

Influence of the *Escherichia coli* Nissle 1917 strain on complications of chronic experimental liver damage

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ABSTRACT: The aim of the study was evaluate the influence of the probiotic *Escherichia coli* Nissle 1917 strain (Mutaflor® suspension, Ardeypharm GmbH, Herdecke, Germany) on bacterial translocation in cases of liver damage, damage to the intestinal mucosa, potential portal hypertension associated with possible development of oesophageal varices and on the bacterial population of the intestine during chronic experimental liver damage in the laboratory rat. Rats with liver damage induced by thioacetamide were divided into an experimental and control group. Experimental and control animals were applied Mutaflor and saline, respectively. Samples of blood, liver, lymph nodes and caecum for microbiological examination, of liver, duodenum and oesophagus for histological examination and of spleen for weight evaluation were collected. There were no significant differences between both groups of animals in the qualitative proportion of *Staphylococcus* spp., *Enterococcus* spp. and *Proteus* spp. cultured from the lymph nodes, blood and liver. The quantitative culture results on *Enterococcus* spp. in the caecum, liver and lymph nodes showed no significant differences between both groups. There was a significant difference between the experimental and control group in the counts of coliform bacteria. No significant differences between both groups were found in the overall damage score of the liver, duodenum and oesophagus. There were no differences in the spleen to body weight ratio of both groups. The application of Mutaflor® suspension for eight days had no recognisable effect diminishing the selected complications of chronic liver damage caused by the administration of TAA to laboratory rats.

Keywords: bacterial translocation; liver cirrhosis; probiotics; spontaneous bacterial peritonitis; thioacetamide

Bacterial infections are a frequent and serious complication of liver cirrhosis. The spontaneous bacterial peritonitis (SBP) is the most frequent one (Cereto et al., 2003). In view of the present knowledge, the features of pathogenesis include bacterial translocation (BT), intestinal bacterial overgrowth (IBO), damage to the intestinal wall permeability and immunosuppression (Fernandez et al., 2000). Antibiotic therapy is the primary choice at present. There is, however, a danger

of bacterial resistance (Dupeyron et al., 1994; Fernandez et al., 2002). Therefore alternatives to the antibiotic therapy have been evaluated. Probiotic products seem to be promising in this respect. Probiotics are preparations including microbial cells or their components of certain health promoting qualities (Salminen et al., 1999). There are some definitions considering probiotics to include live microorganisms only (Ouwehand et al., 1999). The mechanism of the effect of probiotics

is still uncertain. It is only possible to mention several essential ways in which the positive effects become reality such as chemical inhibition and stimulation (e.g. through the production of bacteriocins and acids), competition of nutritional substances, modification of the immune response and competition of adhesion receptors (Bomba et al., 2002; Fuller and Gibson, 2004). Currently used probiotic products include bacteria (most often lactobacilli and bifidobacteria, less often the *Escherichia coli* Nissle 1917 strain, *Streptococcus salivarius*, *Bacillus laterosporus* or *Bacillus subtilis*) or yeasts (*Saccharomyces cerevisiae* CBS 5926 strain) (Kaur et al., 2002).

Lata et al. (2006) published a study concerning the effects of *Escherichia coli* Nissle 1917 strain on intestinal colonisation, endotoxin levels, hepatic encephalopathy and liver function in patients with liver cirrhosis. Their treated group displayed a significant improvement of intestinal colonisation and a trend towards a significant reduction in endotoxin levels and improvement of liver function assessed according to the Child-Pugh classification. Increasing evidence indicates that probiotics and synbiotics can play a role in the treatment of hepatic encephalopathy in humans (Boca et al., 2004; Liu et al., 2004).

The objective of this paper was to test the ability of the *Escherichia coli* Nissle 1917 strain (Mutaflor® suspension, Ardeypharm GmbH, Herdecke, Germany) to influence bacterial translocation in cases of liver damage, damage to the intestinal mucosa, potential portal hypertension associated with possible development of oesophageal varices and the bacterial population of the intestine.

MATERIAL AND METHODS

Experimental animals

A total of 14 outbred SPF laboratory rats of the Wistar strain (males with the body weight ranging from 226 to 262 g) were used. Animals were kept individually in cages made of transparent polypropylene covered by wire netting. Air temperature was kept at 22°C and humidity at 60%. The light regimen included 12 h of light and 12 h of dark. Laboratory rats were fed *ad libitum* a complete feeding mixture Biostan Mypo (Biosta spol. s.r.o., Blucina, Czech Republic). A period of one-week adaptation preceded the experiment.

Substances used

For the experiment we used a magistra liter prepared 0.03% solution of thioacetamide (TAA), physiological saline solution (Infusia, a. s., Horátev, Czech Republic), the probiotic preparation – *Escherichia coli* Nissle 1917 strain, 10⁸ bacteria/ml (Mutaflor® suspension, Ardeypharm GmbH, Herdecke, Germany) and ether (Eter solvens, RNDr. Jan Kulich, Hradec Kralove, Czech Republic).

Methods

Collection of biological samples. Blood samples for microbiological cultures were collected by means of cardiac puncture under general anaesthesia using ether. Following euthanasia samples for the microbiological culture were obtained: a specimen of liver tissue, ileocaecal lymph nodes and a double sutured caecum. After that, we collected samples for histology: liver, duodenum and oesophagus. Finally, the whole spleen for weight measurement was collected.

Microbiological examination. Samples intended for the microbiological culture (blood, liver, ileocaecal lymph nodes and caecal contents) were weighed, homogenized and diluted using the phosphate buffer solution (PBS). The samples were then plated on Columbia blood, Slanecz-Bartley and McConkey agar media. Bacterial colonies were counted and confirmed after 24 to 48 h of incubation at 37°C. Groups of bacteria such as *Escherichia coli*, *Proteus* spp., *Staphylococcus* spp. and *Enterococcus* spp. were evaluated. Colony forming units (CFU) were finally re-counted for the initial volume.

Bacterial translocation was defined as a positive culture result from lymph nodes, liver and/or blood (Berg, 1992).

Histological examination. A 10% formaldehyde solution was used as a fixative for the samples. Cubes of tissue were processed in a standard way to achieve dehydration and then embedded in paraffin. Paraffin mounted tissue slides were stained using haematoxylin and eosin.

Evaluation of histopathological changes in liver. We selected four variables to score histopathological findings in the liver tissue:

- (1) fibrosis
- (2) regressive changes
- (3) nodularity
- (4) regeneration

Each variable was classified by three grades where 0 – no changes, 1 – moderate changes and 2 – fully developed changes.

Evaluation of histopathological changes in duodenum. The following variables were selected for histopathological evaluation of changes in the duodenum:

- (1) hyperaemia of vessels
- (2) oedema within *lamina propria* and submucosis
- (3) inflammatory infiltrates of the intestinal wall
- (4) signs of reactive fibroproduction

Each of the above-listed variables could be classified as 0 – without any changes, 1 – moderate changes and 2 – marked changes.

Evaluation of histopathological changes in oesophagus. Samples of the oesophagus were graded regarding the vascular bed as 0 – without any pathological findings, 1 – hyperaemia with dilatation and 2 – changes recognisable within the vascular wall.

Evaluation of portal pressure. The portal pressure was measured indirectly by means of relating the splenic weight (g) with the body weight of the experimental animal (g).

Statistical data processing

Data analysis was performed using Microsoft® Excel 2000 and Statistica, version 6, StatSoft, Inc. (2003). Results are presented as the mean \pm standard deviation (SD). Mean values were compared using Student's *t*-test. Results were subjected to standard procedures of correlation analysis. The null hypothesis was rejected on the level of significance $P < 0.05$.

Experimental protocol

Following a week of adaptation, body weights of laboratory rats were determined and recorded. A modification of the model by Li et al. (2002) was used to damage the liver of experimental animals. The animals were provided with TAA as the only available source of drinking water for 15 weeks. Body weight was measured every day in individual rats so as to modify the concentration of TAA. The concentration of the stock solution of TAA was that of 300 mg/l. When the body weight grew by 25 g and more, the concentration of TAA was increased

to 450 mg/l. In cases when the body weight dropped by 25 g and more, the concentration of TAA was lowered to 150 mg/l. The respective changes in the solution concentration were performed once a week. Fresh solutions were prepared three times a week.

The application of TAA was finished after 15 weeks and, from that time on, drinking water was provided only. The animals were divided into an experimental and control group, each consisting of seven individuals. For eight days the animals from the experimental group received intragastrically a suspension of Mutaflor in the dose of 1 ml pro toto through a peroral probe inserted under moderate ether anaesthesia. Control animals were given 1 ml of physiological saline solution pro toto in the same way.

Samples were collected and processed properly (see the above-mentioned procedures) after eight days. Spleen weight was determined and recorded.

A special committee of the Faculty of Veterinary Medicine of Veterinary and Pharmaceutical University, Brno, approved the experiment in terms of agreement with Act No. 246/1992 on the Protection of Animals against Cruelty.

RESULTS

Microbiological examination

Tables 1 and 2 present the results of microbiological examinations.

The cultures of lymph nodes evaluated qualitatively revealed *Staphylococcus* spp. in six (85.71%) and five (71.43%) individuals of the experimental and control group, respectively. There were no findings of *Enterococcus* spp. in lymph nodes of any experimental animal. *Enterococcus* spp. was found in lymph nodes of two control animals (28.57%). *Proteus* spp. was cultured from lymph nodes of only one control animal (14.29%).

The cultures from the liver yielded only *Staphylococcus* spp. in four experimental (57.14%) and four control (57.14%) animals. Blood cultures were negative in all experimental laboratory rats. In the control group, blood cultures revealed *Enterococcus* spp. in one animal (14.29%) and *Staphylococcus* spp. in another one (14.29%). Considering the qualitative representation of *Staphylococcus* spp., *Enterococcus* spp. and *Proteus* spp. in cultures from

Table 1. The results of microbiological examination of caecal contents

Group	Caecal contents – G^- rods (coliform bacteria) (CFU/g)	Caecal contents – <i>Enterococcus</i> spp. (CFU/g)
Experimental	4.14 ± 3.39	58.43 ± 59.53
Control	1.00 ± 1.15	120.29 ± 197.00

Table 2. The results of microbiological examination of lymph nodes, liver and blood

Group	Lymph nodes (CFU/150 μ l)	Liver (CFU/150 μ l)	Blood (CFU/100 μ l)
Experimental	55.00 ± 86.10	3.57 ± 4.47	0.00 ± 0.00
Control	22.14 ± 24.59	81.14 ± 178.76	1.29 ± 2.98

lymph nodes, blood and liver specimens, there was not a significant difference between both groups of animals ($P > 0.05$).

No significant differences ($P > 0.05$) between both groups were found in cultures of *Enterococcus* spp. from the caecum, liver and lymph nodes. The experimental and control groups differed significantly ($P > 0.05$) in the number of G^- rods (coliform bacteria) (Figure 1).

The culture findings of *Enterococcus* spp. from the caecum correlated with those from lymph nodes (correlation coefficient 0.69; $P > 0.05$). The same was true of findings of *Enterococcus* spp. in the caecum and blood cultures (correlation coefficient 0.94; $P > 0.05$). Other significant correlation

findings were those of cultures from lymph nodes and blood (correlation coefficient 0.68; $P > 0.05$) and from lymph nodes and liver (correlation coefficient 0.67; $P > 0.05$).

Histological examination

The results of histological examination are presented in Table 3.

There were no significant differences in the overall damage score of liver, duodenum and oesophagus between both groups of animals ($P > 0.05$).

Portal pressure evaluation

The spleen to body weight ratio of experimental and control laboratory rats was 0.00321 ± 0.00069 and 0.00407 ± 0.00166 , respectively. There was no significant difference between both groups of animals ($P > 0.05$).

The spleen to body weight ratio was in correlation with the culture findings in liver (coefficient of correlation 0.78; $P > 0.05$) and *Enterococcus* spp. in lymph nodes (coefficient of correlation 0.84; $P > 0.05$).

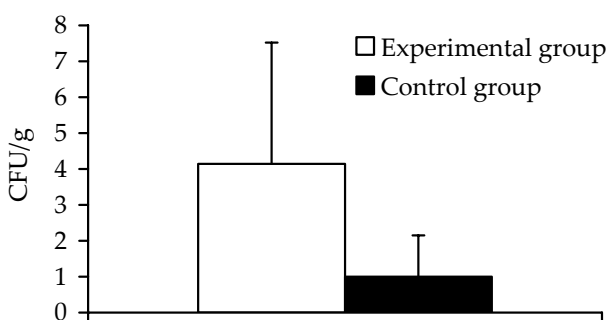
Figure 1. Numbers of G^- rods (coliform bacteria)

Table 3. The overall damage score of liver, duodenum and oesophagus

Group	Liver	Duodenum	Oesophagus
Experimental	6 ± 3	3 ± 2	0 ± 1
Control	7 ± 2	2 ± 1	1 ± 1

DISCUSSION

Bacterial infections frequently complicate both chronic hepatopathies and liver cirrhosis. Many factors take part in their origin. The following ones are currently considered to be the most important: insufficiency of the immune system, impairment of the mucosal barrier of the intestine and intestinal bacterial overgrowth. The above-mentioned three factors probably act in favour of the translocation of bacteria which can be cultured from mesenterial lymph nodes, liver tissue and blood (Albillos and de la Hera, 2002; Guarner and Soriano, 2005; Wiest and Garcia-Tsao, 2005).

The thioacetamide model of liver damage was employed in our experiment because of null mortality prior to achieving the requested morphological changes in the liver parenchyma. We preferred this method of liver damage to the traditional tetrachloromethane model or choledochus ligation. The application of TAA caused profound morphological changes in the liver parenchyma in both groups of animals (Figure 2 and 3) which were not influenced by the subsequent administration of Mutaflor for eight days. Changes in the duodenum and oesophagus were not reduced by the application of Mutaflor either. The portal pressure measured indirectly using the spleen to body weight ratio of the experimental animal was not attenuated by Mutaflor. Such results could be caused by a short period of the application of probiotics. The selection of the

duration of their application was however based on the papers by Adawi et al. (1997) and Bauer et al. (2002).

Higher numbers of G⁻ rods (coliform bacteria) were cultured from the caecal contents in the experimental group receiving Mutaflor compared to the control not provided with the Mutaflor suspension. Even though these coliform bacteria were not identified, we may assume that the higher numbers were caused by *E. coli* from the Mutaflor suspension.

No quantitative and qualitative differences were found in the counts of *Enterococcus* spp. from the caecal contents, lymph nodes, liver tissue and blood of the experimental and control group. According to our results, Mutaflor administered for eight days had no effect on the above-mentioned parameters.

Chiva et al. (2002) tested the influence of the probiotic *Lactobacillus johnsonii* La1 in combination with antioxidants on BT using the tetrachloromethane model of liver cirrhosis in laboratory rats. These authors concluded that it was possible to achieve a decrease in BT into lymph nodes using the combination of *Lactobacillus johnsonii* La1 and antioxidants under specific conditions. The antioxidants alone, administered without probiotics, were also capable of decreasing BT in this experiment. Unfortunately, the above authors did not test the probiotics alone, without antioxidants, so the influence could not be attributed to the probiotics only. The above-mentioned combination decreased the counts of enterobacteria and *Enterococcus* spp.

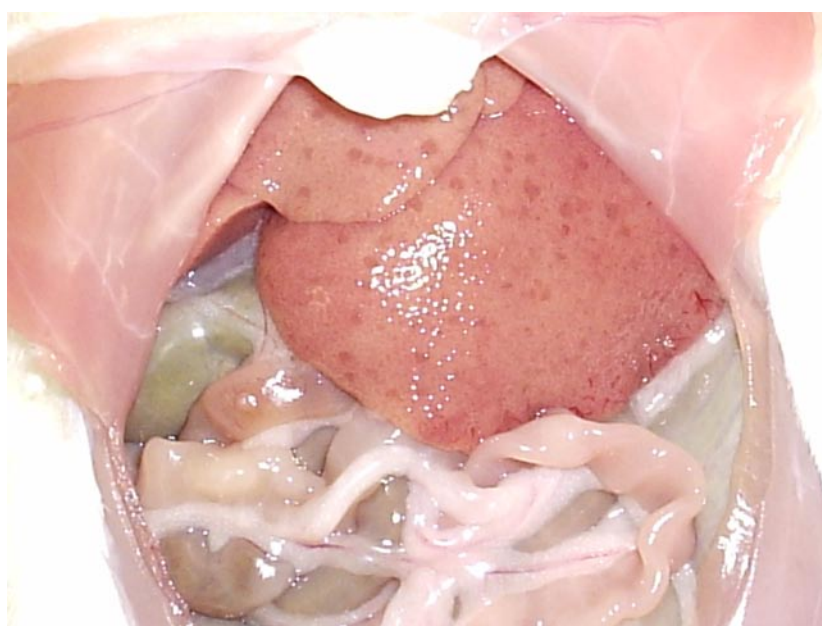


Figure 2. View of the abdominal cavity in a laboratory rat after 15 weeks of TAA administration

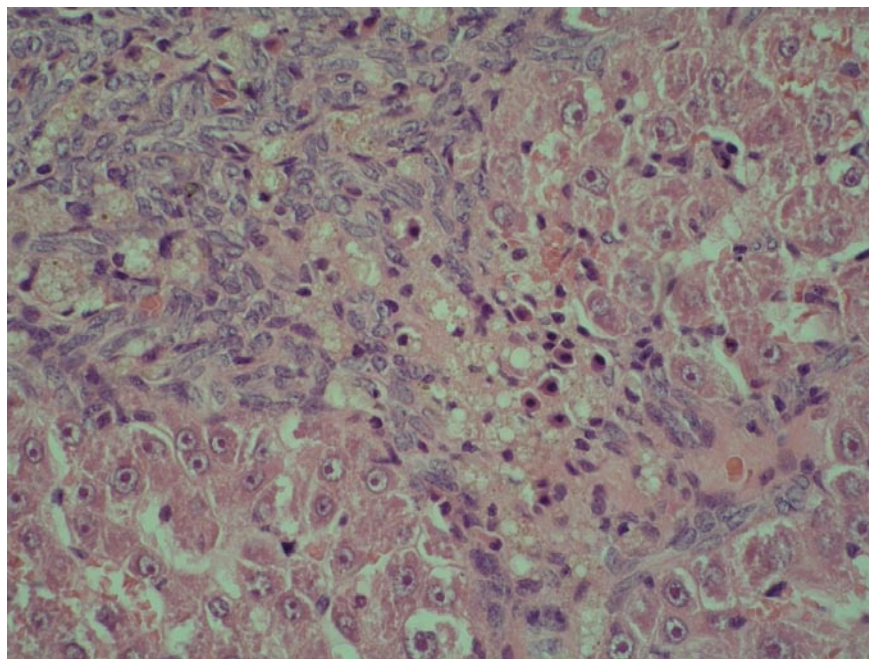


Figure 3. Demonstration of liver parenchyma changes after 15 weeks of TAA administration (photo by Klusakova, magnification 200×)

in the contents of ileum and caecum as well as the level of malondialdehyde – an indicator of oxidative damage. Bauer et al. (2002) used *Lactobacillus rhamnosus*, strain G, as a probiotic and administered it for 8 to 10 days to laboratory rats with liver cirrhosis caused by the inhalation of tetrachloromethane. This procedure did not result in the prevention of IBO, reduction in BT and infection of the ascitic fluid. The translocation of the used probiotics into lymph nodes was a remarkable finding of the above experiment. In our experiment, however, there was no translocation of the used probiotics (*Escherichia coli* Nissle 1917 strain) in any of the experimental animals.

The correlation between *Enterococcus* spp. in the caecal contents and cultures from lymph nodes and blood was an interesting finding of our experiment.

The bacterial translocation was associated with the counts of *Enterococcus* spp. in the caecal contents. It is, however, necessary to state that *Staphylococcus* spp. bacteria were the most frequently translocating ones, followed by *Enterococcus* spp. and only one finding of *Proteus* spp. in a lymph node. Therefore it may be assumed that *Enterococcus* spp. bacteria created conditions for the translocation of staphylococci. *Staphylococcus* spp. as well as *Enterococcus* spp. represent a common kind of microflora of the gastrointestinal tract in the laboratory rat

(Morotomi et al., 1975). Both bacterial species are capable of translocation from the intestine (Wells et al., 1989). Microbial synergism may sometimes create such conditions for an infection that it is caused by organisms not commonly encountered in the disease. This concept also concerns *Enterococcus* spp. (Jett et al., 1994).

In laboratory rats suffering from liver cirrhosis caused by tetrachloromethane Guarner et al. (1997) noticed a translocation of the same kind as that created by IBO and found in the caecal contents of the animal. The counts of *Staphylococcus* spp. from the caecal contents were not determined in our experiment. The counts of *Enterococcus* spp. in the caecal contents were not in correlation with those in the lymph nodes, but showed a strong correlation with the counts of *Enterococcus* spp. cultured from blood samples. Such findings support the above-mentioned theory. Pardo et al. (2000) pointed out the role of the bacterial overgrowth of jejunum by G^+ as well as G^- bacteria in the pathogenesis of BT.

All individuals with positive culture results of the liver and/or blood were also positive for the culture of lymph nodes. The cultures of lymph nodes correlated with those from the liver and blood. There was no correlation of cultures from the liver and blood. Based on this finding we draw a conclusion that the route of BT from the intestine is pri-

marily through the ileocaecal lymph nodes. This statement corresponds with the study by Llovet et al. (1998).

Portal hypertension is a common finding in liver cirrhosis. It is a factor contributing to the damage of the intestinal mucosa (enabling thus BT) as well as to the development of oesophageal varices. We selected the spleen to body weight ratio of the experimental animal as a parameter suitable for the evaluation of portal hypertension (Oren et al., 1999; Dasarathy et al., 2002; Chiva et al., 2003). Chiva et al. (2003) found that the values of the spleen to body weight ratio of the experimental animal in laboratory rats with liver cirrhosis caused by the application of tetrachloromethane were quite comparable with ours. In this study the authors documented that the rats with BT had a significantly higher portal pressure than those without BT. In our experiment, the portal pressure was in correlation with the culture results of *Enterococcus* spp. in lymph nodes and liver, thus corresponding with the above study. Hashimoto and Ohyanagi (2002) studied BT after the induction of acute portal hypertension and found a significant rise of BT into lymph nodes a short time after the induction. On the basis of these findings we can confirm the hypothesis that the portal hypertension plays an important role in the pathogenesis of BT. Unfortunately, the morphological findings of damage were not in correlation with the level of the portal pressure or BT. Misra et al. (1997) described the findings of enteropathy caused by portal hypertension. Their description is based on biopsy specimens obtained from the duodenum and jejunum of human patients. Such et al. (2002) also studied morphological changes in the duodenum of patients suffering from liver cirrhosis and found structural abnormalities. The above-mentioned papers made us select samples from the duodenum for histology in our study. Ersoz et al. (1999) mentioned the increased permeability of the intestine in patients with liver cirrhosis. There were however no differences in the intestinal permeability of patients with the spontaneous bacterial peritonitis and those without this complication. This fact led to a conclusion that the increased intestinal permeability was not the major factor determining BT. This conclusion may be supported by our finding of no correlation between the morphological damage of the intestine and BT.

Oesophageal varices are present in 20% to 60% of patients at the time of liver cirrhosis diagnosis (Fevery and Nevens, 1999). The histological ex-

amination of oesophagus revealed only moderate changes such as hyperaemia and dilatation of veins. In our opinion, prolonged periods of portal hypertension and maybe higher pressures than in our experiment are needed for the development of oesophageal varices.

It may be concluded that eight days of administration of the Mutaflor suspension had no effect diminishing the complications of the chronic liver damage caused by the administration of TAA to laboratory rats.

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Received: 2007–02–02

Accepted after corrections: 2007–02–15

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