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Live and heat-killed probiotic *Lactobacillus casei* Lbs2 protects from experimental colitis through Toll-like receptor 2-dependent induction of T-regulatory response

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ABSTRACT

Inflammatory bowel disease (IBD) is a group of inflammatory disorders of the intestine caused by dysregulated T-cell mediated immune response against commensal microflora. Probiotics are reported as therapeutically effective against IBD. However, variable efficacy of the live probiotic strains, difference in survival and persistence in the gut between the strains and the lack of insight into the mechanisms of probiotic action limit optimal therapeutic efficacy. Our aims were to evaluate the *lactobacillus* strains isolated from the North Indian population for the generation of regulatory cells and cytokines in the intestine, to study their effects on pro-inflammatory mediators in the mouse model of inflammatory bowel disease and to explore the underlying mechanisms of their actions. Among the selected *lactobacillus* strains, *Lactobacillus casei* Lbs2 (MTCC5953) significantly suppressed lipopolysaccharide-induced pro-inflammatory cytokine (TNF-alpha, IL-6) secretion. Both live and heat-killed Lbs2 polarized Th0 cells to T-regulatory (Treg) cells *in vitro*, increased the frequency of FoxP3$^+$ Treg cells in the mesenteric lymph nodes (MLNs) and alleviated macroscopic and histopathological features of colitis in probiotic-fed mice. Moreover, the levels of IL-12, TNF-alpha and IL-17A were suppressed, while IL-10 and TGF-beta levels were augmented in the colonic tissues of Lbs2-treated mice. The induced Treg (iTreg) cells secreted IL-10 and TGF-beta and exerted suppressive effects on the proliferation of effector T-cells. Adoptive transfer of iTreg cells ameliorated the disease manifestations of murine colitis and suppressed the levels of TNF-alpha and IL-17A. Finally, Lbs2 effects were mediated by Toll-like receptor 2 (TLR2) activation on the dendritic cells. This study identified live and heat-killed Lbs2 as putative therapeutic candidates against IBD and highlighted their Toll-like receptor 2-dependent immunomodulatory and regulatory function.

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1. Introduction

Probiotics are live microorganisms, which when consumed in adequate quantities promote human health [1]. Since probiotics are primarily gut-derived commensal microbes, their beneficial role has been most extensively studied in intestinal diseases including infections and inflammatory disorders, such as Crohn's Disease (CD) and ulcerative colitis (UC). Although probiotics are currently not part of the standard treatment regimens for inflammatory bowel disease (IBD), various probiotic strains belonging to the *Lactobacillus* and *Bifidobacterium species* were reported to protect mice and humans from colitis. *Lactobacillus* strains *viz.* LGG, *L. reuteri* ATCC55730, *L. casei* DG, *Bifidobacterium viz. Bifidobacterium breve* strain Yakult and also VSL#3 [a multispecies probiotic formulation] have been shown positive outcome against ulcerative colitis in separate human trials [1–3]. For consistent and optimal therapeutic efficacy, the mechanisms behind the protective role of probiotics must be adequately understood. Current literature suggests that influence on the composition of the gut microbiota and alteration of their metabolic properties and immunomodulatory properties are among the major underlying mechanisms for probiotic actions in IBD [4,5]. The beneficial effects were also reported to be host- and strain-specific [6,7]. However, potential deleterious outcome of live probiotic therapy on immunocompromised individuals and consistent beneficial role of killed organisms have raised a debate whether to use non-viable over viable probiotic strains. Published literature comparing the immunomodulatory functions of live and killed cells of the same probiotic strain is inadequate [8], and more studies are required to reach a realistic conclusion.

Abbreviations: TLR, Toll-like receptor; BMDCs, Bone-marrow derived dendritic cells; TNBS, 2,4,6-trinitrobenzenesulfonic acid; ConA, Concanavalin A; CFSE, Carboxyfluorescein diacetate *N*-succinimidyl ester; IFN, Interferon.

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Inflammatory bowel disease (IBD) is classified into two major clinical phenotypes, Crohn's disease (CD) and Ulcerative colitis (UC). Although the exact etiology of IBD is still unknown, genetic predisposition coupled with breach in the intestinal barrier integrity leading to dysregulated mucosal immune response to the enteric microbiota has been proposed as a key mechanism underlying its development [9–11]. Both innate and adaptive arms of the immune system play a critical role in IBD pathogenesis; two key pro-inflammatory cytokines, TNF-alpha and IL-6, secreted by the myeloid cells and heightened proliferation and function of different effector T-cell subsets, such as Th1, Th2 and Th17 cells have been associated with various clinical manifestations of IBD [12–14]. While Th1 cells and cytokines are traditionally associated with CD and Th2 cells with UC, recent studies have shown that large quantities of IL-23, an IL-12 family member, is produced by macrophages and DCs and play a pivotal role in chronic intestinal inflammation by aggravating Th17 cell response [15]. Immunosuppressive drugs like 5-aminosalicylic acid, azathioprine, mercaptopurine, methotrexate and anti-TNF antibody are the mainstay of current treatment for IBD, but they often fail to induce long term remission and may be associated with unacceptable adverse reactions [2, 10]. Keeping in mind that the disease begins with dysregulated mucosal immune response and often accompanied by altered intestinal flora (dysbiosis) [9], probiotics have drawn a major focus of research in the recent times as a potential treatment modality for IBD.

Immunomodulation by probiotics through the induction of anti-inflammatory and regulatory responses may be particularly important for their role in protection against autoimmune and inflammatory diseases and better understanding of how probiotic interacts with host cells is required for their optimized therapeutic application. Anti-inflammatory role of various *Lactobacilli* (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus*) and *Bifidobacterium* (*B. longum*, *B. infantis*, *B. breve*) has been reported to be mediated by the inhibition of pro-inflammatory cytokines, such as IL-12, TNF-alpha, IL-6 and IFN-gamma [4,16]. A second mechanism could be probiotic-induced generation of regulatory T-cells and cytokines, which has been less extensively studied. Existing literatures suggest a role for the regulatory T-cells in the protection against colitis [3,17], but more investigation is required and especially, the underlying molecular mechanisms need to be explored to achieve optimal benefits of probiotic therapy against autoimmune and inflammatory diseases. In the present study, we evaluated the role of probiotic *lactobacilli* in the modulation of the regulatory arm of mucosal immune response and studied the molecular mechanisms behind the observed functional outcome. This study also compares the immunotherapeutic potential of live and killed probiotics in murine model of colitis through their regulatory activities.

2. Materials and methods

2.1. Reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS), all-trans retinoic acid (ATRA), Concanavalin A, Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), Hexadecyltrimethylammonium bromide (HTAB), *o*-Dianisidine dihydrochloride, 7-Aminoactinomycin-D (7AAD) and Lipopolysaccharide were purchased from Sigma Aldrich, USA. Murine recombinant GM-CSF and IL-2 were purchased from R&D systems, USA. Mouse cytokine ELISA kits for IL-12, TNF-alpha, IL-10 and TGF-beta were procured from eBiosciences. Mouse antibodies, anti-CD11c FITC, anti-CD103 PE, anti-CD4 FITC, anti-CD25 APC, anti-FoxP3 APC, anti-FoxP3 PE, anti-CD62L PE-Cy7, anti-CD3, anti-CD28 and their respective isotypes were purchased from BD Pharmingen, USA.

2.2. Bacterial strains and growth conditions

Lactobacillus strains were procured from Molecular Biology Unit, Dairy Microbiology Department, National Dairy Research Institute, Karnal, India. Two of the strains Lbs2 (MTCC5953) and Lbs4 (MTCC5954) have been deposited at International repository at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh under Budapest Treaty for safe deposit as per ICMR-DBT guidelines. Whole genome sequencing of Lbs2 (MTCC5953) was done and submitted to NCBI (Accession: PRJNA255080; ID: 255080). Gene annotation of Lbs2 (MTCC5953) genome sequence is already published [18]. *Lactobacillus* strains were grown in MRS broth at 37 °C. *Lactobacillus rhamnosus* GG (LGG) was procured from American Type Culture Collection (ATCC 53103). VSL#3 is commercially available, each capsule contains 112.5 billion colony forming units (cfu) of four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus*. For stimulation of BMDCs, probiotic strains were grown till the end of the log phase and resuspended at 1×10^9 cfu/ml in PBS containing 20% glycerol and stored at −80 °C until use for stimulation of cells. For oral treatment of mice, bacteria were grown overnight, washed three times and resuspended in sterile PBS at 5×10^9 cfu/ml. Heat-killed (HK) Lbs2 was prepared by incubating them at 121 °C for 20 min and was confirmed by plating on MRS plate.

2.3. Acid and bile tolerance assay

Lactobacillus strains were grown at 37 °C in MRS broth for 24 h. For acid tolerance assay, 1 ml of culture was spun down and resuspended in 1 ml of MRS broth (pre-adjusted at pH 2.0 with 1.0 N HCl) and incubated at 37 °C for 1–3 h. For Bile tolerance assay, 1 ml of culture was spun down and resuspended in 1 ml of MRS broth supplemented with 0.3% Oxgall (HiMedia) followed by incubation at 37 °C for 1–3 h. 1 ml of culture was collected immediately (0 h) and viable number of *lactobacillus* were enumerated by pour plating after using 10-fold serial dilutions prepared in 0.1% peptone water. Similarly, 1 ml of culture was collected after an interval of 1, 2 and 3 h followed by pour plating. Colony forming units (cfu) were counted after incubating the plates at 37 °C for 24 h.

2.4. Animals and bacterial treatment

Balb/c mice were housed under standard conditions in the animal facility according to protocols approved by Institutional ethical committee (IEC) of National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. All the performed animal experiments were approved by IEC, NICED. 4 week old male Balb/c mice were treated with 1×10^9 cfu/day/mice of *Lactobacillus* strains or VSL#3 (freshly dissolved in 1× PBS) for 2 weeks followed by evaluation for frequency of regulatory cells in MLN cells. For the study of probiotic-mediated anti-inflammatory effect in lipopolysaccharide (LPS)-induced sepsis mice model, 4 week old male Balb/c mice were treated with 1×10^9 cfu/day/mice of *Lactobacillus* strains for 2 weeks followed by intraperitoneal injection of LPS (3 mg/kg wt.). After 2 h of LPS injection, mice were anaesthetized with ketamine and blood were taken from the retro-orbital venous plexus for analysis of cytokines level.

2.5. TNBS-induced murine colitis and treatment with probiotics

TNBS-induced colitic murine model was generated following the protocol earlier described for TNBS presensitization murine model [19]. In brief, mice were presensitized with 1% TNBS solution [in acetone:olive oil (4:1)] on shaved skin. Control mice were treated with vehicle solution without TNBS. After 8 days of presensitization, mice were intra-rectally administered with TNBS solution in 50% ethanol (3 mg/kg wt.) by using 4 cm long catheter. Control mice were intra-rectally instilled with 50% ethanol. For prophylactic evaluation of selected *Lactobacillus* strains against experimental murine colitis, mice were pretreated with live or HK-*lactobacillus* strains, VSL#3 (1×10^9 cfu/day/mice) or vehicle (1× PBS) as oral gavage for 2 weeks before intra-rectal administration of TNBS. Disease phenotypes (macroscopic and

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