Prevention of Gut Mucosa Inflammation by Two Co-cultures of *Lactobacillus plantarum*-*Bifidobacterium longum* and *Streptococcus thermophilus*-*Bifidobacterium longum*

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Received: September 24, 2012 / Published: December 20, 2012.

Abstract: The effect of two fermented milks (FM₁, FM₂) with lactic acid bacteria and *Bifidobacteria* on the intestine mucosa was studied. BALB/c mice were divided in groups of non-sensitized or sensitized by oral route to cow's milk or colonized by FM₁ [10⁷ cfu/mL of *Bifidobacterium longum* (Bfl) and 10⁸ cfu/mL of *Streptococcus thermophilus* (StI)] and sensitized by oral route to cow's milk or colonized by FM₂ [10⁷ cfu/mL of Bfl and 10⁷ cfu/mL of *Lactobacillus plantarum* (LbO)] and sensitized to cow's milk. Blood was sampled and the amount of anti-β-Lactoglobulin (β-Lg) IgG was measured. Mice were sacrificed, fragments of their intestines were isolated to inspect the structural changes of intestinal mucosa. A significant anti-β-Lg response was elicited by oral sensitization in positive control compared to other groups. Inspection of villi structural changes reveal signs of inflammation in challenged group compared with FM₁ and FM₂ groups, which conserved long villi characteristic of negative controls. The colonization of intestines by Bfl-StI and Bfl-LbO, and the evaluation of the residual antigenicity of β-Lg in mice sensitized to bovine milk by oral route followed by histological studies, revealed that FM₁ and FM₂ play protective role and reduce the histological lesions typical of bovine milk allergy.

Key words: Lactic acid bacteria, bovine milk, allergy disease, villi.

1. Introduction

Allergy to cow milk is one of the most common food allergies in children [1]. Although most children out-grow cow milk allergy (CMA) by the age of 4, some retain their allergies for life. CMA may occur in adults usually involving immediate allergic reactions or eczema. The incidence of CMA ranges from 0.3% to 7.5% in population-based studies in different countries [2]. Most studies revealed that caseins and β-lactoglobulin (β-Lg) are the main allergens in cow

milk [3, 4]. Different attempts have been made to reduce the allergenicity of dairy proteins, and various technological processes have been applied. There are many reports showing that beneficial for consumer health effects may be due at least partly, to the immunomodulatory capacities of certain probiotic strains [5, 6]. Lactic acid bacteria (LAB) isolated from human and animal gastrointestinal tract (GIT) are well-known probiotics. When used in large amounts in the preparation of foods, they are able to survive the passage through the upper digestive tract and adhere to intestinal cells, helping to maintain the intestinal balance. Probiotics used in functional dairy products

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belong to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces*. Well balanced intestinal microflora is important in order to maintain GIT in good health, and most LAB survive the transit through the GIT maintaining their viability [7, 8]. The desirable probiotics should have the following properties: resistance to acid and to bile toxicity, adherence to human intestine cells, colonization in human guts, antagonism against pathogenic bacteria, production of antimicrobial substances, and immune modulation properties [9]. The intestinal microflora, the mucosal barrier and the mucosal immune system [the so-called gut-associated lymphoid tissue (GALT)] are all involved in this protection. To produce the desired benefits in foods products, probiotics should be present in the product in viable counts during their whole shelf-life. It has been recommended that the minimal dose able to secure therapeutic effects should be in the range 7-9 log cfu/mL [10]. Oral administration is the normal route by which probiotics are ingested by consumers. Hence, the study of mucosal immunity at the intestinal level is thus essential to understand their effects on the host.

2. Methods

2.1 Experimental Processes

Forty female BALB/c mice weighing 19 ± 0.3 g were studied. They were divided in different groups: (1) non sensitized (negative control), (2) sensitized by oral route to cow milk (positive control), (3) colonized by FM₁ fermented with 10^7 cfu/mL of *B. longum* (BfI) and 10^8 cfu/mL *Str. thermophilus* (StI), (4) colonized by FM₂ fermented with 10^7 cfu/mL of BfI and 10^7 cfu/mL of *Lb. plantarum* (LbO) and sensitized to cow milk. At day 0 (D0), the animals were randomized into four groups: the first group received 9% NaCl during all the duration of experiment, the second group received 9% NaCl during 18 days, then was sensitized by oral route to cow milk, the third group was colonized with 10^7 cfu/mL of FM₁ during 18 days then was sensitized by oral route to cow milk, the fourth group was colonized

with 10^9 cfu/mL of FM₂ during 18 days, then was sensitized to cow milk. Each group was divided into two subgroups of five mice: one group was sacrificed on the 28th day and the second group on the 65th day of experiment.

2.2 Blood Sampling and Determinations of IgG Anti- β -Lg

Blood samples were collected at D0, before sensitization and at days 23, 28, 35 and 65 after sensitization. The blood was centrifuged at 3,500 rpm for 10 min at 4 °C. The serum was collected and stored in aliquots at -25 °C. Specific anti- β -Lg IgG were tested in serum samples using an enzyme linked immunosorbent assay (ELISA) according to Engvall and Perlmann [11]. Multi-well micro-titer plates (Nunc, Polylabo, France) were coated for 1 h at 37 °C with 100 μ L of β -Lg (2 μ g/mL) before being washed with phosphate buffered saline containing 0.05% Tween 20 (pH 7.4). A total of 100 μ L of serially diluted serum (1:30-1:65, 610) was added to each well and the plates were incubated for 90 min at 37 °C and washed. A total of 100 μ L of goat anti-IgG peroxidase conjugate (1:3,000, Sigma) was then added to each well and the plates were left for 1 h at 37 °C. After washing of the plates, peroxidase activity was assayed by adding 0.2 mg/mL of diamino-orthophenylene in 0.05 M citrate buffer, pH 4, containing 0.05% H₂O₂. After external stirring at room temperature in the dark, the reaction was stopped by adding 6 N H₂SO₄ (50 μ L per well) and absorbance was measured at 492 nm using a Uniskan plate reader (Labssystem, Helsinki, Finland). Positive titers were given as the last dilution of optical density exceeding the background.

2.3 Histology

Histological studies were performed according to Ham and Cormack [12] on isolated intestinal fragments from control and cow milk sensitized mice after bacterial colonization by FM₁ and FM₂. Jejunum fragments were removed and fixed in

alcohol-formalin-acetic acid (AFA) (2% formalin, 5% acetic acid, 75% ethyl alcohol solution), embedded in paraffin and stained with hematoxylin-eosin for subsequent histological analyses. These two dyes were used to reveal the nucleus and the cytoplasm, respectively. Paraffin was removed with toluene (56 °C, 2 min) and the tissue fragments were re-hydrated in four successive ethanol/water solutions (100/0, 95/5, 90/10, 70/30, v/v) for 2 min and then washed with water. The fragments were incubated for 3 min in hematoxylin solution (5 g/L hematoxylin, 100 g/L potassium alun, 2.5 g/L mercuric oxide in water), washed with water, incubated for 5 min in eosin solution (20 g/L eosin in ethanol), rinsed twice with 70% ethanol, incubated for 1 min in toluene, and mounted for microscopy. Measurements of the height of villi of the jejunum were performed using optical microscope equipped with micrometer.

2.4 Statistics

The results were analyzed using Student *t*-test and were presented as mean values standard error of the mean (SEM). A *P* value of 0.05 was considered as significant.

3. Results

3.1 Blood Sampling and Anti- β -Lg IgG Determinations

After oral sensitization to cow milk, a significant anti- β -Lg response was detected in sensitized per os with cow milk ($P < 0.05$) group compared with groups FM₁ and FM₂, which showed weaker IgG response. No anti β -Lg antibody responses were detected in control animals during all time of experiment (D₂₃, D₂₈, D₃₅ and D₆₅) (Fig. 1).

3.2 Observations with Optical Microscopy

When scrutinized under optical microscope, the intestinal mucosa in the control group exhibited long, thin villi surrounded by a simple, cylindrical and single-layer epithelium, mainly composed of tall cells with a flat striatus containing regular nuclei at the base

Fig. 1 Measures of residual antigenicity of β -Lg (μ g/mg) in mice sensitized to cow milk per os.

■ Milk challenged group; ■ Group colonized with FM₁; ■ Negative control; ■ Group colonized with FM₂. Values represent mean $\pm$ SE. * $P < 0.05$. $n = 6$. Titer: low level of antibody detected.

corresponding to the enterocytes (Fig. 2A). The intestinal mucosa in groups FM₁ and FM₂ showed weak inflammatory signs with slightly shorter villi (Figs. 2B and 2C, respectively). In contrast, the intestinal mucosa of cow milk sensitized mice was subject to patent atrophy characterized by villi surrounded by a pseudo-stratified epithelium containing cubic cells with dystrophic nuclei and at the level of the chorion inflammatory infiltration was extremely marked (Fig. 2D).

4. Discussion and Conclusions

On the first day of sensitization with cow milk, a significant quantity of IgG anti-bovine β -Lg ($P < 0.05$) was detected in positive serum control. On day 23 (after colonization by FM₁, FM₂ and 2 days after sensitization with cow milk), way lower quantity of IgG anti-bovine β -Lg was observed compared with unprotected with LAB mice, which showed very high and strongly significant ($P < 0.05$) quantities of IgG anti-bovine β -Lg. No anti-bovine β -Lg antibody responses were detected in control animals during all duration of experiment (Fig. 1). These results show pronounced systemic response and they agreed with

Fig. 2A Observation (×20) of jejunal fragments of mice not sensitized (negative control).

Fig. 2B Observation (×20) of jejunal fragments of mice FM₁ group.

Fig. 2C Observation (×20) of jejunal fragments of mice FM₂ group.

Fig. 2D Observation (×20) of jejunal fragments of mice sensitized (challenged group).

previous results of Prioult et al. [13]. It was already reported that some LAB reduce the antigenic response of milk proteins [14]. The reduction of milk protein antigenicity depends on the species of LAB and on conditions of fermentation [15]. Lactic acid fermentation was applied to reduce the β -Lg antigenicity of sweet whey and skim milk, and furthermore, its products were reported to have beneficial effects on the consumer including the

activation of the immune system [16]. Proteolytic systems of lactobacilli are complex and are composed of proteinases and peptidases with different sub-cellular locations. Cellular proteases grown on milk showed similar hydrolytic activities toward α _S- and β -caseins. Proteolysis is often followed by a reduction of the number of epitopes and consequently by a decrease in allergenicity of hydrolyzed proteins or could contribute to preventing allergenic problems frequent in children under 3 years of age due to poor digestion of milk proteins. Protein hydrolysates are also included in specific formulations, as well as hypoallergenic infant formulas to reduce their antigenicity compared with intact protein [3, 17-19]. On day 65, significant difference of IgG serum anti bovine β -Lg rate between mice groups colonized with FM₁ and FM₂, and mice group sensitized with cow milk was observed. These results agree with observations of higher amounts of IgG anti bovine β -Lg antibodies in atopic children compared with non atopic children. Additionally, it has been reported that IgG anti bovine β -Lg expression was associated with atopic dermatitis [20, 21]. It has also been shown that the ingestion of antigenic proteins induced immune responses associated with stimulation of lymphocytes B and T. These authors measured adhesion of *Lactobacillus* sp. and *Bifidobacterium* sp. to intestine mucosa. Johanson et al. [22] found 19 LAB species in jejunum and rectum biopsies after 11 days of feeding with food containing them. Elo et al. [23] reported an important property of probiotics bacteria, which can stick to intestinal membranes. According to Shida and Nanno [24], action of probiotics on intestinal epithelial cells is still poorly understood. The administration of cow milk modifies significantly height of villi. Slides of groups FM₁ and FM₂ sensitized with cow milk were similar to control negative group with long villi. Challenged with cow milk mice group lost brush border, had atrophied villi and hyperplasia of crypts what is in agreement with observations of Addou et al. [25]. It was also reported that probiotics have a crucial

role on induction of tolerance of an antigen, reacting to inflammation intestinal epithelial release IL-8 chemokine and other inflammatory molecules, which cause an acute inflammatory response [26]. It is admitted that β -Lg intolerance of certain children is characterized by a high intestinal permeability to this protein [27, 28]. Addou et al. [25] proposed that the presence of β -Lg modifies considerably the structure of intestinal mucosa in rabbits. Intestinal biopsies of children with CMA reveal almost total atrophy of their villi [29]. These results indicate that the lymphocyte activation could be responsible for histological lesions caused by alimentary diseases. Studies of fetal intestinal cultures showed that the activation of T cells and cytokines released by these cells induced atrophy of villi and crypt hyperplasia [30].

The experimental findings reported in this study indicate that the effect of two fermented milks (FM₁, FM₂) with LAB and *Bifidobacteria* on the intestine mucosa is beneficial. The evaluation of the residual antigenicity of cow milk and the antibody specific β -Lg serum showed low anti β -Lg antibody responses. The results of histological study demonstrate smaller histological damages, weaker inflammatory signs with slightly shorter villi. Hence, it could be concluded that fermented milks (MF₁, MF₂) have a protective effect on murine intestinal epithelium and they reduce the histological lesions due to induced allergy to cow milk proteins.

The answer to the question if the reduction of histological lesions is caused by adhesion of LAB and bifidobacteria to the intestinal mucosa or by another factor would be of great interest.

Acknowledgments

The authors would like to express their gratitude for the support of this work in the frame of TASSILI project No. 10MDU792: Alimentation de l'enfant allergique and for the Algerian Ministry of Higher Education and Scientific Research (MESRS, DGRSDT) for their financial support.

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