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Immunomodulatory effects of *Lactobacillus casei* Shirota

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Abstract

Lactic acid bacteria (LAB) are present in the intestine and the beneficial role played by these microorganisms in humans has been extensively reported. They are frequently used as probiotics to improve some biological functions in the host and various studies have found that probiotic bacteria provide a beneficial effect on gut mucosal function, suggesting their potential effect in the treatment of inflammatory bowel diseases. LAB have been shown to activate both systemic and secretory immune responses via many complex interactions among the different components of the intestinal ecosystem, such as microflora, epithelial cells and other immune cells (Perdigon *et al.*, 2001). There are numerous strains of lactic acid bacteria, but this review will focus particularly on the effects of *Lactobacillus casei* and more specifically *Lactobacillus casei* Shirota, the probiotic used in the commercially available Yakult probiotic drink.

Gut Mucosal Immunity

To understand the effect lactic acid bacteria such as *Lactobacillus casei* Shirota have on gut mucosal function, it is first important to understand the structure of intestinal mucosa and the role of commensal bacteria.

The majority of epithelial surfaces of the body, such as the skin and gut mucosa, are colonized by a vast number of microorganisms; the so-called normal 'microflora' or commensal bacteria (Eckburg *et al.*, 2005). Starting from the first hours after the birth, interaction with micro-organisms begins (Tlaskalova-Hogenova *et al.*, 2004) and the human microbiota is made up of trillions of bacterial cells, the majority of which are found in the gut, particularly in the large intestine (Eckburg *et al.*, 2005). The main route of entry for microbes is the skin and mucosal surfaces of the gastrointestinal, respiratory and urogenital tracts where colonisation of epithelial surfaces takes place and a symbiotic co-existence is formed that is beneficial for the host (Tlaskalova-Hogenova *et al.*, 2011). Most exogenous pathogens also enter their host via mucosal surfaces (Hill and Artis, 2010) and therefore almost 80% of the immunologically active cells of the body belong to the mucosal-associated immune system in the gut. These cells are present in tissues of the gastrointestinal tract, where the prevalence of immunogenic agents, such as food and components of the commensal bacteria, is the highest (Hill and Artis, 2010).

The barrier function of mucosal surfaces in the gut involves a number of complex mechanisms working on several levels. The commensal bacteria themselves form an important part of these natural mechanisms that provide protection against pathogenic microorganisms (Chung and Kasper, 2010; Turner, 2009). They play an important role in pathogen resistance, by direct interaction with pathogenic bacteria and by priming the host immune system, resulting in mucosal tolerance (Turner, 2009; Foey, 2012). When there is an optimal composition of commensal bacteria several events take place: they prevent attachment and multiplication of pathogenic or virulent microorganisms on these surfaces, and inhibit the invasion of these microorganisms into epithelial cells and subsequently the host's circulatory system by outcompeting possible pathogens for nutrients and attachment sites (Chung and Kasper, 2010). The first major barrier between the gut contents and epithelial cells is provided by a class of highly glycosylated macromolecules called mucins, which protect gut epithelial cells from direct contact with commensal bacteria and their components (Linden *et al.*, 2008). Another constituent of barrier defences is the secretion of mucus, which traps pathogenic organisms; it is secreted by goblet cells that are interspersed with epithelial cells in the lining of the gut (Foey, 2012). The epithelial layer of the gut mucosa is also reinforced by junctions, such as tight junctions, adherens junctions and desmosomes, in the paracellular spaces between epithelial cells (Chung and Kasper, 2010). Tight junctions have been shown to act as strictly regulated points of entry that open and close in response to various signals such as cytokines and bacterial components originating in the lumen, lamina propria and epithelium of the gut (Tlaskalova-Hogenova *et al.*, 2011).

Components of innate immunity such as complement, lysozymes, lactoferrin and mannan-binding protein also have a role in gut mucosal immunity (Medzhitov and Janeway, 2000). Epithelial cells facilitate the repulsion of pathogenic organisms by

secreting anti-microbial peptides such as defensins (Foey, 2012). Multiple types of defensins are also produced by Paneth cells, specialized cells present in the crypts of the gut mucosa. In general, innate immune mechanisms to microbes are initiated by phagocytic cells such as macrophages, neutrophils and dendritic cells, via pattern recognition receptors (PRRs), which produce cytokines essential for inflammatory reactions and factors critical for the subsequent initiation of adaptive immunity (Medzhitov and Janeway, 2000). PRRs, such as Toll-like receptors, C-type lectin receptors, RIG-I-like receptors and nucleotide-binding domain (NODs) sense pathogen-associated molecular patterns (PAMPs) and transmit activation signals to their target cells (Tlaskalova-Hogenova *et al.*, 2011). Phagocytic cells such as macrophages and dendritic cells are generally located close to the gut epithelium, so they can quickly recognise invading pathogens and initiate an inflammatory response as well as exert primary defences through phagocytosis (Mannon, 2005). Intestinal adaptive immunity is mediated by Peyer's patches in the small intestine which contain immune cells such as T and B lymphocytes (Mowatt and Viney, 1997). The overlying layer of follicle-associated epithelium of the Peyer's patches contains specialized epithelial cells which have microfolds on their luminal surface, instead of microvilli like on epithelial cells of the intestine, and are known as microfold cells or M cells (Medzhitov and Janeway, 2000). They form a membrane overlying the lymphoid tissue within the Peyer's patch and are adapted to interact directly with molecules and particles within the lumen of the gut (Mowatt and Viney, 1997).

Gut Pathologies

Most of the commensal bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, for example in the case of immunodeficiency, commensal bacteria can cause disease (Tlaskalova-Hogenova *et al.*, 2004). An example of such a pathology is Inflammatory Bowel Disease (IBD) (Tlaskalova-Hogenova *et al.*, 2011), which covers several chronic inflammatory disorders. The two principal forms of these disorders are Crohn's disease and Ulcerative Colitis which affect approximately 0.2% of the population (Tlaskalova-Hogenova *et al.*, 2011). Even though there has been much research in this field, the aetiology and pathogenesis of these diseases remain unclear. However, the combination of immune, environmental and genetic factors is thought to result in the induction of inflammation and the subsequent development of mucosal lesions seen in both diseases (Xavier and Podolsky, 2007). Disruption of T lymphocyte regulatory functions and abnormal mucosal immune responses to commensal bacteria has been shown to play an important role in the pathogenesis of chronic intestinal inflammation (Bengmark, 2007). This suggests that the gut mucosa is a sensitive indicator of immune dysfunction. This abnormal T-cell response against commensal bacteria in IBD suggests that commensal bacteria may initiate the intestinal inflammation seen in this disease (Bengmark, 2007).

Increased interest in the effects of the intestinal microbiota on human health has resulted in attempts to optimise the composition of the microbiota using probiotics (O'Flaherty *et al.*, 2010). The most commonly used microorganisms in probiotic products are lactic acid bacteria (LAB) and various studies have found these probiotic bacteria provide a beneficial effect on gut mucosal function suggesting their potential effect in the treatment or alleviation of inflammatory bowel diseases (O'Flaherty *et al.*, 2010).

Lactobacillus casei and gut pathologies

The effects of LAB such as *Lactobacillus casei* on gut pathologies such as IBD have been established in numerous studies. It has been demonstrated in an IL-10 knockout mice model of colitis that probiotic therapy with *L. casei* is effective in reducing inflammation (Madsen *et al.*, 1999; McCarthy *et al.*, 2003). Madsen *et al.* (1999) demonstrated that by restoring *Lactobacillus* species such as *L. casei* to normal levels in the gut, this reduced levels of pathogenic bacteria and thus prevented the development of colitis. This was substantiated by McCarthy *et al.* in 2003 who found that the production of pro-inflammatory cytokines from Peyer's patches was reduced in the probiotic fed mice, suggesting that *L. casei* had an effect on the mucosal immune system. *L. casei* has also been shown to reduce the inflammatory response in Crohn's disease by significantly reducing the amount pro-inflammatory cytokines such as TNF α . In 2002, Borruel *et al.* obtained ileal samples from the surgery of 10 patients with Crohn's disease and cultured them for 24 hours with different dilutions of heat killed *L. casei*. They found the release of TNF α from inflamed mucosa in Crohn's disease patients was significantly reduced by coculture with *L. casei*. Coculture with *L. casei* was also found to reduce the number of CD4⁺ T cells as well as TNF α expression among intraepithelial lymphocytes from Crohn's disease mucosa compared to no changes in non-inflamed mucosa. It was therefore concluded that the probiotic interacts with immunocompetent cells using the mucosal border and then modulates the production of pro-inflammatory cytokines (Borruel *et al.*, 2002). This was an important discovery as TNF α has been found to be the principal cytokine responsible for the inflammation associated with Crohn's disease (Reimund *et al.*, 2007). Suppressing the action of TNF α using *L. casei* could therefore be used as a possible treatment. In 2005, Herias *et al.* demonstrated that *L. casei* Shirota (LcS) also has a positive effect on Ulcerative Colitis. A murine model of ulcerative colitis was used and the effect of LcS was tested either as a prophylactic 10 days before the onset of the disease, simultaneously with ulcerative colitis induction, or continued 10 days after the disease was induced. Treatment with LcS was found to increase colonic epithelial regeneration in the LcS treated animals when observed in the chronic stage. So although LcS was not able to inhibit the effects of ulcerative colitis on the intestinal epithelium entirely, it contributed by improving the conditions of the animals, in particular when administered at the time of ulcerative colitis induction (Herias, *et al.*, 2005).

Immunomodulatory effects of *Lactobacillus casei*

Probiotics have been shown to interact with a wide variety of cells such as enterocytes, macrophages, dendritic cells, Th1, Th2, and Tregs in the intestine and may modulate the immune response toward a pro- or anti-inflammatory response (Gourbeyre *et al.*, 2011). It has been shown in *in vitro* cell models (enterocytes models, such as HT-29, caco-2) that probiotics such as *L. casei* can influence the production of cytokines by intestinal antigen presenting cells (APC) and initiate the development of the adaptive response (Gourbeyre *et al.*, 2011). The results from several studies on animals showed that treatment with *L. casei* increased the function of phagocytic cells. In 1983, Saito *et al.* described how subcutaneously inoculating mice with *L. casei* stimulated the production of specific antibodies against *Pseudomonas* antigens by increasing the level of circulating IgM antibodies. Then in

1984, Kato *et al.* demonstrated using a murine model that intraperitoneally applied *L. casei* activated macrophages by increasing their phagocytic capacity and enzyme activity, and in addition, this method induced the activation of natural killer cells (NK) which play an important role in preventing tumour development. Hashimoto *et al.* (1985) established using an *in vitro* assay that Kupffer cells and immune cells associated with spleen, lung and peritoneal macrophages were activated by *L. casei* administration. The secretion of lysosomal enzymes from macrophages in mice fed fermented milk containing *L. casei* was found to be more effective compared with feeding the mice fermented milk containing other probiotic bacteria such as *Lactobacillus acidophilus* (Perdigón *et al.*, 1988). Oral treatment with *L. casei* led to macrophage and lymphocyte stimulation and to the release of enzymes from murine macrophages (Perdigón *et al.*, 1986). Subsequent studies by Perdigón *et al.* (1990, 1991) showed that treatment with *L. casei* activated cells in the gut associated lymphatic tissue, even at low doses, which led to significantly higher production of secretory IgA in the intestinal fluid, providing protection against infections such as *Salmonella*. These results illustrate that *L. casei* plays an important role in the prevention of intestinal infections and administration of *L. casei* could therefore be used as an oral adjuvant for preventing intestinal infections.

L. casei Shirota (LcS) has also been shown to enhance natural killer (NK) cell activity (Dong *et al.*, 2010; Takeda *et al.*, 2006). In an *in vitro* study using human peripheral blood mononuclear cells (PBMCs) Dong *et al.*, 2010, found that after incubating the cells with LcS at 10^6 cfu/ml for 24 hours NK cell activity was increased. In another *in vitro* study using PBMCs, heat killed LcS was reported to enhance NK cell activity and induce IL-12 production (Takeda *et al.*, 2006). They combined the ability of LcS to enhance NK cell activity and induce interleukin (IL)-12 production and found that the addition of an anti-IL-12 monoclonal antibody reduced the enhancement of NK cell activity triggered by LcS. It was concluded that these results demonstrate how LcS can enhance NK cell activity *in vivo* and *in vitro* in humans, which may be due to IL-12 (Takeda *et al.*, 2006). In another *in vitro* study, heat-killed LcS was found to stimulate IL-10, IL-12, TNF α and IFN- γ production. It also promoted NK cell activity and activated CD69 expression on NK cells (Shida *et al.*, 2006). In an *in vivo* study by Takeda and Okumura (2007) volunteers drank fermented milk containing 4×10^{10} live cells of LcS everyday for three weeks to find that their NK cell activity had significantly increased suggesting that daily a intake of LcS provides a positive effect on NK cell activity. In contrast to this, in a similar study in 2011, Seifert *et al.*, found that LcS did not modulate NK cell activity due to the fact that participants drank a probiotic drink identical to the commercially available Yakult product for 4 weeks and no significant effect on NK cell numbers or function was observed. This contrast may be due to the fact that the probiotic drink used by Takeda and Okumura (2007) contained more cells than the probiotic drink used by Seifert *et al.* (2011). Also, it must be taken into account that when administering probiotics via a probiotic drink, many of the cells will not reach the intended location of the large intestine as not all will survive passage through the stomach and small intestine. This may explain the positive results seen in Takeda and Okumura's (2007) study, as the high number of cells in the probiotic drink used would increase the chance of a sufficient number of cells reaching the large intestine, where they could exert their effect on the NK cells.

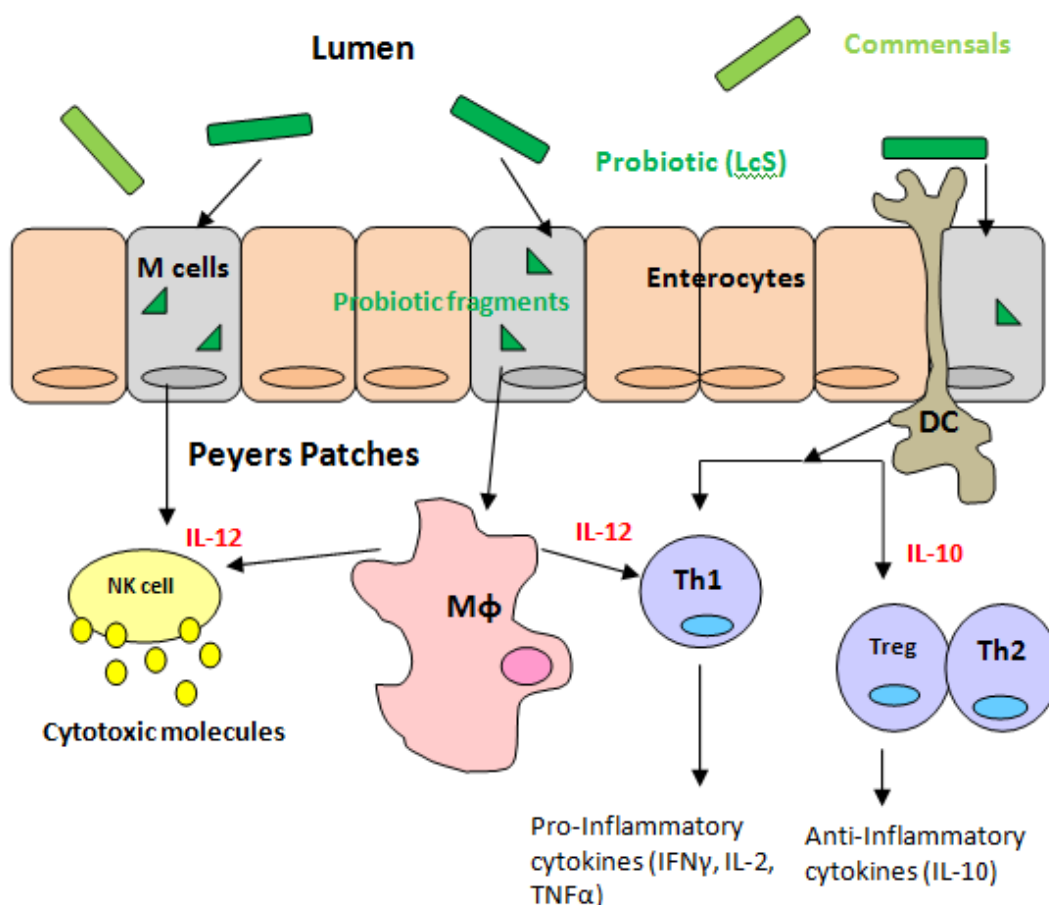


Figure 1: Immunomodulatory effects of probiotic such as *Lactobacillus casei* Shirota. Probiotics have been shown to interact with a wide variety of cells such as enterocytes, macrophages, dendritic cells, Th1, Th2, and Tregs in the intestine. *LcS* has been shown to increase the cytotoxic activity of NK cells and increase the phagocytic capacity of macrophages. *LcS* also has an effect on T cell activation and cytokine production and can modulate the Th1/Th2 balance. Adapted from Shida *et al.* (2011).

L. casei Shirota (*LcS*) has been found to have effects on T cell activation and cytokine production (Dong *et al.*, 2010; Baken *et al.*, 2006). In an *in vitro* study by Dong *et al.* (2010) the effects of *LcS* on immune function using human peripheral blood mononuclear cells (PBMC) was investigated and it was found that *LcS* induced CD69 expression on CD4⁺ and CD8⁺ lymphocytes and CD25 expression on CD8⁺ lymphocytes. There was also significant evidence of preferential activation of CD8⁺ T cells (Dong *et al.*, 2010). It was also established that *LcS* has a modulatory effect on cytokine production; inducing the production of IL-1β, IL-6, TNF-α, IL-12 and IL-10 in the absence of lipopolysaccharide (LPS). However, depletion in monocyte levels significantly reduced the effect of *LcS* on lymphocyte activation and cytokine production (Dong *et al.*, 2010). It has been observed in animal models that *Lactobacillus casei* can also modulate the Th1/Th2 balance toward Th1 activation, which has a pro-inflammatory effect (Gourbeyre *et al.*, 2011). This was substantiated by Shida *et al.* (2002) who used a food allergy model to study the Th2 response in mice and showed that an intraperitoneal injection of heat-killed *L. casei* Shirota

induced a rise in serum IL-12 levels and a skewing of the cytokine profile from Th2 to Th1.

In 2012, Habil *et al.* established that *Lactobacillus casei* Shirota (LcS) also selectively modulates the cytokine production of different macrophage subsets. M1 macrophages (pro-inflammatory) and M2 macrophages (anti-inflammatory) were treated with heat-killed LcS, activated with the LPS and the cytokines that were consequently produced were measured using an ELISA (Habil *et al.*, 2012). They found that heat-killed LcS augmented the production of IL-1 β and IL-8 in M1's, but suppressed the production of TNF α . In M2's, TNF α and IL-1 β production was augmented whereas IL-6 and IL-8 were suppressed (Habil *et al.*, 2012). These results lead them to conclude that LcS differentially regulates macrophage subset cytokine production. Again, this is an important discovery because being able to regulate the production of TNF α using LcS to suppress its production from pro-inflammatory M1's could be used as a possible treatment for Inflammatory Bowel Diseases such as Crohn's disease, where TNF α is the main cytokine associated with the inflammation.

Conclusions

From these studies it has become clear that the modulation of immune responses by LcS can be both anti-inflammatory and pro-inflammatory. LcS has been shown to induce the production of inflammatory cytokines such as IL-6, TNF α and IL-12 (Dong *et al.*, 2010; Takeda *et al.*, 2006) and to modulate the production of inflammatory cytokines by macrophage subsets (Habil *et al.*, 2012). In contrast LcS has also been found to suppress the production of TNF α therefore preventing its inflammatory effects (Borruel *et al.*, 2002). LcS has also been shown to facilitate the development of Th1 cells (Shida *et al.*, 2002) and to enhance NK cell activity (Takeda *et al.*, 2006). These immunomodulatory effects could be used to alleviate the symptoms of Inflammatory Bowel Diseases such as Crohn's Disease and Ulcerative Colitis and could even lead to future treatments.

However, many of these immunomodulatory mechanisms are still not fully understood, and while many positive effects of LcS have been reported, many of these studies have included methods using heat-killed LcS. Using heat-killed bacteria may produce positive results, but the effect of using live probiotics has received less attention so far, and may not deliver such conclusive results.

Further research to investigate the immunomodulatory mechanisms is required and studies using live bacteria are needed to confirm the effects of LcS both *in vitro* and *in vivo*.

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