Trakia Journal of Sciences, Vol. 7, No. 2, pp 33-39, 2009

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

Original Contribution

LACTIC ACID BACTERIA AGAINST PATHOGENIC MICROBES

M. Petrova[1,4], R. Georgieva[1], L. Dojchinovska[2], N. Kirilov[2], I. Iliev[3], S. Antonova[2], N. Hadjieva[4], I. Ivanova[2], S. Danova[1*]

[1] Department of Microbial Genetics, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Science, Sofia, Bulgaria
[2] Department of General and Aplied Microbiology, Biological Faculty, Sofia University, Sofia
[3] Department of Biochemistry & Microbiology, Plovdiv University, Plovdiv, Bulgaria
[4] Department of Clinical Microbiology and Virology, University Hospital Caritca Yoanna - ISUL, Sofia, Bulgaria

ABSTRACT

Background: Lactic acid bacteria (LAB) have become an attractive option of modern medical practice. Recently, attention has been paid to their health-promoting properties. Of particular importance are their probiotic properties and specially the ability to compete with pathogens *in vivo*.
Aim: To determine the antagonistic activity of 46 LAB strains against antibiotic-resistant and other out-patient bacterial strains.
Methods: Different standard protocols conform to the international rules for the data collection on antimicrobial susceptibility and on metabolic activity were applied.
Results: Our hypothesis was that LAB microbiota from complex ecosystems is able to express a broad spectrum of activity. Thus, 12 vaginal lactobacilli and 34 food-originated lactobacilli, lactococci and enterococci were studied. The production of organic acids is clearly involved in antagonistic activity of these bacteria *in vitro.* Eight out of the tested isolates were active against *Staphylococcus aureus* MRSA. The inhibitory activity of two vaginal and four food-originated strains was protease-sensitive and independent of the presence of lactic acid and H_2O_2.
Conclusion: These results are encouraging and prompt further research of the active LAB strains and their possible application to overcome the problem with increasing multi-drug resistance in clinical practice.

Key Words: probiotics, pathogens, antagonistic activity

INTRODUCTION

The Lactic acid bacteria (LAB) are well known probiotics with beneficial effects to human health (1). Their antimicrobial activity is one of the most important probiotic characteristics (2). The production of antimicrobial substances by resident or transit LAB microflora, to remove the pathogens is under extensive study in recent times (3, 4). However, the basis of the inhibition of Gram (-) and the different antibiotic-resistant pathogens have not been well established. Likewise, the widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance (5). This becomes a serious global problem, which constrains scientists to search for new effective therapeutic agents. For their further development the understanding of the mechanisms of competition between "good bacteria" and pathogens is very vital. In the present study we estimate the antagonistic activity of 46 LAB strains, isolated from different habitats, against Gram (+) and Gram (-) human pathogens including out-patient multi-resistant strains. The results of *in vitro* antimicrobial trials could guide microbiologists in the appropriate selection of probiotics for clinical practice and in health care.

*** Correspondence to:** *Dr. Svetla Danova, Department of Microbial Genetics, Institute of Microbiology, Bulgarian Acad. Sci., 26, Acad. Georgi Bontchev str., 1113 Sofia, Bulgaria, tel: (+359 2) 979 31 19; e-mail: std@ microbio.bas.bg*

MATERIALS AND METHODS

Strains and media

Forty six LAB included in this study were isolated from different habitats as follows: First group - 13 strains recently identified as *L. plantarum*- from Bulgarian white brined cheese (6); Second group - 12 *Lactobacillu*s strains from vaginal swab samples of Bulgarian volunteers (7); Third group - 13 new isolates, from home made fermented products, belonging to genus *Lactococcus, Enterococcus* and *Lactobacillus* and Fourth group - 8 strains *L. delbrueckii* subsp. *bulgaricus* from artisanal yogurts (8).

Selective media M17 (*Sharlau, Spain*); *de Man, Rogosa, Sharp* (MRS) broth and agar (*Difco*) and Rogosa agar (*Difco*) were used for pure culture isolations. All cultures were stored at –70°C in MRS or M17 broth supplemented with glycerol 20% v/v.

Ten pathogenic isolates (from the clinical laboratory of University Hospital Carica Joanna, Sofia) and four clinical reference ATCC strains were used as target cultures in the anti microbial tests (**Table 1**).

Antibiotic susceptibility of clinical isolates

The antibiotic susceptibility of each patient's isolates was determined by the standard agar disc diffusion tests (9) applying the widely used antibiotics **(**presented on **Table 1**). The strain's classification as: $^{R-}$ Resistant, $^{S-}$ Susceptible or $^{I-}$ Intermediate, was scored according to recommendation of National Committee for Clinical Laboratory Standards (NCCLS). All test-cultures and determined antibiotic resistance are presented on **Table 1**.

Antimicrobial tests

The inhibitory effect of LAB strains on selected clinical reference and pathogen strains was determined by the well-diffusion method as described previously (7). Two model systems for antimicrobial production were applied: MRS broth and 10% (w/v) Skim milk (*Sharlau, Spain*). The stored LAB strains were subsequently cultivated on MRS broth (pH-6.5), by anaerobiosis (BBL® GasPak Anaerobic system, Baltimore, USA) and transferred in: MRS broth (pH 6.5) and in 10% sterile skim milk, for 24 h at 37°C. Filtered culture supernatants (FCS) and the whey fractions (WF), 100 µl of each, were used as control samples in assays. In order to eliminate the putative effect of produced lactic acid they were additionally buffered with NaOH (5 M) to pH 5.5-6.0. When the inhibition zone was determined around the wells of both control and buffered samples, the inhibitory effect (more than 8 mm zone) was assumed to be due to bacteriocin-like substances (BLS) or H_2O_2.

RESULTS AND DISCUSSION

This *in vitro* study was attempted to evaluate the antagonistic activity of partially characterized and new isolated LAB strains, from different ecological niches. Thus, 12 vaginal and 34 food-originated *lactobacilli, lactococci* and *enterococci* were included in search for new effective probiotic strains**.** In assays with neutralized supernatants antimicrobial activity against the reference ATCC pathogens was shown for 13 out of the 46 strains. These isolates were determined as active due to the observed ability to inhibit the growth of at least one target strain. The data from testing of LAB isolates from human and food origin were compared and summarized (**Figure 1**). The results clearly show that the produced organic acids are involved in antagonistic activity of these bacteria *in vitro.* Only 13 strains were pre-selected for further analyses with test-microorganisms from clinical practice. Ten out-patient isolates belonging to species, often reported in relation with nosocomial infections were included. The high level and diversity of antibiotic resistance, estimated for each of them (presented on **Table 1**), was used as selection criterion. As a result, 9 neutralized cultures (nFCS) and 4 whey fractions (WF) obtained after 24 h cultivation of selected 13 LAB strains in MRS broth and milk respectively were subjected to the tests with ten antibiotic-resistant clinical isolates (**Figure 2**).

Only four *Lactobacillus* isolates were able to inhibit the selected clinical pathogens (**Figure 2A**). Therefore, between the original LAB formed three groups (First group- 13 L. plantarum from cheese; Second- 12 vaginal lactobacilli; Third- 13 other LAB isolated from food) were found strains with a broad spectrum of activity, including Gram (+) and Gram (-) bacteria. In the last 10 years there have been reported activity of lactobacilli against Gram-negative bacteria (10, 11, 12).

Table1 *Test-cultures and antibiotic resistance*

Tests microorganisms	[a] ***Strains and collections***	[b] ***Antibiotic resistance***	***Media and culture conditions***
Reference strains:			
Staphylococcus aureus MSSA	ATCC 25923	[S]-OXA, P, E, CC, G, Cip, Bis	37ºC BBL, Blood agar
Staphylococcus aureus MRSA	ATCC 39592	[R]- OXA, P	37ºC BBL, Blood agar
Escherichia coli	ATCC 25922	[S]-AmC, FOX, CTX, CRO, CAZ, AN, ATM, FEP, BIS, CIP, G, IMP	37ºC BBL, MacConkey agar
Pseudomonas aeruginosa	ATCC 27853	[S]-AN, PIP, FEP, IMP, Sul, Cipro, CAZ, TZP	37ºC BBL, MacConkey agar
Clinical isolates:			
Staphylococcus haemolyticus	CI№ 2788	[R]-P-G, OXA, AmC, CXM, BIS and [S]- G, AN, CIP, E, CC, Va, TCP	37ºC BBL, Blood agar
Steptococcus pyogenes group A	CI№ 2818	[R]- BIS and [S]- P-G, AmC, CXM, CIP, E, CC,VA,TCP	37ºC BBL, Blood agar
Enterococcus faecalis	C№ 2771	[S]- VA, TCP, AmC, G, MEM, LVX, LZD	37ºC BBL, Blood agar
E.coli ESBL	CI№ 2747,	[R]-AmC, AmP, FOX, CTX, CRO, CAZ, AN, ATM, FEP, BIS, CIP, G and [S]- IMP, MEM	37ºC BBL, MacConkey agar
Pseudomonas aeruginosa	CI№ 2792	[S]-PIP, CAZ, FEP, TZP, IMP, MEM, AN, CIP, Sul	37ºC BBL, MacConkey agar
Pseudomonas aeruginosa	CI№ 5	[R]- AmC, AM, AN ATM, CTX, CRO, CAZ, FOX, G, PIP, FEP, TZP, Bis, IMP, MEM, CIP, Sul)	37ºC BBL, MacConkey agar
Klebsiella pneumonie ESBL	CI№ 2766	[R]- AmC, AmP, AN, ATM, CTX, CRO, CAZ, FEP, BIS, CIP, G and [S]-FOX, IMP, MEM	37ºC BBL, MacConkey agar
Serratia marcescens	CI№ 2485	[R]-, AmC, AmP, CXM, AN, ATM, CTX, CRO, CAZ, FOX, G, FEP, BIS, CIP, NIT and [S]-IMP, MEM	37ºC BBL, MacConkey agar
Proteus mirabilis	CI№ 2767	[S]- AmC, CXM, FOX,CTX, CRO, CAZ, FEP, IMP, MEM, ATM, G, AM, BIS, CIP, NIT and [I]- AmP)	37ºC BBL, MacConkey agar
Acinetobacter baumanii	C№ 2762	[R]-AmC, AmP, CXM, FOX, CTX, CRO, CAZ, FEP, ATM and [S]- IPM, MEM, G, AN, BIS, CIP, Sul)	37ºC BBL, MacConkey agar

[a] ***ATCC***- American Type Culture Collection, Virginia, USA; The clinical isolates (C№) were obtained from the Laboratory of Clinical microbiology and virology, University Hospital "Caritca Joanna"Sofia, Bulgaria.
[b] [S]-Sensitive; [R]-Resistant; [I]-Intermediate; Antibiotics (used concentration in µg/disq*):* AmC– Amoxicillin/Clavulanic acid (20/10 µg); Amp– Ampicillin (10 µg); TZP- Piperacillin/Tazobactam (100/10 µg); PIP– Piperacillin (100 µg); CXM- Cefuroxime (30 µg); FOX- Cefoxitine (30 µg); CTX- Cefotaxime (30 µg); CRO– Ceftriaxone (30µg); CAZ – Ceftazidime (30 µg); FEP- Cefepime (30 µg); IMP- Imipenem (10 µg); MEM- Meropenem (10 µg); ATM- Aztreonam (30 µg); G- Gentamicin (10 µg); AN- Amikacin (30 µg); Nitro- Nitrofurantoin (100 µg); BIS- Biseptol (Sulfamethoxazole/Trimethoprim- 23,75/1,25 µg); Cip- Ciprofloxacin (5 µg); Sul- Sulbactam/Cefoperazone (30µg); P-G- penicillin (15 µg), OXA- Oxacillin (1 µg), Erythromycin (15 µg), CC-Clindamycin (2 µg), VA- Vancomycin (30 µg), TCP-Teicoplanin (10 µg), LVX-Levofloxacin (5 µg), LZD- Linezolid (30 µg)

This may be a part of protective mechanisms that allows LAB to dominate in complex ecosystems, such as gastro-intestinal or genital tracts. In the same time, in triple repeated assays with cell-free cultures (FCS and nFCS) and the whey fractions of 13 out of the 46 LAB strains, antibacterial activity against the clinical isolates *Staphylococcus haemoliticus* 2788, *Enterococcus fecalis* 2771 and *Klebsiella pneumonie ESBL* 2766 was not found. Likewise, the WFs, FCS and nFCS obtained after cultivation of lactobacilli isolated from yoghurt (the IV group) in milk and MRS respectively, were not able to inhibit

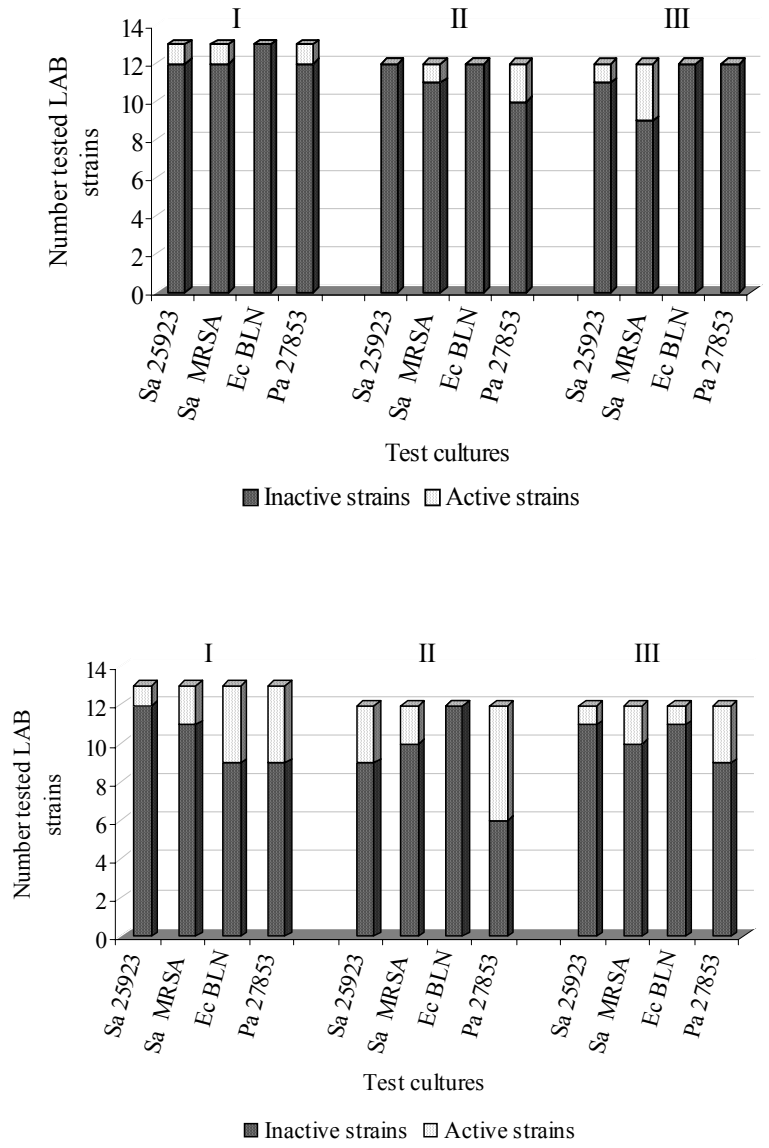

Figure 1. Comparison of activity of three groups of LAB strains against clinical reference pathogens: **group I**- 13 *L. plantarum* strains from white brined cheese; **group II**- 12 vaginal *Lactobacillus* strains, **group III**- non identified to the species level food originated LAB strains from the genus *Lactobacillus*, *Lactococcus* and *Enterococcus*.

Legend: *In vitro* tests were performed with: (A) neutralized filtered culture supernatants (nFCS) and (B) filtered culture supernatants without pH corrections (FCS)
Pa- *Pseudomonas aeruginosa* ATCC 27853, Ec.BLN- *E. coli* ATCC 25922; Sa- *Staphylococcus aureus* ATCC 25923; Sa MRSA- *Staphylococcus aureus* ATCC 39592

the growth of any of tested pathogens. The included 8 strains identified as *L. delbrueckii* subsp *bulgaricus* with high proteolytic activity (10), probably do not produce antimicrobials. Miteva *et al.* (1998) reported 36 *L. delbrueckii* strains from the ELBY *Bulgaricum* collection with a broad spectrum of activity, including closely related LAB species, pathogenic and food spoilage bacteria (13).

When the anti-microbial tests were performed with acid supernatants the inhibitory effect expressed, as diameter of inhibition zones and number of active strains (**Figures 1 and 2**), was higher. Lactic acid production is considered to be the major protection mechanism of resident lactobacilli against invading pathogens. Significant clinical importance was observed in the activity against

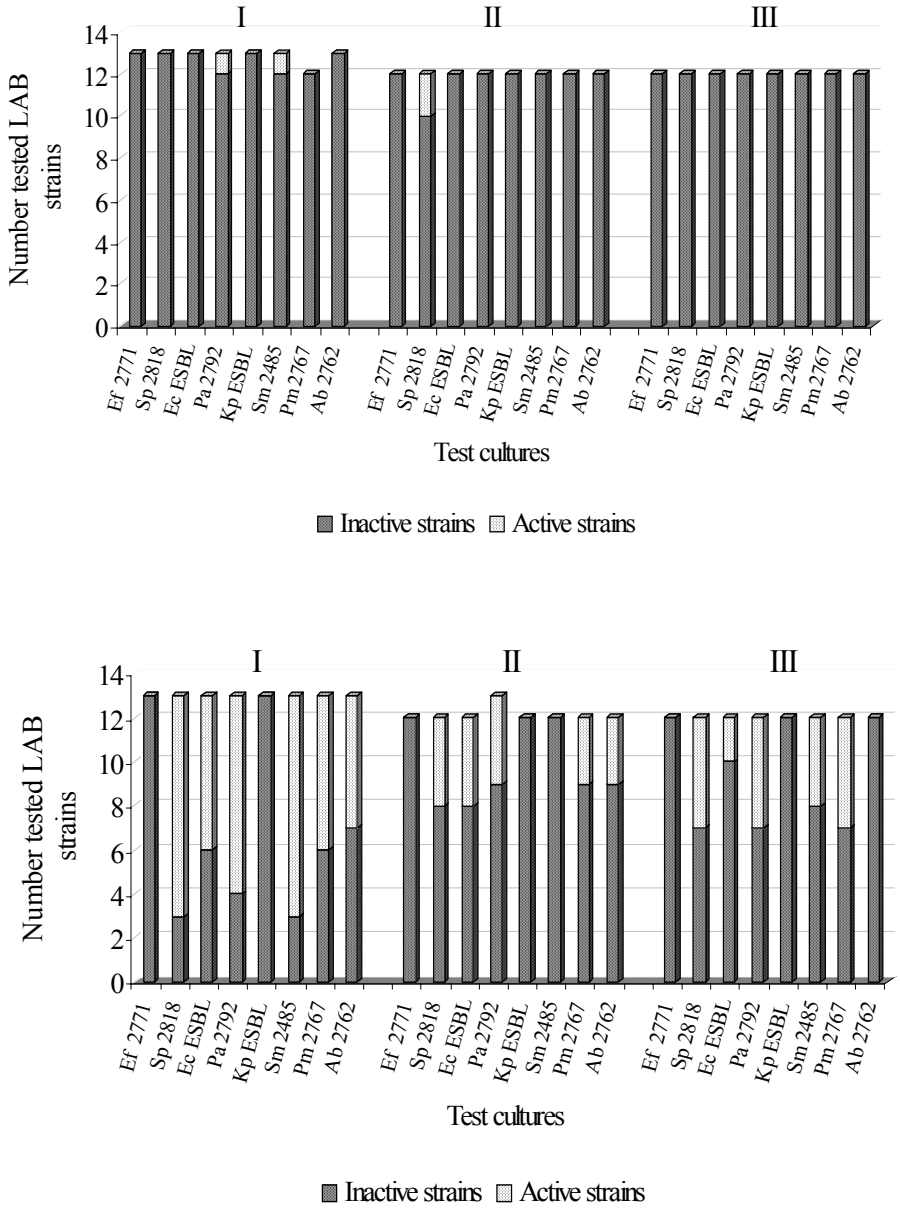

Figure 2: Comparison of activity of three groups of LAB strains against clinical pathogens

Legend: ******In vitro* tests were performed with: **(A)** neutralized filtered culture supernatants (nFCS) and **(B)** filtered culture supernatants without pH corrections (FCS);
Test cultures- clinical isolates: **Ef 2771-*Enterococcus faecalis*; **Sp 2818**- *Streptococcus pyogenes*; **Ec ESBL**-*E. coli*; **Ps.a 2792** *Pseudomonas aeruginosa*; **Kp ESBL 2766**- *Klebsiella pneumoniae*; **Sm 2485**- *Serratia marcescense;* **Pm 2767** – *Proteus mirabilis*; **Ab 2762**- *Acinetobacter baumanii*

Pseudomonas aeruginosa and *Acinetobacter baumanii. Pseudomonas aeruginosa* is naturally resistant to many antimicrobial agents like ampicillin, amoxicillin, first and second-generation cephalosporins, cefotaxime, ceftriaxon, nalidixic acid, nitrofurantoin, trimethoprim. This species is responsible for 10% of all nosocomial infections with more than 50% mortality (14). *Acinetobacter baumanii* have recently been isolated from Bulgarian hospital samples with 7.3% frequency in the control haemocultures between1997-2004 (15). To our knowledge this is the first data for active LAB cultures against these naturally and multiple resistant species, responsible for serious human infections.

In addition, we try to recognize the nature of active components. The activity of FCS from exponential cultures of studied 46 LAB strains was due to the lactic acid production alone. Two vaginal and four food

isolates lost their activity after the proteolytic treatment with proteinase K and pronase E (1 mg/ml). There was no effect on these samples after the catalase action (1 mg/ml, Merck). Observed antagonistic activity after elimination of the putative effects of lactic acid and H_2O_2, raised the question for a possible production of bacteriocin-like substances (BLS) or low-molecular microbocides. Probably selected *Lactobacilli* are bacteriocinogenic and further analyses for characterization of produced BLS are still in progress. Only a few bacteriocins of LAB with activity against Gram-negative bacteria have been reported, viz. thermophilin 81 (4.5 kDa), produced by *Streptococcus thermophilus* (16); a bacteriocin produced by *Lactococcus lactis* B14 (17); plantaricin 35d (4.5 kDa), produced by *Lactobacillus plantarum* (18) etc. Some of these active molecules could be future probiotic therapeutics, appropriate for clinical practice.

CONCLUSIONS

Lactobacilli, despite their origin, have potential to inhibit the growth of pathogens, including problematic antibiotic resistant isolates. The spectrum of activity of each bacteriocinogenic LAB is strain-specific. Therefore, the *in vitro* antimicrobial tests with a large number of target microorganisms are useful and necessary step in the pre-selection of candidates for probiotics. In the time of rapidly growing antibiotic-resistance of pathogens, the well characterized active antimicrobials and their producers could be a mainstay in management of diseases.

ACKNOWLEDGEMENTS

This work was supported by NATO project Sfp 982164 and partially by project TKK 01/442 founded by NSF, Bulgaria.

REFERENCES

1. Klaenhammer, T., Altermann, E., Arigoni, F., Bolotin, A. Breidt, F., Broadbent, J., Cano, R., at al. Discovering lactic acid bacteria by genomics. *Antonie Van Leeuwenhoek*, 82 (1-4):28-59, 2002.
2. Morelli, L., *In vitro* selection of probiotic lactobacilli: a critical appraisal. *Current Issues in Intestinal Microbiology,* 1:59-67, 2000.
3. Amant, D. C. St., Valentin-Bon, I., Jerse, A., Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species commonly isolated from the female genital tract. *Infect Immun,* 70(12):7169–7171, 2002.
4. Tannock, G. W., Probiotics: time for a dose of realism. *Curr Issues Intest Microbiol*, 4(2):33-42, 2003.
5. Beović, B., The issue of antimicrobial resistance in human medicine. *Intern. J. of Food Microbiology*, 112:280–287, 2006.
6. Georgieva, R.N, Iliev I.N., Chipeva V.A.', Dimitonova S.P., Samelis J. and Danova S.T., Identification and *in vitro* characterisation of *L. plantarum* strains from artisanal Bulgarian white brined cheeses, *Journal of Basic Microbiology*, 48:234-244, 2008.
7. Dimitonova, S. P., Bakalov, B. V., Serkedjieva J. P. and Danova S. T., Inhibitory activity and protective properties of vaginal lactobacilli from healthy Bulgarian women. *Anaerobe*, 13:178–184, 2007.
8. Tzvetkova, I., Dalgalarrondo, M., Danova, S., Iliev I., Haertlé, T., Ivanova I.and Chobert J-M., Hydrolysis of major dairy proteins by lactic acid bacteria from Bulgarian yogurts. *Journal of Food Biochemistry*, 31(5):680-702, 2007.
9. Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M., Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 36, 49-52, 1966
10. Ocana, V. S., Pesce de R. Holgado, A. A. and Nader-Macias, M. E., Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. *Appl Environ Microbiol*, 65:5631-5635, 1999.
11. Lee, H. M. and Lee, Y. K., Isolation of *Lactobacillus plantarum* from Kimchi and its inhibitory activity on the adherence and growth of *Helicobacter pylori*. *J. Microbiol. Biotechnol*, 16:1513-1517, 2006.
12. Todorov, S. D., Botes, M., Danova, S. T. and Dicks L. M., T., Probiotic properties of *Lactococcus lactis* subsp. *lactis* HV219, isolated from human vaginal secretions. *Journal of Applied Microbiology*, 103:629-639, 2007.
13. Miteva, V., Stefanova, Tz., Budakov, I., Ivanova, I., Mitev, V., Gancheva, A., and Ljubenov, M., Characterization of bacteriocins, produced by strains from

traditional Bulagarian dairy products. *Syst. Appl. Microbiol*., 21(1):235-244, 1998.
14. Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R.P., Edmond, M.B. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infect. Dis*, 39(3):309-17, 2004.
15. Petrov, M., Study and optimization of the National surveillance on etiology and structure of infections, bacterial resistance and antibiotic treatment in Bulgaria-BulStar. *PhD thesis*, p.132, 2006.
16. Ivanova, I., Miteva, V., Stefanova, T., Pantev, A., Budakov, I., Danova, S. et al., Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. *Intern. J. of Food Microbiology* 42:147-58, 1998.
17. Ivanova, I., Kabadjova, P., Pantev, A., Danova, S. and Dousset, X., Detection, purification and partial characterization of a novel bacteriocins substances produced by *Lactococcus lactis* subsp. *lactis* B14 isolated from boza – Bulgarian traditional cereal beverage. *Biocatalysis-2000: Fundamentals & Applications,* 41:47-53, 2000.
18. Todorov, S. D., Dicks, L. M., *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology,* 36:318-326, 2005.