Antimicrobial activity of probiotic lactobacilli, bifidobacteria and propionic acid bacteria, isolated from different sources

R. Denkova[2], Z. Denkova*[1], V. Yanakieva[1], D. Blazheva[1]

[1] UNIVERSITY OF FOOD TECHNOLOGIES, Department of Microbiology, 26 Maritza Blvd., Plovdiv 4002, Bulgaria
[2] SOFIA UNIVERSITY "ST. KLIMENT OHRIDSKI", Department of Biotechnology, 8 Dragan Tzankov Blvd., Sofia, Bulgaria

80 *Lactobacillus* strains, 10 *Bifidobacterium* strains and 5 *Propionibacterium* strains are isolated from different sources (human gastrointestinal tract, naturally fermented dairy and non dairy products) and are identified using physiological, biochemical and molecular genetic methods. Homo- and heterofermentative lactobacilli, bifidobacteria and propionic acid bacteria with probiotic properties are selected among them. Particular attention is paid to the antimicrobial activity of the selected strains against pathogenic and toxigenic microorganisms, causing food poisoning and toxicoinfections, as well as against bacterial and mold spores. It is shown that the reduction of viable cells of pathogenic microorganisms in mixed populations with probiotic bacteria is strainspecific, but the majority of them die within 72 hours of incubation. It is found that the antimicrobial action of the probiotic cultures is due to the production of short-chain acids (lactic, acetic, propionic), competition for nutrients and production of inhibitory substances with protein and non-protein nature. The high antimicrobial activity of the isolated probiotic bacteria allowed our research team to create the probiotics "Enterosan", functional starter cultures for fermented milk and non-milk foods, starter cultures for meat products, starters for sourdough bread, starter cultures for fermented vegetables as well as to achieve biopreservation of creamy products (cosmetic cream, conditioner, mayonnaise, etc.).

Keywords antimicrobial activity; *Lactobacillus; Bifidobacterium; Propionibacterium;* mixed population; well diffusion method; joint cultivation; starter

1. Introduction

Most foods are unsterile. They can be contaminated with pathogenic and saprophytic microorganisms. Saprophytic microorganisms utilize substrates in food, the metabolites they produce are accumulated, causing microbial spoilage. Pathogens that contaminate food cause foodborne illness and disorders, gastritis, enteritis, peptic ulcer, ulcerative colitis, and others when they enter the gastrointestinal tract. To ensure microbial food safety and to protect public health there is a trend for enrichment of food with lactobacilli, bifidobacteria, propionic acid bacteria with high antimicrobial activity against pathogenic (causing food toxicoinfections and toxemia) and saprophytic microorganisms.

Fermentation with lactic acid bacteria (LAB) has long been used in the processing of different foods. Milk, meat and vegetable products, as well as silage, have been prepared using LAB starters in order to improve the flavour and texture of the products. Lactic acid bacteria have been shown to enhance the stability and nutritional value of food products by preventing the growth of pathogenic and spoilage microbes [1]. Some strains of *Lactobacillus*, *Bifidobacterium* and also some *Propionibacterium* have been introduced as probiotics in food products due to their health-promoting effects [2, 3]. Representatives of these genera can be isolated from their natural habitats (faeces of infants) or naturally fermented products [4, 5]. In the selection of bacterial strains for use in the production of probiotic functional foods, a number of criteria, one of the most important among them being the ability to increase the natural defenses of the host against enteropathogens by the production of antimicrobial substances or through competitive inhibition and expulsion of these pathogens, are required to be met by the probiotic strains [6].

2. Materials and methods

2.1. Studied microorganisms

80 strains homo-and heterofermentative lactobacilli, 10 bifidobacteria strains and 5 propionic acid bacteria strains are isolated from different sources (human gastrointestinal tract naturally fermented vegetables and naturally fermented sourdough and yogurt) and identified by molecular genetic methods (ARDRA, RAPD, 16S rDNA sequencing). The isolated strains are tested for acid synthesis and reproductive activity, ability to survive in the conditions of the gastrointestinal tract (at different values of pH in combination with pepsin, pancreatin, and at different concentrations of bile salts), resistance to the majority of the antibiotics applied in clinical practice, presence of surface S-layer proteins and among them the strains of lactobacilli, bifidobacteria and propionic acid probiotic bacteria in Table 1 are selected.

Table 1 Strains of lactobacilli, bifidobacteria and propionic acid bacteria with probiotic properties.

Strain	Origin
Lactobacillus acidophilus A2	Human origin
Lactobacillus acidophilus Ac	Human origin
Lactobacillus acidophilus Z10	Naturally fermented sourdough
Lactobacillus brevis LBRZ7	Naturally fermented vegetables
Lactobacillus brevis LBRZ8	Naturally fermented vegetables
Lactobacillus paracasei LBRC11	Homemade cheese
Lactobacillus paracasei PX3	Naturally fermented sourdough
Lactobacillus paracasei RN5	Naturally fermented sourdough
Lactobacillus delbrueckii ssp.*bulgaricus* GB	Human origin
Lactobacillus delbrueckii ssp.*bulgaricus* M3	Homemade yoghurt
Lactobacillus delbrueckii ssp.*bulgaricus* B	Human origin
Lactobacillus fermentum LBRH9	Human origin
Lactobacillus fermentum LBRH10	Human origin
Lactobacillus fermentum R10	Naturally fermented sourdough
Lactobacillus plantarum F3	Naturally fermented sourdough
Lactobacillus plantarum LBRZ12	Naturally fermented vegetables
Lactobacillus plantarum X2	Naturally fermented sourdough
Bifidobacterium bifidum 4	Human origin
Propionibacterium freudenreichii ssp. *shermanii* NBIMCC 327	Hard cheese
Propionibacterium freudenreichii ssp. *shermanii* NBIMCC 328	Hard cheese

2.2. Test microorganisms:

2.2.1. Saprophytic microorganisms: *Bacillus subtilis, Bacillus mesentericus, Saccharomyces cerevisiae, Penicillium sp., Rhizopus sp., Aspergillus niger*.

2.2.2. Pathogenic microorganisms: *Escherichia coli* ATCC 25922*, Escherichia coli* ATCC 8739*, Salmonella sp.* (clinical isolate), *Salmonella abony* NTCC 6017*, Staphylococcus aureus* ATCC 25093*, Proteus vulgaris J, Klebsiella pneumoniae, Pseudomonas aeruginosa* ATCC 9027*, Staphylococcus aureus* ATCC 6538T and *Enterococcus faecalis*.

2.3 Media

2.3.1. Sterile skimmed milk with titratable acidity 16-18ºT. Composition (g/dm^3): skimmed milk powder (Scharlau). Sterilization - 15 minutes at 118ºC.

2.3.2. Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

2.3.3. LAPTg10 - broth. Composition (g/dm^3): peptone - 15, yeast extract - 10; tryptone - 10, glucose - 10. pH is adjusted to 6.6 - 6.8 and Tween 80 - 1cm^3/dm^3 is added. Sterilization - 20 minutes at 121ºC.

2.3.4. LAPTg10-agar. Composition (g/dm^3): LAPTg10-broth + 2% agar. Sterilization - 20 minutes at 121ºC.

2.3.5. LBG-agar. Composition (g/dm^3): tryptone – 10, yeast extract - 5, NaCl - 10, glucose – 10, agar - 20. pH is adjusted to 7.5. Sterilization - 20 minutes at 121°C.

2.3.6. Solid medium for bifidobacteria. Composition (g/dm^3): peptone - 10, yeast extract - 10, lactose - 10, $MnSO_4$ – 1, casein hydrolyzate - 8, NaCl – 3.2, CH_3COONa – 1, agar - 20. pH is adjusted to 6.6 - 6.8. Sterilization - 20 minutes at 121°C.

2.4. Determination of the antimicrobial activity against saprophytic microorganisms.

To determine the antimicrobial activity of the tested strains of lactobacilli against saprophytic microorganisms liquid culture (LC), acellular supernatant without pH adjustment (ASN) and neutralized acellular supernatant (NASN) (pH 6.5), obtained from a 48 hour culture of the tested strains are used. The antimicrobial activity is tested against the following test microorganisms: bacteria - *Bacillus subtilis*, *Bacillus mesentericus*; yeasts - *Saccharomyces cerevisiae*,

molds - *Aspergillus niger*, *Penicillium sp*., *Rhizopus sp*. Single strain spore suspensions of each of the test microorganisms (10^6-10^7cfu/cm^3) are plated in Petri dishes applying the pour plate method. The final concentration of the saprophytic microorganisms in the solidified LBG-agar medium is 10^4-10^5cfu/cm^3. After the hardening of the agar wells (d_{well} = 6 mm) are prepared. 60 μl of LC, ASN or NASN are pipetted in the wells of the plates and the plates with the test microorganisms are incubated at 30ºC or 37ºC for 24 to 48 hours. The experiments are performed in quadriplicates and then the average of the four parallel measurements of the inhibition zones in mm are reported.

2.5. Determination of the antimicrobial activity against pathogenic microorganisms.

To determine the antimicrobial activity of the studied strains of lactobacilli or bifidobacteria against pathogens a 48 hour cultural suspension of the *Lactobacillus* or the *Bifidobacterium* strain is used. Separate cultivation of all *Lactobacillus*, *Bifidobacterium* and pathogen strains as well as joint cultivation of each *Lactobacillus* or *Bifidobacterium* strains and each oft he pathogens included in the study are conducted. The following pathogens are used: *E. coli* ATCC 25922, *E.coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella sp*., *Staphylococcus aureus* ATCC 25093 and *Proteus vulgaris J*. For the examination of the joint cultivation 0.5 cm^3 of the suspension of the *Lactobacillus* or the *Bifidobacterium* strain, 0.5 cm^3 of the suspension of the pathogen and 9 cm^3 of culture medium (LAPTg10-broth or skimmed milk) are mixed. In the control of the *Lactobacillus* or the *Bifidobacterium* strain and in the control of each pathogen 9.5 cm^3 of the liquid LAPTg10 medium or skimmed milk are mixed with 0.5 cm^3 of the suspension of the *Lactobacillus* or the *Bifidobacterium* strain or of the suspension of the pathogen, respectively. The joint cultivation of the *Lactobacillus* or the *Bifidobacterium* strain and each of the pathogenic microorganism under static conditions in a thermostat at 37±1ºC for 72 hours, taking samples at 0, 12, 24, 36, 48, 60 and 72 h and monitoring the change of the titratable acidity and the concentration of viable cells of both the pathogen and the *Lactobacillus* or the *Bifidobacterium* strain is performed. Determination of the number of viable cells is done by the spread plate method on LAPTg10-agar (for the enumeration of lactobacilli), on LBG-agar (for the enumeration of pathogens) or the pour plate method in solid medium for bifidobacteria (for the enumeration of bifidobacteria). The titratable acidity is determined according to a standard protocol [7].

3. Results and discussion

The antimicrobial activity of the strains *Lactobacillus acidophilus* A2, *Lactobacillus acidophilus* Ac, *Lactobacillus acidophilus* Z10, *Lactobacillus paracasei* PX3, *Lactobacillus paracasei* RN5, *Lactobacillus paracasei* LBRC11, *Lactobacillus delbrueckii* ssp. *bulgaricus* B, *Lactobacillus delbrueckii* ssp. *bulgaricus* GB, *Lactobacillus delbrueckii* ssp. *bulgaricus* M3, *Lactobacillus plantarum* F3, *Lactobacillus plantarum* X2, *Lactobacillus plantarum* LBRZ12 and *Bif. bifidum* 4 is studied by joint cultivation with pathogenic microorganisms: *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella sp.* (clinical isolate), *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris J*. With representatives of the species *L.acidophilus*, *L.delbrueckii* ssp.*bulgaricus* and *Bif.bifidum* 4 the experiment is performed using skimmed milk, while with the strains of *L.plantarum* and *L.paracasei* - in LAPTg10-broth.

During separate cultivation of the representatives of the genus *Lactobacillus* or the genus *Bifidobacterium* under static conditions at 37±1°C for 12-24 hours they accumulate over 10^{12}-10^{13}cfu/cm^3 viable cells. At the same time the titratable acidity of the medium increases significantly (starting as low as 73.47 and reaching as high as 311.62 at the 72nd hour of separate cultivation). High concentration of live cells of the following pathogens: *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella abony* NTCC 6017, *Salmonella sp.* (clinical isolate), *Staphylococcus aureus* ATCC 25293 and *Proteus vulgaris J* are obtained during the separate cultivation in the same media – up to the 36th hour from the beginning of the process over 10^{11}-10^{14}cfu/cm^3 of the pathogens are accumulated, but the titratable activity of the medium changes very little.

During the joint cultivation of the studied strains of the genus *Lactobacillus* or the genus *Bifidobacterium* under static conditions at 37±1°C an increase in the concentration of viable cells of the probiotic strains from the beginning of the joint cultivation is observed, while the concentration of living cells of each of the pathogens gradually decreases. The reduction in the number of viable cells of the pathogens to a great extent is a result of the generated organic acids, given in Table 2, which lower the pH of the medium, and of the formed antimicrobial substances associated with the cell surface or released into the medium [8].

The antimicrobial effect of lactic, acetic and propionic acid is attributed to their undissociated molecules. Dissociation constants (pKa) are 4.8 for acetic, 4.9 for propionic and 3.8 for lactic acid, respectively. The lower antimicrobial activity of the lactic acid is probably due to its low pKa. The antimicrobial action of the undissociated molecules is obtained by dissociation of the molecules in the cytoplasm after entering through the membrane. The released hydrogen ions during the dissociation reduce the transmembrane gradient and neutralize the proton motive force, change the internal pH and cause denaturation of proteins and loss of viability. However, according to Ray and Bhunia [9] these weak acids exhibit antimicrobial activity due to the combined effects of the undissociated molecules and the dissociated ions. Undissociated molecules and dissociated ions induce cell damage [10].

Table 2 Content of organic acids in the culture medium of lactobacilli, bifidobacteria and propionic acid bacteria.

Organic acid / Strain	Lactic acid, mg/cm^3	Citric acid, mg/cm^3	Acetic acid, mg/cm^3	Tartaric acid, mg/cm^3	Propionic acid, mg/cm^3
L.acidophilus A2	33.47	7.54	-	1.93	-
L.acidophilus Ac	13.31	6.55	-	1.15	-
L.paracasei RN5	23.11	7.75	-	2.18	-
L.d. ssp.*bulgaricus* GB	45.89	-	-	5.25	-
L.d. ssp.*bulgaricus* M3	15.66	7.78	-	1.44	-
L.d. ssp.*bulgaricus* B	12.88	6.25	-	1.18	-
L.plantarum F3	16.69	5.85	-	-	-
Bif. bifidum 4	23.23	12.67	14.09	1.96	-
Prop.freudenreichii ssp. *shermanii* NBIMCC 328	-	-	0.02	-	4.03

-not present in the culture medium

Experimental results, given in Table 3, Table 4, Table 5, Table 6, Table 7 and Table 8 show that the selected bacterial strains inhibit the growth of the pathogenic and toxigenic bacteria and the inhibition is strainspecific. It depends on the very strain of the pathogen as well as of the specific strain of lactobacilli or bifidobacteria.

Table 3 Antimicrobial activity of the selected probiotic strains against *E.coli* ATCC 25922.

Strain	Concentration of viable cells of *E.coli* ATCC 25922 during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$1.3x10^9$	$1.2x10^{12}$	$3.0x10^6$	$1.0x10^5$	$6.0x10^2$	$1.2x10^2$	0
L. acidophilus Ac	$5.0x10^8$	$1.3x10^{11}$	$4.7x10^9$	$3.0x10^4$	$5.0x10^2$	0	0
L. acidophilus Z10	$4.7x10^7$	$8.0x10^5$	$5.0x10^5$	$3.0x10^5$	$1.0x10^5$	0	0
L. paracasei LBRC11	$1.0x10^8$	$5.8x10^{11}$	$1.1x10^{14}$	$1.0x10^8$	$1.0x10^4$	0	0
L. paracasei PX3	$3.7x10^7$	$6.7x10^{10}$	$5.0x10^{11}$	$1.4x10^6$	$1.0x10^4$	0	0
L. paracasei RN5	$5.0x10^8$	$6.0x10^{11}$	$1.0x10^8$	$1.0x10^7$	$1.6x10^4$	0	0
L. d. ssp.*bulgaricus* GB	$1.3x10^9$	$1.3x10^{11}$	$5.0x10^6$	$8.0x10^4$	$5.0x10^4$	0	0
L. d. ssp.*bulgaricus* M3	$5.8x10^7$	$2.2x10^{10}$	$9.0x10^6$	$8.0x10^6$	$2.6x10^6$	$8.4x10^5$	$2.6x10^4$
L. d. ssp.*bulgaricus* B	$2.0x10^7$	$2.0x10^8$	$8.0x10^5$	$1.0x10^4$	$8.0x10^3$	$1.5x10^3$	$1.0x10^1$
L. plantarum F3	$2.0x10^9$	$2.0x10^8$	$1.0x10^6$	$4.5x10^5$	$1.3x10^5$	0	0
L. plantarum LBRZ12	$5.0x10^6$	$2.4x10^9$	$4.8x10^{10}$	$1.6x10^8$	$1.2x10^6$	$2.6x10^5$	$2.0x10^5$
L. plantarum X2	$6.0x10^7$	$1.0x10^8$	$1.1x10^6$	$5.0x10^5$	$1.0x10^5$	0	0
Bif.bifidum 4	$6.7x10^8$	$1.2x10^{11}$	$1.8x10^{10}$	$4.0x10^5$	$6.0x10^3$	0	0

Table 4 Antimicrobial activity of the selected probiotic strains against *E.coli* ATCC 8739.

Strain	Concentration of viable cells of *E.coli* ATCC 8739 during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$2.2x10^8$	$8.0x10^{10}$	$5.1x10^8$	$4.0x10^6$	$3.0x10^5$	0	0
L. acidophilus Ac	$2.2x10^8$	$1.4x10^9$	$1.4x10^9$	$3.0x10^5$	$2.0x10^3$	0	0
L. acidophilus Z10	$7.0x10^6$	$3.3x10^{10}$	$6.0x10^{10}$	$1.0x10^4$	0	0	0
L. paracasei LBRC11	$5.4x10^8$	$3.2x10^{11}$	$2.0x10^{13}$	$1.0x10^9$	$1.6x10^4$	0	0
L. paracasei PX3	$1.2x10^8$	$1.0x10^{13}$	$4.0x10^9$	$1.2x10^6$	$1.5x10^4$	0	0
L. paracasei RN5	$1.0x10^7$	$1.0x10^{13}$	$1.2x10^8$	$1.4x10^7$	$2.0x10^5$	$2.0x10^2$	0
L. d. ssp.*bulgaricus* GB	$2.9x10^8$	$1.1x10^{11}$	$7.0x10^8$	$3.0x10^4$	$5.0x10^2$	0	0
L. d. ssp.*bulgaricus* M3	$1.3x10^6$	$1.2x10^{10}$	$6.0x10^{10}$	$2.4x10^{10}$	0	0	0
L. d. ssp.*bulgaricus* B	$2.8x10^6$	$2.2x10^8$	$4.0x10^5$	$9.0x10^3$	$1.0x10^3$	$5.0x10^2$	$1.0x10^1$
L. plantarum F3	$6.0x10^9$	$3.0x10^{10}$	$1.3x10^6$	$1.6x10^5$	$1.2x10^4$	0	0
L. plantarum LBRZ12	$2.2x10^8$	$2.5x10^{11}$	$2.2x10^{12}$	$3.0x10^7$	$1.9x10^5$	$1.0x10^3$	$4.0x10^0$
L. plantarum X2	$7.1x10^9$	$2.0x10^8$	$1.0x10^8$	$1.7x10^6$	$1.5x10^4$	0	0
Bif.bifidum 4	$2.2x10^9$	$2.4x10^{11}$	$1.6x10^{11}$	$7.0x10^5$	$3.0x10^5$	$2.0x10^4$	0

Table 5 Antimicrobial activity of the selected probiotic strains against *Salmonella abony* NTCC 6017.

Strain	Concentration of viable cells of *Salmonella abony* NTCC 6017 during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$1.0x10^7$	$6.9x10^{10}$	$2.2x10^9$	$1.4x10^9$	$1.0x10^5$	$6,2x10^1$	0
L. acidophilus Ac	$4.0x10^8$	$9.6x10^{11}$	$6.3x10^7$	$6.6x10^4$	$4.8x10^3$	$6.9x10^1$	0
L. acidophilus Z10	$9.0x10^6$	$3.2x10^{11}$	$1.4X10^7$	$1.2x10^4$	$1.5x10^3$	0	0
L. paracasei LBRC11	$4.6x10^8$	$2.0x10^{12}$	$3.0x10^{10}$	$1.5x10^5$	$1.0x10^4$	0	0
L. paracasei PX3	$8.0x10^7$	$3.5x10^{11}$	$2.7x10^{12}$	$2.0x10^{12}$	$6.5x10^6$	$6.0x10^0$	0
L. paracasei RN5	$6.0x10^9$	$4.5x10^{10}$	$4.8x10^{11}$	$1.4x10^5$	$1.5x10^4$	0	0
L. d. ssp.*bulgaricus* GB	$4.0x10^7$	$1.5x10^9$	$1.1x10^9$	$4.8x10^4$	$6.3x10^3$	$5.2x10^1$	0
L. d. ssp.*bulgaricus* M3	$1.2x10^7$	$4.0x10^{11}$	$1.4x10^7$	$1.6x10^4$	$1.5x10^3$	0	0
L. d. ssp.*bulgaricus* B	$1.2x10^6$	$4.6x10^{10}$	$1.6x10^7$	$1.5x10^4$	$1.3x10^3$	$3.0x10^1$	0
L. plantarum F3	$4.4x10^9$	$3.2x10^{11}$	$4.0x10^{10}$	$2.6x10^7$	$1.0x10^7$	0	0
L. plantarum LBRZ12	$2.5x10^9$	$9.1x10^{11}$	$1.0x10^{10}$	$1.2x10^5$	$1.0x10^4$	0	0
L. plantarum X2	$5.7x10^9$	$2.4x10^{11}$	$3.9x10^{11}$	$1.4x10^5$	$1.7x10^4$	$1.0x10^4$	$3.0x10^3$
Bif.bifidum 4	$1.0x10^7$	$2.1x10^{10}$	$7.0x10^9$	$6.0x10^9$	$1.4x10^7$	$7.1x10^1$	0

The representatives of *E.coli*, *Salmonella* and *Proteus vulgaris* are sensitive to the presence of probiotic bacteria and during joint cultivation complete inhibition up to the 60th to the 72nd hour is achieved, which is important for the application of these probiotic strains in the composition of probiotics and probiotic foods. More resistant to the presence of probiotic bacteria in the medium are the cells of *Staphylococcus aureus* ATCC 25293, but this pathogen is sensitive to *Bif.bifidum* 4. The data in Table 7 shows that during joint cultivation of this pathogen and each of the other representatives of the probiotic bacteria the cells of *Staphylococcus aureus* ATCC 25293 decrease but at the 72nd hour they vary within the range of 10^2cfu/cm^3 to 10^5cfu/cm^3.

It can clearly be seen from the results in Table 3, Table 4, Table 5, Table 6, Table 7, Table 8 that acidophilic bacteria and bifidobacteria exibit higher inhibitory activity against the tested pathogens in comparison with the strains of *Lactobacillus delbrueckii* ssp.*bulgaricus*.

Table 6 Antimicrobial activity of the selected probiotic strains against *Salmonella sp.*

Strain	Concentration of viable cells of *Salmonella sp.* during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$8.3x10^9$	$6.0x10^{10}$	$5.6x10^{12}$	$5.2x10^4$	$4.9x10^3$	$6.1x10^1$	0
L. acidophilus Ac	$3.3x10^9$	$6.8x10^{12}$	$8.0x10^{11}$	$7.6x10^6$	$2.0x10^5$	$4.6x10^1$	$1.0x10^1$
L. acidophilus Z10	$8.0x10^8$	$4.6x10^{10}$	$1.3x10^7$	$1.5x10^4$	$1.8x10^3$	0	0
L. paracasei LBRC11	$3.0x10^7$	$2.5x10^{11}$	$8.0x10^{10}$	$1.0x10^6$	$2.5x10^6$	$1.0x10^4$	$5.4x10^3$
L. paracasei PX3	$8.4x10^9$	$1.0x10^{13}$	$1.0x10^{11}$	$5.0x10^9$	$1.3x10^4$	$4.0x10^2$	$8.1x10^1$
L. paracasei RN5	$1.2x10^9$	$5.2x10^{11}$	$5.0x10^{12}$	$1.8x10^{12}$	$1.5x10^4$	0	0
L. d. ssp.*bulgaricus* GB	$1.3x10^9$	$1.7x10^{10}$	$1.0x10^8$	$6.3x10^4$	$7.4x10^3$	$4.0x10^3$	0
L. d. ssp.*bulgaricus* M3	$4.8x10^8$	$1.0x10^{12}$	$1.0x10^9$	$9.0x10^4$	$8.6x10^4$	$8.1x10^4$	$1.4x10^4$
L. d. ssp.*bulgaricus* B	$7.0x10^7$	$1.0x10^{12}$	$5.0x10^9$	$1.3x10^4$	$1.7x10^3$	0	0
L. plantarum F3	$5.0x10^8$	$1.5x10^{12}$	$8.0x10^{10}$	$2.9x10^8$	$1.6x10^4$	0	0
L. plantarum LBRZ12	$7.0x10^9$	$1.5x10^{11}$	$1.0x10^{10}$	$1.4x10^5$	$2.3x10^4$	$1.0x10^4$	$6.4x10^3$
L. plantarum X2	$6.0x10^9$	$1.4x10^{13}$	$1.1x10^{12}$	$2.8x10^7$	$1.3x10^4$	$1.0x10^4$	$5.8x10^3$
Bif.bifidum 4	$7.1x10^8$	$4.8x10^{12}$	$8.0x10^8$	$6.7x10^7$	$1.0x10^5$	$4.7x10^1$	0

Table 7 Antimicrobial activity of the selected probiotic strains against *Staphylococcus aureus* ATCC 25293.

Strain	Concentration of viable cells of *Staph. aureus* ATCC 25293 during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$2.1x10^8$	$5.8x10^{11}$	$1.1x10^{12}$	$1.9x10^8$	$1.0x10^7$	$4.4x10^5$	$3.0x10^2$
L. acidophilus Ac	$2.6x10^8$	$4.5x10^{12}$	$8.0x10^8$	$1.0x10^6$	$7.3x10^4$	$2.0x10^2$	$1.0x10^2$
L. acidophilus Z10	$5.0x10^7$	$1.6x10^{10}$	$6.0x10^9$	$4.0x10^6$	$1.0x10^6$	$6.0x10^5$	$5.0x10^4$
L. paracasei LBRC11	$1.2x10^9$	$6.0x10^9$	$4.0x10^{10}$	$6.0x10^6$	$3.0x10^5$	$2.2x10^5$	$1.3x10^5$
L. paracasei PX3	$3.8x10^8$	$2.5x10^8$	$1.8x10^8$	$2.0x10^6$	$2.5x10^5$	$1.5x10^5$	$1.0x10^5$
L. paracasei RN5	$1.4x10^8$	$1.7x10^8$	$2.0x10^8$	$1.1x10^6$	$1.8x10^4$	$1.4x10^4$	$1.0x10^4$
L. d. ssp.*bulgaricus* GB	$3.5x10^8$	$2.0x10^{10}$	$1.6x10^{10}$	$1.0x10^7$	$9.2x10^6$	$3.0x10^3$	0
L. d. ssp.*bulgaricus* M3	$8.2x10^7$	$8.0x10^9$	$4.0x10^9$	$1.1x10^8$	$7.0x10^7$	$2.0x10^5$	$2.0x10^5$
L. d. ssp.*bulgaricus* B	$2.0x10^7$	$1.5x10^{11}$	$1.3x10^{10}$	$7.0x10^7$	$5.0x10^7$	$6.0x10^5$	$2.8x10^4$
L. plantarum F3	$6.0x10^8$	$5.5x10^9$	$9.5x10^{11}$	$8.0x10^6$	$1.0x10^6$	$1.0x10^6$	$3.8x10^5$
L. plantarum LBRZ12	$1.4x10^9$	$3.6x10^9$	$2.1x10^{11}$	$1.2x10^8$	$1.0x10^6$	$4.0x10^3$	$2.0x10^3$
L. plantarum X2	$5.7x10^8$	$2.4x10^{10}$	$2.0x10^{12}$	$8.4x10^6$	$8.0x10^5$	$4.6x10^4$	$2.4x10^4$
Bif.bifidum 4	$5.0x10^8$	$1.2x10^{11}$	$1.8x10^{11}$	$3.4x10^{13}$	$2.7x10^8$	$3.0x10^3$	0

Table 8 Antimicrobial activity of the selected probiotic strains against *Proteus vulgaris J.*

Strain	Concentration of viable cells of *Proteus vulgaris J* during joint cultivation, cfu/cm^3						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
L. acidophilus A2	$7.2x10^7$	$2.6x10^{10}$	$1.0x10^9$	0	0	0	0
L. acidophilus Ac	$2.4x10^7$	$6.6x10^{10}$	$1.0x10^9$	$5.5x10^4$	0	0	0
L. acidophilus Z10	$8.0x10^7$	$9.0x10^6$	$6.0x10^6$	$1.0x10^3$	$1.0x10^2$	0	0
L. paracasei LBRC11	$8.1x10^7$	$4.2x10^9$	$2.4x10^{11}$	$2.1x10^{11}$	$3.0x10^7$	$1.3x10^5$	$3.0x10^2$
L. paracasei PX3	$9.7x10^8$	$3.3x10^9$	$1.9x10^9$	$1.2x10^7$	$1.6x10^5$	$1.5x10^5$	$4.8x10^2$
L. paracasei RN5	$2.4x10^9$	$3.3x10^9$	$5.0x10^9$	$4.1x10^5$	$1.7x10^4$	$1.5x10^4$	$6.3x10^2$
L. d. ssp.*bulgaricus* GB	$7.0x10^7$	$8.6x10^{10}$	$1.2x10^9$	$3.0x10^2$	$2.0x10^1$	0	0
L. d. ssp.*bulgaricus* M3	$8.3x10^8$	$1.0x10^{10}$	$1.0x10^6$	$3.0x10^5$	$1.0x10^2$	0	0
L. d. ssp.*bulgaricus* B	$4.3x10^8$	$1.0x10^{10}$	$1.0x10^6$	$1.0x10^3$	$1.0x10^2$	0	0
L. plantarum F3	$9.4x10^8$	$2.0x10^9$	$1.1x10^{11}$	$1.7x10^{12}$	$8.2x10^4$	$6.2x10^4$	$5.0x10^0$
L. plantarum LBRZ12	$7.8x10^7$	$1.8x10^9$	$2.0x10^{11}$	$3.0x10^7$	$9.0x10^0$	$5.0x10^0$	$2.0x10^0$
L. plantarum X2	$1.7x10^8$	$1.9x10^{10}$	$8.0x10^5$	$2.8x10^5$	$4.0x10^4$	$3.8x10^4$	$2.0x10^4$
Bif.bifidum 4	$7.0x10^7$	$6.4x10^9$	$3.0x10^9$	$5.0x10^3$	$6.3x10^2$	$1.0x10^2$	$1.0x10^2$

Using the agar diffusion method with wells it is determined that propionic acid bacteria *Prop. freudenreichii* ssp. *shermanii* NBIMCC 328 and *Prop. freudenreichii* ssp. *shermanii* NBIMCC 327 exibit antimicrobial activity against pathogenic microorganisms as well.

Table 9 Inhibitory activity of *Pr. freudenreichii* ssp. *shermanii* NBIMCC 328 and *Pr. freudenreichii* ssp. *shermanii* NBIMCC 327 on the growth of pathogenic and toxigenic microorganisms. The values are in mm. d_{well} - 6 mm.

Pathogenic microorganisms	Concentration of viable cells of pathogenic microorganisms, cfu/cm^3	*Prop. freudenreichii* ssp. *shermanii* NBIMCC 327	*Prop. freudenreichii* ssp. *shermanii* NBIMCC 328
Salmonella sp.	$1.2x10^{12}$	13	12.5
Candida albicans NBIMCC 74	$5.0x10^{8}$	9	8
Proteus vulgaris J	$5.0x10^{11}$	9	8
Enterococcus faecalis	$2.2x10^{11}$	10	10
Staphylococcus aureus ATCC 6538T	$1.0x10^{11}$	9	8
Pseudomonas aeruginosa ATCC 9027	$7.0x10^{10}$	9	9
Klebsiella pneumoniae	$1.0x10^{11}$	21	21
Escherichia coli ATCC 8739	$8.6x10^{11}$	13	9
Titratable acidity, (ºT)	-	100	95

Table 9 shows that *Pr. freudenreichii* ssp. *shermanii* NBIMCC 328 and *Pr. freudenreichii* ssp. *shermanii* NBIMCC 327 supress the growth of the pathogens by the metabolites they produce, which is essential for the suppression of the unwanted microflora during the ripening of hard cheeses.

The antimicrobial activity of the strains *Lactobacillus brevis* LBRZ7, *Lactobacillus brevis* LBRZ8, *Lactobacillus paracasei* LBRC11, *Lactobacillus paracasei* PX3, *Lactobacillus paracasei* RN5, *Lactobacillus fermentum* LBRH10, *Lactobacillus fermentum* LBRH9, *Lactobacillus fermentum* R10, *Lactobacillus plantarum* F3, *Lactobacillus plantarum* LBRZ12 and *Lactobacillus plantarum* X2 against saprophytic microorganisms is determined using the well diffusion method. The results are given in Table 10.

Lactobacillus brevis LBRZ7 and *Lactobacillus brevis* LBRZ8 demonstrate low antimicrobial activity against *Penicillium sp.* and high inhibitory activity against *Aspergillus niger* and *Rhizopus sp. Lactobacillus brevis* LBRZ7 exibits antimicrobial activity against *Bacillus mesentericus* and *Bacillus subtilis* at 30°C but not at 37°C. In contrast, *Lactobacillus brevis* LBRZ8 is active against *Bacillus mesentericus* and *Bacillus subtilis* at 37°C but not at 30°C. Both strains do not supress *Saccharomyces cerevisiae*.

Table 10 Antimicrobial activity of *Lactobacillus brevis* LBRZ7, *Lactobacillus brevis* LBRZ8, *Lactobacillus paracasei* LBRC11, *Lactobacillus paracasei* PX3, *Lactobacillus paracasei* RN5, *Lactobacillus fermentum* LBRH10, *Lactobacillus fermentum* LBRH9, *Lactobacillus fermentum* R10, *Lactobacillus plantarum* F3, *Lactobacillus plantarum* LBRZ12 and *Lactobacillus plantarum* X2 - against saprophytic microorganisms. The values are in mm. Diameter of the wells - 7 mm. Liquid culture (LC), acellular supernatant without pH adjustment (ASN) and neutralized acellular supernatant (NASN) (pH 6.5).

Saprophyte			*Bacillus subtilis* $1.9x10^5$cfu/cm^3		*Bacillus mesentericus* $4.0x10^4$cfu/cm^3		*Saccharomyces cerevisiae* $9.2x10^4$cfu/cm^3		*Aspergillus niger* $1.2x10^5$cfu/cm^3		*Rhizopus sp.* $1.8x10^5$cfu/cm^3	*Penicillium sp.* $5.2x10^5$cfu/cm^3
Temperature of incubation			30°C	37°C	30°C	37°C	30°C	37°C	30°C	37°C	30°C	30°C
Lactobacillus brevis	**LBR Z7**	LC	11	-	13	9	-	-	10	13	14.5	8
		ASN	10	-	9	-	-	-	10	10	12	8
		NASN	10	-	9	-	-	-	10	9	12	-
	LBR Z8	LC	-	10	-	10.5	-	-	9	11	10	8
		ASN	-	10	-	9	-	-	8	9	9	8
		NASN	-	9	-	9	-	-	-	-	-	-
Lactobacillus paracasei	**LBR C11**	LC	-	-	10	12	-	15	10	16	14.5	10
		ASN	-	-	10	10	-	-	9	10	11	8
		NASN	-	-	9	10	-	-	9	-	8	8
	PX3	LC	+single colonies in the clear zone	13	13	10	-	15	12.5	10	13.5	8
		ASN	-	11	10	9	-	-	10	9	10	-
		NASN	-		7	8.5	-	-	10	9	10	-
	RN5	LC	18	15	11.5	13	-	11	11	11	15	13
		ASN	13	13	11	12	-	10	9	10	10.5	8
		NASN	10	-	9	10	-	9	9	9	8	8
Lactobacillus fermentum	**LBR H10**	LC	10	12	-	10.5	-	-	11.5	10	12	-
		ASN	-	10	-	10	-	-	10	-	9.5	-
		NASN	-	10	-	9.5	-	-	10.5	-	-	-
	LBR H9	LC	13	10.5	-	10	-	-	9	10	11	-
		ASN	-	10	-	10	-	-	9	9	11	-
		NASN	-	10	-	10	-	-	9	9	11	-
	R10	LC	10	9	9.5	10	-	+single colonies in the clear zone	12	10	13.5	10
		ASN	9	-	9	9	-	-	10	9	12	9
		NASN	8	-	9	-	-	-	10	-	9	8

Lactobacillus plantarum	LBR Z12	LC	-	-	9	11.5	-	-	12	12	14	13
		ASN	-	-	9	11	-	-	10	12	10.5	11
		NASN	-	-	9	11	-	-	10	9	-	10
	F3	LC	13	10.5	12	12	-	20	9	14	14.5	10
		ASN	12.5	10	10	11.5	-	13	8	10	12	9
		NASN	-	-	8.5	9	-	-	8	9	10	8
	X2	LC	11	9	12	11	-	14.5	16	13	16	12
		ASN	-	-	10	10	-	10	12	9	12	10
		NASN	-	-	8	8	-	-	11	9	11.5	-

Lactobacillus paracasei LBRC11, isolated from cheese, *Lactobacillus paracasei* PX3 and *Lactobacillus paracasei* RN5, isolated from naturally fermented sourdough, inhibit the growth of the fungi *Rhizopus sp*., *Penicillium sp*. and *Aspergillus niger*, and the bacteria *Bacillus mesentericus* at 30°C and 37°C. The liqiud cultures of the three strains exhibit antimicrobial activity against *Saccharomyces cerevisiae* only at 37°C. *Lactobacillus paracasei* LBRC11 does now supress the growth of *Bacillus subtilis. Lactobacillus paracasei* PX3 inhibits the growth of *Bacillus subtilis* at 30°C and at 37°C. Only single colonies in the clear zone around the wells with the liquid culture at 30°C are observed, while at 37°C there are clearly established clear zones. *Lactobacillus paracasei* RN5 possesses antimicrobial activity against *Bacillus subtilis* at 30°C and at 37°C. The highest inhibitory activity against *Bacillus subtilis* demonstrates *Lactobacillus paracasei* RN5, against molds *Aspergillus niger*, *Rhizopus sp*. and *Penicillium sp*. - *Lactobacillus paracasei* LBRC11, followed by *Lactobacillus paracasei* RN5 and *Lactobacillus paracasei* PX3. As for *Bacillus mesentericus* and *Bacillus subtilis Lactobacillus paracasei* RN5 has the highest inhibitory effect, followed by *Lactobacillus paracasei* PX3 and *Lactobacillus paracasei* LBRC11.

Lactobacillus fermentum LBRH10 and *Lactobacillus fermentum* LBRH9 (human origin) and *Lactobacillus fermentum* R10 (from naturally fermented sourdough) inhibit the growth of the fungi *Rhizopus sp*. and *Aspergillus niger* as well as of the bacteria *Bacillus subtilis* at 30°C and 37°C. *Lactobacillus fermentum* R10 exhibits antimicrobial activity against *Penicillium sp*. and *Bacillus mesentericus* at 30°C and 37°C, while *Lactobacillus fermentum* LBRH10 and *Lactobacillus fermentum* LBRH9 inhibit *Bacillus mesentericus* only at 37°C. *Lactobacillus fermentum* R10 exhibits antimicrobial activity against *Saccharomyces cerevisiae* at 37°C and only single colonies are observed in the clear zone around the wells with the liquid culture.

Lactobacillus fermentum R10 possesses the highest inhibitory activity against *Rhizopus sp*., *Penicillium sp*., *Aspergillus niger* and *Bacillus mesentericus*, followed by *Lactobacillus fermentum* LBRH10 and *Lactobacillus fermentum* LBRH9. As far as the inhibitory activity against *Bacillus subtilis* is concerned, the highest antimicrobial activity has *Lactobacillus fermentum* LBRH10, followed by *Lactobacillus fermentum* LBRH9 and *Lactobacillus fermentum* R10.

Lactobacillus plantarum LBRZ12, *Lactobacillus plantarum* F3 and *Lactobacillus plantarum* X2 possess antimicrobial activity against *Rhizopus sp.*, *Penicillium sp*., *Aspergillus niger* and *Bacillus mesentericus* at 30°C and at 37°C. *Lactobacillus plantarum* LBRZ12 inhibits the growth of *Bacillus mesentericus* at 30°C and 37°C, the values for the diameters being comparable, but does not influence the growth of *Saccharomyces cerevisiae* and *Bacillus subtilis* at both temperatures.

Lactobacillus plantarum F3 and *Lactobacillus plantarum* X2 demonstrate antimicrobial activity against *Bacillus subtilis* at 30°C and at 37°C as well as against *Saccharomyces cerevisiae*, but *Lactobacillus plantarum* X2 supresses *Saccharomyces cerevisiae* only at 37°C. *Lactobacillus plantarum* X2 shows the highest inhibitory activity against *Rhizopus sp*. and *Penicillium sp*. As far as *Aspergillus niger* is concerned the highest activity at 37°C has *Lactobacillus plantarum* F3, followed by *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12, while at 30°C the highest antimicrobial activity demonstrates *Lactobacillus plantarum* X2, followed by *Lactobacillus plantarum* LBRZ12 and *Lactobacillus plantarum* F3. For *Bacillus mesentericus Lactobacillus plantarum* F3 shows the highest antimicrobial activity at 30°C, followed by *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12, but at 37°C the highest antimicrobial activity possesses *Lactobacillus plantarum* F3, followed by *Lactobacillus plantarum* LBRZ12 and *Lactobacillus plantarum* X2. The strain *Lactobacillus plantarum* F3 shows higher antimicrobial activity than *Lactobacillus plantarum* X2 against *Saccharomyces cerevisiae*. The highest inhibitory activity against *Bacillus subtilis* exibits *Lactobacillus plantarum* F3, followed by *Lactobacillus plantarum* X2 at both temperatures of incubation. Against the molds *Aspergillus niger*, *Rhizopus sp*. and *Penicillium sp*. the strain *Lactobacillus plantarum* X2 has the highest inhibitory activity, followed by *Lactobacillus plantarum* LBRZ12 and *Lactobacillus plantarum* F3. As for *Bacillus mesentericus* and *Bacillus subtilis* the highest antimicrobial effect shows *Lactobacillus plantarum* F3, followed by *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12.

In the study of the antimicrobial activity of each strain of the species *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus fermentum* against any of the saprophytes, included in the study, the culture liquid exhibits greater antimicrobial activity than the supernatant, which means that the *Lactobacillus* strains' inhibition of the saprophytes is due not only to the formation of lactic acid and other organic acids, resulting in decreased pH, but there is also a competition for nutrients between the the *Lactobacillus* strain and the saprophyte as well as production of metabolites with antimicrobial effect by the *Lactobacillus* strain.

The observed differences between the strains of the species *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus fermentum* confirm the need for mandatory evaluation of the antimicrobial activity of the strains against saprophytes, because the inhibition proved to be strainspecific. It also underlines the need to combine different probiotic strains in order to achieve complete inhibition of the growth of bacterial and mold spores. As a result of the conducted studies strains of lactobacilli, bifidobacteria and propionic acid bacteria with high antimicrobial activity are selected and they are included in the composition of a number of products (Table 11): probiotics and starter cultures for the production of functional foods (yogurt; bioyogurt; bread with extended shelf-life; raw-dried meat products; soft, hard and semi-hard cheeses; organic preservation of cosmetic creams, etc.).

Table 11 Application of the selected probiotic strains

Strain	Application
Lactobacillus acidophilus A2	In the composition of the probiotics "Enterosan" For microbiological preservation of cosmetic creams
Lactobacillus acidophilus Ac	
Lactobacillus acidophilus Z10	
Lactobacillus brevis LBRZ7	In the composition of starters for sourdough for bread
Lactobacillus paracasei LBRC11	In the composition of starters for sourdough for bread In the composition of the starters for cheese
Lactobacillus paracasei PX3	In the composition of starters for sourdough for bread
Lactobacillus paracasei RN5	In the composition of starters for sourdough for bread
Lactobacillus delbrueckii ssp.*bulgaricus* GB	In the composition of starters for bioyoghurt with high concentration of viable cells In the composition of starters for cheese
Lactobacillus delbrueckii ssp.*bulgaricus* B	
Lactobacillus fermentum LBRH9	In the composition of starters for sourdough for bread
Lactobacillus fermentum R10	In the composition of starters for sourdough for bread
Lactobacillus plantarum F3	In the composition of starters for sourdough for bread
Lactobacillus plantarum LBRZ12	In the composition of starters for sourdough for bread In the composition of starters for raw dried meat products For microbiological preservation of cosmetic creams
Lactobacillus plantarum X2	In the composition of starters for sourdough for bread
Bifidobacterium bifidum 4	In the composition of the probiotics "Enterosan" For microbiological preservation of cosmetic creams
Propionibacterium freudenreichii ssp. *shermanii* NBIMCC 328	In the composition of starters for hard cheese with high temperature of secondary heating; For microbiological preservation of cosmetic creams

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References

[1] Samot J, Badet C. Antibacterial activity of probiotic candidates for oral health. *Anaerobe* 2013; 19: 34-38.
[2] FAO-WHO. Guidelines for the evaluation of probiotics in food. 2002; Working Group Rep. Food and Health Agricultural Organization of the United Nations and World Health Organization, Washington, DC.
[3] Lee LK, Salminen S. The coming of age of probiotics. *Trends in Food Science and Technology*. 1995; 6: 241–246.
[4] Delavenne E, Mounier J, Deniel F, Barbier G, Le Blay G. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. *International Journal of Food Microbiology*. 2012; 155: 185-190.
[5] Solis G, Reyes-Gavilan CG, de los Fernandez N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breastmilk and the infant gut. *Anaerobe*. 2010; 307-310.
[6] Morelli L. In vitro selection of probiotic lactobacilli: a critical appraisal. *Current Issues in Intestinal Microbiology*. 2000; 1: 59-67.
[7] Denkova Z. Production and application of probiotics. DSc thesis. Plovdiv, Bulgaria. 2005: 82 – 86.
[8] Macrae R, Robinson RK, Sadler M. Encyclopaedia of Food Science. *Food Technology and Nutrition*, Academic Press Inc. San Diego. 1993; 5: 3082.
[9] Ray B, Bhunia A. *Fundamental Food Microbiology*, fourth edition. 2007; 176-184.
[10] Jay SM, Loessner MS, Golden DA. *Modern Food Microbiology*, Copyright Springer. 2005; 376.

