

## Effects of *Bacillus subtilis* 'PB6' (ATCC - PTA 6737) on *Clostridium difficile* Associated Diarrhea (CDAD) and Inflammatory Bowel Disease (IBD) in Animal Models

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**Abstract:** The administration of probiotic bacteria is emerging as a potential means of preventing the onset or recurrence of *Clostridium difficile* associated diarrhea (CDAD) and of attenuating inflammatory activity and preventing relapses in inflammatory bowel disease (IBD). We evaluated the efficacy of *Bacillus subtilis* 'PB6' (ATCC – PTA 6737) in a hamster model of antibiotic-induced CDAD and in a rat model of IBD. CDAD was induced in male Golden Syrian hamsters using *C. difficile* and clindamycin. These hamsters received either nothing or, by gavage, vancomycin (5 days) or PB6 (low, middle and high dose, 6 days). Diarrhea, body weight loss and mortality were observed in all groups in which CDAD was induced. Intensity of diarrhea and body weight loss was least in the groups treated with vancomycin or with the highest dose of PB6. At the end of the treatment period, vancomycin and the highest dose of PB6 were equally efficient in preventing mortality in this hamster model of CDAD. No adverse effects of PB6 treatment were observed in healthy animals. In male Wistar rats, colitis was induced using a single intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS). Treatments consisted of PB6 (low, middle and high dose), *Saccharomyces boulardii*, mesalazine, infliximab, or no treatment. A possible benefit of the prophylactic use of PB6 was also tested. At the end of the treatment period significant differences in body weight gain, in colon inflammatory edema and in gross morphology of the colon intestinal lining were observed between groups. The groups treated with high dose PB6 could not be distinguished from the colitis-free negative control group nor from the group treated with mesalazine. The data presented are suggestive of possible therapeutic effectiveness of PB6 in CDAD and IBD in humans.

**Key words:** *Bacillus subtilis*, *Clostridium difficile* associated diarrhea (CDAD), inflammatory bowel disease (IBD), animal model

### INTRODUCTION

In 1965 the term “probiotics” was used for the first time to indicate “substances produced by microorganisms which promote the growth of other microorganisms”<sup>[1]</sup>. Since then, the definition of probiotics has been modified several times. A widely used definition, developed by the FAO/WHO, calls probiotics “defined, live microorganisms administered in adequate amounts which confer to a beneficial physiological effect in the host”.

Research and trials have shown that probiotics are or may become an effective therapy for gastrointestinal infections, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)<sup>[2]</sup>.

PB6 (ATCC-PTA 6737, Anaeban™, Kemin Pharma) is a unique and natural *Bacillus subtilis* strain,

that has shown *in vitro* to produce antimicrobial substances broadly active against various strains of *Campylobacter* sp. and *Clostridium* sp. and more specifically against *C. difficile* (27 November 2002, US Patent Office).

*Clostridium difficile* was first isolated by Hall and O' Toole in 1935<sup>[3]</sup> and originally named *Bacillus difficilis*. In 1938 this species was reclassified as *C. difficile* (type strain ATCC 9689). Only in the late 1970's, it was associated with antibiotic-related diarrhea. *C. difficile* is a spore-forming gram-positive anaerobic bacillus. Toxigenic strains can produce at least two exotoxins, virulence factors responsible for *C. difficile* disease: toxin A, primarily an enterotoxin and toxin B, a cytotoxin. Colonization occurs via the fecal-oral route. Ingested spores of *C. difficile* survive the gastric acid barrier and germinate in the colon<sup>[4,5]</sup>.

*C. difficile* is the primary cause of hospital-acquired infectious diarrhea and colitis when patients receive antibiotics, chemotherapeutics or other drugs that alter their normal intestinal flora. Antibiotics most frequently associated with the infection are clindamycin, ampicillin, amoxicillin and cephalosporins, however all antibiotics might predispose a patient to *C. difficile* infection<sup>[6, 7]</sup>. The clinical presentation varies from asymptomatic colonization to mild diarrhea to severe debilitating disease, with high fever, severe abdominal pain, pseudomembranous colitis, toxic megacolon, colonic perforation, or even death<sup>[8-10]</sup>. *Clostridium difficile* associated diarrhea (CDAD) is currently treated with metronidazole or vancomycin. Although both are highly effective, there is a strong medical need for alternative and better therapeutic options to combat CDAD. The main factors for this are undesirable side effects associated with their use, reported metronidazole-resistant *C. difficile* isolates, spread of vancomycin-resistant enterococci in vancomycin-treated patients and the fact that approximately 15-35% of the patients experience relapse after initial therapy<sup>[11-13]</sup>.

The minimal inhibitory concentration of a PB6 fermentation broth diethyl ether crude extract against *C. difficile* ATCC 9689 was between 5 and 10 ppm. The antimicrobial actives identified in the PB6 fermentation product were surfactins. These are amphiphatic cyclic lipopeptides belonging to the nonribosomal peptide family of natural products that also includes other important therapeutic agents, such as vancomycin and daptomycin. Surfactins have exceptional emulsifying but also antibacterial, antiviral and antitumoral properties<sup>[14-18]</sup>. As inhibitors of cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>), surfactins are also putative anti-inflammatory agents<sup>[19]</sup>. Oral bacteriotherapy with PB6 therefore could deliver a potential therapeutic benefit in pathologies involving inflammation of the intestinal wall.

Based on the association of intestinal inflammation with luminal bacteria, considerable research is focused on modifying the intestinal flora with probiotic bacteria to antagonise pathogenic bacteria and/or to attenuate inflammatory activity, induce remission and prevent relapses in ulcerative colitis, Crohn's disease and pouchitis<sup>[20-24]</sup>.

Ulcerative colitis, Crohn's disease and pouchitis are chronic inflammatory disorders of the bowel categorised as inflammatory bowel diseases. In genetically susceptible subjects the mucosal immune system displays an unbalanced response towards luminal antigens. Extensive research has well established that gut microbial flora is essential for the

induction of IBD, but no specific microorganism has been convincingly associated with the pathogenesis. Permeability defects in the intestinal epithelium, as primary event or secondary to the inflammation, may play an important role in the onset or perpetuation of IBD. Although the exact mechanism of IBD is not known, most probable inducers are commensal bacteria and several immune and inflammatory mediators are thought to be involved<sup>[25-29]</sup>. Current methods of treatment vary from eliminating luminal bacteria by antibiotics (e.g. metronidazole, ciprofloxacin), over modulating one or more of these immune or inflammatory mediators (e.g. corticosteroids, infliximab, mesalazine), to immunosuppression (e.g. 6-mercaptopurine). When antibiotics, anti-inflammatories, immunomodulators or -suppressors do not help, surgical removal of the affected areas serves as a last resort. Bacterial resistance, undesirable side effects, high costs of production, *in vivo* instability, poor bioavailability and limited routes of administration are major drawbacks of the current treatment drugs.

In a first trial, PB6 was evaluated, in comparison to vancomycin, for its efficacy in a hamster model of clindamycin-induced CDAD, a well established and highly sensitive model of this infection<sup>[30]</sup>. Clindamycin induces in hamsters a lethal colitis that is accompanied by severe diarrhea and body weight loss. This colitis in hamsters is more extreme, but similar to antibiotic-associated colitis in man.

In three other trials the efficacy of PB6 in treating TNBS-induced colitis in a rat model of IBD was determined and evaluated in comparison to the probiotic *S. boulardii* and the standard drugs mesalazine and infliximab. The TNBS-induced colitis model is a well established, reliable model that is being widely used to examine the efficiency of drugs aimed at treating IBD<sup>[31]</sup>.

## MATERIALS AND METHODS

### Hamster model of clindamycin-induced CDAD

**Study design:** The study was done on behalf of Kemin Pharma bvba at the test facility of Kemin Nutritional Technologies (Gummidipundi, India) and has complied with all relevant federal guidelines and institutional policies. 42 male Golden Syrian hamsters were obtained from the National Centre for Laboratory Animal Sciences, NIN (Hyderabad, India). At the beginning of the treatment period, the animals were 12 to 14 weeks old. Upon their arrival at the test facility, the animals were given a complete clinical examination under the supervision of a veterinarian to ensure that they were in good condition. The animals were

acclimatized to the study conditions for a period of at least 7 days. Body weights were recorded before allocation of the animals into the study groups at the start of the trial. The animals were housed individually in polycarbonate cages (290x220x140 mm, LxWxH). The animal room and test room conditions were set as follows: temperature:  $22 \pm 4^\circ\text{C}$ , relative humidity:  $50 \pm 20\%$ , light/dark cycle: 12 hr / 12 hr (light 07.00-19.00) and ventilation: approximately 7 cycles  $\text{hr}^{-1}$  of filtered, non-recycled air. All animals had free access to Hamster Pellet Feed (NIN, Hyderabad) and Aquaguard purified water *ad libitum*. Animals were allocated to one of 7 study groups (A to G). In groups A to E *C. difficile* associated diarrhea was induced by administering 10000 CFU's of toxigenic *C. difficile* ATCC 9689 orally on day 0, followed by a subcutaneous injection, in the trunk region just behind the ears, with clindamycin  $100 \text{ mg kg}^{-1}$  on day 1. Group A received no further treatment. Group B was treated once daily from days 2 to 6 with vancomycin  $50 \text{ mg kg}^{-1}$  by oral gavage. Groups C, D and E were treated with PB6 at a dose of  $1.5 \times 10^9$  (PB6 high),  $1.5 \times 10^7$  (PB6 medium) and  $1.5 \times 10^6$  CFU  $\text{kg}^{-1}$  (PB6 low), 3 times daily (tid, ter in die) with 4 hrs interdose (first dose at 9.30 am) from day 1 to 6 by oral gavage. On day 1 the first dose of PB6 was given 1 hr after the injection of clindamycin. The treatments with PB6 were started 1 day earlier than the vancomycin treatment to allow for the transport of the bacterial cells, more than 90% of which were in spore form, to the colon and for their probable germination<sup>[32]</sup> and production of antimicrobials. In groups F and G CDAD was not induced and the animals were given PB6 at a dose of  $1.5 \times 10^9$  CFU  $\text{kg}^{-1}$ , tid with 4 hrs interdose, from day 1 to 6 by oral gavage. Twice daily, up to day 15, observations were made for clinical signs and mortality. Signs of diarrhea were scored as being mild, moderate or severe. Body weights of animals were recorded on day 0, 7 and 14. For groups A to E fecal samples were tested on days 1, 2 and 7 for the presence of Clostridium toxin A and B using ImmunoCard Toxins A&B (Meridian life sciences). On day 1 fecal samples were taken before clindamycin administration. On days 2 and 7 fecal samples were taken between the second and third treatment dose.

**Study preparations:** A Culti-loop (Oxoid, Basingstoke, England) containing the toxigenic *C. difficile* ATCC 9689 (type strain) was inoculated according to the manufacturer's instructions and the broth was diluted with saline to obtain 10000 CFU  $\text{mL}^{-1}$ . *C. difficile* ATCC 9689 is an A+/B+ strain producing both toxins A and B. Clindamycin

hydrochloride (Pharmacia, Puurs, Belgium) and vancomycin (Neon Laboratories, Mumbai, India) were suspended in double distilled water to a concentration of 10 and  $50 \text{ mg mL}^{-1}$  respectively. Dose volumes were 10 and  $1 \text{ mL kg}^{-1}$  body weight for clindamycin and vancomycin respectively. PB6 Dry (Kemin Health, Des Moines, USA), a *Bacillus subtilis* 'PB6' fermentation broth dried on a malto- and cyclodextrin carrier, was suspended in double distilled water to concentrations of  $1.5 \times 10^8$ ,  $1.5 \times 10^6$  or  $1.5 \times 10^5$  CFU  $\text{mL}^{-1}$ . Dose volume was  $10 \text{ mL kg}^{-1}$  bodyweight. More than 90% of the viable cells in PB6 Dry were present as spores. Fresh preparations were made prior to each administration. Clindamycin, vancomycin and PB6 were administered on the basis of the last individual body weight measured. All preparations were stirred vigorously before each dosing.

#### Rat model of IBD

**Study design:** Three consecutive trials were done on behalf of Kemin Pharma at the test facility of Kemin Nutritional Technologies (Gummidipundi, India) in compliance with all federal government guidelines and institutional policies. The first trial was intended to search for a possible effect of PB6 on 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats. The next two trials served to evaluate the effect found, in comparison with mesalazine and infliximab. In all 3 trials the day of induction of colitis was set as day 1.

Male Wistar rats were obtained from the National Centre for Laboratory Animal Sciences (Hyderabad, India) (trial 1 and 2) or from the Department of Animal Medicine, TANUVAS (Chennai, India) (trial 3). At the beginning of the treatment period, the animals were 12 to 15 (trial 1) and 10 to 12 (trials 2 and 3) weeks old. Upon their arrival at the test facility, the animals were given a complete clinical examination under the supervision of a veterinarian to ensure that they were in good condition. The animals were acclimated to the study conditions for a period of at least 7 days. Per trial, animals were randomised and allocated to one of the study groups. Each group consisted of 5 animals. The animals were housed per study group in polycarbonate cages (421x290x190 mm, LxWxH). The animal room and test room conditions were set as follows: temperature:  $22 \pm 3^\circ\text{C}$ , relative humidity:  $50 \pm 20\%$ , light/dark cycle: 12 hr / 12 hr (light 07.00-19.00) and ventilation: approximately 7 cycles  $\text{hr}^{-1}$  of filtered, non-recycled air. All animals had free access (except for the overnight fasting prior to TNBS administration) to rat pellet feed from the National Institute of Nutrition (NIN, Hyderabad, India) and Aquaguard purified water *ad libitum*.

Colitis was induced, after an overnight fast, using a single intrarectal administration of TNBS at 100 mg kg<sup>-1</sup> body weight, 8 cm proximal to the anus. The colitis-negative control rats were given saline intrarectally (0.5 ml per animal once) on day 1. Colitis-negative and colitis-positive control groups were given distilled water orally at 10 mL kg<sup>-1</sup>, tid with 4 h inter dosing, starting on day 1 and up to and including day 7. In the first trial one group was given PB6 (8 x 10<sup>7</sup> CFU kg<sup>-1</sup>), tid with 4 hrs inter dosing, starting 7 days before induction of colitis and up to and including day 7. The other groups were treated with PB6 (8 x 10<sup>7</sup> or 1.5 x 10<sup>9</sup> CFU kg<sup>-1</sup> respectively), tid with 4 hrs inter dosing, starting on day 1 and up to and including day 7. In the second trial, groups with TNBS-induced colitis were treated with PB6 (1.5 x 10<sup>8</sup> or 1.5 x 10<sup>9</sup> CFU kg<sup>-1</sup> respectively), tid with 4 hrs inter dosing, starting on day 1 and up to and including day 7; with mesalazine (250 mg.kg.day<sup>-1</sup>), starting on day 1 and up to and including day 7; or with infliximab (3 mg kg<sup>-1</sup>) as a single dose on day 1. The third trial partially repeated the second one concerning the PB6 (1.5 x 10<sup>8</sup> CFU kg<sup>-1</sup>), mesalazine and infliximab treatments and included an additional treatment with *S. boulardii* (1.5 x 10<sup>8</sup> CFU kg<sup>-1</sup>), tid with 4 hrs inter dosing, starting on day 1 and up to and including day 7. The first or only (in case of infliximab) dose of treatment was given within 2 (distilled water, PB6, *S. boulardii* and mesalazine) or 3 (infliximab) hrs after administration of TNBS. Except for infliximab, which was injected intravenously, all treatments were administered by gavage. Twice daily observations were made for clinical signs and mortality. Body weights of animals were recorded on days -7, 4 and 8 (trial 1), or days 1, 4 and 7 (trials 2 and 3). Percent body weight gain was calculated based on the first and last recording. On day 8 animals were sacrificed and a 5 cm long segment of the colon (from 10 to 5 cm proximal to the anus) was excised. These segments were opened longitudinally. Contents were removed by washing with saline and gross morphology was scored using the following scale: 0. no ulcers or inflammation, 1. no ulcers only local hyperaemia, 2. ulceration without hyperaemia, 3. ulceration and inflammation at one site only, 4. two or more sites of ulceration and inflammation and 5. ulceration extending more than 2 cm. The weight of each 5 cm colonic segment was also recorded to assess inflammatory induced edema.

**Study preparations:** TNBS 5% (w/v) in water (Sigma-Aldrich, St Louis, USA) was diluted to a 2.5% solution with ethanol 50%. Dose volume was 4 mL kg<sup>-1</sup> body weight. PB6 Dry (Kemin Health, Des Moines, USA), a *Bacillus subtilis* 'PB6' fermentation broth dried on a

malto- and cyclodextrin carrier, was suspended in distilled water to a concentration of 8 x 10<sup>6</sup>, 1.5 x 10<sup>7</sup> or 1.5 x 10<sup>8</sup> CFU mL<sup>-1</sup>. Dose volume was 10 mL kg<sup>-1</sup> body weight. *S. boulardii* (Enterol®, Biodiphar, Brussel, Belgium) was suspended in distilled water to a concentration of 1.5 x 10<sup>7</sup> CFU mL<sup>-1</sup>. Dose volume was 10 mL kg<sup>-1</sup> body weight. Mesalazine (5-aminosalicylic acid) (Mesacol®, Sun Pharmaceutical Ind. Ltd, Mumbai, India) tablets were powdered using pestle and mortar and a solution in distilled water was prepared containing 25 mg 5-aminosalicylic acid per ml. Dose volume was 10 mL kg<sup>-1</sup> body weight. Infliximab (Remicade®, Centocor B.V., Leiden, The Netherlands) was first reconstituted with 10 ml water for injection and was further diluted to 2 mg mL<sup>-1</sup> concentration using sterile saline. Dose volume employed was 1.5 mL kg<sup>-1</sup> body weight. All body weight dependant doses were administered on the basis of the last individual body weight taken. Fresh preparations were made prior to each administration. The preparations were stirred vigorously before each dosing.

## RESULTS

### CDAD hamster trial

#### Effect of PB6 on *C. difficile*-associated diarrhea:

About 6 hrs after clindamycin was administered mild diarrhea was observed in 3 animals of the no treatment group (A) and in 1 animal of the group to be treated with vancomycin (B). In the groups C, D and E, in which the animals received their first dose of PB6 about 1 hr after the administration of clindamycin, none of the animals showed any sign of diarrhea that same day. The number of animals suffering from diarrhea and the severity of diarrhea evolved differently between treatment groups the following 2 days. All groups in which CDAD was induced, showed signs of diarrhea. In groups F and G, without induction of CDAD, no diarrhea was observed. On day 3 the number of animals with diarrhea was 6, 2, 4, 6 and 6 in groups A to E. The intensity of diarrhea was less in the group treated with vancomycin and the group treated with the high dose of PB6. In the no treatment group as well as in the low and mid dose PB6 groups the stool was very watery and whole the abdominal area was wet.

#### Effect of PB6 on *C. difficile*-associated mortality:

At the end of day 3 all animals of groups in which CDAD was induced were still alive but several of them were showing signs of severe diarrhea. On day 4, three days after clindamycin administration, the first animals died.

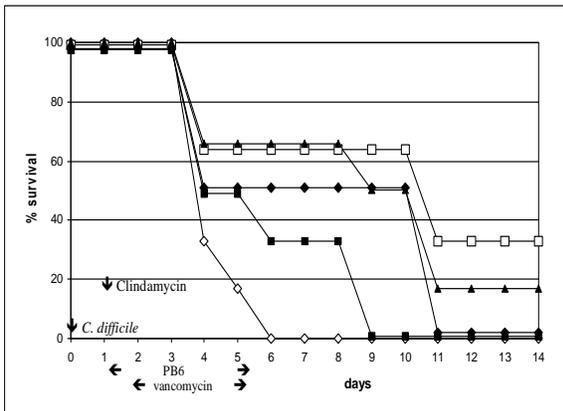


Fig. 1: Percent survival in groups in which CDAD was induced. (No treatment ◇, vancomycin □, PB6 high ▲, PB6 medium ◆, PB6 low ■)

At the end of the treatment period, on day 6, all hamsters in the no treatment group had died. Survival was highest in the vancomycin and PB6 high dose treatment groups, where 4 out of 6 had survived (Fig. 1). In groups F and G, treated with a high dose of PB6 without prior induction of CDAD, no animals died.

**Effect of PB6 on *C. difficile*-associated weight loss:**

On day 7 a decreased average body weight was observed in all groups in which CDAD was induced. There was a clear inverse dose response relation with PB6 concerning this decrease in weight (Table 1). The animals receiving a low dose of PB6, on average lost 3 times more weight than the animals which received a high dose. Weight loss was least with vancomycin treatment. The average body weight in the two groups treated with a high dose of PB6 in which CDAD was not induced, had slightly increased over the same time period.

***C. difficile* toxins in fecal samples:** The presence of Clostridium toxin A and B in fecal samples of the animals in which CDAD was induced, was checked on days 1, 2 and 7. As expected, groups showing high mortality and a high decrease of average body weight also had a higher percentage of animals tested positive for these toxins. The number of animals tested positive in groups A to E respectively (n=6) was on day 1: 3, 0, 0, 0, 0 and on day 2: 5, 2, 3, 3, 5. On day 7 all animals in group A had died and in groups B to E 1, 2, 2, 2 animals out of 4, 4, 3, 2 survivors tested positive respectively.

**Post-treatment effect of PB6:** Last day of treatment was day 6. In all groups in which CDAD was induced,

more animals surviving on day 6 died post-treatment. All animals of the PB6 low dose treatment group were dead at day 9, those of the PB6 medium dose treatment group at day 11. Vancomycin and PB6 high dose, with respectively 2 and 1 survivor, were the only treatments that prevented a 100% mortality at day 14 (Fig. 1).

**IBD rat trials**

**Trial 1:** Shortly after induction of colitis with TNBS several animals showed signs of diarrhea. The number of observations of diarrhea recorded (and the number of animals affected) in the different treatment groups from day 1 up to and including day 7 were 5 (2), 38 (5), 21 (3), 26 (4) and 12 (3) for colitis-negative control, colitis-positive control, PB6  $8 \times 10^7$  CFU  $\text{kg}^{-1}$  tid pre- and post-induction treatment, PB6  $8 \times 10^7$  CFU  $\text{kg}^{-1}$  tid post-induction treatment and PB6  $1.5 \times 10^9$  CFU  $\text{kg}^{-1}$  tid post-induction treatment respectively. The colitis and accompanying diarrhea, in some cases probably tempered by the treatment, also reflected in the groups mean body weight and body weight gain data (Table 2).

Due to the administration of TNBS, preceded by an overnight fasting and to the onset of colitis, body weight gain retarded or animals even started losing weight. On days 4 and 8 after induction of colitis, statistically significant differences in mean body weight were observed between groups. At the end of the treatment period, only the colitis-negative control group and the group treated for 7 days post-induction with  $1.5 \times 10^9$  CFU  $\text{kg}^{-1}$  tid had an average body weight and body weight gain that was clearly higher than the colitis-positive control group ( $P < 0.05$ ) (Table 2).

Statistically significant differences were also observed between groups in score for gross morphology of the intestinal wall of a 5cm colon segment and in the wet weights of these segments (Table 3). The only groups that could be clearly distinguished from the colitis-positive control group ( $P < 0.05$ ) were again the colitis-negative control group and the group treated with PB6  $1.5 \times 10^9$  CFU  $\text{kg}^{-1}$  tid. All other groups showed an increased colon segment weight (as a measure of inflammatory edema) and a clear ulceration of the colon wall comparable to that of the colitis-positive control group. The macroscopical colon wall health status of the group treated with PB6  $1.5 \times 10^9$  CFU  $\text{kg}^{-1}$  tid for 7 days was comparable to that of the colitis-negative control group. PB6 at a dose of  $8 \times 10^7$  CFU  $\text{kg}^{-1}$  tid for 7 days was possibly close to the minimal dose effective to suppress colitis as induced by TNBS in rats in this trial. Some of the animals in this group had clear signs of colitis while in others these

Table 1: The effect of treatment on body weight and body weight gain in male Golden Syrian hamsters with clindamycin-induced *C. difficile* associated diarrhea

Treatment	Body weight (g)		Body weight gain (%)
	Day 0	Day 7	Day 0 to 7
CDAD Induced			
No treatment	219.0 ± 5.1 <sup>a</sup> (n=6)	<sup>q</sup>	<sup>q</sup>
Vancomycin	218.0 ± 4.6 <sup>a</sup> (n=6)	215.8 ± 1.7 <sup>ab</sup> (n=4)	-1.7 ± 1.8 <sup>bc</sup>
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	218.8 ± 4.0 <sup>a</sup> (n=6)	206.5 ± 5.7 <sup>ab</sup> (n=4)	-5.0 ± 2.1 <sup>c</sup>
PB6 1.5 x 10 <sup>7</sup> CFU kg <sup>-1</sup> tid	217.8 ± 3.2 <sup>a</sup> (n=6)	189.0 ± 2.7 <sup>b</sup> (n=3)	-12.9 ± 0.7 <sup>d</sup>
PB6 1.5 x 10 <sup>6</sup> CFU kg <sup>-1</sup> tid	217.2 ± 4.5 <sup>a</sup> (n=6)	176.5 ± 2.1 <sup>b</sup> (n=2)	-17.9 ± 1.2 <sup>d</sup>
No CDAD induced			
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	217.8 ± 2.3 <sup>a</sup> (n=6)	230.0 ± 7.6 <sup>a</sup> (n=6)	5.6 ± 3.1 <sup>a</sup>
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	219.2 ± 3.9 <sup>a</sup> (n=6)	225.8 ± 4.8 <sup>ab</sup> (n=6)	3.1 ± 3.2 <sup>ab</sup>

Data are expressed as mean ± SD. <sup>q</sup> no data, all animals had died. <sup>a,b,c,d</sup> values within the same column not bearing a common superscript are significantly different (P<0.05, Tukey-Kramer multiple comparisons procedure[33]). tid, ter in die (three times a day).

Table 2: The effect of treatment on body weight and body weight gain in male Wistar rats with TNBS-induced colitis. Trial 1.

Treatment	Body weight (g)			Body weight gain (%)
	Day -7	Day 4	Day 8	
Colitis-negative control	253 ± 15	287 ± 5 <sup>*</sup>	288 ± 8 <sup>*</sup>	14.1 ± 5.2 <sup>*</sup>
Colitis-positive control	252 ± 16	238 ± 14	237 ± 13	-5.7 ± 5.3
PB6 8 x 10 <sup>7</sup> CFU kg <sup>-1</sup> tid (pre- and post-induction)	254 ± 12	257 ± 2	262 ± 9	3.1 ± 7.7
PB6 8 x 10 <sup>7</sup> CFU kg <sup>-1</sup> tid	256 ± 11	248 ± 19	248 ± 31	-3.1 ± 11.7
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	255 ± 8	263 ± 11 <sup>*</sup>	277 ± 10 <sup>*</sup>	8.4 ± 1.8 <sup>*</sup>

Data are expressed as mean ± SD. The day of induction of colitis is defined as day 1. PB6 was administered for 7 days post-induction. One group received PB6 also prophylactically for 7 days pre-induction. <sup>\*</sup> Significantly different from the positive control group (Dunnett, P<0.05). tid, ter in die (three times a day).

Table 3: The effect of treatment on the gross morphology score for the colon wall and the wet weight of a 5 cm colon segment in male Wistar rats with TNBS-induced colitis. Trial 1.

Treatment	Gross morphology score	Wet weight (g)
Colitis-negative control	0.2 ± 0.4 <sup>*</sup>	0.465 ± 0.147 <sup>*</sup>
Colitis-positive control	3.2 ± 1.1	0.858 ± 0.152
PB6 8 x 10 <sup>7</sup> CFU kg <sup>-1</sup> tid (pre- and post-induction)	2.0 ± 1.4	0.692 ± 0.135
PB6 8 x 10 <sup>7</sup> CFU kg <sup>-1</sup> tid	2.4 ± 1.3	0.745 ± 0.231
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	0.6 ± 0.5 <sup>*</sup>	0.504 ± 0.172 <sup>*</sup>

Data are expressed as mean ± SD. The day of induction of colitis is defined as day 1. PB6 was administered for 7 days post-induction. One group received PB6 also prophylactically for 7 days pre-induction. <sup>\*</sup> Significantly different from the positive control group (Dunnett, P<0.05). tid, ter in die (three times a day).

were almost absent. This was also the case in the group treated both pre- and post-induction with that same dose. An additional prophylactic treatment with PB6 8 x 10<sup>7</sup> CFU kg<sup>-1</sup> tid for 7 days did not improve the results of a 7 days treatment post-induction.

**Trials 2 and 3:** In the second trial one of the rats treated with infliximab died on day 2. This animal had been given a second injection on day 1 because at the first one drug solution oozed out. Also in these trials some animals showed mild signs of diarrhea after induction of colitis with TNBS. The number of observations of diarrhea recorded (and the number of

animals affected) in the different treatment groups from day 1 up to and including day 7 were in the second trial 0, 22 (2), 4 (1), 0 (0), 0 (0) and 8 (1) for colitis-negative control, colitis-positive control, PB6 1.5 x 10<sup>8</sup> CFU kg<sup>-1</sup> tid, PB6 1.5 x 10<sup>9</sup> CFU kg<sup>-1</sup> tid, mesalazine 250 mg.kg.day<sup>-1</sup> and infliximab 3 mg kg<sup>-1</sup> single dose respectively; and in the third trial 0, 42 (4), 10 (1), 34 (3), 12 (1) and 24 (2) for colitis-negative control, colitis-positive control, PB6 1.5 x 10<sup>8</sup> CFU kg<sup>-1</sup> tid, *S. boulardii* 1.5 x 10<sup>8</sup> CFU kg<sup>-1</sup> tid, mesalazine 250 mg.kg.day<sup>-1</sup> and infliximab 3 mg kg<sup>-1</sup> single dose respectively.

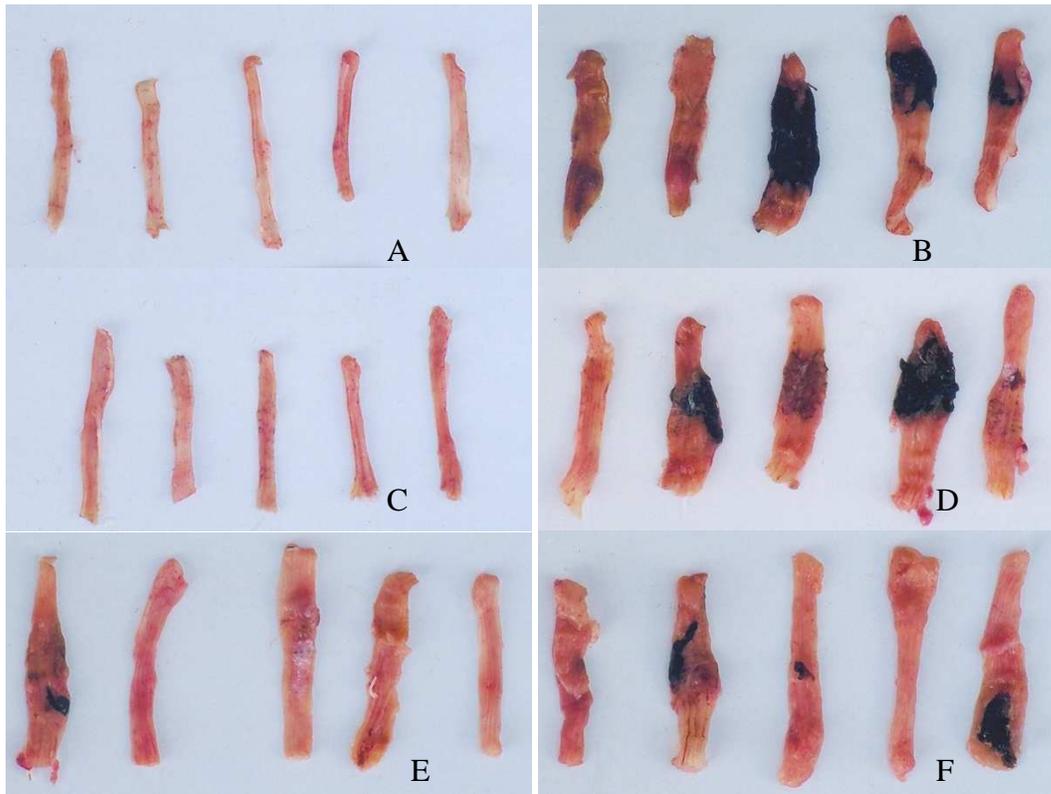


Fig. 2: Longitudinally opened colon segments of the colitis-negative control group (A), the colitis-positive control group (B) and of the groups treated for 7 days post-induction with PB6 ( $1.5 \times 10^8$  CFU  $\text{kg}^{-1}$  tid) (C), *S. boulardii* ( $1.5 \times 10^8$  CFU  $\text{kg}^{-1}$  tid) (D), or mesalazine ( $250 \text{ mg.kg.day}^{-1}$ ) (E) or treated with a single dose of infliximab ( $3 \text{ mg kg}^{-1}$ ) (F) post-induction on day 1. Photographs of trial 3 colon segments

Table 4: The effect of treatment on body weight and body weight gain in male Wistar rats with TNBS-induced colitis. Trials 2 and 3.

Treatment	Body weight (g)			Body weight gain (%)
	Day 1	Day 4	Day 7	
<b>Trial 2</b>				
Colitis-negative control	178 ± 17	191 ± 13 *	203 ± 13 *	14.3 ± 4.5 *
Colitis-positive control	182 ± 15	167 ± 16	159 ± 18	-13.0 ± 4.5
PB6 $1.5 \times 10^8$ CFU $\text{kg}^{-1}$ tid	180 ± 13	186 ± 13	195 ± 10 *	8.8 ± 8.0 *
PB6 $1.5 \times 10^9$ CFU $\text{kg}^{-1}$ tid	180 ± 16	187 ± 15	199 ± 17 *	10.3 ± 1.1 *
Mesalazine $250 \text{ mg kg}^{-1}$	181 ± 11	187 ± 10	196 ± 10 *	8.8 ± 1.4 *
Infliximab $3 \text{ mg kg}^{-1}$	180 ± 14	183 ± 7	186 ± 6 *	1.3 ± 5.4 *
<b>Trial 3</b>				
Colitis-negative control	207 ± 20	210 ± 19	214 ± 17	3.5 ± 1.8 *
Colitis-positive control	208 ± 21	196 ± 21	193 ± 19	-7.5 ± 3.2
PB6 $1.5 \times 10^8$ CFU $\text{kg}^{-1}$ tid	206 ± 23	206 ± 23	212 ± 26	2.9 ± 3.1 *
<i>S. boulardii</i> $1.5 \times 10^8$ CFU $\text{kg}^{-1}$ tid	207 ± 17	196 ± 16	195 ± 24	-5.7 ± 5.6
Mesalazine $250 \text{ mg kg}^{-1}$	207 ± 12	199 ± 18	201 ± 20	-3.3 ± 5.5
Infliximab $3 \text{ mg kg}^{-1}$	208 ± 15	197 ± 14	197 ± 13	-5.2 ± 3.4

Data are expressed as mean ± SD. The day of induction of colitis is defined as day 1. PB6, *S. boulardii* and mesalazine were administered for 7 days post-induction. Infliximab was administered on day 1 only. \* Significantly different from the positive control group (Dunnett,  $P < 0.05$ ). tid, ter in die (three times a day).

Table 5: The effect of treatment on the gross morphology score for the colon wall and the wet weight of a 5 cm colon segment in male Wistar rats with TNBS-induced colitis. Trials 2 and 3.

Treatment	Gross morphology score	Wet weight (g)
Trial 2		
Colitis-negative control	0.2 ± 0.4 *	0.378 ± 0.047 *
Colitis-positive control	3.8 ± 0.8	1.406 ± 0.209
PB6 1.5 x 10 <sup>8</sup> CFU kg <sup>-1</sup> tid	1.0 ± 0.0 *	0.443 ± 0.063 *
PB6 1.5 x 10 <sup>9</sup> CFU kg <sup>-1</sup> tid	0.6 ± 0.5 *	0.513 ± 0.317 *
Mesalazine 250 mg kg <sup>-1</sup>	0.8 ± 0.4 *	0.435 ± 0.052 *
Infliximab 3 mg kg <sup>-1</sup>	3.0 ± 1.4	1.055 ± 0.490
Trial 3		
Colitis-negative control	0.0 ± 0.0 *	0.274 ± 0.049 *
Colitis-positive control	3.4 ± 1.1	0.832 ± 0.216
PB6 1.5 x 10 <sup>8</sup> CFU kg <sup>-1</sup> tid	0.6 ± 0.5 *	0.280 ± 0.039 *
<i>S. boulardii</i> 1.5 x 10 <sup>8</sup> CFU kg <sup>-1</sup> tid	2.8 ± 1.3	0.735 ± 0.221
Mesalazine 250 mg kg <sup>-1</sup>	1.2 ± 0.8 *	0.445 ± 0.145 *
Infliximab 3 mg kg <sup>-1</sup>	2.0 ± 0.7	0.657 ± 0.076

Data are expressed as mean ± SD. The day of induction of colitis is defined as day 1. PB6, *S. boulardii* and mesalazine were administered for 7 days post-induction. Infliximab was administered on day 1 only. \*Significantly different from the positive control group (Dunnett, P<0.05). tid, ter in die (three times a day).

The colitis-positive control groups showed substantial body weight loss accompanying the colitis. In trial 2 this loss was higher than in trial 3, what may be related to a difference in genetic background and a possible difference in age and growth rate at the moment of induction of colitis. In trial 2 there was a lower average body weight in general at the start and a higher body weight gain in terms of percentage over the trial period of the colitis-negative control group. While common treatments in trial 2 all significantly suppressed the negative effect of colitis on body weight gain, in trial 3 this was only the case with PB6 treatment (Table 4).

The greater impact of colitis on the animals in trial 2 probably left more room for improvement by any of the treatments. PB6 and mesalazine always resulted in a colon segment weight and a gross morphology score for the colon wall that could be clearly distinguished from those of the colitis-positive control group (Table 5).

The health status of the colon wall in rats with TNBS-induced colitis treated with PB6 and mesalazine was macroscopically the same as that in colitis-free rats. For an unknown reason mesalazine treatment was somewhat less effective in trial 3, resulting in some ulcerations. Macroscopic pictures of the longitudinally opened colon segments clearly show the ulcerations and areas of necrotic tissue present in the positive control, the *S. boulardii* and the infliximab treatment groups and the absence thereof in the PB6 group (Fig. 2).

Common treatments in trial 2 resulted in colon segments with the same macroscopical appearance as in trial 3. At the end of the treatment period the average body weight gain and colon segment weight of rats treated with infliximab were intermediary to those of the colitis-negative and the colitis-positive control groups in both trials. This probably indicates that there

was some effect of infliximab, although not statistically significant in these trials. In the colon segments of rats treated with PB6 there were no ulcerations. Only hyperaemia was observed in the majority of these segments. PB6 clearly attenuates inflammation in the rat colon wall as induced by intrarectal administration of TNBS. The efficacy of a 7 days post-induction treatment with 1.5 x 10<sup>9</sup> CFU kg<sup>-1</sup> tid observed in trial 1 was confirmed in trial 2. While a 7 days post-induction treatment with PB6 8 x 10<sup>7</sup> CFU kg<sup>-1</sup> tid from the first trial was concluded to be ineffective, an increase of the CFU kg<sup>-1</sup> tid to 1.5 x 10<sup>8</sup> proved in both following trials to be sufficient to reduce inflammation to an extent that the data of this treatment could no longer be statistically distinguished from those of the colitis-negative control group.

## DISCUSSION

**CDAD hamster trial:** Vancomycin and in first place metronidazole are the drugs of choice for the treatment of *C. difficile* infection. Trials have repeatedly indicated that in hamsters death from clindamycin-induced colitis can be delayed by either of these two as long as they are administered. Death almost invariably occurs however after treatment is stopped<sup>[34-37]</sup>. Also in this trial, at the end of the observation period and 8 days after the end of the treatment, half of the surviving animals treated with vancomycin had died. Vancomycin and metronidazole fail to correct the predisposing conditions in the gut and being antibiotics, might even aggravate disruption of the intestinal flora which could account for the relapse of colitis in the hamster model and in humans once the antibiotic treatment is stopped. Agents that help to maintain or are able to restore the

microbiological equilibrium within the intestinal tract may prevent proliferation of *C. difficile* and the occurrence of CDAD. The prophylactic use of probiotics in humans to help prevent possible diarrhea and other gastrointestinal disturbances associated with antibiotic treatment is well established and may constitute a valuable treatment support<sup>[38, 39]</sup>. Studies conducted to date on a possible protective or therapeutic effect of probiotics against CDAD in humans however provide insufficient evidence for their routine clinical use with this infection<sup>[40]</sup>. Studies on the efficiency of probiotics in an animal model of CDAD are very scanty. Colonization with nontoxicogenic *C. difficile* strains has proven to be highly effective in preventing CDAD in hamsters<sup>[41]</sup>. Although *C. difficile* is usually not associated with probiotics, the results obtained suggest that use of a probiotic strategy for CDAD prevention might be beneficial. The probiotic yeast *Saccharomyces boulardii* at  $8 \times 10^{10}$  CFU.kg.day<sup>-1</sup>, as a 5% suspension of a lyophilized powder in drinking water, has been shown to help prevent extensive overgrowth of *C. difficile* and to reduce clindamycin-induced cumulative mortality in hamsters by 10 to 35% when consumption of this yeast started prophylactically during at least 3 days before clindamycin exposure. Clindamycin doses exceeding 0.6 mg kg<sup>-1</sup> however obliterated the protective effect<sup>[42]</sup>. In current trial a hundredfold higher, single subcutaneous dose of clindamycin (100 mg kg<sup>-1</sup>), 24 hr after a single oral administration of  $10^4$  CFU of toxigenic *C. difficile* ATCC 9689, was used to induce CDAD in hamsters. The difference in the number of animals that tested positive for *C. difficile* toxins observed at the end of day 1 between the no treatment and the vancomycin groups, might seem to indicate that induction of CDAD failed in the latter. The onset of diarrhea in this group at the end of day 1, the presence of toxin positives on day 2 and the further trial course is convincing of the opposite. Hamsters that were not treated after induction of CDAD this way all got severe diarrhea, lost weight and died within 3 to 5 days. The administration of PB6 in this trial started on the same day as the administration of clindamycin and not several days before, as was the case with *S. boulardii* in the aforementioned trial. Additionally, the fact that PB6 is susceptible to clindamycin might have impaired the effect of the initial doses. Nonetheless PB6 ( $1.5 \times 10^9$  CFU kg<sup>-1</sup> tid for 6 days) reduced cumulative mortality over the treatment period by 66%, to the same level as vancomycin (Fig. 1). A strong indication for the therapeutic potential of PB6 is the inverse dose response relation observed with *C. difficile*-associated weight loss. Animals receiving the low dose of PB6

( $1.5 \times 10^6$  CFU kg<sup>-1</sup> tid) suffered most from diarrhea and lost the most weight. The weight loss with animals receiving the high dose ( $1.5 \times 10^9$  CFU kg<sup>-1</sup> tid) was much less distinct and almost at the same level as with vancomycin treatment (Table 1). Under the conditions used in this trial, treatment with PB6 three times daily at  $1.5 \times 10^9$  CFU kg<sup>-1</sup> for 6 days could not prevent further animal deaths after cessation of treatment (Fig. 1). The hamster model however represents an extreme form of CDAD of which the counterpart in humans, in most instances, is a milder condition.

The lack of knowledge on their mode(s) of action or the fact that these are at least not well defined, is something most probiotics have in common. Lactic acid bacteria, so far, are the best-documented probiotics. In contrast, the mechanisms responsible for the beneficial effect of other probiotics, especially the *Bacillus* species, have remained relatively unexplored. Additional experiments are needed to provide insight into the mechanism(s) responsible for the probiotic effect of PB6. At the moment we can only hypothesize based on the data we have. The production of surfactins active against *Clostridium* certainly could be an element in it. The biological activity of surfactins is supposedly related to its ability to destabilize and permeabilize membranes at very low concentrations, leading to membrane disintegration at higher concentrations. Micrographs from transmission electron microscopy have shown that when *C. perfringens* was incubated *in vitro* with PB6 culture supernatans centrifuged at 14000g for 15 min and filter-sterilized, the cell wall of the bacteria was disrupted and cytoplasmic contents leaked into the exterior (Fig. 3). Disruption of viral lipid membranes and mycoplasma membranes by surfactin also has been observed by electron microscopy<sup>[16, 17]</sup>.

Another possible mechanism is immunomodulation. As described in this article, PB6 is very effective in reducing the severeness of inflammation in trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats. This may be due to the putative anti-inflammatory activity of surfactin but may also be an indication that PB6 has immunomodulatory properties<sup>[19]</sup>.

In summary, our results indicate that in hamsters treatment of clindamycin-induced CDAD with PB6 resulted in a dose related response. Diarrhea, body weight reduction and mortality were least with the highest dose of PB6. At treatment stop, the highest dose of PB6 showed to have been equally efficient as vancomycin in helping hamsters to survive clindamycin-induced CDAD.

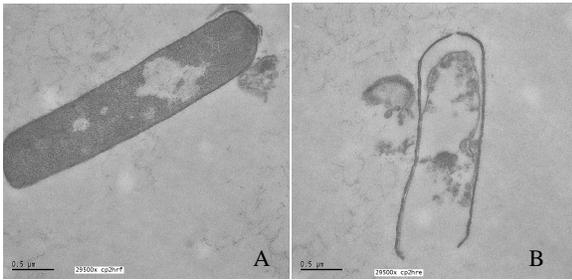


Fig. 3: The effect of PB6 (ATCC – PTA 6737) culture supernatant on *Clostridium perfringens* at 37°C. (A) disruption of cell wall and loss of cytoplasmic contents into the exterior after 1 hr; (B) rupture and death of cell after 4 hrs (transmission electron micrograph: X 29500)

**IBD rat trials:** Studies conducted with animal models of colitis demonstrate that probiotic treatment can sometimes reduce the development of colitis and treat or attenuate established disease. Immunomodulation, improvement of epithelial barrier function, competitive exclusion of gastrointestinal pathogens and secretion of antimicrobial compounds which suppress the growth of harmful enteric bacteria, are all basic mechanisms by which probiotics can exert an effect. Overall studies in animal models indicate that various probiotic species have different effects in selected hosts and inflammatory conditions, linked to the likely involvement of multiple mechanisms of action<sup>[43-50]</sup>. In the current study *Bacillus subtilis* 'PB6' and the standard drug mesalazine showed to be very efficient in suppressing TNBS-induced colitis in a rat model of IBD. Mesalazine has *in vitro* and *in vivo* pharmacologic effects that decrease leukotriene production, scavenge for free radicals and inhibit leukocyte chemotaxis. While its exact mode of action is currently unknown, any of these biochemical mechanisms may play a role in the clinical effectiveness of mesalazine. Infliximab, the other standard drug we used as a reference in our evaluation, is a genetically engineered hybrid monoclonal antibody manufactured using cells containing human and mouse antibody genes. The drug works by binding to TNF- $\alpha$ , a promoter of inflammation and essentially removing it from circulation. Our results with infliximab are comparable to those of a similar trial in rats where infliximab was administered at the same single dose of 3 mg kg<sup>-1</sup> i.v. 2 days before induction of colitis with TNBS. In that trial, 8 days after induction, infliximab was shown to have some effect on body weight gain and colon gross morphology but not to reduce the level of edema significantly below that of the colitis-positive control group<sup>[51]</sup>. For the anti-inflammatory effect observed with the oral administration of PB6 we hypothesize that this may be due to the probable germination of PB6 spores in the intestines, followed by bacterial growth and production of surfactins<sup>[32, 52]</sup>. The demonstrated suppression of anti-inflammatory responses by surfactin possibly relates to the selective inhibition of cytosolic

PLA<sub>2</sub>, which is known to contribute in the biosynthesis of several inflammatory mediators<sup>[19, 53]</sup>. By its action on membrane phospholipids PLA<sub>2</sub> releases arachidonic acid and lysophospholipids; precursors of proinflammatory mediators, such as prostaglandins, thromboxanes, leukotrienes and platelet-activating factor. Diverse of these have been found to be strong mediators of intestinal inflammation<sup>[54]</sup>. Arachidonic acid also upregulates P38 mitogen-activated protein kinase (MAPK)-induced production of TNF- $\alpha$  and associated proinflammatory mediators<sup>[55]</sup>. Inhibition of cytosolic PLA<sub>2</sub> therefore suppresses both the release of precursors of proinflammatory mediators and the upregulation of the P38 MAPK-induced production of proinflammatory mediators. Inhibitors of PLA<sub>2</sub>, such as quinacrine, p-bromophenacyl bromide and carboxymethylcellulose-linked phosphatidylethanolamine have been shown to attenuate intestinal inflammation in chemically induced and immunologically mediated animal models of IBD<sup>[56, 57]</sup>. Surfactins have shown their anti-inflammatory activity in different animal models. They ameliorated both tetradecanoyl phorbol-acetate- and acetic acid-induced mouse ear edema, carrageenan-induced rat paw edema and showed a potent dose dependant analgesic effect in the acetic acid-induced abdominal constriction assay in mice when administered intraperitoneally. In the latter test surfactins outperformed indomethacin, a common non-steroidal anti-inflammatory drug, by 10-fold<sup>[19]</sup>. Taking into account firstly the literature findings on the pivotal role of PLA<sub>2</sub> in the inflammation process, on the suppression of inflammation by PLA<sub>2</sub> inhibitors and on the inhibition of PLA<sub>2</sub> by surfactin and secondly the production of the latter by PB6, it can be reasonably hypothesised that PB6 in the intestine mediates inhibition of PLA<sub>2</sub> through the production of surfactins, thus reducing the deteriorating effects of TNBS-induced inflammation. Future studies, focusing on the fate of PB6 in the intestines, the possible production of surfactins *in situ* and the effect of PB6 on the levels of proinflammatory mediators, should verify this hypothesis. In humans, clinical trials suggest that selected probiotic species can prevent recurrent intestinal inflammation and possibly treat active IBD. The most convincing results to date are obtained with VSL#3 in maintaining remission in recurrent or refractory pouchitis<sup>[23, 58]</sup>. VSL#3 is a probiotic-mixture, consisting of four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus*. VSL#3 decreases the level of proinflammatory cytokines and the activity of inducible nitric oxide synthase and matrix metalloproteinase<sup>[59]</sup>. Its actual mechanism of action and whether all eight bacteria are required for its efficacy still remains under investigation. In ulcerative colitis and Crohn's disease results with probiotics are less convincing or mixed<sup>[20-22, 27, 60]</sup>. The results obtained with *Bacillus subtilis* 'PB6' in TNBS-induced colitis in rats, a well established, reliable model of human IBD, are suggestive of possible therapeutic effectiveness of PB6.

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