Original article

Colonic infusion with *Propionibacterium acidipropionici* reduces severity of chemically-induced colitis in rats

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Abstract – Propionibacteria are cheese starters, also studied for their probiotic potential. An interesting feature is the ability of some strains to synthesize nitric oxide (NO) from nitrate reduction. NO production is hypothesized to be the mechanism by which some probiotics improve the mucosal barrier in rats. We therefore investigated the ability of two *Propionibacterium acidipropionici* strains (TL15 & TL223), which differ in their in vitro production of NO, to improve colitis in rats. Three groups of rats in which colitis was induced by trinitrobenzene sulfonic acid (TNBS), received intracolonic infusions of 9 g·L^{-1} NaCl (n = 6), or 0.9×10^{10} CFU·d^{-1} of TL15 (n = 7), or 1.4×10^{10} CFU·d^{-1} of TL223 (n = 7), for 7 days. Following treatment, animal weight, food consumption, inflammatory score and myeloperoxidase (MPO) activity, together with changes in intestinal bacteria and SCFA concentrations, were investigated. As expected, both TL223 and TL15 supplements induced detectable counts of *P. acidipropionici* in colonic contents of rats (7.67 ± 0.67 and 7.43 ± 0.50 log eq. CFU·g^{-1}, respectively, as determined by real-time PCR). Food consumption and body weight of rats receiving propionibacteria were overall higher than those of rats with NaCl (P = 0.007 and P = 0.004). Moreover, both propionibacteria infusions similarly and dramatically reduced the gross score for inflammation (P = 0.004) as compared with NaCl (0.6 ± 0.3 and 0.7 ± 0.5 vs. 4.8 ± 1.5). A similar effect was observed for MPO activity (P = 0.056), which reached 2.50 ± 1.37 U·g^{-1} of mucosal tissue for NaCl vs. 0.05 ± 0.02 and 0.26 ± 0.18 U·g^{-1} for TL223 and TL15, respectively. This study shows for the first time the potential benefit of propionibacteria in colonic mucosa healing, but the mechanism involved needs to be elucidated.

Propionibacterium / probiotic / colonic ulceration / nitric oxide / real-time PCR

Résumé – L'administration colique de *Propionibacterium acidopropionici* atténue la sévérité de la colite induite chez le rat. Les bactéries propioniques, utilisées comme levains fromagers, sont également étudiées pour leurs propriétés probiotiques. Une caractéristique intéressante concerne la capacité de certaines souches à synthétiser du monoxyde d'azote (NO) lors de la réduction des nitrates. La production de NO est évoquée comme mécanisme d'action de certains probiotiques capables de restaurer la muqueuse intestinale chez des rats. Nous avons donc déterminé l'impact de deux souches de *Propionibacterium acidipropionici* (TL15 & TL223) présentant des capacités de synthèse de NO différentes in vitro, sur des colites induites chez le rat. Trois groupes de rats à colite induite par le trinitrobenzene sulfonic acid (TNBS) ont reçu pendant 7 j des infusions intracoliques de solution

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saline (9 g·L^{-1} NaCl), ou de suspensions de TL15 (0,9 × 10^{10} UFC·j^{-1}) ou de TL223 (1,4 × 10^{10} UFC·j^{-1}). Les effets des traitements sur le poids des animaux, leur consommation alimentaire, le score inflammatoire, l'activité myéloperoxydase (MPO) et sur les concentrations intestinales en bactéries et en acides gras à chaîne courte ont été recherchés. Comme attendu, les infusions de TL223 et de TL15 ont induit des niveaux de population détectables de *P. acidipropionici* dans les contenus coliques des rats (respectivement 7,67 ± 0,67 et 7,43 ± 0,50 log éq. UFC·g^{-1}, déterminés par PCR quantitative). La consommation alimentaire et le poids des animaux recevant les propionibactéries étaient globalement supérieurs à ceux des animaux recevant le NaCl ($P = 0,007$ et $P = 0,004$). Le score macroscopique inflammatoire observé en présence de NaCl (4,8 ± 1,5) était très nettement ($P = 0,004$) diminué par la supplémentation en propionibactéries, sans différence entre les 2 souches (0,6 ± 0,3 et 0,7 ± 0,5 pour TL223 et TL15). Un résultat similaire a été obtenu pour l'activité MPO ($P = 0,056$), qui atteignait 2,50 ± 1,37 U·g^{-1} de tissu mucosal pour NaCl alors qu'elle n'était que de 0,05 ± 0,02 et 0,26 ± 0,18 U·g^{-1} pour TL223 et TL15, respectivement. Cette étude démontre pour la première fois le bénéfice potentiel des propionibactéries pour la cicatrisation de la muqueuse colique, mais le mécanisme mis en jeu reste à déterminer.

Propionibacterium / **probiotique** / **ulcération colique** / **oxyde nitrique** / **PCR quantitative**

1. INTRODUCTION

Dairy propionibacteria are largely used as starters in cheese technology. Besides this application, they are also studied for their probiotic properties. Indeed, *Propionibacterium freudenreichii* is able to stimulate the growth of bifidobacteria in vitro [15, 29] and some selected strains partially survive during their transit in the human gut and display bifidogenic activity [5, 12]. *Propionibacterium acidipropionici* CRL 1198 is able to survive in mice intestine, adhering to the epithelium, and induces positive effects on lipid metabolism, on intestinal flora activity [35, 36, 48] and on immune response [2, 27, 35].

Moreover, some strains belonging to *P. freudenreichii* and *P. acidipropionici* species are known to reduce nitrate [7]. Nitrous oxide (N$_2$O) [16] and more recently nitric oxide (NO) are described as final products of nitrate and nitrite reduction. Such NO production has been demonstrated in vitro for a particular strain of *P. acidipropionici* [3]. NO synthesis has been proposed as the potential mechanism by which some probiotics could reduce inflammation or improve the barrier function in animal models of colonic dysfunctions [1, 19]. NO synthesis naturally occurs in the intestine due to both epithelial cells and gut-associated immune cells. In this environment, it participates in the defence mechanisms of the intestinal mucosa against exogenous compounds [37].

Because significantly higher NO concentrations and NO synthase (NOS) activities have been detected in patients suffering from inflammatory bowel diseases (IBD) as compared with healthy subjects [38], NO has first been suggested as being involved in the inflammatory process. This deleterious impact is supported by the biological properties of NO. Indeed, NO can modulate COX-2-dependent production of prostaglandins, promote leucocyte chemotaxis, stimulate production of proinflammatory cytokines and activate lymphocytes. NO is also supposed to favor the production of ONOO$^-$, which is a potent oxidant, through interaction with reactive oxygen species such as O$_2^{\bullet}$ [34].

However, experimental studies aimed at assessing the benefit of inhibiting NO production on mucosal healing are far from being conclusive [18]. The administration of selective (L-NIL and 1400w) or nonselective NOS inhibitors (L-NAME and L-NMMA) has been reported to attenuate colonic injury in animals with chemically-induced colitis [26]. However, in other studies, these inhibitors were ineffective at attenuating colitis in different animal models [43]. Furthermore, other findings suggest that NO could be of benefit in colitis therapy: L-NAME treatment worsened dextran-sulfate-induced inflammation in rats [47], L-NMMA increased colonic damage when administered in the first 3 days of colitis development [9], and knock-out mice,

Table I. List of strains used in the study and growth conditions. Strains of *Propionibacterium* spp. were obtained from different international collections: TL, the collection of the dairy research laboratory UMR-STLO of INRA (Rennes, France); CIP, Bacterial Collection of Pasteur Institute (Paris, France); DSM, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). YEL: Yeast Extract Lactate medium [22]; PYG: Peptone Yeast Glucose medium (DSM culture conditions).

Collection name	Species	Culture medium and temperature
TL223, TL15, TL2, TL54, TL9	*P. acidipropionici*	YEL, 30 °C
CIP 103029[T]	*P. thoenii*	YEL, 30 °C
CIP 103028[T]	*P. jensenii*	YEL, 30 °C
CIP 103027[T], TL14, TL147, TL162, TL166	*P. freudenreichii* subsp. *shermanii*	YEL, 30 °C
CIP 103026[T], TL3	*P. freudenreichii* subsp. *freudenreichii*	YEL, 30 °C
DSM 13435[T]	*P. microaerophilum*	PYG, 37 °C
CIP 101941[T]	*P. propionicus*	PYG, 37 °C
CIP 53117[T]	*P. acnes*	PYG, 37 °C
CIP 103262[T]	*P. granulosum*	PYG, 37 °C
CIP 103261[T]	*P. avidum*	PYG, 37 °C

which are deficient in iNOS, not only develop a more severe experimental colitis than wild-type mice, but also exhibit delayed healing of their colonic mucosa [25]. Such a beneficial effect of NO has also been suggested from the increased anti-inflammatory activity of NO-mesalamine, as compared with mesalamine alone [44].

Taking all this into account, we investigated whether propionibacteria could improve the mucosal healing in rats in which colitis was induced by trinitrobenzene sulfonic acid (TNBS) and whether this property was related to their NO synthesis ability. As first steps in this goal, different dairy *Propionibacterium* strains were screened in vitro with respect to their ability to produce NO from nitrate, and a specific and quantitative method for *P. acidipropionici* enumeration by real-time PCR was developed.

2. MATERIALS AND METHODS

2.1. Bacterial strains

All the strains of propionibacteria used in this work and their culture conditions are listed in Table I. Cells were stored at –80 °C in broth culture containing 15% glycerol. Prior to use, they were subcultured twice (2% inoculum) as indicated.

2.2. Nitrate reduction and analysis of NO production in bacterial cultures

Propionibacteria were grown on YEL medium for 3 d at 30 °C. Then, subsequent cultures were carried out for 72 h on KNO_3-containing YEL medium (final concentration 650 $\mu mol \cdot L^{-1}$) under a helium atmosphere. At the end of the incubation, NO production was assessed by isotope ratio mass spectrometry (IRMS) according to [3] and residual nitrates and nitrites were quantified in the cell-free supernatants of cultures using a colorimetric test (Boehringer-Mannheim, Meylan, France). Assays were carried out in duplicate.

2.3. Preparation of bacterial suspensions for in vivo experiment

Strains TL223 and TL15 were cultivated in YEL medium supplemented with

500 µmol·L^{-1} KNO$_3$ for 3 d at 30 °C. After centrifugation (8000 rpm, 20 min, 4 °C), the pellets were washed and suspended in NaCl 9 g·L^{-1} (cell concentration × 4 compared with initial cultures). Suspensions were aliquoted into 30-mL portions and frozen at –20 °C before use. From enumeration on YEL agar, counts of propionibacteria before and after freeze were, respectively, 1.4×10^{10} and 7.1×10^9 CFU·mL^{-1} for TL223 and 7.0×10^9 and 4.3×10^9 CFU·mL^{-1} for TL15.

2.4. Animals and treatments

Twenty male Wistar rats (Janvier, Le Genest Saint Isles, France), with an initial mean weight of 250 g, were housed individually in suspended cages with wire-mesh bottoms and maintained at 23 °C in an animal room with a 12-h light:dark cycle. Food and water were consumed ad libitum, and food intake and body weight were recorded daily. Individual values obtained from day 1 to day 7 were averaged. The diet contained (g·kg^{-1}): gelatinized cornstarch (626), soluble casein (204), lard (58.5), a mineral mixture (43.7), cellulose (40), corn oil (18.5), a vitamin mixture (5.6) and DL-methionine (3.7). The composition of the mixtures has been detailed previously [20]. The experiment was in accordance with the recommendations of the local Animal Care and Use Committee of Nantes (France).

The rats were surgically equipped with a chronic catheter (1.0 mm i.d., 1.8 mm o.d., Tygon® Tubing, Fisher Bioblock Scientific, Illkirch, France) fixed in the proximal colon, 1.5 cm beyond the ceco-colonic junction according to [6]. After 5 d of post-operative recovery, the animals were submitted to colitis induction with trinitrobenzene sulfonic acid (TNBS, 80 mg·kg^{-1}, dissolved in ethanol 400 g·L^{-1}) as described in [6]. The rats were then randomly assigned to 3 groups. Each group was administered with one of the following treatments: saline (9 g·L^{-1} NaCl), TL223 suspension or TL15 suspension for the next 7 d. The treatments (1 mL) were administered through the catheter twice daily (08:00 a.m. and 05:00 p.m.). The TL223 and TL15 suspensions thus delivered 1.4×10^{10} and 0.9×10^{10} CFU per day, respectively.

Fresh stools were collected from the anus of each animal on the day before TNBS infusion and on day 6 (i.e., 1 day before slaughtering) and immediately processed for bacterial enumeration.

At the end of the treatment, the rats were killed by cervical dislocation, 6 h after the last administration. The large bowel was dissected free from the fat and mesentery, removed, and opened along the mesenteric border.

An aliquot of caecal content was collected and added to 1 mL of a HgCl$_2$/H$_3$PO$_4$ solution (1 g·L^{-1} and 50 g·L^{-1}, respectively), then frozen (–20 °C) before SCFA analysis.

The colonic contents were collected and divided into 2 parts: (1) approx. 0.5 g was collected into a sterile tube for immediate total anaerobe enumeration, and (2) residual material was frozen (–80 °C) in view of *Propionibacterium acidipropionici* quantification by real-time PCR. Then, the mucosa was carefully cleaned with cold sterile saline and colonic damage and inflammation were assessed as described below. Two colonic tissue samples (1 cm^2) were collected for immediate enumeration of mucosa-associated aerobes and anaerobes and for myeloperoxidase (MPO) activity measurement, respectively. The latter was frozen before analysis (–80 °C).

2.5. Control of *P. acidipropionici* delivery

The 16S-23S rDNA intergenic spacer region was used to select primers specific to *P. acidipropionici* species. The primers used (QPACIDI for: 5' GATGGTTCGGTT-CAGGCTGTC 3' and QPACIDI rev: 5' CCATAGCCACATTCCACTCAG 3') were newly designed using the Primer Express® software (PE Applied Biosystems, Foster City, CA, USA) and were synthesized by Qiagen Operon Europe (Cologne, Germany). The expected size of the amplified DNA was 69 bp. DNA was extracted from 2-day-old pure cultures of different *Propionibacterium* species (strains TL223, TL15, CIP 103029^T, CIP 103028^T, CIP 103027^T, CIP 103026^T, DSM 13435^T, CIP 101941^T, CIP 53117^T, CIP 103262^T and CIP 103261^T, Tab. I) and from the colonic

content of the rats, using the QIAamp tissue kit and QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. Real-time PCR was conducted using an Applied Biosystems 7000 instrument (Applied Biosystems, Foster City, CA, USA). Reaction mixtures consisted of a total volume of 25 μL containing 12.5 μL 2 X SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 8 pmol of each specific primer. Genomic DNA was added at a level of 10 ng or 50 ng for specificity or calibration studies, respectively. In both cases, the conditions for amplification were 1 cycle at 95 °C for 10 min, followed by 35 cycles of 15 s at 95 °C and 60 s at 60 °C. The purity of the amplicon was verified by analyzing the melting curve over a range of 60 to 99 °C, with incrementations set at $0.2\ °C \cdot s^{-1}$. Assays were carried out in triplicate for each DNA sample. To quantify *P. acidipropionici*, a standard curve was constructed and used to interpolate the number of copies present in the rat colonic samples. For this, 100 μL of ten-fold successive dilutions of the bacteria were added to 200 mg of the colonic content of the rats without *P. acidipropionici* flora, and thoroughly mixed before DNA extraction.

2.6. Assessment of colonic damage and inflammation

Gross colonic damage was scored by an independent observer, unaware of the treatments, according to the scale described by [30]. Each colon was assigned a score ranging from 0 (normal) to 15 (severe damage) indicative of adhesions, ulcerations, gross inflammation of the colonic wall and diarrhea.

The activity of myeloperoxidase (MPO), a specific enzyme marker of polymorphonuclear neutrophil primary granules, was measured using the method of [17] as modified by [6]. Activity was expressed in units per milligram of tissue.

2.7. Analysis of luminal and mucosa-associated floras

All samples were added (5 mL for mucosal samples and 9 mL for stools and for intra-colonic contents) to sterile and anoxic peptone water ($7.5\ g \cdot L^{-1}$, Oxoid, Dardilly, France) containing cysteine ($0.5\ g \cdot L^{-1}$, Sigma, St-Quentin-Fallavier, France) immediately after collection. Mucosal sample suspensions were homogenized under N_2 flux using an ethanol-sterilized Polytron for 5 min. Fecal and colonic samples were homogenized by vortex for 3 min. All samples were serially diluted ten-fold in the same diluant. 100 μL of the appropriate dilutions were inoculated on duplicate plates using unselective media for the enumeration of total anaerobes (Wilkins-Chalgren agar, Oxoid, Dardilly, France) and total aerobes (Nutrient agar, Oxoid, Dardilly, France). Plates were incubated at 37 °C in anaerobic (H_2:CO_2:N_2 – 5:10:85) or aerobic conditions, as appropriate, for 72 h. Single colonies were then counted and the results were expressed as $\log CFU \cdot g^{-1}$ (wet weight) or $\log CFU \cdot cm^{-2}$, as appropriate.

2.8. SCFA analysis

SCFA were quantified by gas chromatography [14] on supernatants of thawed samples of cecal contents centrifuged at 8000 g for 10 min. 4-methyl valeric acid was used as an internal standard. Concentrations were expressed as $mmol \cdot L^{-1}$.

2.9. Statistical analysis

Statistical analysis was performed using the Statview 5.0 package (SAS institute, Berkeley, CA, USA). Data are expressed as means ± sem. One-way ANOVA was used to assess the effects of treatments. When a significant effect was observed ($P < 0.10$), individual means were compared by Fisher's Protected Least Significant Difference test. The paired Student's t-test was used to compare the total anaerobe counts enumerated in stools before and after treatment.

3. RESULTS

3.1. In vitro NO production and strain selection

NO production by the different propionibacteria cultivated in the presence of KNO_3 was strain-dependent (Figs. 1a, 1b).
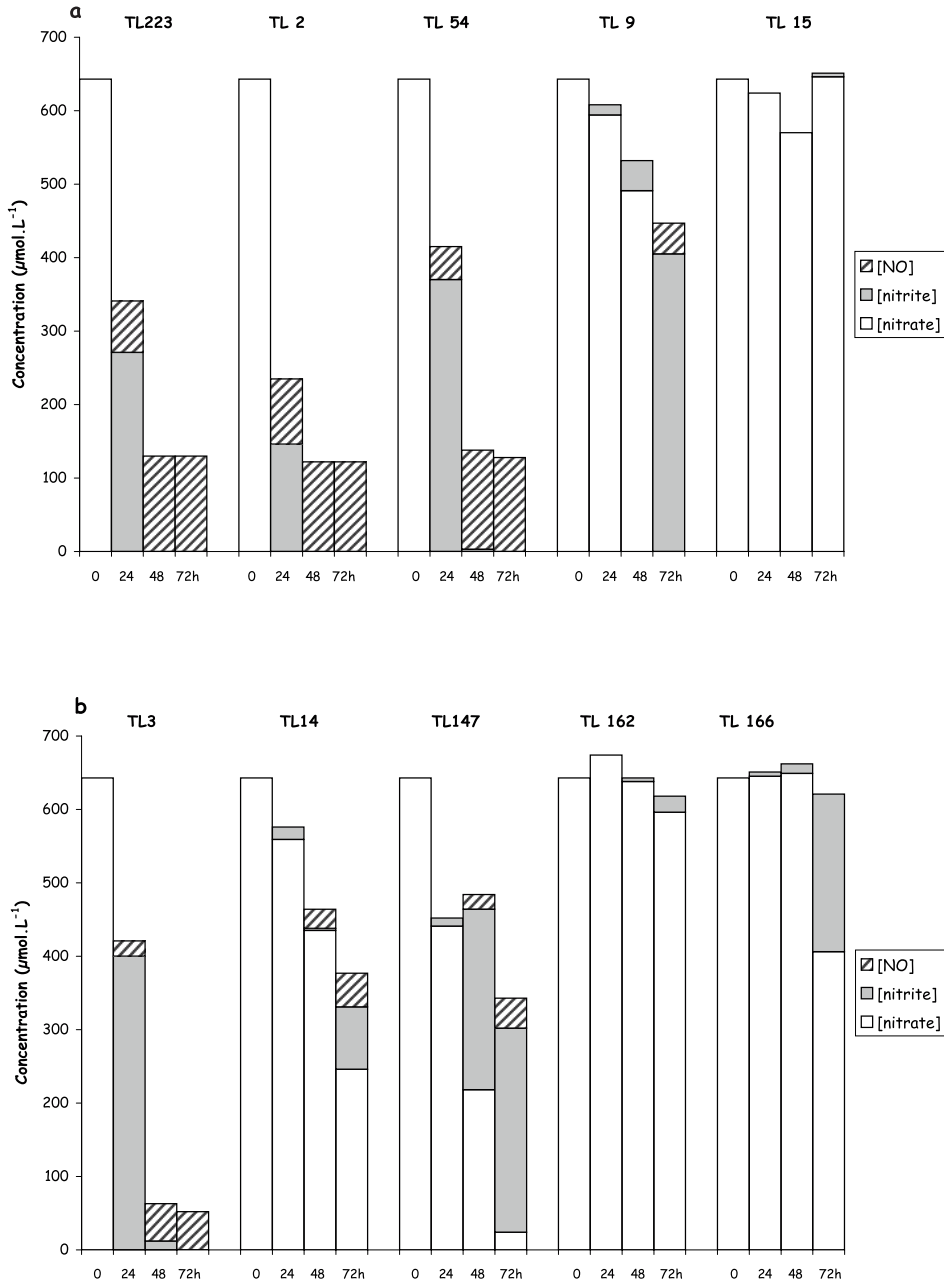
Figure 1. Concentration of nitrate and nitrite in cell-free supernatant of bacterial cultures at 0, 24, 48 and 72 h of incubation at 30 °C in YEL medium containing 650 $\mu mol \cdot L^{-1}$ nitrate, and concentration of NO accumulated in the tubes of culture (expressed as $\mu mol \cdot L^{-1}$ of culture) for strains of *P. acidipropionici* (a) and *P. freudenreichii* (b).

The greatest accumulation of NO occurred after 48 h with strains of *P. acidipropionici* rapidly reducing nitrate then nitrite (TL223, TL2, TL54) and, to a lesser extent, with one strain of *P. freudenreichii* (TL3). Some strains were unable to use nitrate (TL15 and TL162) or reduced it to nitrite very slowly (TL166). In this case, no accumulation of NO was observed. The other strains (TL14, TL147 and TL9) slowly reduced nitrate and nitrite but accumulated significant amounts of NO after 72 h.

In view of these results, TL223, which produced high amounts of NO, and TL15, which was unable to convert nitrate into NO, were selected for the in vivo experiment.

3.2. In vivo experiment

3.2.1. Control of P. acidipropionici delivery

Using genomic DNA from different propionibacteria species and the newly designed primer set, PCR amplification was only obtained for *P. acidipropionici*. This illustrates the specificity of the method developed towards this particular species among propionibacteria. The equation of the calibration curve was $Ct = -3.37 \log (eq. CFU) + 38.34$, with the coefficient of correlation (r^2) being 0.997. The PCR efficiency was thus calculated as 97.6% and the detection threshold was 10^5 eq. $CFU \cdot g^{-1}$ of content.

When this method was applied to the colonic contents of all experimental animals, no signal was obtained in the control group, indicating that the level of *P. acidipropionici* was lower than 10^5 eq. $CFU \cdot g^{-1}$. Conversely, high and similar numbers of *P. acidipropionici* were detected in the colonic contents of rats receiving TL223 and TL15 (7.67 ± 0.67 and 7.43 ± 0.50 $CFU \cdot g^{-1}$, respectively, $P = 0.773$).

3.2.2. Effects of treatments on rats' status and inflammation severity

Before colitis induction (day 0), all animals exhibited similar daily food intakes (20.8 ± 1.3 $g \cdot d^{-1}$, $P = 0.50$). However, following colitis induction (day 1 to day 7), the rats receiving saline consumed significantly lower amounts of food (17.6 ± 1.1 g on average on the whole follow-up duration) than those receiving TL223 (23.8 ± 1.1 g, $P = 0.004$) or TL15 (23.3 ± 1.5 g, $P = 0.006$), which did not differ from each other ($P = 0.79$). Similarly, although comparable body weights were observed for all rats before TNBS treatment (day 0: 287.8 ± 2.1 g, $P = 0.18$), the mean body weight of rats receiving saline (280.3 ± 4.9 g) was significantly lower than that of rats receiving TL223 (295.5 ± 5.5 g, $P = 0.03$) or TL15 (292.4 ± 2.6 g, $P = 0.08$) following colitis induction (day 1 to day 7). Again, there was no statistical difference ($P = 0.63$) between the two *P. acidipropionici* strains.

Both TL223 and TL15 infusions considerably reduced inflammation compared with NaCl after only 7 d of treatment. Indeed, the colonic mucosa of rats receiving NaCl were severely inflamed at this stage, as indicated by their high macroscopic damage score (4.8 ± 1.5) and by the elevated MPO activity of their mucosa (2.50 ± 1.37 $U \cdot g^{-1}$, Fig. 2). The beneficial impact of TL223 and TL15 infusions was illustrated by the significantly lower values observed for both the macroscopic score (0.6 ± 0.3, $P = 0.003$ for TL223 and 0.7 ± 0.5, $P = 0.003$ for TL15) and MPO activity (0.05 ± 0.02, $P = 0.029$ for TL223 and 0.26 ± 0.18, $P = 0.043$ for TL15), while no difference was detected between the two strains ($P = 0.83$ and $P = 0.90$ for the macroscopic score and MPO activity, respectively).

3.2.3. Effects of treatments on intestinal flora and SCFA concentrations

Neither the total anaerobe counts in the colonic contents nor the total anaerobe and total aerobe counts associated with the colonic mucosa were affected by TL223 or TL15 infusions (Tab. II). Before colitis induction, all animals exhibited similar total anaerobe counts in their stools ($P = 0.90$, Fig. 3). However, on the 6th day after colitis induction, the rats receiving saline exhibited significantly lower total fecal anaerobe counts than those receiving TL223
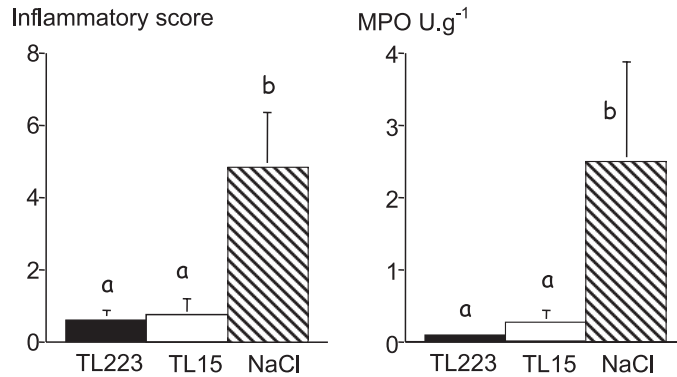
Figure 2. Macroscopic damage scores and myeloperoxidase (MPO) activity in the colonic mucosa of rats with TNBS-induced colitis after intracolonic treatment with NaCl, *P. acidipropionici* strain TL15 or *P. acidipropionici* strain TL223. Values are means ± sem. Bars with a different letter differ significantly ($P < 0.1$).

Table II. Intracolonic and mucosa-associated bacterial counts from rats with TNBS-induced colitis, after intracolonic treatment with *P. acidipropionici* strain TL223, *P. acidipropionici* strain TL15 or NaCl. Values are means ± sem.

	TL223	TL15	NaCl	*P* value
Total anaerobes in colonic content (log CFU·g^{-1})	8.98 ± 0.46	9.74 ± 0.28	9.21 ± 0.20	0.285
Total anaerobes on mucosa (log CFU·cm^{-2})	7.41 ± 0.33	6.97 ± 0.20	6.96 ± 0.42	0.522
Total aerobes on mucosa (log CFU·cm^{-2})	5.74 ± 0.58	6.19 ± 0.44	6.15 ± 0.62	0.810

($9.13 ± 0.24$ versus $9.80 ± 0.10$ log CFU·g^{-1}, $P = 0.017$) while those receiving TL15 showed an intermediate value ($9.52 ± 0.17$). Interestingly, t-test comparison on paired data between these two days showed that the decrease in total fecal anaerobe numbers which occurred with NaCl was not observed when TL223 and TL15 were infused (Fig. 3).

Intracolonic pH was not affected by treatments (Tab. III). Conversely, total SCFA concentration was significantly lower in animals infused with TL223 and TL15 as compared with controls, with no difference ($P = 0.84$) between the two *P. acidipropionici* strains (Tab. III). Similarly, lower concentrations of propionate were observed for animals receiving the bacterial suspensions.

4. DISCUSSION

This study was carried out in order to assess the ability of some propionibacteria to relieve TNBS-induced colitis in rats and to evaluate whether this beneficial effect could be related to the ability of the strain to produce NO from nitrate.

In this respect, we first determined the in vitro denitrifying ability of different strains of *P. acidipropionici* and *P. freudenreichii*. Our results indicated that nitrate-reducing propionibacteria were able to produce high and steady amounts of NO and that this property appeared more pronounced in *P. acidipropionici* species, although it was strain-dependent. In NO-producing cultures, this compound accounted for up to

Table III. pH values and short-chain fatty acid concentrations in cecal contents of rats with TNBS-induced colitis, after intracolonic treatment with *P. acidipropionici* strain TL223, *P. acidipropionici* strain TL15 or NaCl. Values are means ± sem. Within a same raw, values with a different letter differ significantly ($P < 0.1$).

	TL223	TL15	NaCl	*P* value
n	7	7	6	
Cecal pH	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	0.615
Total SCFA $\mu mol \cdot g^{-1}$	125.3 ± 7.6 a	123.6 ± 3.7 a	143.8 ± 6.2 b	0.065
Acetate $\mu mol \cdot g^{-1}$	87.7 ± 8.6	80.2 ± 7.3	90.7 ± 11.1	0.700
Propionate $\mu mol \cdot g^{-1}$	14.6 ± 0.9 a	19.3 ± 0.9 b	22.1 ± 0.8 c	< 0.0001
Butyrate $\mu mol \cdot g^{-1}$	20.6 ± 5.2	21.7 ± 5.0	28.7 ± 8.5	0.630
Minor[†] SCFA $\mu mol \cdot g^{-1}$	2.4 ± 0.2	2.3 ± 0.4	2.3 ± 0.4	0.978

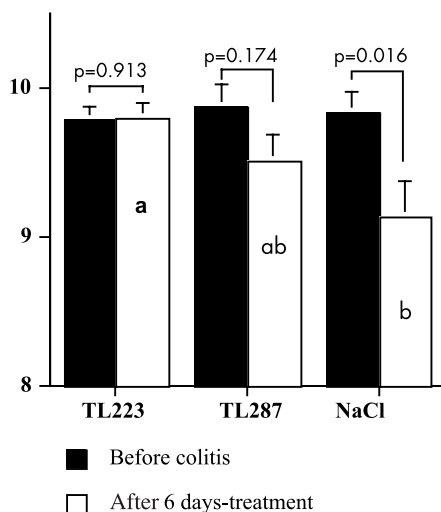[†] Sum of isobutyrate, isovalerate and valerate.

Figure 3. Total anaerobe counts in feces of rats with TNBS-induced colitis before and after intracolonic treatment with NaCl, *P. acidipropionici* strain TL15 or *P. acidipropionici* strain TL223 for 6 d. Values are means ± sem. Bars from day 6 with a different letter differ significantly ($P < 0.1$).

20% of the initial pool of nitrate. The apparent loss of 80% of the initial nitrate was probably due to nitrous oxide production, which could not be quantified in our experimental conditions, since such N_2O accumulation has been reported by [16] for both *P. acidipropionici* and *P. freudenreichii* strains.

Production of bacterial NO has been extensively described in established environmental denitrifiers such as *Pseudomonas* and *Paracoccus* [49] but it is very unusual for bacteria which are encountered in the intestine. Indeed, the commensal intestinal flora can efficiently reduce nitrate and nitrite but this phenomenon occurs mainly via a dissimilatory reaction where ammoniac is the final product of nitrite reduction [33]. One exception is some enteric bacteria, which can produce small amounts of NO from nitrite reduction [13]. As far as food or probiotic bacteria are concerned, the ability to produce NO from nitrate has only been reported for *Lactobacillus farciminis* [45] and *Lactobacillus fermentum* [46].

Anyhow, the aim of this NO-synthesis screening was to select the strains which would be assessed in vivo with respect to their healing ability against induced colitis. We therefore chose the TL223 and TL15 strains because they were typified in our in vitro conditions as "NO-positive" and "NO-negative" strains, respectively, and because they belong to the same species (*P. acidipropionici*) which will minimize differences in their phenotypic characteristics.

A specific and quantitative method for *P. acidipropionici* enumeration was thus required to control the comparability of *P. acidipropionici* delivery in both animal groups infused with the two bacterial

strains. By targeting the 16S-23S rDNA intergenic spacer region, we developed in this work a molecular approach which allowed the specific quantification of *P. acidipropionici* species in a digestive environment. Both the PCR efficiency and linearity were satisfactory. The detection limit obtained in this work (10^5 CFU·g^{-1}) is intermediate between that described in human stools for the detection of *Lactobacillus helveticus* (10^4 CFU·g^{-1}) and various species of *Bifidobacterium* (10^6 CFU·g^{-1}) [24, 40]. When applied to the colonic contents of experimental animals, this method revealed that *P. acidipropionici* supplementation was strictly comparable in both animal groups infused with the bacteria.

In this context, our in vivo data demonstrated the beneficial impact of the two investigated strains of *P. acidipropionici* towards colonic inflammation in the TNBS-induced colitis rat model. Indeed, intracolonic infusions of TL223 and TL15 suspensions in NaCl restored both the daily food intake and the animal body weight as compared with NaCl alone. Weight loss and the slight anorexia observed with saline are typical of colonic inflammations, as previously observed in animals with chemically-induced colitis [6, 23] or in patients suffering from inflammatory bowel diseases [39]. Administration of both bacterial strains efficiently reduced these symptoms immediately after colitis induction, suggesting that *P. acidipropionici* may exert beneficial effects on the onset of the inflammation. Moreover, this administration significantly reduced the severity of the intestinal inflammation induced by TNBS, producing less damage to the mucosa and decreasing MPO activity in the colonic wall. These observations confirm previous data indicating that some probiotics may protect against experimental intestinal inflammation [6, 19, 21, 23, 31], against inflammatory bowel diseases [41] or against dysfunction of the intestinal barrier [1]. Some of these studies have hypothesized that this benefit originates from NO synthesis by probiotics [1, 19]. This suggestion cannot be supported by our findings since the two bacterial suspensions infused were similarly efficient at reducing colonic inflammation while differing in their in vitro ability to convert nitrate into NO. However, the contribution of bacterial NO synthesis cannot be definitively excluded, since in vivo NO production was not quantified in this study. In vivo conditions may have affected bacterial NO production by propionibacteria as compared with in vitro ones, and have stimulated NO production from TL15. Alternatively, considering that the commensal intestinal bacteria intensively reduce nitrate to ammonia [33], the in vivo conditions may also have inhibited NO production by TL223 due to competition for nitrate.

Numerous other mechanisms have been proposed in order to explain the benefit of probiotics towards colonic inflammation [41]. Among them is the interaction between the probiotic and the colonic flora, resulting either in modulation of colonic mucosal adherent flora or of the luminal flora [21]. Such a possibility needs to be considered for propionibacteria since some of these bacteria have been reported to be highly adherent to epithelial cells both in vitro and in vivo [10, 32, 48] while another study has demonstrated that propionibacteria administration to humans increases the fecal numbers of *Bifidobacterium* sp. [5]. In our study, neither the luminal nor the mucosa-associated bacteria numbers were affected by propionibacteria supplementation. However, this does not exclude some modulation of the bacterial composition which has not been characterized here. The only detected effect of *P. acidipropionici* infusions on intestinal flora was a restoration of the initial bacterial density in feces after 6 d of treatment. Taking into account that stools from animals receiving NaCl alone were more watery than those from animals receiving the propionibacteria suspensions (data not shown), the change in fecal bacterial numbers induced by TL15 and TL223 probably results from a lower dilution of the bacteria in the feces. It would therefore be subsequent rather than initiating in the process of mucosa healing.

Other potential mechanisms of probiotics are: (i) antagonism to pathogenic bacteria through acidification of the ceco-colonic lumen; (ii) production of SCFA which beneficially affect numerous key functions of

the colonic epithelium [4, 11], and (iii) interaction with the immune response [41]. From our observations, neither of the two former mechanisms seems involved. Indeed, administration of propionibacteria suspensions did not affect the luminal acidity of the hindgut and SCFA concentration was not increased but decreased when TL15 and TL223 were infused. Although apparently contradictory to the well-known propionate production by *Propionibacterium* sp. [7], such a decrease in intracolonic SCFA concentrations has already been observed in TNBS-induced colitic rats which were treated with a mix of lactic acid bacteria as compared with those treated with NaCl alone [6]. In this case, the decrease in SCFA concentrations was observed only after 14 d of administration, i.e. at the stage where the benefit of the treatment was maximal. Considering this, one could hypothesize that the lowering impact of propionibacteria on the intracolonic concentration of SCFA would be subsequent to their healing effect on the mucosa, through restoration of its uptake potential. This scenario is supported by the fact that inflamed colonic mucosa exhibit a decreased SCFA absorption ability together with a decreased SCFA metabolism as compared with a healthy one, but these decreases are limited to the acute phases of inflammation and are rapidly compensated with healing [8, 28].

Whether or not modulation of the immune response is involved in the beneficial impact of TL15 and TL223 could not be answered in this study since no immunological parameters were followed up. However, this possibility needs to be considered since some *P. acidipropionici* strains increase phagocytose and IgA production in both in vitro and in vivo conditions [2, 27, 36], two properties which are supposed to be involved in the protective effect of probiotics against intestinal inflammation [41, 42].

5. CONCLUSION

Finally, our results demonstrated for the first time a beneficial impact of *P. acidipropionici* on colonic inflammation in the TNBS-induced colitis rat model, with this benefit appearing to be independent from the in vitro ability of the strains to produce nitric oxide from nitrate. Thus, the involved mechanisms of *P. acidipropionici* need to be further investigated.

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