

Full Length Research Paper

# Patterns of colonization and immune response elicited from interactions between enteropathogenic bacteria, epithelial cells and probiotic fractions

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The purpose of this study was to investigate by *in vitro* studies the antimicrobial activity of eight lactic acid bacterial (LAB) strains belonging to *Lactobacillus paracasei* spp. *paracasei*, *L. plantarum* and *L. rhamnosus* species against *Salmonella enteritidis*, *Shigella flexneri* and EPEC pathogenic strains isolated from pediatric diarrhoea cases, simultaneously with the assessment of the cytotoxicity and immunomodulatory potential of the respective strains. The study of the adherence capacity to the cellular substrate represented by HeLa cells was performed by Cravioto's adapted method. The cytotoxicity was determined on HeLa cells and the level of soluble pro- and anti-inflammatory cytokines was assessed by ELISA. Our *in vitro* studies are demonstrating that the selected probiotic strains are inhibiting the adherence and colonization of HeLa cells by the enteropathogenic strains isolated from pediatric diarrhoea mainly by direct competition for adherence sites, demonstrating their potential use in the treatment of pediatric gastro-intestinal disorders, as an alternative to/in association with antibiotics. A great advantage of the selected probiotic strains is their low cytotoxicity and ability to trigger a beneficial cytokine response in the epithelial cells, which potentiates their antimicrobial activity by stimulating the occurrence of a rapid immune response following the intestinal injury.

**Key words:** Antimicrobial activity, immunomodulatory, cytokines, invasive bacteria, *Lactobacillus*, enteropathogenic bacteria, antimicrobial effect, cytotoxicity, probiotics.

## INTRODUCTION

In recent years, several articles have reviewed the efficacy, mechanism of action, and safety of probiotics in the treatment of infectious disease (Vanderhoof and Young, 2002; Alvarez-Olmos and Oberhelman, 2001; Salminen and Arvilommi, 2001; Borriello et al., 2003; Ishibashi and Shoji, 2001).

The term probiotic refers to a product or preparation

containing viable, defined microorganisms in numbers thought to be sufficient to alter the host's microbiota (by implantation or colonization) and thereby exert beneficial effects (Havenaar and Huis, 1992). The microorganisms most frequently used as probiotic agents are lactic acid bacteria (species of *Lactobacillus*, *Enterococcus* and *Bifidobacterium*) and nonpathogenic, antibiotic-resistant, ascosporic yeasts, especially *Saccharomyces boulardii* (FAO, 2001). *Lactobacillus rhamnosus* strain GG (ATCC 53103), which was originally isolated from human intestinal flora, is the most widely studied probiotic agent for adults and children (Gorbach, 2000). *L. rhamnosus* strain GG can prevent diarrhoea and atopic diseases among children (Szajewska and Mrukowicz, 2001; Kalliomaki et al., 2001; Majamaa and Isolauri, 1997). A

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**Abbreviations:** LAB, Lactic acid bacterial; IL, interleukins; ATCC, american type culture collection; FCS, foetal calf serum; EPEC, Enteropathogenic *E. coli*.

recent meta-analysis suggested that *Lactobacillus* is a safe effective treatment for children with acute infectious diarrhoea, serious infections attributable to probiotic lactobacilli being extremely rare (Van Neil et al., 2002; Rautio et al., 1999; Mackay et al., 1999; Bradley, 2006).

For the best use of the probiotic microorganisms, the mechanisms by which they work should be better understood. The selection of an appropriate probiotic strain for its inclusion in a probiotic preparation should be made on the basis of its capacity to induce an improved gut immune response without modification of the intestinal homeostasis. To achieve this task, probiotic strains should have the following properties: (i) high cell viability, thus they must be resistant to low pH and bile acids; (ii) ability to persist in the intestine even if the probiotic strain cannot colonize the gut (continuous or prolonged administration may be necessary); (iii) adherence to the gut epithelium to avoid the flushing effects of peristalsis; In this last aspect, there are many relevant literature reports of the adhesive property of the probiotic bacteria to epithelial cells proved by *in vitro* studies (iv) also, they should be able to interact or send signals to the immune cells associated with the gut (Perdigon et al., 1995). There are reports from *in vitro* assays that show the activation of immune cells after stimulation by probiotics (Spanhaak et al., 1998).

The mechanisms of probiotics action are most probably multi-factorial, involving a variety of effector signals, cell types and receptors (Jack et al., 1995; Link-Amster et al., 1994; Schiffrin et al., 1995). But, strains may differ in their respective ability to trigger these signals, which is depending on both immunocompetence and the intestinal epithelial cells (Schiffrin et al., 1997; De Simone et al., 1992).

It is commonly suggested that probiotics must "persist and multiply" in the target ecosystem to be efficient.

However, the interaction of orally ingested probiotics with the intestinal epithelium or other immunologically active intestinal cells has just begun to be rigorously studied. However, for requirement of probiotics persistence to be efficacious, it depends on the specific immunological activities (prophylactic or therapeutic) and mechanisms by which ingested probiotics prevent or cure enteric disorders (Malin et al., 1996; Majamaa and Isolauri, 1997; Link-Amster et al., 1994).

It has been proposed that some probiotics are able to prevent reduction of or restore intestinal homeostasis after a prolonged antibiotherapy in connection with immunological disorders, improving mucosal barrier functions as well as down-regulation of the inflammatory responses (Malin et al., 1996). It was observed that strong strain-specific variations of the *in vitro* cytokine induction profiles after stimulation of immuno-competent host cells (Schiffrin et al., 1995; Schiffrin et al., 1997).

Our objective in this study was to investigate by *in vitro* studies the antimicrobial activity of some lactic acid bacterial (LAB) strains against pathogenic bacterial strains isolated from pediatric diarrhoea, simultaneously

assessing the cytotoxicity and immunomodulatory potential of the respective strains.

## MATERIALS AND METHODS

### LAB strains

Eight (8) LAB strains, that is, 6 *Lactobacillus paracasei* subsp. *paracasei* strains encoded CMGB 18, CMGB 19, CMGB 20, CMGB 21, CMGB 22 and CMGB 23 isolated from infant child feces, one *L. plantarum* strain CMGB 24 isolated from fermented vegetal debris and one *b. rhamnosus* CMGB 29 strain isolated from milk products were studied. The strains were isolated and grown in Man Rogosa Sharp (MRS) medium. All strains were identified by standard morphological, cultural and biochemical (API 50 CHL) features and stored in the collection of the Center for Research, Education and Consulting in Microbiology, Genetics and Biotehology - MICROGEN (acronym CMGB) at -70°C in appropriate medium represented by MRS and supplemented with 20% glycerol.

Fresh cultures were obtained and thereafter cultivated in MRS liquid medium in order to obtain mid-logarithmic phase cultures that were further used in our experiments (Smarandache et al., 2004; Lazar et al., 2004).

### Enteropathogenic strains

The pathogenic strains used in our study were recently isolated from acute diarrhoeal cases in children under 2 years of age and identified following stool sample enrichment and seeding on specific selective media (EMB Levine, SS). The biochemical identification was performed comparatively by classical and API 20E microtests (BioMérieux) and the species were serologically confirmed by agglutination with polyvalent and monovalent sera as: two *Shigella flexneri*, one *Salmonella enteritidis* and one enteropathogenic *Escherichia coli* (EPEC) strain.

### Study of the adherence and invasion capacity to the cellular substrate represented by HeLa cells (Cravioto's adapted method) (Smarandache et al., 2004; Lazar et al., 2004)

In this purpose, HeLa cells were routinely grown in Eagle's minimal essential medium (Eagle MEM) supplemented with 10% heat-inactivated (30 min at 56°C) foetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamycin (50 µg/ml) (Gibco BRL) and incubated in a 5% CO<sub>2</sub> humidified atmosphere, at 37°C for 24 h (Kalliomaki et al., 2001). HeLa cell monolayers grown in 6 multi-well plastic plates were used at 80 - 100% confluence. Bacterial strains from an overnight culture on 2% nutrient agar were diluted at 10<sup>7</sup> CFU/ml in Eagle MEM with no antibiotics. The HeLa cell monolayers were washed 3 times with Phosphate Buffered Saline (PBS) and 2 ml from the bacterial suspension were inoculated in each well. The inoculated plates were incubated for 3 h at 37°C. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 min), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and incubated for 30 min. The plates were washed, dried at room temperature overnight, examined microscopically (magnification, ×2500) with the immersion objective (IO) and photographed with a Contax camera (Company, City, Country) adapted for Zeiss (AxioLab 459306) microscope (Zeiss, City, Country).

For the quantitative assay of adhesion and invasion capacity, the infection step was performed in duplicates for each strain, and after

**Table 1.** The results of testing the adherence ability to HeLa cells of the enteropathogenic strains.

Enteropathogenic microbial strains	Adherence pattern	Adherence index (%)
EPEC	Aggregative (AggA)	40
<i>S. enteritidis</i> 361	Localized (LA)	5
<i>S. flexneri</i> 29833	Diffuse-aggregative	10
<i>S. flexneri</i> 29834	Diffuse (DA)	5

3 h incubation of the HeLa monolayer in the presence of microbial strains, the first well plates were washed four times in PBS, the cells were permeabilized by Triton X 1% (Sigma) and incubated for 5 min at 37°C for the release of intracellular invasive bacteria. Thereafter, serial ten-fold dilutions in saline solution were performed and 20 µl from each dilution was spotted in triplicates on solid media; in the second plate, after 2 h of incubation the monolayer was washed 4 times in PBS and 1 ml of 100 mg/ml gentamycin solution was added; the plates were further incubated for 1 h, in order to kill all adherent extra-cellular bacteria. Thereafter, the second plate was treated as the first one.

It is to be mentioned that in the case of lactic acid bacteria, the adherence capacity was investigated in three variants: 1) integral mid-logarithmic phase cultures; 2) microbial suspensions obtained from the washed sediment; and 3) heat inactivated microbial suspensions (30 min at 100°C).

In order to investigate how the probiotic strains influence the adherence and invasion of the HeLa cells by the pathogenic strains, the adherence and invasion assays were performed following the steps mentioned above, the monolayer infection being done in the presence of equal volumes of different LAB culture fractions (that is, integral cultures, washed sediments and heat-inactivated washed sediments).

#### Qualitative screening of the antimicrobial properties of the LAB culture supernatants

The qualitative screening was performed by an adapted disk diffusion method. Petri dishes with Mueller Hinton medium were seeded with bacterial inoculums as for the classical antibiotic susceptibility testing disk diffusion method (Kirby-Bauer); a 5 µL drop of the LAB culture supernatants were placed on the seeded medium, at 30 mm distance. The plates were left at room temperature for 20 - 30 min and then incubated at 37°C for 24 h. The positive results were read as the occurrence of an inhibition zone of microbial growth around the dried liquid spot (CLSI, 2008; Lazar et al., 2005).

#### Cytotoxicity of LAB strains

An approximate quantity of  $5 \times 10^3$  HeLa cells were seeded in each well of a 96-well tissue culture plate, in DMEM supplemented with 10% foetal calf serum (FCS). After 24 h the pH adjusted culture supernatants and respectively heat-inactivated microbial suspensions obtained from washed sediments were added. The cytotoxicity effect was read using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), according to manufacturer's indications (Balotescu et al., 2005).

#### Annexin-V immunostaining and flow cytometric analysis

HeLa cells were treated for 24 h with pH adjusted LAB supernatants in DMEM with 10% foetal bovine serum (1:1). Cells

from the supernatant and monolayer were harvested and  $5 \times 10^5$  cells were stained with annexin V and propidium iodide using the Immunotech Annexin V-FITC Kit and following the manufacturer's instructions (Beckman Coulter Company, France). Cells were analyzed by flow cytometry using a Coulter EPICS XL flow cytometer (Beckman Coulter). Green fluorescence (525 nm; FITC annexin V) and red fluorescence (613 nm; propidium iodide) were measured (Balotescu et al., 2005).

#### Immunomodulatory activity of LAB strains

In order to assess the potential immunomodulatory activity of the studied LAB strains, as well as correlate the obtained results with a certain fraction (that is, soluble fraction located in supernatants or cell-associated fraction located in the bacterial sediment) the levels of the main soluble pro- and anti-inflammatory cytokines, that is, IL-1, IL-2, IL-6, IL-8, IL-10, TNF-alpha and INF-gamma induced by different fractions of probiotic cultures were assessed by ELISA (Pierce Endogen kit) according to manufacturer's indications.

## RESULTS

### Adherence to the cellular substrate represented by HeLa cells and competition studies

Our results showed that the enteropathogenic tested strains exhibited different adherence abilities for colonizing the HeLa cells, as demonstrated by different adherence patterns and rates (Table 1).

It is to be mentioned that the adherence rates observed for these pathogenic strains are only apparently low and they are not reflecting a reduced ability to colonize the cellular substratum, but on the contrary, their ability to be rapidly internalized in the host cell following the initial adherence step. This hypothesis was confirmed by our quantitative adhesion and invasion assay studies, showing high invasion indexes for the selected enteropathogenic strains (data not shown).

Concerning the LAB strains, the ability to adhere to HeLa cells was different, depending on the analyzed fraction, that is, integral mid-logarithmic phase cultures, microbial suspensions obtained from the washed sediment as well as heat inactivated microbial suspensions (Table 2).

The live cells from the integral cultures generally exhibited higher adherence rates than the washed sediment and heat-inactivated cells.

However, all strains exhibited adherence abilities, with higher adherence rates for *L. paracasei* ssp. *paracasei*

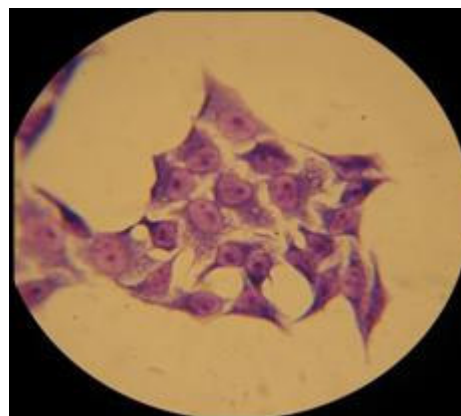
**Table 2.** The results of testing the adherence ability to HeLa cells of different fractions of the LAB cultures.

Microbial strain	Adherence pattern			Adherence		
	Integral fraction	Washed sediment	Heat inactivated washed sediment	Integral fraction (%)	Washed sediment (%)	Heat inactivated washed sediment (%)
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 18	Diffuse - aggregative	Diffuse-localized	Aggregative	100	100	100
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 19	Aggregative	Localized - aggregative	Aggregative	50	70	80
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 20	Diffuse - aggregative	Localized - aggregative	Aggregative	100	10	25
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 21	Aggregative	Localized - aggregative	Localized	80	10	90
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 22	Aggregative	Diffuse-aggregative	Diffuse-localized	50	50	50
<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 23	Aggregative	Diffuse	Diffuse	80	100	90
<i>L. plantarum</i> CMGB 24	Diffuse - localized	Diffuse	Diffuse	10	10	10
<i>L. rhamnosus</i> CMGB 29	Diffuse	Non-adherent	Non-adherent	5	0	0

(50 to 100%) than for *L. rhamnosus* and *L. plantarum* (5 to 10%) (Table 2).

Three distinct patterns of adherence have been investigated during this study: localized adherence (LA), in which bacteria attach to and form microcolonies in distinct regions of the surface; diffuse adherence (DA), in which bacteria adhere evenly to the whole cell surface, and aggregative adherence (AggA), in which aggregated bacteria attach to the cell in a stacked-brick arrangement.

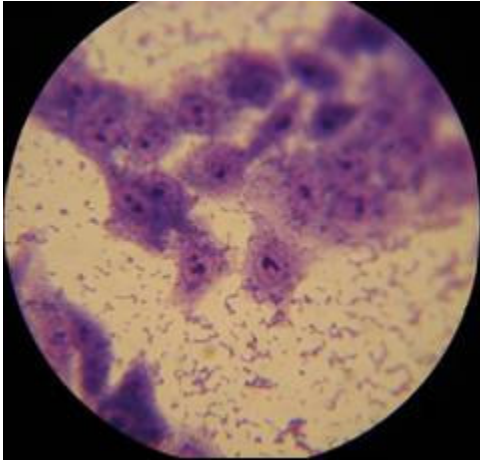
The adherence index was expressed as the ratio between the numbers of the eukaryotic cells with adhered bacteria: 100 eukaryotic cells counted on the microscopic field. The adherence pattern of the *L. paracasei* whole cultures was predominantly aggregative, while for *L. plantarum* and *L. rhamnosus* a diffuse one (Figures 1-4). However, *L. paracasei* exhibited a significantly higher ability to colonize the cellular substrate compared with the other two tested species. *L. paracasei* exhibited the highest adherence for all tested fractions (100%) (Table 2). The adherence pattern was not significantly different for different fractions of the same strain. The slight changes in the adherence patterns consisted in the shift from aggregative (observed for the integral cultures) to a diffuse pattern (when other fractions were used) in case of *L. paracasei* strains and the loss of adherence ability in the case of *L. plantarum* strain (Table 2).



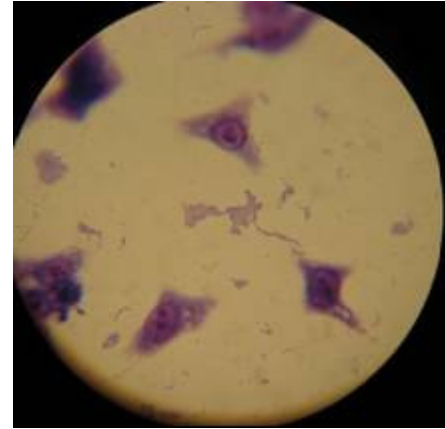
**Figure 1.** HeLa cells control (Giemsa staining, x2500).

### Competition studies

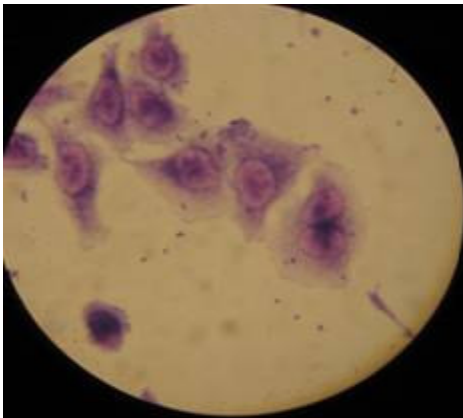
All tested fractions of the selected probiotic strains, that is, whole bacterial culture, washed viable cells and heat-inactivated bacterial cell suspensions inhibited almost totally the adherence ability of the pathogenic strains to the cellular substrate (Figures 5 -10).



**Figure 2.** HeLa cells infected with *L. paracasei* ssp *paracasei* CMGB 18 (Giemsa staining, x2500).



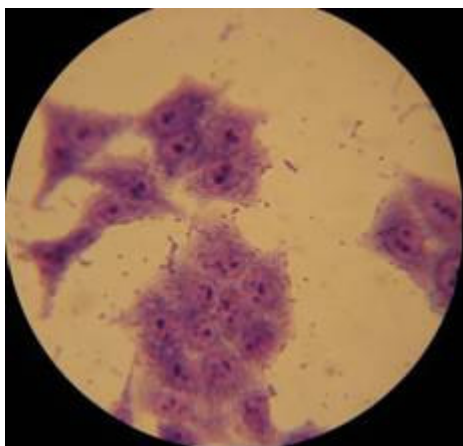
**Figure 5.** HeLa cells infected with *EPEC* (Giemsa staining, x2500).



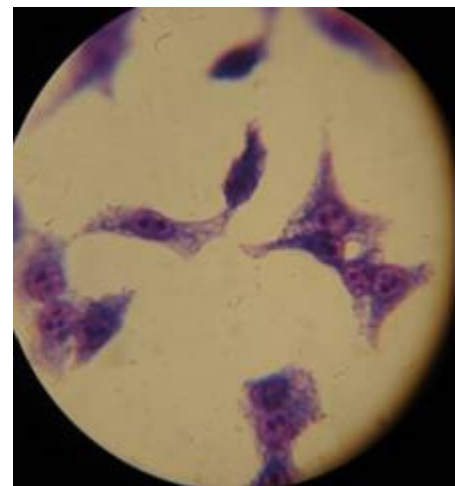
**Figure 3.** HeLa cells infected with *L. plantarum* CMGB 24 (Giemsa staining, x2500).



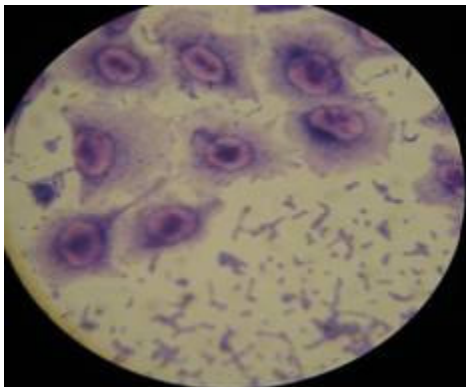
**Figure 6.** HeLa cells infected with *Salmonella enteritidis* (Giemsa staining, x2500).



**Figure 4.** HeLa cells infected with *L. rhamnosus* CMGB 29 (Giemsa staining, x2500).



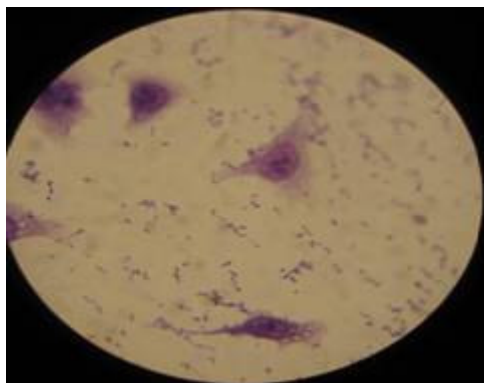
**Figure 7.** HeLa cells infected with *S. flexneri* 29834 (Giemsa staining, x2500).



**Figure 8.** HeLa cells infected simultaneously with *EPEC* and whole culture of *L. paracasei ssp paracasei* CMGB 18 (Giemsa staining, x2500).



**Figure 9.** HeLa cells infected simultaneously with *Salmonella enteritidis* and washed viable cell suspension *L. paracasei ssp paracasei* CMGB 19 (Giemsa staining, x2500).



**Figure 10.** HeLa cells uninfected simultaneously with *S. flexneri* 29834 and washed viable cell suspension of *L. paracasei ssp paracasei* CMGB 21 (Giemsa staining, x2500).

### Antimicrobial activity of the LAB cultures supernatants

The tested strains did not exhibit any quantifiable antimicrobial activity, so they probably did not produce bacteriocins or other soluble factors with intrinsic antimicrobial activity.

### Cytotoxicity

All tested supernatants with adjusted neutral pH and heat inactivated LAB washed cells suspension showed no cytotoxicity when tested by Cell Titer method. However, when assessed by Annexin V immunostaining, it was noticed that they are inducing the apoptosis of HeLa cells (Table 3).

### Immunomodulatory activity of LAB supernatants and heat-inactivated LAB washed cells suspension

One of the purposes of this study was to investigate the influence of different probiotic fractions on the secretory pattern of the HeLa cells, by quantifying the most important pro - and anti-inflammatory cytokines.

Our results have demonstrated that the cellular fractions of tested lactic bacteria specifically modulate the expression of pro and anti-inflammatory cytokines in epithelial HeLa cells.

The level of the IL-1, the major pro-inflammatory cytokine and respectively IL-10 which have anti-inflammatory properties such as *in vitro* suppression of production of other pro-inflammatory cytokines could not be detected by our experimental model.

The general effect of bacterial cells and supernatants was the increase in TNF-alpha expression (Figure 11). The expression level of this cytokine was different, depending on the probiotic strain and fraction, the highest level being induced in the presence of two probiotic strains supernatants, that is, *L. plantarum* CMGB 24 and *L. paracasei paracasei* CMGB 19.

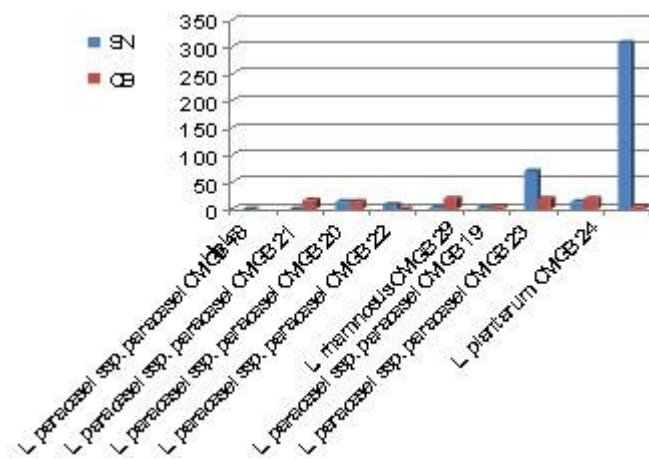
The increase of TNF-alpha level and the subsequent pro-inflammatory effect is balanced by the decreased levels of IL-6 (Figure 2) and IL-8 (Figure 13), also observed for all tested fractions, regardless of the analyzed strain.

Our results showed that the probiotic fractions are inhibiting the expression of IL-8 (interleukin with chemotactic properties) (Figure 12).

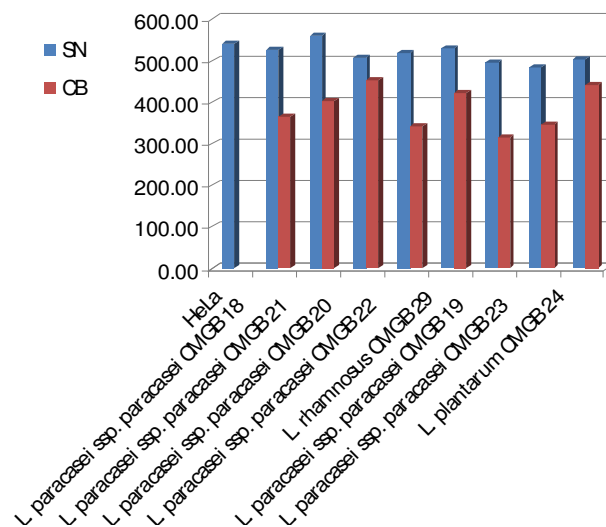
It should also be noted the positive correlation between increasing expression of IL-2, TNF-alpha and IFN gamma-induced by the cellular fractions, as well as by supernatants of three *L. paracasei ssp paracasei* CMGB 18, 19, 23 strains, having in mind that IL-2 stimulates

**Table 3.** The effect of the probiotic strains supernatants on HeLa cells quantified by flow cytometry.

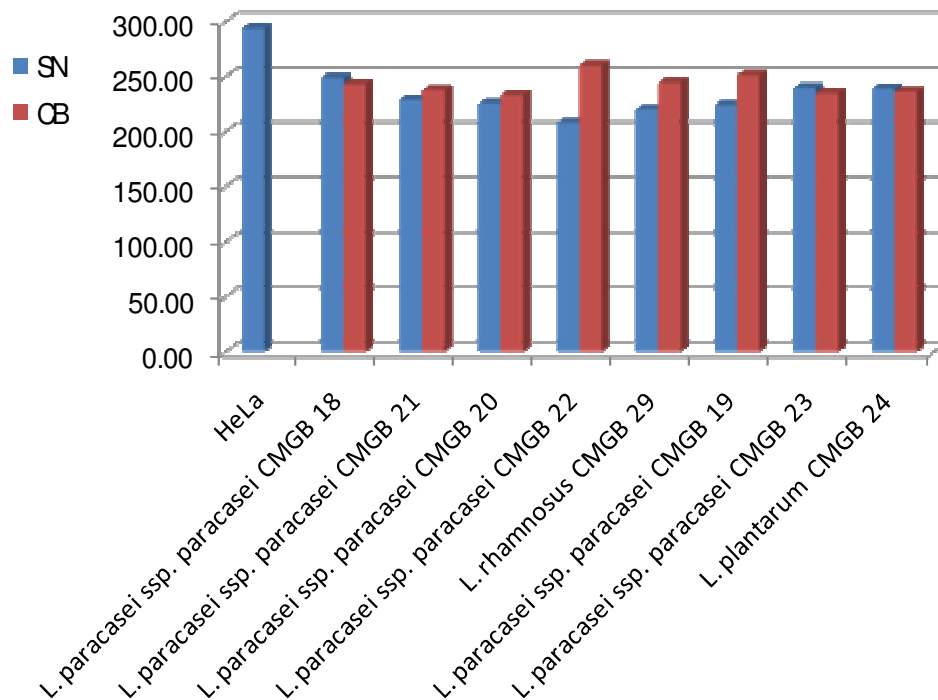
Crt. No.	Microbial strains	Viable cells (%)	Apoptosis	Advanced apoptosis	Necrosis
1.	HeLa cell control	99.20	0.37	0.34	0.10
2.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 18	83.36	12.93	3.47	0.23
3.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 19	84.34	10.02	2.89	2.76
4.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 20	83.46	11.02	4.72	0.20
5.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 21	81.34	13.52	4.76	0.23
6.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 22	89.19	7.29	0.76	2.76
7.	<i>L. rhamnosus</i> CMGB 29	84.10	12.80	3.06	0.07
8.	<i>L. paracasei</i> ssp. <i>paracasei</i> CMGB 23	84.19	11.52	3.96	0.33
9.	<i>L. plantarum</i> CMGB 24	84.55	9.10	5.85	0.50



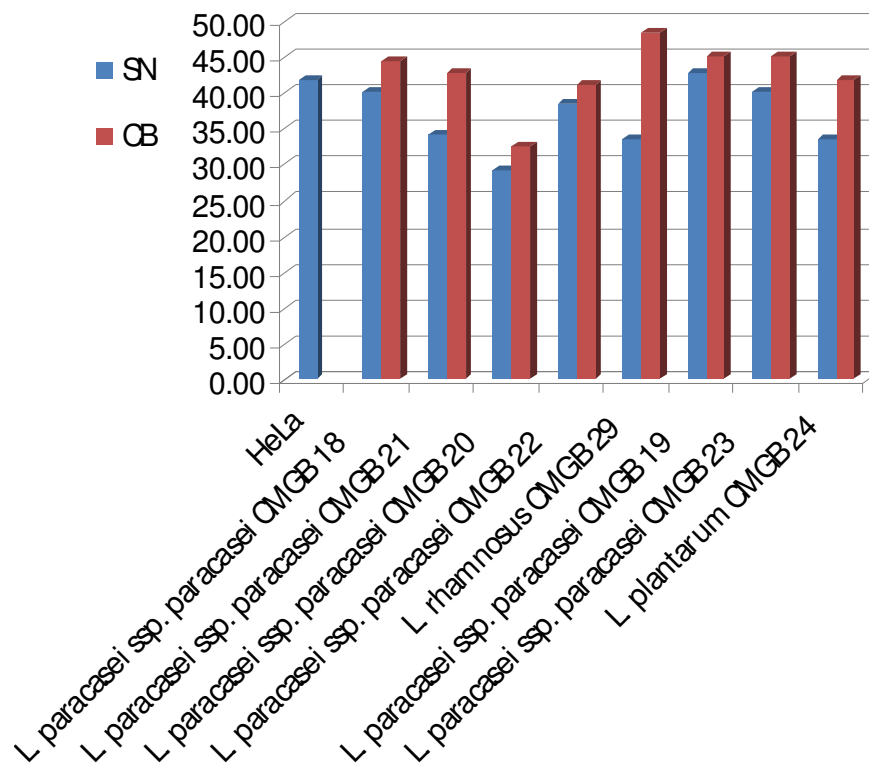
**Figure 11.** The comparative representation of TNF-alpha expression levels in HeLa in the presence of LAB supernatants (SN) and heat-inactivated LAB washed cells suspension (CB).



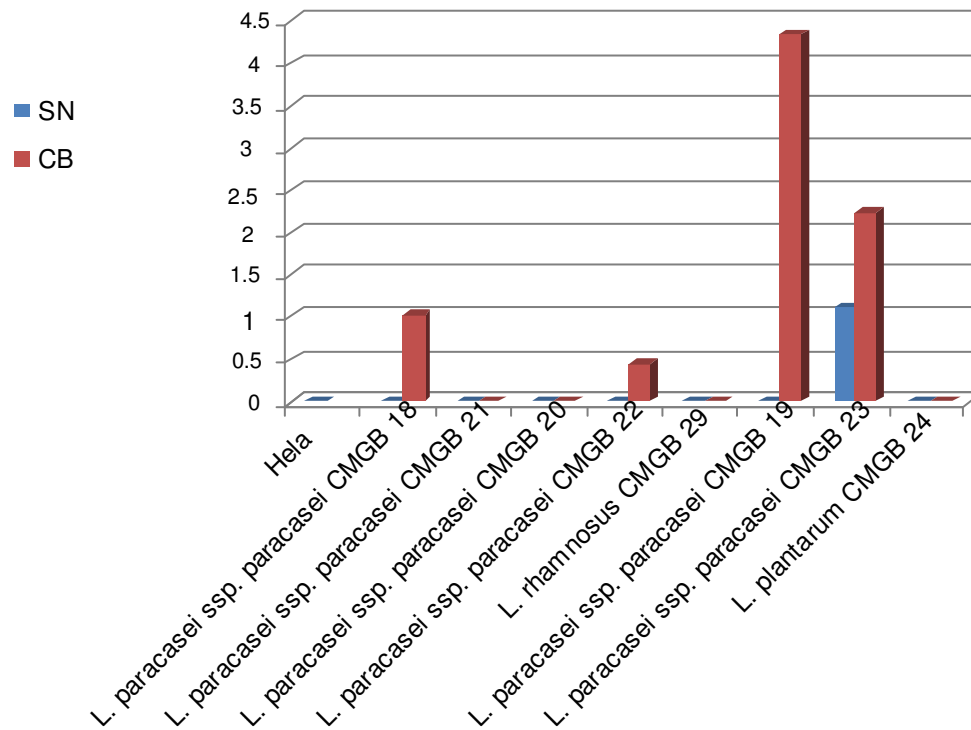
**Figure 12.** The comparative representation of IL-6 expression levels in HeLa in the presence of LAB supernatants (SN) and heat-inactivated LAB washed cells suspension (CB).



**Figure 13.** The comparative representation of IL-8 expression levels in HeLa in the presence of LAB supernatants (SN) and heat-inactivated LAB washed cells suspension (CB).



**Figure 14.** The comparative representation of IL-2 expression levels in HeLa in the presence of LAB supernatants (SN) and heat-inactivated LAB washed cells suspension (CB).



**Figure 15.** The comparative representation of IFN-gamma expression levels in HeLa in the presence of LAB supernatants (SN) and heat-inactivated LAB washed cells suspension (CB).

production of interferon gamma (IFN  $\gamma$ ), IL-4, TNF by *T. lymphocytes* (Figures 11, 14 and 15). The induction of this secretory pattern in the epithelial cells by probiotic fractions might decrease the time required for the activation of *T. lymphocytes* and the occurrence of a specific immune response, which could speed up the elimination of infectious agent from the host organism.

In conclusion, the stimulation of TNF-alpha, while decreasing expression of IL-6 and IL-8, can be considered a beneficial immunomodulating effect. With special reference to *L. paracasei* ssp. *paracasei* CMGB 18, 19, 23 strains, the induction in the epithelial cells of a secretory pattern similar to that required for the activation of lymphocytes could be of significant advantage in probiotic administration in therapeutic purposes at the beginning of infection, in order to stimulate the rapid occurrence of a specific immune response mediated by *T. lymphocytes*.

## DISCUSSION

The probiotic arsenal includes multiple mechanisms for preventing infection and enhancing the immune system, but each mechanism of action is strain-dependent (Majamaa and Isolauri, 1997; Link-Amster et al., 1994).

Our study is clearly demonstrating that one of the main mechanisms of probiotic antimicrobial activity is direct

competition with the probiotic bacterial cells for the adhesion sites at the intestinal level triggering the pathogens exclusion.

The reduced cytotoxicity of the tested probiotic strains is representing an important advantage in the selection process of probiotic products to be administered in children. The cytotoxicity was assessed by flow cytometry using Annexin-V assay carried out in conjunction with vital dye Propidium Iodide (PI) staining in order to distinguish between apoptosis and necrosis, because PI staining can detect DNA that has leaked from the necrotic cell (Liu et al., 2003). Annexin V is a 35-to 36-kD calcium-dependent, phospholipid binding protein that has an affinity for phosphatidyl serine and bind to cells with exposed phosphatidyl serine. Because the membrane phospholipid phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane at the early stage of apoptosis, an Annexin-V assay can identify apoptosis at an earlier stage than the TUNNEL assays based on nuclear changes such as DNA fragmentation. In additions, Annexin-V is conjugated to fluorochrome FITC that served as a sensitive probe for flow cytometric analysis in this Annexin-V assay.

The first stage of infectious process is the adherence of pathogenic microorganisms to epithelial cells or extracellular matrix molecules, which will lead to the colonization at the entrance gate.

Host colonization involves complex cell to cell

communication systems, both between bacterial cells, and cross communication between bacterial and host cells. One of the aspects of this cell to cell communication is the modulation of the epithelial cells secretory pattern (Wilson et al., 1998).

It is to be noticed that the eukaryotic cells are never exposed to a single cytokine and different cytokines could act synergistically or be antagonist; their combined action could result in a certain behavior of the target cells, having as consequence the amplification of inhibition of different biological activities.

Interaction between bacterial pathogens and host cells invariably results in the release of one or more cytokines, the actual cytokines produced depending mainly on the nature of the bacterium and host cells involved. Several bacterial pathogens have the capacity to alter host cell cytokine synthesis, degrade pro-inflammatory cytokines, or use cytokine receptors as portals of entry for cellular invasion (Link-Amster et al., 1994). Manipulation of the cytokine networks to the advantage of the invading pathogen offers a further example of the importance of pro-inflammatory cytokines in the protection against microbial invasion. The resulting cytokine network, of course, constitutes an important part of the innate immune response and represents the host's attempt to deal with that particular organism (Eckmann et al., 1993). As such, therefore, the ability of probiotic bacterial components (supernatants or bacterial cells) to modulate the cytokine release from host cells can be regarded as an aspect of improving the host response to pathogenic bacteria aggression and to decrease the intensity and/or chronicity of infectious diseases pathology.

All tested strains increased the level of tumor necrosis factor (TNF) expression. It is well known that TNF acts synergistically and have similar biological activities with IL-1, which is the major mediator of the acute phase response, inducing the production of other cytokines during infection, the expression of adhesion molecules and chemokines secretion by endothelial cells (Jung et al., 1995).

Regarding IL-6, one of the main *in vivo* roles of this cytokine is to initiate the acute phase response by inducing the production of acute phase proteins by hepatocytes (Barton, 1997; Barton et al., 1996). IL-6 is detected in blood of patients with inflammation, thus the decreasing of IL-6 expression by probiotics, could prevent the occurrence of an intense inflammatory response with lesional effects on host.

A wide variety of cells produce IL-8 as a response to IL-1 $\beta$ , TNF, LPS, microbial adhesins, radiation, infection, other cytokines and retinoic acid. The decrease induced by probiotics in IL-8 level could be considered as a positive effect, having in view that increased levels of the IL-8 were observed in case of gastro-intestinal infections with invasive bacteria such as *Shigella* spp. or *Salmonella* spp. (Eckmann et al., 1993; Schulte et al., 1996).

Taken together, the immunomodulatory studies allowed

us to state that the most appropriate immunomodulatory activity was exhibited by three strains belonging to the same species, that is, *L. paracasei* ssp. *paracasei* (Malin et al., 1996; Majamaa and Isolauri, 1997; De Simone et al., 1992), the strains modulating the expression of the most important cytokines in the development of the anti-infectious immunity against enteric pathogens. The immunomodulation findings are also supported by the good results exhibited by these strains in the competition studies and also by their low cytotoxicity on HeLa cells, this last aspect being of great importance having in view that we intend to develop a probiotic product for the infant use.

## Conclusion

Our results showed that the interactions between epithelial cells and probiotic bacteria are complex and involve complex cross-talk communication mechanisms involving both the physical interaction between bacterial and epithelial cells that is, adhesions and complementary receptors and the modulation of pro-inflammatory and anti-inflammatory molecules. Our *in vitro* studies are demonstrating that the selected probiotic strains are antagonizing the adherence and colonization of HeLa cells by the enteropathogenic strains isolated from pediatric diarrhoea mainly by direct competition for the adherence sites, demonstrating their potential use in the treatment of pediatric gastro-intestinal disorders, as an alternative or in association with antibiotics. A great advantage of the selected probiotic strains is their low cytotoxicity and ability to induce a beneficial cytokine response in the epithelial cells, which potentiates their antimicrobial activity by stimulating the rapid immune response following the intestinal injury. Different probiotic fractions have shown specific features, demonstrating the utility of this approach for the selection of the most appropriate LAB culture fraction, in order to maximize their beneficial, antimicrobial and immunomodulatory activities and to reduce the side effects on the host.

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