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Efficiency of metabiotics from lactic acid bacteria against pathogens

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A B S T R A C T

Purification of culture broth obtained from endemic strains of lactic acid bacteria *Lactobacillus rhamnosus* BTK-2012, *Lactobacillus plantarum* BTK-66 and *Enterococcus faecium* BTK-64 by the ion-exchange chromatography method allowed obtaining purified metabiotics. The quantitative antimicrobial activity and activity spectrum of the studied metabiotics against Gram-negative and Gram-positive pathogens, such as *Salmonella sp., E.coli, Proteus mirabilis, Staphylococcus aureus, Shigella spp., Yersinia enterocolitica, Bacillus cereus*, *Listeria monocitogenes spp, Yersinia pseudotuberculosis* were shown. Applied titration method showed that the growth inhibition depends on concentration, time of incubation and from the genera and the species belonging of pathogens, as well as from the genus of bacteria from which the metabiotics were obtained. It was shown, that *Lactobacillus rhamnosus* BTK-2012 endemic strain differ by their antimicrobial activity spectrum from well known LAB strains, belonging to same genera and species, described in literature sources.

Introduction

The growing problem of the prevalence of pathogenic bacteria resistant to antibiotics, motivated to search alternative natural microbial preparations, including on the basis of probiotic lactic acid bacteria and its metabiotics. For the last two decades, the problem acquired more actual character, as the number of antibiotic-resistant pathogenic bacteria had increased, that led to the spreading of infections both among the populace and animals. Biopreservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocins (BCN) have received increasing attention, since these bacteria have generally been regarded as safe (GRAS) and have been used as starter cultures in the fermentations of many food products [Bari et al., 2005]. It has been shown that some strains of LAB possess interesting health-promoting properties, such as the potential to combat gastrointestinal pathogenic bacteria *Helicobacter pylori*, *Escherichia coli* and *Salmonella.*

One of the most promising new concepts in antimicrobial technology is the use of natural antimicrobial preparations (AMP), such as bacteriocins, metabiotics and peptides as antimicrobial agents. AMPs represent bactericides (cell killers) or bacteriostatics (bacteria growth inhibitors), have a broad range of activity and are excellent candidates for development of new prophylactic and therapeutic substances to complement or replace conventional antibiotics [Kristiansen et al., 2010]. Metabiotics are the structural components of probiotic microorganisms and/or their metabolites with a determined chemical structure. Metabiotics of LAB can contains bacteriocins and other low molecular weight antimicrobial molecules, short chain fatty acids, various other fatty and organic acids, polysaccharides, peptidoglycans, antioxidants, different proteins including enzymes, peptides with various activities, amino acids and other [Shenderov, 2013]. Bacteriocins are ribosomally synthesized peptides with broad spectrum of antimicrobial activity. Some of their properties (significant potency, high stability, low toxicity, broad spectrum of activity) make them suitable compounds for using them as a basis for development of antimicrobial agents of new generation [Ken-ichi Okuda, 2013].

Some investigators have isolated and partially purified bacteriocins from different species of *lactobacilli* predominantly from food and some in human feces. For example, bacteriocins ST28MS and ST26MS, produced by *Lactobacillus plantarum* isolated from molasses inhibited the growth of *Escherichia coli* and *Acinetobacter baumanii* [Torodov and Dicks, 2005]. *Enterococcus faecium* CRL35, a strain isolated from regional Argentinean cheese, produces a bacteriocin called enterocin CRL35. It possess activity against the food borne pathogen *Listeria monocytogenes*, and by its efficiency, this peptide has potential as antimicrobial agent in foods [Minahk et al., 2000]. Strains *L.rhamnosus* ST 461BZ and ST462BZ were isolated from Boza, witch produces a bacteriocins with a broad range of activity [Todorov and Dicks, 2005].

The new endemic strains of LAB from dairy products from different alpine households of Armenia were isolated and investigated by us. The technologies of cultivation of endemic LAB strains were developed. Further purification of their cell free culture broth (CFC) by the method of ion-exchange chromatography allowed obtaining metabiotics with broad spectrum of antimicrobial activity [Tkhruni et al., 2013].

The aim of presented article is investigation of efficiency of obtained metabiotics, isolated from cultural broth of *Lactobacillus rhamnosus* BTK-2012, *Lactobacillus plantarum* BTK-66 and *Enterococcus faecium* BTK- 64 strains on the growth of different pathogenic bacteria.

Materials and Methods

Bacterial cultures and media

The endemic LAB cultures *L.rhamnosus* BTK-2012 (MDC 9631), *L.plantarum* BTK-66 (INMIA 9619), *Ent.faecium* BTK-*64* (INMIA 9620) were used. Strains were deposited with the Department of Center of Microbial Depository at SPC "Armbiotechnology" NAS of Armenia. Pure cultures of LAB were maintained as frozen stocks at -20°C in the MRS broth containing 40% Glycerol. Strains were cultivated at 37°C in MRS broth (Merck, Germany).

Test cultures growth

Gut microbiota pathogenic bacteria, such as G-negative *Salmonella enteritidis, S.typhimurium Salmonella* spp.,

Pseudomonas aeruginosa, *Klebsiella pneumonia, Proteus mirabilis* and G-positive *Staphylococcus aureus* were isolated from infected patients in the "Nork" Infections Hospital (Yerevan, RA).

Food contaminating pathogenic bacteria *Pseudomonas aeruginosa, Staph. aureus*, *Esherichia coli* strains were isolated from different food products in the National Bureau of Expertise (Yerevan, RA). Isolated bacteria stored in the microorganism depository of the Institute of Epidemiology, Microbiology and Parasitology of Ministry of Health, Armenia.

The following bacteria from the Department of Center of Microbial Depository (CMD) of SPC "Armbiotechnology" NAS RA were used: *E.coli* K12, *E.coli* ATCC 11303, *Ps.fluorescens* INMIA 9068, *Ps. aerugines* INMIA 9056, *Salmonella typhimurium* G 38, *Bacillus subtilis* 17-89. Test cultures were grown on solid Nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 30°C, then harvested and suspended in the Nutrient broth at the concentration 2.2x10^{6} CFU/ml.

G-positive *Listeria monocytogenes* 1691, 35 and 37, G-negative *E.coli* 2529 and 2859, *Yersinia pseudotuberculosis* 2143 and 28, *Yersinia enterolitica* 373 and 19 from "Center for Prophylaxis for Especially Dangerous Infections„ (CPEDI, Yerevan, RA) were investigated.

L.monocytogenes, E.coli and *Yersinia pseudotuberculosis* were grown overnight respectively on nutrient and sugar agar pH 7.3 at 37°C. The cells were harvested and suspended at the concentration 1.0 x 10^{9} CFU/ml. *Yersinia enterolitica* was cultivated during 24 hours on MPA pH 7.3 at 37°C, harvested and suspended at concentration 1.0x10^{6} CFU/ml.

Inoculum preparation and obtaining of cell free culture broth

Single colonies of each LAB strain were grown in five ml of MRS broth (37° C, 24 hrs) and when were transferred into 100 ml-Erlenmeyer's flask containing 50 ml of MRS broth and incubated overnight at 37° C in the thermostat. At the end of culture growth cell concentration achieved (7±2) x10^{8} CFU/ ml, DM (dry matters) - 6% and pH reduced to 3.5- 4.2 and antimicrobial activity was 800-1000 AU/ml. Prepared inoculum was used for growing larger volumes of LAB.

Determination of antimicrobial activity

The spot-on-lawn method was applied. Antimicrobial activity was assessed by measuring the size of the inhibition zone (diameter) of test culture growth (Ø, mm) after 24 hrs incubation in thermostat at 30°C. The antimicrobial activity is expressed in arbitrary units (AU/ml). One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition [Parente,Brienza et al., 1995]. For calculation of antibacterial activity the average results against *Salmonella typhimurium* G 38 and *Bacillus subtilis* 17-89 test cultures were used.

Growth inhibition of pathogenic bacteria

For broth tube dilution method, test cultures were grown overnight on appropriate Nutrient broth at 30 ^{o}C or 37°C depending from test culture. 0.5 ml of suspension was added to experimental flask containing 4.5 ml of appropriate liquid Nutrient broth. 100 µl aliquots of investigated samples were individually added to each experimental flask inoculated with each test culture. The flasks were incubated for 1, 3 and 24 hrs at 30 ^{o}C or 37°C. After incubation, 100 µl aliquot of the suspension was serially diluted, seeded on solid nutrient medium,

and grown during 24 hrs at appropriate temperature depending from test culture. The control flasks contained test cultures but no bactericidal substances were added. The bactericidal efficacy of the samples in liquid medium was calculated as the number of viable colonies after the incubation with samples and was expressed in lg of CFU/ml.

Purification of CFC broth by ion-exchange chromatography

Cell free culture broth was purified by combination of adsorbtion-desorbtion [Yang et al., 1998] and ion-exchange chromatography methods [Aghajanyan et al., 2006]. As a result, partially purified antimicrobial preparations (AMP) were obtained. Further purification of AMP was carried out by fractionation with gel filtration method, conducted on Sephadex G-25 (Superfine) equilibrated with 0.1M NaCl. Elution was carried out with 0.1 M NaCl. Bactericidal activity was examined in each of the 2-ml fractions. Fractions having bactericidal properties were collected and vacuum evaporated at temperature 50-55° C, residual pressure 0.01 MPa. The content of DM of fractions reached to approximately 30%. Antibacterial activity determined as described above.

Purification of bacteriocins

Separation of the bacteriocins was performed with using of high-performance liquid chromatography (HPLC) Shimadzu LC-20 analytical C_{18} column (4.6 by 250 mm, Symmetry, USA), with a detector Diode array SPD-20a, auto-sampler. Sample injection volume of 100 µl. The column was eluted with a linear gradient of water/ trifluoroacetic acid/ acetonitrile at a flow rate of 1.5 ml/ min. Elution was monitored at different wavelength range 190-400 nm. Detection was performed at 210, 254, 280 nm wavelengths [Aslam et al., 2011]. Fractions eluted from the column were freeze-dried, dissolved in 150 µl of bi-distilled water and tested for antibacterial activity against *Salmonella typhimurium* G 38 and *Bacillus subtilis* 17-89 test cultures. Fractions, showing maximal antimicrobial activity have been selected.

For determination of molecular weights of bacteriocins SDS-PAAG polyacrylamide separating gel (T-15%,C- 2,6%, in Tris Cl buffer) with standard staining with Coomassie brilliant blue solution was used by comparison with low molecular weight protein Standards (Mol. weight =1,500 - 14,000 kDa, Sigma) on the same gel.

Result and Discussion

Previously we have shown the morphological and physiological features, carbohydrate fermentation, probiotic properties, specific growth rate and broad spectrum of antimicrobial activity of LAB cultures against Gram-positive and Gram-negative organisms [Tkhruni et al., 2013]. It was shown, that the LAB antimicrobial properties depend on the growth conditions (temperature, time, composition of nutrient medium) [Karapetyan et al., 2008]. For purification of antimicrobial substances from CFC broth, obtained after growth of selected strains, the method of ion-exchange chromatography followed by fractionation by gel filtration was applied. Purification stages are shown in Table 1.

It was shown presence of one fraction with maximal activity for *L.plantarum* 66 and *Ent.faecium* 64 strains. After purification of *L.rhamnosus* 2012 two fractions were obtained also, which inhibited the growth of Gram-positive and Gram-negative bacteria differently.

Thus, applied purification method allows isolating substances with antimicrobial activity from LAB CFC broth. At the same conditions of purification, purification factor, yield and number of fractions differ and depend on species belonging of strains of LAB. Determination of molecular weights of substances with antimicrobial activity containing in the fractions by SDS PAAG electrophoresis shown, that F1 obtained from *L.rhamnosus* 2012 contain one bacteriocin (BCN) with molecular weight approximately 2,0 kDa, F2 contains two active bacteriocins with molecular weight approximately 1,5-1,0 kDa, *L.plantarum 66* and *Ent. faecium* 64 produces a substances with a molecular weight about 2,0 and 1,0 kDa respectively.

For determination of bactericidal or bacteriostatic effect of AMP, the experiments were performed to study a capability of AMPs obtained from researched LAB (500 AU/ml) to inhibit the growth of *Listeria monocytogenes* 1691 and *Salmonella typhimurium* G 38 test cultures in the liquid medium during different time of incubation. Summary results are shown in Figure 1.

Our results also confirm that partially purified antimicrobial preparations, obtained from different LAB inhibited the growth of *S.typhimurium* and *L.monocytogenes* strains differently.

The results presented in Table 2 demonstrated concentration- and time-dependent bactericidal or bacteriostatic efficacy of AMPs, obtained from *Ent.faecium* 64 and *L.plantarum* 66 toward two pathogenic strains of *L.monocytogenes* in the liquid nutrient media. As it seen from given results, low concentrations of preparations 125 and 250 AU/ml for 3 hours inhibited the growth of the studied bacteria differently. The AMP of *L.plantarum* 66 strain was inhibiting the growth of pathogenic bacteria *L.monocytogenes* after one hour of incubation with the 500 AU/ml concentrations. Bactericidal effect of the growth inhibition was observed after 3 hours of incubation at 250 AU/ml concentrations with AMP of the *Ent. faecium* 64 strain.

AMP obtained from *L.rhamnosus* 2012 inhibited the growth of *L.monocytogenes sp*. in higher concentrations (≥1000 AU/ml). Concentrations, mentioned in the table, doesn't affect on the growth of *L.monocytogenes* strains.

The AMPs of investigated LAB strains were studied for their ability to inhibit the growth of different pathogenic bacteria. Table 3 presents results of inhibition of growth of those pathogenic bacteria by the same AMP with final bactericidal activity of 500 AU/ml in the incubation mixture. As it seen from the given results, after the first hour of incubation the growth of studied *Y.Pstbc* 28, *Y.Pstbc* 2143 the inhibition of the growth observed. After three hours of incubation with the preparation from *Ent.faecium* 64 and *L.plantarum* 66 with this concentration the growth of the pathogenic bacteria *E.coli* was not inhibited - a bacteriostatic effect was observed. The AMP of *L.rhamnosus* 2012 possessed bactericidal effect after 3hrs of incubation. However, after 24 hours of incubation bactericidal effect was observed for *Ent. faecium* 64 and *L.rhamnosus* 2012, but AMP of *L.plantarum* 66 shown bacteriostatic effect.

L.rhamnosus 2012 demonstrates bacteriostatic effect on two strains of *Y.enterolitica*. The bactericidal effect was observed after 1 hour of incubation of the *Y.enterolitica* with AMP of *L.plantarum* 66 and *Ent. faecium* 64.

Thus, inhibition of different strains of pathogenic bacteria depends from species belonging of LAB strains, from which the preparations were obtained.

It was shown that the AMP, obtained from of *L.rhamnosus* BTK-2012 strain, inhibited the growth of some antibiotic-resistant Gram-positive and Gram-negative bacteria with different efficiency [Melik-Andreasyan et al., 2013]. The inhibition effect depended on genus of the examined bacteria of gut microbiota. The diverse efficacy of growth inhibition may probably relate to the different mechanisms of action of the substances towards bacteria cell membrane. This is confirmed by the fact that these preparations contain different bacteriocins which can possess both bacteriostatic and/or bactericidal properties.

It was shown by us , that *L.rhamnosus* BTK-2012 contains 2 active bacteriocins of peptide nature (BCN 1 and BCN 2) with molecular weight 1,427 Da, 602.6 Da respectively. The bactericidal activity of two bacteriocins against some G-positive and G-negative pathogens belonged to different genus were investigated. The results summarized in Table 4.

Very similar host specificity of the BCN 1 and 2 was observed in the experiments with G-positive and G-negative microorganisms from *Bacillus, Staphylococcus, Pseudomonas, Klebsiella, Proteus,* and *Escherichia* genus. The results presented in the Table 4 demonstrate bactericidal effect of both BCNs toward different microorganisms. The BCN 2 was slightly more effective than the BCN1 in growth inhibition of all tested cultures.

As following from the results presented in the Table 4, out of 6 tested *E.coli* strains five of them were resistant to the BCN 2, while different strains from *Bacillus* genus were all susceptible to both bacteriocins. The number of the species of the other examined genus was limited and therefore, the range of the host specificity of the BCN 1 and 2 within *Staphylococcus, Pseudomonas, Klebsiella,* and *Proteus* genus remained unclear. Bactericidal efficacy of the BCN 1 and 2 was compared with the efficacy of conventional antibiotics [not published]. The diverse efficacy of growth inhibition may probably relate to the different mechanisms of action of the bacreriocins towards bacteria cell membrane; the mechanism of action described for bacteriocin involved a phenomenon of adsorption of bacteriocins on the cell wall. Above all, it can be explained with the presence of specific receptor proteins required for binding to bacteriocins and their transport into the bacteria.

Comparison of these data with our results shows that activity spectrum and quantitative antimicrobial activity of the studied *L rhamnosus* 2012 recognize from the data cited in the articles. [Weese S.J and Anderson, M.E.C. 2002. Ambalam P, Prajapati JB,2009, Dimitrijevic´ R., M. Stojanovic 2009]. Titration method showed that the growth inhibition depends on concentration, time of incubation, the species of pathogenic bacteria and of genus of bacteria from which the cationic fraction is obtained.

There are much of publications on bacteriocins produced by *L.plantarum* [Torodov and Dicks, 2005]. However, for the first time it is shown that from the first hours of incubation the partially preparation from *L.plantarum* 66 inhibit the growth of some SDI causing pathogenic strains. Received data show that the difference is growh inhibition spectrum may be conditioned both by the nature of the test strain, as well as the

nature of the synthesized bacteriocine. For the first time it is shown that the isolated strain *Ent. faecium* 64 inhibits the growth of pathogenic bacteria from the first hours of incubation.

As it can be seen from the obtained data, partially purified antimicrobial preparation, obtained from *L.rhamnosus* 2012 broth by ion exchange chromatography and gel filtration methods, can inhibit the growth of gram-positive and gram-negative bacteria. According to reference sources, for the evaluation of the growth suppression spectrum the spot-test method is prevalently applied and determination is carried out after 24 hours of incubation in the thermostat. To determine the growth inhibition effectiveness, the titration method was used. For the first time it is shown that after 1-3 hour contact of the examined pathogenic bacteria with preparation the growth inhibition (bactericidal effect) was observed. For example, inhibition of the growth of *L.monocytogenes* depends on fraction concentration, time of contact and the test culture titer. The results of inhibition of pathogenic strains do not repeat the data presented in the cited articles which suggests that the endemic strain isolated from the brine cheese in Armenia is not identical to the strains described in the literature.

The AMP of the two other strains *L.plantarum* 66 and *Ent. faecium* 64,obtaided by application of purification methods developed by us shown that they also inhibit the growth of some pathogenic bacteria, but there is a difference in the spectrum of inhibition and this difference does not repeat the data available in the literature.

Thus, data on inhibiting growth of some pathogenic strains are promising for further studies in various applied and fundamental areas.

Table.1 Comparative results of purification of CFC broth from different strains of LAB (test culture B. subtilis G17-89)

LAB strains	CFC broth		AMP after ion-exchange chromatography		Fractions after gel filtration			Purification factor
	Total activity, AU/ml	V, ml	Total activity, AU/ml	V, ml	No	V, ml	Total activity, AU/ml	
L.rhamnosus 2012	$5,5x10^5$	3,000	$0.9x10^5$	100	F1 F2	24 70	$9.6x10^3$ $4.9x10^4$	$0.7x10^1$ $1.4x10^1$
Ent. faecium 64	$1.2x10^5$	1,000	$1.8x10^4$	23	F1	24	$3.3x10^4$	$1.8x10^1$
L.plantarum 66	$6.0x10^4$	1,000	$5.0x10^4$	26	F1	30	$3.6x10^4$	$8.8x10^1$

Table.2 Inhibition of *L.monocytogenes* sp. growth in dynamics by various concentrations of AMP from studied strains of LAB

Pathogenic strains	LAB strains	AU/ml	Time of incubation with AMP, hrs		
			1	3	24
			n x 10^3CFU/ml		
L.monocytogenes 35	*L.plantarum* 66	0	6.0	12.0	45
		125	5.8	4.3	0
		250	4.6	4.0	0
		500	0	0	0
	Ent.faecium 64	125	4.9	1.4	0
		250	4.2	0	0
		500	0	0	0
L.monocytogenes 37	*L.plantarum* 66	0	7.6	14.0	48
		125	6.1	5.3	0
		250	5.2	4.8	0
		500	0	0	0
	Ent. faecium 64	125	5.0	4.2	0
		250	4.1	0	0
		500	0	0	0

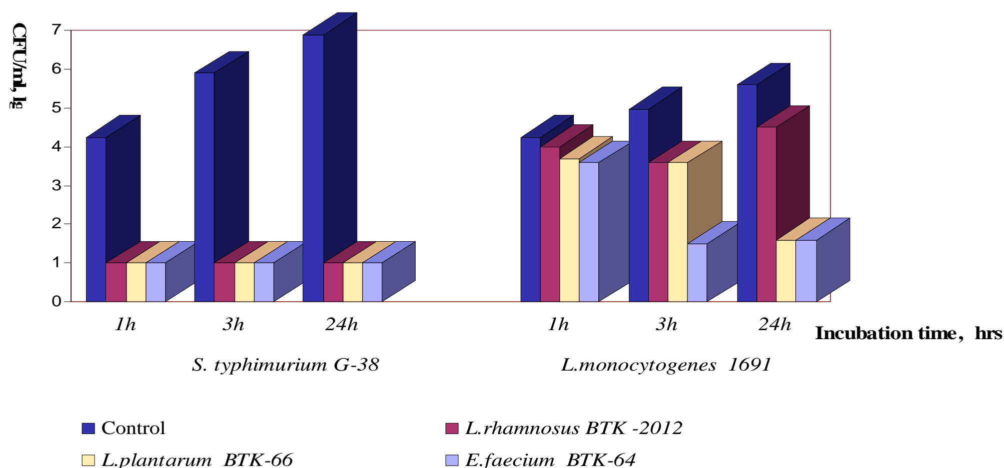
Table.3 Inhibition of the growth of pathogenic bacteria with the AMP of the studied strains (500 Au/ml, CFU/ml x 103)

Pathogenic bacteria	AMP of strains	Time of incubation with AMP, hrs					
		1		3		24	
		Cont	Exp.	Cont	Exp.	Cont	Exp.
E.coli 2529	*L.plantarum* 66	9.5	30	31.5	33.2	growth	18.4
	Ent. faecium 64		22		20		0
	L.rhamnosus 109		2.4		13		0
E.coli 2859	*L.plantarum* 66	9.0	20	36	22.2	growth	20
	Ent. faecium 64		17.9		17.,9		0
	L.rhamnosus 109		0		0		0
Y.Pstbc 28	*L.plantarum* 66	3.0	2.2	3,4	1.9	growth	0
	Ent. faecium 64		0		0		0
	L.rhamnosus 109		0		0		0
Y.Pstbc 2143	*L.plantarum* 66	2.6	0	3.0	0	growth	
	Ent. faecium 64		0		0		0
	L.rhamnosus 109		0		0		0
Y. enterolitica 19	*L.plantarum* 66	6.0	0	12	0	growth	0
	Ent. faecium 64		0		0		0
	L.rhamnosus 109		2.3		0.7		0
Y. enterolitica 373	*L.plantarum* 66	10	0	30	0	growth	0
	Ent. faecium 64		0		0		0
	L.rhamnosus 109		1		3.0		0

Table.4 Growth inhibition of G- positive and G-negative pathogens by two bacteriocins of L.rhamnosus BTK-2012 (Ø, mm, ±0,5)

Test cultures		BCN 1	BCN 2
		80 AU/ml	
G-positive	*Staphylococcus aureus* 13	10	20
	Bacillus subtilis G17-8	15	14
	B. subtilis INMIA 626	10	14
	B. subtilis ATCC 6633	12	16
	B. cereus INMIA 620	9	16
	B. cereus INMIA 614	10	12
	B. thuringiensis subsp. galleriae INMIA	16	14
	B. thuringiensis ATCC 19265	6	14
	B. mesentericum INMIA 78	8	19
	B. megaterium ATCC 14581	6	14
G-negative	*Pseudomonas aeruginosa* 12	10	14
	Ps. aeruginosa INMIA 9056	9	18
	Ps. fluorescence INMIA 9068	8	8
	Klebsiella sp.	7	14
	Proteus mirabilis 597	14	13
	Pr. mirabilis 22	12	12
	Escherichia coli 10	9	14
	E. coli 2	10	none
	E. coli 5	7	none
	E. coli 7	5	none
	E. coli K12	none	none
	E. coli ATCC 11303	none	none
	Salmonella enteritidis	none	14
	Salmonella spp.	none	12

Fig.1 Influence of the AMPs on the growth dynamics of test cultures



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