Digestive Diseases and Sciences, Vol. 51, No. 2 (February 2006), pp. 390–400 (© 2006)
DOI: 10.1007/s10620-006-3143-x

Recommendations for Improved Use of the Murine TNBS-Induced Colitis Model in Evaluating Anti-inflammatory Properties of Lactic Acid Bacteria: Technical and Microbiological Aspects

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Probiotic bacteria have been shown to exert promising beneficial effects in different types of intestinal disorders, including chronic inflammation. In this context, animal models of inflammatory bowel disease are useful in studying the possible prophylactic role of candidate probiotic strains. This study aimed at evaluating the critical technological and microbiological parameters as well as the robustness of the murine trinitrobenzene sulfonic acid (TNBS)–induced model of colitis, after intragastric administration of lactic acid bacteria (LAB) preparations. A standardized methodology was applied to assess the protective effect achieved by various bacterial concentrations and culture conditions of the reference strain *Lactobacillus plantarum* NCIMB 8826. Not only was protection found to vary in function in different levels of colitis, but also repeated experiments showed a clear bacterial dose-dependent attenuation of colitis. The physiological stage of bacteria was shown to impact as well, with substantial, mild, or reduced improvement of inflammatory scores for exponentially growing, stationary-phase, or killed bacteria, respectively. A recombinant strain, secreting murine interleukin-10 (IL-10) and previously reported to successfully treat colitis in two different models of murine colitis (dextran sulfate sodium [DSS] and IL-10-deficient mice), was used to validate the final experimental conditions. In conclusion, we identified and optimized some of the key parameters that need to be controlled in order to ensure reliable comparison of results generated over a long period of time or independent experiments. The recommendations for an improved model presented here will prove to be helpful for reproducible, independent comparison of the anti-inflammatory potential of wild-type or recombinant candidate probiotic strains, whether administered as pure cultures or as blends.

KEY WORDS: probiotics; TNBS mice model; standardization; strain evaluation; inflammatory bowel disease.

It is now well established that the enteric microflora is involved in abnormal inflammatory responses observed in diverse animal models for inflammatory bowel disease (IBD). This fact is supported by the reduction or absence of intestinal inflammation in trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS) models of colitis using antibiotic-treated and germ-free animals (1–4). However, although mechanisms are only partially known, modification of the commensal flora, including a prophylactic treatment with probiotics (5), was recognized as one effective way to control the immune regulation of IBD

Manuscript received February 16, 2005; accepted April 12, 2005.

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(6). Different mechanisms can explain the effect of probiotics: through direct influence on the immune system (see Ref. 7 for review), by signaling through Toll-like receptors (TLRs) on epithelial and dendritic cells (8, 9), and by modification of the composition and activities of the resident flora (10).

Numerous experiments in rodent models of colitis and a few clinical studies led to promote the use of probiotic bacteria, both to prevent the onset of colitis and to reduce established mucosal damages (3, 11–14). Strikingly, most of these studies have not been driven by a clear strain selection strategy, e.g., the use of an animal model to select the most promising strain for a clinical study and the validity of such a screening approach could only be confirmed a posteriori. Anti-inflammatory strains for potential human applications could most probably be detected through the use of a proper animal model. However, current literature shows that the disease model, the animal species, and the study design used may have a serious impact on the results. In addition, parameters such as bacterial viability, growth status, dose, and frequency of administration may all influence the outcome of a clinical or experimental study. It is thus essential to define a standardized and reliable model, which offers acceptable homogeneity and reproducibility in terms of inflammatory scores and allows reproducible measurement of protective intervention. Such standardization is independent of the underlying mechanisms and is a prerequisite to objectively assess and compare the protective effects of bacterial strains, to assess the influence of certain microbiological and technological parameters, and to ensure reliable comparison of results generated in different experiments as well as in distinct animal models.

In both ulcerative colitis (UC) and Crohn's disease (CD), the inflamed colonic mucosa is infiltrated by neutrophils, macrophages, and lymphocytes. Major effects induced in mice through the use of TNBS include focal ulcers, distortion of crypts, goblet cell depletion, and, most remarkably, the presence of granulomas (15). This experimental model, which is mainly driven by Th1 cells, also shares other immunological features with CD (16–18) and has been used extensively to test the efficacy of anti-inflammatory drugs (19), nutritional and chemical compounds (20), prebiotic fibers (21, 22), probiotic strains (23, 24), or bacterial components (9, 25, 26). *Lactobacillus plantarum* strains have already been studied for their probiotic properties (27–30). Our laboratory previously selected *L. plantarum* NCIMB 8826 as a probiotic candidate strain based on in vitro studies (31) and human feeding trials (32). More recently, we used a mouse model approach to study the persistence and the anti-inflammatory properties of this strain and reported that it attenuated colitis symptoms (33). Therefore, we chose *L. plantarum* NCIMB 8826 as a reference strain for the present standardization study. The aim of this work was first to assess the long-term reproducibility of a TNBS-induced colitis mouse model and to define criteria that allow comparative analysis of bacterial strains under standardized levels of colitis. In this respect, we analyzed the protective potential of strain NCIMB 8826 against weak, moderate, strong, or severe colitis as a function of bacterial doses and culture conditions. Using the standardized TNBS model, we confirmed the protective effect of a recombinant *Lactococcus lactis* strain secreting murine interleukin-10 (*LL*-mIL-10), which was previously reported to improve colitis in two other mouse models, i.e., DSS- and IL-10-deficient (IL-$10^{-/-}$) mouse models (34). This indirectly validated the use of the well-standardized TNBS mouse model to screen or compare the anti-inflammatory potential of candidate probiotic strains.

MATERIALS AND METHODS

Chemical Reagents. Chemicals and reagents were purchased from Sigma-Aldrich Chemical, France, unless mentioned otherwise in the text.

Animals. Animal experiments were performed at an accredited establishment (no. A59107; animal facility of the Institut Pasteur de Lille, France) according to French government guidelines (no. 86/609/CEE). Adult female BALB/C mice, aged 7–8 weeks and maintained under conventional conditions, were purchased from Iffa Credo (Saint-Germain sur l'Arbresle, France). Mice were group-housed (8–10/cage), kept under filter-top hoods, and had free access to tap water and standard rodent chow. They underwent at least 1 week of acclimatization before any intervention. Groups of 8 to 12 mice were used in each experiment.

Preparation of Bacterial Cultures and Administration to Mice. The human isolate *L. plantarum* NCIMB 8826 (strain isolated from saliva; National Collection of Industrial and Marine Bacteria, Aberdeen, UK) was grown at 37°C in MRS medium (Difco, Detroit, MI, USA) under limited aeration. Exponential and stationary growth phases were reached at optical densities, measured at 600 nm (A_{600}), of 1–2 and 4–4.6, respectively. The correspondence between absorbance and bacterial counts was established (1 ml of culture at $A_{600} = 1$ contains about 10^8 colony forming units [CFU]). The number of CFU administered was routinely verified by plating. Strains were harvested by centrifugation at 3000g for 10 min, washed twice with phosphate-buffered saline (PBS; pH 6.8), and resuspended at the appropriate cell concentration in 0.2 M $NaHCO_3$ buffer, pH 8.5, containing 2% glucose (gavage buffer). One hundred microliters of the various doses of bacteria used in the study (CFU varying from 10^5 to 10^9) was administered daily by intragastric gavage.

A *Lactococcus lactis* (LL) strain secreting the biologically active murine IL-10 was previously engineered by transforming the strain MG1363 with the mIL-10 recombinant plasmid (*LL-mIL-10*). The corresponding control strain carries only the nonexpressing plasmid construct (*LL-TREX1*) (34). Standard growth media for both constructions were M17 (Difco) with 0.5% glucose for overnight culture and pH 8.5 $NaHCO_3$-buffered

minimal medium (BM9) with 0.5% glucose for optimal secretion and preservation of the IL-10 cytokine (34, 35).

Preparation of UV-Killed Bacterial Cultures. UV-killing of *L. plantarum* NCIMB 8826 and LL-mIL-10 strains was achieved by UV irradiation for 30 in (312 nm, 90 J/cm^2, on a Fluolink table; Vilber Lourmat, Marne-la-vallée, France). The efficiency of the irradiation process was verified by plating the bacteria on agar medium and was found to be >99%.

TNBS Induction of Acute Colitis and Study Design. Bacterial suspensions were given to mice from day 5 before to day 1 after induction of colitis. Mice were anesthetized by intraperitoneal injection of 3 mg of ketamine (Imalgene 1000; Mérial Lyon, France), 46.7 μg of diazepam (Valium; Roche Diagnostics, France), and 15 μg of atropine (Aguettant Laboratory, Lyon, France) dissolved in 0.9% NaCl. TNBS (Fluka, France) at doses varying from 50 to 150 mg/kg body weight was dissolved in 0.9% NaCl/ethanol (50/50, v/v) and 50 μl was administered intrarectally 4 cm proximal to the anus, using a 3.5F catheter (EO 3416-1; Biotrol, Chelles, France). "Negative control" mice received only 50% ethanol ("ethanol mice"). "TNBS-positive control" mice were gavaged with $NaHCO_3$ buffer before TNBS treatment, while "treated" mice received bacteria and TNBS. Mortality rate and inflammation scores (macroscopic and histological; see below) were assessed 48 hr after TNBS administration. Mice were weighed prior to TNBS administration and at sacrifice by cervical dislocation.

Macroscopic Assessment of Colitis. The colon was removed, dissected free of fat and mesentery, carefully opened, and cleaned with PBS. Colonic damage and inflammation were assessed according to the Wallace criteria (36). These criteria for macroscopic scoring (scores ranging from 0 to 10) have been well established in mouse studies (37) and reflect (i) the intensity of inflammation, (ii) the thickening of colonic mucosa, and (iii) the extent of ulceration. Mice were scored blindly by two researchers.

Histological Assessment of Colitis. For histological assessment, a colon sample located in the most damaged area was fixed in 4% paraformaldehyde acid and embedded in paraffin. Four-micrometer sections were stained with May Grünwald–Giemsa (Fluka, France) and examined blindly by two researchers according to the well-defined Ameho criteria (20). The grading on a scale from 0 to 6 takes into account the extent of inflammatory infiltrates, the presence of erosion, ulceration, or necrosis, and the depth and surface of the lesion.

Statistical Analysis. Results were analyzed by the nonparametric one–way analysis of variance and Mann–Whitney U test (XLSTAT software: http://www.xlstat.com). Differences were judged to be statistically significant at $P < 0.05$. Pearson's regression was used to calculate the correlation between macroscopic and histological scores. Dead mice were not included in the statistical analyses.

RESULTS

Correlation Between the Ameho and the Wallace Scoring Systems. In this work, the histological Ameho score has systematically been compared with the macroscopic Wallace score in successive experiments, performed over a period of 3 years. A confident correlation (Pearson's regression value of $r^2 = 0.8016$ over $n = 521$ inflamed mice intestines) was obtained between the macroscopic and the histological assessment of colitis (Figure 1). Consequently, only the macroscopic damage measurements are reported for all experiments described below.

Relationships Between TNBS Dose and Colitis Severity. Figure 2 represents a general overview of 30 distinct colitis experiments, displaying individual Wallace scores and group mean values, for various doses of TNBS. In all experiments, colons of mice receiving exclusively ethanol showed a normal macroscopic appearance, confirmed by a histological score of 0. A TNBS dose of 50 mg/kg body weight resulted in a very low level of colitis ("histological colitis"), associated with a mean Wallace

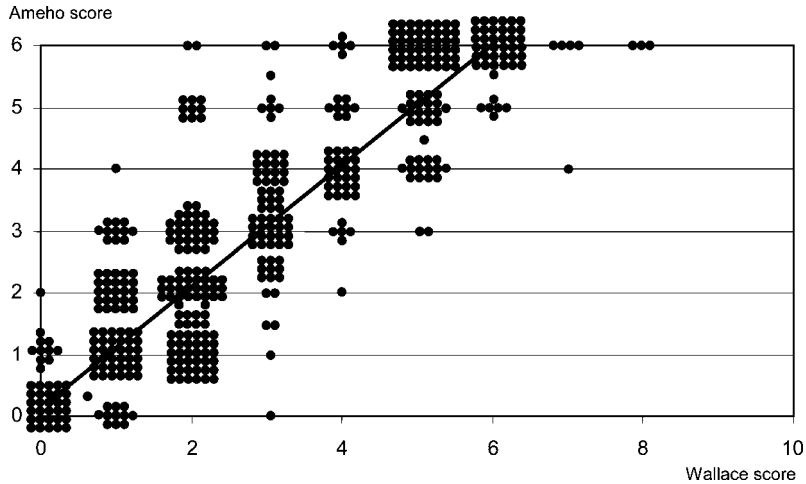

Fig 1. Correlation between macroscopic (Wallace) and histological (Ameho) scores. Distribution and correlation of 521 individual macroscopic and histological evaluations through distinct colitis experiments. Linear equation: $Y = 0.9398X + 0.453$. $r = 0.89$; $P < 0.05$; $r^2 = 0.8016$.

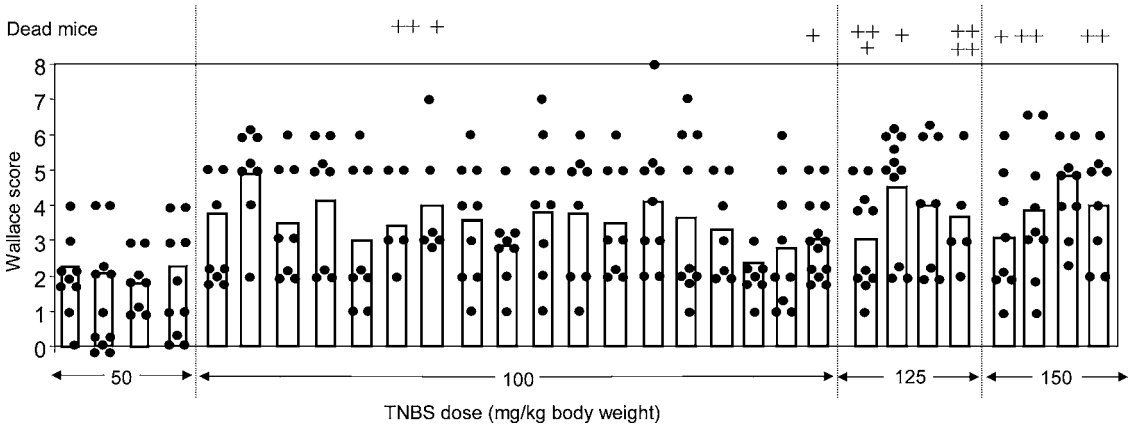

Fig 2. Wallace score of 30 distinct colitis experiments induced with different TNBS doses. Macroscopic damage is characterized by the mean Wallace score per group (columns) obtained from 30 colitis experiments, using various doses of TNBS: 50, 100, 125, and 150 mg/kg body weight. Individual values of TNBS-treated mice (filled circles) are represented for each experiment. Ethanol-control mice always displayed Wallace scores of 0. Mortality corresponds to numbers of individual dead mice (+).

score of about 2, while a dose of 150 mg/kg body weight induced a 20 to 30% mortality rate, associated with high to very high levels of colitis (minimum Wallace score of 5). The mean macroscopic scores did not show marked differences in the range of 100 to 150 mg TNBS/kg. Death resulted mainly from an excessive inflammatory reaction, as assessed by postmortem autopsy.

For a TNBS dose varying from 100 to 150 mg/kg, with 10 mice per group, the reproducibility of the mean Wallace score as well as the spread per experimental group appeared to be quite stable, with an average score of 3.6 ± 0.6, ranging from 2.8 to 4.9 (for 26 experiments). In experiments with the standard dose of 100 mg/kg, more than 90% of the mice showed a slight thickening of the colon, with focal hyperemia and intestinal ulcerations (Wallace score ≥ 3) in approximately 65% of the animals. Consequently, in each experiment, a small proportion of mice can be expected to display low levels of inflammation, while another small number of animals will show "close-to-death-scores" or will die during the 48 hr following TNBS administration. This observation leads us to recommend the use of a minimum of 10 mice per group. This was confirmed by a power calculation value of 80%, meaning that a "significant" decrease of 30% of a mean Wallace score of 4, with a standard deviation (SD) of around 1.2, will require at least 10 mice per group in order to guarantee that this effect is detected in 80% of cases with a P value of 0.05. The difference in responsiveness has been observed before in this model (15, 38) as well as in many other animal models, i.e., TNBS in rats (39), DSS in mice (40, 41), and transgenic mice models (42–44), but was never thought to have a major influence on the final average score, provided that a sufficiently high number of mice was included in each group. The observed variation, even within a given experiment, could probably be attributed to differences in individual responsiveness of the mice rather than to a lack of standardization of the experimental parameters in the model.

The observed distribution of the scores of individual mice within a given experimental setup was independent of the TNBS dose administered and did not change significantly with prolonged exposure time (comparison of damage at 24, 48, and 72 hr for some representative samples; data not shown). While no weight losses were generally seen with a TNBS dose of 50 mg/kg, doses varying from 100 to 150 mg/kg increased the individual body weight loss concomitantly from 10 to 20%.

In order to better define the severity of colitis, linked to the TNBS dose used, we summarized the relationship among the different experimental parameters such as TNBS dose, mortality rate, weight loss, and mean Wallace score (Table 1). Despite relative overlaps and realistic ranges of values, four levels of colitis could be established, corresponding to weak, moderate, strong, or severe colitis. Table 1 can be used to facilitate interpretation of results obtained in such a model, where variation of TNBS-positive controls is inherent and should, therefore, ensure that only results of equivalent ranges of colitis are compared.

Assessment of LAB-Derived Protection: Relation Among Probiotic Dose, Level of Inflammation, and Protection. As mentioned before, equivalent distributions were noted for all bacteria-fed groups, confirming the usefulness of the average SD values in estimating data significance. SD values obtained for all Wallace scores over a 3-year period (Figure 2) established that within a single experiment, a strain can only be considered as significantly

TABLE 1. COLITIS SEVERITY CRITERIA IN TNBS-INDUCED INFLAMMATION IN MICE (RANGES OF PARAMETERS ARE DEDUCED FROM 40 INDEPENDENT EXPERIMENTS)

	Weak	*Moderate*	*Strong*	*Severe*
TNBS dose range (mg/kg body weight)	50 to 100	100 to 125	125 to 150	125 to 150
Mortality rate (%)	0	0 to 10	0 to 20	20
Weight loss (%)	0 to 10	5 to 15	10 to 20	20
Wallace mean score	3	3 to 4.5	3.5 to 5	5

protective if a >30% difference is measured in the average macroscopic score between the TNBS-positive control and the mice treated with a given strain. This protection, expressed as a percentage, together with its statistical significance, was found to be a very convenient way to compare two groups of mice not only within but also, more importantly, between experiments. The percentage relative protection was calculated as $100 \times$ [(average Wallace score of TNBS-positive control group–average Wallace score of treatment group)/average Wallace score of TNBS-positive control group]. When using this "protection rate," the mean Wallace score variation between independent experiments was minimized, for both the TNBS-positive control and the treatment groups.

The protective effect of different doses of *L. plantarum* NCIMB 8826 against weak, moderate, or strong levels of colitis was measured in distinct experiments (Figure 3A) and is expressed as percentage protection. The lowest dose, 10^5 CFU/day, was found to be ineffective, even in weak and moderate levels of colitis, whereas 10^7 CFU/day generally led to a 20 to 60% reduction of the Wallace score. This protection level was seen to decrease progressively with more severe colitis. Similar observations were made at doses of 10^8 and 10^9 CFU/day (Figure 3A). In cases of severe colitis, the protection levels varied substantially and, in at least one case, led to unresponsiveness with a limited worsening of the symptoms, indicating that extreme damage cannot be reversed by oral administration of *L. plantarum* NCIMB 8826. For weak levels of colitis (mean Wallace score of 2.1 ± 0.66), a positive dose–response curve was found for doses varying from 10^5 to 10^9 CFU/day (Figure 3B). In this situation, the highest bacterial dose (10^9 CFU/day) led to a total abrogation of macroscopic and histological damage (data not shown). Therefore, this very weak level of inflammation appeared less suitable for quantifying and comparing the protective effect for different LAB strains. For a moderate or high level of colitis, obtained with TNBS doses of 100 and 125 mg/kg body weight, respectively, protection levels exceeding 50% were rarely observed (Figures 3A and B). This indicates that even with increasing doses of bacteria, inflammation cannot completely be prevented. In summary, the NCIMB 8826 dose that offered maximal protection against a moderate or high level of TNBS-induced colitis was found to be 10^9 CFU/day. However, to discriminate between different strains, we recommend using a bacterial dose of close to 10^8 CFU/day.

Influence of Viability and Bacterial Growth Phase on Moderate Colitis. The protective potential of a stationary-phase (overnight) culture of *L. plantarum* NCIMB 8826 was compared to that of (i) UV-killed bacteria, (ii) late stationary-phase bacteria (48 hr of culture), and (iii) bacteria in their midexponential growth phase ($A_{600} < 2$) (Figure 4). Whether or not the attenuation of colitis was substantial, protection was abolished when nonviable bacteria of *L. plantarum* NCIMB 8826 were used. Late stationary-phase cultures consistently led to lower protection values compared to those obtained with cells collected in the early stationary phase. This difference was particularly noteworthy at the dose of 10^7 CFU/day, where protection against colitis was significantly better for exponential-phase cultures versus overnight-grown strains (70% decrease in mean Wallace score [$P < 0.001$] versus limited protection, respectively).

***L. lactis* mIL-10 Strain–Induced Protection in the TNBS Model.** Figure 5 shows the protective effect on moderate colitis of 10^9 CFU/day of the recombinant strain *L. lactis* MG1363 secreting murine IL-10 (*LL*-mIL-10). No effect was observed when using UV-killed recombinant bacteria or the live nonproducing control strain *LL*-TREX1 at 10^9 CFU, which even seemed to aggravate intestinal damage. The protective effect of the *LL*-mIL-10 strain was analyzed in three distinct experiments with varying levels of colitis. In the case of strong and severe colitis, a clear dose–response effect of the strain *LL*-mIL-10 was observed (Figure 6), leading to 50% protection levels only at the dose of 10^9 CFU/day ($P < 0.05$). Moderate colitis was still attenuated by 10^7 CFU of *LL*-mIL-10, but this dose became less effective with increased colitis severity and, finally, led to a worsening of symptoms in very severe colitis.

DISCUSSION

Animal models of IBD have proven to be useful in studying the mucosal immune response in intestinal

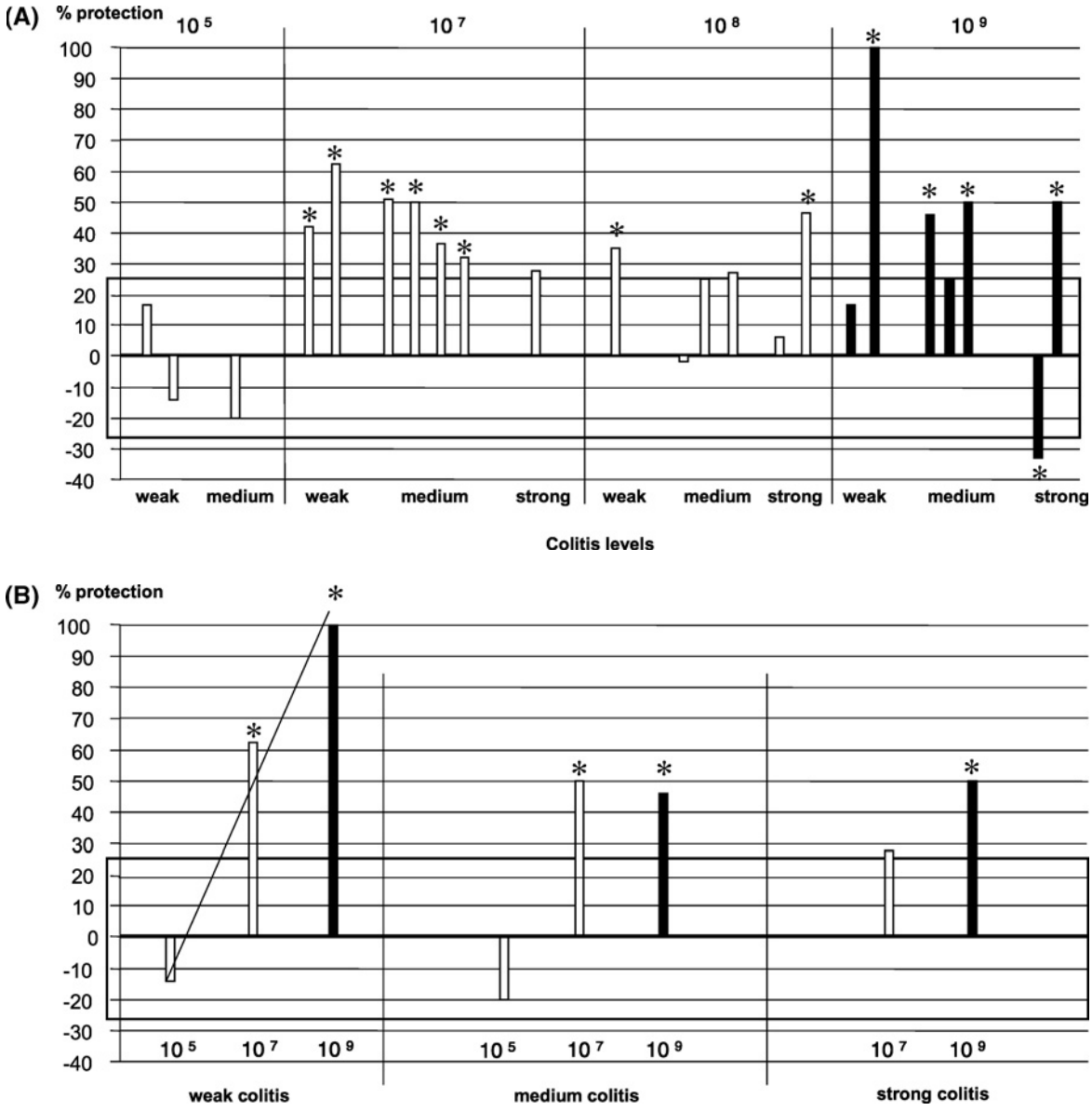

Fig 3. Dose-related protection of *L. plantarum* NCIMB 8826. The impact of different doses of bacteria (10^5 to 10^9 CFU/day) on the macroscopic damages induced by TNBS is shown for weak, moderate, and strong colitis (A) in distinct experiments and (B) within the same experiment. Results are expressed as percentage protection (see text for further explanation), where each bar represents an independent experiment including control ($n = 10$) or LAB-treated ($n = 10$) mice. The statistically uncertain area is delimited by the grey frame. *Significantly different from the corresponding TNBS-positive control group ($P < 0.05$).

inflammatory pathogenesis and in defining the role of probiotic bacteria in the protection process. It is extremely important, however, that critical technical parameters of the model (45) and of the bacterial preparations are properly controlled. Table 2 summarizes the recommendations resulting from our work.

Given the favorable correlation we obtained between macroscopic and histological evaluation, the macroscopic Wallace score, when performed under well-controlled conditions, can directly account for the severity of colitis. This macroscopic score therefore becomes an easy-to-use visual marker of inflammation as far as subjective evaluation is reduced by a blind registration, preferably by two individuals. Since the purpose of the TNBS model is to compare possible bacteria-induced protection against colitis, the reduction of the macroscopic damage observed in

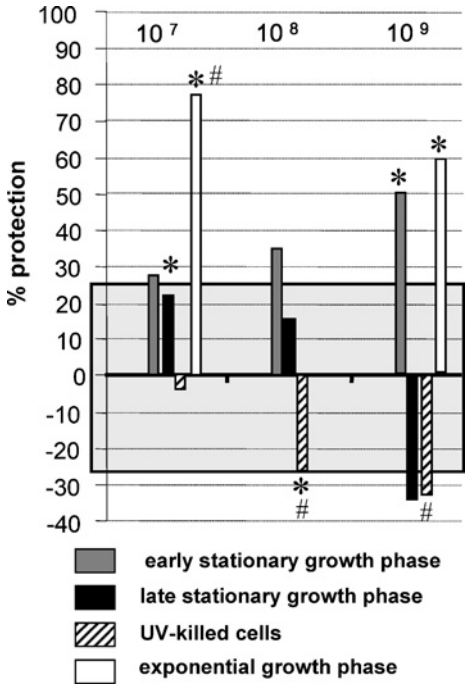

Fig 4. Percentage of protection as a function of the *L. plantarum* NCIMB 8826 physiological state. The impact of different physiological stages of the bacteria, i.e., UV-killed treatment (hatched bars), late stationary-phase (48 hr of culture; black bars) and exponential-phase (white bars) bacteria, at different doses (from 10^7 to 10^9 CFU/day), is compared with the corresponding *L. plantarum* early stationary-phase culture (gray bars). Results are expressed as percentage protection (see text for further explanation), where each bar represents an independent experiment including control ($n = 10$) or LAB-treated ($n = 10$) mice. The statistically uncertain area is delimited by the grey frame. *Significantly different from the corresponding TNBS-positive control group ($P < 0.05$); #significantly different from the early stationary-phase culture of *L. plantarum* ($P < 0.05$).

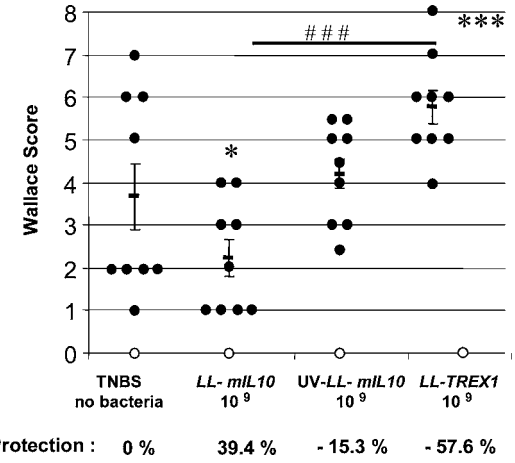

Fig 5. *Lactococcus lactis* (*LL*-mIL-10) effects on the macroscopic damage induced by TNBS (moderate colitis). The impact of 10^9 CFU/day bacteria on colitis is shown for live *LL*-mIL-10, UV-inactivated *LL*-mIL-10 (UV-*LL*-mIL-10), and control (*LL*-TREX1) strains. Results are expressed as individual Wallace scores and mean values of group scores. Percentage protection values are also indicated for each group of mice. *Significantly different from the corresponding TNBS-positive control group, at $P < 0.05$; ***significantly different from the corresponding TNBS-positive control group, at $P < 0.001$); ###significantly different from the corresponding *LL*-mIL-10 TNBS-positive control group ($P < 0.001$). Black points, individual values of TNBS-treated mice; white points, individual values of ethanol-treated mice.

LAB-treated mice compared to the TNBS-positive control is a useful criterion for strain evaluation. Obviously, other markers of inflammation such as myeloperoxidase (MPO) activity, cytokine release (data not shown), and histological assessment (Figure 1) may yield additional information on inflammation and should corroborate with the macroscopic damage registered. These approaches, however, are much more time-consuming and therefore less suitable for a first classification of a large number of strains.

The current study showed that the protection by LAB against TNBS colitis in mice is quantitative and reproducible as long as certain parameters of the model are controlled, i.e., intensity of colitis, bacterial dose, and growth phase. Accepting the inherent response variation observed between individual mice, the statistical limitation of the model was identified at a minimal protection of 30%. For comparing different LAB strains and selecting the most promising "anti-inflammatory strain," microbial parameters such as bacterial growth phase and viable counts should be carefully calibrated and a moderate level of colitis should be induced in mice. A meaningful comparative percentage protection can only be calculated when the same reference strain is included in each experiment. Moreover, in order to measure efficiently the differences in the anti-inflammatory potential of different LAB strains, the colon inflammation induced in the TNBS-positive control group should result in a macroscopically detectable but sublethal level of colitis not surpassing the immunomodulatory potential of the LAB strain tested. Our results clearly indicate that it is important and possible to determine the "adequate" level of colitis at which reliable and quantitative comparison of potential protective effects of different bacterial strains can be measured. Weak colitis does not allow us to draw significant conclusions; colitis that is too severe can lead to extensive mucosal damages and high levels of translocation (46) resulting in dramatic inflammatory responses (19, 47), which disturbs all final readings. In our experimental settings, optimal TNBS doses varied from 100 to 125 mg/kg body weight.

It is noteworthy that for severe levels of colitis, a plateau was reached at which further reduction of the symptoms was no longer achieved, independently of the amount of bacteria administered. Consequently, it seems more

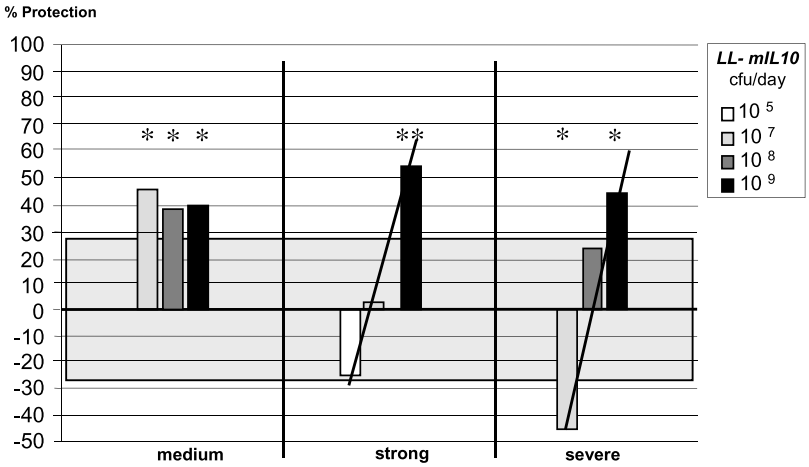

Fig 6. *Lactococcus lactis* (*LL*-mIL-10) protection against different levels of colitis. The impact of different doses of strain *LL*-mIL-10 (10^5 to 10^9 CFU/day) is shown for five distinct experiments, at three different levels of colitis (moderate, strong, and severe colitis experiments). Results are expressed as percentage protection (see text for further explanation), where each bar represents an independent experiment including control ($n = 10$) and LAB-treated ($n = 10$) mice. The statistically uncertain area is delimited by the grey frame. Significantly different from the corresponding TNBS-positive control group at $^*P < 0.05$ and $^{**}P < 0.01$.

appropriate to talk about "attenuation" and "reduction" of colitis rather than real "prevention," since we never induced 100% protection in moderate and severe colitis. Nevertheless, under optimal conditions (moderate colitis and 10^9 exponentially grown bacteria), *L. plantarum* NCIMB 8826 induced a reduction of up to 60% in colitis symptoms, which is close to the values reported for other probiotics in a rat TNBS model (20) or in other mouse

TABLE 2. RECOMMENDATIONS FOR THE IMPROVED USE OF THE MURINE MODEL OF TNBS-INDUCED COLITIS TO ENSURE RELIABLE COMPARISON OF ANTI-INFLAMMATORY PROPERTIES OF LACTIC ACID BACTERIA

Possible point of intervention	*Recommendations*
Technical considerations of the TNBS model	
Registration of classical inflammation parameters Weight loss Cytokine levels MPO activity Histological score Macroscopic score	Analyze several parameters but apply only the macroscopic damage score (Wallace score) for comparable and reproducible analysis of a large number of independent experiments. Scoring should be blind and performed by two researchers
Duration of the experimental model	One week for the acute colitis TNBS model
Dealing with variable levels of colitis severity, classically obtained between independent experiments	Use an a posteriori classification of a positive control group to normalize colitis levels (e.g., low, moderate, strong, severe colitis)
Adjustment of the colitis level, allowing optimal discrimination of anti-inflammatory effects	Best differentiation is obtained with moderate and strong colitis; moderate colitis is preferred to strong colitis
Dealing with response variation between individual mice and statistical significance	Standardize maximally all mice characteristics (species, sex, age, gender, provider, etc.) and use $n \geq 10$ mice per group; repeat experiment ($n > 2$ experiments/strain)
Normalization of results	Calculate strain efficiency as a percentage of protection, including the same positive control group in each experiment; only a protection % >30 should be taken into account. Include protective strain as internal reference
Microbiological considerations	
Bacterial viability and physiological stage	Use bacteria collected at equivalent growth stages
Bacterial dose	Use comparable doses of bacteria and check CFU/ml; Reproducible discriminative results have been obtained with 10^8 CFU/dose in moderate colitis (stationary or exponential phase)

models of colitis (5, 11, 48). Total prevention of colitis using well-known anti-inflammatory drugs or other nutritional or chemical compounds has rarely been noticed either, and the best reported protection did not exceed 50 to 70% (19, 20, 25, 34, 36). The factors that actually limit the level of protection are currently not clear. It would therefore be interesting to study the cumulated effect of traditional anti-inflammatory drugs and probiotics in order to establish if they exhibit a synergistic effect in the acute phase of the TNBS mouse model.

Our study also showed that viability of bacteria might be an important factor in protection against colitis, despite a few recent generalizing claims (8, 9) that waive the need for viable probiotic bacteria. It was recently shown that the effective protection induced by a *Lactobacillus* strain in the DSS experimental colitis model was abolished when using heat-killed bacteria (49). Moreover, a γ-irradiated but not a heat-killed probiotic cocktail (VSL#3) was reported to attenuate the severity of DSS-induced colitis (9). We could thus have expected that UV-killed *L. plantarum* NCIMB 8826 might induce some degree of protection in the TNBS-induced colitis model, which was not found to be the case. Thus, the method of bacterial inactivation (UV treatment, heat-killing, or formalin fixation) undoubtedly plays an important role.

In order to validate the standardized TNBS model, we assessed the immunomodulatory impact of a strain previously demonstrated to prevent or treat inflammation in the more time-consuming DSS and IL-10-deficient mouse models. The recombinant strain *LL*-mIL-10, previously reported to improve colitis in these two models of colitis (34), also exhibited a significant protection in the TNBS model, for both moderate and high levels of colitis. As the DSS model is often associated with crypt distortion and adenomas, and mimics human UC (50), related to a relative deficiency of IL-10 (51), the TNBS model in BALB/c mice (18) and the IL-10-deficient mouse model (52, 53) are usually regarded as Th1 models that mimic the acute phase of CD. Strikingly, results in the mouse TNBS model fully corroborate those obtained in the chronic DSS and spontaneous IL-$10^{-/-}$ models, possibly pointing to a common mechanism of inflammation reduction. In this context, further experiments will need to assess whether the protective capacity of recombinant strains might be improved by combining IL-10 secretion with a carrier strain that naturally exhibits intrinsic protective capacity against chronic intestinal inflammation (e.g., *L. plantarum* NCIMB 8826). We recently started to use the standardized TNBS model to study strains reported to exert either a positive or no effect in human IBD clinical trials. Preliminary results point to a rather good correlation with animal data and are presently being completed.

In summary, by compiling various results for a single reference strain (*L. plantarum* NCIMB 8826) collected in a standardized mouse model of TNBS-induced colitis, we were able to identify and optimize some of the key parameters that need to be controlled in order to ensure reliable comparison of results generated in different experiments. The fact that the model yields results similar to those obtained in the much more tedious DSS and IL-$10^{-/-}$ models, without the need to perform the labor-intensive Ameho scoring or cytokine identification assays, makes this model an excellent and fast tool to compare the anti-inflammatory potential of bacterial strains. The standardization was considerably improved through calculation of the percentage protection, measured under well-known conditions of bacterial growth and viability, and use of controlled levels of colitis. The use of the model as described above allows the construction of long-term databases which can serve as a reference for future strain comparisons, knowing that mechanistic studies will require analysis of many more parameters. The applicability of such a database including strains already used in existing clinical trials will promote a future screening strategy based on the above animal model to select the most interesting strains for future clinical trials.

ACKNOWLEDGMENTS

This work was supported by the EU Grant QLK1-2000-00146 DEPROHEALTH Research Program, Institut Pasteur de Lille funding, and funds from DANISCO France.

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