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Lactic Acid Bacteria and their Effect on the Immune System

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Abstract

Lactic acid bacteria (LAB) are present in the intestine of most animals. The beneficial role played by these microorganisms in the humans and other animals, including the effect on the immune system, has been extensively reported. They are present in many foods and are frequently used as probiotics to improve some biological functions in the host. The activation of the systemic and secretory immune response by LAB requires many complex interactions among the different constituents of the intestinal ecosystem (microflora, epithelial cells and immune cells). Through different mechanisms they send signals to activate immune cells. Thus the knowledge of the normal intestinal microflora, the contribution of LAB and their role in the numerous functions in the digestive tract as well as the functioning of the mucosal immune system form the basis for the study and selection of a probiotic strain with immunostimulatory properties. In the selection of LAB by their immunostimulatory capacity it helps to know not only the effect which they have on the mucosal immune system, but the specific use to which these oral vaccine vectors are being put. Although there are reports of the protection of animals and humans against diseases such as microbial infections and cancer, more work remains to be done on the factors affecting the design of oral vaccine vectors and the use of LAB for therapeutic purposes. The basic knowledge of LAB immunostimulation and the criteria for selection of LAB by their immunostimulatory capacity, will be extensively discussed and appraised in this review.

Introduction

All warm-blooded vertebrates live in symbiotic association with a complex population of microorganisms which inhabits their gastrointestinal tract. One of the benefits which the host animal derives from this relationship is an enhanced resistance to infectious diseases (Fuller, 1992, 1997). Thus conventional animals with a complete gut microflora are more resistant to infection than are germfree animals. The detailed basis for this difference is not known but it seems certain that changes in immunity are likely to be involved. The gut microflora stimulates mainly a local response at the gut wall. This mucosal immunity is an important element of the animal's immune status because it is responsible for the control of infections as well as inducing tolerance to environmental and dietary antigens.

Under natural conditions the level of immunity is adequate, but under domesticated conditions, stress factors cause deficiences to occur which render the animal vulnerable to infection. Under these circunstances, supplementation with live microorganisms to repair the deficiences in the composition of the gut microflora can stimulate an immune response and restore the animal's resistance to infection. These supplements known as probiotics have been defined as: "live microbial food supplement which benefically affects the host animal by improving its intestinal microbial balance" (Fuller 1989).

This definition includes not only preparations specifically designed to act as probiotics but also the traditional yogurts and other fermented products where the benefits confered on the consumer may be incidental to their primary role as a tasty and nutritious food. By far the most commonly used microorganisms in probiotic products are the lactic acid bacteria (LAB) and it is important to know how these LAB affect the immune status of the consumer.

The probiotic approach is attractive because it is a reconstitution of the natural condition; it is a means of repairing a deficiency rather than the addition of foreign chemicals to the body which may have toxic consequences or, as in the case of antibiotics induce resístance and compromise subsequent therapy.

The discovery that probiotics can stimulate an immune response (Fuller and Perdigón, 2000) provides a scientific basis for some of the observed probiotic effects. This is an important function of probiotic preparations and a rapidly developing area of research. The scientific results and their practical implications will be reviewed and discussed in the remainer of this review.

The Role of Mucosal Immunity

The intestine is the largest immunological organ in the body. It contains 70-80% of all the IgA producing cells which exceeds the total production of all other immunoglobulin classes in the body.

The most important factor for local immunity is the cellular migration of specific activated B and T cells from the Peyer's patches (PP) which are the inductive sites to the distant mucosal sites such as respiratory, genitourinary tract and various secretory glands. They also return to the intestinal lamina propria and epithelium (Phillips-Quagliata

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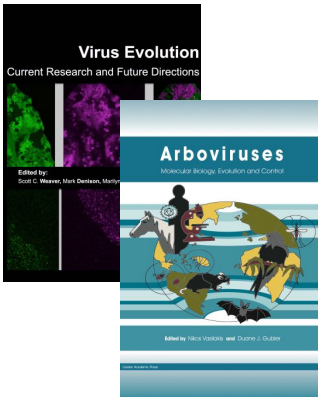

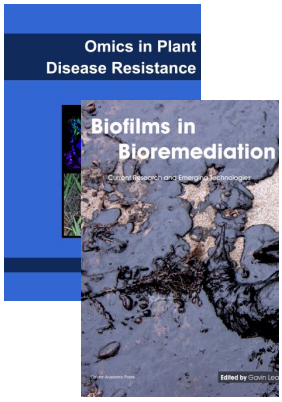

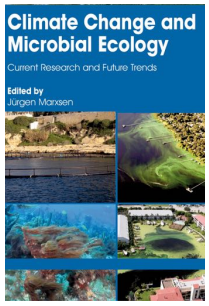

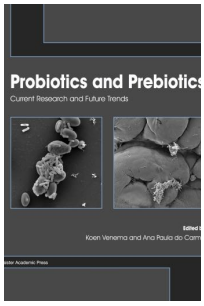

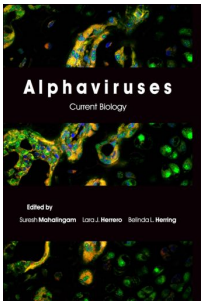

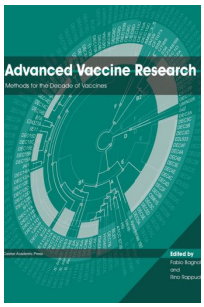

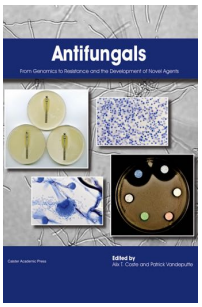

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and Lamm, 1988, Weisz-Carrington *et al.,* 1979). The specific migration of these immune cells is the basis of the Common Mucosal Immune System (Scicchitano *et al.,* 1988, Mestecky *et al.,* 1994).

The structure and function of the intestinal mucosa are designed to supply different biologically active molecules such as gastrointestinal peptides, enteroglucan, trefoil peptides, hormones, prostaglandins, growth factors (Levis *et al.,* 1992, Wright, 1995, Jankowsky *et al.,* 1994), and mucus produced for the goblet cells.

External secretions such as tears, saliva, milk, intestinal, genital and bronchial fluids have non-specific antiviral, antibacterial and antiparasitic activity due to inorganic and organic acid, lysozyme, lactoferrin, peroxidases and interferons (Mc Ghee and Kiyono 1992, Brandtzaeg, 1989, Kilian *et al.,* 1988, Mestecky *et al.,* 1988, Strobel, 1995). Intestinal peristalsis is another important mucosal mechanism of defense for the host preventing the colonization of the gut by foreign microorganisms ingested with the food. The intestinal epithelium is also a barrier for the antigens present in the daily diet. This barrier is maintained by the tight junction at the apical site of the epithelial cell, by phospolipids and proteins that cover the microvilli of these cells.The lamina propia of the intestine is rich in immune cells such as lymphocytes, plasma cells, neutrophils, eosinophils, macrophages and mast cells. The lymphocytes are associated not only with the lamina propria but also with the epithelium: intraepithelial lymphocytes (IEL). They are active in maintaining the hyporesponsiveness at the intestinal level.

Lymphoid nodes are present in the small and large intestine. In the small intestine they constitute the Peyer's patches where microvilli are absent and mucus production is reduced.

When an antigen is orally administered the main immune response induced is in the form of hyporesponsiveness. It is known as oral tolerance and avoids an increase in the inflammatory immune response that can lead to an enhancement in intestinal permeability.

In general, soluble antigens give a strong oral tolerance. Particulate antigens, especially bacteria or virus, favour the induction of the immune response. The hyporesponsiveness is related to the dose of the antigen administered. Lower or higher doses of antigens can induce oral tolerance through supression of cytokines such as interleukin 10 (IL10) or transforming growth factor β (TGFβ) or by clonal deletion (Elson and Zivny, 1996)). However, oral tolerance can be abrogated and an immune response induced.

The entry of the antigens by the oral route is essential to induce a mucosal immune response. This fact was determined in germfree mice receiving a diet free of antigen where it was demonstrated that the presence of a microflora increased the number of IgA secreting cells in the lamina propria of the intestine, mesenteric node or in the bone marrow (Hoojkaas *et al.,* 1984, Bos *et al.,* 1987). In similar germfree studies with antigen free diet the levels of the IgM, IgG and IgA in the serum were also diminished (Wostmann and Pleasants, 1991). They concluded that the level of IgA is mainly dependent on the presence of microflora, whereas for IgG the diet is the more important factor. It would seem that the IgM is not influenced by environmental antigens.

The gut associated lymphoid tissue (GALT) is characterized by the development of a systemic hyporesponsiveness or oral tolerance. This suppressor immune response avoids immunological reactions induced by chronic stimulation by the microbial and other antigens contained in the diet. Oral tolerance is mainly induced by antigens of T dependent cells; the presence of $CD8^+$ T cells is required in this process (Challacombe and Tomasi, 1980, Mowatt, 1987, Mattingly and Waksman, 1978). The lipopolysacharide (LPS) originating in the normal enteric microflora is involved in the maturation of the T cell precursors of the T suppressor cells responsible for oral tolerance at the gut level. This was demonstrated in germfree mice that are unable to induce oral tolerance, but this can be reconstituted by the intestinal colonization with the enteric microflora or by LPS ingestion (Kiyono *et al.,* 1982, Michalek *et al.,* 1982, Michalek *et al.,* 1983). It is also known that the maturation of lymphocytic function is controlled by the normal microflora and by the non-colonizing, non-pathogenic exogenous bacteria that pass through the gastrointestinal tract. The maintainence of a constant number of IgA secreting cells in the intestine, in normal conditions, has been atributed to the Gram negative microflora specially *Bacteroides* spp. (Porter and Allen, 1989) It was also demonstrated that the muramyl dipeptide (MDP) of the Gram positive cell wall activates immune cells such as macrophages, and B and T lymphocytes (Lise and Audibert, 1989) asssociated with the intestinal mucosa.

Induction of Mucosal Immunity

To achieve an effective oral immune response the participation of almost all of the immune cells associated with the gut is necessary. Macrophages, regulatory T cells and effector B and T lymphocytes induce the protective IgA associated with the mucosal surfaces. This process can be divided into inductive sites where antigen is encountered and initial responses are induced, and effector sites where IgA plasma cells are found and where the production of s-IgA antibodies results in local immune protection. Although physically separated they are functionally interconnected (Mestecky and Mc Ghee, 1992, Czerkinsky *et al.,* 1993).

The IgA inductive sites have been extensively studied in GALT, which is represented by the Peyer's patches (PP), the appendix and the small lymphoid nodules in the large intestine. The bronchus associated lymphoid tissue (BALT) (Bienestock and Clancy, 1994) shares many similarities with GALT and it is also probable that lymphoid nodules are present in the urogenital tract.

The PP contains a "dome" region enriched by lymphocytes, macrophages and some plasma cells. This area is covered by a unique epithelium that contains specialized cells termed microfold (M) cells, which have short microvilli, small amounts of cytoplasm and few lysosomes. Its function is the uptake and transport of lumenal antigens and small parasites. The antigen uptake by M cells does not result in degradation, but delivers intact antigens into the underlying lymphoid tissue (Mc Ghee and Kiyono, 1992, Neutra and Krahenbuhl, 1996).

Peyer's patches are considered germinal centres

where B cells change IgM to IgA and affinity maturation occurs. In this center the majority of the cells are B cells producing IgA. The switch of IgM to IgA is induced by T helper ($CD4^+$) cells of type Th2. The 60% of T lymphocytes present in PP are $CD3^+$, $CD4^+$, $CD8^-$ with properties of T helper and the T cell receptor (TCR) in α/β form, the type $CD8^+$ T cells are also present in PP. The difference between Th1 and Th2 populations with the same $CD4^+$ phenotype is in the cytokines produced. Th1 cells release Interleukin (IL) 2, IL3 and interferon γ (IFNγ). The Th2 cells produce IL4, IL5, IL6, IL10 (Mosmann and Coffman, 1987). The accesory cells such as dendritic cells and macrophages present in the IgA inductive sites are the antigen presenting cells (APC) and they are engaged in regulation of humoral and cellular immune responses for mucosal protection.

Following antigen stimulation in PP and its presentation to B and T cells, the antigen induced B and T cells ($CD4^+$ and $CD8^+$) are able to migrate *via* efferent lymphatics and through the mesenteric node; they reach the systemic circulation through the thoracic duct and repopulate not only the lamina propria of the intestine but other distant mucosal sites such as respiratory, urogenital, mammary and salivary glands. The result of this process is that by oral stimulation, distant mucosal sites can be repopulated with IgA producing cells to protect these surfaces. This phenomenon has been termed the Common Mucosal System (Cebra *et al.,* 1991). However, in spite of the scientific evidence of the ability of the common mucosal system to induce a good local mucosal response, local stimulation is also required.

In the process of antigen uptake by M cells the antigen must associate with the M cells in the apical membrane of PP which have abundant glucoconjugates. These lectin binding sites coat the antigens. Most of the bacteria have adhesin on their surface that permits the adhesion to the M cells and their subsequent contact with the immune cells of the PP (Bye *et al.,* 1984, Clark *et al.,* 1993, Falk *et al.,* 1994) stimulating a mucosal immune response.

Although in the past it has been suggested that PP is the only site of mucosal immune response induction, recent studies have demonstrated that the epithelial intestinal cells are another important component of the mucosal immune system. These cells were extensively studied for their essential role in the secretory and absorptive processes (Kagnoff, 1996). However, many studies showed that the intestinal epithelial cells can be regarded as non-professional antigen presenting cells. Epithelial cells express histocompatibility antigen (HLA) class I and II molecules (Mayer *et al.,* 1991, Blumberg *et al.,* 1991) and they can release interleukins such as IL6 and IL8 (Mulder *et al.,* 1990, Reinecker and Pololsky, 1995). The epithelial cells communicate with other mucosal cells *via* a spectrum of mediators that act on the intestinal epithelial cells as well as on the intraepithelial lymphocytes, lymphoid cells, mononuclear phagocytes, neutrophils, mast cells and eosinophils present in the lamina propria. The cascade of mediators is regulated both to induce or to down-regulate appropiate host immune and inflammatory responses at mucosal surfaces.

If the antigen interacts with the epithelial cells, it can be partially taken up degraded and presented to the immune cells. Another possibility is that undigested particles are eliminated through the intercellular space by the portal circulation and carried to the liver where they are cleared (Walker and Sanderson, 1995).

Whatever the route of the secretory immune response induction, the main immune response is the humoral immune response by IgA^+ cells and secretory IgA production. This immunoglobulin is present in its two subclasses IgA1 and IgA2 and they have an important role in the protection of mucosal surfaces against pathogens. Secretory IgA (s-IgA) is a dimeric molecule bound by the join chain "J" produced by the plasma cells and in the secretions it has a small glycoprotein of 80 Kd called the 'secretory component' which in responsible for resistance to gastric juice, fatty acids and bile acids. This secretory component is synthetized by the epithelial cells and is combined also with IgM antibodies (Brandtzaeg, 1995).

When IgA is secreted from plasma cells in the lamina propria, it can enter enterocytes by receptor-mediated endocytosis and the IgA is transported in vesicles through the cell into the luminal secretions, where it has the oportunity to complex antigens. IgA can also act at the intraepithelial level neutralizing viruses that are infecting the cells (Mazanec *et al.,* 1992, Mazanec *et al.,* 1995) and in the lamina propria it can bind antigens. This complexed antibody is internalized and transported to the lumen in a manner similar to that of free IgA. This is an excretory function of IgA (Lamm *et al.,* 1996). In the mucosal immune response the release of cytokines by the immune cells associated with the mucosal and intestinal epithelial cells play an important role in triggering the immune response or in stimulating the inflammatory immune response. As the profile of cytokines is different in different immune cells, alteration of the ratio of one type such as $CD4^+$or $CD8^+$ in the number of these immune cells present in the lamina propria can lead to an increase in the inflammatory immune response. It has been demonstrated that tumour necrosis factor (TNF), IFNγ and IL4 have a modulatory effect on the secretory component expression (Phillips *et al.,* 1990).

The cytokines released by Th2 cells are involved in the induction of the IgA immune response. The IL6 and IL8 released by the epithelial cells are proinflammatory, so an intense epithelial stimulation can favour an inflammatory immune response.

Although it has been shown that the pattern of cytokines produced by T helper cells associated with the lamina propria is the same as that of those produced in the PP, they are functionality less active (Williams *et al.,* 1997).

IgA synthesis involves a complex network of signals between antigen, immune cells and cytokines. Weiner (1997) proposed three ways of interaction with the intestinal cells to evoke an immune response: 1) through M cell from PP, 2) through the epithelial cell with processing and presentation or not of the antigen 3) interaction with the epithelial cells and elimination of the antigen by portal circulation or by inducing a local immune response activated by the release of cytokines.

To enhance mucosal immunity a number of different compounds have been found to have adjuvant properties when given orally together with antigen. These antigen delivery systems, designed to stimulate the mucosal immune response, have been studied almost exclusively

in experimental animals (Michalek *et al.,* 1994). It has been proposed that such systems include incorporation of antigens into particles (liposomes, proteasomes, immunostimulatory complexes, ISCOM, chemical or biological linkage of antigens to cholera toxin (CT) or fraction β (CTβ), expression of antigens in recombined viruses, (poliovirus, adenovirus and vaccinia) bacteria (*Salmonella*, BCG and lactobacilli) or plants (potatoes, tomatoes, spinach) or mucosal immunization with DNA.

However, the efficiency in humans of the mucosal antigen delivery system is rather limited. Only liposomes, microspheres, recombinant *Salmonella* and adenovirus have been used in human trials (Mestecky *et al.,* 1997). The other mucosal antigen delivery systems mentioned await evaluation in human vaccines.

Liposomes and biodegradable microspheres have been used for systemic and mucosal immunization of animals (Kersten and Crommelin, 1995, Childers *et al.,* 1994, Mestecky *et al.,* 1994, Tacket *et al.,* 1992, Lise and Audibert, 1989). Their use in humans is limited to enteral immunization.

Cholera toxin (CT) is a potent mucosal immunogen and enteric adjuvant and its non-toxic β subunit (CTβ) in pure or recombinant form has been approved for use in humans (Jertborn *et al.,* 1992). It enhances the secretory IgA antibody response to the coupled antigen, as well as circulating IgG antibody.

The generation of the mucosal immune response through the common mucosal immune system as well as their compartments has led to the use of individual vectors by virtue of their ability to colonize or infect selected inductive sites such as the upper respiratory tract mucosa, the gut or the female genital mucosa.

Live vectors by themselves are better antigens than the inserted gene product from other microorganisms. However, they can induce a greater immune response against the vector that may lead to its elimination restricting the use of the live-vector based vaccines.

In vivo assessment of mucosal immune response is limited if the studies can only be performed in animals or in human beings. Evaluation of the effect of the different adjuvants on the local and generalized mucosal immune response could be assessed by many *in vitro* techniques.

Effect of LAB on the Immune Response

Enhancement of Systemic Immune Response

The role played by lactic acid bacteria in various biological functions of the host has been extensively reported. Numerous studies have demonstrated that LAB and milk fermented with LAB had antitumour activity and that they were able to prevent intestinal infection; these observations imply an active participation of the immune system.

Early studies (Bloksma *et al.,* 1979) demonstrated that viable *Lactobacillus plantarum* administered intraperitoneally stimulated the delayed type hypersensitivity (DTH) reaction, whereas non-viable bacteria acted as adjuvants for antibody production. Kato *et al.* (1983) demonstrated that *Lactobacillus casei* inoculated intraperitoneally activated peritoneal macrophages, increasing their phagocytic capacity. They also observed an enhanced activity of the mononuclear phagocytic system measured by an increase in the colloidal carbon clearence index rate. This would mean that *L. casei* has an immunopotentiator effect. Saito *et al.* (1983) demonstrated that subcutaneous administration of *L. casei* induced an increase in the production of circulating antibodies against *Pseudomonas aeruginosa* and sheep red blood cells (SRBC). The intravenous or intraperitoneal inoculation of *L. casei* induced the activation of natural killer cells (NK) which play an important role in preventing tumour development (Kato *et al.,* 1984). The strain of *L. casei* assayed in this study proved to be as effective as other microorganisms used as immunopotentiators. However, unlike *Propionibacterium acnes* (previously *Corynebacterium parvum)* or *Mycobacterium bovis*, *Bacillus Calmette-Guerin* (BCG), it did not produce hepatomegaly or splenomegaly which are very common side effects of these immunomodulators (Yasutake *et al.,* 1984b).

Hashimoto *et al.* (1985) demonstrated by *in vitro* assay that Kupffer cells and the immune cells associated with spleen or lung and peritoneal macrophage were activated by *L. casei* administration. De Simone *et al.* (1986) observed that the yogurt given to humans induced production of the cytokine interferon γ (IFN γ) when their circulating blood lymphocytes were stimulated with concavalin-A. In spite of these beneficial properties described for the LAB, there are other reports indicating that LAB do not always produce beneficial effects on the host. Sharpe *et al.* (1973), observed that *Lactobacillus rhamnosus* was associated with endocarditis or abscesses. Some strains of *Lactobacillus acidophilus* and *L. plantarum* may possess undesirable properties. It was also demonstrated (Iwasaki *et al.,* 1983) that *Lactobacillus arabinosus* administration to mice bearing chemically induced intestinal tumour could enhance the tumour effect of the carcinogen.

Although these previous studies encouraged the use of certain lactobacilli as immunopotentiators for therapeutic purposes, there is still much to be discovered. For example a) it is important to know the type of immune cells that the LAB are able to stimulate, to know if the immune response induced will be beneficial or not for the host (inflammatory or specific immune response) b) which is the most active strain, c) the dose required for maximum effect, d) when it should be administered and e) is it safe to use LAB or fermented milks in an immunosuppressed host?. We must also be reassured that substances or bacteria used as immunomodulators do not have harmful effects on the host, for example can we be certain that side effects will not occur as a result of long term administration.

Since lactic acid bacteria are usually ingested with the daily diet it is also important to know the mechanisms of action of these bacteria not only on the systemic immune system but, on the mucosal immune system. All of these parameters mentioned before must be taken into account for the optimal induction of the mucosal immune response.

In previous studies in our laboratory, using mice as the experimental model, we demonstrated that the oral administration of *L. casei* CRL 431, *L. acidophilus* CRL 924, *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423 and *Streptococcus thermophilus* CRL 412 were able to increase the non-specific immune response measured by

the phagocytic activity of peritoneal macrophages and by the release of lysosomal enzymes such as β glucuronidase and β galactosidase (Perdigón *et al.,* 1986a). We also demonstrated that LAB increased the phagocytic activity of the mononuclear phagocytic system measured by the colloidal carbon clearence test (Perdigón *et al.,* 1986b). The effect observed on the non-specific immune response when the LAB were administered by the oral route was comparable to those obtained when the LAB were injected intraperitoneally.

When we analyzed the influence of the oral administration of the LAB under study on the specific systemic immune response, we found that the LAB stimulation induced an increase in the IgM levels against sheep red blood cells (SRBC) measured by the plaque forming cells test (PFC) (Perdigón *et al.,* 1986c, Perdigón *et al.,* 1987, Perdigón and Alvarez, 1992). We also analyzed the effect of a mixture of *L. acidophilus* and *L. casei* in a fermented milk and conventional yogurt (mixture of *L. debrueckii* ssp. *bulgaricus* and *S. thermophilus*) on the systemic immune response. We found that the mixture of *L. acidophilus* and *L. casei,* microorganisms were better able to survive in the intestinal tract and were more effective than yogurt (Perdigón *et al.,* 1988, Perdigón *et al.,* 1989, Perdigón, Alvarez and Medici, 1992). We also investigated whether or not the long term administration of LAB induced side effects. We observed that after treatment for 7 consecutive days at a dose of 10^9 cell/day/mouse the bacteria did not induce hepato- or splenomegaly.

Antitumour Activity

During the last two decades, the anticarcinogenic properties of LAB and of yogurt have been extensively studied. A great emphasis has been laid on the antitumour property exerted by yogurt. Most of the scientific evidence came from animals models but no evidence has been reported from humans.

Shahani *et al.* (1983) demonstrated in mice fed with fermented colostrum that the growth of an experimental tumour was inhibited. Reddy *et al.*(1983) and Ayebo *et al.* (1982) studied whether the antitumour effect observed was exerted by the presence of LAB in the fermented milks or by components of their cell wall, or by products produced as a consequence of the fermentation process. Ayebo (1981) isolated a dialysable antitumour component from yogurt. Goldin and Gorbach (1980) showed in mice that *L. acidophilus* orally administered induced a decrease in the incidence of the colon cancer caused by 1-2 dimethylhydrazine dihydrochloride (DMH).

Kato *et al.* (1981, 1985) found that the intraperitoneal administration of *L. casei* inhibited tumour growth in syngeneic and allogenic mice, and that the effect was dose dependent. Yasutake *et al.* (1984a) observed that the intratumour administration of *L.casei* produced a total inhibition of the tumour, while simultaneous injection of *L. casei* in different body sites had no effect on tumour growth.

Matsuzaki *et al.* (1985) showed tumour inhibition by intravenous administration of *L. casei* in syngeneic mice and guinea pigs with carcinoma of the lung and liver tumour respectively. Bogdanov *et al.* (1975) and Sekine *et al.* (1985) demonstrated that the antitumour capacity of *L. delbrueckii* subsp. *bulgaricus* or *Bifidobacterium infantis* respectively was related to a fraction of the cell wall (peptidoglycan).

Several papers have reviewed the evidence for the supression of carcinogenesis by LAB (Friend and Shahani, 1982, Fernandes *et al.,* 1987, Hosono, 1988, Gilliland, 1990 a, Adachi, 1992, Ballongue, 1993, Nadathur *et al.,* 1994) based on research concerning antimutagenic and anticarcinogenic activity. There is also evidence in humans that oral supplements of *L. acidophilus* reduce acitivities of faecal bacterial enzymes such as β- glucuronidase, nitroreductase and azoreductase that are involved in procarcinogen activation (Gilliland, 1990 b, Hosoda *et al.,* 1992).

Many researchers have studied the biological basis of the antitumour effect of dietary LAB in various animal models for human cancer. The understanding of the suppression of antitumour activity has led to the conclusion that the LAB could act by modulation of the immune response. However, the mechanisms are not the same for different types of tumour. In addition, therapeutic antitumour effect by LAB differs as a function of dose, time of administration and the route chosen.

The mechanisms that could be involved are: a) LAB induce an increase in the cytotoxic capacity of macrophages or $CD8^+$ T cells, b) LAB are cytotoxic for tumour cells, c) LAB induce a non-specific local inflammatory reaction inducing a host-mediated immunological response against the tumour, d) LAB enhance the cytokines released which are involved with the cellular apoptosis induction, e) LAB give rise to specific immunity to the tumour.

Bifidobacteria are not LAB, but they are frequently included in probiotic preparations and they have shown antitumour activity when administered as preventive or therapeutic agents. These microorganisms are capable of inhibiting tumour growth or causing a complete regression of solid tumours (Kohwi *et al.,* 1978, 1982). In the case of *B. infantis,* active components isolated from the cell wall were characterized (Tsuyuki *et al.,* 1991, Yasui *et al.,* 1995). This cell wall preparation (whole peptidoglican (WPG) not only exhibited a high capacity to suppress the tumour growth, but also reduced the tumour incidence (Hosono *et al.,* 1997). In most of these animal models the antitumour activity of LAB was demonstrated using different routes of administration, but not the oral route. The finding that the oral administration of dairy lactic acid bacteria or fermented milk exerted a therapeutic or antitumour effect would provide a very attractive form of therapy in humans.

As regards the therapeutic aspect, different authors (Reddy *et al.,* 1983, 1973, Friend *et al.,* 1982, Ayebo *et al.,* 1981) have demonstrated in mice that feeding with yogurt or yogurt components for 7 consecutive days after intraperitoneal inoculation of Ehrlich ascitic tumour cells, produced significant antitumour activity. Asano *et al.* (1986) observed tumour regression of a carcinoma of the bladder by the daily administration of a fermented milk containing *L. casei.* An antitumour effect of *L. casei* was also observed on a primary colon tumour by oral administration (Kato *et al.,* 1994). In all of these studies viable bacteria were more effective than non-viable ones in producing the antitumour effects.

In our laboratory we demonstrated using mice

(Perdigón *et al.,* 1993 b, Perdigón *et al.,* 1994, Perdigón *et al.,* 1998, Valdez *et al.,* 1997, Perdigón and Oliver, 2000), that oral administration of *L. casei* and yogurt were able to inhibit the tumour growth of a chemically induced fibrosarcoma or a carcinoma respectively.

In the inhibition of fibrosarcoma (non-intestinal tumour) by *L. casei,* we observed that the size of the dose was important, and that *L. casei* was more effective when it was administered as a preventive rather than for therapeutic purposes.

We studied the possible mechanisms involved in this antitumour effect by measuring peritoneal macrophage activity, as well as cytotoxic capacity (Perdigón *et al.,* 1995c). We observed an increase in the macrophage activity and also an enhancement of the cytolitic activity of the serum on tumour cells in the animals treated with *L. casei.*

However, we could not demonstrate the exact mechanisms by which *L. casei* inhibited tumour growth in sites remote from the immunomodulator penetration route. Perhpas this effect was mediated by cytokines released as a consequence of the oral activation of the immune cells associated with the gut.

We also studied (Perdigón *et al.,* 1998) in mice the effect of yogurt on the inhibition of colon tumours induced by DMH. We determined the importance of the dose needed to induce the antitumour effect of yogurt, and found the effect more evident after 7 or 10 days of feeding. We demonstrated an increase in the IgA^+ B cells and $CD4^+$ T cells present in the large intestine, and a diminution of the $CD8^+$ T lymphocytes and β- glucuronidase enzyme measured in the intestinal fluid. We also determined an increase in the phagocytic capacity of macrophages infiltrating the tumour (Valdez *et al.,* 1997). We suggest that one of the mechanisms by which yogurt exerts tumour inhibition is through its immunomodulatory activity causing a reduction in the inflammatory immune response. We believe that yogurt also induces the release of different cytokines able to send signals to the proliferative cells inducing apoptosis, with an inhibition of tumour growth.

Effect of LAB Against Intestinal Infections

Lactic acid bacteria and bifidobacteria have their probiotic effects by influencing the biochemical, physiological and antimicrobial activities or changing the composition of the autochtonous or allochthonous intestinal microfloras.

The demostration that LAB are able to inhibit, under *in vitro* conditions, the growth of pathogens including *Salmonella enteritidis* serovar Typhimurium (Gilliland and Speck, 1977) stimulated work on the mechanisms involved in this antibacterial effect. A bacteriocin produced by *L. delbrueckii* subsp. *bulgaricus* has been identified (Pulverer *et al.,* 1993, Nord, 1993) as bulgarican which posseses a wide, *in vitro,* antibacterial activity. In addition, live microbial therapy has been shown in some reports to be more effective than antibiotic administration for treating *Salmonella* infections (Hitchins *et al.,* 1985). This effect may be due to the enhancement of a specific immune response. The protection against *Salmonella* can be mediated by macrophages and specific immunity. Yogurt feeding enhances murine defences against serovar Typhimurium (De Simone *et al.,* 1988, De Simone *et al.,* 1986) through several proposed mechanisms such as a) by increasing antibacterial activity of mononuclear cells against serovar Typhimurium, b) by specific IgA antibody production, c) by increasing the number of polymorphonuclear cells, as first line of defence of the host, against *Salmonella* infection, d) by increasing the proliferative response of both T and B cells. One or more of these mechanisms would produce a strong reduction of serovar Typhimurium growth in liver and spleen resulting in improved survival of animals treated with LAB.

LAB contribute to the maintenance of colonisation resistance, mainly against *Listeria monocytogenes, Escherichia coli, Salmonella enteritidis* serovars Typhimurium and Enteritidis (Fernandes *et al.,* 1988, Chateau *et al.,* 1993). There is evidence from experimental and clinical studies which indicate that LAB administration could lead to significant changes in the intestinal microflora (Johansson *et al.,* 1993, Lidbeck and Nord, 1993). Futhermore it is well known that disturbances in the normal intestinal microflora lead to gastrointestinal disorders often resulting in diarrhoea (Johansson *et al.,* 1993). The ability of LAB to affect the systemic and mucosal immune responses suggests that these microorganisms would contribute to the recovery from infections. *Lactobacillus* GG has been shown to promote the recovery of children with rotavirus diarrhoea *via* augmentation of local and systemic immune response (Kaila, M. *et al.,* 1992). The exact mechanisms by which some LAB prevent enteric infections is unknown.

In virus infections of the gastrointestinal tract, there is a strong possibility that probiotics exert immunomodulatory mechanisms inducing a high level of specific secretory IgA which is a major immunological barrier against viruses. Yasui *et al.* (1995) showed protection against rotavirus, using *Bifidobacterium brevis*. Similar mechanisms may be responsible for protection against bacterial infections. Another possibility is that cytokine production may mediate immunostimulation (Solis and Lemmonier, 1993, Kitazawa *et al.,* 1994).

In human studies Link-Amster *et al.* (1994) demonstrated serum specific IgA against an attenuated *S. enteritidis* serovar Typhimurium given to volunters who consumed *L. acidophilus*.

Priming of GALT may be an important part of this protective mechanism. However, the competition for binding sites on epithelial cells resulting in competitive exclusión could be another way in which LAB increase the host's resistance to infection.

The mechanism of the host's response to probiotics is unclear. Much of the work indicates that probiotics exert a "barrier effect" against colonic pathogens, by the induction of specific IgA antibodies.

There are other studies in childhood diarrhoea using lactobacilli especially *L. acidophilus* and *L. casei* (Isolauri *et al.,* 1991, Saxelin *et al.,* 1998) which have been used to reduce *rotavirus*, *Salmonella* and *Shigella* infections. Attempts have been made to use the non- pathogenic forms of certain organisms to induce bacterial interference against the virulent forms. Although the competitive exclusion between non-pathogen and pathogen for the colonisation site could be effective, consumers may be reluctant to ingest this type of preparation.

Protection against some enteropathogens can be obtained by vaccination, but at present oral vaccines using bacteria are not available.

There is now a renewed interest in the use of LAB as food additives to prevent diarrhoea. LAB are considered by the Food Drug Administration (FDA) in USA as GRAS (Generally Regarded As Safe) microorganisms. These bacteria are often used in the prevention of diarrhoea of farm animals specially newborn piglets (Underdahl *et al.,* 1982). It is important to find a treatment that will increase resistance to disease of newborn animals, including the human baby.

Oral stimulation with particulate antigens such as bacteria can induce a mucosal immune response, and LAB could enhance the mucosal immunity in the host.

In an attempt to analyze the protective effect of some LAB against a *Salmonella* infection we performed *in vivo* experiments using mice as the experimental model. LAB were administered prior to, or together with, the pathogen. The protective capacity was determined by culture of the liver and spleen to detect the pathogen, and measuring the levels of anti-enteropathogen s-IgA in the intestinal fluid by an ELISA test. If the LAB were effective, the invasive capacity of the pathogen should be supressed at the intestinal level. Naturally these speculations are not valid in the case of the immunosuppressed host in which the reactive capacity of the system is diminished.

We studied the protective capacity against *Salmonella enteritidis* serovar Typhimurium infection of the following LAB: *L. casei* CRL 431, *L. acidophilus* CRL 924 *L. rhamnosus* CRL 74, *L. delbrueckii* ssp. *bulgaricus* CRL 423, *Lactococcus lactis* CRL 526, *S. thermophilus* CRL 412 We observed that only some doses of *L. casei*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were able to protect against the pathogens (Table 1).

We determined that only *L. casei* induced high levels of specific secretory IgA but the effect was dose dependent with lower doses being more effective than the higher one (Perdigón and Alvarez, 1992, Perdigón *et al.,* 1991, Perdigón *et al.,*1990 a, 1990b). We also observed that the other LAB assayed induced an increase in the number of cells associated with the inflammatory immune response. We studied the increase of this inflammatory immune response by measuring the increase in intestinal permeability, for the antigen ovoalbumin orally administered at tolerogenic dose. We measured the levels of anti-ovoalbumin antibodies present in the serum by the ELISA test. We demonstrated that with LAB at doses that were not able to protect against *Salmonella,* intestinal permeability was increased. We are currently studying the mechanisms by which *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus,* which do not induce a specific s-IgA response, are able to protect against the pathogen.

We selected *L. casei* CRL 431 as the most appropriate strain with which to prevent enteric infection, and we examined the effect of administering it simultaneously with the pathogen, and after challenge, to study the therapeutic effect. In the latter case we determined the effect of repeated stimulation with *L. casei* (Perdigón *et al.,* 1993 a, 1993c). We found that *L. casei* administered simultaneously with the pathogen was ineffective indicating that *L. casei* was not able to inhibit the pathogen by competitive exclusion. We demonstrated that *L. casei* administered during infection with *Salmonella* is beneficial for the host, but the effect was dose dependent. The increase in the synthesis of s-IgA (Alvarez *et al.,* 1998) was significant compared with the control when the time of administration was for periods of no more than 5 or 7 consecutive days. Thus the importance of the oral administration of an appropriate LAB used as probiotic is mainly in the prevention of enteric infection; their use for therapeutic purposes is still limited.

Stimulation of the Secretory Immune System by LAB

Effect of LAB on the Gut Mucosa

LAB are part of the normal microflora and inhabit the intestinal tract of humans, pigs, fowl and rodents. Many factors have been shown to affect the prevalence and distribution of LAB in the gastrointestinal tract. In humans it has been shown that the stomach acidity reduces bacterial colonization, with the microflora mainly restricted to the distal small intestine increasing along its length into the colon where the largest microbial community develops.

The involvement of mucosal lymphoid tissue in host defense mechanisms has been extensively studied. GALT is the pivotal site for the induction of mucosal immune response in the gut, including the generation of oral tolerance.

The IgA antibodies can bind antigen and minimise its entry with a consequent reduction in inflammatory reactions, which avoids a potentially harmful effect on the tissue. The induction of a local gut immune response may affect the secretory and absorptive functions of enterocytes

Table 1. Effect of the different LAB orally administered on the protection against *Salmonella enteritidis*subsp. *typhimurium* infection

Strains	Days of feeding	Log. Nº of bacteria/organ
	2	2.2±0.5
L. rhamnosus	5	3.4±0.7
	7	3.4±0.4
Lac. lactis	2	3.2±0.1
	5	4.7±0.5
	7	3.3±0.2
L.acidophilus	2	3.1±0.8
	5	4.1±0.3
	7	4.9±0.4
L. casei	2	0
	5	4.2±0.5
	7	0
*L. delb.*ssp. *bulgaricus*	2	1.3±0.2
	5	3.1±0.5
	7	0
S. thermophilus	2	1.1±0.7
	5	0
	7	0
L.plantarum	2	2±0.6
	5	1.5±0.5
	7	4.2±0.7
Control		5.4±0.4

Animals were treated with the LAB for 2, 5 or 7 consecutive days, at the end of each administration period they were challenged with 20 LD50 *Salmonella.* On the seventh day post-challenge, animals were killed and the liver and spleen were removed for colonization assays. Results represent the average of *Salmonella* number found in liver and spleen on the 7th day post challenge. Control are animals untreated with the LAB but challenged with *Salmonella.* Values are mean of n=6±SD.

as well as the motility of the gut, because the cytokines produced by the immune cells modulate several functions of the enterocytes both directly and indirectly *via* the recruitment of inflammatory cells. These may induce the release of proinflammatory cytokines such as IL-1 (interleukin-1) and TNFα (tumour necrosis factor α), that increase the secretion of electrolytes and water into the human intestine (Perdue and McKay, 1993). Furthermore TNFα modulates the expression of the secretory component of IgA antibodies thereby regulating the transport of IgA across the epithelium (Kvale *et al.,* 1988). Other proinflammatory cytokines such as IFNγ also induce the expression of Major Histocompatibility Complex (MHC) class II antigens on the enterocyte surface which contribute to the amplification of the local immune response. On the other hand IFNγ (interferon γ) and TNFα can directly mediate the killing of epithelial cells (Deem *et al.,* 1991). Thus the immune reaction can activate enterocytes in terms of cell mitosis and cytokine expression. Furthermore activated T lymphocytes from the lamina propria can enhance the proliferation rate of intestinal epithelial cells (Ferreira *et al.,* 1990). In addition cytokines released during the inflammatory immune response drive mesenchymal cells (fibroblasts, smooth muscle cells and endothelial cells) to produce eicosanids, other cytokines and chemotactic and growth factors (Perdue and McKay, 1993). Another important amplifying mechanism of the intestinal inflammatory response is the induction of gene expression for endothelial and macrophage adhesion molecules which act in synergy with other chemoattractants to increase the infiltration of granulocytes, monocytes and lymphocytes into the inflammatory focus (Gundel and Letts, 1994).

How does the LAB modulate the gut immune response, especially the cytokine release which prevents an increase in the inflammatory immune response? Although it has been suggested that Gram-negative bacteria are the most efficient stimulus for driving the production of macrophage derived cytokines (Nicaise *et al.,* 1993), other reports have demonstrated that Gram-positive bacteria, specifically LAB, can also induce proinflammatory cytokines. For example it has been described that *L. acidophilus* induces the production of IFNα/β by murine peritoneal macrophages (Kitazawa *et al.,* 1992, Kitazawa *et al.,* 1994). *L delbrueckii* ssp. *bulgaricus* and *S thermophilus* have been shown to induce the production of IL-1β, TNFα and IFNγ but not IFNα and IL-2 (interleukin-2) by peripheral mononuclear cells (PBMC) from humans (Pereyra and Lemonnier, 1993) after ingestion of bacteria in yogurt or sterile milk.

The age-related decline in the production of cytokines including IFNs is common and it has been demonstrated that supplementing the diet of ageing mice with live *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* restores completely the levels of IFNγ and IFNα compared with the control animals (Muscettola *et al.,* 1994). There is also evidence that LAB, particulary *S. thermophilus*, can induce production of proinflammatory cytokines such as IL-1 β, IL-6 (interleukin-6) and TNFα (Aattouri and Lemonnier, 1995), and an oral preparation from *L. delbrueckii* ssp. *bulgaricus* stimulated the production of proinflammatory cytokines such us IL-1 and TNFα (Popova *et al.,* 1993). De Simone *et al.* (1993) showed an increase in the NK cell cytotoxic activity by oral administration of *L. delbrueckii* ssp. *bulgaricus* and *S thermophilus*. Similar results were found using *L. acidophilus*, *L. casei* and *L. plantarum* (De Simone *et al.,* 1986). Even if the LAB administration under *in vivo* conditions strongly enhances the production of IFNγ, this could be beneficial to the host, because that IFNγ production is involved in mediating the increased resistance towards pathogens (De Simone *et al.,* 1988), as well as in the enhancement of the expression of the secretory component, contributing to an increase in the external transport of dimeric IgA. On the basis of previous results, LAB could modulate several functions of GALT. Available data also indicate that LAB such as *L. acidophilus* which can persist in the gastrointestinal tract, may act as adjuvants to the immune response (Link-Amster *et al.,* 1994, Schiffrin *et al.,* 1995).

The capacity of LAB to induce production of cytokines, means that they probably also affect other immune functions of the cells such as macrophages and granulocytes associated with mucosal sites. The possibility that LAB modulate the expression of cell-surface molecules that are involved in bacterial uptake by leucocytes should be investigated.

There are studies that show the importance of LAB administration in the preservation of intestinal integrity and stabilization of the gut mucosal barrier (Salminen *et al.,* 1988, Salminen *et al.,* 1996a). The intact intestinal epithelium with the normal intestinal microflora represents a barrier to antigens and other noxious substances from the gut lumen. In health this barrier is stable protecting the host and providing normal intestinal function. When either the normal microflora or the epithelial cells are disturbed by triggers such as dietary antigens, pathogens, chemicals or radiation, defects in the barrier mechanisms are induced with alteration in permeability, and mucosal inflammation (Isolauri, 1995).

Even under physiological conditions, a quantitatively unimportant but immunologically important fraction of antigens bypasses the defense barrier. They are absorbed across the epithelial layer by tran-scytosis along two functional pathways. The main one is the degradative pathway which reduces immunogenicity, and diminishes the antigen load by more than 90%. A minor pathway allows the transport of antigens inducing an immune response. The integrity of the defense barrier is necessary to prevent inappropriate and uncontrolled antigen transport.

As a result of local intestinal inflammation, a greater amount of antigens may traverse the mucosal barrier and the routes of transport will be altered; this immunogenic stimulus favours allergic reactions (Fargeas *et al.,* 1995). Foreign antigens such as viruses, dietary antigens or pathogenic or non-pathogenic bacteria (LAB) can induce local inflammation in the intestinal mucosa. As a consequence of this inflammatory response the intestinal functions are modified and an increase in IgG^+ immune cells are observed, and translocation of the normal microflora may occur. Thus for successful use of LAB to improve secretory immune system functions, all of these previous considerations must be taken into account especially if, as is currently believed, the viability of the LAB strain is critical in determining the capacity of LAB to induce immune stimulation. LAB as probiotics have a great potential in the prevention and treatment of some clinical

disturbances (Salminen and Deighton, 1992), however, each strain must be carefully selected and controlled studies in animals and humans must be performed to detected possible intestinal side effects.

In our laboratory, we use a mouse model and LAB which are frequently used in the food industry. We studied the effect of the oral administration of these LAB on: 1) side effects such as translocation of normal microflora to the liver and spleen, 2) which immune cells associated with the gut are activated, 3) effects on inflammatory immune response (Vintiñi *et al.,* 2000), 4) conditions required for use as an effective mucosal adjuvant (Alvarez *et al.,* 1998), 5) effect of oral administration of LAB on distant mucosal sites such as the bronchus (Perdigón *et al.,* 1999a), 6) determination of anti-LAB antibodies, 7) develop hypotheses on the possible mechanisms by which LAB could interact at the intestinal level and induce a secretory immune response. We found that only high doses of LAB can induce translocation of normal microflora to the liver and spleen.(Perdigón *et al.,* 1999 b, Perdigón *et al.,* 2000).

Study of the Immune Cells Involved in the Nonspecific Mucosal Immune Response

These tests were performed on histological slices from the small intestine. The immune cells involved in the inflammatory immune response (e.g. macrophages, neutrophils and eosinophils) were studied for different LAB over different periods of administration (2, 5 or 7 days). Only some of the LAB such as *L. rhamnosus* and *Lactococcus lactis* for 5 and 7 days increased the number of macrophages, neutrophils and eosinophils and the inflammatory immune response. This effect was also observed in histological slices from the small intestine stained with haematoxilin-eosin where oedema within the villi was observed. We also determined the inflammatory immune response by measuring of IgG secreting cells and $CD8^+$ T cells present on the lamina propria of the small intestine as a marker of inflammation.

The number of IgG^+ B cells and $CD8^+$ T cells was assessed by direct immunofluorescence using monospecific and monoclonal antibodies respectively. With the exception of *L. plantarum* fed for 2 days, none of the other LAB assayed increased the number of IgG^+ B cells or $CD8^+$ T cells. These results are shown in Table 2.

Effect of LAB on the Specific Mucosal Immune Response

In this study we analyzed the number of IgA^+, IgM^+ B cells and $CD4^+$ T cells associated with the lamina propria of the small intestine. The immune cells were determined on histological slices by direct immunofluorescence. The number of IgM^+ cells was studied to determine if LAB interaction induced modification in the number of IgM^+ cells by induction of a switch from IgM^+ to IgA^+ as a result of the cytokines released by $CD4^+$ activated T cells.

The number of IgM^+ cells was increased by 2 days of feeding *Lactococcus lactis*, 2 and 5 days of *L. acidophilus,* and 2 and 7 days of *L. plantarum*. This could mean that no switch was induced and that LAB stimulation only evoked

Table 2. Effect of oral administration of LAB on IgG^+ B cells and $CD8^+$ T cells associated with the lamina propria of the small intestine

Strains	Days of feeding	IgG^+ B cells (Number/10 fields)	$CD8^+$ T cells (Number/10 fields)
	2	11±1	18±4
L. rhamnosus	5	22±1	12±4
	7	10±1	17±5
	2	48±3	64±6
L. acidophilus	5	33±2	40±6
	7	25±1	39±5
	2	31±1	50±5
L. casei	5	39±3	55±6
	7	32±1	47±5
	2	30±3	35±7
L.delb.ssp.bulgar.	5	23±3	41±6
	7	26±1	50±7
	2	33±3	49±5
S. thermophilus	5	20±3	31±5
	7	19±2	38±7
	2	52±5	41±6
Lac. lactis	5	36±3	54±5
	7	18±1	38±5
	2	71^{*}±1	107^{**}±8
L. plantarum	5	23±2	38±7
	7	12±2	33±6
Normal control		45±1	58±5

Histological slices from the small intestine were prepared at the end of each period of administration. The IgG^+ B cells and $CD8^+$ T cells were determined by direct immuno-fluorescence using monospecific γ chain and monoclonal anti-CD8 antibodies labeled with fluorescein, respectively. Values are mean of $n = 5 \pm SD$. ** $P < 0.01$ * $P < 0.05$. Controls are mice without LAB administration.

the clonal expansion of those cells present on the lamina propria. We observed that all the LAB assayed were able to increase the number of IgA^+ cells favouring a good intestinal mucosal immune response. The effect was dose dependent. With the exception of *L. casei* the increase of IgA^+ cells was not correlated with an increase in CD4+ T cells.

L. casei was the best inducer of a specific gut immune response accompanied by an increase in IgA^+ and $CD4^+$ T cells. Therefore, we attempted to answer the following questions: a) how long does the immune response last and, b) when a booster is given to maintain an optimal mucosal state is it able to prevent enteric infection?

We demonstrated that: a) the importance of the size of the dose of *L. casei* that was administered for 2 days to obtain an effective protection against *Salmonella* infection, b) that the number of IgA^+ cells must be slightly increased to avoid harmful effects, as was shown in coeliac disease (Brandtzaeg *et al.,* 1993), c) to have a good mucosal immune response the rate between $CD4^+/CD8^+$ should be maintained at 50 to 50 similar to the control values, d) boosting with a single dose (10^9 cells) each 15^{th} day is necessary to protect against *Salmonella* infection (Perdigón *et al.,* 1995 a, Alvarez *et al.,* 1998).

Effect of LAB on IgA^+ Cells Associated With the Bronchus

We determined whether the oral administration of LAB induced an increase in the IgA^+ B cells, in distant sites such as the bronchus. We determined the number of IgA^+ cells present in the bronchus for each LAB and after 2, 5 or 7 days of feeding was determined. All of the LAB with the exception of *L. acidophilus,* increased the number of IgA^+ cells in the bronchus (Perdigón *et al.,* 1999a). The effect was dose dependent. The oral administration of LAB increased the number of IgA^+ cells entering into the IgA cycle repopulating other mucosal sites such as the bronchus. This observation is important, because the most frequent portal of entry for pathogens is the respiratory tract; oral ingestion of LAB could protect the respiratory mucosa.

Study of the Anti-LAB Immune Response

This study was performed to determine if the LAB that interact at different levels of the intestinal tract (Peyer's patches or epithelial cells) were processed and presented as antigen, inducing specific anti-LAB antibodies. These antibodies were determined in the intestinal fluids by an ELISA test. We observed that *L. casei, L. rhamnosus, S. thermophilus* and *L. plantarum* induced specific IgAantibodies against their epitopes, meaning that those microorganisms were processed and presented as antigen by the immune cells associated with the gut mucosa. However, we do not know the exact role played by these anti-LAB antibodies, neither why not all the LAB assayed were not processed as antigen. We think that the pathway of internalization of LAB to interact with the immune cells associated with the intestine is important in understanding these results.

The Possible Mechanisms of Interaction of the LAB With the Gut.

Taking into account the results obtained on IgA^+ cells present in the small intestine and in the bronchus, and observations concerning $CD4^+$ T cells and anti-LAB antibodies, we suggested the following mechanisms of LAB interaction with the small intestine (Perdigón *et al.,* 1999b).

a) *L. casei* and *L. plantarum* would interact at the Peyer's patches level because these microorganisms induce cell migration and increase the IgA^+ cell cycle. As $CD4^+$ T cells are enhanced in the lamina propria of the intestine, this would mean that $CD4^+$ T cells enter into the IgA cycle and repopulate the lamina propria. The interaction in Peyer's patches is the only way to induce the migration of T cells. These LAB were processed as antigen because anti-LAB antibodies were detected.

b) *L. rhamnosus, S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis* would interact with the epithelial cells of the small intestine or with the epithelial cells associated with follicle FAE in Peyer's patches. This possibility is supported by the fact that these LAB increased the number of IgA^+ cells at intestinal and bronchus level but not CD4+ T cells. The IgA cycle can also be increased by stimulation of the IgA^+ cells from the mesenteric node (Weiner, 1997). The interaction with epithelial cells can induce activation of T cells by cytokines released from them, inducing production of other cytokines from T cells associated with the lamina propria. At the epithelial cell level, the LAB may or may not be processed as antigen.

c) *L. acidophilus* would induce only a local gut immune response because the only parameter increased was intestinal IgA^+ cells. Because of the ecological niche which it occupies, *L. acidophilus* would also be expected to interact with the epithelial cells of the large intestine.

We demonstrated that the LAB can interact at different levels in the small intestine (Peyer's patches, FAE or with the epithelial cells), or with the large intestine (Perdigón *et al.,* 2000). The different ways of interaction might explain why the mucosal immunostimulation by LAB is not the same for all of them. In some cases they induce an inflammatory immune response and in others a specific mucosal immune response. The immune stimulation by LAB cannot be generalized for genera or species; this property may be strain specific.

Even if our hypothesis of interaction of the LAB with the gut is confirmed, other important questions should be answered:

a) Why, if all the LAB have the same muramyldipeptide antigen, mucosal stimulation is not the same with all of them?
b) What are the conditions required by LAB to bind to M cells of Peyer's patches and to induce a complete immune response?
c) Is the production of specific anti-LAB IgA an advantage or not?
d) Is the site interaction of the LAB with the intestine restricted to the ecological niche?
e) Can the selection of a strain of LAB with immunopotentiation capacity, to be used as a probiotic, be compromised by adverse side effects?
f) Can a strain of LAB with immunological activity, be used as a mucosal adjuvant or as a vehicle for oral

vaccines? This last aspect will be discussed in the following section.

LAB as Vaccine Vectors

The existence of a common mucosal immune system can be exploited to design vaccines capable of protecting mucosal surfaces that are less accessible to mucosal immunization. Accoding to Jennings *et al*. (1998), an ideal mucosal vaccine should: a) promote an effective contact of the antigen with the immune system; b) stimulate specific humoral and cell-mediated immunity responses; c) elicit a long-lasting protection after a single-dose in early infancy; and d) be stable and non-toxic.

Recent work has highlighted the potential of lactic acid bacteria (LAB) as antigen presenting vehicles suitable for mucosal administration (Mercenier, 1999; Wells *et al.,* 1995, 1996). They have no recorded toxic or pathogenic activity (i.e.: no harmful effect was observed after subcutaneous or oral administration of mice with up to 10^9 live cells of *Lactococcus lactis*. The studies for the safety assessment of LAB have been summarised by Salminen *et al*. (1996b). On the other hand, some species of lactobacilli are maintained transiently or are able to colonise the gut mucosa, and certain strains have intrinsic adjunvant activity which may promote the immunogenicity of heterologous antigens (Perdigón *et al.,* 1995 b; Pouwels, 1996). The dairy industry has a long experience with these cultures, and their preparation on a large scale for a live LAB vaccine would be cost-effective (feasible and a cheap alternative to other delivery systems).

Several genera of LAB are being tested as vaccine delivery vehicles, but here attention will only be given to the work with *Lactococcus lactis* and certain species of lactobacilli. To develop LAB as live vaccines, they have to be genetically transformed. Protocols for the transformation of LAB have been optimised and last-generation food-grade vectors for expression, secretion, and surface- anchoring of antigens are available (Klaenhammer, 1995, Kleerebezem *et al.,* 1997, Kok, 1996). Limited progress has been made within the genus *Lactobacillus* since some species are refractory to transformation and often show a strong strain-specificity in gene expression. Production of recombinant proteins (antigen) in this genus can be achieved in three different ways: intracellulary, extracellulary and surface-bonded. Selecting one of these alternatives is of paramount importance because the size, nature, molecular weight and organisation of the antigen may affect humoral and cellular immune responses. Several studies showed that recombinant *Lactococcus lactis* strains are suitable for oral administration to stimulate responses at mucosal surfaces: a protective humoral response was elicited against a bacterial antigen (tetanus toxin) after nasal or oral immunisation of mice (Wells *et al.,* 1993).

Highly efficient expression vectors have been developed which successfully expressed and secreted heterologous fusion proteins in *Lactococcus*. In these vectors, expression of heterologous proteins is driven by either a strong constitutive promoter (i.e.:lactococcal phage P1 promoter) or an inducible system (*Escherichia coli* bacteriophage T7 RNA polymerase or nisin expression system), while secretion is driven by signal sequences known to be functional in several LAB (i.e.: signal sequences from PrtP, a cell-envelope associated proteinase found in *Lactococcus lactis* SK11 and *L. paracasei* subsp. *paracasei*, and from usp45, a secreted protein found in *Lactococcus lactis*). Tetanus toxin fragment C (TTFC), the B subunit of cholera toxin (CT), or protective epitopes (i.e.: the gp41E) can be expressed and presented to the immune system in an immunogenic form (Agren *et al.,* 1999) In *Lactococcus lactis*, up to 22% of soluble TTFC was expressed intracellularly and about 2.9 mg of TTFC was secreted (Wells *et al.,* 1993).

Live lactococci have been developed as mucosal vaccine delivery vectors for recombinant proteins associated with microbial virulence. It has been shown that *Lactococcus* vaccines elicit protective antibody and cell mediated immune responses in the host after either parenteral or mucosal immunization. Intranasal (i.n.) or oral administration of recombinant *Lactococcus lactis* expressing TTFC to C57 BL/6 mice elicited mucosal s-IgA and serum IgG responses (primarily of the IgG1 and IgG2a subclasses), which suggested involvement of both Th1 and Th2 $CD4^+$ T cell activity. In addition, secretory antibody responses in the lung and nasal tissues were elicited after intra-nasal inoculation in the presence of the adjuvant (Norton *et al.,* 1997). Further, the vaccine elicited protective immunity against lethal challenge of mice with tetanus toxin. Both killed and live recombinant strains induced similar immune responses and no requirement for either colonization or invasion of the mucosa was observed (Robinson *et al.,* 1997; Wells, 1996).

Recombinant lactococci can also deliver cytokines to the immune system. Secretion of recombinant murine interleukin-2 (mIL2) or mIL6 (shown to be the most effective terminal differentiation factor for IgA-committed B cells to become IgA-producing cells, in both human and murine systems) were achieved in *Lactococcus lactis* using the secretion signal leader of the lactococcal usp45. The rIL-2 showed the same specific biological activity as mIL-2 (Steidler *et al.,* 1998a). An enhanced immune response against TTFC was observed in mice immunised with live recombinant *Lactococcus lactis* strains which expressed both interleukins and TTFC.

Progress in the use of *Lactobacillus* strains as live vaccines is limited (Pouwels *et al.,* 1996). Several strains have been evaluated for their ability to produce and secrete the B subunit of cholera toxin (CTB), alpha-amylase, or an epitope from human immunodeficiency virus (gp41 protein) under the control of a set of expression or expression/ secretion signals from various lactic acid bacteria (Hols *et al.,* 1997, Piard *et al*, 1997). The capacity to secrete heterologous protein varied between different species, the highest level being detected in *L. plantarum* NCIMB 8826 (levels as high as 10 mg l-1 of the M6-gp41 fusion protein were secreted; Hols *et al.,* 1997). Secretion of CTB molecules by this strain was also efficient, but no folding of the B toxin subunits in pentamers, and therefore GM1 ganglioside binding activity, was found.

The development of new expression systems designed for cell surface display of chimeric antigens on LAB, using signals of the lactococcal usp45 secretion peptide and of the cell wall anchoring of protein A from *Staphylococcus aureus* or protein M6 from *Streptococcus pyogenes*, have

been recently described. Streptavidin monomers fused to protein A (Steidler *et al.,* 1998b) and several M6-fusion proteins (Hols *et al.,* 1997) have been successfully expressed, secreted and anchored to the cell wall of several LAB. The M6-fusion proteins were successfully secreted into the growth medium, at levels of 5 mg/l, by recombinant *Lactococcus lactis* cells containing a low copy plasmid (about 10^5 molecules per cell), even though most of M6-fusion recursors accumulated in the cytoplasm. The highest amount of a heterologous protein secreted by LAB has been reported by Savijoki *et al.* (1997); 80 mg/ml of a reporter gene (ß-lactamase) were secreted into the growth medium by S-layer signals from *L. brevis*, both in *Lactococcus lactis* and *Lactobacillus brevis*. Application of this efficient system in antigen presentation shows considerable potential for enhancement of immune responses.

Finally, protein A is also well known for its strong binding to IgG subclasses. Recently, it has been shown that the enzymatically active toxin A1 subunit fused to two recombinant Ig-binding domains of staphylococcal protein A was primarily targeted to B cells with comparable adjuvant ability to that of native cholera toxin (Agren *et al.,* 1999). Furthermore, it was not toxic. The potential use of this system to target antigen delivery by LAB vaccines to B cells deserves special consideration.

Conclusions

In the selection of immunomodulating strains of LAB capacity, it is important to know if they induce good mucosal immunostimulation without inducing side effects such as bacterial translocation or a strong inflammatory immune response which can alter intestinal permeability. It is also necessary to control the levels of IgA; these can be influenced by the dose administered. Depending on the LAB interaction with the intestine (Peyer's patches, FAE or epithelial cells) the immune response obtained will be different at different mucosal sites. In spite of the multiple screening needed to check immunopotentiator activity of LAB, we only can predict their behaviour in the complex interactions within the intestinal ecosystem and their influence on the immune cells. Thus if some LAB induce IgA^+ B cell and $CD4^+$ T cell migration we can predict that this LAB would have local and systemic effects, but if the LAB do not induce an increase in the IgA migration their action would be only at the gut level and could be used to increase intestinal mucosal immunity.

The development of effective mucosal vaccines relies almost entirely on our understanding of the mucosal immune system. However, much remains to be learnt about the cellular and molecular mechanisms involved in the control of the mucosal immune system: i.e., antigen presentation, IgA B-cell differentiation, T-cell regulation, and development of long term immunological memory. Although there are many reports characterising LAB as delivery systems for oral antigen administration, the development of recombinant lactic acid bacteria vaccines is still in its early stages. Advances are expected in the construction of improved delivery systems for oral administration of antigens that are immunogenic and targeted to specific areas or cells in the gut. The nature of the vehicle delivery system plays an important role in the type of cells induced with subsequent mucosal or systemic antibody responses. Therefore, more appropriate basic knowledge has to be acquired about the type of antibody responses (e.g., Th1 or Th2), the nature and roles of cytokines, or phagocytic cellular functions elicited by recombinant lactic acid bacteria. This information can provide the scientific foundation that will be useful in designing rational guidelines for development of efficacious LAB live vaccines. Finally, before these LAB vaccines are used for humans, the pharmacokinetic properties and rigorous clinical trials are necessary to assess their efficacy and safety.

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