobacteriaceae family members, including the probiotic *E. coli* Nissle 1917 found to secrete small antimicrobial peptides called microcins, which specifically target and kill related competitors, including pathogenic organisms [31, 32]. Secretion of antimicrobial peptides and specialized toxins are another means of competition between beneficial commensals and pathogens within the gut. Recently, [33] reported presence of heat-resistant small peptides in *L. rhamnosus* GG CFCs, two of which have NPSRQERR and PDENK sequences, which display anti-bacterial activity against enteroaggregative *E. coli* (EAEC) strain 042 and *Salmonella enterica* serovar *Typhi*. Other than production of antimicrobial peptides, Bacteroidetes phylum, mammalian-associated polymicrobial commensal community were found to display type VI secretion system (T6SS) for inter bacterial antagonism *via* toxin production vying for the same ecological niche [34, 35].

## 5. By competition for nutrients

Preferential consumption of metabolic essential nutrients is found to be alternative strategy utilized by the commensal microbial community for competing with pathogenic bacteria. This type of competition was primarily seen between metabolically related bacteria especially in Enterobacteriaceae family. Commensal *E. coli* was commonly found in nutrient base interactions and completion with diarrheal EHEC for organic acids and amino acids like proline [36, 37]. *Bifidobacterium* spp too exhausts iron content in gut which is necessary for pathogen survival [38]. After consumption of limited nutrients competing pathogens were forced for starvation. Probiotic strain *E. coli* Nissle 1917 have beneficial property of impeding the colonization of pathogen *Salmonella typhimurium* in gut and used for a probiotic preparation used for Inflammatory bowel diseases. *E. coli* Nissle 1917 have redundant iron transporters which may be the perception behind the competition [39, 40].

This phenomenon of competition was not much effective and trendy among different groups of commensals because gut pathogens like pathogens like EHEC, *Salmonella typhimurium*, and *C. difficile* are better evolved to exploit greater or newer varieties of metabolites than gut commensals especially in altered condition of gut [41, 42]. Recently, [4] have shown antibiotic treatment of mice induces gut alteration which increase in specific global metabolic profile like carbon

sources including primary bile acid taurocholate for germination, and carbon sources such as mannitol, fructose, sorbitol, raffinose and stachyose that support the germination and growth of *C. difficile*.

## 6. Competitive exclusion

Competitive exclusion is the property of elimination of one species from a habitat or specific region under influence of another species. A probiotic strain with exclusion property has good competitive edge against pathogen both in case of prophylactic and therapeutic treatment. Exclusion property of probiotic strains depends on their autoaggregation and coaggregation affinity. Intestinal epithelial cells express receptor for adhesion of bacteria, both pathogens and probiotic or beneficial commensal compete for same sites. Better probiotic strains having good adhesion ability blocks the connection between intestinal epithelium and pathogenic bacteria [12]. Blocking epithelium and pathogenic bacteria interaction is strain specific property and most commonly characterized during prophylactic based approach [43, 18]. During *in vitro* experiments, when HT29-MTX mucus secreting cell line was infected with *H. pylori*, the adhesion of the pathogen was decreased by *L. acidophilus* LB CFCS [44]. Similarly, *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus johnsonii* strain P47-HY inhibited ETEC attachment on IPEC-J2 intestinal epithelial cell line model [45]. Competitive exclusion effects of probiotic *Lactobacillus* strains are also seen against bacterial pathogens in animal infection models. Administration of *L. rhamnosus* GG in newly born rats pups decreases colonization of enteroinvasive *E. coli* [46]. Prominent probiotic strains like *L. johnsonii* NCC 533, *L. casei* Shirota and *L. acidophilus* LB was found to restrict *H. pylori* infection and rate of gastritis in mice models [47, 48].

## 7. Indirect effects against pathogens

Probiotic strains on colonization with gut commensal can indirectly control establishment of pathogen by up regulating host barrier property [49, 50], enhancement of immunity [51, 52] and silencing the virulence expression [53] through variety of mechanism like mucus layer development, SCFA production, IL-1 and IL-22 mediated placement of neutrophil cells, induction of defensins production by host, T-cells differentiation and sIgA secretion at pathogen proliferation site.

## 8. Enhancement of epithelial barrier function

Enhancement of barrier function within the epithelial lining is one of the key mechanisms by which probiotics strive colonization resistance. Epithelial layer acts as first arsenal defense for avoiding initiation of infection and separates beneficial commensals and pathogens from bottom line immune cells. Adherence to mucosal surface is the first step for pathogens to colonize in gut but the presence of thick mucus layer prevents their attachment. Development of proper mucus layer is dependent on the interaction between host and commensals [54]. Mucin protects epithelial cells from any mechanical, enzymatic or microbial damage. In humans, till now about 18 types of mucin glycoproteins was found, and of these MUC3 and MUC2 are predominant ileo-colonic mucins [55]. Probiotics induces over expression of mucin, contributing to exclusion of pathogens and barrier function [56]. During *in vitro* experiments, probiotics like *L. plantarum* 299v and *L. rhamnosus* GG strains have shown induction of mucin gene expression on HT29 intestinal epithelial cell line [57]. In another experiment on Caco-2 cell model LGG strain increased expression of MUC2 mRNA and restricts translocation of pathogen to intestine [58]. Some strains like *Lactobacillus reuteri* were found to maintain the integrity of mucosal barrier despite dysfunctioning of mucus layer [59].

Probiotics also enhance functionality of tight junction and up regulates heat shock proteins to preserve the barrier function [60, 61]. Intestinal epithelial junction barrier is maintained by intercellular junctional complexes and specific proteins like zonula occludens (ZO-1, ZO-2, and ZO-3), claudins, occludins, beta-catenin and E-cadherin. The tight junction acts as gates which checks vectorial paracellular transport across the cell barrier. Enterovirulent pathogens mainly target these junctional domains of the intestine and can delocalized or alter cytoskeletal arrangement [62]. Probiotic *L. acidophilus* and *S. thermophilus* were found to mend invasion effects of enteroinvasive *E. coli* (EIEC) by increasing trans-epithelial resistance with maintenance and enhancement of cytoskeletal and tight junctional protein phosphorylation [63]. Exposure of T84 cells with EPEC and probiotic *Escherichia coli* Nissle 1917 demonstrates that probiotic strain altered expression, distribution of zonula occludens-2 (ZO-2) protein and restored barrier integrity. Recently, [45] observed attenuation of pathogen mediated disruption of ZO-1 in tight junctions on co-culturing of IPEC-J2 cells with novel *Lactobacillus reuteri* strain and ETEC.

## 9. Inhibition of virulence factor expression

Pathogenic potential of any pathogens for infection can be attributed to expression of virulence factors. It is a complex process which is regulated by diverse environment present around within host including acid, bile, nutrient presence,

peptides, mucus and especially competitive microbiota in the intestine. Research studies on probiotics anti-pathogenic strategies have shown influence of probiotics and gut commensals on virulence gene expression of specific pathogens. Metabolites released by probiotics plays a significant role in modulating the pathogen environment with the help of host. Recently, co-culturing studies of probiotic strain *Escherichia coli* Nissle 1917 and entero-hemorrhagic *Escherichia coli* (EHEC) displayed down regulation of Shiga toxin (stx) gene expression [64]. Earlier [53] also demonstrated decrease in stx2A gene expression on co-culturing with LGG and concluded that the down regulation in gene expression is dependent upon amount of organic acid formed upon fermentation. Probiotic *L. acidophilus* La-5 strain was found to inhibit expression of the LEE virulence genes and lowers quorum sensing necessary for *E. coli* O157:H7 cell in infection [65]. This action was further validated in animal model where *E. coli* O157:H7 infection was impeded [66]. [67] found reduction of Salmonella SPI-1 gene expression in chicken cecum by probiotic *Lactobacillus* spp. In the same theme, earlier *Bacteroides thetaiotaomicron*, was found to inhibit transcription of Shiga toxin gene in EHEC O157:H7 [68]

Pathogenic bacteria regulate virulence factor production and coordinate gene expression through quorum sensing (QS) via autoinducers molecules. Pathogens like *Escherichia coli* O157:H7, *C. difficile* and *Clostridium perfringens* synthesize autoinducer-2 (AI-2) which control virulence in them [69, 70]. Recently, [71] concluded that cell extract of probiotic *L. acidophilus* GP1B can interfere QS in *C. difficile* by restricting AI-2 molecule production and can down regulate virulence toxin genes like in *C. difficile* *tcdA* and *tcdB* which encodes for their toxin formation. In future, experiments focused on characterization of inhibitory compounds and signalling molecules between commensal and pathogen should be needed to be performed to know unknown pathways and mechanisms linked with virulence.

## 10. Enhancement of immunity

Gut is known to be the largest immunological organ of the body. It plays an important role in maintenance, maturation of immune cells and interaction with different microbiota [72, 73]. Immunomodulation and Immunostimulation are most probable action mechanisms of probiotics via the mucosal immunity of the gut against bacterial pathogens [74]. Expression of antigen receptors on T and B lymphocytes and its clonal proliferation initiates for adaptive immune response. Its induction depends upon direct antigen identification by receptors or signal transmitted by innate immune cells [75, 76]. Although, gut commensal and pathogenic microbes share much similarity in their surface characteristics but still intestine with the help of immune cells distinguishes between self and non self on the basis of their surface antigens. Probiotics with innate and adaptive immunity work in synergistic manner to prevent invading pathogens. Interaction of probiotics with intestinal enterocytes induces host response through immunomodulatory molecules in a strain-dependent manner modulating the expression of various pro and anti-inflammatory molecules [74].

Innate immunity is considered as rapid first line of defense but non-specific in their ability to recognize the pathogens. They include macrophages, neutrophils and dendritic cells (DC) and natural killer (NK) cells as immune cell arsenals for early protection action in host. They possess specialized Toll-like receptors (TLRs) as membranous Patterns Recognition Receptors on surface of immune cells which interacts with Pathogen-Associated Molecular patterns (PAMPs). The ultimate objective of these transmembrane molecules is the production of type I interferons or NF- $\kappa$ B activation via signalling adapter molecules like myeloid differentiation factor 88 (MyD88), interferon regulatory factor (IRF) and TIR domain-containing adaptor protein [77] Probiotic strains *L. rhamnosus* HN001 and *B. lactis* HN109 was found to enhance polymorphonuclear and mononuclear phagocytosis activity and boost cytotoxic potential of NK cells on their consumption. Probiotics increases phagocytic capacity and up regulates expression of phagocytosis receptors like CR1, CR3, Fc $\gamma$ RIII and FC $\alpha$ R in neutrophils of healthy person [80]. In the same context, [81] also found enhanced phagocytosis activity of granulocytes cells in human volunteers after intake of *L. plantarum* 299v, *L. paracasei*, *L. plantarum* and *L. fermentum* as probiotics strains.

Recently, probiotic formulation VSL#3 was found to prevent intestinal inflammation by stimulation of innate immune responses in intestine by increased production of epithelial tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), activation of NF- $\kappa$ B in vitro and restoration of epithelial barrier function *in vivo* [76]. Enterovirulent pathogens can induce release of inflammatory cytokines such as IL-6, IL-8, IL-1 $\beta$ , TNF-  $\alpha$  and TNF-  $\beta$ . During *in vitro* studies, LGG was found to decrease IL-8 production in both live and inactivated form [82]. Same strain was also found to lowers IL-8 production after *V. cholerae*, EHEC and *Salmonella* infection on HT -29 and T84 cells [83]. [84] found application of *L. casei* DN-114 001 down regulates genes linked to proinflammatory and adherence molecules in *Shigella flexneri* infected Caco-2 cells. In mice models, administration of *L. casei* has shown to activate innate immune response with increase in CD-206 markers and TLR -2 cells [85, 86]. Boosting adaptive immunity, probiotics stimulates the IgA production by B cells which further bind antigens thereby protect epithelium. Secretory IgA interfere pathogens at their early step of infection as it can trap them in mucus or block their secreted toxins. During *in vitro* experiments on T84 cell monolayers, SIgA was found to bound cholera toxin in dose dependent manner. The same SIgA during *in vivo* test protected mice from severe diarrhea symptoms on oral challenge by *Vibrio cholerae* [87, 88, 89].

## 11. Postbiotics

Postbiotics refers to the metabolic by products like enzymes, peptides, teichoic acid, peptidoglycan derived muropeptides, exopolysaccharides, cell surface and secreted proteins, bacteriocins and organic acids generated by a probiotic organism during its lifespan [90, 91]. Probiotics are living organisms, which eventually die but they secrete these postbiotic as nutritive patrimony that continues to improve health. Postbiotics avoids risks linked with the administration of live probiotic bacteria. Despite of proven benefits of probiotics, there are many concerns about their side effects like spreading of antibiotic resistance gene, virulence factor in particular strain, translocation to tissues or blood, risk of sepsis in premature infants, hindrance to normal colonization of other microflora [92]. Opposite to that, postbiotics have advantage due to their clear chemical structure, safety dose parameters and longer shelf life which can influence physiological function of host [93]. Postbiotic substances contain various metabolites and signalling molecules which display broad antibacterial spectrum and immunomodulatory actions [94, 95, 96, 97]. Recently, [98] demonstrated that supplementation of postbiotics with inulin leads to decrease in proliferation of pathogenic bacteria. CFCS of *Bifidobacterium breve* CNCM I-4035 found to decrease pro-inflammatory cytokines through TLR activation when human dendritic cells were infected with *Salmonella typhi* [99]. Probiotics cell remains are also found instrumental as postbiotics in bacterial interference and immune regulatory functions [91]. [100] demonstrated that teichoic acid D -Alanylation of *Lactobacillus plantarum* WCFS1 have positive impact on generation of regulatory T-cells in healthy mice. In same tune, [90] analyzed activity of probiotic strains and their postbiotics on organ culture system and found protective against *Salmonella* invasion which also down regulated ongoing inflammation in inflammatory bowel disease (IBD) tissue. In future, identification and ongoing studies on postbiotics not only provide opportunity to understand mechanism of action against pathobionts but provide a path to develop newer pharmabiotic products and pharmacological strategies devoid of live cells having better specific physiological effects.

## 12. Prebiotics

Prebiotics contribute to the nutrition of the host, inhibit the growth of potential pathogens and promote beneficial microbiota. The latter causes fermentation of non-digestible fibers, save energy, synthesize vitamins B and K, produce short chain fatty acids (SCFA) and polyamines, leads to improvement in GI motility and function, reduce the level of cholesterol and stimulate the immune system. Other benefits of prebiotic consumption includes, reduction in the prevalence and duration of infectious and antibiotic-associated diarrhea; reduction in inflammation and symptoms associated with inflammatory bowel disease, protective effects for prevention of colon cancer, enhancement of the bioavailability and uptake of minerals, including calcium, magnesium, and possibly iron; lowering in some risk factors for cardiovascular disease; and promotion of satiety and weight loss and prevention of obesity.

Prebiotics provide their health benefits by two main mechanisms, one is by selective proliferation of beneficial bacteria especially bifidobacteria and lactobacilli in the gut, which provide resistance against colonization of pathogens thereby reducing exogenous and endogenous intestinal infections. These beneficial organisms modulate the immune system and suppress IBD inflammation. The other mechanism is by production of SCFA. Metabolism of prebiotics leads to the production of SCFA. These SCFA show various beneficial effects including reduction of cancer risk, increase in mineral absorption, improvement in bowel habit, control of serum lipid and cholesterol level, and reduce cancer risk and IBD inflammation.

## 13. Selective proliferation of beneficial bacteria

Prebiotics act like growth factor to particular commensal bacteria, which inhibit the adherence and invasion of pathogens in the colonic epithelia by competing for the same glycoconjugates present on the surface of epithelial cells, altering the colonic pH, favoring the barrier function, improving the mucus production, producing short-chain fatty acids and inducing cytokine production [101]. Bifidobacteria and lactobacilli are known to be potentially beneficial for health of hosts. Human milk is found to have a number of oligosaccharides which are bifidogenic in nature. Studies have shown that bifidobacteria is the dominating species in the intestinal microflora of breast-fed infants, whereas in case of formula fed infants many harmful organisms like clostridia and enterococci and harmful chemicals like ammonia, amines, and phenols are found. The prevalence of bifidobacteria in breast-fed babies is thought to result from their abilities to utilize oligosaccharides in breast milk, including GOS. In some other studies, it was reported that feeding of a mixture of 10% long- chain FOS and 90% GOS to preterm infants resulted in an increase in intestinal bifidobacteria and lactobacilli, with a gut microbiota and fecal fermentation product composition more resembling that of breast-fed infants [102]. The administration of a GOS mixture (3.6 g/d) containing mainly  $\beta 1 \rightarrow 3$ , as well as  $\beta 1 \rightarrow 4$  and  $\beta 1 \rightarrow 6$  linkages, proved to have a better bifidogenic effect than a GOS mixture (4.9 g/d) containing mainly  $\beta 1 \rightarrow 4$ , as well as  $\beta 1 \rightarrow 6$ , after 1 wk of intake by healthy humans [103]. Both mixtures had mainly di- and trisaccharides. Both these GOS mixtures had low polymerization degree with  $DP \geq 4$  accounting for less than 12% and 19% of total saccharides, respectively.

Probiotic strains have been shown to prevent the attachment and invasion of pathogens in cell culture, resulting in the inhibition of enteropathogens *in vitro* and enhancement the immune response. Considering this, there is at least the possibility that the use of probiotics may decrease reliance on antimicrobials. Proposed mechanisms by which probiotic cultures may act in infection control include competition for nutrients, secretion of antimicrobial substances, reduction of pH, blocking of adhesion sites, attenuation of virulence, blocking of toxin receptor sites, immune stimulation, and suppression of toxin production. Probiotics are also used in the treatment of infections of the upper GIT, such as those caused by *Helicobacter pylori*. *H. pylori* an important etiological agent in peptic ulcers and has an involvement in gastric cancer. Studies have demonstrated the *in vitro* inhibition of this pathogen by *Lactobacillus acidophilus* and also by other LAB [12]. Studies have also reported that probiotics can inhibit the attachment of *Escherichia coli* O157:H7 [104].

#### 14. Prebiotics and immune modulation

The colonic microbiota is important for development and maturation of the immune system. Diet is one of the major factors that can influence the immune system in the gastrointestinal tract as well as intestinal microbial composition and metabolic product formation. Currently, there is increasing interest in the use of functional foods to modulate the gut immune system, with the aim of improving health and well-being. The gut contains a major part of the body's immune system, termed the gut-associated lymphoid tissue. Experimental obtained data so far suggest that immune modulation of the gastrointestinal tract can occur through the use of functional foods such as prebiotics [105]. To date, few studies have been made on interactions between fermentable carbohydrates and the immune system, or whether they exert direct or indirect modulatory effects. Increased SCFA production, and increase in immunogenic bacteria such as lactobacilli and bifidobacteria are the 2 main methods by which prebiotics can exert their effects on the immune system.

Feeding of GOS to mice challenged with pathogen was reported to increase the concentration of IgA and IgG in mice [106]. Fermentation of GOS (degradation of GOS by intestinal microflora) result in the production of butyrate, which serves as a fuel for colonic epithelial cells stimulates apoptosis suppress both cytokine-induced and constitutive expression of the transcription factor NF- $\kappa$ B in HT-29 cell lines and may be a protective factor in carcinogenesis [107]. Prebiotic fermentation in large intestine also produces propionate, which has been shown to be anti-inflammatory with respect to colon cancer cells [108]. A very recent study investigated the effect of prebiotic oligosaccharide on microbiota composition and immune function (NK cells, phagocytosis, and cytokines) in healthy elderly volunteers. This study showed that administration of prebiotic led to a significant decreases in the number of less beneficial bacteria (*Bacteroides*, *Clostridium perfringens*, *Desulfovibrio* spp., and *E. coli*) and a significant increase in the number of beneficial bacteria especially bifidobacteria [109]. The study also found significant positive effect on immune response, evidenced by an improvement in NK cell activity and phagocytosis, increased secretion of the anti-inflammatory cytokines, IL-10, and decreased secretion of proinflammatory cytokines (IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ). A number of studies reported the beneficial effect of prebiotics on immune system [110, 111]. Role of FOS and Inulin in modulation of immune system has been comprehensively reviewed by [112].

Although data from human studies is still scarce, the results from recent animal studies clearly suggest that prebiotics benefit the gastrointestinal tract by stimulating the immune system. The metabolites from bifidobacteria and lactic acid bacteria seem to cause positive regulation of the immune system, and thus reducing the incidence and severity of inflammatory diseases and allergies through the modulation of the functions of resident cells, particularly in the down regulation of the production of pro-inflammatory cytokines in those areas. Nutritional intervention with prebiotics is an attractive option for modulating both the gastrointestinal tract and the immune system, particularly for some divisions of the population such as infants, elders and the immunosuppressed. However, well designed clinical studies in humans are still necessary in order to investigate the optimal dosage, duration of treatment, and the specific effects of each prebiotic in different dietary matrices, in different populations showing different composition of the intestinal microbiota and immune response.

#### 15. Anti-pathogenic activity of prebiotics

Various studies have suggested that having prebiotics in diet protect the gut from infection and inflammation by inhibiting attachment and/or invasion of pathogenic bacteria or their toxins to colonic epithelium. This attachment is mediated by glycol-conjugation glycoproteins and lipids present on the microvillus membrane. Prebiotic especially GOS contain structures similar to those found on microvillus membrane that interfere with the bacterial receptor by binding to them and thus prevent bacterial attachment to colonic epithelium. Prebiotics, present in human milk, are known to have antiadhesive properties and be capable of toxin neutralization [113].

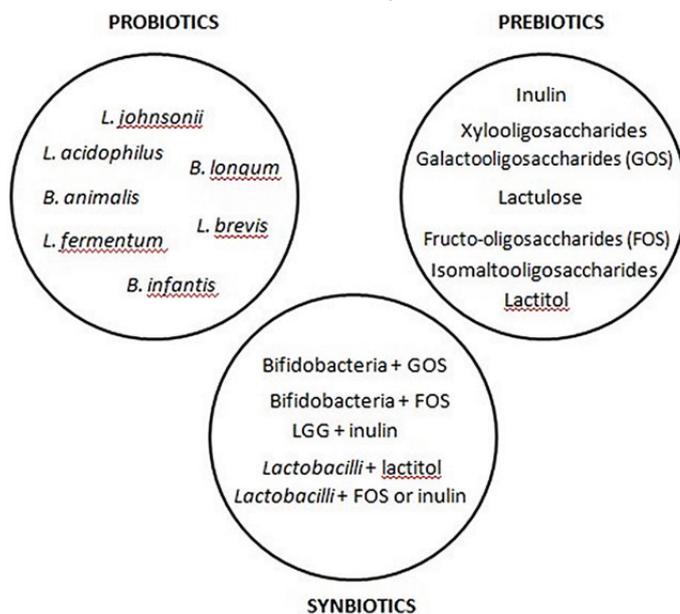
B-GOS contains an oligosaccharide in alpha anomeric configuration, and it was shown to significantly decrease the attachment of enteropathogenic *E. coli* (EPEC) and *Salmonella enterica* serovar *Typhimurium* to HT-29 epithelial cell line [114, 115]. It was shown that the animals fed the prebiotic mixture did not develop clinical symptoms of salmonellosis, even though the pathogen could be recovered in the feces. Furthermore the histopathology and structure

of the epithelium were completely protected and translocation of the pathogen to other organs was limited compared to placebo. In another study, different prebiotics (Inulin, FOS, GOS, lactulose and raffinose) were shown to inhibit the adhesion of EPEC to Hep-2 and Caco-2 epithelial cell lines [116]. Similar results were obtained with *Listeria monocytogenes*. Feeding of prebiotic preparation to mice challenged with *L. monocytogenes* resulted in the lowering of severity of infection along with modulation of immune system [106].

## 16. Synbiotic approach: Blending probiotics and prebiotics

In the new health concerned world, development of synbiotics is a very promising area in nutraceutical or functional food. Preparation of a synbiotic mixture without calibrating the dose amount, compatibility or type of prebiotic sugar (or non digestive oligosaccharides) with probiotic strain is never been a success. The combination of prebiotic and probiotic has good synergistic effects if they are blended after studies comprising growth rate and fermentation profile of different probiotics strain in presence of particular oligosaccharide. Added prebiotic not only stimulate probiotic counterpart strains in combination, it also encourage growth of existing strains of beneficial bacteria in the colon [117, 118]. Probiotics, prebiotics, and synbiotics that may be suitable for human consumption are listed in Table 1.

**Table 1** Some useful combinations as synbiotics.



Now days, synbiotic supplements not only given as feed supplements or pharmaceuticals but increasingly in suitable food specimens such as dairy products, fruit juices and chocolates. The synbiotic concept has been widely used by European dairy drink and yoghurt manufacturers such as Aktifit (Emmi, Switzerland), Proghurt (Ja Naturlich Naturprodukte, Austria), Vifit (Belgium, UK) and Fysiq (Netherlands) [119].

Administration of synbiotics as a food supplement is safe, simple, and convenient. Therefore, characterizing a new and novel synbiotic combination would find multifaceted use in disease prophylaxis and management for human use. Nowadays, the term integrated synbiotics is more preferable than synbiotics alone, when we consider specific synergies between probiotics and prebiotics [120].

## 17. Studies pertaining to the effect of synbiotics on pathogens

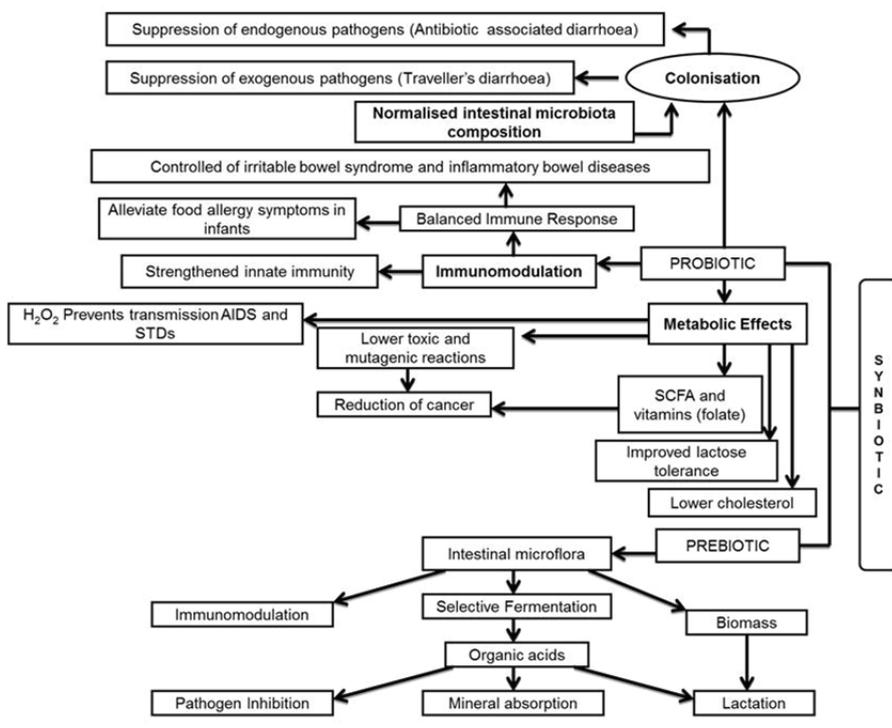
Though, there is growing interest in the development of new functional foods with synbiotics, combination of prebiotics and probiotics into a synbiotic has been studied to a limited extent and needs further investigations, because of the aforementioned different substrate requirements for individual probiotic LAB species and strains. Only a few human studies have been carried out on the effectiveness of synbiotics [121, 122, 123, 124, 125].

In a comparative *in vitro* study [126], it was concluded that strains of *Bifidobacterium longum*, *Bifidobacterium catenulatum* and *Bifidobacterium animalis* grew best on Fructooligosacchride (FOS) with significant increase in bifidobacteria and while a decrease was seen in coliform population. *Lactobacillus reuteri* has been investigated as a component in a synbiotic with soygerm powder [127]. Soygerm powder (4 g l<sup>-1</sup>) increased resistance of *L. reuteri* to bile salts. In addition, the lactobacilli cleaved the isoflavone glycosides to liberate the aglycone isoflavone, by increasing its

bioavailability [128]. One of the principal benefits of synbiotics is believed to be increased persistence of the probiotics in the GI tract. A synbiotic preparation of *Lactobacillus acidophilus* (probiotic strain 74-2) and FOS has been studied in an *in vitro* model of the human gut [129].

Increase in levels of propionate and butyrate and in  $\beta$ -galactosidase while decrease was seen in  $\beta$ -glucuronidase levels. Therefore, the development of synbiotics might be more important for strains of probiotic with poorer survival properties. Although, *Lactobacillus reuteri* is known to produce a bacteriocin reutrin associated as antimicrobial multi-compound against potential pathogens like *E. coli* has shown a significant increase in growth rate and bactericidal action when short chain FOS is used as only carbon source [130, 131]. SCFA (Short chain fatty acid) concentration has direct impact on harmful pathogens [132], which directly links with the type of prebiotic used in synbiotic combination [133]. Earlier studies shows increase in levels of propionate and butyrate while decrease was seen in  $\beta$ -glucuronidase levels in synbiotic combinations. The ability of a synbiotic preparation (*Bifidobacter breve* Yakult together with GOS) to protect against *Salmonella* infection in mice has been investigated [134]. Mice were treated with streptomycin to conciliate the gut flora by selective removal to undetectable levels of bifidobacteria, lactobacilli and enterobacteria. Feeding with *B. breve* at  $10^8$ CFU mice<sup>-1</sup>day<sup>-1</sup> or the synbiotic preparation which additionally contained GOS at a concentration of 2–50 mg mice<sup>-1</sup>day<sup>-1</sup>, resulted in recolonisation of the gastrointestinal tract with *B. breve*. Mice fed with probiotic and synbiotic displayed reduced faecal excretion of *Salmonella enterica* serovar *typhimurium* after pathogen challenge. In addition, the synbiotic blocked extra-intestinal translocation of the pathogen, whereas GOS alone did not.

A combination of probiotic and prebiotics (*Bifidobacterium* sp + chicory) and (*Bifidobacterium* sp + Inulin) for their antimicrobial activity against array of pathogenic bacteria like *Esherichia coli*, *Proteus mirabilis*, *Klebsiella* sp, *Pseudomonas aeruginosa* and *Serratia marcescens* was investigated. The synergistic inhibitory effect of synbiotic (*Bifidobacterium* sp + chicory) and (*Bifidobacterium* sp + inulin) on pathogenic bacteria was found higher than the effect of *Bifidobacterium* spp. alone, chicory and inulin alone. Results of the study showed the antimicrobial activity of *Bifidobacterium* sp against pathogenic bacteria may be enhanced due to the production of organic acids (acetic and lactic) that lowered the pH of the medium [135, 136] and production of other antimicrobial compound such as bacteriocin that act as antibiotic agent [137]. The antimicrobial activity of chicory extract and may be due to the chicory content, while on the other hand, inulin acts as "prebiotic" promoting selective development of beneficial microorganisms "probiotic". It supports growth of *Bifidobacterium* spp. and enhanced it to produce antimicrobial compounds *viz* acetic, lactic and benzoic acid and bacteriocin type compound. On the other hand, inulin reduces the amount of harmful bacteria such as Bacteroides, Fusobacteria and Clostridia. It was concluded that *Bifidobacterium* spp. had the highest inhibitory effect against pathogenic bacteria, followed by chicory and Inulin.



**Fig. 1** Outline of Prebiotics, Probiotics and Synbiotics.

The study was done to examine the efficacy and safety of seven different probiotic cultures of lactobacilli and child specific *Bifidobacterium infantis* along with prebiotic (FOS) in reducing the frequency of stool excretion and stool consistency in acute watery diarrhea. In the management of diarrhea studied the effect of probiotic agents, for the treatment and control of antibiotic associated diarrhea [138]. The product used in this study is a combination of multi-strains of *Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Streptococcus thermophilus*. Prebiotic FOS

(Fructooligosaccharide) was also included as a diet for the friendly bacteria [139]. In the synbiotic group, a more rapid improvement in the stool texture and average stool remission time was seen significantly better as compared to control group. While, the average duration of stool remission time in the synbiotic group was 41.53 hours and this was significantly different when compared to control group average time of 74.94 hours. Many strains of probiotic bacteria

that produce lactose also produce a substance like bacteriocin having antibiotic properties for eradicating pathogens that cause diarrhea. While no side effects were observed during the active treatment period with the use of synbiotic, this also highlights the high safety profile [140]. In a study, the role of some probiotic strains in treatment of infants and young children with infectious diarrhea, as well as in prevention of antibiotic-associated diarrhea has been reported. Results of randomized, double-blind, placebo-controlled study showed that supplementation with three probiotic strains and prebiotic fructooligosaccharides reduced ear -nose-throat, broncho-pulmonary or gastric disorder during winter. Furthermore, the combination of probiotic strains and prebiotic fibre reduced the incidence of childhood infections by about 25%. Even though, fortification with prebiotic and probiotic together may provide one of the potential interventions to reduce the burden of common childhood morbidities [141].

The efficacy of synbiotic supplementation in reducing common winter diseases in children was studied [142]. A randomized, double-blind, placebo-controlled study was conducted in young school-age children (3-7 years old) during a winter period. Participants were supplemented daily with either a synbiotic preparation (*Lactobacillus helveticus* R0052, *Bifidobacterium infantis* R0033, *Bifidobacterium bifidum* R0071, and fructooligosaccharides or a matched placebo for 3 months. Though, there was a significant 25% relative risk reduction and this difference was due to a decrease in the number of children who suffered from at least one ENT, respiratory tract or gastrointestinal disorder (50.0% with synbiotic group versus 67.1% with placebo). It was concluded that 3-month supplementation with synbiotic preparation can decrease the risk of occurrence of common infectious diseases in children and limits the risk of school day loss.

The effects of selected probiotic microorganisms, in combination with prebiotics, on certain human intestinal food-borne pathogens were investigated [143]. Probiotics grown with different carbohydrate sources were observed to inhibit growth of *Escherichia coli*, *Campylobacter jejuni* and *Salmonella enteritidis*. Prebiotics identified as being preferentially utilized by the probiotics tested were oligofructose (FOS), inulin, xylo-oligosaccharide (XOS), and mixtures of inulin: FOS (80:20 w/w) and FOS: XOS (50:50 w/w). Two of the probiotics, *Lactobacillus plantarum* and *Bifidobacterium bifidum* were selected for further co-culture experiments. Each was combined with the selected prebiotics, and was observed to inhibit pathogen growth strongly. It was found that acetate and lactate were directly conferring antagonistic action, which was not necessarily related to a lowering of culture pH. This study has shown that lactobacilli and bifidobacteria species can inhibit some important pathogenic species as this antagonism was influenced by the carbohydrate provided *in vitro*. Also, the inhibitory mechanism is due the production of SCFA as the underlying mechanism of inhibition of enteropathogens.

The *in vitro* antimicrobial activity of *Lactobacillus fermentum* and *Bifidobacterium longum*, isolated from faeces of healthy elderly individuals, was investigated against enterohaemorrhagic *Escherichia coli* (*E. coli* O157:H7) and enteropathogenic *E. coli* (*E. coli* O86) [144]. Antimicrobial activity of the putative probiotics and synbiotics was examined by a microtitre method using cell-free culture supernatants (CFCS). Results of the antimicrobial assay showed that both putative probiotic strains produced compounds at pH 5 lead to higher lag phases of both *E. coli* O157:H7 and *E. coli* O86. When half the quantity of cell-free culture supernatants of both probiotic strains was used at pH 5, *B. longum* maintained the same antimicrobial effect against both strains of *E. coli*, whereas *L. fermentum* lead to a higher lag phase of *E. coli* O86 only. A short chain fructooligosaccharide (scFOS) and an isomaltooligosaccharide (IMO) proved to be the most effective substrates, enhancing antimicrobial activity for *L. fermentum* and *B. longum* respectively. The adhesion of the synbiotic combinations showed that *L. fermentum*, exhibited higher percentage of adhesion when grown on glucose and as a synbiotic combination with scFOS whereas *B. longum* exhibited lowest percentage of adhesion when grown on both glucose and IMO.

*Lactobacillus kefir*, isolated from kefir grains for its properties associated with probiotic bacteria were examined [145]. The strain was found to be fully resistant to bile at 0.4% (w/v), but showed only moderate resistance to acid. It grew well on media supplemented with prebiotic compounds (two fructooligosaccharides, galactooligosaccharide (GOS) and lactulose) and significant inactivation of *Listeria monocytogenes* was observed when GOS was added to the co-culture medium, whereas *Escherichia coli* was not significantly affected. *Lb. kefir* show potential as a probiotic, which is a first step towards the development of a functional product and it would be more effective in a synbiotic combination with GOS.

The effect of probiotics and synbiotics consumption based on microbiota of human gut was carried out *in vivo* study [146]. Three groups, P (consuming probiotics), S (consuming synbiotics) and C (control group) of 22 healthy adults were used for this experiment. P and S groups had 10 days long adaptation phase without consuming probiotics and consequently they consumed yoghurt for another 21 days. Control group did not consume yoghurt during the experiment. Faecal samples were collected 10<sup>th</sup> day of the adaptation phase and then 7, 14 and 21<sup>th</sup> day of yoghurt consumption phase and finally 26 and 28<sup>th</sup> day of wash out period. Effect of probiotics and synbiotics on *Clostridium* sp. and *Escherichia coli* (*E. coli*) counts in human digestive system have been reported. Consumption of probiotics decreased of *E. coli* count and consumption of synbiotics increased of both *E. coli* count and *Clostridium* sp. in human digestive system.

So far, while investigating the efficacy of synbiotic administration commercially available products have been used. The results are indeed encouraging, and there seems to be scope for further studies and more widespread application of

synbiotics in these patients. However, the lack of placebo and proper controls (e.g., prebiotic only, probiotic only) to elucidate the mechanism of the effects prevent the proper evaluation of the synbiotic effects.

## 18. Conclusion and future demands

Antipathogenic action is an inborn characteristic of most of a probiotic strain. Till date several studies shown its benefits over pathogen but they are more restricted towards preventive approach and same effect in *in vivo* antimicrobial studies are scare in reports. Similarly, *in vivo* mechanistic data on exclusion principle is almost negligible. Hence, molecular mechanisms of both probiotics and pathogenic strains should be studied more precisely to know sensing mechanism behind their communication and production of antimicrobial peptides for their own colonization. The robustness of probiotic strain to bypass pathogenic effects should be increased through pathobiotechnological approach and preparation of designer strains. Well planned clinical studies are still needed to know the dose and duration effects in different hosts which vary due to age, immune status, living condition and genetic makeup. Newer antimicrobial metabolite gene cluster like sactibiotics producing machinery can be exploited to produce best metabiotics for antimicrobial use. Currently known prebiotic sugars have good boosting effect on probiotics but studies regarding their compatibility with good and bad colonizers should be clearer. The structural composition for utilization of prebiotics is less studied and should be viewed in magnified form to bond level as their chain length plays important role in anti-pathogenic action. Prebiotics having capacity to adhere pathogenic metabolites like toxins are needed to be explored. To gain the effect of prebiotic and probiotic as synbiotic reinforcement more perfect combination are needed to examine with clinical trials.

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