Antibiotics influence on lactic acid bacteria inhabiting gastrointestinal tract

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Summary

Lactic acid bacteria (LAB) are common inhabitants of the gastrointestinal (GI) tract and have important role in maintaining the equilibrium of GI flora, which can be influenced by various factors like diets, antimicrobials and stress. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of 6 antibiotics, commonly used in human medicine for 8 selected lactobacilli strains were determined by macrodilution and microdilution methods in liquid media and by diffusion method on agar plates. The effects of Penicillin G and Ampicillin on intestinal LAB were tested in vivo on mice as well. Lactobacilli were sensitive to Penicillin G, (penicillines and their derivatives) and Erythromycin (macrolides) by in vitro testing. Clyndamycin (pyranosid) showed moderate inhibitory effect. All lactobacilli strains were resistant to Kanamycin and Neomycin (aminoglycosides), while L. salivarius IM 124 has shown extra resistance to Erythromycin and Clyndamycin. The influence of orally administered Ampicillin showed no significant influence on LAB count in mice faeces. The effect of Penicillin G on mice LAB total count was significant, while no effect of orally administered lactobacilli was determined.

Key words: *antibiotics, inhibitory effect, lactic acid bacteria, gastrointestinal tract*

Introduction

Medical treatment with antibiotics causes non-selective elimination of part of the microbial population from the gastrointestinal tract, which in this way are weakened and present an easy target for microorganisms dangerous to health. To prevent such a threatening situation and to restore the normal state of intestinal microflora, consumption of biologically active fermented foods

containing probiotic bacteria during and/or after antibiotic intake is recommended. By their definition probiotics are live microbial feed/food supplement which beneficially affects the composition of the host intestinal microflora (Ouwehand et al., 1999). Therefore, probiotic LAB present an appropriate adjunct in antibiotic therapy, especially if they posses intrinsic resistance to antibiotics. The acquired resistance might sometimes be of safety concern as it could be transmissible among bacteria, as in the case with vancomycin resistance in enterococci (Mattila-Sandholm et al., 1999). For that reason the selection of probiotic strains for fermented foods must be completed with a high degree of caution.

The most common way to analyse the susceptibility of a bacterium to antibacterial substance is via detecting the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). MIC is defined as the concentration of the antibiotic at which there is no visible growth, where MBC refers to the lowest drug concentration that gives no growth on the agar (Piddock, 1990). Both, MIC and MBC are expressed as μg of antibiotic/ml. Another method is agar diffusion test which is performed by using discs impregnated with antibiotic and the sensitivity of a microorganism is determined according to the zone of inhibition. This procedure was developed and standardised by Bauer et al. (1966). As the method was optimised for rapidly growing pathogens such as *Staphylococcus* sp. and *Enterobacteriaceae*, results cannot just simply be interpreted in the same way in our study in which bacteria tested belong to the *Lactobacillus* genus. Recently, an extended study on disc diffusion antibiotic susceptibility assay with refinements for *Lactobacillus* species application was published (Charteris et al., 1998). A susceptibility of 46 potentially probiotic lactobacilli to 44 antibiotics was surveyed, what enabled to determine the reaction of lactobacilli species to each agent in a sense of their selection for use as microbial adjunct nutrition and disease management (prophylaxis and therapy). At the same time, authors defined the antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing in terms of resistance, moderate susceptibility or susceptibility.

The aim of our study was to analyse in vitro susceptibility of 8 selected lactic acid bacteria to 6 antibiotics. In this way we wanted to establish which strains of LAB tested were enough resistant and potentially help to maintain the microbial balance in intestinal tract during and after the antibiotic

treatment. The second part of our experiment employed in vivo testing of two of the most wide-used antibiotics on the mice intestinal population of lactic acid bacteria with simultaneous intake of probiotic lactic acid bacteria.

Material and methods

Microorganisms and antibiotics

Selected strains of lactobacilli and two non-lactobacilli reference strains, growth medium, propagation temperature and atmosphere conditions used in this study, are listed in Table 1, while antibiotics and their potencies are presented in Table 2. *Lactobacillus acidophilus* LF221 is from our collection, and the rest of the strains from commercial collections. Strains were stored at -20 °C in an appropriate broth with 20 % glycerol stock solutions.

Some of the *Lactobacillus* species such as *L. acidophilus*, *L. casei*, *L. johnsonii*, *L. reuteri* and *L. salivarius* are typical members of intestinal microflora.

In vitro tests

Antibiotic diffusion test

Agar diffusion test was performed as described by Bauer et al. (1966). Briefly, discs impregnated with specific concentrations of antibiotics (Becton Dickinson, BBL Sensi-Disc Antimicrobial Susceptibility Test Discs) were applied to the surface of the MRS (Merck) or Mueller-Hinton (MH; Merck) agar, where 0.1 ml of test or reference culture with recommended cell concentration of 10^5 CFU/ml had already been spread out. Following incubation for 16-18 h at 37 °C, plates were examined and zones of inhibition surrounding the discs were measured. Zone diameters of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were compared with established zone size ranges for individual antimicrobial agents as indicated by the Bauer-Kirby procedure (Bauer et al., 1966).

Table 1: Tested bacteria and growth conditions
Tablica 1: Ispitivane bakterije i uvjeti rasta

Strain Soj	Growth medium Hranjiva podloga	Temperature (°C) Temperatura	Atmosphere Atmosfera
L. acidophilus LF221	MRS	37	microaerophilic
L. casei ATCC 393	MRS	37	microaerophilic
L. johnsonii ATCC 11506	MRS	37	microaerophilic
L. helveticus ATCC 15009	MRS	42	microaerophilic
L. plantarum IM 42	MRS	37	microaerophilic
L. reuteri DSM 20016	MRS	37	microaerophilic
L. sakei ATCC 15521	MRS	30	microaerophilic
L. salivarius IM 124	MRS	37	microaerophilic
Escherichia coli ATCC 25922	MH	37	aerobic
Staphylococcus aureus ATCC 25923	MH	37	aerobic

Legend:
IM: Culture Collection of the Institute of Dairying, University of Ljubljana, BTF, Slovenia
ATCC: American Type culture Collection, Rockville, USA
DSM: Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany

Determination of minimal inhibitory concentrations (MIC)

MICs were determined via two methods: macro and micro broth dilution method. In tests employing liquid media the inoculum density is of great importance, and should be of 10^5 CFU/ml. Heavy inoculum can contain a few mutant cells which would multiply during the growth and lead up to false susceptibility.

For macro dilution method, a series of two-fold dilutions of the antibiotic stock solutions were prepared in the liquid MRS media in which test bacteria were grown. Tubes with 10 ml of MRS broth were then seeded with standardised inoculum of LAB and incubated for 16-20 h. Tubes indicating no turbidity were recognised as MIC for the particular strain.

Broth microdilution method was performed similarly. This time the two-fold dilutions of antibiotics were done in broth media in a 96-well microtitre plate and broth was inoculated with 10^5 CFU/ml of the tested organism. After incubation for 16-20 h, MIC was described as concentration in the first well in which no visible growth was observed.

All tests were performed 4 times or as many times needed to obtain reproducibility.

Determination of minimal bactericidal concentration (MBC)

The experiment was set up as described for MIC determination (broth microdilution method) with some modifications. Shortly, the antibiotic concentrations used in this experiment were much higher than those used in determining the MIC. After the microtitre plates incubation the loopful from each clear microtitre well was subcultured to MRS (Merck) agar plate and incubated for 48 h at the temperature recommended for the particular strain. The lowest concentration of an antibiotic that resulted in no visible colonial growth was recorded as MBC.

In vivo testing

Two antibiotics, ampicillin and penicillin G were applied for the *in vivo* experiments. Female mice (NIH, KRKA, Novo Mesto, Slovenia), weighing 8-10 g, were divided into 5 groups of 3 mice each for each antibiotic. In a 15-days-experiment they all were fed with mouse feed (M-K, Eksperimentalna mešanica Homec, Slovenia) *ad libitum*. First group was treated as a control group, while other four as experimental groups receiving *L. acidophilus* LF221 cells once a day for 14 days, *L. acidophilus* LF221 cells once a day for 14 days and antibiotic twice a day for 10 days, antibiotic twice a day for 10 days, and the last group receiving cells prepared from *L. acidophilus* LF221, *L. casei* ATCC393, *L. johnsonii* ATCC11506 and *L. sakei* ATCC15521 once a day for 14 days with antibiotic intake twice a day for 10 days. Bacteria and/or antibiotics were dosed directly into the mouse mouth, at the concentration of cells $1x10^9$, while ampicillin and penicillin intake was 0.1 mg/g and 200 IU/g of live mouse weight, respectively. The antibiotic doses were chosen according to the literature and corresponded those given to children.

Microbiological analyses

Faecal samples from each mouse were aseptically collected just before the start of the experiment (sample 0) and then on 4^{th}, 7^{th}, 11^{th}, and 15^{th} day. Samples from each group were homogenised and further 10-fold serially diluted in Ringer solution. Diluted samples were plated on MRS (Merck) and Rogosa (Merck) agar, and incubated at 37 °C for 48 h. Further, colonies grown on MRS and Rogosa were counted and expressed as total lactic acid bacteria (LAB) and lactobacilli population, respectively. Monitoring of the bacterial populations in faecal samples were performed equally in control and experimental groups within either ampicillin or penicillin trial. Results were presented as log of number of bacteria/ g of faeces.

Results

Agar diffusion method

As it is evident from the results of agar diffusion method, lactobacilli were the most sensitive to penicillin, ampicillin and erythromycin where zones of inhibition reached 22 up to 37 mm (Table 1). Less inhibitory was clyndamycin resulting in smaller zones of 9 to 24 mm. The only exception seemed to be *L. salivarius* which showed susceptibility to neither erythromycin nor clyndamycin. Neomycin inhibited 4 out of 8 lactobacilli tested. Test was performed with control strains of *E. coli* and *S. aureus*. Results obtained with these two strains were compared to those published by the NCCLC (National Committee for Clinical Laboratory Standards). As our results agreed with those of NCCLS we approved that the method was properly performed. Similar preliminary experiments with control bacteria were done using by microdilution and macrodilution methods to confirm the regularity of the accomplishment.

Table 2: Antibiotic resistance patterns interpreted as zone diameters obtained by agar diffusion method.
Tablica 2: Primjeri antibiotičke rezistencije interpretirani kao promjeri zona dobivenih sa agar difuzijskom metodom

	Zone diameter (mm) / Promjeri zona (mm)					
STRAIN/ SOJ	Am	E	K	Cc	N[a]	P
L. acidophilus LF221	34-S	28-S	0-R	9-MS	9	36-S
L. casei ATCC 393	26-S	22-S	0-R	9-MS	0	35-S
L. johnsonii ATCC 11506	28-S	25-S	0-R	17-S	0	34-S
L. helveticus ATCC 15009	37-S	29-S	0-R	14-S	11	34-S
L. plantarum IM 42	35-S	25-S	0-R	24-S	8	36-S
L. reuteri DSM 20016	28-S	22-S	0-R	23-S	0	30-S
L. sake ATCC 15521	27-S	24-S	0-R	24-S	10	29-S
L. salivarius IM 124	26-S	0-R	0-R	0-R	0	33-S
E. coli ATCC 25922	20-S[1]	0-R[1]	17-MS[1]	0-R[1]	17-S[1]	0- R[1]
S. aureus ATCC 25923	30-S[1]	22	19-S[1]	24	18-S[1]	33-S[1]

Legend:
Am – ampicillin; E – erythromycin; K – kanamycin; Cc – clyndamycin; N – neomycin; P – penicillin.
Susceptibility expressed as R (resistant), MS (moderately susceptible), or S (susceptible) according to Charteris et al., 1998; [a] no zone interpretation data available for neomycin.
R[1] (resistant), MS[1] (moderately sensitive), or S[1] (susceptible) adopted by NCCLS (National Committee for Clinical Laboratory Standards).

MIC

Microdilution broth method was another procedure used to determine the susceptibility of lactobacilli to antibiotics. When results were compared to those obtained using disc analysis, antibiotics demonstrated similar efficacy. The lowest MICs were observed with ampicillin, erythromycin and penicillin ranging between 0.125 - 4 µg/ml, while clyndamycin resulted in slightly higher MICs as 0.125 - 16 µg/ml. *L. salivarius* was the least sensitive to erythromycin and clyndamycin (Table 3).

The results of both microdilution and macrodilution methods were exactly the same (data not shown) indicating that there was no need for further

procedure using macrodilution method, which is anyway considered as time and material consuming test.

Table 3: Minimal inhibitory concentrations (MIC) of selected antimicrobial substances for Lactobacillus strains.

Tablica 3: Minimalne inhibicijske koncentracije (MIC) izabranih antimikrobnih supstancija za Lactobacillus sojeve.

	M I C (µg/ml)					
STRAIN/ SOJ	Am	E	K	Cc	N	P
L. acidophilus LF221	1	0.5	312.5	16	2500	0.25
L. casei ATCC 393	1.5	4	2500	8	312.5	0.5
L. johnsonii ATCC 11506	1	2	1250	2	625	0.125
L. helveticus ATCC 15009	0.25	0.5	156.25	8	78.125	0.5
L. plantarum IM 42	0.25	1	2500	0.125	312.5	0.25
L. reuteri DSM 20016	1	2	2500	0.062	625	0.5
L. sake ATCC 15521	2	1	156.25	0.25	78.125	2
L. salivarius IM 124	0.5	5000	2500	5000	625	0.25
E. coli ATCC 25922	8	156.125	1.83	62.5	1.22	39
S. aureus ATCC 25923	0.076	0.61	0.305	/	0.305	0.019

Legend:
Am – ampicillin; E – erythromycin; K – kanamycin; Cc – clyndamycin; N – neomycin; P – penicillin.
/: no data

MBC

Results of MBCs appeared comparable to those already obtained using previous two methods in terms of susceptibility patterns, although the values of MBCs were much higher than MICs. MBCs for all strains except *L. salivarius* were highest with kanamycin and neomycin, and lowest with penicillin (Table 4).

Table 4: Minimal bactericidal concentrations (MBC) of selected antimicrobial substances for Lactobacillus strains.
Tablica 4: Minimalne baktericidne koncentracije (MBC) izabranih antimikrobnih supstancija za Lactobacillus sojeve.

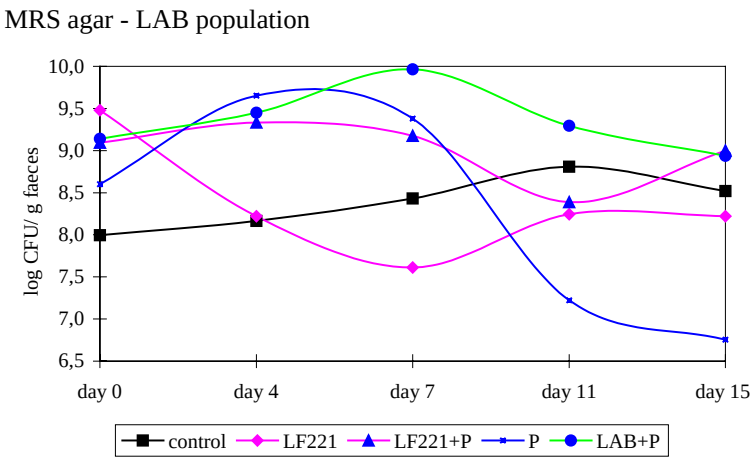
	M B C (µg/ml)					
STRAIN / SOJ	Am	E	K	Cc	N	P
L. acidophilus LF221	156.25	312.5	312.5	250	2500	156.25
L. casei ATCC 393	156.25	625	2500	>500	1250	156.25
L. johnsonii ATCC 11506	312.5	312.5	1250	/	1250	78
L. helveticus ATCC 15009	78.125	312.5	312.5	125	156.25	3.8
L. plantarum BI 42	31.125	2500	5000	16	312.5	<4.35
L. reuteri DSM 20016	312.5	312.5	2500	32	625	<4.35
L. sake ATCC 15521	312.5	312.5	1250	/	1250	156.25
L. salivarius IM 124	250	5000	2500	>500	625	9.76
E. coli ATCC 25922	625	1250	117.2	500	312.5	2500
S. aureus ATCC 25923	19.53	312.5	4.88	/	1.22	4.88

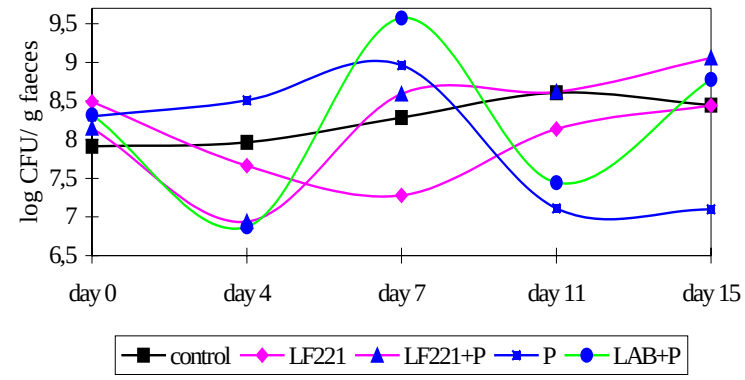
Legend:
Am – ampicillin; E – erythromycin; K – kanamycin; Cc – clyndamycin; N – neomycin; P – penicillin.
/: no data

In vivo testing

Total LAB and lactobacilli count in feeding trial with penicillin G experiment is presented in Figure 1. As observed from the diagram, the LAB or lactobacilli count in control group showed slight increase during the feeding trial, whereas in groups receiving antibiotic the population of LAB or lactobacilli drastically decreased after the slight increase at the beginning. Significant differences in LAB numbers were determined between control group and experimental group receiving penicillin, and between control group and test group receiving the mixture of 4 lactobacilli and penicillin.

Figure 1: Effect of different LAB and penicillin administration on faecal microflora of mice.
Slika 1: Utjecaj različitih BMK (bakterija mliječne kiseline) i penicilina na mikrofloru u fecesu miševa.



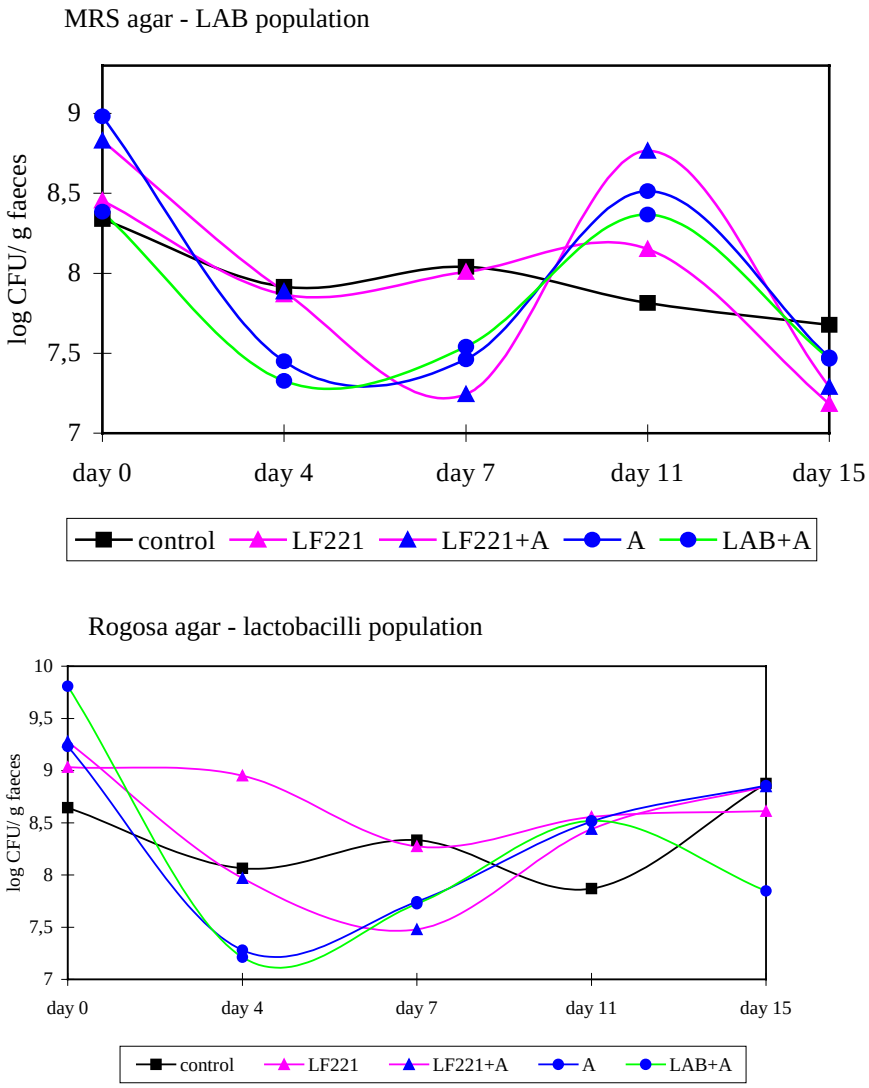


In an ampicillin trial, total counts of LAB and lactobacilli populations in mice faeces were traced as well (Figure 2). When MRS and Rogosa agar plates were examined for oscillation of LAB or lactobacilli in collected samples of faeces, there were no drastic differences observed between control group and the groups taking ampicillin. In all groups examined the LAB or lactobacilli count slightly decreased in the first few days of the experiment,

and later increased. In the groups receiving antibiotic and bacteria the decrease was more evident.

Figure 2: Effect of different LAB and ampicillin administration on faecal microflora of mice.

Slika 2: Utjecaj različitih BMK (bakterija mliječne kiseline) i ampicilina na mikrofloru u fecesu miševa.



Discussion

The rate of antibiotic resistant bacteria has increased lately, where resistance among bacteria common to the human GI tract is of no exception. Drug resistance presents a health concerning issue, especially if resistance is pointed towards clinical antibiotics and when genetic elements carrying resistance genes are mobile and as such possibly transmitted to health harming microbes. As it is well known, LAB naturally shows both antibiotic resistance and susceptibility and since they have a long history of safe use with no indications of transfer resistance to other species, especially species of lactobacilli, leuconostoc and pediococci, they still remain leading microbes in fermentation processes and as human and animal probiotics. Besides all the properties that define a strain as a good probiotic, the antibiotic resistance and the ability of such strain to act as a donor of antibiotic resistance genes must be carefully assayed as well. Thus, combination of antibiotic and probiotic intake can give a promising advantage in treating bacterial disorders with simultaneous recovering of a weakened intestinal microflora (Mattila-Sandholm et al., 1999).

At the beginning of our experiment we have focused to the in vitro sensitivity testing of 8 selected strains of *Lactobacillus* genus to 6 antibiotics, as lactobacilli are among the most wide-spread inhabitants of the intestinal tract. Tests were performed using standardised methods on either solid or liquid media. A comparison of antibiotic susceptibility pattern presented in our work with previous studies relating probiotic LAB shows certain similarities in terms of sensitivity to penicillin, ampicillin and clyndamycin (Bayer et al., 1978; Vescovo et al., 1982; Charteris et al., 1998) and resistance to kanamycin and neomycin (Vescovo et al., 1982; Charteris et al., 1998). When LAB isolated from ecological niches other than gastrointestinal tract, such as plants, meat and fermented dairy foods, were subjected to susceptibility tests, similar sensitivity/resistance patterns were observed (Müller et al., 1993; Ametrano Vidal and Collins-Thompson, 1987; Orberg and Sandine, 1985). Independently of the place of origin, LAB were, in general, sensitive to ampicillin, penicillin and erythromycin, and resistant to kanamycin and neomycin. Therefore, various species of LAB seem not to differ much in sensitivity/resistant pattern indicating that antibiotic susceptibility cannot serve as a criterion for LAB classification. Similar statements were recently reported by Hamilton-Miller and Shah (1998)

where among 42 different lactobacilli strains tested for susceptibility no obvious differences between species were determined with regard to antibiotics used. Only in the case of vancomycin, there was a clear-cut separation along species lines. Namely, all *L. acidophilus* and *L. delbrueckii* strains examined were sensitive to vancomycin, while the other species were resistant. As authors suggested, a vancomycin susceptibility could serve as a useful tool in the lactobacilli identification.

Our task was concluded by *in vivo* examination of combined probiotic/antibiotic treatment in mice. The well-known side effect of antibiotic treatment is weakening of the natural intestinal microflora, which might result in disease such as diarrhoea and other digestive tract damage caused by opportunistic microorganisms. In order to avoid the risk of the post-antibiotic infections, the original intestinal population must be restored as efficiently as possible. The penicillin therapy (Figure 1) revealed that after decrease at the beginning, the total count of LAB and lactobacilli resulted in remarkable increase in mice taking antibiotic in combination with either *Lactobacillus acidophilus* LF221 or lactobacilli mixture. Moreover, *L. acidophilus* LF221 strain itself had no greater effect on bacterial population in faeces, while only penicillin intake resulted in drastic drop of the LAB and lactobacilli population. De Petrino *et al*. (1997) came with similar conclusions when tested the behaviour of the intestinal microflora in ampicillin treated mice with the effect of orally administered *L. casei*, *L. acidophilus*, *L. delbrueckii* and *Str. salivarius*. In their studies, application of *Lactobacillus* strains together with ampicillin improved intestinal microflora. Some scientists even suggested (Nousiainen and Setälä, 1998) that combination of probiotic lactic acid bacteria and antibiotic treatments might have certain advantages. Namely, natural flora resists the invasion of both harmful and probiotic bacteria. And if the natural flora is weakened by antibiotic, probiotic bacteria may more easily colonise the guts of target animals. Furthermore, by combined treatment the level of required antibiotics could be reduced.

On the contrary, ampicillin administration did not drastically influence bacterial populations. Although the drop of LAB or lactobacilli populations were more expressed in experimental groups with regard to control group (Figure 2), oscillations of LAB and lactobacilli populations in either control or experimental groups were similar and therefore no statistically significant differences between groups were noticed. This might be due to extremielly low ampicillin doses which could not provoke any drastic changes in bacterial

counts. In our studies, antibiotic concentrations used were identical to ones used in the therapy of children but not adults (De Petrino *et al.*, 1997).

Nevertheless, the present study gives promising results concerning the potential use of probiotic lactic acid bacteria with intention to improve the intestinal microbial environment when disrupted by the antibiotic treatment. Prior to use in human application probiotic lactic acid bacteria must be carefully selected emphasiting their beneficial effects on the host without causing any side or even harmful effects.

Conclusions

In vitro test

From the experiments performed we have concluded that selected strains of lactobacilli were sensitive to penicillin G, ampicillin and erythromycin, moderately sensitive to clyndamycin and resistant to kanamycin and neomycin. Moreover, *L. salivarius* IM 124 was an extra resistant to erythromycin and clyndamycin suggesting this strain as possible recovering agent in helping restoring the damaged intestinal microflora after antibiotic treatment. On the other hand, human isolate of *L. acidophilus* LF221 did not deviate in antibiotic susceptibility compared to other lactobacilli. Finally, equally important, results of our experiment are comparable with results from the literature.

In vivo test

In mouse feeding trial the antibiotic consumption differed in the impact on microbial population in mice faeces. Penicillin G significantly reduced the number of LAB in mice faeces which cannot be affirmed in the case of ampicillin dosage. The intake of the mixture of lactobacilli or *L. acidophilus* LF221 alone, had no statistically significant influence on the size of the intestinal microflora as well.

UTJECAJ ANTIBIOTIKA NA BAKTERIJE MLIJEČNE KISELINE PROBAVNOG SUSTAVA

Sažetak

Bakterije mliječne kiseline (BMK) su uobičajeni sudionici crijevne mikroflore ljudi i životinja. One imaju važnu ulogu u održavanju ravnoteže gastrointestinalne (GI) flore, na koju mogu utjecati različiti faktori poput prehrane (hrane) antimikrobnih supstancija i stresa. Pomoću makrodilucijske i mikrodilucijske metode u tekućoj podlozi te metode difuzije na agar pločama, određene su minimalne inhibicijske koncentracije (MIC) i minimalne baktericidne koncentracije (MBC) šest (6) antibiotika koji se svakodnevno upotrebljavaju u humanoj medicini za 8 sojeva BMK. Učinak penicilina G i ampicilina na BMK probavnog sustava testiran je također in vivo na laboratorijskim miševima. Laktobacili su se pokazali osjetljivim na penicilin G, na ampicilin (derivati penicilina) i na eritromicin (makrozidni antibiotik) prilikom testiranja in vitro. Klindamicin (piranozidni antibiotik) je pokazao umjereni inhibicijski učinak. Svi sojevi BMK pokazali su se otpornim na kanamicin i neomicin (aminoglikozidni antibiotici). Od svih testiranih BMK posebno se otpornim na eritromicin i klindamicin pokazao L. salivarius IM 124. Učinak oralno primjenjenog ampicilina, na broj BMK u stolici miševa nije statistički dokazan. Utjecaj penicilina G na broj BMK kod miševa pokazao se signifikantnim, ali to nije bio slučaj s oralno primijenjenim BMK.

Ključne riječi: antibiotici, inhibicijski učinak, bakterije mliječne kiseline, probavni sustav

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