



Original Contribution

ANTIMICROBIAL ACTIVITY OF *LACTOBACILLUS HELVETICUS* STRAIN 50P1

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ABSTRACT

Few years ago the effectiveness of different *Lactobacillus* strains, with activity against different pathogens, was intensively studied. In particular, by producing metabolites such as acetic and lactic acid and thus lowering the pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them. In this study were included original Bulgarian dairy lactobacilli. The antibacterial activity against 8 test cultures and 8 clinical isolates was estimated by the agar diffusion method. We observed the inhibitory effect of growth of *Bacillus subtilis*, *Bacillus alvei*, *Escherichia coli* and *Pseudomonas aeruginosa* strains. The cells-free exponential cultures of non-neutralized (pH 3.9) and neutralized forms of the strain *Lactobacillus sp.* 50P1 showed highest activity. The active strain was identified as *L. helveticus* by classical and biochemical tests (API LAB 50CH kit). In addition to some technologically relevant properties the active strain 50P1 showed strong antibacterial effect against clinically isolated *Pseudomonas aeruginosa* and *Bacillus alvei* – an agricultural important bee pathogen. In conclusion, our results will form the basis for additional studies and possible further application of characterized *L. helveticus* 50P1 strain in clinical practice and/or veterinary medicine.

Key Words: *Lactobacillus helveticus*, antimicrobial activity, probiotics

INTRODUCTION

Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of food. Improving shelf-life and nutritional quality of food and feed, they are able to stabilize gut microflora of consumers, improve the digestion of proteins and fats and actively reduce toxins production. The probiotic health benefits include also the metabolic stimuli of vitamin synthesis and enzyme production, reduction of serum cholesterol by assimilation mechanisms; boosting of the immune system and tumour suppression by modulation of cell-mediated immunity; prevention of diarrhoea from various causes; prevention and decreased risk of colon cancer by detoxification of

carcinogens; and reduction of the risk of inflammatory bowel movements (1).

In the selection of microbial strains for probiotic use, several criteria must be considered, which include bio-safety aspects, production and processing aspects, the method of administering the probiotic, the location on/in the body where the microorganisms of the probiotic product must be active, survival and/or colonization in the host, and the tolerance for bile (2, 3). One of the most significant criteria for a probiotic selection is the capability to enhance innate host defences by production of antimicrobial substances, and the growth inhibition and/or competitive exclusion of the enteric pathogens (4). Probiotics control intestinal pathogens by production of antibacterial compounds, including lactic and acetic acid and antibiotic-like substances, competition for nutrients and adhesion sites, increased and decreased enzyme activity, increased antibody levels and increased macrophage activity (5).

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The effectiveness of selected *Lactobacillus* strains with activity against different pathogens were first investigated a few years ago (3, 6). In particular, by producing metabolites such as acetic and lactic acid and thus lowering the pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them (1). Recently the other mechanisms of antagonism have been also intensively studied.

The aim of present study was to determine the antibacterial activity of original Bulgarian dairy lactobacilli against clinically important test cultures and isolates.

MATERIALS AND METHODS

Microorganisms, cultures and conditions

Two dairy strains (*Lactobacillus sp. 50P* and *Lactobacillus sp. 51PP*- from the collection of Department of Biotechnology, Sofia, Bulgaria) and two reference type strains (*Lactobacillus helveticus* ATCC 15009^T and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T) were pre-selected for this study. They were cultivated in MRS broth (Merck) 24 h at 37°C, in micro-aerophilic conditions. Stock cultures in MRS broth supplemented with 20% v/v glycerol were stored at -20°C.

Antimicrobial activity determination

Cell-free supernatant (CFS) and neutralized (NCFS), obtained after 24 h cultivation in MRS, were assayed for inhibitory activity against different Gram positive and Gram negative microorganisms as follows: *Staphylococcus aureus* MSSA ATCC 25923,

Staphylococcus aureus MRSA ATCC 39592, *Bacillus subtilis* ATCC 6633, *Escherichia coli* HB 101, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853; *Bacillus alvei* - a bee isolate; the clinical isolates: *Enterococcus faecalis*, *Streptococcus pyogenes* group A, , *E. coli* ESBL, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus mirabilis*, *Acinetobacter baumani*. The test microorganisms, used in this study, were obtained from National Bank for Industrial Microorganisms and Cell Cultures and the laboratory collections of the Department of Microbiology and Virology (University Hospital "Tsaritsa Joana"), Institute of Microbiology (Bulgarian Academy of Science) and Department of Biotechnology, Faculty of Biology, University of Sofia.

Overlays of each test strain (10⁶ CFU/ml) were prepared on agar plates and allowed to dry. Wells (8 mm) were made in the agar plates and CFS and NCFS (100 µl) was placed in the wells and allowed to diffuse through the agar for 20-40 min at room temperature prior to incubation for 24 h after which inhibitory zones were measured.

RESULTS AND DISCUSSION

Dairy lactobacilli usually are active against closely related species. In this study the antibacterial activity of selected dairy strains was estimated by the agar diffusion method. A strong inhibitory effect of the growth of *Bacillus subtilis*, *Bacillus alvei*, *E. coli* and *Pseudomonas aeruginosa* was detected (Figure 1, Table 1).

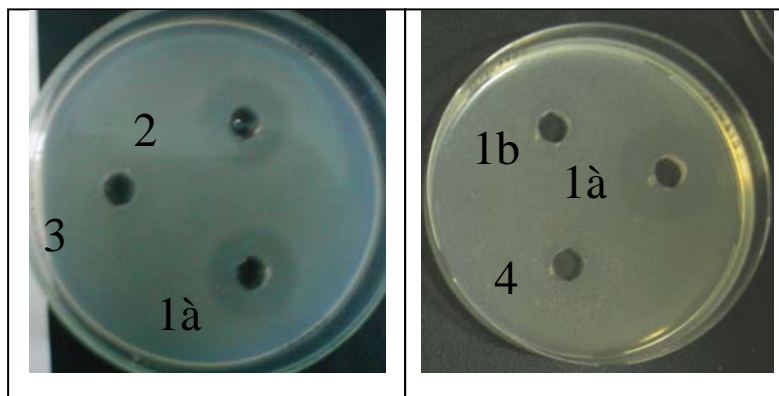


Figure 1. Antimicrobial activity of *Lactobacillus sp. 50P* (1a – CFS; 1b – NCFS), *Lactobacillus sp. 51PP* (4 – CFS) and reference strains *Lactobacillus helveticus* ATCC 15009^T (2 – CFS), *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (3 – CFS) against *Bacillus alvei* (an isolate)

Table 1. Antimicrobial activity of tested *Lactobacillus* strains*

Test - microorganisms	CFS from <i>Lactobacillus</i> sp. 50 P1		CFS from <i>Lactobacillus</i> sp. 51PP		CFS from <i>Lactobacillus</i> helveticus ATCC 15009		CFS from <i>Lactobacillus</i> delbrueckii subsp. bulgaricus ATCC 11842	
	acid	neutralized	acid	neutralized	acid	neutralized	acid	neutralized
<i>Staphylococcus aureus</i> (MSSA) ATCC 25923	NA	NA	NA	NA	nd	nd	nd	nd
<i>Staphylococcus aureus</i> (MRSA) ATCC 39592	NA	NA	NA	NA	nd	nd	nd	nd
<i>Bacillus subtilis</i> ATCC 6633	+ (13 mm)	NA	NA	NA	+ (11 mm)	NA	NA	NA
<i>Bacillus alvei</i> (isolate)	+ (24 mm)	NA	NA	NA	+ (23 mm)	NA	B	NA
<i>Enterococcus faecalis</i> (clinical isolate)	NA	NA	NA	NA	nd	nd	nd	nd-
<i>Streptococcus pyogenes</i> grup A (clinical isolate)	B	NA	NA	NA	NA	NA	NA	NA
<i>Escherichia coli</i> HB 101	+ (14 mm)	NA	NA	NA	+ (14 mm)	NA	NA	NA
<i>Escherichia coli</i> ATCC 25922	NA	NA	NA	NA	NA	nd	NA	nd
<i>Pseudomonas aeruginosa</i> ATCC 27853	NA	NA	NA	NA	NA	nd	NA	nd
<i>E. coli</i> ESBL (clinical isolate)	B	NA	NA	NA	NA	NA	NA	NA
<i>Pseudomonas aeruginosa</i> (clinical isolate)	+ (8 mm)	B	NA	NA	+ (6 mm)	NA	NA	NA
<i>Klebsiella pneumoniae</i> ESBL (clinical isolate)	NA	NA	NA	NA	NA	nd	NA	nd
<i>Serratia marcescens</i> (clinical isolate)	NA	NA	NA	NA	NA	nd	NA	nd
<i>Proteus mirabilis</i> (clinical isolate)	NA	NA	NA	NA	NA	nd	NA	nd
<i>Acinetobacter baumani</i> (clinical isolate)	NA	NA	NA	NA	NA	nd	NA	nd

*Activity of cells-free supernatants was presented as follows: Bactericidal activity against the test culture + (in mm sterile zone); B- Bacteriostatic activity ; NA- No activity; nd- not determined

The strain *Lactobacillus* sp. 50P1 showed a broad spectrum of activity in assays with non-neutralized (pH 3.96) and neutralized exponential CFS (pH-6.5). The inhibitory effects were higher in tests performed with acid CFS from *Lactobacillus* sp. 50P1 and *Lactobacillus helveticus* ATCC 15009^T. Probably, the lactic acid production is the one of primary mechanism of expression of LAB activity against pathogens. It has recently been reported that *Lactobacillus* sp. strains inhibit the growth of Gram-negative pathogenic bacteria (3). This activity, like the observed effects of CFS (Table 1), has generally been attributed to the production of lactic acid during the *Lactobacillus* growth. In addition, some of lactic acid bacteria produce

bacteriocin-like substances. The findings from our triple repeated assays confirmed that the antimicrobial effect of some strains may be completed with the production of relevant concentration of lactic acid in the microenvironment, which in combination also inhibit the growth of Gram-negative pathogenic bacteria (7). Therefore, the reported higher antagonistic activity detected with non-neutralized supernatants of both strains, was also important because the effect would be exerted in dairy products.

Such broad spectrum of activity is an unusual phenomenon reported only recently for lactobacilli. Atassi et al. (2006) investigated *in vitro* the antibacterial activity of the *Lactobacillus helveticus* strain KS300

against vaginosis-associated bacteria including *Gardnerella vaginalis* and *Prevotella bivia*, uropathogenic *Escherichia coli*, and diarrhoeagenic *Salmonella enterica* serovar *Typhimurium* (8). The authors found that KS300 strain inhibited the growth of *G. vaginalis*, *P. bivia*, *S. typhimurium*, and pathogenic *E. coli*. The other strains belonging to the species *Lactobacillus acidophilus*, *Lactobacillus casei* subsp. *rhamnosus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* inhibited the growth of clinical isolates of *H. pylori*, while *L. casei* subsp. *rhamnosus* strain Lcr35 reduced the growth of enteropathogenic and enterotoxigenic *Escherichia coli* and *Klebsiella pneumoniae* (2).

The dairy strain *Lactobacillus sp.* 50P1 was chosen as active and the species affiliation was initially determined. According to classical and biochemical tests (using API LAB 50 CH BioMérieux, France) *Lactobacillus* 50P1 strain belong to the species *L. helveticus* (unpublished data).

In our *in vitro* study the *L. helveticus* 50P1 strain has been selected, due to the expressed broad spectrum of activity, against microorganisms accepted as food-spoilage, agriculture- spoilage bacteria or serious clinical pathogens (Table 1). *Bacillus alvei* (so-called *Bacillus* "of a beehive") is one of the earliest described species of the genus *Bacillus*. *Bacillus alvei* has been isolated from soil, honeybee larvae and honeycombs of infected bees and presently is accepted as pathogen with importance for agriculture production (especially bee-honey) (9). In addition, the sensitive Gram (-) isolates were multi-resistant to the widely used antibiotics as follows: (i) *E. coli* *ESBL*: Amoxicillin/Clavulanic acid, Ampicillin, Cefoxitine, Cefotaxime, Ceftriaxone, Ceftazidime, Amikacin, Aztreonam, Cefepime, Biseptol, Ciprofloxacin, Gentamicin and (ii) *Pseudomonas aeruginosa*: Amoxicillin/Clavulanic acid, Ampicillin, Amikacin, Aztreonam, Cefotaxime, Ceftriaxone, Ceftazidime, Cefoxitine, Gentamicin, Piperacillin, Cefepime, Piperacillin/Tazobactam, Biseptol, Imipenem, Meropenem, Ciprofloxacin, Sulbactam/Cefoperazone. *Pseudomonas aeruginosa* has emerged as an important human pathogen (10) and also a common nosocomial contaminant. It causes between 10% and 20% of infections in most hospitals. *P. aeruginosa* have been traced to many items in the hospital environment. In addition to its pathogenicity, this bacterium has minimal

nutritional requirements and can tolerate a wide variety of physical conditions (11).

A recent problem in clinical practice, with regard to *Enterobacteriaceae*, is the increasing number of strains expressing extended-spectrum β -lactamases (ESBLs). Under normal physiological conditions, resistant bacteria are repressed by the dominant digestive flora (12). Thus, the discovery of active strains which could suppress the growth of problematic pathogens is a promising way for new therapeutic approaches. *In vivo*, by competition for nutrition and by production of lactic acid and antimicrobial substances, a probiotic strain could prevent the colonization of resistant strains.

CONCLUSION

We report a dairy strain *L. helveticus* 50P1 with combined antibacterial activities against the clinical isolate, *Pseudomonas aeruginosa* and bee pathogen *Bacillus alvei* (an isolate). Further evaluation of other probiotic characteristics is necessary and is still in progress. Such *in vitro* trials accumulate new data concerning the mechanisms of antimicrobial activity expressed from LAB, which are appropriate for the probiotic application.

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