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Research Article

Probiotic Treatment does not Change Sulphasalazine and its Metabolites Excretion in Patients with Inflammatory Bowel Disease

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ABSTRACT

Background: The manipulation of gut microflora composition and activity by probiotics could modify the enzymatic activity of intestinal bacteria. In this study, we sought to investigate the influence of probiotic treatment on sulphasalazine (SSZ) excretion in inflammatory bowel disease (IBD) patients.

Methods: Newly diagnosed IBD patients were randomised in two groups; half of subjects were treated with SSZ and other half were treated with combination of SSZ and probiotics. At the each visit, patients were assesed clinically andfecal samples and total volume of 24 h urine was measured and noted. Urine samples were collected and analized by liquid chromatography-mass spectrometry/mass spectrometryfor determination of SSZ and its metabolites. The enzymatic activity of azoreductase by intestinal bacteria in the fecal contents was determined spectrophotometrically.

Results: Urinary levels of SSZ and its metabolites showed no statistically significant changes after probiotic administration. Azoreductasa activities, in both experimental groups, decreased comparing with pretreatment values in both cultivation conditions. Transient colonization with *Bifidobacterium* BB12 was confirmed in 22% of samples. *Lactobacillus rhamnosus* LGG did not show transient colonisation of the digestive tract.

Conclusions: Co-administration of probiotics in patients treated with SSZ did not change the amounts of execreted SSZ and its metabolites.

Keywords: Probiotic; Gut microflora; Drug metabolism; Inflammatory bowel disease

INTRODUCTION

Sulphasalazine (SSZ) is an old drug which have been used in the treatment of inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) [1]. The molecule of sulfasalazine contains 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP) linked together by an azo bond. Intestinal microflora plays a crucial role in the metabolism and pharmacokinetics of sulfasalazine. Only a small fraction of orally administered sulphasalazine is absorbed. The majority of the ingested dose turns through the small intestine to the colon where it undergoes bacterial azo reduction yielding two major metabolites SP and 5-ASA. SP appears to be the active moiety in rheumatoid arthritis due to its antibacterial and immunomodulating effects, whereas 5-ASA is the active agent in IBD [2].

Because the intestinal microflora plays an important role in physiological, nutritional, metabolic and immunological processes in the human body, there is currently some interest in the manipulation of its composition and activity by probiotics. Probiotics can be used to modify the enzymatic activity of intestinal bacteria. According to the definition formulated by the World Health Organization (WHO) probiotics are ' live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' [3]. Bacteria of the genus *Lactobacillus* and *Bifidobacterium* are commonly used probiotics in humans. Due to the link between intestinal microflora and IBD, there are currently numerous studies with probiotics [4]. They are trying to introduce probiotics as new drugs that shouldreplaceor at least enhance the effects of

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standard drugs used in the treatment of IBD [5]. Nevertheless, the exact mechanism of action of probiotics is not fully understood, and there is still a lot of controversisin the literature.

In this study, we sought to explore the influence of probiotic treatment on sulfasalazine elimination in IBD patients.

MATERIALS AND METHODS

Patients

Twenty nine patients (15 female, 14 male) newly diagnosed with mild to moder at IBD were recruited into the study. All patients fulfilled diagnostic criteria for IBD (Baron score 1-2) and no one had been on SSZ or probiotics treatment, previously. In addition, all participants had a normal haematological and renal and liver function tests. Study exclusion criteria included age below 18 years, Clostridium difficile colitis, a positive results of stools on pathological bacteria, fungi, protozoa or parasites, the existence of malignancy, metabolic disease, heart failure, renal or hepatic insufficiency, significant psychiatric disease, known allergy to sulphasalazine or probiotics, pregnancy or lactation, earlier use of systemic corticosteroids more than 10 days in the previous 2 months. Patients were randomised in two groups; sulphasalazine (SSZ only) and sulphasalazine and probiotics (combination of SSZ and probiotics).

Study design and treatment

Study was performed as a monocentric, randomized, open, controlled phase IV study with parallel groups for 8 weeks. Sulphasalazine (Krka-pharmaceutical company, Slovenia) was provided to all patients as a 500 mg tablets. Fixed combination of probiotics with the tradename Normia (Jadran Galenski Laboratorij-pharmaceutical company, Croatia) was provided to patients ascapsulas, containing viable *Lactobacillus rhamnosus* GG (3 x 108 to 3 × 10^{10} CFU) and *Bifidobacterium* BB 12 (3 × 10^{8} to 3 × 10^{10}CFU) each. All patients were dosed with 1 g of SSZ three times a day for eight weeks. Controls are conducted every 2 weeks (total 5 controls). Participants in Sulfasalazine group were treated only with sulfasalazine, while participants in Sulfasalazine and probiotic group were treated with sulfasalazine for 8 weeks and probiotics during 4 weeks. In this group, therapy with probiotics is started 2 weeks after initiation with sulfasalazine therapy (period after 1. control), and lasted two weeks. After that, probiotic therapy is interrupted during the following 2 weeks, then re-included (period after 3. control) and lasted until the end of the study. Patients were asked not to consume any youghurt or additional probiotic or prebiotic preparation throughout the study period. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University Clinical Centre of the Republic of Srpska, number of approval 01- 5-105.2/11.

Clinical follow-up and sample collection

Patients were assesed clinically at baseline and every two weeks until completion of the study (W 0, 2, 4, 6, 8.) wherein the samples of stool and 24 h urine were collected. The enzymatic activity of azoreductase by intestinal bacteria in the fecal contents was determined spectrophotometrically. Total volume of 24 h urine (collected from 08: 00 a.m. to 08: 00 a.m. at next day prior to clinical visits) was measured and noted and samples of 10 mL of urine were stored at -20°C for the further analysis.

Identification of probiotic bacteria in the fecal content was carried out by PCR method and microchips electrophoresis using the mehod of **Walter J. et al (2000)**[6].

Assay of SSZ and metabolites in urine

After thawing at room temperature 100 µL of urine samples were pipetted to a 1.5 mL Eppendorf polypropylene tube and 0.3mL of methanol with I.S. working solution (dimenhydrinate, 50 ng/mL) was added. The contents were vortex mixed for 1min. After centrifugation at 15,000g for 5min, a 100 µL aliquot of clear supernatant was mixed with 100 µL of water in polypropylene tubes and transferred to the autosampler. A volume of 10 µL was injected into the LC-MS/MS system using the method of Guang-Zhi Gu et al (2011) for SSZ and metabolites [7].

The results were used for the calculation of the quantity of SSZ and metabolites 24h urinary excretion.

Assay of azoreductase

A suspension of fecal and 0.1M potassium phosphate buffer pH 7.2 was prepared in a ratio of 1: 10. The suspension was stirred at a vortex (30s / cycl in min about 50% max). The reaction was started by adding 0.9 ml of a fecal suspension in 30 µl of amaranth solution (77.5 mM). Samples were mixed in vortex and incubated on 37°C, in a water bath, aerobic and anaerobic. Samples in an amount of 0.1 ml were collected at 10, 20 and 30 minutes and the reaction was interrupted by the addition of 0.9 ml of sodium azide (1 mM). The samples were then stirred at a vortex, centrifuged 15000 rpm for 3 min and a UV absorption of the supernatant at 520 nm was determined.

The calibration standards were prepared by making a series diluting amorphous solution dilution to a concentration of 1.21, 2.42, 4.84, 9.68, 19.38, 38.75 and 77.5 µM. After the correction for dilution, the enzyme activity is expressed in ammonia micromoles disposed per gram of fecal suspension per hour. Spectrophotometric determination of azo reductase enzymatic activity was performed using the method described in Goldin BR (1990) [8].

Molecular detection and identification of probiotics bacteria

The detection and partial molecular characterization of the tested fecal samples was performed using the Polymerase Chain Reaction (PCR) method.

DNA samples were extracted from the tested samples by using Norgen Stool DNA Isolation Kit.

For the detection of bacteria from isolated DNA, a PCR method with one pair of primers for the Bifidobacter lactis Lm3 and

Lm26 type was applied [9] as well as a universal set of detectors for bacteria in HDA1-GC-HDA2 faecal samples, [7] as well as a set of specific primers Prl-RhaII for the detection of the species *Lactobacillus rhamnosus* [10]. Visualization of the obtained products was performed by electrophoretic separation using electrophoresis on microchips.

Statistical methods

To compare the mean values the Student's t test for paired samples (if observed characteristics have normal distribution) or nonparametric Wilcoxon's W test (if observed characteristics do not have a normal distribution) were used. Differences were considered significant at P<0.05. Statistical analysis was performed using IBM SPSS Statistics 21.0 and MS Office Excel 2010.

RESULTS

There are not statistical significant changes (p<0.05) between amounts of sulphasalazine excreted in urine in controls, neither sulphasalazine nor sulphasalazine and probiotic groups (Figure1).

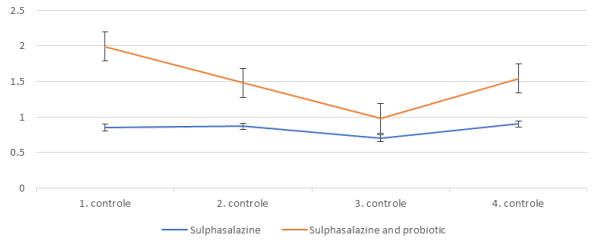

Figure 1: Amounts of sulphasalazine (mg) excreted in 24 h urine on different controls in both groups.

Comparing the amounts of sulphasalazine between the experimental groups did not show statistical significant changes (p<0.05) (Table 1).

Table 1: Amounts of sulphasalazine (mg) excreted in 24 h urine on different controls between groups.

Controls	Sulphasalazine group		Sulphasalazine and probiotic group		p
	n	median	n	median	
1.	14	0.85 (0.63, 1.10)	13	1.99 (0.48, 2.68)	0.244
2.	14	0.87 (0.36, 1.29)	15	1.48 (0.71, 1.85)	0.190
3.	12	0.70 (0.46, 1.04)	15	0.98 (0.74, 3.05)	0.118
4.	13	0.90 (0.54, 1.55)	13	1.54 (0.93, 2.62)	0.061

There are not statistical significant changes (p<0.05) between amounts of mesalazine excreted in urine in controls, neither sulphasalazine nor sulphasalazine and probiotic groups (Figure 2).

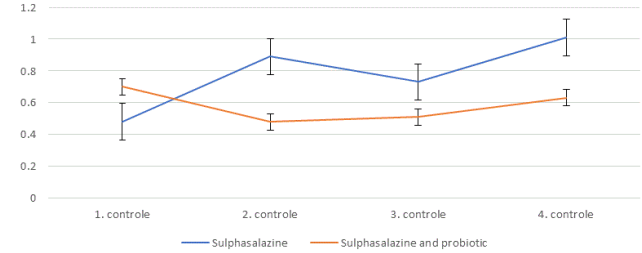

Figure 2: Amounts of mesalazine (mg) excreted in 24 h urine on different controls in both experimental groups.

Comparing the amounts of mesalazine between the experimental groups did not show statistical significant changes (p<0.05) (Table 2).

Table 2: Amounts of mesalazine (mg) excreted in 24 h urine on different controls between groups.

Controls	Sulphasalazine group		Sulphasalazine and probiotic group		p
	n	median	n	median	
1.	14	0.48 (0.36, 0.58)	13	0.70 (0.35, 0.77)	0.427
2.	14	0.89 (0.48, 1.40)	15	0.48 (0.22, 0.66)	0.089
3.	12	0.73 (0.52, 1.56)	15	0.51 (0.39, 1.03)	0.239
4.	13	1.01 (0.42, 1.19)	13	0.63 (0.43, 1.03)	0.713

There are not statistical significant changes (p<0.05) on amounts of sulphapiridine (SP) excreted in urine on different controls, neither sulphasalazine nor sulphasalazine and probiotic groups (Figure 3).

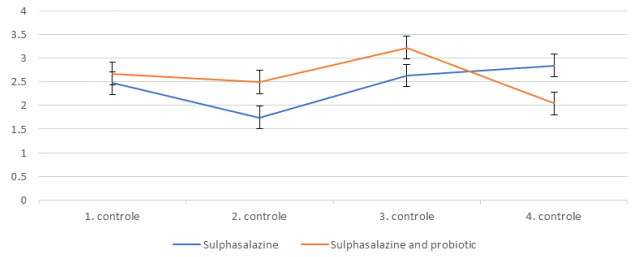

Figure 3: Amounts of sulphapiridine (mg) excreted in 24 h urine on different controls in both groups.

Comparing the amounts of sulphapiridine between the experimental groups did not show statistical significant changes ($p<0.05$) (Table 3).

Table 3: Amounts of sulphapiridine (mg) excreted in 24 h urine on different controls between groups.

Controls	Sulphasalazine group		Sulphasalazine and probiotic group		p
	n	median	n	median	
1.	14	2.47 (1.46, 4.57)	13	2.67 (1.10, 10.23)	0.679
2.	14	1.74 (1.13, 3.81)	15	2.49 (0.77, 7.12)	0.760
3.	12	2.63 (1.03, 4.64)	15	3.22 (1.80, 6.92)	0.581
4.	13	2.84 (1.13, 5.07)	13	2.04 (0.81, 9.08)	0.908

Azoreductasa activities, in both experimental groups, were decreased comparing with pretreatment values in both cultivation conditions (Figure 4-Figure 7).

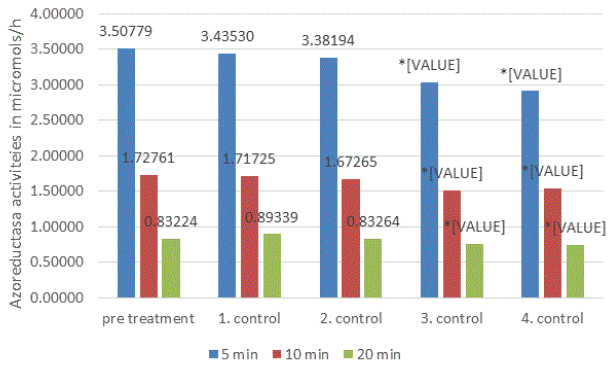

Figure 4: Values of azoreductase activities in Sulphasalazine group under aerobic conditions on different controls.

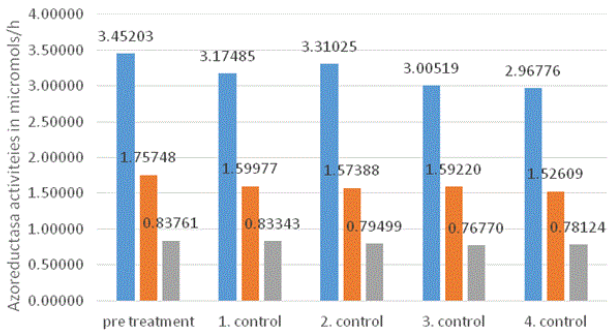

Figure 5: Values of azoreductase activities in Sulphasalazine group under anaerobic conditions on different controls.

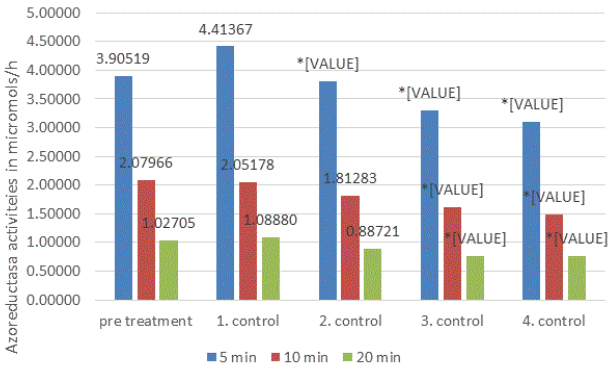

Figure 6: Values of azoreductase activities in Sulphasalazine and probiotic group under aerobic conditions on different controls.

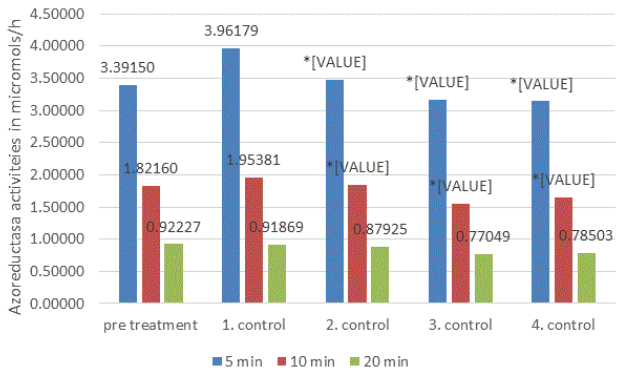

Figure 7: Values of azoreductase activities in Sulphasalazine and probiotic group under anaerobic conditions on different controls.

There were not statistical significant changes ($p<0.05$) in azoreductase activities between experimental groups neither aerobic or anaerobic condition (Tables 4 and 5).

Table 4: Azoreductasa activity (micromol/h) in aerobic condition mesaured in fecal samples on different controls between groups.

Azoreductasa (micromol/h)	After	Sulphasalazine		Sulphasalazine and probiotic group		p
		n	median	n	median	
Pretreatment	5 min	14	4.13224 (3.45393, 4.81056)	15	3.90519 (3.34780, 4.46257)	0.614
	10 min	14	2.05599 (1.71662, 2.39537)	15	2.16536 (1.87010, 2.46063)	0.636
	20 min	14	0.83224 (0.77686, 1.41349)	15	1.02705 (0.78523, 1.27206)	0.861
1st control	5 min	14	3.43530 (2.76704, 5.59940)	15	4.40305 (3.55717, 5.48151)	0.150
	10 min	14	2.02982 (1.67876, 2.38089)	15	2.07733 (1.78149, 2.37316)	0.840
	20 min	14	0.89339 (0.75296, 1.44456)	15	1.08880 (0.84100, 1.39237)	0.337
2nd control	5 min	14	3.38194 (3.01874, 4.27083)	15	3.38672 (3.19556, 4.49704)	0.793
	10 min	14	1.83582 (1.56767, 2.10397)	15	2.04929 (1.75347, 2.34511)	0.306
	20 min	14	0.83264 (0.72507, 1.10115)	15	0.88721 (0.78961, 1.17684)	0.359
3rd control	5 min	14	2.95228 (2.76547, 3.13909)	15	3.29337 (2.85815, 3.72859)	0.174
	10 min	14	1.50658 (1.30945, 1.70053)	15	1.60813 (1.56353, 1.74274)	0.138
	20 min	14	0.76092 (0.65774, 0.82307)	15	0.76889 (0.72706, 0.85694)	0.419
4th control	5 min	13	2.93602 (2.74665, 3.12539)	14	3.10191 (2.94173, 3.26210)	0.200
	10 min	13	1.52162 (1.43656, 1.60669)	14	1.51358 (1.42157, 1.60558)	0.901

	20 min	13	0.78290 (0.71705, 0.84874)	14	0.77342 (0.73283, 0.81400)	0.809

Table 5: Azoreductasa activity (micromol/h) in anaerobic condition mesaured in fecal samples on different controls between groups.

Azoreductasa (micromol/h)	After	Sulphasalazine		Sulphasalazine and probiotic group		p
		n	median	n	median	
Pretreatment	5 min	14	4.04099 (3.31102, 4.77095)	15	4.04888 (3.36343, 4.73432)	0.988
	10 min	14	2.02180 (1.65232, 2.39128)	15	2.04621 (1.70758, 2.38483)	0.925
	20 min	14	0.83761 (0.69161, 1.40751)	15	0.92227 (0.74499, 1.26130)	0.527
1st control	5 min	14	3.17485 (2.95024, 5.03866)	15	3.96179 (3.29114, 5.39231)	0.138
	10 min	14	1.59977 (1.50698, 2.58464)	15	1.95381 (1.71964, 2.68659)	0.162
	20 min	14	0.83343 (0.74698, 1.43420)	15	0.91869 (0.72109, 1.19915)	0.827
2nd control	5 min	14	3.56206 (2.99292, 4.13120)	15	3.83467 (3.20986, 4.45948)	0.534
	10 min	14	1.57388 (1.46317, 2.31702)	15	1.83753 (1.54123, 2.54561)	0.295
	20 min	14	0.79499 (0.71113, 1.16569)	15	0.87925 (0.76730, 1.27724)	0.326
3rd control	5 min	14	3.00519 (2.85306, 3.37397)	15	3.16370 (2.96776, 3.60177)	0.266
	10 min	14	1.59220 (1.44326, 1.66787)	15	1.55716 (1.42892, 1.71009)	0.965
	20 min	14	0.76770 (0.69639, 0.82507)	15	0.77049 (0.70236, 0.84538)	0.513
4th control	5 min	13	3.01506 (2.83160, 3.19852)	14	3.14435 (2.90932, 3.37939)	0.408
	10 min	13	1.57296 (1.47140, 1.67453)	14	1.61018 (1.49639, 1.72398)	0.639
	20 min	13	0.76316 (0.72428, 0.80204)	14	0.78836 (0.75131, 0.82541)	0.366

Detection of probiotics in the faecal samples by PCR electrophoresis on the microchip

Fecal samples from patients in the group Sulfasalazine and probiotic (n=15) were analyzed by microchip method for the detection of probiotic bacteria. The PCR product analysis was performed after the electrophoretic separation of the obtained products using electrophoresis on the MultiNA Shimadzu Biotech microchips (Shimadzu, Japan). DNA bands that correspond with those of the probiotics used in the study were detected and identified by comparing the migration distance obtained from the reference strains of individual probiotics (*Lactobacillus rhamnosus* LGG and *Bifidobacterium lactis* BB-12).

The presence of *Bifidobacterium lactis* BB-12 was confirmed in 22% of the analyzed samples. There is a noticeable increase in the number of positive samples in the controls that followed after the two-week period of probiotics application (Figures 8 and 9).

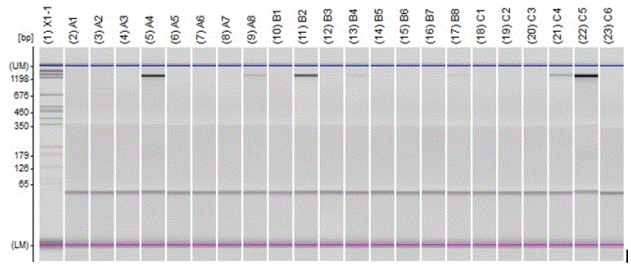

Figure 8: Gene multiplication profile obtained by electrophoresis on the microchip specific for Bifidobacterium lactis BB-12 obtained in PCR reactions with the primer pair Lm3 and Lm26. Line 1: Base pair height; lines 2-21: examined samples; line 22: positive control; line 23: negative control.

The presence of *Lactobacillus rhamnosus* LGG was not confirmed in any of the tested samples.

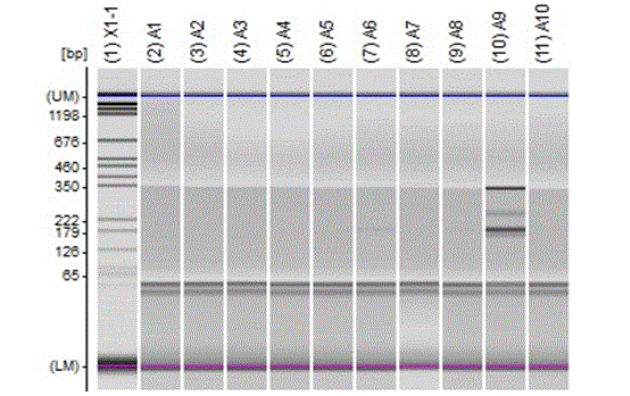

Figure 9: Gene multiplication profile obtained by electrophoresis on a microchip specific to Lactobacillus rhamnosus LGG obtained in PCR reactions with the Prl-RhaII primer pair. Line 1: Base pair height; lines 2-9: examined samples; line 10: positive control; line 11: negative control.

DISCUSSION

Gut flora plays a crucial role in the metabolism and pharmacokinetics of SSZ and in the efficacy of the drug in IBD. The main aim of our clinical trial was to investigate the effects of probiotic treatment on SSZ and its metabolites excretion. All patients included in the study were taking SSZ doses 3g per day comparable to doses taken in other trials of its use in IBD and rheumatoid arthritis [11-13]. After oral administration, probiotics exhibit a specific enzymatic activity that exerts its effects on the intestine, which may affect the increase or decrease of metabolic activity of the gut flora. In most previous trials with probiotic, daily doses were comparable to those (3×10^8-10^{10}CFU) used in the trial [14-17].

Previous study on animals showed potential beneficial effects of probiotics on SSZ metabolism after 3 days of probiotic treatment [2]. Mikov et al. proved that administration of probiotics significantly enhance bacterial mediated reduction of sufasalazine to SP and 5-ASA in colon content. Therefore, probiotics (*Lactobacillus acidophilus, Bifidobacterium lactis*, and *Lactobacillus rhamnosus*) can be used to modify the enzymatic activity of intestinal bacteria by increasing azoreductase activity, thereby producing more extensive metabolism of sulfasalazine. The other study on animals with different probiotics showed increases azoreductase activity in the gut microbiota but did not confirm the effect on plasma levels of SSZ and SP following a subsequent oral dose of SSZ [14]. Lee et al. confirmed a significant increase in azoreductase activity in *ex-vivo* colon contents with a corresponding increase in sulfasalazine metabolism, after treatment of rats with oral doses of a mixture of the three probiotics. Clinical study on patients with rheumatoid arthritis did not demonstrate a significant effect of short term co-administration of probiotic on the metabolism of SSZ [11]. In our investigation we did not demonstrate a significant effect of co-administration with probiotics comparing with previous results of other autors [2,7,10]. Urinary excretion of SSZ and its metabolites showed no statistically significant changes after probiotic administration. It is interesting that quantities of sulphasalazine and sulphapiridine elimination via urine were higher in the group treated with SSZ and probiotic compared with SSZ only treated group. This finding indicates that probiotics did not provide an increase in the amount of enzyme needed for the metabolism of sulphasalazine or did not induce the increase of azoreductase activity that comes from already existing intestinal commensal in gut flora of the subjects. Human studies have shown that the impact of probiotics on the activity of bacterial enzymes is strain specific [18,19]. The first reason for that kind of finding could be the fact that probiotic showed different effects on enzymatic activity, probably due to application of different lactobacilli or *bifidobacteria* strains as probiotic, as well as differences in amount or the duration of the probiotic intake [20]. The other reason for this finding could be the fact that we confirmed only the presence of *Bifidobacterium* BB12 DNA in the examined stool samples, while DNA of *Lactobacillus rhamnosus* LGG was not confirmed. Identification of probiotic DNA was performed by using species specific PCR primers and electrophoresis on microchip [7].

CONCLUSION

Co-administration of probiotics in patients treated with SSZ did not change the amount of execreted SSZ and its metabolites. Applied probiotics did not provide an increase in the amount of enzyme needed for the metabolism of sulphasalazine or did not induce the increase of azoreductase activity that comes from already existing intestinal commensal in gut flora of the subjects. Also we could not confirmed the presence of DNA of *Lactobacillus rhamnosus* LGG, which implies potential problems in the survival of the bacterium in this way of administration. New pharmaceutical formulations are necessary for the delivery of live probiotic bacteria to the inflamed bowel. The clinical studies on probiotic bacteria influence on IBD and the faith of the drugs used in IBD is necessary before broad application of probiotic formulations.

AUTHOR CONTRIBUTIONS

Investigation, N.S and S.T; methodology, M.M, R.S and S.SS; formal analysis, S.V; writing-original draft preparation N.S.;

writing-review and editing, M.M, R.S and S.SS; supervision, M.M and S.T.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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