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Antimicrobial Activity of *Lactobacillus Plantarum* Strains Against *Escherichia Coli* Strains

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Running title: **Antimicrobial Activity of *Lactobacillus Plantarum* Against *Escherichia Coli***

Abstract
The antimicrobial activity of *Lactobacillus plantarum* BZ1 and *Lactobacillus plantarum* BZ2 against *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 8739 in co-culture and at single strain culture at a temperature of 37±1°C was examined. During co-cultivation an increase in the concentration of viable lactobacilli cells by the 24th h was established and it reached above 1.10^{13}cfu/cm^3, and then remained relatively constant. The concentration of viable pathogenic cells was reduced in a strain-specific manner and by the 36th h no living pathogen cells were detected. The observed antimicrobial activity was due to a great extent to the acidification of the medium because of the production and accumulation of lactic acid and other oraganic acids. The demonstrated antimicrobial activity is a prerequisite for further research on the probiotic potential of the two *Lactobacillus plantarum* strains for their inclusion in the composition of probiotic preparations.

Practical applications
One of the requirements for probiotic strains is to exhibit antimicrobial activity against pathogenic microorganisms. The observed antimicrobial activity of the examined *Lactobacillus plantarum* strains against *Escherichia coli* strains is a prerequisite for further research on the probiotic potential of the two *Lactobacillus plantarum* strains for their inclusion in the composition of probiotic preparations and functional foods.

Key words: probiotic, *Lactobacillus plantarum*, pathogen, antimicrobial, co-culturing, *Escherichia coli*

Introduction

Escherichia coli and *Staphylococcus aureus* are food pathogens that cause severe food poisonings. Many food-poisoning outbreaks of *Escherichia coli* have been associated with contaminated food, such as beef, pork, chicken and water. Another public health concern is associated with the increased incidence of antibiotic-resistant strains isolated from poultry meat. Due to the widespread use of antimicrobials in chicken and pig growth units, the development of resistant strains that can infect humans via the food chain has increased. As a result, contamination of pathogenic microorganism is recognized as a potential public health concern. As more bacteria become resistant to traditional antibiotics, this leads to emergence and re-emergence of multidrugresistant pathogens (Wang, 2008; Bachir and Benali, 2012; Mihaiu et al., 2014; Dan et al., 2015).

Lactic acid bacteria have been used in the production of varieties of fermented dairy, vegetable and meat products for many centuries now. Recent research revealed that lactic acid bacteria can produce antibacterial substances including organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins to inhibit the growth of a wide range of intestinal pathogens. Bacteriocins are defined as antimicrobial peptides or proteins observed in many genera of bacteria, including many strains of lactic acid bacteria, which mainly inhibit the growth of related species. Lactic acid bacteria are very important in ensuring the safety of various foods by production of bacteriocins and other antimicrobial substances. Bacteriocins produced by *Lactobacillus plantarum* are known as plantaricins (Omar et al., 2008; Garcia-Ruiz et al., 2013; Man et al., 2014; Anyogu et al., 2014; Chang et al., 2016).

Lactic acid bacteria inhibit the growth of pathogenic microorganisms due to the formation of short chain fatty acids, lactic and other organic acids and bacteriocins which change the environmental conditions or affect the cell walls of the developing pathogenic cells. But pathogen inhibition is a strain-specific property which requires the mandatory examination of the antimicrobial activity of each potentially probiotic strain before its inclusion in the composition of probiotic preparations and functional foods.

The purpose of the present work was to study the antimicrobial activity of *Lactobacillus plantarum* BZ1 and *Lactobacillus plantarum* BZ2 against two strains of *Escherichia coli*: *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 8739.

Materials and methods

1. Microorganisms

L. plantarum BZ1 and *L. plantarum* BZ2, isolated from salad dressings;

test pathogenic microorganisms *E. coli* ATCC 25922 and *E. coli* ATCC 8739.

Media

1. MRS (Man, Rogosa and Sharpe) – broth medium

Composition (g/dm^3): peptone from casein - 10; yeast extract - 4; meat extract - 8; glucose - 20; K_2HPO_4 - 2; sodium acetate - 5; diammonium citrate - 2; $MgSO_4$ - 0.2; $MnSO_4$ - 0.04; Tween 80-1 cm^3/dm^3; pH = 6.5. Sterilization - 15 minutes at 118ºC.

2. LAPTg10 (Laboratorios Conda S.A., Cat. #: 2070.05) – agar medium

Composition (g/dm^3): peptone - 15; yeast extract - 10; tryptone - 10; glucose – 10; Tween 80-1 cm^3/dm^3, agar - 15. pH=6.6. Sterilization - 20 minutes at 121ºC.

3. LBG (Laboratorios Conda S.A., Cat. #: 2070.05) – agar medium

Composition (g/dm^3): tryptone - 10; yeast extract - 5; NaCl - 10; glucose - 10; agar - 15; pH = 7.5. Sterilization - 20 minutes at 121°C.

Determination of the antimicrobial activity against pathogenic microorganisms - by co-culturing

To determine the antimicrobial activity of the studied strains of lactobacilli against pathogens, a 48 h cultural suspension of each *L. plantarum* strain was used. Separate cultivation of the two *L. plantarum* and the two *E. coli* strains as well as co-cultivation of each of the two *L. plantarum* strains and each *E. coli* strain included in the study were conducted. For the examination of the co-cultivation, 0.5 cm^3 of the suspension of the *L. plantarum* strain, 0.5 cm^3 of the suspension of the *E. coli* strain and 9 cm^3 of culture medium (MRS-broth medium) were mixed. In the control of each *L. plantarum* strain and in the control of each pathogen 9.5 cm^3 of the MRS-broth medium were mixed with 0.5 cm^3 of the

suspension of the *L. plantarum* strain or of the suspension of the *E. coli* strain, respectively. The culturing was conducted under static conditions in a thermostat at 37±1°C for 72 hours, taking samples at 0, 12, 24, 36, 48, 60 and 72 h. Monitoring the change of the titratable acidity and the concentration of viable cells of both the pathogen and the *L. plantarum* strain was performed. Determination of the number of viable cells was done by the spread plate method on LAPTg10-agar (for the enumeration of lactobacilli), and on LBG-agar (for the enumeration of pathogens). The titratable acidity was determined according to a standard protocol (Denkova et al., 2013).

Modeling of kinetics

For the modeling of the kinetics of growth of the studied strains, the logistic curve equation (Verhulst equation (1)) was used:

$$\frac{dX}{d\tau} = [\mu - \beta X]X \qquad (1)$$

Results and discussion

In a study of the antimicrobial activity of lactic acid bacteria against pathogenic microorganisms by the method of co-cultivation, it is important to identify the specific growth rates of both the *Lactobacillus* strains and the pathogens. The maximum specific growth rates of the two *L. plantarum* and the two *E. coli* strains as single-strain cultures and in a mixed population by the equation of the logistic curve were calculated (Table 1). The dynamics of the change in the number of viable cells and in the titratable acidity were monitored (Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7 and Fig. 8).

In the single-strain cultivation of each *L. plantarum* strain and each *E. coli* strain, high concentrations of viable cells were achieved by the 12th h and they were maintained by the end of the cultivation. In the co-cultivation of each *L. plantarum* strains and each *E. coli* strains, the *Lactobacillus* strains was not significantly influenced by the presence of any of the *E. coli* strains. But the number of viable cells of the pathogens was greatly reduced in a strain-specific manner.

The two *L. plantarum* strains developed at a relatively high maximum specific growth rates (0.340 h^{-1} for *L. plantarum* BZ1 and 0.418 h^{-1} for *L. plantarum* BZ2), the coefficient of internal population competition being low (Table 1). They both entered the stationary growth phase at the 24th h, reaching maximum concentration of viable cells - 10^{14}cfu/cm^3 (Fig. 1, Fig. 2, Fig. 5 and Fig. 6). Meanwhile, the titratable acidity of the medium in the single strain culturing of each of the two lactobacilli strains reached 200°T (Fig. 3, Fig. 4, Fig. 7 and Fig. 8). A similar trend was established in the single-strain culturing of each of the two *E. coli* strains. The maximum specific growth rate of the two *E. coli* strains was 0.381 h^{-1} for *E. coli* ATCC 25922 and 0.337 h^{-1} for *E. coli* ATCC 8739. Therefore, *E. coli* ATCC 25922 *and E. coli* ATCC 8739 entered the stationary growth phase at the 24th h, reaching concentrations of viable cells about 10^{12} cfu/cm^3 (Fig. 1, Fig. 2, Fig. 5 and Fig. 6). The titratable acidity of the medium for both strains was about 50°T.

In co-cultivation it is important for the lactobacilli to retain higher maximum specific growth rate and their development to be slightly influenced by the presence of the pathogen. The maximum specific growth rates of the two *L. plantarum* strains in the co-culturing with each of the two pathogens was calculated (Table 1). In both *L. plantarum* strains there was a slight decrease in the specific growth rate compared to single-strain culturing, but it remained high (from 0.219 h^{-1} to 0.342 h^{-1}) for the co-culturing with *E. coli* ATCC 25922 and *E. coli* ATCC 8739, respectively. The coefficient of internal population competition increased slightly for both *L. plantarum* strains, but it remained low (0.05 cm^3/(cfu.h) to 0.09 cm^3/(cfu.h)), which demonstrated that the presence of the pathogen and the metabolites it produced had little impact on the growth of the lactobacilli strains. The high concentration of viable cells of the two *L. plantarum* strains - over 10^{13}cfu/cm^3 served as a proof (Fig. 3, Fig. 4, Fig. 7 and Fig. 8). A similar trend was established for the *E. coli* strains, when comparing the coefficient of internal population competition in the single-strain culturing and co-culturing of the two *E. coli* strains with the two *L. plantarum* strains.

In co-culturing of *E. coli* ATCC 25922 and each of the two *L. plantarum* strains a reduction in the maximum specific growth rate was established. The rate of reduction of the concentration of viable pathogen cells was high (0.507 s^{-1} for the co-culturing with *L. plantarum* BZ1 and 0.529 s^{-1} for the co-culturing with *L. plantarum* BZ2), and thereafter its reciprocal value was low (1.97 and 1.89). This indicated that *E. coli* ATCC 25922 was more sensitive to the conditions created by the

growth of *L. plantarum* BZ2 compared to those created by *L. plantarum* BZ1 (Fig. 1, Fig. 2 and Table 1).

In the co-culturing of *E. coli* ATCC 8739 with the two *L. plantarum* strains a continuous decrease in the concentration of viable cells of the pathogen was observed. Nearly identical rate of reduction of the living pathogen cells in the co-cultivation of *E. coli* ATCC 8739 with the two *L. plantarum* strains was established (0.563 s^{-1} for the co-culturing with *L. plantarum* BZ1 and 0.550 s^{-1} for the co-culturing with *L. plantarum* BZ2). The same trend was observed for the inverse values of the reduction constant (1.78 and 1.82). These data and the lack of active pathogen cells at the end of the process showed that *E. coli* ATCC 8739 was very sensitive to the adverse conditions created by the two *L. plantarum* strains (Fig. 5 and Fig. 6).

The observed antimicrobial activity of the two *L. plantarum* strains included in the present study was due to the production and accumulation of lactic and other organic acids. According to Helander et al., *L. plantarum* produce a variety of low molecular mass compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide and other metabolites. Many of these metabolites have a broad activity spectrum against other species, and their production is largely affected by the food matrix itself. Bacteriocins inhibit only closely related species or other Gram-positive and Gram-negative microorganisms.

In co-cultivation of *L. plantarum* BZ1 or *L. plantarum* BZ2 and the two *E. coli* strains, an increase in the concentration of viable cells of lactobacilli was observed, while that of the two *E. coli* strains was maintained during the first 12 h, then the number of living cells of the pathogen was reduced and at the 36th h no viable cells of the pathogen were found. The data is sustainable with the results, obtained with *L. plantarum* X2 and *E. coli* ATCC 25922 at 37±1°C (Denkova et al., 2013).

L. plantarum BZ1 or *L. plantarum* BZ2 supressed the two *E. coli* strains from the beginning of the co-cultivation (Fig. 1, Fig. 2, Fig. 5 and Fig. 6), while *L. plantarum* NBIMCC 2415 inhibited the growth of the pathogen after the 12th h of co-cultivation. Moreover, in the co-cultivation of the *E. coli* strains and *L. plantarum* BZ1 or *L. plantarum* BZ2 no living cells of the pathogen were found at the 36th h, while in the co-cultivation of *E. coli* ATCC 8739 and *L. plantarum* NBIMCC 2415 at the 72nd h the number of living cells of the pathogen was about 10^{11}cfu/cm^3 (Denkova and Nedelcheva, 2009).

Conclusion

L. plantarum BZ1 and *L. plantarum* BZ2 maintained high concentration of viable cells in single-strain culturing and in co-culturing with each of the two *E. coli* strains at a temperature of 37±1°C. Both *L. plantarum* strains inhibited significantly the growth and development of the pathogens. The observed antimicrobial activity was due to a great extent to the acidification of the medium because of the production and accumulation of lactic and other oraganic acids. The demonstrated antimicrobial activity is a prerequisite for further research on the probiotic potential of the two *L. plantarum* strains for their inclusion in the composition of probiotic preparations.

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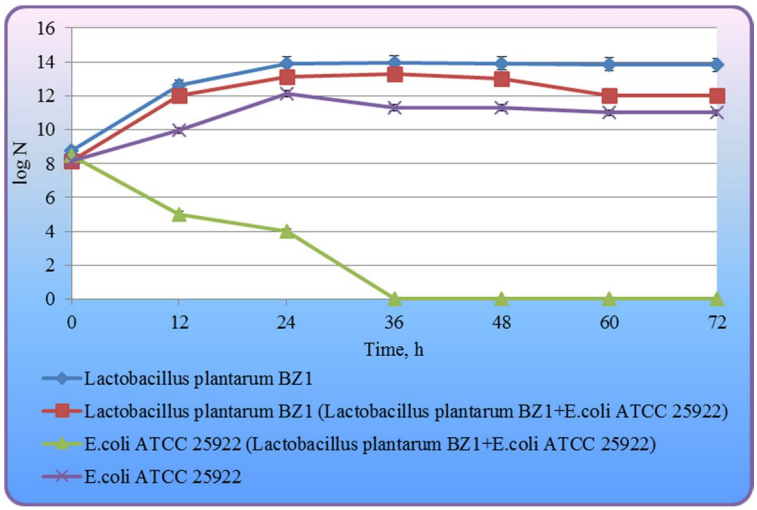

Figure 1. Survival of *Lactobacillus plantarum* BZ1 and *Escherichia coli* ATCC 25922 in single-strain culturing and in co-culturing at 37±1°C

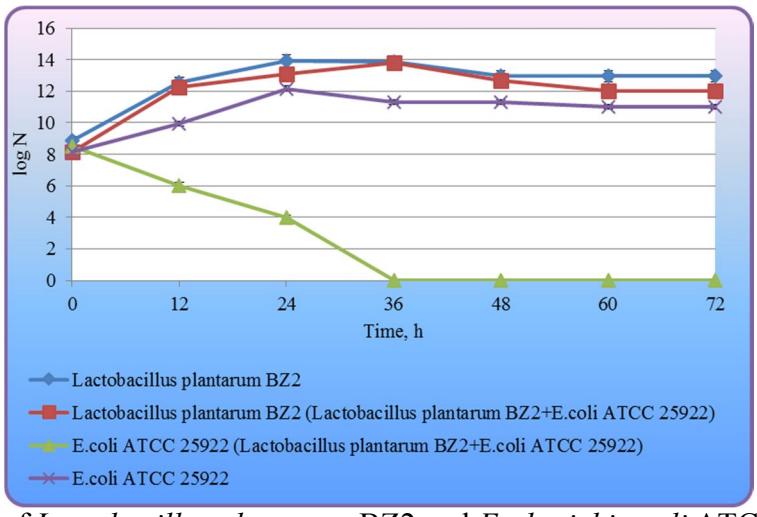

Figure 2. Survival of *Lactobacillus plantarum* BZ2 and *Escherichia coli* ATCC 25922 in single-strain culturing and in co-culturing at 37±1°C

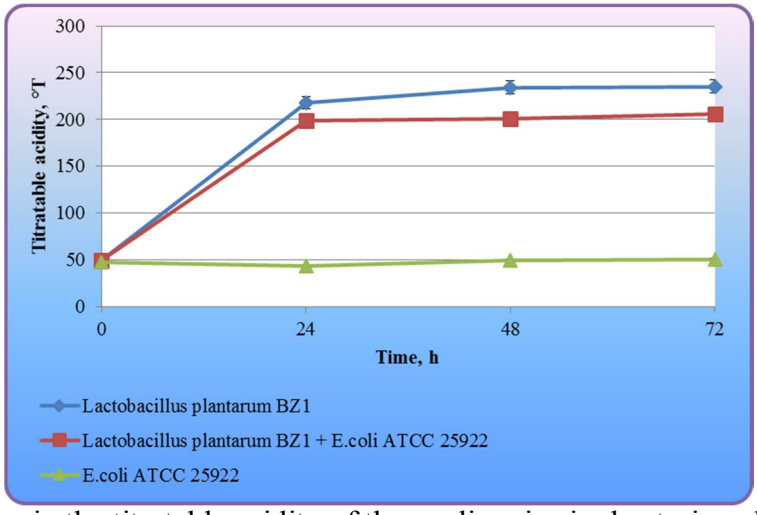

Figure 3. Changes in the titratable acidity of the medium in single-strain culturing and in co-culturing of *Lactobacillus plantarum* BZ1 and *Escherichia coli* ATCC 25922 at 37±1°C

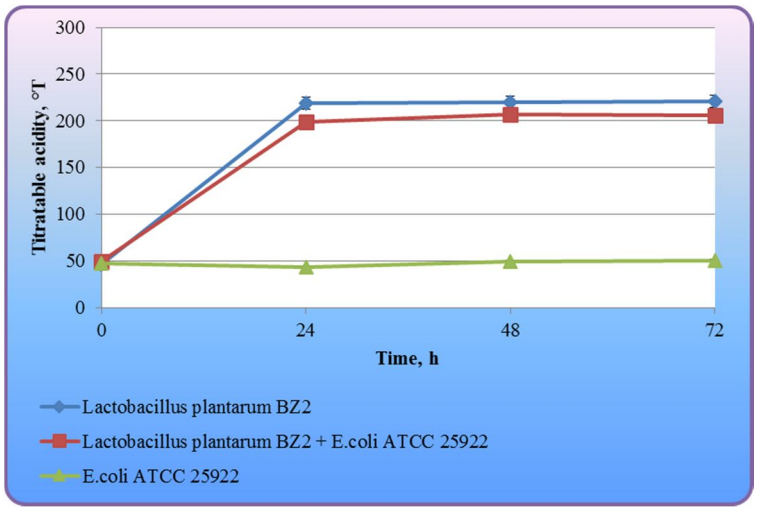

Figure 4. Changes in the titratable acidity of the medium in single-strain culturing and in co-culturing of *Lactobacillus plantarum* BZ2 and *Escherichia coli* ATCC 25922 at 37±1°C

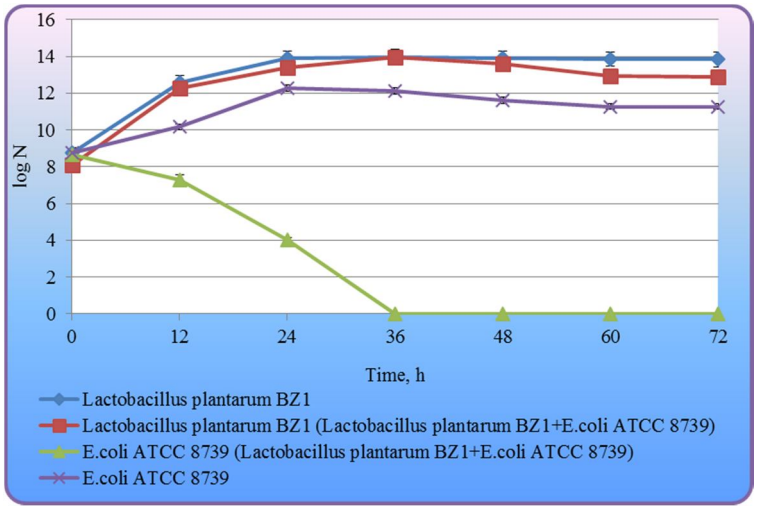

Figure 5. Survival of *Lactobacillus plantarum* BZ1 and *Escherichia coli* ATCC 8739 in single-strain culturing and in co-culturing at 37±1°C

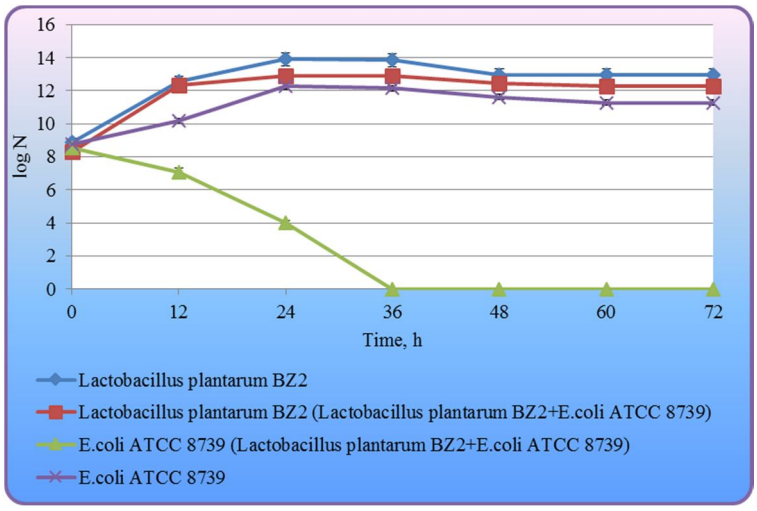

Figure 6. Survival of *Lactobacillus plantarum* BZ2 and *Escherichia coli* ATCC 8739 in single-strain culturing and in co-culturing at 37±1°C

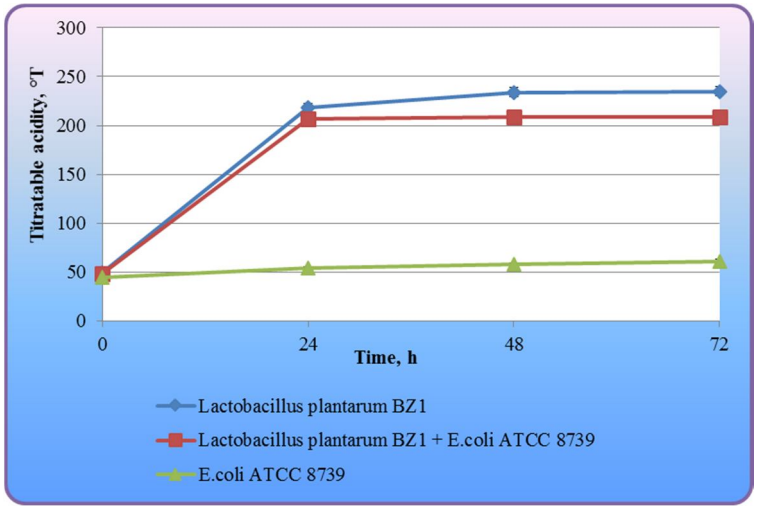

Figure 7. Changes in the titratable acidity of the medium in single-strain culturing and in co-culturing of *Lactobacillus plantarum* BZ1 and *Escherichia coli* ATCC 8739 at 37±1°C

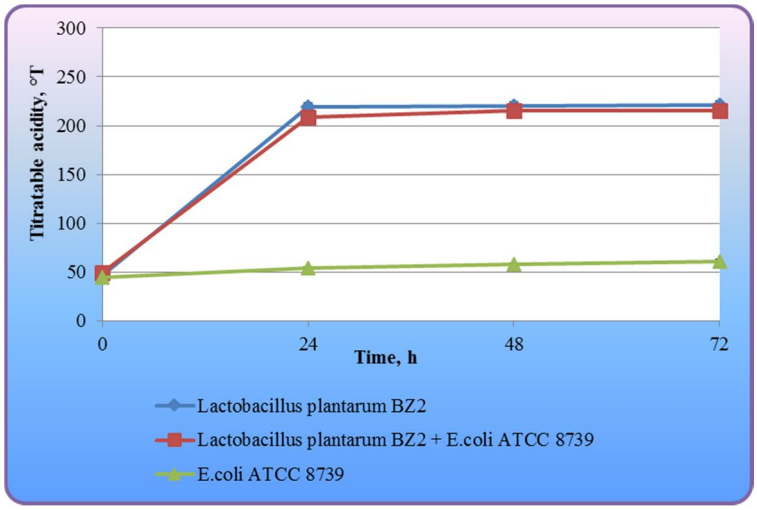

Figure 8. Changes in the titratable acidity of the medium in single-strain culturing and in co-culturing of *Lactobacillus plantarum* BZ2 and *Escherichia coli* ATCC 8739 at 37±1°C

Table 1. Kinetic parameters of the processes of single-strain culturing and co-culturing of *Lactobacillus plantarum* and *Escherichia coli* strains

Strain	μ, h^{-1}	β, cm^3/(cfu.h)	k, s^{-1}	1/k, s
L. plantarum BZ1	0.340	0.02		
L. plantarum BZ2	0.418	0.03		
E. coli ATCC 25922	0.381	0.03		
E. coli ATCC 8739	0.337	0.03		
L. plantarum BZ1 (*L. plantarum* BZ1 + *E. coli* ATCC 25922)	0.219	0.09		
L. plantarum BZ1 (*L. plantarum* BZ1 + *E. coli* ATCC 8739)	0.254	0.05		
L. plantarum BZ2 (*L. plantarum* BZ2 + *E. coli ATCC 25922*)	0.342	0.05		
L. plantarum BZ2 (*L. plantarum* BZ2 + *E. coli ATCC 8739*)	0.296	0.09		
E. coli ATCC 25922 (*L. plantarum* BZ1 + *E. coli* ATCC 25922)	-	-	0.507	1.97
E. coli ATCC 25922 (*L. plantarum* BZ2 + *E. coli* ATCC 25922)	-	-	0.529	1.89
E. coli ATCC 8739 (*L. plantarum* BZ1 + *E. coli* ATCC 8739)	-	-	0.563	1.78
E. coli ATCC 8739 (*L. plantarum* BZ2 + *E. coli* ATCC 8739)	-	-	0.550	1.82