IN VITRO AND *IN VIVO* STUDIES ABOUT PROBIOTIC CHARACTER OF *LACTOBACILLUS BREVIS 16GAL* STRAIN

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Abstract: *Lactobacillus brevis* 16GAL strain has been isolated from wheat epiphyite microbiota, characterized from the morphological, physiological and biochemical point of view and identified by means of API 50CHL microtest systems. By the qualitative method, carried out by Craviato et al., modified by Nataro and Kaper in 1998, 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 (with 4 batches of holoxenic rats) to study the protective role of the *Lb. brevis* 16GAL pure culture on the intestinal microbiota against infection with *Salmonella enterica* serovar. *enteridis*.

Keywords: *Lactobacillus brevis*, probiotic, *in vitro*, *in vivo*

INTRODUCTION

Because of commercial interest for functional food containing probiotics, the scientific interests in these products has also incresed. Recently, the researches in the food biotechnologies are centred 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,

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and by producing vitamins and enzymes, they have a positive effect on host human metabolism. By producing antimicrobial compounds they can bring 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.

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 eukariote 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). *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.

MATERIALS AND METHODS

Specificity of *Lactobacillus brevis* 16GAL strain ahesion to eukariote 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 principle consists of infecting the single-layer cellular cultures, with a junction of 80%, with tested strains, their 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 slurry (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 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 analysed strain slurry 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, are fixed with cold ethanol for 8–10 minutes, are coloured with Giemsa solution (1:20) (Merck, Darmstadt, Germany) and are maintained for 20 minutes, at a temperature of 37°C. Plates are washed, dried at room temperature over night, are examinated by an immersion lens microscope (magnification 2500X) and photographed by a Contax camera adapted to the microscope.

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 of 5 holoxenic rats each: **batch 1-**negative control, **batch 2**-infection control with administration, by oral route, a single dose of 2ml/animal of *Salmonella*

enterica serovar *enteritidis*, suspension containing ~10^8CFU/ml, **batch 3**-probiotic control with administration 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, jejun, 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 buffered 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 37^0C 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;

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* 16 GAL 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, 5. Competitive inhibition has been quite obvious compared to *E. coli, Salmonella* sp. and *Staphylococcus* sp. it is very good (+++) and less obvious (++) compared to *Listeria* sp.

Figure 1. Type of diffuse adherence developed by *Lb.* brevis 16 GAL strain

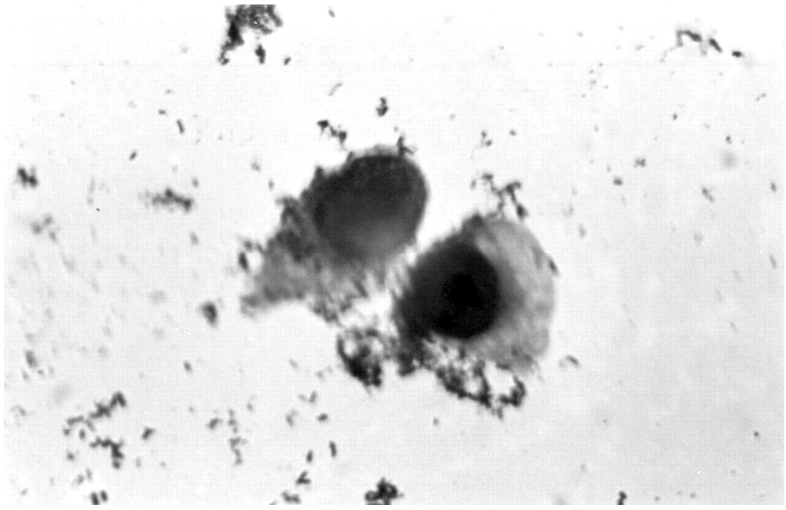

Figure 2. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *E. coli*

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. 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, V., 2004).

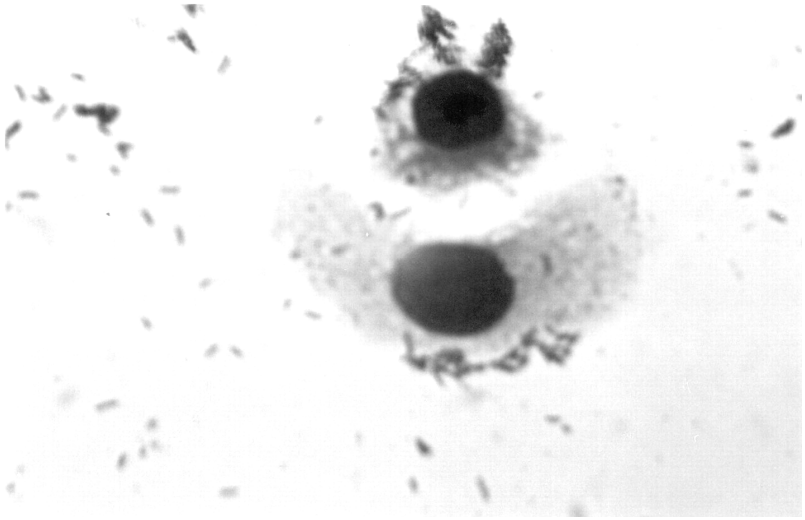

Figure 3. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Salmonella* sp.

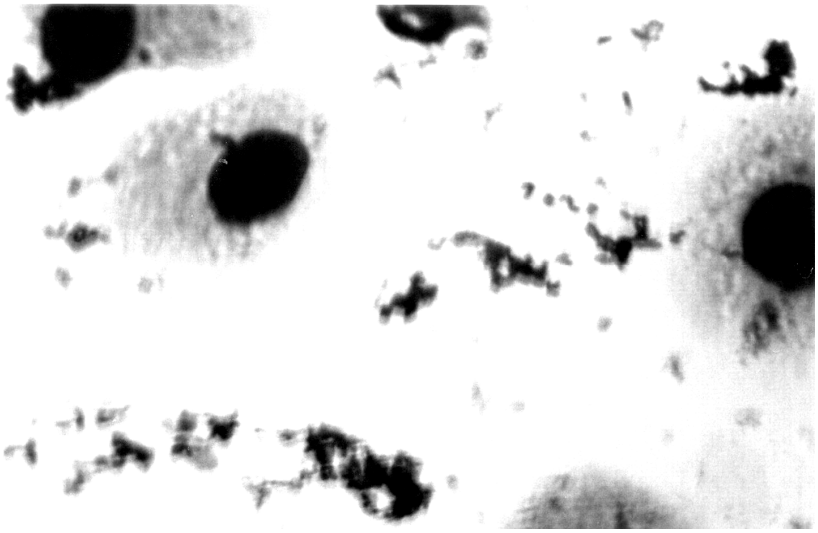

Figure 4. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Staphylococcus* sp.

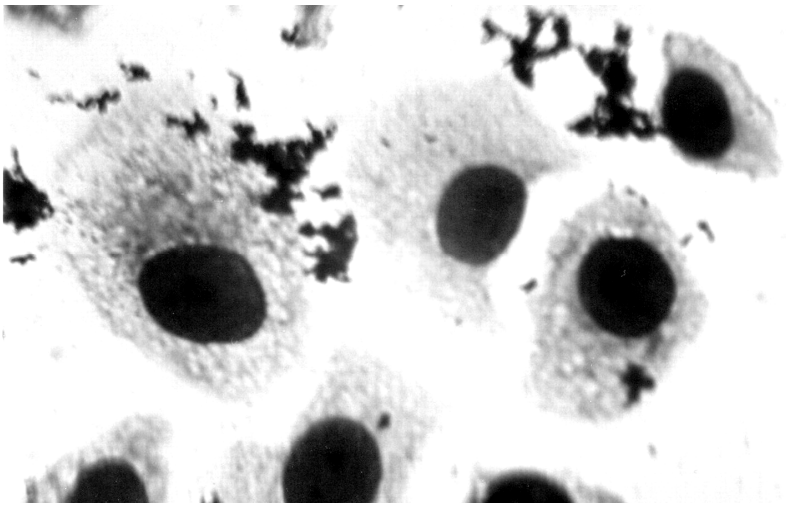

Figure 5. Type of localized-aggregative adherence developed by *Lb. brevis* 16 GAL strain in competition with *Listeria* sp.

Results of the ***in vivo*** **test with pure cultures *Lb. brevis* 16 GAL** showed that normal microbiota eliminated in faeces is represented exclusively by lactose-fermentative microorganisms (LFM), when plated on MacConkey agar exhibiting densities of 10^7CFU/g of faeces. When plated on MRS solid medium, the faeces of control animals showed high densities of LAB ($9x10^{11}$CFU/ml) (Table 1). 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 (~10^3CFU/g), but less dominant comparatively to the lactose-fermentative microbiota, found in high densities in ileum ($7x10^2$CFU/ml), as well as in colon (~$5x10^4$CFU/g). The cultivation of intestinal specimens on MRS solid medium revealed high LAB counts in duodenum ($7x10^6$CFU/g), as well as in colon ($6x10^7$CFU/g), the LAB microbiota being absent in ileum (Table 2).

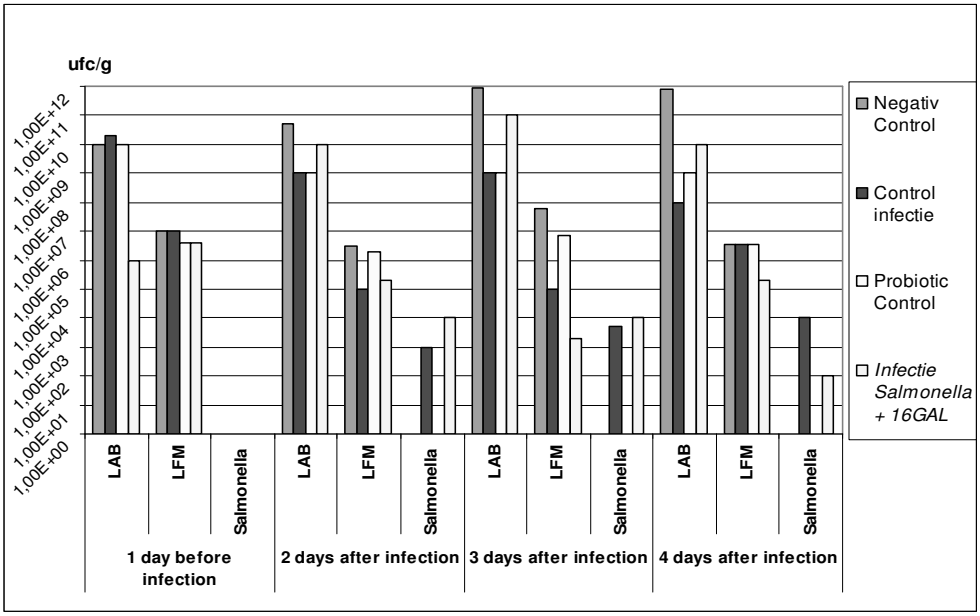

Table 1. Microbiological analysis for LAB, LFM and *Salmonella* sp. in faeces of the rats

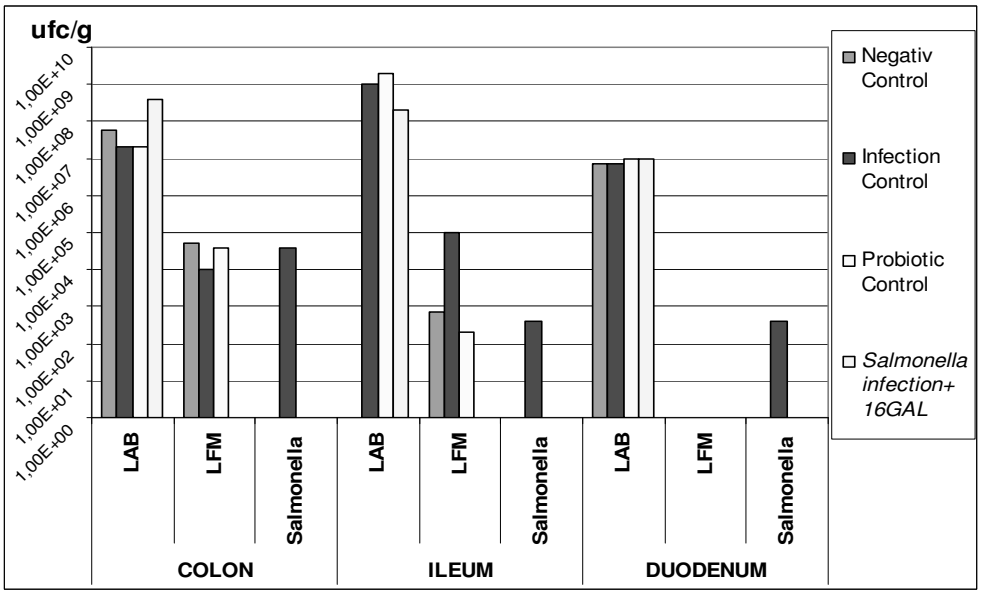

Table 2. Microbiological analysis for LAB, LFM and *Salmonella* sp. in different segments of the intestinal mucosa

Concerning the infection control batch, the removal of pathogenic agent in faeces after infection has been noticed (10^3CFU/g in the second day, $5x10^3$CFU/g in the third day and $1x10^4$CFU/g in the fourth day) and was done by a rate of ~ 40-50% from the infection dosage (~10^8CFU/g).
The removal of pathogenic agents took place in conjunction with a decrease of the lactic acid bacteria removal rate (10^8–10^9CFU/g compared with the negative control (10^{10}–$9x10^{11}$CFU/g). The LFM removal rate decreases as well from 10^7 to 10^5CFU/g (fig.6.). When animals are slaugthered and fragments of intestinal tract are sampled, it was noticed that the pathogenic bacteria colonizes the duodenum and ileum with similar rates ($4x10^2$CFU/g intestinal content), and the colon with higher rates ($4x10^4$CFU/g) (Table 2).
In contrast with the negative control batch, a redistribution of the lactic acid bacteria population was noticed; these population colonizes with high densities (>10^6CFU/g) all the 3 sections analysed (duodenum– $7x10^6$CFU/g, ileum – 10^9CFU/g, colon – $2x10^7$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 existance of some inhibitors of microorganisms growth. If for the LFM negative control batch, they reach a density of $7x10^2$ CFU/g in ileum, after the pathogenic agent supply they reach densities of 10^5CFU/g in ileum and $5x10^4$CFU/g in colon. LFM were not found in duodenum (Table 2).

For the probiotic control batch, 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} – $9x10^{11}$CFU/g faeces for the negative control batch to 10^9CFU/g for the probiotic product control batch) (fig.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, being present only in ileum and colon, but with a

lower density than for the negative control batch due to the change of physico-chemical 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–$2x10^9$CFU/g intestinal content (duodenum: 10^7CFU/g intestinal content, ileum: $2x10^9$CFU/g intestinal content, colon: $2x10^7$CFU/g intestinal content) (Table 2). 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 experimental infection has been simultaneously done with the start of probiotic product supply (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 (Table 2).
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.

CONCLUSIONS

- When pathogens are absent, *Lb. brevis* 16 GAL strain shows a diffuse adherence pattern (+++) to HeLa cell underlayer, and in competition with

pathogenic species, and adherence phenotype, it turns into localized-aggregative.

- 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, and 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 therapeutical supply (repeated dosages, post-infection). This allowing the selection of some different supply methods concerning these products as probiotics, depending on various economic or process reasons.

- As for the impact of lactobacilli supplied in probiotic preparations over the native lactic acid mictrobiota 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.

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