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Evaluation of the Intestinal Colonizing Potential and Immunomodulating Capacity of *Lactobacilli* Microspheres

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ABSTRACT

Lactobacilli species get degraded by acidic conditions in the stomach. Thus, the objective of this study was to (1) formulate and characterize gastro-resistant *Lactobacilli* microspheres and (2) evaluate the ability of *Lactobacilli* microspheres to colonize the intestine and their capacity to have an immunomodulating effect *in vivo*. The product yield and the encapsulation efficiency were 45% and 100%, respectively. The average microsphere particle size was 5 μm. *Lactobacilli* microspheres were most stable at 4°C and showed a better suspendibility in distilled water. Without encapsulation, the viability of bacteria decreased within 30 min. In the case of *Lactobacilli* microspheres, no *Lactobacilli* were released in the first 3 h, and highest release was observed at 4 h, thus, suggesting the significance of encapsulation of *Lactobacilli*. *Lactobacilli* microspheres maintained intestinal colonization only during the dosing period, and the serum IgG, serum IgA, fecal, intestinal, nasal IgA, and the serum interleukin-1β levels were higher in the *Lactobacilli* microsphere group compared with the blank microsphere and the *lactobacilli* solution group, suggesting that the *Lactobacilli* microspheres were more gastro-resistant and, hence, showed positive effects compared with the *Lactobacilli* solution. However, the *Lactobacilli* microspheres did not have a significant effect on the tumor necrosis factor-α levels.

Introduction

An expert panel commissioned by the Food and Agriculture Organization of the United Nations and the WHO in 2001 defined "probiotics" as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." The probiotic bacteria most commonly studied include members of the genera *Lactobacillus* and *Bifidobacterium*.

Important physiological functions like the functioning of the immune system can be enhanced and optimized by attractive and noninvasive means like dietary intervention.[1]

The optimization of the immune function by dietary supplementation is particularly more important among those groups of individuals who may have an underdeveloped or poorly functioning immune system, such as infants, immunocompromised subjects, and the elderly.[2,3] A sub-optimally functioning immune system can adversely affect health parameters, such as the ability to combat secondary microbial infections and the ability to produce protective responses to novel foreign material (e.g., in tumor control).[4-7] Consumption of fermented foods is necessary for the maintenance of good health.[8,9] Fermented food contains lactic acid bacteria known as probiotics. Probiotics may be an effective means of disease prevention. Probiotics are live micro-organisms that have beneficial effects on the host.[10,11] Both scientific and commercial groups are interested in "probiotic" bacteria. This interest is because of positive health effects of these bacteria. Some of the positive health effects include cholesterol lowering and prevention of cancer recurrence.[9,11-15] The beneficial effects of probiotics continues to grow and includes nutrient processing,[16] regulation of intestinal angiogenesis,[17] development of gut-associated lymphoid tissues (GALT),[18] induction of oral tolerance,[19] and diversification of the preimmune antibody repertoire.[20] It is also becoming clear that the lack of proper interactions between bacteria and the human host may lead to the occurrence of allergies and Crohn's disease in developed countries.[21,22]

The primary effector arm of the immune system is the so-called innate immune system, which includes nonspecific immune protection mediated by monocytes, macrophages, and dendritic cells. The cells of the innate immune system have an important role as

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antigen-presenting cells. The innate immune system further regulates the function of the antigen-specific adaptive immune system, such as the functional balance of immune response related to cytokine and chemokine receptor profiles. Defective maturation of immune competence in association with poor microbial stimuli may thus lead to dysregulation of both innate and adaptive immune systems. Intestinal microbes have been suggested to be important regulators of the function and development of immune and epithelial cells.

Studies have shown that intestinal flora is required for GALT development. It was indicated that follicular development was arrested in rabbit appendices that had been surgically ligated at birth to prevent microbial colonization. When the ligated appendix was reconnected with the intestinal lumen, follicular development was restored.[23] The appendices of germ-free rabbits were shown to be markedly underdeveloped and contained reduced numbers of lymphoblasts and lymphocytes.[18] It was also observed that these rabbits lacked natural antibacterial and hemolytic antibodies and were either unresponsive or poorly responsive to immunization with several antigens.[24] These observations suggest that intestinal microflora is essential for B-cell expansion, GALT development, and generation of a normal antibody repertoire in rabbit. This signifies the importance of intestinal colonization by beneficial bacteria like *Lactobacilli*.

IgA is an antibody playing a critical role in mucosal immunity. More IgA is produced than all other types of antibody combined.[25] It is only found in small amounts in blood. However, IgA is the main immunoglobulin (Ig) found in mucous secretions, including tears, saliva, colostrum, intestinal juice, vaginal fluid, and secretions from the prostate and respiratory epithelium. Because IgA is resistant to degradation by enzymes, secretory IgA can survive in harsh environments, such as the digestive and respiratory tracts, to provide protection against microbes that multiply in body secretions.[26] One of the key roles of the gut mucosa is to exclude and eliminate potentially harmful dietary antigens and micro-organisms, while providing selective absorption of nutrients.[27] The ability of the gut mucosa to produce secretory IgA and mucus influences this antigen exclusion.[28,29] Secretory IgA prevents the adherence of enteral antigens to the mucosal surface, and mucus prevents microbial infestation.

Cytokines serve as messengers of the immune system. They are a group of low–molecular weight regulatory proteins, which regulate the intensity and duration of the immune response. This is done by stimulating or inhibiting the activation, proliferation, and differentiation of various cells and by regulating the secretion of antibodies or other cytokines. Thus, cytokines are critical components of both humoral and cell-mediated immune responses.[30]

It is, thus, well established that different components of the immune system act together to mediate protection. Second, the activation of both natural and acquired (antibody and cell mediated) immune responses influences the most successful host immune responses.[31,32]

Considerable research interest has been dedicated to the encapsulation of bacterial cells for the growing and promising potential in therapeutic applications, such as in kidney failure uremia, cancer therapy, diarrhea, cholesteremia, and other diseases.[33-35] Micro-organisms used as probiotic adjuncts are commonly delivered in the food system. However, when these micro-organisms are ingested, their activity and viability are reduced under highly acidic conditions of the stomach.[36] In addition, the usual starter organisms in yogurt are not bile tolerant and do not colonize the intestines. Hence, there is a need for lactic acid bacteria products that are resistant to the stressful conditions of the stomach and the upper intestine, both of which contain bile.[37] Second, for these bacteria to exert positive health effects, they have to reach their site of action alive and establish themselves in certain numbers.[38] However, a major barrier to the survival of ingested micro-organisms is the acidic environment (pH 2.0) of the stomach.[39] Thus, problems inherent with oral delivery have made the goal of oral delivery of live bacterial cells very challenging. Providing probiotic living cells with a physical barrier against adverse environmental conditions is, therefore, an approach currently receiving considerable interest.[40]

Second, the quality of probiotics has received very little attention. Quality issues for probiotic supplement include the viability of micro-organism in the product and the stability under different storage conditions. Analysis of products in several different countries has confirmed that probiotic strains exhibit poor survival in traditional probiotic foods, such as yogurt and fermented milks.[41]

Thus, the objectives of this study are

1) to formulate gastro-resistant *Lactobacilli* microspheres,
2) to characterize the *Lactobacilli* microspheres *in vitro*,
3) to evaluate the effect of *Lactobacilli* microspheres on intestinal colonization,
4) to evaluate the effect of the *Lactobacilli* microspheres on inducing systemic immunity by checking its effect on serum IgG levels *in vivo* using Sprague–Dawley rats,
5) to evaluate the effect of the *Lactobacilli* microspheres on inducing mucosal immunity by checking its effect on IgA levels in fecal, nasal, and intestinal wash samples *in vivo* using Sprague–Dawley rats, and
6) to determine the effect of *Lactobacilli* microspheres on cytokine production.

Materials and Methods

Materials

The materials included bovine serum albumin (BSA) and hydroxy propyl methyl cellulose acetate succinate (HPMCAS) obtained from Fisher Scientific (Norcross, GA), ethyl cellulose (EC) obtained from FMC Biopolymer, a mini spray dryer (Buchi 191) with a high-efficiency cyclone, a freeze dryer (Labconco Centrivap Concentrator), Distek dissolution apparatus (Distek 2100C), Spectrex PC 2000 Laser particle counter, refrigerator (Kelvinator), an incubator (Type 37900 Culture Incubator), and Zetasizer (Malvern Instruments). MRS (deMan, Rogosa, and Sharpe) agar, culture plates, fisher-inoculating turntables, and inoculating loops used for cultivating bacteria were obtained from Fisher Scientific. A Napco E series model 302 CO_2 incubator was used to grow bacteria. *Lactobacilli* were extracted from iFlora capsules obtained from Sedona Labs (Providence, UT). *In vivo* studies were carried out in Sprague–Dawley rats from Charles River Laboratories. Purified mouse anti-rat IgA monoclonal antibody, rat IgG standard, goat anti-rat IgG-UNLB, rat IgA standard, goat anti-rat IgG-biotinylated, goat anti-rat IgA-biotinylated, and ABTS powder (2,2′-azino-bis-(3-benzthiazoline-6-sulfonic acid)) were obtained from Southern Biotech (Birmingham, AL). Rabbit anti-biotin-horseradish peroxidase (HRP)–conjugated antibody obtained from Bethyl Labs Inc. (Montgomery, TX) and purified anti-rat IgA (A93-3) obtained from BD Pharmingen were all used for enzyme-linked immunosorbent assay (ELISA). PBS buffer containing protease inhibitor was used to emulsify the fecal matter to get fecal extract.

Methods

Preparation of Gastro-Resistant Lactobacilli *Microspheres*

HPMCAS, EC, and BSA were used as the polymer matrix to make *Lactobacilli* microspheres. Because HPMCAS is insoluble in gastric