Colonization of intestinal mucosa and barrier effect of *Lactobacillus brevis* 16GAL

Vasilica BARBU* and Carmen BALOTESCU**

* Galati *Dunarea de Jos* University, Faculty of Food Science and Engineering, Bioengineering Dept., 47, Domneasca St., 800008 Galati, Romania., Tel./Fax: +40 236 460165, e-mail: vasilica.barbu@ugal.ro
** University of Bucharest, Faculty of Biology, MICROGEN – Center for Research in Genetics, Microbiology and Biotechnology, 1-3 Aleea Portocalilor, Sector 6, 060101 Bucharest, Romania

Abstract
Lactobacillus brevis 16GAL strain has been isolated from wheat epiphyite microbiota, characterized and identified by means of API 50CHL microtest systems. By the qualitative method, the specificity of probiotic strain adhesion to eukariote cells has been proved, using as underlayer – the *HeLa*.stabilized cellular line. Its competitivity for adherence sites has been compared to different pathogen species: *E. coli*, *Listeria* sp., *Staphylococcus* sp., *Salmonella* sp. It was elaborated an *in vivo* experimental design to study the protective role of the *Lb. brevis* 16GAL pure culture on the intestinal microbiota against infection with *Salmonella enterica* serovar. *enteridis*.

Key words: *Lactobacillus brevis* 16GAL, adherence pattern, *HeLa* cells, barrier effect, intestinal mucous.

Résumé
La souche *Lactobacillus brevis* 16 GAL a été isolé du microbiote épiphyte du blé, caractérisé et identifié à l'aide des systèmes microtest API 500CHL. Par la méthode qualitative, on a démontré la spécificité de l'adhésion du souche probiotique à des cellules eucaryotes, utilisant comme sous-couche – la ligne cellulaire établie *HeLa*. On a comparé la compétitivité de celle-ci pour les situs d'adhésion par rapport à des différentes espèces pathogènes *: E coli*, *Listeria* sp., *Staphylococcus* sp., *Salmonella* sp. On a élaboré un modèle expérimental *in vivo* pour étudier le rôle prophylactique de la culture pure de *Lb.brevis* 16 GAL sur le microbiote intestinal envers l'infection avec *Salmonella enterica* serovar. *enteridis*.

Mots clé : *Lactobacillus brevis* 16 GAL*,* adhérence, cellules *HeLa*, effet de barrière muqueuse intestinale.

Rezumat
Tulpina *Lactobacillus brevis* 16GAL a fost izolata din microbiota epifita a graului, caracterizata si identificata cu ajutorul sistemelor microtest API 50CHL. Prin metoda calitativa, s-a demonstrat specificitatea adeziunii tulpinii probiotice la celule eucariote, utilizand ca substrat - linia celulara stabilizata *HeLa*. S-a comparat competitivitatea acesteia pentru situsurile de aderare, fata de diferite specii patogene: *E. coli*, *Listeria* sp., *Staphylococcus* sp., *Salmonella* sp. S-a elaborat un model experimental *in vivo* pentru a studia rolul profilactic al culturii pure de *Lb. brevis* 16GAL asupra microbiotei intestinale fata de infectia cu *Salmonella enterica* serovar. *enteridis*.

Cuvinte cheie: *Lactobacillus brevis* 16GAL, aderență, celule *HeLa*, efect de barieră, mucoasa intestinală.

1. Introduction

Because of commercial interest for functional food containing probiotics, the scientific interests in these products has also increased. Recently, the researches in the food biotechnologies are centered on careful isolation and selection of new „safety" strains of *Lactobacillus* that could ensure the microbiological security of the food and bring benefits to the consumer's health. The species of *Lactobacillus* belong to human intestinal microbiota, and by producing vitamins and enzymes, they have a positive effect on host human metabolism. By producing antimicrobial compounds they can bring

University *Dunarea de Jos* of Galati
Faculty of Food Science and Engineering
The paper has been presented at the International Symposium **Euro - aliment 2007** 20 – 21 September 2007

therapeutic benefits to the host body checking the pathogen proliferation. Probiotics are effectively being used in the food industry, agriculture and human and veterinary medicine (Tancrède, 1992, Vaughan, 1999).

Adherence is a key factor concerning the colonization of some specific sites and the survival of microorganisms in different habitats depends on their ability to adhere to various surfaces or underlayers. The adherence process involves an interaction between complementary molecules on the surface of microorganisms (adhesines) and underlayer (receivers). Biofilm cells that are built are entirely different from the phenotype point of view compared to their planktonic shape having a higher strength to stress conditions, achieving an elementary "homeostasis", a metabolic, physiologic cooperation, being similar to tissues made of eukaryote cells (they show integrality – a key property of a biological system) (Costerton, 1995). In order to adhere, cell viability is not indispensable. Thus the adherence and invasion ability of some pathogenic cells to human intestinal cells cultivated *in vitro* by the preadhesion of some *Lb. acidophilus* cells, alive or heat-killed, has been inhibited. The experiment has proved that when lactobacilli occupy the attachment sites, these sites are not available for enteropathogen adherence (Coconier et al., 1993, Coconier et al., 1997). *De novo* synthesis of adhesines is carried out with energy consumption and that is why cells in the exponential stage of growth adhere more quickly to underlayers than those in the steady stage, that are old or dead.

In line with the current research concerning scientific, economic and especially medical importance of probiotics, the present study approaches the adherence of a *Lactobacillus brevis* 16GAL strain, based on the elaboration of some *in vitro* and *in vivo* experimental models, to certify its probiotic character.

2. Materials and methods

Specificity of *Lactobacillus brevis* 16GAL strain adhesion to eukaryote cells and tissues through *in vitro* studies has been proved by using the HeLa stabilized cellular line as cellular underlayer. In order to test the adherence ability the qualitative method has been used (Craviato at al. method, 1979, changed by Nataro and Kaper, 1998), and evaluation has been done by optic microscopy. Bacteria growth on continuous single-layer cellular cultures ("immortalized" tumoural cells that can be maintained for an indefinite number of generations) is an economic true but time-consuming method. The method's principle consists of infecting the single-layer cellular cultures, with a junction of 80%, with tested strains, thermostation and coloration through the Giemsa method, followed by the examination with an immersion lens microscope in order to set the adherence pattern. Microscopic analysis also allows a semiquantitative analysis of intensity extent related to bacteriological adherence to cellular underlayer, evaluated by 1-3 „+" signs. Strains marked with „++" or „+++" are considered intensively adherent.

The tested strain comes from a 24-h fresh culture, in MRS broth used for preparing the working suspension (in Phosphate Buffered Saline) with a density comparable to Mc Farland 0,5 standard (corresponding to 10^8 cells/ml).

The *HeLa* cells have been cultivated in Eagle *Minimum Essential Medium* MEM environment (EMEM, Gibco) enriched with 10% bovine fetal serum (Gibco BRL) thermal inactivated (30 min. at 56°C), with 0.1 mM inessential amino-acids (Gibco BRL) and with 0.5 ml gentamicin (50 mg/ml) (Gibco BRL). The *HeLa* cells are cultivated in *multi-well* plastic plates with 6 rooms, up to a junction of 80-100% (24-h thermostation, at a temperature of 37°C).

The *HeLa* cellular single-layers are washed three times in PBS, phosphate buffer sterile. Fold 1 remains the reference fold (without strain to be tested), In fold 2, 2 ml of strain to be tested are added, and in folds 3 – 6 only 1 ml of analyzed strain suspension is added because, 1 ml from different cultures of pathogenic strains is added: in 3 - *E. coli*, in 4 – *Listeria* sp., in 5 - *Salmonella* sp., in 6 – *Staphylococcus* sp. (to evaluate competition compared to pathogens for adherence sites) and they are thermostated for 2h, at a temperature of 37°C. Single-layers are washed 3 times with PBS, fixed with cold ethanol for 8–10 minutes, coloured with Giemsa solution (1:20) (Merck, Darmstadt, Germany) and maintained for 20 minutes, at a temperature of 37°C. Plates are washed, dried at room temperature over night, examined by an immersion lens microscope (magnification 2500X) and photographed by a Contax camera adapted to the microscope.

University *Dunarea de Jos* of Galati
Faculty of Food Science and Engineering
The paper has been presented at the International Symposium **Euro - aliment 2007** 20 – 21 September 2007

It was elaborated an *in vivo* experimental design to study the protective role of the *Lb. brevis* 16GAL pure culture against infection with *Salmonella enterica* serovar. *enteridis*.

Conventional (holoxenic) rats, 6-8 weeks old, purchased from Darvari farm (Bucharest) were used in these experiments. In order to obtain comparative results the animals were grouped in different batches, each with 5 holoxenic rats: **batch 1**-negative control, **batch 2**-infection control with oral administration, of a single dose of 2ml/animal of *Salmonella enterica* serovar *enteritidis*, suspension containing ~10^8CFU/ml, **batch 3**-probiotic control with administration of a daily dose of 2ml/animal of probiotic strain *Lb. brevis* 16GAL, in liquid form, represented by mid-logarithmic phase culture. The animals from **batch 4** have received simultaneously the infection dose of *Salmonella enterica* serovar *enteritidis* as well as the probiotic (gived and 3 days in succession, after infection). All the animals were fed normally by solid, dehydrated food (300 g/day).

During that experiment were determined: a daily microbiological analysis in faeces; a final microbiological analysis, in different regions of the intestine (duodenum, jejune, colon), drawn after the animals were euthanized, 4 days after infection. The faeces, as well as the intestinal tissue specimens were weighted and homogenised in sterile phosphate buffer saline (PBS). Serial ten-fold dilutions from homogenate were plated (0,1 ml/plate) onto different media, as follows:

- MRS solid medium for the isolation and counting of LAB, incubated at 37°C for 24 hrs;
- MacConkey medium (Microbiologycal Manual, Merck, 2000) for the isolation and counting of lactofermentative microbiota (LFM) component, as well as of *Salmonella enterica* serovar *enteritidis* incubated at 37^0C for 24 hrs.

3. Results and discussion

Results of the ***in vitro* test with pure cultures *Lb. brevis* 16 GAL** showed it has a diffuse adherence model (+++), when pathogens are absent, forming a uniform border on the entire surface of *HeLa* cell plasmatic membrane and blocking the pathogen adherence sites as illustrated in Figure 1.

Competition with pathogens for cellular underlayer represented by the *HeLa* cells has driven the change of adherence phenotype and its intensity. The probiotic strain has the ability to inhibit the pathogen adherence to *HeLa* cell underlayer in a proportion of 5-25%. The *Lb. brevis* 16GAL strain shows a localized-aggregative adherence pattern in competition with pathogens forming microcolonies or aggregates by overlapping cells like “bricks” in certain areas on the cell surface, where they find specific receivers. This adherence model can be noticed in Figures 2, 3, 4 and 5.

Figure1. Type of diffuse adherence developed by *Lb.* brevis 16 GAL strain

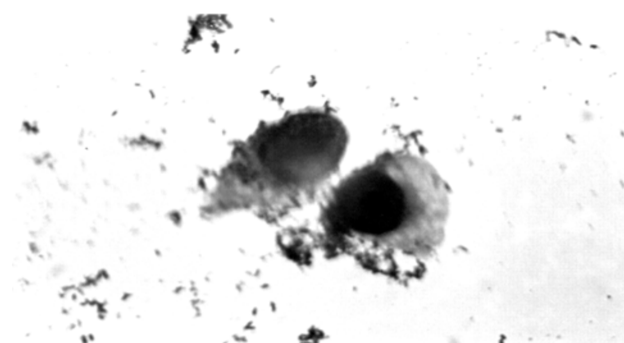

Figure 2. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *E. coli*

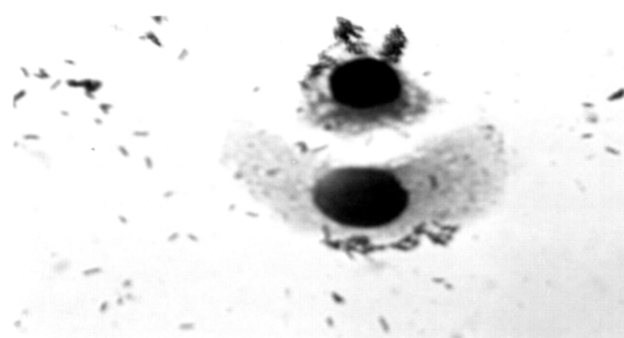

Figure 3. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Salmonella* sp.

Competitive inhibition has been quite obvious compared to *E. coli, Salmonella* sp. and *Staphylococcus* sp. it is very good (+++) and less

University *Dunarea de Jos* of Galati
Faculty of Food Science and Engineering
The paper has been presented at the International Symposium **Euro - aliment 2007** 20 – 21 September 2007

obvious (++) compared to *Listeria* sp. In the colonization stage, cells irreversibly linked to underlayer, but not between them, form a continuous single-layer, and those linked either to underlayer and between them, form microcolonies and biofilms.

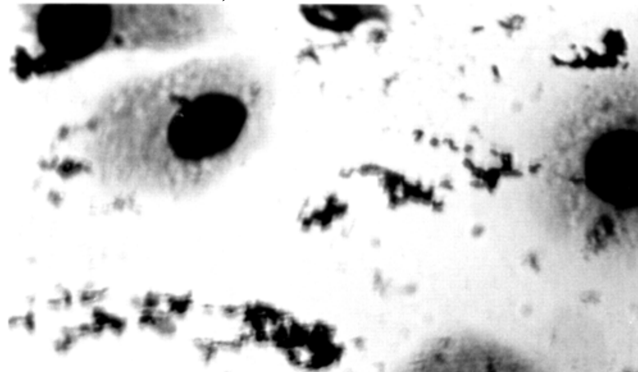

Figure 4. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Staphylococcus* sp.

Microorganism adherence is a slow phenomenon followed by their multiplication with higher growth rates compared to those of planktonic microorganisms. Biofilms become multilayered afterwards, their thickness may vary from a few µm to a few mm and facilitate colonization of other microorganisms unable to do it alone (Lazar, 2004).

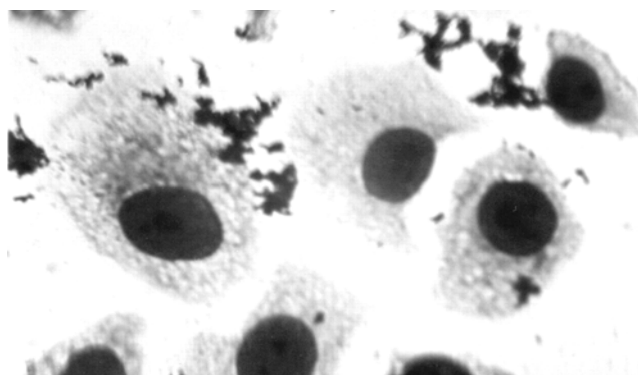

Figure 5. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Listeria* sp.

Results of the ***in vivo*** **test with pure cultures *Lb. brevis* 16GAL** showed that normal microbiota eliminated in faeces is represented exclusively by lactose-fermentative microorganisms (LFM), when plated on MacConkey agar exhibiting densities of 10^7 CFU/g of faeces. When plated on MRS solid medium, the faeces of control animals showed high densities of LAB (9×10^{11} CFU/ml) (figure 6.).

Qualitative and quantitative characterisation of normal intestinal microbiota colonising different segments of the the intestinal mucosa (duodenum, jejun, colon) in rats showed that the lactose non–fermentative microbiota (LNF) colonizes especially the colonic intestinal segment ($\sim10^3$ CFU/g), but less dominant comparatively to the lactose-fermentative microbiota (LFM), found in high densities in ileum (7×10^2 CFU/ml), as well as in colon ($\sim5\times10^4$ CFU/g). The cultivation of intestinal specimens on MRS solid medium revealed high LAB counts in duodenum (7×10^6 CFU/g), as well as in colon (6×10^7 CFU/g), the LAB microbiota being absent in ileum (fig.7.).

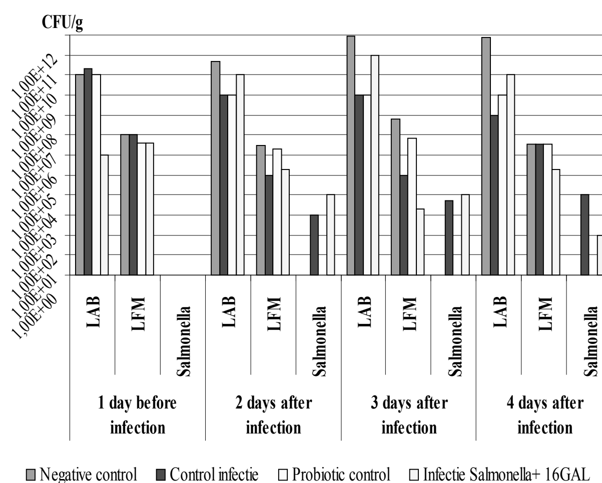

Figure 6. Microbiological analysis for LAB, LFM and *Salmonella* sp. in faeces of the rats

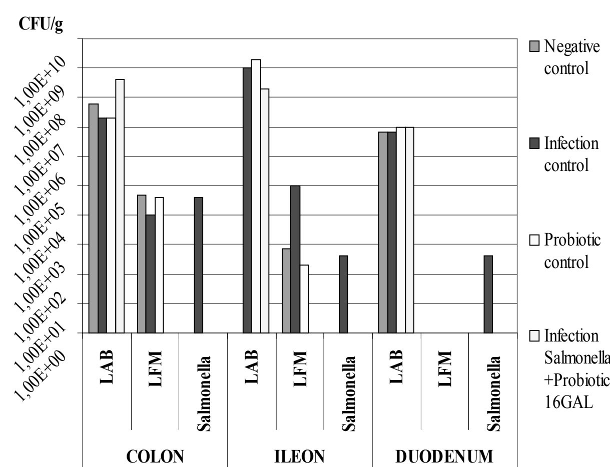

Figure 7. Microbiological analysis for LAB, LFM and *Salmonella* sp. in different segments of the intestinal mucosa

As far as the infection control batch is concerned, the removal of pathogenic agent in faeces after infection has been noticed (10^3CFU/g in the second day, 5×10^3 CFU/g in the third day and 1×10^4 CFU/g

in the fourth day) and was done by a rate of ~ 40-50% from the infection dosage (~10^8 CFU/g). The removal of pathogenic agents took place in conjunction with a decrease of the lactic acid bacteria removal rate (10^8–10^9 CFU/g compared with the negative control (10^{10}–9×10^{11} CFU/g). The LFM removal rate decreases as well from 10^7 to 10^5 CFU/g (fig.6.). When animals are slaughtered and fragments of intestinal tract are sampled, it was noticed that the pathogenic bacteria colonizes the duodenum and ileum with similar rates ($4\text{x}10^2$CFU/g intestinal content), and the colon with higher rates ($4\text{x}10^4$CFU/g) (fig.7.).

In contrast with the negative control batch, a redistribution of the lactic acid bacteria population was noticed; these population colonizes with high densities (>10^6 CFU/g) all the 3 sections analysed (duodenum– 7×10^6 CFU/g, ileum – 10^9 CFU/g, colon – 2×10^7 CFU/g). Therefore, these results could suggest that following the entrance of an infectious allochthonous agent into the alimentary duct, a sudden increase of the normal microbiota density and its homogenous distribution take place on the whole alimentary duct, in order to ensure the lock of colonization sites. A rise of lacto-positive microorganisms density can also be noticed in regions close to the intestinal tract (ileum), that are normally less colonized with microorganisms due to the presence of some restrictive factors of multiplying bacteria in the small intestine represented by: gastric acidity, intestinal peristaltis ensuring the relatively quick transit to the large intestine and existence of some inhibitors of microorganisms growth. If for the LFM negative control batch, a density of 7×10^2 CFU/g was reached in ileum, after the pathogenic agent supply densities of 10^5 CFU/g were reached in ileum and 5×10^4 CFU/g in colon. LFM were not found in duodenum (figure 7).

For <u>the probiotic control batch</u>, the supply of the probiotic prepared represented by the culture of *Lactobacillus sp.* 16GAL in the logarithmic stage, produces a quantity change in the lactic acid bacteria population released (from 10^{10} – 9×10^{11} CFU/g faeces for the negative control batch to 10^9 CFU/g for the probiotic control batch) (figure 6.). After animal slaughtering and quantity analysis of microbiota that colonizes various sections of the alimentary duct, it results that lacto-positive microbiota is not present in the duodenum; it is present only in ileum and colon, but with a lower density than for the negative control batch due to the change of physicochemical parameters of the intestinal biotope under the action of supplied lactobacilli, leading to the decrease of allochthonous microorganisms viability. In exchange, all analysed sections of the intestinal tract were colonized with lactic bacteria with densities of 10^7–2×10^9 CFU/g intestinal content (duodenum: 10^7CFU/g intestinal content, ileum: 2×10^9 CFU/g intestinal content, colon: 2×10^7 CFU/g intestinal content) (figure 7). As a result of the probiotic supply, the lactic acid bacteria also colonizes the ileum with higher densities, this intestinal section being poorly represented in microorganisms normally (due to the higher peristaltis as well as due to high levels of digestive enzymes).

As far as the animal batch is concerned where <u>experimental infection</u> <u>has been simultaneously done with the start of probiotic product supply</u> (that has been continued for 3 days), it was noticed that lactobacilli supplied favor a quicker removal of the *Salmonella* cells, from 10^3CFU/g of the infection control batch to 10^4 in the first days (II, III) simultaneously with a reduction by 10–100 times of the removed normal microbiota density, that probably sticks to intestinal sites, in order to lock the pathogenic agent adherence. The analysis of microbial composition related to different sections of alimentary duct has confirmed these results, showing the decrease of the lacto-positive and lacto-negative microbiota density in all reviewed sections, with the preservation of a higher lactic bacteria density on the entire intestinal tract, these bacteria showing strength to the new physico-chemical conditions produced (figure 7). The adherence of lactic acid bacteria to these sites could explain the absence of pathogenic bacteria at experimental batches where the pathogenic agent and probiotic product have been supplied while the pathogenic agent densities for the infection control batch in all intestine segments are high. Therefore, the lactic bacteria prevent enteropathogenic colonization of the intestinal mucous.

4. Conclusions

- When pathogens are absent, *Lb. brevis* 16 GAL strain shows a diffuse adherence pattern (+++) to *HeLa* cell underlayer, in competition with pathogenic species, and adherence phenotype, it turns into localized-aggregative.

University *Dunarea de Jos* of Galati
Faculty of Food Science and Engineering
The paper has been presented at the International Symposium **Euro - aliment 2007** 20 – 21 September 2007

- The supply of lactobacilli culture determines the *Salmonella* removal in faeces, in a significant ratio, reducing simultaneously the removal of normal microbiota, that adheres and colonizes more intensively the intestinal tract, on its entire length, shutting off the pathogen's adherence sites. This effect is shown both during the prophyilactic supply (repeated dosages), simultaneous supply (in sole dosage) and in case of therapeutically supply (repeated dosages, post-infection). This allows the selection of different supply methods concerning these products as probiotics, depending on various economic or process reasons.
- Concerning the impact of lactobacilli supplied in probiotic preparations over the native lactic acid microbiota composition, it was noticed that lactobacilli supply leads to a rise of lactic acid bacteria density, both of those colonizing different sections of intestinal tract, and those luminale, eliminated in faeces.
- Lactic acid bacteria are able to produce antipathogenic adesyne called *biosurfactants* with inhibiting effect over a wide range of pathogenic agents including the *Salmonella* species. Perhaps these proteic character biosurfactants are produced *in situ,* process favoured by a low pH of the medium created by producing acids e.g. lactic acid, acetic acid, ethylene dicarboxylic acid, phenyl-lactic acid.
- The anti-*Salmonella* bacteriostatic or even antibacterial effect showed by probiotic strain is cancelled by the pH neutralization proving this is the major mechanism of the antimicrobial activity and not that of producing bacteriocine.

References

Coconnier, M.H., Bernet, M.F., Chauviere, G. and Servin, A.L. 1993. Adhering heat-killed human *Lactobacillus acidophilus* strain LB, inhibits the process of pathogenity of diarrhoeagenic bacteria in cultured human intestinal cells, *J. Diarrhoeal Research*, **11 (4)**, 235-242.

Coconnier, M.H., Bernet, M.F., Hudault, S., and Servin, A.L. 1997. Antibacterial Effect of the Adhering Human *Lactobacillus acidophilus* Strain L.B., *Antimicrobial Agents and Chemotherapy*, **41 (5)**, 1046-1052.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections*, Science*, **284**, 1318 – 1322.

Lazar, V., Bezirtzoglou, E., Balotescu, C., Cernat, R. Bulai, D, Vadineanu, E. and Tache, E. 2004. Study of adhesive and invasion capacity of some opportunistic enterobacterial strains and interaction with probiotics, *Roum. Biotech. Lett.,* **9 (3)**, 1705-1711.

Tancrède, C. 1992. Role of human microflora in health and disease, *Eur. J. Clin. Microbiol. Inf. Dis*., **11**, 1012-1016.

Vaughan, E.E., Mollet, B., Willem, M. de Vos. 1999. Functionality of probiotics and intestinal lactobacilli : light in the intestinal tract tunnel. *Current Opinion in Biotechnology*, **10**, 505-510.

University *Dunarea de Jos* of Galati
Faculty of Food Science and Engineering
The paper has been presented at the International Symposium **Euro - aliment 2007** 20 – 21 September 2007