PERIODICO di MINERALOGIA Volume 89, No. 2, 2020

In vitro study of the interference of some *Enterococcus* strains with the adhesion of human and poultry enteropathogens to HeLa cells

VERONICA LAZAR, MARIANA CARMEN BALOTESCU, DIANA SMARANDACHE, TATIANA VASSU, ELENA SASARMAN*, LIA MARA PETRACHE, MARIANA ORASANU, RAMONA CERNAT**

Faculty of Biology, Microbiology Department, Ale. Portocalilor 1-3, Sector 5, 77206-Bucharest, ROMANIA
** MICROGEN (Center for Research in genetics, Microbiology and Biotechnology), University of Bucharest, Faculty of Biology, Ale. Portocalilor 1-3, Sector 5, 77206-Bucharest, ROMANIA*
***Cantacuzino Institute, Spl. Independentei 103, Sector 6, POB 1-525, Romania*

Abstract

Few studies, so far have addressed the adhesion of enterococci to the intestinal mucosa and their interference with the adhesion of pathogens, although more than 60% of probiotic preparations in the market contain strains of enterococci. The objective of this study was to investigate if some Enterococcus faecium strains isolated from Robinia pseudacacia and Chelidonium majus flowers and grass and previously selected for strong adherence capacity to the cellular substrate have the ability to inhibit the adhesion of some intestinal pathogens as Escherichia coli, Listeria monocytogenes, Staphylococococcus aureus and Salmonella typhimurium to HeLa cells. Antimicrobial properties of enterococci have been determined by using three methods: the washed bacterial cells, supernatant, or original culture of Enterococcus faecium strains. They were tested separately to identify which part was involved in the inhibition.

Our results showed that the original Enterococcus cultures remarkably reduced the attachment of the tested pathogenic strains to HeLa cells, compared with washed cells or culture supernatant which inhibited the adhesion of tested pathogens to a lesser degree.

A strong lytic effect against pathogenic strains was noticed when culture supernatants were tested. Paradoxically, this lytic effect was not observed when original bacterial cultures were used. These results are pleading for the existence of complex regulatory and signalling mechanisms in original cultures, that are lost when bacterial supernatants are used. However, indepth further studies on a large number of pathogenic strains are needed in order to confirm these results.

Keywords: *Enterococcus*, probiotics, inoculant for silage, pathogenic bacteria, competition for adherence sites

Introduction

Antibiotics are routinely used in an attempt to control pathogens, but the organisms are becoming resistant to the more commonly used treatments, making antibiotic therapy unreliable. Furthermore, the use of antimicrobial growth promoters may cause the

development of resistance in a number of important pathogenic bacterial species. Recently, the European Union decided to ban the use of four widely used antibiotics, i.e., tylosin, virginiamycin, spiramycin, and zinc bacitracin, as growth promoters from July 1999 (Witte, 1998). As a consequence, there is an urgent need to seek an alternative to antibiotics for the purpose of enhancing the health status and production performance of domestic animals (Matilla-Sandholm et al., 1999).

Probiotics have been used to reduce the colonization of the intestines of animals by pathogens (Nikoskelainen et al., 2000). This, in turn, reduces the prophylactic use of antibiotics as feed additives in animal production (Jin et al., 1997; Isolauri et al., 2000).
Most probiotic bacteria are of intestinal origin and belong to the lactic acid-producing bacteria (LAB) such as bifidobacteria, lactobacilli, and enterococci. Probiotics may have antimicrobial, but also immunomodulatory, anticarcinogenic, antidiarrheal, antiallergenic and antioxidant activities (Majamaa & Isolauri, 1997; Dugas et al., 1999; Lyn & Yen, 1999; Arunachalam et al., 2000; Saavedra, 2000). The mechanisms of action of probiotics have been sintetized in four possibilities: i. antagonism through production of inhibitory substances (*Short Chain Fatty Acids*-SCFA, lytic enzymes and bacteriocins); ii. competition with the pathogen for adhesion sites or nutrients; iii. immunomodulation of the host; and iv. inhibition of toxins.

Many studies have focused on lactobacilli and bifidobacteria and have been carried out to elucidate the mechanism of bacterial adhesion and the ability of these bacteria to inhibit the adhesion of pathogens to the intestinal mucosa (Ewing & Hasign, 1989; Fulle, 1989).

However, only few studies, have addressed the adhesion of enterococci to the intestinal mucosa and their interference with the adhesion of pathogens, although more than 60% of probiotic preparations in the market contain strains of enterococci (Mack et al., 1999).

The objective of this study was to investigate if some *E. faecium* strains isolated from *Robinia pseudacacia* and *Chelidonium majus* flowers and grass and previously selected for strong adherence capacity to the cellular substrate have the ability to inhibit the adhesion of some intestinal pathogens as *E. coli*, *L. monocytogenes*, *S. aureus* and *S. typhimurium* to HeLa cells.

Material and methods

Bacteria and culture conditions *E. faecium* strains no. VL 43, VL 47 and GM-8 were used. These bacteria were originally isolated from *Robinia pseudaccacia* and *Chelidonium majus* flowers and grass and grown in MRS medium (Merck, 2000). All strains were stored in the laboratory collection at -70°C in appropriate medium represented by MRS supplemented with 20% glycerol Primary cultures were obtained and after cultivated in MRS liquid medium in order to obtain mid-logarithmic phase cultures that were further used in our experiments.

Pathogenic microorganisms were represented by strains of *E. coli* and *S. aureus* (isolated from diarhhoeal illness), *L. monocytogenes* and *S. gallinarum* (isolated from poultry). The strains were identified by conventional biochemical tests and stored in the laboratory collection at room temperature in appropriate preservation medium.

Competition for adherence sites capacity was assayed by Cravioto's adapted method. HeLa cells were routinely grown in Eagle's minimum essential medium (MEM) (MEM enriched supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamicin (50 mg/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% confluency. For the competion assay, bacterial mid-logarithmic phase cultures of *Enterococcus faecium* tested strains were centrifuged at

4000 rpm/min for 10 minutes, and the pellet was washed three times in phosphate buffered saline (PBS) and resuspended in Eagle MEM. Bacterial suspension density was adjusted at 10^7 CFU/ml. Bacterial suspensions in Eagle MEM adjusted at 10^7 CFU/ml were also obtained from the pathogenic cultures obtained on specific solid media. The HeLa cell monolayers were washed 3 times with PBS; 1 ml of *Enterococococcus* and respectively, 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.

Results and discussion

It is well known that the presence of enterococci is important for the maintenance of the intestinal microbial ecosystem. They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp. and others (McKay, 1990; Giraffa et al., 1995; Harris et al., 1989). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins, etc.) or to competitive adhesion to the epithelium (El-Ziney & Debevere, 1998). In order to survive in and colonize the gastrointestinal tract, probiotic bacteria should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (Bielecka et al., 1998). For this reason, together with the other LAB, i.e., lactobacilli and bifidobacteria, enterococci are widely used in probiotic products due to their high bile resistance.

Eukariotic HeLa cells have been successfully used for *in vitro* studies on the mechanism of cellular adhesion of probiotic bacteria. Moreover, this cell line has been used to examine the mechanism of cellular adhesion and invasion of pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli* (Gaillard et al., 1987; Finlay & Falkow, 1990). In the present study, we used HeLa cells to examine the antimicrobial activity of some *Enterococcus faecium* strains against pathogenic bacteria.

The pathogenic bacterial strains have been selected for their adhesive properties to the cellular substratum. Our results showed that *Staphylococcus aureus* exhibited a mixt adherence pattern (diffuse and localized) and an adherence index of 50-60%. Some cytotoxic effects were also noticed represented by cell vacuolisation, picnosis and the presence of internalized bacteria (Fig. 1-2).

Fig.1. HeLa cells with picnotic aspect consequently to *S. aureus* infection; it could be noticed the mixed diffuse and localized adherence pattern (Giemsa staining, x 2500)

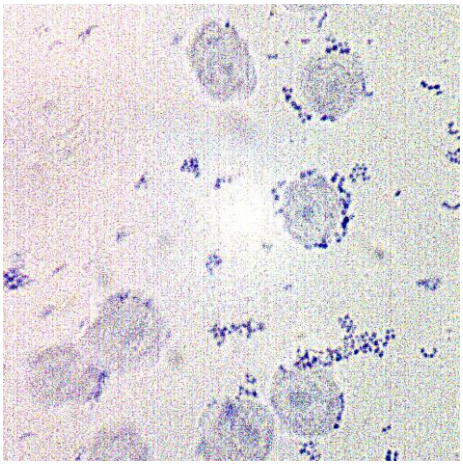
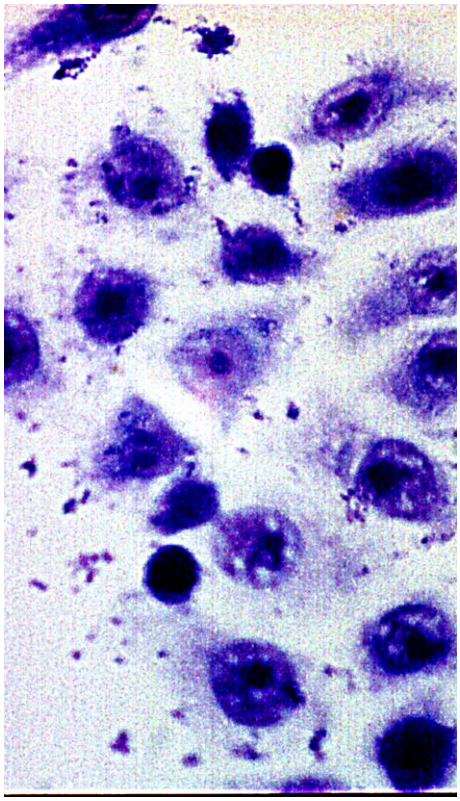
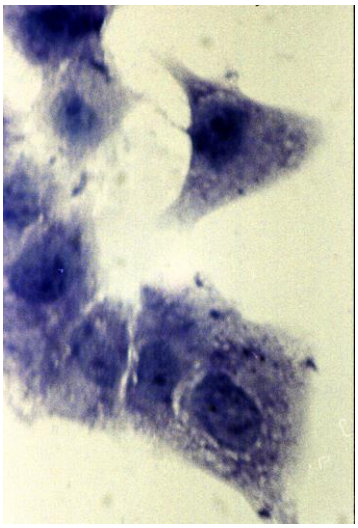


Fig.2. HeLa cells infected with *S. aureus*; it can be noticed the presence of endocytic vacuoles and internalized bacteria (Giemsa staining, x 2500)



Listeria monocytogenes strain, similarly with *Salmonella gallinarum*, exhibited a diffuse adherence pattern and an adherence index of 15-20% (Fig. 3).

Fig.3. HeLa cells infected with *L. monocytogenes;* the presence of internalized bacteria could be noticed (Giemsa staining, x2500).



The relatively reduced adherence index observed for *Salmonella* and *Listeria* could be explained by the subsequent internalization of the adhered bacterial cells.

The *Escherichia coli* strain exhibited a diffuse pattern of adherence and an adherence index of 10%.

Antimicrobial properties of enterococci have been determined by using three methods: the washed bacterial cells, supernatant, or original culture of *E. faecium* strains. They were tested separately to identify which part was involved in the inhibition.

Our results showed that the original *Enterococcus faecium* (EF) cultures, washed bacterial cells, or culture supernatant remarkably reduced the attachment of the tested pathogenic strains to HeLa cells (5 to 10% adherence rate of pathogenic bacteria in the presence of the mentioned EF preparations), but compared to the original EF cultures (under 1% adherence rate of pathogenic bacteria in the presence of EF whole cultures), washed cells or culture supernatant inhibited the adhesion of tested pathogens to a lesser degree.

However, this inhibiting activity was recovered after the washed cells and the supernatant were recombined, with the recombined mixture having a similar inhibitory ability to that of the original *E. faecium* culture (pathogenic bacteria not adhered to HeLa cells). This result may imply that the substances from both *E. faecium* cells and the spent culture supernatant contribute to the inhibition of adhesion of pathogens to the HeLa strains.

Somewhat surprisingly to our knowledge, this is the second report of its kind, the first one being reported by Jin et al., (2000).

The inhibitory effects of LAB on adherence capacity of pathogens have been attributed to steric hindrance of binding sites, pH values, or certain components of the lysed cell wall (El-Ziney & Haresign, 1989). The results of the present study support the idea that *E. faecium* cells or the substances released in its culture might occupy the binding sites, although these binding sites are not necessarily targeting the same epitopes in pathogenic strains.

The culture pH has been proposed to be an important factor in the inhibition of adhesion of pathogens to the mucosa (Matilla –Sandholm et al., 1999). In the present study,

the inhibiting effect was not solely a pH effect, since considerable inhibitory action was demonstrated after washing the bacterial cells and resuspending them in Eagle MEM and adjusting the bacterial suspension used for inoculation of cell monolayer to pH 7.0.

In the case of culture supernatants, we have to mention that a strong lytic effect was noticed on pathogenic bacterial cells, that was not recovered when washed bacterial cells or original cultures were used. This could be due to the presence in bacterial culture supernatants of lytic enzymes. Paradoxically, this lytic effect was not observed when original bacterial cultures were used. These results are pleading for the existence of complex regulatory and signalling mechanisms present in original cultures, that are lost when bacterial supernatants are used. However, indepth further studies on a large number of pathogenic strains are needed in order to confirm these results.

In conclusion, the present study found that: (i) living *Enterococcus faecium* cultures efficiently inhibited the adhesion of *E. coli, S. aureus, L. monocytogenes* and *S. gallinarum* to HeLa cells; (ii) the substances from both the intact *E. faecium* cells and the spent culture supernatant contribute to the inhibition of adhesion of pathogenic strains to the HeLa cells; (iii) the inhibition of adhesion pathogenic strains by *E. faecium* cultures or their supernatants might occur by steric hindrance, modified pH and lytic enzymes.

Selective references

1. Arunachalam K, Gill HS, Chandra RK. Enhancement of natural immune function by dietary consumption of bifidobacterium lactis. *Eur J Clin Nutr*. 2000; 54:263-267.
2. Bielecka M, Biedrzycka E, Biedrzycka E, et al. Interaction of Bifidobacterium and Salmonella during associated growth. *Int J Food Microbiol.* 1998; 45:151-155.
3. Dugas B, Mercenier A, Lenoir-Wijnkoop I, et al. Immunity and probiotics. *Immunol Today*. 1999; 20:387-390.
4. El-Ziney M, Debevere J. The effect of reuterin on *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and cottage cheese. *J. Food Prot*. 1998; 61:1275-1280
5. Ewing W, Haresign W. Probiotics UK. Chalcombe Publications, Bucks, United Kingdom,1989.
6. Finlay BB, Falkow S. *Salmonella* interactions with polarized intestinal Caco-2 epithelial cells. J. Infect. Dis. 1990; 162:1096-1106
7. Fuller R. Probiotics in man and animals. J. Appl. Bacteriol. 1989; 66:365-378
8. Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti JP. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. 1987; 55:2822-2829
9. Giraffa G**.** Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. Food Microbiol. 1995; 12:291-299.
10. Harris LJ, Daechsel MA, Stiles ME, Klaenhammer TR**.** Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J. Food Prot. 1989; 52:384-387.
11. Isolauri E, Kirjavainen P V, Salminen S Probiotics: a role in the treatment of intestinal infection and inflammation?. *Gut*, 2002; 50: iii54-59
12. Jin LZ, Ho YW, Abdullah N, Jalaludin S. Probiotics in poultry: mode of action. *World's Poult. Sci. J*. 1997; 53:351-363
13. Jin Z, Marquardt RR., Zhao XA. Strain of *Enterococcus faecium* (18C23) Inhibits Adhesion of Enterotoxigenic *Escherichia coli* K88 to Porcine Small Intestine Mucus Applied and Environmental Microbiology 2000; 66, 10: 4200-4204
14. Lin M-Y, Yen C-L. Antioxidative ability of lactic acid bacteria. *J Agric Food Chem*. 1999; 47:1460-1466.

15. Mack DR, Michail S, Wei S, et al. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am J. Physiol*. 1999; 276(4 Pt 1):G941-G950.
16. Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol.* 1997; 99:179-185.
17. Matilla-Sandholm T, Blum S, Collins JK, et al. Probiotics: towards demonstrating efficacy. *Trends Food Sci Technol*. 1999; 10:393-399.
18. Mattila-Sandholm T. The PROBDEMO project: demonstration of the nutritional functionality of probiotic foods. *Trends Food Sci Technol*. 1999; 10:385-386.
19. McKay AM. Antimicrobial activity of *Enterococcus faecium* against *Listeria* spp. Lett. Appl. Microbiol. 1990; 11:15-17.
20. Nikoskelainen S, Salminen S, Bylund G, Ouwehand AC. Characterization of the Properties of Human- and Dairy-Derived Probiotics for Prevention of Infectious Diseases in Fish. *Applied and Environmental Microbiology*, 2001, 67 (6): 2430-2435
21. Saavedra J. Probiotics and infectious diarrhea. *Am J Gastroenterol*. 2000; 95 (1 Suppl): S16-S18.
22. Witte W. Medical consequences of antibiotic use in agriculture. *Science* 1998; 279:996-997