

## Study of adhesive and invasion capacity of some opportunistic enterobacterial strains and interaction with probiotics

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### Abstract

*The specific interaction between pathogenic or opportunistic bacteria and the host cells could determine in certain conditions the occurrence of infectious diseases. A great number of E. coli strains could induce a great variety of infectious diseases in adult and infant population as: intestinal, urinary tract infections or septicemia. Opportunistic and pathogenic bacteria exhibit specific adhesins, which mediate the bacterial adhesion to susceptible host cells having as consequence intestinal mucosa colonization. Bacterial attachment to epithelial cells represents the first step in bacterial pathogenesis. Many studies have shown that Lactobacillus and Bifidobacterium species frequently isolated from the small intestine and colon could prevent bacterial adherence and invasion of susceptible cellular substratum, so different strains of these species have been selected and introduced in different probiotic products.*

*The purpose of this study was to investigate invasion and adhesion capacity of some E. coli strains to CaCo-2 cells comparatively in the presence/absence of Lactobacillus acidophilus and Bifidobacterium bifidum, which could exhibit an antagonistic effect by preventing the attachment of opportunistic bacterial strains to the host cells by steric hindrance. HEp-2 cells were cultivated in 80% confluent monolayers in 24 multiwell plates and subsequently infected with mixed bacterial suspensions. The results were appreciated by semiquantitative assay in optic microscopy and quantitative assay by CFU counting. Our results showed that the ability of enterobacterial strains to adhere and invade the cellular substratum was decreased or minimized in the presence of anaerobic strains. The inhibitory effect of the studied probiotic strains were different depending on the tested E. coli tested strain, which is meaning that the trials of a strain as a candidate for the probiotic status must be extensive, using a lot of strictly pathogenic and opportunistic microbial strains, with different origins.*

**Keywords:** enterobacterial opportunistic strains, Lactobacillus acidophilus, Bifidobacterium bifidum, probiotic products

## Introduction

Intact intestinal epithelium together with normal intestinal microbiota constitute an efficient barrier against pathogenic bacteria protecting the host and assuring the normal functioning of the intestine. When normal microbiota or epithelial cells undergo modifications, alterations of barrier effect could occur and the increased epithelial cell permeability facilitates the invasion of the intestine by pathogenic bacteria (Tancrede, 1992). The interaction between pathogenic and opportunistic bacteria and susceptible host cells could have as consequence the occurrence of infectious diseases (Chauviere et al., 1992; Bouloche et al., 1994). *E. coli* strains, as well as other opportunistic bacteria could determine a great number of infections with different localization, some of them being acquired by endogenic contamination, especially in immunocompromised patients. Enterobacterial strains which determine intestinal infections exhibit specific adhesins that mediate bacterial adhesion and subsequent colonization of intestinal mucosa (Salyers and White, 1994; Low, 1994, Bezirtzoglou, 1997).

Nonpathogenic anaerobic bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera could inhibit the adhesion and invasion capacity of some enteropathogenic enterobacterial strains. Some of these strains have recently been selected and introduced in different probiotic products due to their beneficial biological effects (Wilson, 1995). The adhesion of *Lactobacillus* to the epithelial intestinal cells is a key property because it prevents their clearance from the intestine on one side and on the other side the adherence of pathogenic bacteria by competition for the adherence sites (Reinheimer, 1990; Salminen et al., 1996).

In the small intestine this property could determine the occurrence of a transitory barrier effect (Andremont, 1990; Bezirtzoglou, 1997), as consequence of some physiological properties, especially the production of lactic acid and low pH, acting as inhibitors of associated bacteria growth rate. *Lactobacillus* are also able to produce other antibacterial compounds as hydrogen peroxide, bacteriocin-like substances and biosurfactants (Wilson, 1995, Velraeds, 1996)

The probiotic strains of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* could improve the anti-infectious host defense mechanisms both at gastric and intestinal level (Falk, 1998; Granatto, 1999).

The purpose of this study was to determine by qualitative and quantitative assays the adherence and invasion capacity of some *E. coli* strains to the cellular substratum represented by HEp-2 cells in the presence and also in the absence of some *Lactobacillus acidophilus* and *Bifidobacterium bifidum* strains, as well as the possible antagonistic effect that these probiotics could develop against the opportunistic enterobacterial strains adherence capacity to the host cells.

## Materials and methods

### Bacterial strains

In this study we analyzed five opportunistic *E. coli* strains with the following isolation sources: *E. coli* 86 and *E. coli* 89 from urine cultures; *E. coli* 111 and *E. coli* 115 from stool cultures from patients with acute diarrhea; *Enteroadgregative E. coli* 42-3 reference strain provided by Dept. of Health – Microbial Disease Laboratory, Berkeley, California, USA).

The anaerobic bacterial strains used as probiotics were *Lactobacillus acidophilus* as a liophilised product containing live cells named “Enterolactil”, made by Bactolact S.A.

Bucharest (being in clinical study) and *Bifidobacterium bifidum* - Microbiology Laboratory Medical School University of Ioannina, Greece.

## Methods

The enterobacterial strains were cultivated on nutritive agar and passed on 20 ml nutrient broth and incubated for 24 h at 37°C. These overnight cultures were centrifuged and the sediment was taken in PBS and adjusted to an optical density value of 2, corresponding to  $1 \times 10^9$  CFU/ml. This suspension was diluted in EAGLE MEM to a final concentration of  $1 \times 10^7$  CFU/ml.

Anaerobic bacteria were cultivated in Agar Columbia (Oxoid) medium and incubated at 37°C in anaerobic chambers with Gas Packs (BBL Anaerobic System, Becton Dickinson). The CaCo-2 cell line was cultivated in EAGLE MEM (supplemented with 10% fetal calf serum, 1% glutamine, 1% penicillin 200 IU/ml, 100 µg/ml streptomycin, 20 I.U./ml fungizone), I.T.S. (*Insuline – Transferrine – Selenium*, a Sigma growth promoter) and 80% confluency monolayers were obtained after 48h of incubation at 37°C in 24 multiwell plates (Cell-Cult, UK).

*In vitro* testing of the influence of anaerobic bacteria on adhesion and invasion capacity of *E. coli* strains was performed by using a modified Cravioto method (Cravioto, 1979). For each strain two 24 multiwell plates were used, one of them with circular coverslips of 13mm diameter (Chance Propper Ltd. –England), subsequently used for optic microscopic examination of infected cell monolayers.

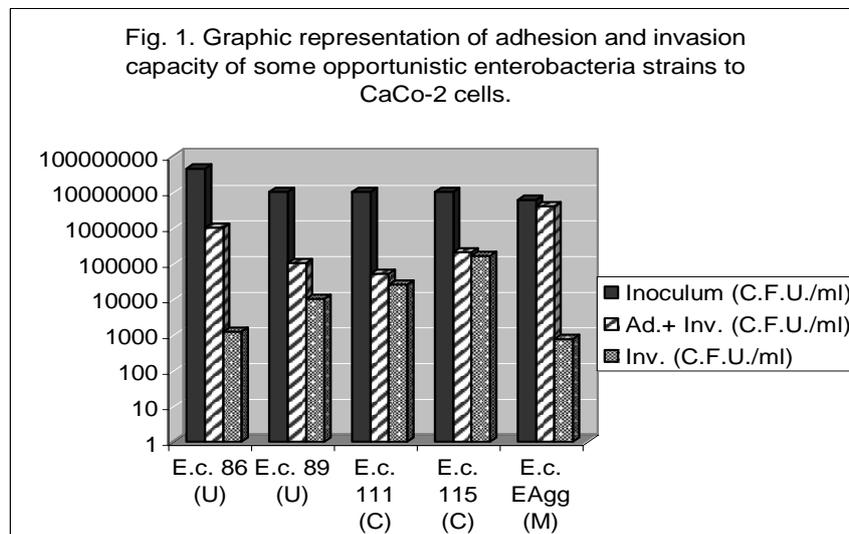
The cell monolayers were infected firstly with 1ml enterobacterial suspension/well after growth medium removal and, in a second experiment, with mixed suspensions of 1:1, v/v enterobacteria and anaerobic bacteria, respectively, prepared in EAGLE MEM without antibiotics. For each strain two wells were used on each plate. The plates were incubated for 2 h at 37°C and after the monolayers were washed 4 times in PBS and 1 ml EAGLE MEM without antibiotics was added in the wells destined for adhesion plus invasion assay and respectively 1 ml EAGLE MEM supplemented with gentamycin was added in the wells destined to invasion assay. The plates were incubated again for 1h at 37°C and after washed one time in PBS.

In the first plate 1 ml of TRITON X-100 (1% solution in PBS) was added in all wells and the plate was incubated for 5 minutes at 37°C. In this step TRITON X-100 induced the epithelial cells lysis and the release of all adherent and internalized bacteria. From the resulted suspensions there were prepared serial dilutions in PBS (from  $10^{-1}$  to  $10^{-8}$ ) which were seeded on nutritive agar for CFU counting. The second plate was similarly processed, but without adding TRITON X-100. The coverslips were taken out of the wells, fixed in methanol, GRAM stained making possible the differentiation between GRAM positive anaerobic bacteria and GRAM negative enterobacteria, and examined in optic microscopy, to determine the pattern of adherence. The photographs were performed using a Contax camera adapted to microscope.

## Results

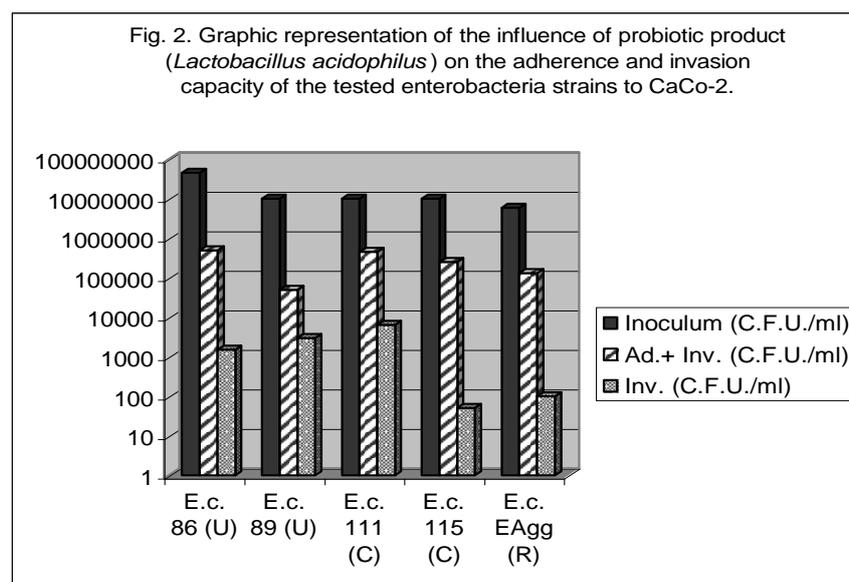
Our study was performed in the purpose of determining by qualitative and quantitative assays the adhesion and invasion capacity of some *E. coli* strains to the cellular substratum in the absence and in the presence of some anaerobic bacterial strains. The results of quantitative assays of adhesion and invasion capacity showed that the EAggEC reference strain exhibited

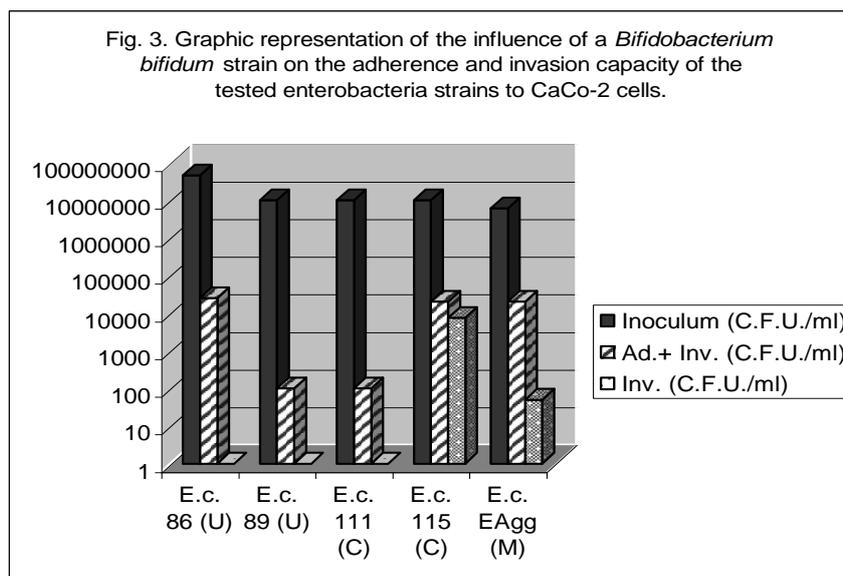
the greatest adherence, the invasion capacity being more significant to the strains isolated from stool cultures (fig. 1) and which also presented an intense diffuse pattern of adherence.



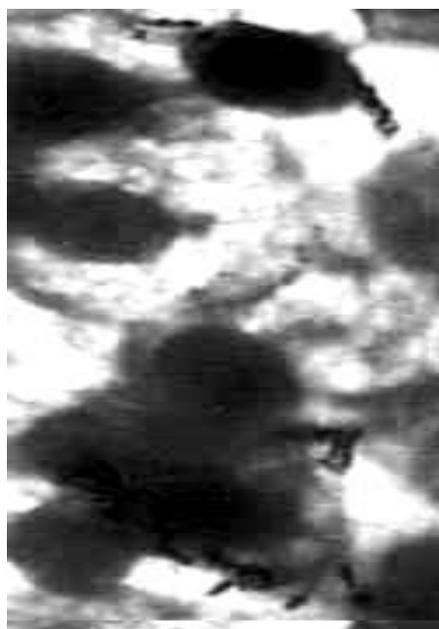
The simultaneous adding of probiotic bacteria in this experiment showed that *Lactobacillus acidophilus* and *Bifidobacterium bifidum* significantly decreased the adhesion and invasion ability of all tested enterobacterial strains, but the inhibitory effects of the tested probiotic strains were different depending on the tested *E. coli* strain.

*Lactobacillus acidophilus* significantly reduced the invasion capacity of all strains (fig. 2) compared to *Bifidobacterium bifidum* which selectively exhibited an inhibitory effect on enterobacterial strains isolated from stool cultures. *Bifidobacterium bifidum* also exhibited a strong inhibition of the invasion ability, especially of the urinary strains, in these cases the invasion being even abolished an intensified inhibitory effect of adherence ability of EAggEC reference strain (fig. 3).

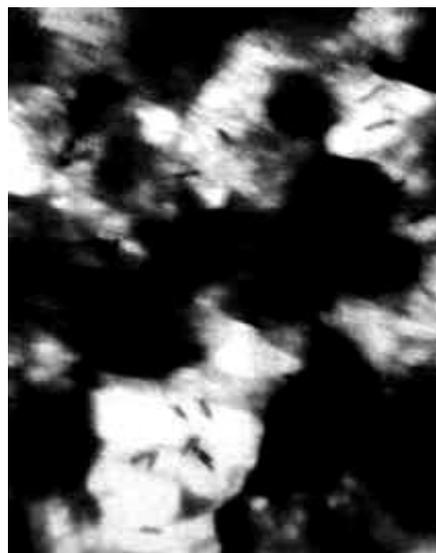




The inhibitory effect of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* strains could be attributed to the competition for attachment sites at the level of intestinal epithelium as demonstrated by microscopy (fig. 4-5) and probably due to some metabolic products. This last mechanism is not valuable for *Lactobacillus acidophilus*, because the live bacterial cells contained in the tested product, although in viable state, did not exhibited any growth on Columbia medium.



**Fig.4.** Optic microscopy showing the interspecific competition between *EAgg E. coli* and *Lactobacillus acidophilus* with the reduction of *EAggEC* (reference strain) adherence capacity (Gram stained smear) (x2500)



**Fig.5.** Optic microscopy showing the interspecific competition between *E. coli* and *Bifidobacterium bifidum* with the reduction of *E. coli* 115 adherence to the cellular substratum (Caco-2 cells) in the presence of *Bifidobacterium bifidum* (Gram stained smear) (x2500)

## Conclusion

The inhibitory effects of anaerobic bacteria on adhesion and invasion capacity of enteropathogenic opportunistic strains is pleading for the use of these strains in probiotic products in order to prevent infectious diarrheal diseases, acting by improving the anti-infectious intestinal barrier and by inhibiting enterobacterial strain multiplication in the normal intestinal microbiota.

Our study also proves that CaCo-2 cells constitute a more appropriate *in vitro* model, for the study of interspecific competition between enterobacterial and anaerobic strains with probiotic potential, being more permissive comparatively with similar studies on HEp-2 cells previously performed.

The inhibitory effect of the studied probiotic strain were different depending on the tested *E. coli* strain, which is meaning that the trials of a strain as a candidate for the probiotic status must be extensive, using a lot of strictly pathogenic and opportunistic microbial strains, with different origins.

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