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Alternative ecological strategies used in the prevention and treatment of infections produced by antibotic multiresistant bacterial strains

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Abstract

The dual effect of antibiotics, beneficial-antiinfectious, and negative – the emergence of antibiotic resistance, should determine the rational use of these drugs, careful monitoring of their impact on the natural bacterial community, and also the research on new ecological strategies used for treating animal and human infectious diseases and especially for increasing the biomass. Probiotic microorganisms and prebiotic substances represent one crucial ecological alternative for the prevention and treatment of gastrointestinal and urogenital infections. The mechanisms of probiotic action are not completely elucidated but it is generally accepted that probiotics exhibit an inhibitory antagonistic effect on pathogenic bacteria mucosal colonization by competition for adhesion sites. The in vitro methods used for probiotics and prebiotics selection include hemaglutination and haemaglutino-inhibition tests, qualitative and quantitative adherence and invasion assay, the evaluation of protective antimicrobial effect of lactic acid bacteria (LAB) and mananooligosaccharides by the study of their influence on pathogenic bacteria adherence capacity to the cellular substratum and also by qualitative assay of pathogenic bacteria growth in the presence of potentially probiotic or prebiotic products. This study details the application of these methods for the study of the influence of some mananooligosaccharide-containing products (comercialised or proposed for patenting) on the adherence capacity of some pathogenic bacteria to HeLa cells.

Keywords: prebiotics, manooligosaccharide-containing products, *in vitro* selection, adhesion and invasion, HeLa cells

Introduction

The microbial population and their animal hosts are forming an ecological system (eubiosis state) which is absolutely required for the human and animal health [2,4]. The ecological system microbiota/intestine could be changed by the administration of some antibiotics which kill the sensitive microorganisms generating a disbiosis state which favourise the selection of antibioresistant strains [3]. The long use of antibiotics as growth promoters in subinhibitory concentrations enforced the selection of antibioresistant bacterial strains in the normal microbiota. For example, the increased rates of vancomycin resistance in human enterococci in Europe are the consequence of avoparcin (glycopeptidic antibiotic as well as the vancomycin) use as growth promoter in pigs and poultry farms in the 70's. Tilozin is a 16 C atoms macrolide used as growth promoter in Europe and USA and recently demonstrated to be implicated in the selection of enterococci resistance to all macrolides.

Starting with 1997, EU has forbidden the avoparcin use as growth promoter, and the rapid consequence was the decrease not only of the vancomycin resistance rates in

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enterococcal strains, but also of macrolides and tetracyclins resistance, suggesting that the reduction of the selective pressure could generate the reduction of bacterial strains resistance to other classes of antibiotics. Similar results were noted in Danemark [1], where the restricted use of virginiamycin as food aditive in 1997 was followed by the reduction of virginiamycin resistance from 66,2% in 1997 to 33% in 2000.

The misuse of the antibiotics could also represent an additional selective pressure for the occurrence and persistance of antibiotic resistant bacterial strains in the microbial community, being rapidly disseminated by human, animals, water, food etc. Recent epidemiological studies are concluding that the mankind could face very soon a situation similar to that existent before the antibiotic era, without any efficient antibiotic for at least a five years period, while a lot of antibiotic producers will give up the search for new antibiotic substances due to economical reasons.

The dual effect of antibiotics, beneficial-antiinfectious, and negative – the emergence of bacterial resistance and multiresistance to antibiotics, should determine the rational use of these drugs, careful monitoring of their impact on the natural bacterial community and also the intensive research in the field of finding new antimicrobial agents or new preventive and therapeutic ecological strategies for the infectious diseases, which still remain a major public health problem at global level.

Probiotic microorganims represent one of the ecological alternatives for the treatment of gastro-intestinal as well as urinary tract infections. "*Probiotic"* concept was introduced in 1965 (Lilly and Stillwell) to denominate the substances produced by a protozoan (*Tetrahymena pyriformis*) favourising the growth of other protozoan (*Stylonychia* sp.). In 1974 (Parker) defines as probiotic any food additive exhibiting a positive effect on a host by inducing changes in the intestinal microbiota. Nowadays, the generally accepted definition is that of Fuller, 1989 [13], stating that a probiotic is a live microorganism which addition to food exhibits a beneficial effect on the host organisms by the improvement of the intestinal microbial balance. The probiotic products are using one or more bacterial strains (*Lactobacillus bulgaricus, L. acidophilus, L. casei, L. helveticus, L. lactis, L. salivarius, L.* p*lantarum, Bifidobacterium sp., Streptococcus termophilus*), introduced in the animal food in different forms.

The features of a good probiotic strain are represented by: lack of pathogenicity and toxicity, the survival capacity in the intestinal environment and the induction of a beneficial effect on the receiving host, economic accessibility, stability by preservation.

The mechanisms of probiotics action are not well known, but it is considered that probiotics exhibit a direct, antagonistic effect at the mucosal level by the production of different metabolites such as organic acids, H_2O_2 or macromolecular substances with antimicrobial activity (*bacteriocine – like* substances, antibiotics, biosurfactants), by competition for nutrients and by blocking the mucosal colonization by pathogenic bacteria through competitive inhibition of adherence sites on the surface of intestinal epithelium (Figure 1) as well as an indirect effect, manifested by induction of changes in intestinal microbiota metabolic activity, by enzyme activity modulation and by immunostimulatory effect (macrophages activity stimulation and the increase of sIgA concentration) [5].

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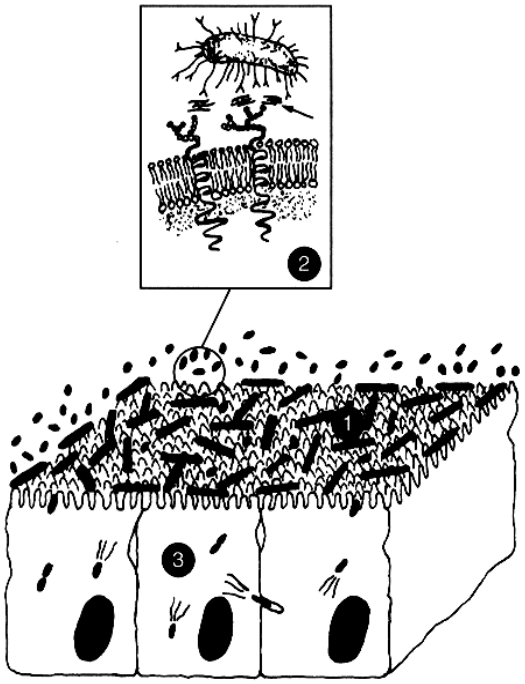

Figure 1. The mechanism of action proposed for the probiotic product represented by *Lactobacillus acidophilus LB* (of human origin), killed by heat. Adherent cells of *L. acidophilus LB* are inducing a steric masking (1) and/or release active compounds, preventing the pathogen adherence to the cellular receptors (1 and 2) and consequently, the invasion of the cellular substratum by enteroinvasive bacteria is diminished (after Coconnier et al., 1993).

One of the main concerns regarding the probiotic administration is to avoid or minimalise the risk of pathogenicity, virulence and resistance genes transfer to the endogenous or pathogenic bacteria present in the receiving organisms. The most controversial probiotic species are represented by *Enterococcus* due to its high incidence in the aetiology of nosocomial infections and its high resistance to different classes of antibiotics.

For example, *Enterococcus faecium* is naturally resistant to: aminoglycosides (gentamycin) due to the secretion of antibiotic inactivating enzymes; beta-lactams (piperacillin, amoxicillin and oxacillin, cefotaxime), by target modification; macrolides by ribosomal methylation. *E. faecium* also exhibit a high level of vancomycin resistance (*van*A operon).

It was demonstrated that *E. francium van*A operon is localized on an R plasmid encoding a sex pheromone synthesized also by *S. aureus*, aspect that could facilitate the transfer of *van*A operon from resistant enterococci to staphylococci, that explaining the emergence of *S. aureus* vancomycin resistant strains by the same mechanism as in *E. faecium.*

The aquired resistance to cloramphenicole is predominantly plasmidial in enterococci and is due to *cat* gene (*cloramphenicole acetylating enzyme*) homologous to staphylococcal *cat* genes. The presence of this gene confers the resistance phenotype Cmr-Emr (resistance to cloramphenicole and eritromycin).

The screening of antibiotic resistance could be a useful tool for detecting the extrachromosomal resistance genes which could be transmitted by horizontal transfer to the intestinal microbiota.

As consequence, in the Report of Working Group for Drafting Guidelines for Evaluation of Probiotic Administration in Food (*FAO/WHO Working Group*) (2002) it is recommended the investigation of antibioresistance *patterns* in all species intended to be used as probiotics, including those Generally Recognized as Safe (GRAS).

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The concept of *prebiotic* [12] defines some food components (usually oligosacharides as inulin, fructooligosaccharides, lactulose, alpha-galactooligosaccharides, alpha-glucosaccharides, beta-gluco-saccharides and mananooligosaccharides) which cannot be degraded by the digestive enzymes present in the superior regions of the mammalian gastro-intestinal tract.

Once arrived in colon, these compounds are hydrolysed by some lactic acid bacteria and resulting short chain fatty acids which are rapidly absorbed from the intestine and used as energetic source by colonic mucosa, hepatic, muscular and nervous tissues and leading to pH decrease, which contributes to the barrier effect exhibited by the intestinal microbiota.

Methods for the *in vitro* study of the efficiency of probiotic and prebiotic compounds

The *in vitro* screening methods for the selection of probiotic strains and prebiotic potential compounds are including: haemaglutination and haemaglutinoinhibition tests, qualitative and quantitative methods for the investigation of antimicrobial protection ability of some probiotic lactic acid bacteria and mananooligosaccharides for the colonisation of cellular substratum with pathogenic bacteria.

The haemaglutination and haemaglutinoinhibition tests allow the selection of enteropathogenic strains exhibiting adhesive structures represented by manose-sensitive, type 1 fimbriae that will be used for the study of the antimicrobial protective action of manose-based prebiotics.

The qualitative and quantitative methods for the investigation of adherence and invasion capacity of the cellular substratum are used for investigating the interactions between pathogenic and opportunistic adherent bacterial strains and probiotic strains (e.g. *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. casei*) or different prebiotic products (e.g. galactomanan, yeast cell wall extract) [8,10,11]. The qualitative assay was performed on HeLa cells which were routinely grown in Eagle MEM-Minimal Essential Medium enriched 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 at 37°C for 24 hrs. HeLa cells monolayers grown in 6 multi-well plastic plates were used at 80-100% confluence. Bacterial suspensions in Eagle MEM adjusted at 10^7 CFU/ml were obtained from cultures obtained on specific solid media (MacConkey's medium). The HeLa cell monolayers were washed 3 times with PBS (Phophate Buffered Saline) and 2 ml pathogenic bacteria were inoculated in each well. The inoculated plates were incubated for 2 hrs at 37ºC. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 minute), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and left to incubate for 30 min. The plates were washed, dried at room temperature overnight, and examined microscopically (magnification, ×2500) with I.O. and photographed with a Contax camera adapted for microscope Zeiss.

For the quantitative assay of adherence (Ad.) and invasion (Inv.) capacity the Cravioto's adapted method (Figure 2) was used [6,7,9]. The eukaryotic cells were cultivated in 24- *multiwell* plates were used (*Cel-Cult-U.K.*) (2 plates /experiment):

I) – the well of the first plate were used for:
   a) - 1, 2 (duplicate) for Ad. +Inv.;
   b) - 3, 4 (duplicate) for Inv.;

II) – the second plate – circular slides are introduced in each well-used for:
   a) - 1, 2 (duplicate) for Ad. +Inv;
   b) - 3, 4 (duplicate) for Inv.

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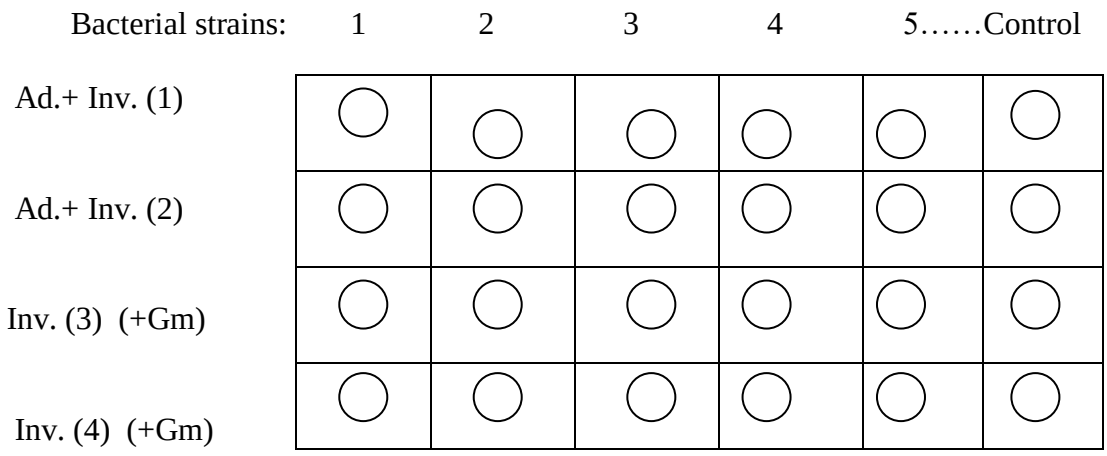

Figure 2. The schematic representation of the experimental model for the quantitative study of bacterial adherence and invasion capacity performed in 24 -*multiwell* plates (Gm-gentamycin)

The first experimental steps are similar to the qualitative method, with the addition that in the wells used for Inv. assay, the cell monolayers, after the incubation with the bacterial strains, are treated for 1 hr with gentamycin that will kill the extracellular bacteria, thus only the invasive, intracellular ones will survive.

After incubation, in the first plate 1 ml Triton X-1% in PBS/ well were added and the plate was incubated for 5 min at 37ºC for the release of the intracellular bacterial cells; thereafter, tenfold dilutions in sterile saline were performed for each well (till $1/10^8$) and 20µl of each suspension was spotted on nutritive agar, in duplicate for viable cell count in order to establish the Colony Forming Units (CFU).

II) The wells from the second plate were Giemsa stained and the circular slides were taken off and fixed on microscopic slides and examined in optic microscopy with immersion oil.

Study of the influence of mananooligosaccharidic fractions on the pathogenic bacteria adherence capacity

This method was used in our laboratory for the *in vitro* study of some prebiotic products (commercial or submitted for patenting).

The study of the influence of glucidic fractions extracted from *Hansenula polimorpha* cell wall demonstrated that they exhibited variable effects on the adherence and invasion capacity of pathogenic bacterial strains on cell cultures (table no. 1). The inhibitory effect was observed for 8A1, 8B3, 6B2, 6B3 (table no. 1), while the other ones easily stimulated the adherence capacity (table no. 2), that could be explained by:

1. oligosaccharidic and mananooligosaccharidic fractions contained in these products could constitute a nutritive resource exploited by the pathogenic bacteria;
2. the existent fractions are functioning as intermediate ligands between the bacterial adhesions and the epithelial cell receptors, increasing the bacterial adherence capacity to the cellular substratum.

It is to be mentioned that in the presence of the inhibitory fractions the pathogenic bacterial strains have no more affected the morphology of the eukaryotic cells, demonstrating that the releasing of cytotoxic and/or cytopathogenic bacterial factors is blocked by the prebiotic compound, this constituting one of their antimicrobial role.

Table no. 1 – Screening of oligosaccharidic and mananooligosaccharidic fractions of *Hansenula polimorpha* cell wall effects on pathogenic bacterial strains adherence

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Tested fraction	The effect on adherence capacity
8A1	**Inhibitory**
8A2	Stimulatory
8A3	Stimulatory
8A4	Stimulatory
6B1	No effect
8B2	Stimulatory
8B3	**Inhibitory**
8C	Stimulatory
6A2	Stimulatory
6A3	No effect
6A4	Stimulatory
8B1	Stimulatory
6B2	**Inhibitory**
6B3	**Inhibitory**
6C	Stimulatory
D	Stimulatory
E	No effect

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Table No. 2. The influence of the selected oligosaccharidic and mananooligosaccharidic inhibitory fractions of *Hansenula polimorpha* cell wall on the adherence of pathogenic bacteria

Fraction	*Staphylococcus aureus*				*Escherichia coli*				*Salmonella gallinarum*				*Listeria monocytogenes*			
	a.i..	i.s.	c.s.	Obs.	a.i..	i.s.	c.s.	Obs.	a.i..	i.s.	c.s.	Obs.	a.i..	i.s.	c.s.	a.i..
8A1	0	0	0	Intact cells	<10%	+	+		0	0	0		<1%	+	+	
6B2	<1%	0	<1%	Contracted cells	0	0	0		0	0	0		0	0	0	
6B3	0	0	0		0	0	0		0	0	0		0	0	0	

Legend:

a.i.= adherence index
c.s.= cellular substratum
i.s.= inert substratum
obs.=observation

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The commercial prebiotic products tested for their influence on the adherence capacity to the cellular substratum of some lactic acid bacterial strains and respectively pathogenic bacterial strains were represented by the atomised products obtained from *Hansenula polimorpha* cultivated on cereal substratum and one mananooligosaccharidic cell wall fraction of *Saccharomyces cerevisiae* (BIOMOS).

Our results demonstrated that BIOMOS inhibits unspecifically the adherence capacity to the cellular substratum of lactobacilli as well as of pathogenic bacteria.

The atomised product obtained from *Hansenula polimorpha* is represented by yeast cell aggregated that could represent an inert adherence substratum for lactobacilli and respectively, pathogenic bacteria (figure 3).

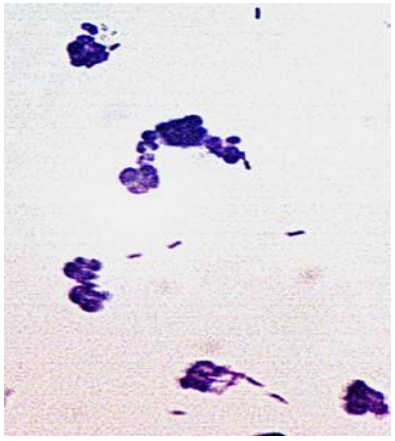

Figure 3. Atomised *Hansenula polimorpha* product with adherent *Escherichia coli* cells (x2500).

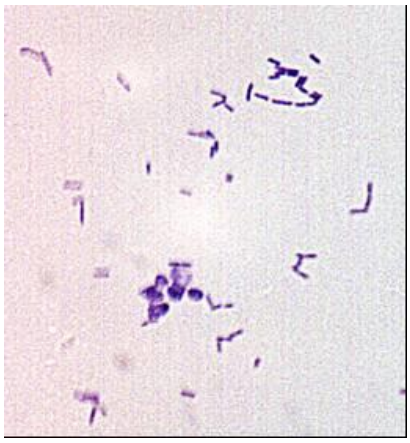

Figure 4. HeLa cells infected with lactobacilli in the presence of atomised *Hansenula polimorpha* product (x2500).

This last aspect could be exploited in a positive way, i.e. by offering an adherence substratum for lactobacilli, this product, in its intestinal transit could favour the colonisation with lactobacilli and consequently, the production and releasing in the intestinal lumen of soluble products with probiotic effect. Moreover, the adherence of pathogenic bacterial strains to these aggregates is diminishing the pathogenic bacteria to the intestinal epithelial cells, blocking the first step of the gastro-intestinal infections.

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Conclusion

The oligosaccharidic and mananooligosaccharidic fractions extracted from *Hansenula polimorpha* cell wall exhibited various effects on the adherence capacity of the pathogenic bacterial strains to the cellular substratum represented by HeLa cells, and the inhibitory fractions blocked also the releasing of soluble cytotoxic and cytopathogenic factors. Our results are proving the necessity of in depth studies regarding the antagonistic interactions between probiotic/prebiotic compounds and pathogenic microorganisms for the selection of effective ecological strategies to the use of the antibiotics for the treatment and prevention of bacterial infections with resistant microorganisms.

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