Study on the effect of sublethal concentrations of antimicrobials on the growth and development of probiotic lactobacilli

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Abstract. The objective of this study was to investigate the effect of sublethal concentrations of 5 antibiotics (tetracycline, erythromycin, penicillin G, lincomycin, ciprofloxacin) and 2 preservatives (benzoic and sorbic acid) on the growth and development of 8 probiotic *Lactobacillus* strains. All lactobacilli were subjected to a large range of concentrations, growth curves were plotted and MICs were determined for each antimicrobial. The results showed correlation between the sublethal concentrations and the growth rate of the studied microorganisms. Based on the obtained data two groups of strains could be defined – sensitive and relatively resistant. The first group includes *L. bulgaricus* S2. *L. bulgaricus* S4. *L. bulgaricus* S19 and *L. gasseri* S20. The most resistant of this group was *L. gasseri* S20. The second group showed more sensitivity and the most affected by the antimicrobials was *L. bulgaricus* S28.

1 Introduction

The main group of probiotic bacteria in application today are species belonging to the genus *Lactobacillus*. They have many beneficial effects on the host, which are well documented in reference literature [1, 2, 3]. A useful trait in a probiotic strain is its resistance to the effect of different antimicrobials, such as antibiotics and preservatives. Benzoic and sorbic acids are commonly used in foods as pH adjusters and preservatives, preventing the growth of the undesirable microflora [4. 5]. Antibiotics could be ingested in low concentrations with food as residue from the treatment of farm animals [6], or as part of the medical treatment of different diseases. Such antimicrobials potentially disrupt the balance of the intestinal flora, which is linked to various medical conditions – gastrointestinal or infectious diseases, allergies, etc. [7]. The use of probiotic supplements, containing strains, resistant to antimicrobials, could treat dysbacteriosis and prevent its negative health effects.

One the other hand, antibiotic resistance may affect the safety of a probiotic. The antibiotic resistance of a microorganism could be intrinsic, due to a chromosomal mutation or to the acquisition of genetic material through transposons or plasmids [8, 9]. According to some authors non-pathogenic bacteria, such as *Lactobacillus,* could serve as a reservoir of antibiotic resistance genes, which can be transferred to other microorganisms, including human pathogens [8, 10]. Because of the taxonomic complexity of genus *Lactobacillus* more data is needed for the antimicrobial resistance of its representatives and the mechanisms behind it.

The objective of this study is to investigate the effect of sublethal concentrations of 5 antibiotics (tetracycline, erythromycin, penicillin G, lincomycin, ciprofloxacin) and 2 preservatives (benzoic and sorbic acid) on the growth and development of 8 probiotic *Lactobacillus* strains.

2 Materials and methods

2.1 Microorganisms

Eight probiotic strains from genus *Lactobacillus* were used: *Lactobacillus acidophilus* S11. *Lactobacillus delbrueckii subsp. bulgaricus* S2. *Lactobacillus delbrueckii subsp. bulgaricus* S4. *Lactobacillus delbrueckii subsp. bulgaricus* S6. *Lactobacillus delbrueckii subsp. bulgaricus* S19. *Lactobacillus delbrueckii subsp. bulgaricus* S28. *Lactobacillus gasseri* S20 and *L. helveticus* S3. The strains were isolated from fresh homemade cheese and yogurt. All microorganisms were cultivated in MRS broth (Oxoid, UK) and stored as 15% (w/v) glycerol stock cultures at -30°C.

2.2 Preparation of active bacterial culture

Active bacterial cultures were prepared by inoculation of 1 ml MRS broth (Oxoid, UK) with 50 μl glycerol stock culture, which were cultivated at 37°C until they reach optical density of 1.000 at λ = 600 nm [11].

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2.3 Determination of the optical density of the bacterial cultures

The optical density was determined by using a micro-plate reader Spectrostar Nano (BMG Labtech, Germany) against MRS broth as blank at λ = 600 nm. The sample volume was 200 µl with correction to 1 cm path length. Every value is presented as the mean of nine measurements (three samples, measured three consecutive times each) [12].

2.4 Determination of the minimal inhibitory concentrations of antibiotics and preservatives

MRS broth samples (850 µl) with double-fold decreasing concentrations of antibiotic/ preservative - erythromycin (Merck, Germany) 0.38 - 200 µg/ml, lincomycin (Merck, Germany) 0.38 - 100 µg/ml, ciprofloxacin (Merck, Germany) 0.38 - 100 µg/ml, penicillin G (Merck, Germany) 0.38 - 100 µg/ml, tetracycline (Merck, Germany) 0.38 - 200 µg/ml, benzoic acid (Merck, Germany) 0.25 - 8 mg/ml, and sorbic acid (Merck, Germany) 0.125 - 8 mg/ml were inoculated with 50 µl active bacterial culture and cultivated at 37°C for 24 h. The optical density at λ = 600 nm was determined for each sample.

3 Results and discussion

The effect of sublethal concentrations of 5 antibiotics and 2 preservatives was studied during 24 h cultivation and the minimal inhibitory concentrations (MICs) were determined for all antimicrobials.

Lactobacillus bulgaricus S6 was sensitive towards all antibiotics, except ciprofloxacin (Fig. 1 – Fig. 5) of the effective antibiotics the highest MIC was determined for erythromycin - 3.06 µg/ml.
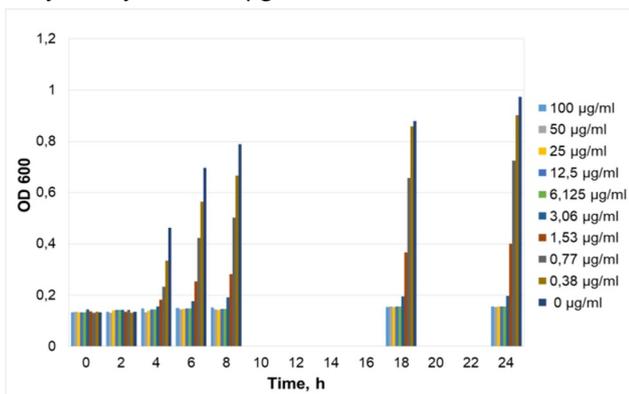

Fig. 1. Effect of sublethal concentration of erythromycin on the growth and development of *Lactobacillus bulgaricus* S6

All of the tested lincomycin concentrations inhibited the growth of *Lactobacillus bulgaricus* S6 (Fig. 2). The actual MIC for this antibiotic was below 0.38 µg/ml.

The strain exhibited resistance towards all of the ciprofloxacin concentrations included in the experiment (Fig. 3).
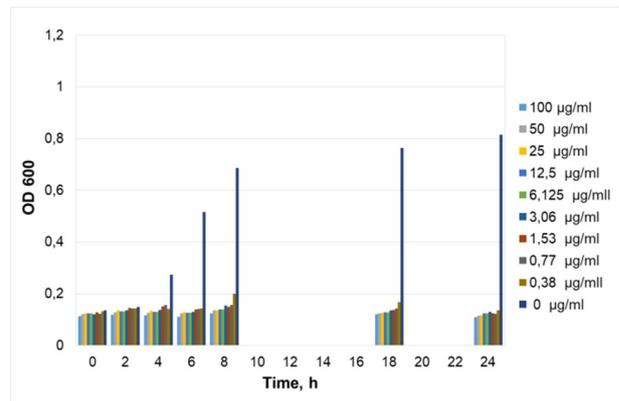

Fig. 2. Effect of sublethal concentration of lincomycin on the growth and development of *Lactobacillus bulgaricus* S6

This was the only antibiotic, which did not influence the development of *Lactobacillus bulgaricus* S6 even in 100 µg/ml concentration.
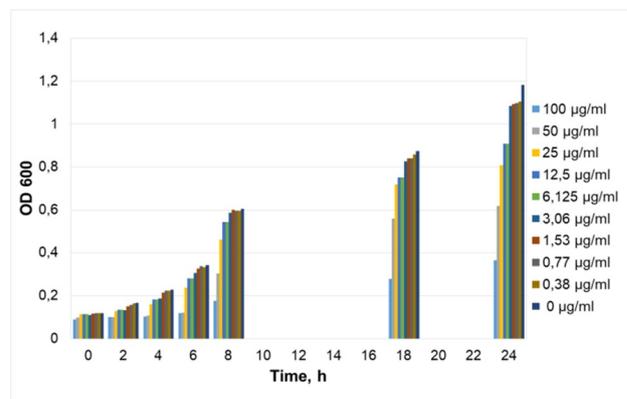

Fig. 3. Effect of sublethal concentration of ciprofloxacin on the growth and development of *Lactobacillus bulgaricus* S6

The other antimicrobial, which inhibited the growth of *Lactobacillus bulgaricus* S6 completely, was penicillin G (Fig. 4). The exact MIC was below 0.38 µg/ml.
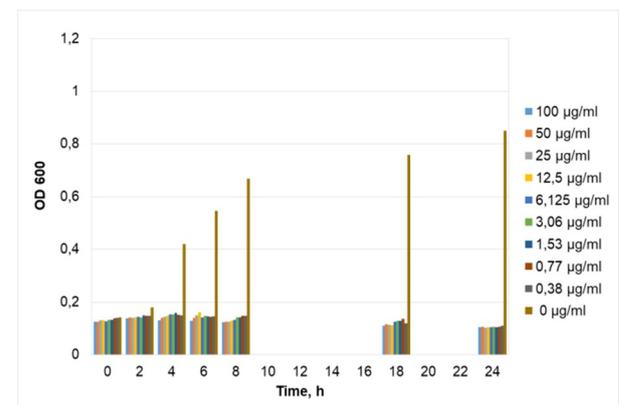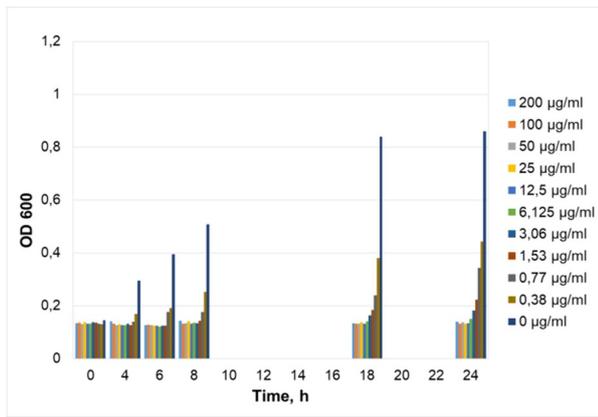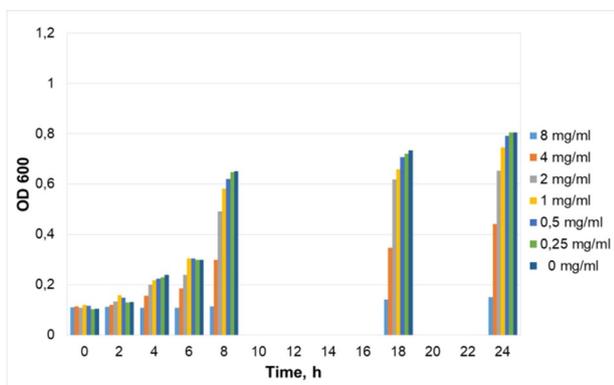

Fig. 4. Effect of sublethal concentration of penicillin G on the growth and development of *Lactobacillus bulgaricus* S6

Lactobacillus bulgaricus S6 was more sensitive towards tetracycline in comparison with erythromycin. The MIC was 1.53 µg/ml (Fig. 2).
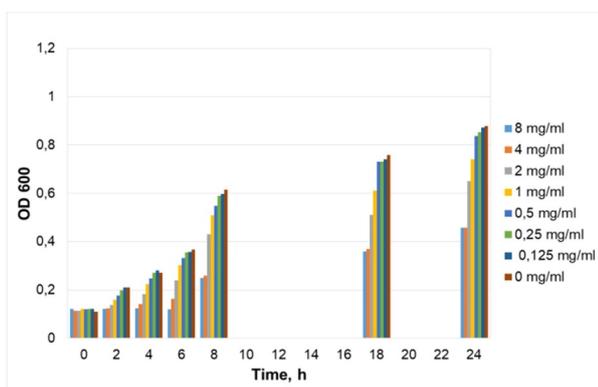
Fig. 5. Effect of sublethal concentration of tetracycline on the growth and development of *Lactobacillus bulgaricus* S6

On the other hand, it was resistant to benzoic and sorbic acids (Fig. 6 and Fig. 7). The MIC of both preservatives was 8 mg/ml.

Fig. 6. Effect of sublethal concentration of benzoic acid on the growth and development of *Lactobacillus bulgaricus* S6

Fig. 7. Effect of sublethal concentration of sorbic acid on the growth and development of *Lactobacillus bulgaricus* S6

The MICs of all antimicrobials for every *Lactobacillus* strain, included in this study, are presented in Table 1. Since some compounds showed the same MICs for multiple strains, Table 1 also includes the optical density of the microbial suspension at sublethal concentrations (OD at 0.5 MIC). This gave us better

understanding of the effect of the antibiotics and preservatives in the development of the lactobacilli.

All strains were susceptible to the activity of penicillin G in all tested concentrations, which was reported in multiple studies in reference literature [9, 13]. All eight lactobacilli were resistant towards ciprofloxacin in the tested concentrations. This result is supported by the work of other authors, according to which most *Lactobacillus* species are resistant to inhibitors of the nucleic acid synthesis, such as ciprofloxacin, nalidixic acid and metronidazole [13].

Strain and species specific differences in the effect of the rest of the antibiotics were observed. *Lactobacillus acidophilus* S11 was most resistant towards tetracycline with MIC of 6.125 µg/ml and was most sensitive to lincomycin (MIC 0.77 µg/ml). Sharma et al. also report resistance to tetracycline for 7 *Lactobacillus acidophilus* strains [14]. *Lactobacillus bulgaricus* S2 showed the exact same response to the influence of these antibiotics, while *Lactobacillus bulgaricus* S4 was a little more sensitive towards tetracycline and the MIC for this strain was 3.06 µg/ml.

Lactobacillus bulgaricus S19 exhibited its highest resistance towards erythromycin, which had MIC of 3.06 µg/ml. On the other hand, *Lactobacillus bulgaricus* S28 was equally sensitive towards tetracycline and erythromycin with MIC for both antibiotics 1.53 µg/ml.

Lactobacillus gasseri S20 demonstrated the highest tolerance towards the tested antimicrobials. The MICs of erythromycin and lincomycin was 12.5 µg/ml and for tetracycline – 6.25 µg/ml. In contrast, *L. helveticus* S3 was one of the more sensitive strains. The highest MIC of 3.06 µg/ml was observed for erythromycin.

The prevailing resistance overall for the tested strains was towards tetracycline and erythromycin, which is in compliance with the results of other studies on the antimicrobial resistance of *Lactobacillus* strains [15-20]. For the rest of the antibiotics, included in the study, various sensitivities were reported in the reference literature. This could be due to the facts that the mechanisms of resistance could be both intrinsic and acquired [9].

There were two distinct groups in regard to the effect of the preservatives on the growth and development of the lactobacilli. The first group, including *Lactobacillus bulgaricus* S6. *Lactobacillus bulgaricus* S19. *Lactobacillus bulgaricus* S28 and *Lactobacillus gasseri* S20. exhibited better tolerance towards both benzoic and sorbic acid. The detected MIC was 8 mg/ml for benzoic acid and above 8 mg/ml for sorbic acid. The rest of the tested strains were more sensitive and the MIC of both antimicrobials for this group was 4 mg/ml. Despite this fact, all MICs for the preservatives were above the allowed concentration in food according to the EU regulations, which is up to 2 mg/ml.

Table 1. Effect of sublethal concentrations of antibiotics and preservatives on the growth and development of the *Lactobacillus* strains

		Strains	*L. acidophilus* S11	*L. bulgaricus* S2	*L. bulgaricus* S4	*L. bulgaricus* S6	*L. bulgaricus* S19	*L. bulgaricus* S28	*L. gasseri* S20	*L. helveticus* S3
Antibiotics	erythromycin	MIC, µg/ml	1.53	1.53	1.53	3.06	3.06	1.3	12.5	3.06
		OD at 0.5 MIC	0.190	0.362	0.270	0.400	0.356	0.595	0.729	0.265
	lincomycin	MIC, µg/ml	0.77	0.77	0.77	< 0.38	< 0.38	0.77	12.5	0.77
		OD at 0.5 MIC	0.616	0.668	0.648	-	-	0.545	0.791	0.125
	ciprofloxacin	MIC, µg/ml	100	100	100	over 100	over 100	over 100	over 100	100
		OD at 0.5 MIC	0.437	0.439	0.488	0.620	0.549	0.477	1.030	0.553
	penicillin G	MIC, µg/ml	< 0.38	< 0.38	< 0.38	< 0.38	< 0.38	< 0.38	< 0.38	< 0.38
		OD at 0.5 MIC	-	-	-	-	-	-	-	-
	tetracycline	MIC, µg/ml	6.125	6.125	3.06	1.53	1.53	1.53	6.125	1.53
		OD at 0.5 MIC	0.450	0.487	0.319	0.343	0.333	0.351	0.596	0.408
Preservatives	benzoic acid	MIC, mg/ml	4	4	4	8	8	8	8	4
		OD at 0.5 MIC	0.263	0.230	0.235	0.441	0.520	0.447	0.588	0.267
	sorbic acid	MIC, mg/ml	4	4	4	over 8	over 8	over 8	over 8	4
		OD at 0.5 MIC	0.365	0.322	0.342	0.458	0.420	0.459	0.567	0.352

4 Conclusion

The study on the effect of five antibiotics and two preservatives on lactobacilli with probiotic properties showed that based on the obtained data the eight strains could be divided into two groups – more resistant strains and more sensitive towards the antimicrobials.

The first group includes *Lactobacillus bulgaricus* S2. *Lactobacillus bulgaricus* S4. *Lactobacillus bulgaricus* S19 and *Lactobacillus gasseri* S20. The most resistant of this group was *Lactobacillus gasseri* S20. The second group showed more sensitivity and the most affected by the antimicrobials was *Lactobacillus bulgaricus* S28.

Unlike the effects of penicillin G and ciprofloxacin, which are well established for the entire genus *Lactobacillus,* most other antimicrobials affect different species and even strains to a different extent. This variation in resistance necessitates the investigation of each strain before its application.

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