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ANTIMICROBIAL ACTIVITY OF *LEUCONOSTOC LACTIS* STRAIN BT_17, ISOLATED FROM A SPONTANEOUSLY FERMENTED CEREAL BEVERAGE (BOZA)

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ABSTRACT

The antimicrobial activity of *Leuconostoc lactis* BT_17, isolated from a spontaneously fermented cereal beverage (boza) against some test microorganisms (*Escherichia coli* ATCC 25922, *Salmonella* sp. and *Klebsiella pneumoniae*) was determined by co-culturing of the strain *Leuconostoc lactis* BT_17 with each of the test microorganisms under static conditions. It was found that the strain *Leuconostoc lactis* BT_17 inhibited the growth of the test microorganisms and no viable cells of *Escherichia coli*, *Salmonella* sp. and *Klebsiella pneumoniae* were detected at the 48^{-th} hour of the co-culturing. The reduction of the number of viable cells in the mixed populations was a result of the produced lactic acid by *Leuconostoc lactis* BT_17 in the culture medium, which modify the conditions unlikely to growth of the test microorganisms.

Keywords: *Leuconostoc lactis*, lactic acid bacteria, antimicrobial activity, co-culturing

INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. They consist of many genera including *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* **(Hladikova *et al.*, 2012)**.
LAB are widely used as starter cultures for the production of a number of fermented milk and non-milk products. The fermentation process leads to improving of some organoleptic qualities of these products and extends their expiry date by inhibition of the growth of saprophytic and pathogenic microorganisms **(Hancioglu and Karapinar, 1997).** A large number of lactic acid microorganisms belonging to different bacterial genera have been isolated from fermented milk and non-milk products. One of these fermented non-milk products is boza - a traditional beverage in the countries of the Balkan region. Boza is a drink, produced from cereals fermented with LAB of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and some yeasts such as *Saccharomyces* sp. **(Todorov *et al.*, 2007; Cholakov *et al.*, 2016).**
LAB are known to produce a large number of antimicrobial compounds including organic acids (lactic, acetic and propionic), diacetyl, acetoin, acetaldehyde, hydrogen peroxide, ethanol and other metabolites. Besides them, some LAB spp. synthesize a wide range of substances with proteinaceous nature (bacteriocins and bacteriocin-like substances - BLIS) that have antagonistic activity against a number of closely or distantly related species and thus exert more or less antibiotic-like activity **(Todorov and Dicks, 2004, 2006; Thakur and Roy, 2009; Hladikova *et al.*, 2012)**. Besides various substances with antimicrobial activity that possess favorable characteristics for application as food biopreservatives, it is also proven that some *Leuconostoc* strains exhibit probiotic properties such as resistance to low pH (gastric juice), bile salts, certain antibiotics and others **(Zhang *et al.*, 2013)**.
Leuconostoc is a genus of Gram-positive, facultative anaerobic bacteria, placed within the family of *Leuconostocaceae*. The members of this genus are ovoid cocci or coccobacilli, arranged singly, in pairs or forming long chains. They form round shape colonies with smooth edges, convex surface, whitish or cream-colored, sticky consistency and size of 1 - 4 mm **(Cholakov *et al.*, 2016).** *Leuconostoc* spp. are catalase-negative and oxidase-negative, and these biochemical properties distinguish them from staphylococci (which are catalase-positive and oxidase-negative). All species within this genus are heterofermentative and are able to produce dextran from sucrose. *Leuconostoc* spp. are intrinsically resistant to vancomycin. The temperature range of growth is between 1°C and 37°C, but the optimum is between 20°C and 30°C **(Yang *et al.*, 2015).**
Presently there are no enough data reported in the scientific literature for the antimicrobial activity of *Leuconostoc* spp., in particular of *Leuconostoc lactis*. Therefore, the aim of the present study was to evaluate the antimicrobial potential of *Leuconostoc lactis* BT_17, isolated from a naturally fermented cereal beverage (boza) against *Escherichia coli*, *Salmonella* sp. and *Klebsiella pneumoniae* by co-culturing of the strain with each of the test microorganisms under static conditions.

MATERIAL AND METHODS

Microorganisms

In the present study the following microorganisms from the collection of the Department of Microbiology, University of Food Technologies, Plovdiv, Bulgaria were used:

Studied microorganism: *Leuconostoc lactis* BT_17, isolated from a naturally fermented cereal beverage (boza).

Test microorganisms: *Escherichia coli* ATCC 25922, *Salmonella* sp. (clinical isolate) and *Klebsiella pneumoniae* (clinical isolate).

Media

MRS-broth: Composition (g/L): peptone proteose – 10.0; yeast extract – 4.0; meat extract – 8.0; glucose – 20.0; K_2HPO_4 – 2.0; sodium acetate – 5.0; triammonium citrate – 2.0; $MgSO_4$ - 0.2; $MnSO_4$ - 0.05; Tween 80 - 1 mL. The final pH was adjusted to 6.5 and the medium was autoclaved for 15 minutes at 121ºC.

MRS-agar medium: Composition (g/L): MRS–broth; agar – 20.0. The medium was autoclaved for 20 minutes at 121ºC.

LBG-agar medium: Composition (g/L): tryptone – 10.0; yeast extract – 5.0; NaCl – 10.0; glucose – 10.0; agar – 20.0. The final pH was corrected to 7.5 and the medium was autoclaved for 20 minutes at 121ºC.

Antimicrobial assay

To determine the antimicrobial activity of *Leuconostoc lactis* BT_17 against the test microorganisms, a 48-hour culture of the studied strain was used. In the experimental procedure, 0.5 mL of the suspension of the strain *Leuconostoc lactis* BT_17, 0.5 mL of the suspension of the relevant test microorganism and 9 mL of MRS-broth were mixed. For preparation of the controls, 0.5 mL of the suspension of *Leuconostoc lactis* BT_17 with 9.5 mL of MRS-broth and 0.5 mL of the suspension of the relevant test microorganism with 9.5 mL of MRS-broth were mixed.
Co-culturing of the strain *Leuconostoc lactis* BT_17 and each of the test microorganisms was carried out under static conditions in a thermostat at 37±1°C for 72 hours. Samples at the 0^{-th}, 12^{-th}, 24^{-th}, 36^{-th}, 48^{-th}, 60^{-th} and 72^{-nd} hour were taken and monitoring of the changes in the titratable acidity and the number of viable cells was conducted.
Determination of the number of viable cells of *Leuconostoc lactis* BT_17 was done by the spread plate method on MRS-agar medium for selection of lactic acid bacteria **(Zhang *et al.*, 2013)**. Determination of the number of viable cells of the test microorganisms was done by the spread plate method on LBG-agar medium **(Cholakov *et al.*, 2014)**. The titratable acidity was determined according to the standard protocol **(Denkova, 2005).**

RESULTS AND DISCUSSION

The interaction between *Leuconostoc* spp. and bacteria, members of *Enterobacteriaceae* family, some of which are important pathogens and causative agents of food toxicoinfections, is of major scientific interest. In addition, this study provides more complete biological characterization of the isolated strain *Leuconostoc lactis* BT_17 and assists in the selection of appropriate strains for solving specific problems.
During the separate culturing under static conditions, the strain *Leuconostoc lactis* BT_17 and the test microorganisms reached the number of viable cells of $1x10^{14}$ cfu/mL by the 48^{-th} hour of the culturing (Fig. 1a, Fig. 2a, Fig. 3a). It was found that the titratable acidity of the medium for all of the three test microorganisms was lower compared to the titratable acidity of the studied strain (Fig. 1b, Fig. 2b, Fig. 3b).

Antimicrobial activity of *Leuconostoc lactis* BT_17 against *Escherichia coli* ATCC 25922

During the process of co-culturing under static conditions of *Leuconostoc lactis* BT_17 with *Escherichia coli* ATCC 25922, an increase in the viable cells for both studied strain and test microorganism in the first 24 hours was observed. Then, the number of the viable *Leuconostoc lactis* BT_17 cells remained unaffected ($1x10^{12}$ cfu/mL), while the number of the viable *Escherichia coli* ATCC 25922 cells gradually decreased and no viable cells were detected at the 48^{-th} hour (Fig. 1a).
The change of titratable acidity indicates that the acidity of test microorganism was significantly lower than the acidity of *Leuconostoc lactis* BT_17 and the titratable acidity of the mixed population (*Leuconostoc lactis* BT_17 + *Escherichia coli* ATCC 25922). During the first 24 hours, the titratable acidity of the mixed population was commensurable with the titratable acidity of the studied strain, while the acidity of the test microorganism was significantly lower, reaching 60°T, and this trend continued until the end of their co-culturing (Fig. 1b).

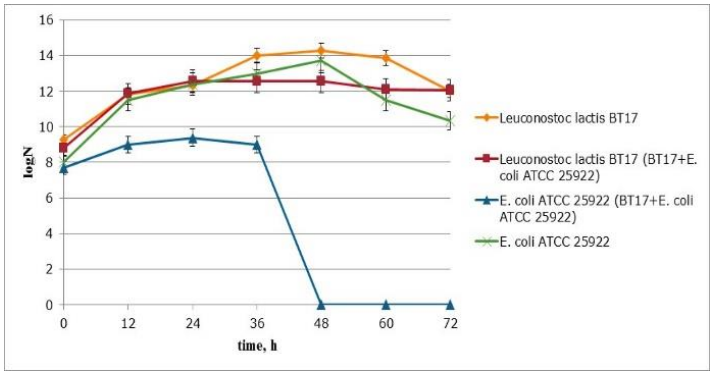

Figure 1a Survival of *Leuconostoc lactis* BT_17 and *Escherichia coli* ATCC 25922 during separate culturing and culturing in a mixed population at 37±1°C.

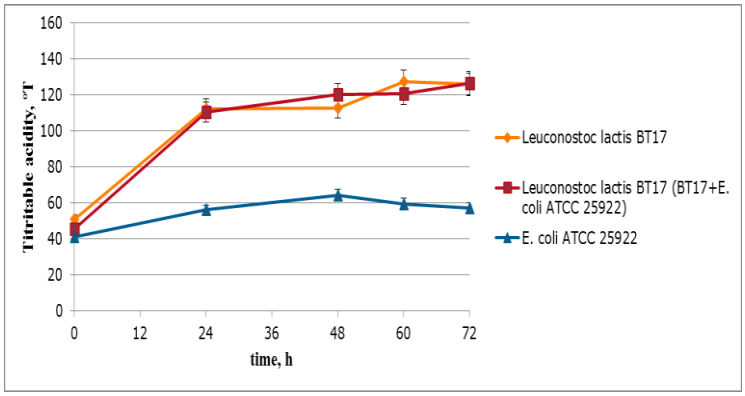

Figure 1b Changes in the titratable acidity of the medium during separate culturing and co-culturing of *Leuconostoc lactis* BT_17 and *Escherichia coli* ATCC 25922 at 37±1°C.

Antimicrobial activity of *Leuconostoc lactis* BT_17 against *Salmonella* sp.

During the process of co-culturing under static conditions of *Leuconostoc lactis* BT_17 with *Salmonella* sp., an increase in the number of viable cells for both studied strain and test microorganism in the first 12 hours was observed. Then, the number of the viable *Leuconostoc lactis* BT_17 cells continue to increase (reaching $1x10^{13}$ cfu/mL at 48^{-th} hour), while the number of the viable *Salmonella* sp. cells gradually decreased and no viable cells were observed at the 48^{-th} hour (Fig. 2a).
The change of titratable acidity indicates that the acidity of test microorganism was significantly lower than the acidity of *Leuconostoc lactis* BT_17 and the titratable acidity of the mixed population (*Leuconostoc lactis* BT_17 + *Salmonella* sp.) for each hour of the evaluation (Fig. 2b).

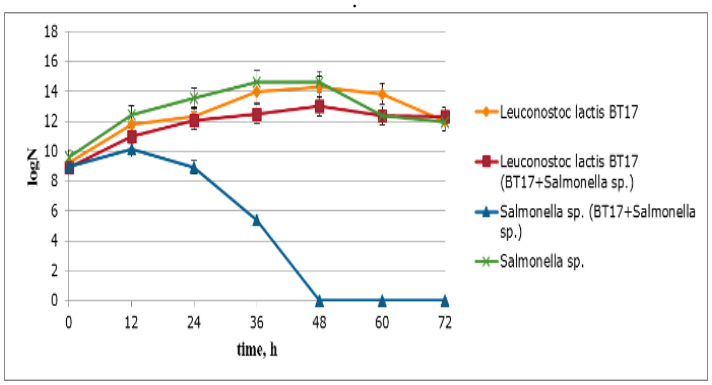

Figure 2a Survival of *Leuconostoc lactis* BT_17 and *Salmonella* sp. during separate culturing and culturing in a mixed population at 37±1°C.

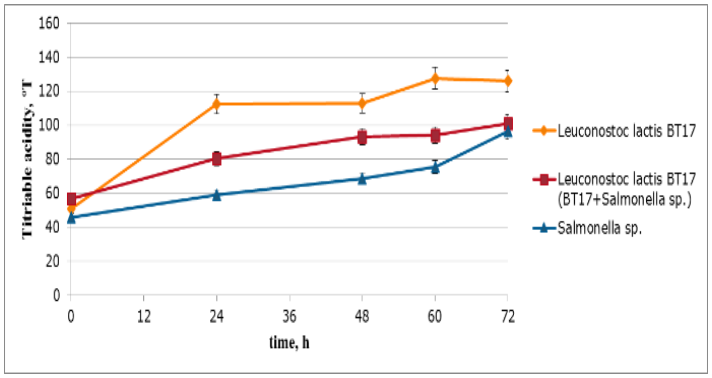

Figure 2b Changes in the titratable acidity of the medium during separate culturing and co-culturing of *Leuconostoc lactis* BT_17 and *Salmonella* sp. at 37±1°C.

Antimicrobial activity of *Leuconostoc lactis* BT_17 against *Klebsiella pneumoniae*

During the process of co-culturing under static conditions of *Leuconostoc lactis* BT_17 with *Klebsiella pneumoniae*, an increase in the number of viable cells for both studied strain and test microorganism in the first 12 hours was detected.

Then, the number of the viable *Leuconostoc lactis* BT_17 cells continue to increase (reaching $4.1x10^{13}$ cfu/mL at 60^{-th} hour), while the number of the viable *Klebsiella pneumoniae* cells gradually decreased and no viable cells were detected at the 48^{-th} hour (Fig. 3a).
The change of titratable acidity indicates that the acidity of test microorganism was significantly lower than the acidity of *Leuconostoc lactis* BT_17 and the titratable acidity of the mixed population (*Leuconostoc lactis* BT_17 + *Klebsiella pneumoniae*) (Fig. 3b).

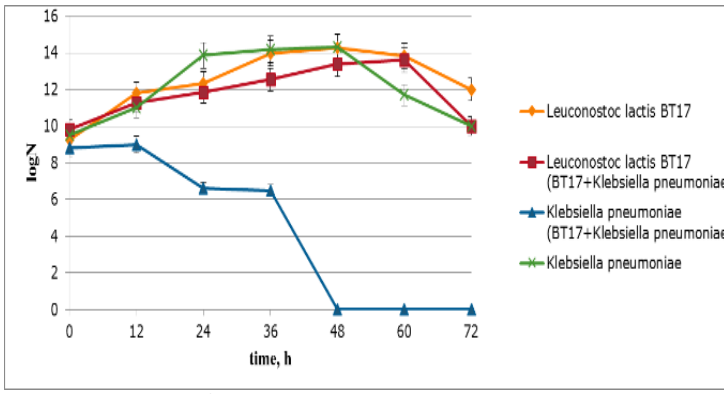

Figure 3a Survival of *Leuconostoc lactis* BT_17 and *Klebsiella pneumoniae* during separate culturing and culturing in a mixed population at 37±1°C.

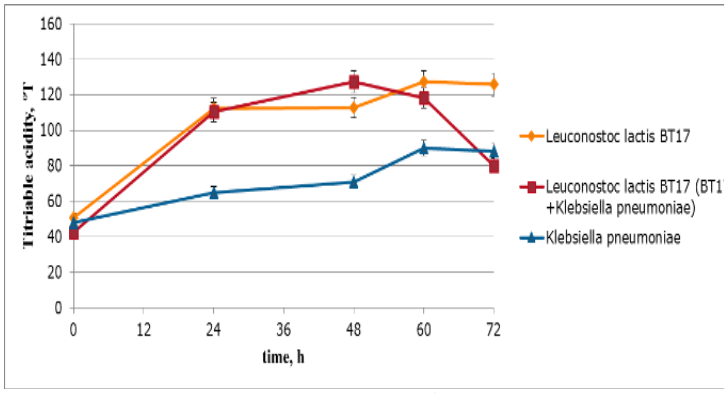

Figure 3b Changes in the titratable acidity of the medium during separate culturing and co-culturing of *Leuconostoc lactis* BT_17 and *Klebsiella pneumoniae* at 37±1°C.

As seen from the results above, *Leuconostoc lactis* BT_17 possesses high antimicrobial activity against the indicator microorganisms used. The strain effectively inhibited the growth of the microorganisms *Escherichia coli* ATCC 25922, *Salmonella* sp. and *Klebsiella pneumoniae*. During the co-culturing of *Leuconostoc lactis* BT_17 with three test microorganisms, the strain retained a high number of viable cells, while the number of living cells of the test microorganisms was significantly reduced. The observed reduction in the number of living cells of the test microorganisms to a great extent was a result of the production and accumulation of lactic acid in the culture medium. Similar antimicrobial effect on the same test microorganisms was observed in our previous experiments with *Lactobacillus plantarum* strain BG24, also isolated from boza **(Cholakov *et al.*, 2014).**
A couple of studies revealed that the inhibitory activity was not only due to the presence of organic acids and other metabolites in the culture medium, but also to specific antimicrobial compounds (bacteriocins and bacteriocin-like substances - BLIS) produced by *Leuconostoc* spp. as a result of their metabolic activity. **Daba *et al.* (1991)** investigated the production of a bacteriocin, referred as mesenterocin 5 – a heat stable protein, synthesized by *Leuconostoc mesenteroides* strain UL5, isolated from Cheddar cheese. This bacteriocin has been secreted from the cells during the late exponential phase of growth and displayed high inhibitory activity against some pathogens such as *Listeria* spp., but with no effect on several useful lactic acid bacteria from *Lactobacillus* spp., *Lactococcus* spp. and *Leuconostoc* spp. **Van Laack *et al.* (1992)** reported for the physico-chemical properties and antimicrobial activity of carnosin - a bacteriocin produced by *Leuconostoc carnosum* strain LA44A, isolated from vacuum packaged sausages. Carnosin showed the highest antagonistic effect against various Gram-positive microorganisms - lactic acid bacteria, *Enterococcus faecalis*, *Enterococcus faecium* and *Listeria* spp. **Thakur and Roy (2009)** described the antibacterial effect of a bacteriocin-like substance leuconocin produced by a strain *Leuconostoc lactis* isolated from fresh raw cattle milk. Leuconocin has shown high inhibitory activity against Gram-positive and Gram-negative microorganisms such as *Escherichia coli*, *Pseudomonas putida*, *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*.

Although the lack of scientific data, concerning the antimicrobial activity of *Leuconostoc* spp., the results from these investigations as well as the results from the current study demonstrated the great antimicrobial capacity of the genus *Leuconostoc*, in particular of *Leuconostoc lactis* BT_17. The high inhibitory activity makes the studied strain attractive for application in the food industry as potential natural food preservative against several spoilage bacteria and pathogenic microorganisms.

CONCLUSION

The results we obtained demonstrated the great antimicrobial potential of *Leuconostoc lactis* BT_17, which makes the studied strain suitable for use as biopreservative in the preparation of fermented foods and beverages. Furthermore, the antimicrobial effect of *Leuconostoc lactis* BT_17 on some members of *Enterobacteriaceae* family and the industrially important characteristics it possesses (safe and non-pathogenic; possibility for accumulation of high numbers of viable cells and production of organic acids), makes the studied strain very perspective for application in the composition of starters for probiotics, probiotic foods and beverages along with other lactic acid bacteria such as *Lactobacillus plantarum* and *Pediococcus pentosaceus*.

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