

Recombinant Probiotic Therapy in Experimental Colitis in Mice

(probiotic therapy / IL-10 / colitis / inflammatory bowel disease / oxidative stress)

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Abstract. Recently, high interest has been attracted to the research of inflammatory bowel diseases (IBD). Recombinant probiotic bacteria may represent an interesting way to influence the course of IBD. Their benefits include cheap and simple production and easy manipulation of the genetic material. Several gene therapy and probiotic approaches already showed promising results in the past. The aim of this study was to test the probiotic potential of IL-10-expressing *Escheria coli* Nissle 1917 in a mouse model of IBD and to compare it with control bacterial strains. The dextran sulphate sodium (DSS) model of colitis was examined for this purpose. Animals received control probiotic bacteria or modified probiotics (expressing IL-10) via gastric gavage. Body weight, stool consistency, food and water consumption were monitored. At the end of the experiment, the parameters of inflammation, oxidative stress and carbonyl stress were analysed in the samples and statistical analysis was performed. We prepared an anti-inflammatory probiotic *Escherichia coli* strain that we designated Nissle 1917/pMEC-IL10 and proved its anti-inflammatory properties, which are similar to those of the control probiotic strains Nissle 1917 and *Lactococcus lactis*/pMEC-IL10 *in vivo*.

The probiotic therapy was successful according to several parameters, including colon length, and oxidative and carbonyl stress. Bacterially produced IL-10 was detected in the plasma. The potential of bacterial anti-inflammatory therapy of IBD using modified probiotics was outlined. The results opened a way for upcoming studies using modified probiotics for therapy of systemic diseases.

Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organization of the United Nations, 2001). The mechanisms of action of probiotic strains are still not fully understood. Their beneficial effect can be based on the ability to change the composition of bacterial communities in gastrointestinal tract (GIT) biofilm and on displacement of pathogenic microorganisms and/or modification of immune system reactions through different mechanisms (de Vrese and Schrezenmeir, 2008). Currently, several products with probiotic content are available on the market. Whether these products are actually beneficial for the patients' health or not is not clear, and without clinical studies it is hard to come to any conclusion.

Several clinical studies using probiotic therapy in IBD patients were recently published; however, the results have thus far been inconsistent (Matthes et al., 2010; Tursi et al., 2010; D'Inca et al., 2011; Wildt et al., 2011). Modification of the GIT microflora composition may favourably influence some issues related to antibiotic and drug administration in IBD. In a double-blind randomized placebo-controlled trial the positive effects of the consumption of a probiotic drink (*Lactobacillus casei*, *L. bulgaricus* and *Streptococcus thermophilus*) on diarrhoea caused by antibiotic treatment and *Clostridium difficile* infection were clearly proved (Hickson et al., 2007). In this study, the probiotic milk decreased the amount of patients with antibiotic-associated diarrhoea in comparison with the placebo (sterilized milk) group. The anti-inflammatory effect of probiotic yogurts was proved in a study with 20 healthy volunteers and 20 IBD patients (Lorea Baroja et al., 2007). Both groups were supplemented with probiotic yogurts (*Lactobacillus*

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Abbreviations: ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radical), AGEs – advanced glycation end products, AOPP – advanced oxidation protein products, BSA – bovine serum albumin, CFU – colony-forming units, DSS – dextran sulphate sodium, FRAP – ferric reducing ability of plasma, GIT – gastrointestinal tract, GM – genetic modifications, IBD – inflammatory bowel diseases, IL-10 – interleukin 10, MDA – malondialdehyde, PBS – phosphate-buffered saline, TAC – total antioxidative capacity.

rhamnosus GR-1 and *L. reuteri* RC-14) during one month. The supplementation had significant anti-inflammatory effects. Further, ten Crohn's disease patients without surgery have every day consumed 4.5×10^{10} CFU of probiotic bacteria on average (mainly genus *Bifidobacterium* and *Lactobacillus*) during 13 months (some of them were also supplemented with prebiotics) (Fujimori et al., 2007). There were no side effects and the disease activity was also significantly decreased in comparison to the base line. Six patients achieved a complete response, one had a partial response and three had no response. In two studies by Bibiloni et al. (2005) and Huynh et al. (2009), remission was achieved in 77 % and 56 % of patients with ulcerative colitis, respectively, after several-week-long consumption of probiotic preparation VSL#3.

Similarly to studies mentioned above, the most frequently used probiotic strains in experimental conditions include bacterial genera *Bifidobacterium* and *Lactobacillus*. However, owing to well-defined laboratory procedures and gene manipulations currently available, the probiotic strain *Escherichia coli* Nissle 1917 is of high experimental and clinical importance as well. Its positive therapeutic effects were proved in clinical conditions in diarrhoeal diseases of both newborns (Henker et al., 2007) and adults (Kruis et al., 2004).

The beneficial effect of probiotic therapy can further be augmented by genetic modifications (GM) of the bacterial strains. The genetic modifications were originally employed because of food industry requirements, including stability of the strains or better resistance to external factors during production of the food (Ahmed, 2003). There is a large amount of data from animal experiments regarding GM probiotics, their efficiency and mode of action. However, only a few of these bacterial strains got further into clinical trials. In animal experiments, the most commonly used probiotic therapy is based on bacterial production of anti-inflammatory agents or proteins modifying the inflammatory status. Huibregtse et al. (2007) described triggering of antigen tolerance to ovalbumin and increased IL-10 production using ovalbumin-producing *Lactococcus lactis*. Following this approach, virtually any pro-inflammatory antigen could be used to trigger tolerance to it. Combining the antigen tolerance triggering, immunostimulatory effect of the probiotic strains, their competitive colonization of the GIT and production of certain therapeutic proteins (e.g. anti-inflammatory cytokines) by the strain could lead to the generation of a potent and effective GM probiotic strain intended for the use in treating diseases.

The GM probiotics possibly represent the next level in the treatment of IBD. Several studies have been published regarding IL-10-producing *L. lactis* probiotic strains for alleviation of bowel inflammation in animal IBD models (Steidler et al., 2000, 2003; Waeytens et al., 2008), as well as in clinical trial (Braat et al., 2006). Apart from lactococci, the strain *Escherichia coli* Nissle 1917 has been proposed as a potent and safe instrument to produce anti-inflammatory agents in the bowel (Wes-

tendorf et al., 2005). The excellent colonization properties render this strain an ideal candidate as a carrier organism for gut-focused *in situ* synthesis of therapeutic molecules. Despite a considerable amount of literature describing the beneficial use of *E. coli* Nissle 1917 for gastrointestinal indications, thus far no genetic modifications have been shown to improve its properties for treatment of IBD, either in experimental or clinical settings. The aim of this study was to test the probiotic potential of IL-10-expressing *E. coli* Nissle 1917 in a mouse model of IBD and to compare it with control bacterial strains.

Material and Methods

Bacterial strains and plasmids

The bacteria *L. lactis* and *L. lactis* with plasmid pMEC-IL10 were obtained from Dr. Catherine Daniel, Bactéries Lactiques et Immunité des Muqueuses, Institut Pasteur de Lille, Lille, France (Foligne et al., 2007). The bacterial strain *E. coli* Nissle 1917 was obtained from Dr. Ulrich Sonnenborn, ARDEYPHARM GmbH., Herdecke, Germany (Cukrowska et al., 2002). *E. coli* Nissle 1917 was electroporated with plasmid pMEC-IL10 using Gene Pulser X-cell electroporation system (Biorad, Hercules, CA) following the manufacturer's instructions. The strain *Escherichia coli* ER2738 was obtained from New England Biolabs (Ipswich, MA) and was used as a non-probiotic control (Woodcock et al., 1989). The overview of the bacterial strains and plasmids is provided in Table 1. Bacterial cultures were grown overnight in LB broth (BD Microbiology, Heidelberg, Germany) at 37 °C. To determine the exact bacterial titres, colony-forming units (CFU) were determined by colony dilutions of the respective overnight cultures on LB agar with appropriate antibiotics (ampicillin, streptomycin, tetracycline or chloramphenicol). Bacteria were washed in saline and diluted to a titre of 4×10^9 CFU/ml in phosphate-buffered saline (PBS), pH 7.4.

Animals and DSS protocol

Animals were obtained from Charles River Laboratories (Wilmington, MA). They were kept in separate cages in a controlled environment with 12:12 light-dark cycle and *ad libitum* access to water and food. The animal experiments were approved by the local Ethics Committee.

Male C57BL/6 mice (N = 60, age 10 weeks) were divided into 6 groups: CTRL, DSS, DSS+ER2738, DSS+Nissle, DSS+Nissle/IL10 and DSS+*L. lactis*/IL10. In DSS-treated groups mice were fed *ad libitum* with 2% DSS (MW 36,000–50,000; MP Biomedicals, Santa Ana, CA) for 7 days. During DSS treatment mice in DSS+ER2738, DSS+Nissle, DSS+Nissle/IL10 and DSS+*L. lactis*/IL10 groups received 10^9 bacteria daily via gastric gavage in 0.25 ml phosphate-buffered saline (PBS) with 15% glycerol. Animals in CTRL and DSS groups received 0.25 ml PBS with 15% glycerol. Body

Table 1. Bacterial strains and plasmids

Bacterial strain	Full name	Purpose	Source
ER2738	<i>Escherichia coli</i> ER2738	control bacteria	New England Biolabs, Ipswich, MA (Woodcock et al., 1989)
<i>L. lactis</i>	<i>Lactococcus lactis</i>	probiotic	Dr. Catherine Daniel (Foligne et al., 2007)
<i>L. lactis</i> /pMEC-IL10	<i>Lactococcus lactis</i> bearing plasmid pMEC-IL10	probiotic	Dr. Catherine Daniel (Foligne et al., 2007)
Nissle1917	<i>Escherichia coli</i> Nissle 1917	probiotic	Dr. Ulrich Sonnenborn (Cukrowska et al., 2002)
Nissle1917/pMEC-IL10	<i>Escherichia coli</i> Nissle 1917 bearing plasmid pMEC-IL10	probiotic	this work
Plasmid	Encoded gene	Purpose	Source
pMEC-IL10	interleukin 10	anti-inflammatory	isolated from <i>L. lactis</i> /pMEC-IL10

weight, water and food intake and stool consistency (0 – normal, 1 – soft formed, 2 – watery, 3 – stool with blood) were monitored every day. On day 7 the animals were sacrificed and samples of blood and colon were taken for further analyses. Plasma was obtained by centrifugation of blood samples.

Collection of colon samples

The whole colon was cut longitudinally and washed with PBS, pH 7.4. Two samples, approximately 100 mg each, were taken from the aboral end for cytokine assay and biochemical measurements. The pieces were immediately frozen in liquid nitrogen and 10% homogenates in PBS (weight per volume) were prepared prior to analyses.

Protein measurement

The concentration of proteins in the samples was measured using Lowry assay with Biuret reagent and Folin & Ciocalteu's reagent (Lowry et al., 1951) in 96-well plates using bovine serum albumin (BSA) as a standard. The absorbance was measured with a microplate reader (Tecan Sapphire II, Salzburg, Austria) at 660 nm.

Malondialdehyde measurement

Malondialdehyde (MDA) was measured in colon homogenates using a spectrophotometric assay after derivatization with 0.67% thiobarbituric acid in acidic solution of acetic acid (95 °C, 45 min). After derivatization the coloured product was extracted to n-butanol and measured with a microplate spectrophotometer at 535 nm. MDA concentration in colon homogenates was expressed in $\mu\text{mol/g}$ of proteins based on the calibration curve using 1,1,3,3-tetramethoxypropane as a standard.

Cytokine assay

The levels of the IL-10 cytokine were measured in plasma and bacterial cultures using an ELISA kit (Bender MedSystems GmbH, Vienna, Austria). IL-10 concentrations were expressed as pg/mg of proteins.

Total antioxidative capacity

Total antioxidative capacity (TAC) of colon homogenates was measured using the protocol of Erel (2004), which is based on the decolourizing reaction with 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid cation radical (ABTS). Absorbance was taken at 660 nm before and 5 min (at 37 °C) after the addition of ABTS reagent. TAC was expressed in AU/g of proteins or as trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent in mmol/g of proteins.

Advanced glycation end products

Advanced glycation end products (AGEs) were measured in colon homogenates. The samples were diluted 20 times in PBS, pH 7.4 and fluorescence was measured using a microplate reader (Tecan Sapphire II, Salzburg, Austria) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. As a standard for calibration curve, the mixture of BSA and AGE-BSA was used. The results were expressed as AGE-BSA equivalent in g/g of proteins.

Advanced oxidation protein products

Advanced oxidation protein products (AOPP) were measured in colon homogenates. The samples were diluted 5 times in PBS, pH 7.4. Absorbance was measured using a microplate reader (Tecan Sapphire II) at a wavelength of 440 nm after acidizing with acetic acid. The AOPP level was expressed as chloramine-T equivalent in $\mu\text{mol/g}$ of proteins.

Ferric reducing ability of plasma

Ferric reducing ability of plasma (FRAP) is a method for measuring the antioxidant power of plasma (Benzie and Strain, 1996) or tissue homogenates. The samples and standards were mixed with fresh FRAP reagent (acetate buffer, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 2,4,6-tripyridyl-s-triazine) at 37 °C and after 4 min the absorbance was measured using a microplate reader (Tecan Sapphire II) at a wave-

length of 593 nm. FRAP was expressed as Fe^{2+} equivalent in $\mu\text{mol/g}$ of proteins.

Fructosamine

Fructosamine is a product of the reaction between fructose and ammonia or an amine. It is formed when a carbonyl group of glucose reacts with an amino group of a protein. Fructosamine was measured using colour reaction of samples with nitro blue tetrazolium salt at 37°C after 15 min using a microplate reader (Tecan Sapphire II) at a wavelength of 530 nm. Results were expressed as 1-deoxy-morpholino-D-fructose equivalent in mmol/g of proteins.

Statistical analysis

Data were analysed using one-way ANOVA or (Pearson) χ^2 test (P value in the figure headlines). Bonferroni *t*-test was used to evaluate the differences between groups. P values less than 0.05 were considered significant. The calculations were performed using XLstatistics and Microsoft Excel 2007 software.

Results

The ability of Nissle 1917/pMEC-IL10 bacteria to express the IL-10 cytokine

The ability of Nissle/IL10 to express the IL-10 cytokine was proved by ELISA. A significant increase in IL-10 production was observed in Nissle/IL10 compared to the control Nissle strain (Fig. 1). However, the level of IL-10 produced by Nissle/IL10 was approximately six times lower than by *L. lactis*/IL10. Measurements of IL-10 were performed in four independent cultures of each bacterial strain.

Clinical monitoring and macroscopic examinations

We monitored the weight loss and stool consistency during the whole experiment. The most important changes were observed on day 7. DSS treatment caused

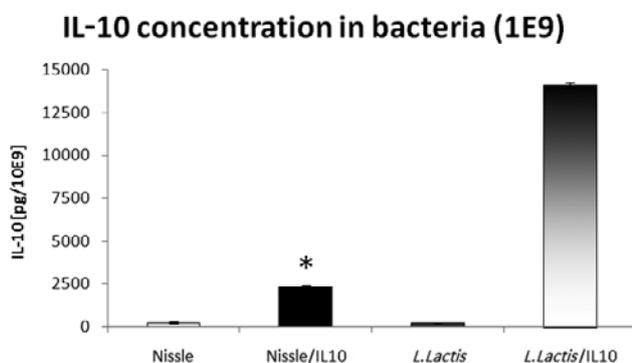


Fig. 1. IL-10 levels of bacterial cultures. IL-10 was measured in Nissle, Nissle/IL10, *L. lactis* and *L. lactis*/IL10 bacterial LB cultures after dilution to the same titre. A significant increase in IL-10 production was observed in Nissle/IL10 compared to control Nissle strain (* $P < 0.05$). The values are expressed in $\text{pg}/10^9$ of bacteria.

significant weight loss in ER2738 and Nissle/IL10 groups. On the contrary, the treatment with non-modified control strain Nissle 1917 maintained body weight of the animals (Fig. 2). All treated groups improved the stool quality significantly in comparison to DSS and ER2738 groups (Fig. 3). The food and water intake did not change significantly. Two animals died during the experiment due to incorrect gavage application (group CTRL and Nissle/IL10).

At the end of the experiment, we measured the colon length of each animal as a marker of inflammation-caused injury. There was a significant shortening of the colon induced by DSS treatment. All groups with the bacterial treatment showed significant improvement in colon length in comparison to the DSS group. Surprisingly, the group with the control non-modified strain Nissle 1917 showed the highest improvement (Fig. 4). No significant differences were observed between the respective bacterial treatment groups.

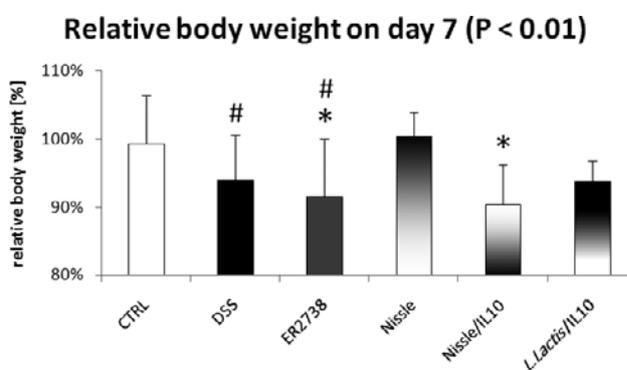


Fig. 2. Relative body weight on day 7 expressed in % of the initial body weight. Significant differences were found between the CTRL group and ER2738 and Nissle/IL10 groups (* $P < 0.05$) and between the Nissle group and DSS and ER2738 groups (# $P < 0.05$).

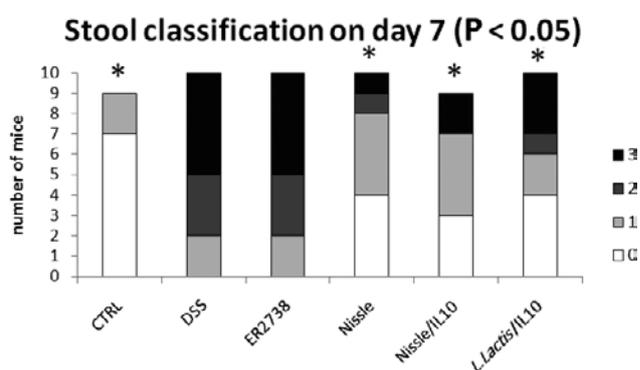


Fig. 3. Stool classification on day 7. Stool consistency was classified as follows: 0 – normal stool, 1 – soft formed, 2 – watery, 3 – stool with blood. The DSS treatment worsened the score in groups DSS and ER2738 equally. Significant differences were found between these groups and CTRL, Nissle, Nissle/IL10 and *L. lactis*/IL10 groups (* $P < 0.05$). Statistical analysis was performed in XLStatistics using (Pearson) χ^2 test.

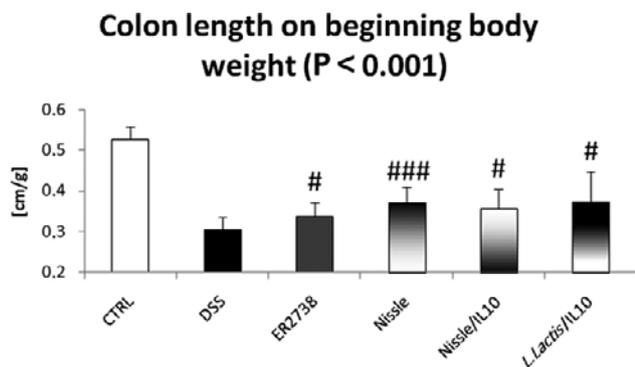


Fig. 4. Colon length on body weight at the beginning. The colon was significantly shortened in all DSS-receiving groups in comparison to the CTRL group. Treatment with bacteria improved the shortening of colon in comparison to the DSS group (# $P < 0.05$, ### $P < 0.001$). No differences were observed between the bacterial treatment groups.

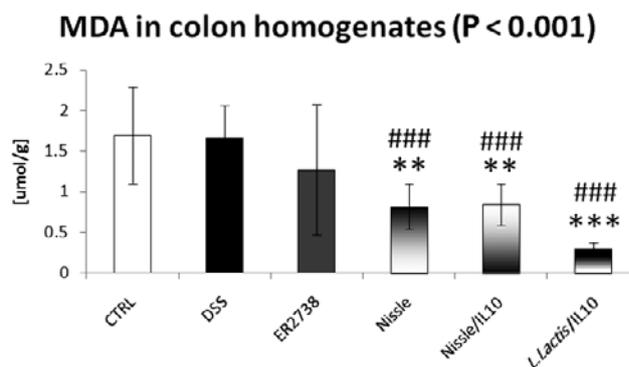


Fig. 6. MDA levels in colon homogenates. The values are expressed in $\mu\text{mol/g}$ of proteins. Significant differences between the CTRL group and all probiotic-treated groups were found in the MDA parameter (** $P < 0.01$, *** $P < 0.001$). Significant differences were also found between the DSS group and all probiotic-treated groups (### $P < 0.001$).

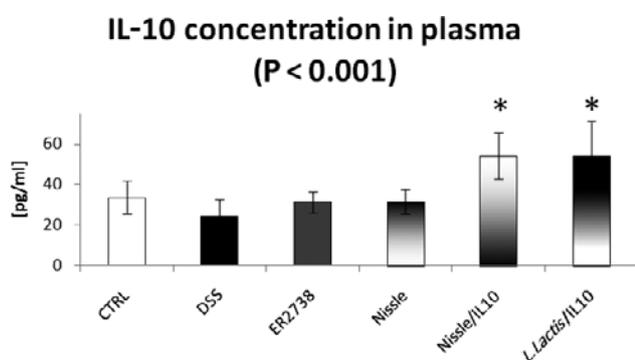


Fig. 5. IL-10 levels in plasma. The values are expressed in pg/mg of plasma proteins. Significant differences between groups Nissle/IL10, *L. lactis*/IL10 and all other groups were found (* $P < 0.05$).

Cytokine assay

Plasma IL-10 concentration was measured in each animal at the end of the experiment. The levels of the IL-10 cytokine were the same in all groups without IL-10 supplementation by probiotic bacteria. Nissle/IL10 and *L. lactis*/IL10 groups showed significantly higher plasma levels of IL-10 than other groups (Fig. 5).

Oxidative and carbonyl stress parameters in colon homogenates

The concentration of MDA did not increase in the DSS group in comparison to the CTRL group. However, all probiotic bacterial therapies improved this parameter significantly when compared to CTRL and DSS groups (Fig. 6). AOPP as a marker of the effect of oxidative stress on proteins was not increased by DSS treatment, but administration of all *E. coli* strains (*i.e.* ER2738, Nissle and Nissle/IL10) lowered this parameter (data not shown). The treatment with *L. lactis*/IL10 had no significant effect on AOPP. However, the parameters of antioxidant capacity – TAC and FRAP – were not increased by any bacterial treatment (data not shown).

Fructosamine in colon homogenates (P < 0.001)

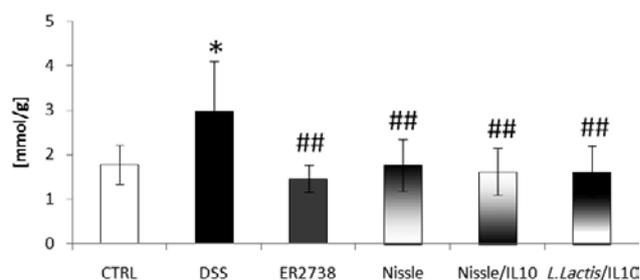


Fig. 7. Fructosamine in colon homogenates. The values are expressed in mmol/g of proteins. Significant differences in this parameter were found between the CTRL group and DSS group (* $P < 0.05$). All bacteria-fed groups had lower fructosamine levels than the DSS group (## $P < 0.01$).

Further, no significant differences were found in AGES between the groups. On the other hand, DSS treatment increased fructosamine levels in colon homogenates and all groups receiving bacteria had significantly decreased fructosamine levels, which were comparable to the CTRL group (Fig. 7).

Discussion

Here, we prepared the bacterial strain *E. coli* Nissle 1917/pMEC-IL10 that is able to produce IL-10. The potential of Nissle 1917 for delivering therapeutic molecules was outlined already some time ago (Westendorf et al., 2005). However, our study is the first to describe the use of this strain for the production of a cytokine. In a very interesting recent study a remote control of gene expression in this strain was described for regulation of therapeutic protein expression in the intestine, the gallbladder and the tumour (Loessner et al., 2009). Such mechanism might possibly be exploited in the treatment of IBD via activation of the therapeutic protein expres-

sion during a relapse phase. The duration of Nissle 1917 colonization in the colon was previously shown to be in the range of 7 to 40 days, depending on the number of administered CFU (Westendorf et al., 2005; Remer et al., 2009).

In the current experiment, DSS administration and probiotic treatment led to significant changes in the stool quality as a marker of bowel discomfort and injury. All of the therapeutic probiotic bacteria used in our study showed some beneficial effect in this parameter. The colon length, as a marker of inflammation in the colon, was decreased by DSS treatment in all groups compared to the control group, as is well known from the literature (Rumi et al., 2004; Geier et al., 2007). However, administration of all of the bacterial strains showed some protective effect.

We confirmed the *in vivo* production of IL-10 by measurement of IL-10 levels in plasma samples. It is known that administration of the probiotic strain itself can increase the IL-10 production in plasma (Imaoka et al., 2008), as well as in inflamed bowel (Ulisse et al., 2001), but this is probably not the case in our study because there was no increase in IL-10 plasma levels in the group receiving control Nissle 1917 bacteria. Our results clearly show that the bacterially produced IL-10 is active systemically. This fact indicates that the orally administered modified probiotic bacteria could possibly be used for therapeutic modulation of the processes that are not directly associated with the gastrointestinal pathology.

Interestingly, the DSS treatment did not increase the oxidative stress parameters as compared to the control group. However, the bacterial treatment lowered the oxidative stress, as is evident from the MDA and AOPP levels. Lipid peroxidation was only lowered by the probiotics, but the protein oxidation parameter AOPP was also lowered by laboratory strain ER2738. Our data suggest that the administration of non-pathogenic bacteria may have positive effects on bowel inflammation. However, in our study the antioxidative effects of bacterial administration were not caused by higher antioxidative capability, since FRAP and TAC were not increased in these groups (data not shown).

The parameter of general carbonyl stress AGES was not significantly changed by DSS treatment or by the therapy, but fructosamine levels in colon homogenates brought up interesting results. Fructosamine as a part of carbonyl stress products was increased by DSS treatment and lowered by administration of bacteria. We are the first describing the effect of bacteria on the DSS-mediated carbonyl stress. This carbonyl stress can be either a direct effect of DSS treatment or a consequence of oxidative stress and inflammation (Blackburn et al., 1999; Negre-Salvayre et al., 2008).

In some parameters (stool consistency, body weight, colon length) the control Nissle 1917 showed the same or better effect on amelioration of DSS-caused injuries as compared with the IL-10-producing strains. Similar positive effects of Nissle 1917 were described in previ-

ous successful studies (Grabig et al., 2006; Schlee et al., 2007). It is not clear why the modification of this strain (transformation with plasmid pMEC-IL10) did not lead to any additional beneficial effects. Therefore, in view of our results, the beneficial effect of the production of IL-10 by Nissle seems to be questionable. On the other hand, the increase of plasma IL-10 after administration of IL-10-producing strains may be of importance for the treatment of other diseases where systemic IL-10 could have a therapeutic effect.

Surprisingly, the laboratory strain ER2738 also showed positive effects. It is known that bacterial DNA is able to modulate immune response by decreasing inflammatory cytokine levels or by increasing IL-10 levels (Jijon et al., 2004; Imaoka et al., 2008). However, why ER2738 had these positive effects and whether other non-pathogenic strains will work in the same way are questions for the following studies. In conclusion, the results of our study prove some therapeutic potential of (modified) probiotic bacterial strains. The benefit of the newly prepared strain *E. coli* Nissle 1917 expressing IL-10 over the control strains, however, was not shown. The future studies will focus on the potential of IL-10-expressing probiotics in the treatment of systemic diseases.

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