Scientific Papers. Series D. Animal Science. Vol. LXV, No. 1, 2022
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

ADHESION AND ANTAGONISTS PROPERTIES OF *ENTEROCOCUS* MONOCULTURES AND THE OPPORTUNITY OF THEIR USE AS PROBIOTICS

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Abstract

The adhesion and antagonist capacity of some Enterococcus strains isolated from human and animal intestinal contents was investigated. The obtained results demonstrated the increased adhesive capacity of the studied Enterococcus strains, especially those specific to the human digestive tract. At the same time, the high activity of enterococci monocultures in the control of Escherichia and Salmonella bacteria has been established, which indicates their antagonistic property. Based on the adhesive capacity and high antagonistic activity of enterococci, new microbial associations containing enterococci were investigated. The obtained results revealed a beneficial action of the new microbial preparations, which prevented the appearance and development of diarrheal dysfunctions, which argues the opportunity to include enterococci in the composition of associations or probiotic microbial preparations.

Key words: *adhesion capacity, antagonistic activity, Enterococcus, probiotics.*

INTRODUCTION

For the production of probiotics, one of main criterion in the selection of useful strains of microorganisms is the ability to colonize the digestive tube (McNaught & MacFie, 2001; Alp & Kuleasan, 2019).

This process is quite complex and depends on several factors. These factors include the adhesion property of probiotic microorganisms, which is important for the interaction between probiotic strains and the host organism and underlies antagonism to pathogens (Nishiyama et al., 2016; Hanifeh et al., 2021).

The mechanism of adhesion is determined by the interaction between molecular complexes (proteins, oligosaccharides) from the surface of bacteria and intestinal glycoconjugates of epithelial intestinal cells, more precisely their mucus. The peculiarities of the formation of associations between bacterial proteins and components of intestinal mucus establish differential adhesion, which might be employed as a host strategy to possibly select for particular strains or species (Ouwehand & Salminen, 2003; Garcia-Gonzalez et al., 2018; Monteagudo-Mera et al., 2019; Hanifeh et al., 2021).

Some representatives of enterococci, namely specific strains of *Enterococcus faecalis* and *Enterococcus faecium* have been used as probiotics or feed additives (Becquet, 2003; Araújo & de Luces Fortes Ferreira, 2013; Hanchi et al., 2018). Enterococci are a component part of the human and animal intestinal micro-biocenosis, having an important role in the vital activity of the host organism (Sivieri et al., 2008; Araújo & de Luces Fortes Ferreira, 2013).

Some strains of enterococci have been used as triggers in food fermentation and as food preservatives. At the same time, strains of enterococci were tested as probiotics (Araújo & de Luces Fortes Ferreira, 2013; Hanchi et al., 2018). But the development of new enterococcal probiotics requires a more rigorous study in safety aspects in order to select harmless enterococcal strains for safe application (Ben Braïek & Smaoui, 2019).

As it was mentioned, one of the characteristics that apply to the selection of probiotic microorganisms is adhesion to the intestinal mucosa.

Adhesion capacities of *E. faecalis* and *E. faecium* to the intestinal mucosa have been studied in some agricultural and domestic animals.

Some studies confirm the efficacy of selected strains of enterococci as probiotics (Sivieri et al., 2008). Thus, the strain *Enterococcus faecium* WEFA23, isolated from Chinese infant feces, is able to exclude or displace the adhesion of *Escherichia coli* O157:H7, *Salmonella typhimurium* ATCC 13311, *Listeria monocytogenes* CMCC54007, *Staphylococcus aureus* CMCC26003, and *Shigella sonnei* ATCC 25931 to Caco-2 cells (He et al., 2019).
The strain of *Enterococcus faecium* OV3-6 with probiotic properties and its secreted active peptides is able to survive in simulated gastric and small intestinal conditions (Choeisoongnern et al., 2021). It is known that one of the basic characteristics in the selection of probiotics is resistance to gastric juice and bile salts (Araújo & de Luces Fortes Ferreira, 2013) and production of antimicrobial compounds such as enterocin (Franz et al. 1999; Araújo & de Luces Fortes Ferreira, 2013). This strain denotes α-hemolysis and is susceptible to most clinically relevant antibiotics (Choeisoongnern et al., 2021) and reduces the adhesion of *E. coli* and *S. typhimurium* on Caco-2 cells. The strain can prevent the growth of Gram-positive strains belonging to the genera *Bacillus*, *Carnobacterium*, *Listeria* and *Staphylococcus*. (Choeisoongnern et al., 2021).
However, representatives of enterococci are involved in several nosocomial infections due to virulence factors and antibiotic resistance. Thus, the development of new enterococcal probiotics requires strict evaluation in terms of safety aspects for the selection of harmless enterococcus strains (Ben Braïek & Smaoui, 2019).
At the same time, the use of certain strains of microorganisms as probiotics is regulated by the respective decision-making bodies. Thus, the European Food Safety Authority determined that enterococci did not meet „Qualified Presumption of Safety" status (Becquet, 2003; Wang et al., 2020). No enterococcal probiotic has been approved by the United States Food and Drug Administration for the treatment, cure, or amelioration of human disease and enterococcus strains used or proposed for use as probiotics should be carefully screened for efficacy and safety (Wang et al., 2020).
However, *E. faecium* is still used as feed supplements in the USA and China, termed as direct-fed microorganisms (Wang et al., 2020).
The efficacy and safety of microorganisms as probiotics, including enterococci, should be based on rigorous studies that would reveal all aspects of the action of the bacteria investigated. In the paper it was proposed to study the adhesion and antagonist capacities of enterococci, as these properties are considered a crucial step for intestinal bacteria to colonize and further interact with the host epithelium and the immune system and appears to be an important feature for probiotics.

MATERIALS AND METHODS

The human and animal intestinal contents served as study material. *In vitro* conditions, using classical microbiological methods, the single strains of enterococci with enhanced antagonistic and adhesive properties were isolated, identified and selected.

Table 1. Distributions of *Enterococcus* strains by sources

Source	The selected strains (number)
Human intestinal content	18; 25; 32; 46; 49; 58; 67; 70; 82; 85; 89; 93; 108; 112; 116
Animal intestinal content	13; 21; 37; 43; 55; 64; 74; 77; 97; 101; 129

The adhesion capacity of enterococci was studied according to the method of Brilis (1986) using erythrocytes as a model of the macroorganism cells. The mixture of human, bovine and porcine erythrocytes was incubated at 30°C for 30 min, being stirred regularly, then the smear was prepared, lyophilized, fixed and colored according to the Romanovschi-Ghimze technique. The adhesion study was performed using a light microscope. The calculation was performed on the basis of 25 erythrocytes, analyzing 5 erythrocytes in the visual field, using the adhesion index of microorganisms. After this index, the adhesion level of the microorganisms is determined.
The antibacterial (antagonistic) activity of the culture supernatant of these strains were tested against *Escherichia* and *Salmonella*. For this purpose, the agar well diffusion method was used to test the antagonistic activity of the isolated strains of enterococci against the selected human pathogens (using nutrient agar media for testing bacteria). Then the plates were incubated for 24 h at 37°C. The zones of inhibition of pathogenic bacteria were measured by a transparent ruler. Three replicates for each

test were done for every evaluated pathogenic species (Bhat & Nalawade, 2016).

Based on the selected enterococcal strains, associations of enterococci with bifido- and lactobacilli were prepared. The newly developed associations were investigated in comparison with other existing microbial preparations in laboratory conditions on laboratory animals (white mice). For this purpose, the laboratory animals were divided into five groups of ten (10) animals each.

Group I received Bifidobacterin (based on bifidobacteria); group II - Lacidophil-WM (containing lactobacilli); group III - Bifi.form (includes bifidobacteria and enterococci); group IV - the new association of bacteria, developed for the first time based on microorganisms of the genus *Enterococcus* (*E. faecium*) and those of the genera *Bifidobacterium* and *Lactobacillus*; group V served as a control.

All preparations were administered orally, on the background of intestinal dysmicrobism, for 6 days, of 1 ml of microbial suspension (1 billion microbial cells) to 1 animal per day (before morning feeding).

Diluted intestinal contents samples were studied at the beginning and end of the experiment (before and after 6 days of administration of the studied microbial preparations). Quantitative indices of microorganisms of the genera: *Bifidobacterium*, *Lactobacillus*, *Escherichia*, *Proteus*, *Enterococcus* were determined.

The samples were subjected to research using classical microbiological methods (Garmasheva & Kovalenko, 2010).

Inoculation was performed on elective agar nutrient media for each genus of bacteria, with subsequent incubation at 37 ± 1ºC, for 24-72 h, under aerobic and anaerobic conditions. The final results are expressed in decimal logarithms (log) (GOST 30518-97, 2000).

RESULTS AND DISCUSSIONS

The results of investigations regarding the selection of enterococci strains with high adhesion properties and antagonistic activity, revealed that streptococci isolated from human intestinal contents belong mostly to the genera *Streptococcus*, *Lactococcus* and *Enterococcus*, and from the intestinal contents to animals - from the genera *Streptococcus* and *Enterococcus* (data are not presented in this article).

The enterococci strains isolated from human and animal intestinal contents were tested for their adhesion and antagonist capacities.

The results of the research regarding the adhesive capacity of selected *Enterococcus* strains on native human, porcine and bovine erythrocytes (Table 2) show that the adhesion index was it was quite high (within 2.81 - 4.67 c. u.).

Table 2. The adhesive capacity of enterococci specific to the human and animal digestive tract

Number of isolated and tested enterococcal strains	The number of microbial cells adhering to the surface of 25 native erythrocytes	Adhesion index c. u.
From the human intestinal content		
18	102.50±3.50	3.18
25	106.75±3.25	4.27
32	103.75±1.25	4.15
46	102.50±2.50	4.10
49	103.50±3.50	4.14
58	103.00±2.00	4.12
67	102.00±3.00	4.08
70	104.00±3.00	4.16
82	102.75±2.25	4.11
85	103.50±2.50	4.14
89	99.50±3.50	3.98
93	103.00±4.00	4.12
108	102.50±3.50	4.10
112	104.00±3.00	4.16
116	116.75±2.25	4.67
From the animal intestinal content		
13	79.50±2.50	3.18
21	89.75±2.25	3.59
37	86.25±2.75	3.45
43	81.00±3.00	3.24
55	70.25±1.75	2.81
64	86.00±4.00	3.44
74	93.00±2.00	3.72
77	96.25±1.75	3.85
97	89.25±3.75	3.57
101	78.00±3.00	3.12
129	91.25±3.75	3.65

A higher adhesion capacity was identified in enterococcus strains from human intestinal contents, ranging from 3.98 to 4.67 c. u. The highest value of the adhesion index was found at strain no. 116, namely 4.67 c. u.

The adhesion index to the selected enterococcal strains from the animal intestinal contents is in the range of 2.81-3.85 c. u. The highest value (3.85 c.u.) was detected at strain no. 77.

Data on antagonistic capacity (Table 3A and B) indicate that high antagonistic activity against pathogens (*Escherichia* and *Salmonella*) shows the selected enterococcal strains from the human intestinal contents, compared to those in the animal digestive tract.

Table 3A. The antagonistic activity of isolated enterococcal strains, specific to the human and animal digestive tract (part I)

Number of selected entero-coccal strains	Absolute number of microbial cells per 1 ml of microbial suspension in decimal logarithms (lg), inoculated jointly with bacteria of the genera			
	Escherichia		*Salmonella*	
	at the beginning	*at the finally*	*at the beginning*	*at the finally*
From the human intestinal content				
18	3.90±0.09	2.74±0.07	3.46±0.1	2.77±0.07
25	3.82±0.08	2.88±0.06	4.07±0.11	3.13±0.12
32	3.80±0.08	2.87±0.06	3.77±0.05	3.04±0.13
46	3.46±0.05	2.70±0.00	3.82±0.04	3.00±0.19
49	3.70±0.08	2.84±0.16	3.76±0.17	3.04±0.12
58	3.95±0.12	3.07±0.13	3.70±0.12	2.98±0.15
67	4.11±0.11	3.23±0.12	3.62±0.26	2.88±0.13
70	3.69±0.11	2.72±0.09	3.54±0.14	2.64±0.17
82	3.77±0.13	2.87±0.13	3.46±0.08	2.88±0.13
85	4.07±0.12	3.04±0.05	3.84±0.09	2.94±0.04
89	4.20±0.10	3.32±0.1	3.68±0.11	2.92±0.07
93	3.79±0.11	2.88±0.04	3.62±0.13	2.81±0.12
108	4.07±0.13	3.14±0.13	3.64±0.12	2.92±0.09
112	4.23±0.14	3.20±0.10	3.77±0.15	2.90±0.11
116	3.98±0.10	2.70±0.08	3.72±0.13	2.77±0.07
From the animal intestinal content				
13	4.25±0.09	3.46±0.06	3.84±0.09	3.13±0.14
21	3.60±0.07	2.90±0.08	3.41±0.13	2.92±0.08
37	3.64±0.11	2.98±0.1	4.17±0.13	3.46±0.09
43	3.50±0.1	2.90±0.13	3.60±0.07	3.07±0.04
55	3.70±0.04	3.00±0.12	3.65±0.06	3.11±0.13
64	3.78±0.13	3.11±0.11	3.77±0.07	3.17±0.13
74	3.75±0.08	3.14±0.12	3.49±0.08	2.96±0.12
77	3.67±0.07	2.96±0.14	3.50±0.04	2.86±0.15
97	4.17±0.09	3.43±0.07	3.54±0.14	2.98±0.14
101	4.04±0.07	3.46±0.12	3.76±0.08	3.25±0.10
129	4.13±0.08	3.41±0.11	3.80±0.10	3.23±0.12

Table 3B. The antagonistic activity of isolated enterococcal strains, specific to the human and animal digestive tract (part II)

Number of selected enterococcal strains	The control coefficient for pathogens, %	
	Escherichia	*Salmonella*
From the human intestinal content		
18	29.74	19.94
25	24.60	23.09
32	24.47	19.36
46	21.96	21.45
49	23.24	19.14
58	22.27	19.45
67	21.41	20.44
70	26.28	25.42
82	23.87	19.76
85	25.30	23.43
89	20.95	25.85
93	24.01	22.37
108	22.85	19.78
112	24.34	23.07
116	32.16	25.53
From the animal intestinal content		
13	18.58	18.48
21	19.44	14.36
37	18.13	17.02
43	17.14	14.72
55	18.91	14.79
64	17.72	15.91
74	16.26	15.18
77	19.34	18.28
97	17.74	15.81
101	14.35	13.56
129	17.43	15.00

Thus, the antagonistic activity of enterococcal strains specific to the human digestive tract varies from 20.95% to 32.16% (Table 3B), the most effective in this regard being strain no. 116, for which the coefficient of control of *Escherichia* and *Salmonella* constituted respectively 32.16% and 25.53%.

Among the enterococcal strains specific to the animal digestive tract, the highest activity to combat pathogenic microorganisms was detected in strain no. 77, at which the control coefficient of *Escherichia* and *Salmonella* was 19.34% and 18.28% respectively.

Thus, it is observed a strong correlation between adhesion capacity and antibacterial activity of studied strains of *Enterococcus*. The strain no. 116 from human intestinal content and strain no. 77 from animal digestive tract, which a high adhesion capacity, are also more efficient in fighting against pathogenic bacteria, and capable in this way preventing infectious diseases.

Based on the data obtained, it can be stated that all enterococcal strains, isolated for the first time, highlighted the probiotic potential, expressed by the increased adhesion capacity, high level of antagonistic activity.

The investigations *in vivo* conditions on laboratory animals (white mice) were aimed to elucidate the action of the new developed association based on microorganisms of the genus *Enterococcus* (*E. faecium*) and those of the genera *Bifidobacterium* and *Lactobacilli* compared to other microbial preparations on the quantitative indices of some intestinal microbial representatives (*Bifidobacterium*, *Lactobacillus*, *Escherichia*, *Proteus*, *Enterococcus*) (Table 4).

Analyzing the data from table 4, it was observed that for all the animals (experimental groups I-IV), after 6 days of probiotic preparation administration, the state of the intestinal eubiosis is present, and in those from the control group - of dysmicrobism. The state of dysmicrobism is characterized by high quantitative indices of *Escherichia* and *Proteus* bacteria and the state of intestinal eubiosis - by the low values of these indices respectively and high indices of *Bifidobacterium*, and *Lactobacillus.*

At the same time, all tested preparations had a beneficial effect on the animal body, preventing the occurrence and development of diarrheal dysfunction (in 100% of tested animals), while

in the control group such disorders were recorded in 80% of animals and the remaining 20% - intestinal dysmicrobism.

Table 4. Quantitative indices of intestinal microbiocenosis (of experimental animals) at administering of various microbial preparations

The experimental groups	Types of microorganisms	Number of microbial cells per 1 g of intestinal contents, decimal logarithm (log)	
		at the beginning	*at the finally*
I	*Bifidobacterium*	7.17±0.08	9.41±0.07
	Lactobacillus	6.88±0.12	7.72+0.09
	Escherichia	8.84±0.13	7.46±0.12
	Proteus	3.11±0.09	2.07±0.10
	Enterococcus	6.77±0.11	7.90±0.14
II	*Bifidobacterium*	7.53±0.11	8.04±0.13
	Lactobacillus	6.32±0.12	8.32±0.10
	Escherichia	8.96±0.10	7.54±0.14
	Proteus	3.00±0.13	1.23±0.12
	Enterococcus	6.84±0.15	7.04±0.13
III	*Bifidobacterium*	7.23±0.13	9.20±0.12
	Lactobacillus	6.63±0.11	7.70±0.13
	Escherichia	8.68±0.12	6.82±0.10
	Proteus	3.07±0.11	1.17±0.10
	Enterococcus	6.54±0.14	8.62±0.10
IV	*Bifidobacterium*	7.60±0.10	9.47±0.13
	Lactobacillus	6.50±0.11	8.65±0.09
	Escherichia	8.46±0.12	5.79±0.11
	Proteus	3.17±0.08	0
	Enterococcus	6.32±0.13	8.88±0.12
V	*Bifidobacterium*	7.20±0.12	7.82±0.10
	Lactobacillus	6.11±0.11	6.77±0.11
	Escherichia	8.53±0.11	9.49±0.12
	Proteus	3.04±0.10	4.14±0.08
	Enterococcus	6.49±0.12	7.23±0.13

The association developed for the first time based on microorganisms of the genus *Enterococcus* (*E. faecium*) and those of the genera *Bifidobacterium* and *Lactobacilli* has a greater action of inhibiting pathogenic bacteria (*Escherichia* and *Proteus*).

The antagonistic relations between the bacteria are of interest in their use in the fight against pathogenic genes and the infections triggered by them. According to several studies, the antagonistic activity is based on the adhesion capacity of bacteria and the production by microorganisms of substances with antagonistic/antibacterial action (lactic acid, bacteriocins, etc.). Use as a probiotic of lactic acid bacteria - LAB; is recognized by the FAO and the WHO, being recognized as safe status (GRAS) (Zielińska& Kołożyn-Krajewska, 2018; Ben Braïek & Smaoui, 2019).

It has been established that enterococci produce lactic acid and enterocin (a substance with antibacterial action) (Nami et al., 2019), which make some enterococcus strain promising for probiotics, with their application in diarrhea treatment in association with antibiotic medication, viral infection, chemotherapy and diseases originated from food-borne pathogens (Lau & Chamberlain, 2016).

While, probiotic effect of *Enterococcus* is strain dependent. The use of enterococci stains as probiotics must be based on research proving their safety, as well as the lack of virulent factors (Nascimento et al., 2019).

Based on the obtained data, it is argued that the selected strains of enterococci in the composition of associations or microbial preparations with probiotic action, but the investigations on adhesion capacity and antagonistic activity is a first step in the selection of probiotic strains of *Enterococcus*.

CONCLUSIONS

Enterococci are part of the digestive tract microbiocenosis, having a special role in the normal functioning of the digestive tract.

The strains of enterococci isolated for the first time from the intestinal contents of humans and animals have shown useful properties for the organism, confirmed by increased indications of antagonistic activity and adhesive capacity.

Human digestive tract-specific enterococcal strains showed a higher adhesion and antagonist capacity compared to those specific to the animal digestive tract.

Experimentally, the inclusion of enterococcal strains isolated for the first time in the composition of associations and microbial preparations intended to strengthen the health of the digestive tract (in case of intestinal dysmicrobism and diarrheal dysfunction) has been argued.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Institute of Physiology and Sanocreatology and also was financed from Project 15.817.04.01A, and doctoral studies project.

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