

Control of Acute, Chronic, and Constitutive Hyperammonemia by Wild-Type and Genetically Engineered *Lactobacillus plantarum* in Rodents

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Hyperammonemia is a common complication of acute and chronic liver diseases. Often accompanied with side effects, therapeutic interventions such as antibiotics or lactulose are generally targeted to decrease the intestinal production and absorption of ammonia. In this study, we aimed to modulate hyperammonemia in three rodent models by administration of wild-type *Lactobacillus plantarum*, a genetically engineered ammonia hyperconsuming strain, and a strain deficient for the ammonia transporter. Wild-type and metabolically engineered *L. plantarum* strains were administered in ornithine transcarbamoylase-deficient Sparse-fur mice, a model of constitutive hyperammonemia, in a carbon tetrachloride rat model of chronic liver insufficiency and in a thioacetamide-induced acute liver failure mice model. Constitutive hyperammonemia in Sparse-fur mice and hyperammonemia in a rat model of chronic hepatic insufficiency were efficiently decreased by *Lactobacillus* administration. In a murine thioacetamide-induced model of acute liver failure, administration of probiotics significantly increased survival and decreased blood and fecal ammonia. The ammonia hyperconsuming strain exhibited a beneficial effect at a lower dose than its wild-type counterpart. Improved survival in the acute liver failure mice model was associated with lower blood ammonia levels but also with a decrease of astrocyte swelling in the brain cortex. Modulation of ammonia was abolished after administration of the strain deficient in the ammonium transporter. Intestinal pH was clearly lowered for all strains and no changes in gut flora were observed. **Conclusion: Hyperammonemia in constitutive model or after acute or chronic induced liver failure can be controlled by the administration of *L. plantarum* with a significant effect on survival. The mechanism involved in this ammonia decrease implicates direct ammonia consumption in the gut. (HEPATOLOGY 2008;48:1184-1192.)**

Hyperammonemia (HA) is a well-known complication of acute and chronic liver diseases and plays a central role in the pathogenesis of hepatic encephalopathy (HE).¹⁻⁵ This neurological dysfunction

results, at least in part, from an increase in plasma ammonia level and the severity of the symptoms correlates with blood ammonia level.⁶⁻⁹ Animal models used in studying hyperammonemic disorders are multiple: fulminant hepatic failure,¹⁰ chronic liver failure,¹¹ or urea cycle deficiency.¹² Ammonia is produced by many tissues but its major external source results from deaminase and urease activities of the gut flora. Although its absorption occurs through the intestinal epithelium, ammonia is carried by the portal vein into the liver where it is metabolized into urea.¹³

Classical treatments of HE, except through liver transplantation and liver replacement therapies, consist in decreasing the ammonia production of urease-positive bacteria by antibiotics or decreasing the ammonia absorption into the gut by acidifying the colon content with nonabsorbable sugars such as lactulose.^{4,13-15} However, these treatments are insufficient due to their side effects, toxicities, poor compliance by patients, and the lack of clear effect on survival.¹⁶⁻¹⁸

Abbreviations: AlaD, alanine dehydrogenase; ALT, alanine aminotransferase; AmtB, ammonium transporter; AST, aspartate aminotransferase; CCL₄, carbon tetrachloride; CFU, colony-forming unit; HA, hyperammonemia; HE, hepatic encephalopathy; sp., species; spf, sparse-fur; TAA, thioacetamide.

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Increasing evidence indicates that probiotics, such as *Lactobacillus*, promote the growth of non-urease producing species, lower the intestinal pH and reduce the absorption of ammonia in the colonic lumen,¹⁹ and could be efficient to treat HA and HE.^{20,21} In this context, clinical or experimental studies have shown positive effects of probiotic or synbiotic preparations on mild HE.^{21,22} Probiotics have already been used safely in intestinal bowel diseases²³ such as ulcerative colitis or in chronic pouchitis,²⁴ to decrease symptoms in patients with irritable bowel syndrome,²⁵ or even to improve the clinical status of children with stable Crohn's disease.²⁶ In addition, new recombinant strains of probiotics are used as novel therapeutic strategies to deliver vaccines²⁷ or biologically active compounds such as interleukin-10 in murine colitis.^{28,29}

Lactobacillus plantarum is a nonpathogenic, noninvasive, Gram-positive bacterium found in the normal mucosal flora of the human mouth and intestine and frequently associated with lactic acid fermented foods of plant origin. In this study, ammonia absorption was modulated in the gut by the administration of a wild-type strain of *L. plantarum*, NCIMB8826, and a genetically engineered ammonia hyperconsuming *L. plantarum* strain, able to efficiently convert ammonia to alanine in three rodent models of hyperammonemia.¹⁰⁻¹² An *L. plantarum* strain deficient for the ammonium transporter was also constructed in order to evaluate its implication in ammonia metabolism and consumption.

Materials and Methods

Bacterial Strains, Plasmids, Media, and Growth Conditions. *Lactobacillus plantarum* NCIMB8826 (National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, UK) and derivatives were grown in Mann, Rogosa, Sharpe (MRS) medium (Becton Dickinson, Cockeysville, MD) at 37°C without shaking. When appropriate, chloramphenicol or erythromycin was added to the medium at a final concentration of 10 µg/mL.

Strains VL103 (LDH⁻) and VL113 (LDH⁻, NisRK⁺) are derived from NCIMB8826 and are both deficient for L-lactate dehydrogenase and D-lactate dehydrogenase (LDH). VL113 contains the nisin regulatory two-component system (NisRK), which was not exploited during this study. Plasmid pCNR52 contains the alanine dehydrogenase gene (*alaD*) of *Bacillus natto* under the control of a strong promoter (a gift from H. van der Kaaij and B. Mollet, Nestlé). Plasmid pNZ8048 was used as a negative control.

Lactobacillus plantarum strains were grown in a 2-L batch reactor (BIOFLO1000, New Brunswick Scientific, Nijmegen, The Netherlands). The fermentation medium

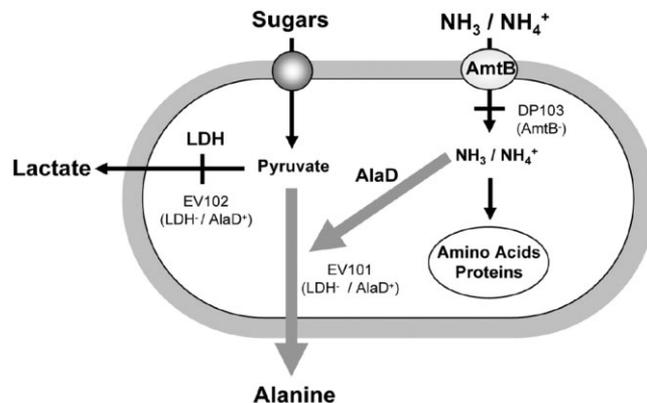


Fig. 1. Metabolic engineering of ammonia metabolism in *L. plantarum* NCIMB8826. The ammonia hyperconsumer strain EV101 (LDH⁻/AlaD⁺) is able to use ammonia via the transfer of an alanine dehydrogenase (AlaD), which catalyzes the conversion of pyruvate into alanine, while the inactivation of lactate dehydrogenases (LDH) prevents the transformation of pyruvate in lactate. The isogenic strain EV102 (LDH⁻/AlaD⁻), which is deprived of AlaD, was used as control. The ammonia transporter-deficient strain DP103 (AmtB⁻), which is unable to consume ammonia, was obtained by an inactivation of the ammonia transporter AmtB.

of strain EV101 that produces alanine was supplemented with 150 mM ammonium sulfate, pH was maintained at 5.5, and the culture was stirred at 150 rpm. After 24 hours of growth, cells were centrifuged, washed, and suspended at 10¹¹ colony forming unit (CFU)/mL in the vehicle solution (casein hydrolysate 5%, glucose 0.5%, NaHCO₃ 0.2 M). Aliquots were stored at -80°C and bacterial viability was measured before each *in vivo* experiment.

Construction of Recombinant *L. plantarum* Strains. Electrotransformation of *L. plantarum* was performed as previously described.³⁰ The ammonia hyperconsumer strain EV101 (LDH⁻/AlaD⁺) of *L. plantarum* was constructed by electroporation of pCNR52 into strain VL103. Strain VL113 harboring plasmid pNZ8048 was used as negative control and was named EV102 (LDH⁻/AlaD⁻). Strain EV101 is able to use ammonia via AlaD, which catalyzes the conversion of pyruvate into alanine, while the inactivation of LDH prevents the transformation of pyruvate into lactate (Fig. 1). The consumption of ammonia at a high level and the production of alanine from glucose fermentation were validated with cell suspensions (conversion of glucose to alanine > 95%) and batch fermentations with growing cells (up to 60% conversion) (data not shown).

In order to obtain an *L. plantarum* strain unable to consume ammonia, the ammonia transporter AmtB was inactivated (Fig. 1). The *amtB* knockout mutant was constructed by a two-step homologous recombination procedure as described.³¹ The disruption vector containing the 5' and 3' recombination fragments of the *amtB* gene, with the chloramphenicol resistance marker inserted in

between, was constructed as follows. The different DNA fragments were amplified by polymerase chain reaction, digested with appropriate restriction enzymes, and cloned in a triple ligation event at the EcoRI and BamHI sites of plasmid pJDC9. This suicide vector was used to delete the *amtB* gene in *L. plantarum* NCIMB8826, yielding a mutant strain designated DP103 (*AmtB*⁻). The *amtB* mutant genotype was confirmed by polymerase chain reaction amplification (data not shown).

Animals. B6EiC3Sn ornithine transcarbamoylase-deficient Sparse-fur mice (*spf*), X-linked metabolic disease model of the urea cycle, and wild-type control males from The Jackson Laboratory (Bar Harbor, ME) were used at 8 to 10 weeks of age. Ten-week-old to 12-week-old male C57BL/6 mice and 4-week-old male Lewis rats were purchased from Charles River Laboratories (Brussels, Belgium). Animals were maintained in our animal facilities and received care in compliance with the national legal requirements and National Institutes of Health guidelines.

Experiments. In the constitutive HA model, *spf* mice received daily orally and intrarectally 10⁹ CFU in 100 μL of viable *L. plantarum* strains or vehicle for 3 days (day -2 to day 0). Blood samples were analyzed every 2 days to control ammonia level and mice were sacrificed 7 days after the last bacteria administration.

In thioacetamide (TAA)-induced acute liver injury, TAA (Sigma-Aldrich, Bornem, Belgium) was injected intraperitoneally at 250 mg/kg body weight in NaCl 0.9% on day 0 and day 1. Mice were daily preadministered orally and intrarectally either with 100 μL of 10⁷, 10⁸, or 10⁹ CFU of viable *L. plantarum* strains, either with lactulose 6 g/kg in water (Acros Organics, Geel, Belgium)²² or with vehicle solution for 3 days (day -2 to day 0). Mice were killed on day 2 by cervical dislocation; blood, liver, and colon were removed.

Chronic liver insufficiency was induced using phenobarbital and carbon tetrachloride (CCL₄; Sigma-Aldrich) following the protocol described by Kobayashi et al.¹¹ Briefly, rats were given phenobarbital (0.5 g/L) in the drinking water. Two weeks later, CCL₄ (diluted 1:9 in olive oil) was given twice a week by gavage. Initial dose of CCL₄ was 0.2 mL/kg and adjusted weekly according to change in body weight. After 5 months, phenobarbital and CCL₄ were discontinued. Rats were daily administered orally and intrarectally with 500 μL of 10¹⁰ CFU of viable *L. plantarum* strains for 4 days (day 1 to day 4 and day 11 to day 14). At the end of the experiment, blood, liver, and brain were removed after ketamine overdose and exsanguination.

Assessment of Blood Ammonia, Fecal Ammonia, pH, and Liver Injury. Blood ammonia levels were

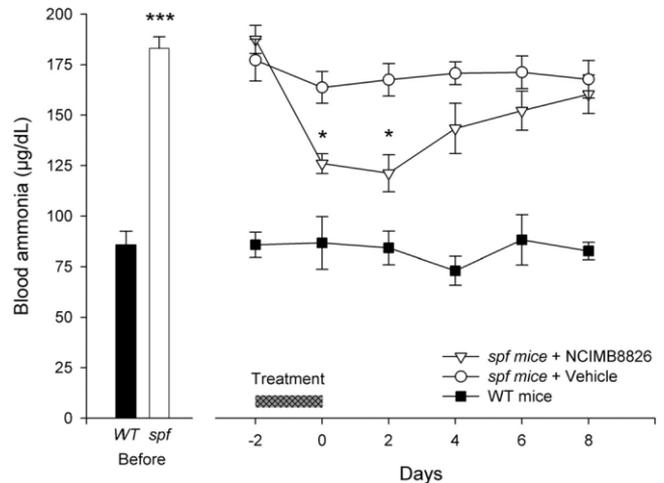


Fig. 2. Blood ammonia in Sparse-fur mice treated with NCIMB8826 (∇ ; $n = 8$) or with vehicle (\circ ; $n = 4$) and in wild-type (WT) controls (\blacksquare ; $n = 4$). Mice received a dose of 10⁹ CFU orally and intrarectally the first 3 days and blood ammonia concentrations were monitored during 11 days. Results are expressed as means \pm SEM. * $P < 0.05$.

measured on a Blood Ammonia Checker II (Menarini Diagnostics, Belgium). Alanine and aspartate aminotransferases (ALT, AST) and intraluminal fecal ammonia were freshly analyzed using commercial kits (Roche Diagnostics, Belgium) based on methods recommended by the International Federation of Clinical Chemistry. Ammonia levels represent NH₃ and NH₄⁺ concentrations. After homogenization in 1 mL of distilled water, aliquots of feces were centrifuged at 3000g for 10 minutes and the pH was determined on the supernatant (Sartorius Basic Meter PB-11, Belgium). Liver tissue samples were fixed for 48 hours in formalin and embedded in paraffin. Serial tissue sections were cut and stained with hematoxylin/eosin and Masson's Trichrome. Histological grade of acute liver injury was determined blindly by a semiquantitative method²²: grade 0, normal liver; grade 1, edema in the liver cell; grade 2, changes as balloon in liver cell; grade 3, necrosis as dots in liver cell; grade 4, necrosis as small pieces.

Immunohistochemistry. Immunohistochemistry analysis of glial fibrillary acidic protein (GFAP) was performed on sections of brain from frontal cortex areas. After rehydration, endogenous peroxidases were blocked (0.3% H₂O₂ in methanol) and antibody nonspecific binding was inhibited using 10% horse serum. Mouse monoclonal anti-GFAP (GA5, Sigma-Aldrich) was incubated overnight, followed by biotinylated horse anti-mouse immunoglobulin G. Staining included avidin-biotin-peroxidase (Vector Labs, Lab Consult, Belgium) and diaminobenzidine (Dako, Belgium), with hematoxylin counterstaining.

Quantitative Bacteriological Analysis of Fecal Samples. Fecal samples for quantitative culture were col-

lected in sterile tubes at baseline and on day 2. Chromagar Orientation (BioAgar, Belgium) media was used to determine viable counts of *Staphylococcus* sp., *Enterococcus* sp., *Escherichia coli*, *Pseudomonas* sp., *Klebsiella* sp., *Citrobacter* sp., and *Proteus* sp. The survival of recombinant *L. plantarum* strains in the gut was assessed by plating on MRS medium with chloramphenicol. Bacterial counts were expressed as the logarithm of CFU per gram of feces.

Statistical Analyses. Results are expressed as means \pm standard error of the mean (SEM). Statistical significance was assessed by analysis of variance, multiple comparisons post-hoc test, and Wilcoxon nonparametric test. Survival curves were analyzed by the Kaplan-Meier method using the log-rank test. Statistics were computed with SPSS 11.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as statistically significant.

Results

Effect of *L. plantarum* NCIMB8826 on Constitutive HA in *spf* Mice. In order to validate *L. plantarum* for its capacity to modulate blood ammonia, the *spf* mouse model of constitutive HA was used for our initial trials. At basal levels, plasma ammonia was almost two-fold higher in *spf* mice compared to wild-type mice ($P < 0.001$) (Fig. 2). Two groups of *spf* mice were subjected to administration of either *L. plantarum* NCIMB8826 or the vehicle. Administration of NCIMB8826 induced a significant decrease in blood ammonia levels compared to the group receiving only the vehicle (Fig. 2). Although the lowest values of blood ammonia in *spf* mice after NCIMB8826 treatment did not reach values of wild-type mice, this decrease was statistically significant ($P < 0.05$) on days 0 and 2.

Effect of *L. plantarum* NCIMB8826 on HA-Associated CCL₄-Induced Chronic Liver Failure in Rats. We assessed the effect of *L. plantarum* NCIMB8826 on a rat model of cirrhosis associated with hyperammonemia. After 5 months of CCL₄ challenge, animals progressively developed a chronic liver insufficiency. At sacrifice, all livers exhibited multiple nodules and bridging fibrosis consistent with cirrhosis (Fig. 3A,B). Biological parameters showed slightly elevated aminotransferases ($P < 0.01$) and constant hyperammonemia ($P < 0.001$) for the time of the experiment (Fig. 3C,D). NCIMB8826 was administered daily orally and intrarectally for 4 days at a dose of 10^{10} CFU. Blood ammonia levels were measured before and 1 day after the last administration. NCIMB8826 administration induces a significant decrease in blood ammonia levels compared to rats receiving only the vehicle ($P < 0.05$) (Fig. 3D). Blood ammonia

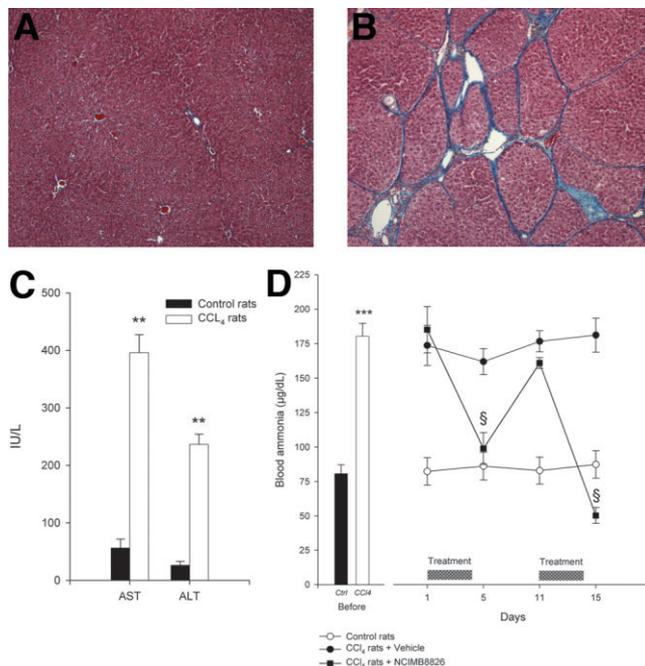


Fig. 3. Chronic liver insufficiency with hyperammonemia. Animals were treated for 5 months with phenobarbital and CCL₄. Masson's Trichrome staining from (A) normal rat liver and from (B) CCL₄ rat liver (original magnification X50). Rats treated with CCL₄ ($n = 11$) exhibited elevated hepatic aminotransferases (C) with significant hyperammonemia (D) compared to control rats (\circ ; $n = 5$). A 4-day administration of *L. plantarum* NCIMB8826 (\blacksquare ; $n = 4$) significantly reduced blood ammonia levels in hyperammonemic rats (D) compared to rats receiving the vehicle solution (\bullet ; $n = 2$). Results are expressed as means \pm SEM in International Units per liter (IU/L) for aspartate and alanine aminotransferases (AST, ALT) and $\mu\text{g/dL}$ for blood ammonia. ** $P < 0.01$, *** $P < 0.001$; § $P < 0.05$, day 5 versus day 1 and day 15 versus day 11.

levels returned to initial values after cessation of probiotics administration, and a second round of *Lactobacillus* administration was able to modulate hyperammonemia similarly.

Effect of *L. plantarum* NCIMB8826 and Lactulose on Blood Ammonia in TAA-Induced HA. The effect of NCIMB8826 on HA associated with acute liver failure was then addressed using the mouse model of TAA-induced acute liver injury. As shown in Table 1, ammonia and ALT serum levels increased progressively from day 0 to day 2 ($P < 0.05$ and $P < 0.001$, respectively) following TAA injections. For the following experiments, we only analyzed liver injury and ammonia on day 2, because acute liver failure and HA were obvious with highest values for blood ammonia, ALT, and a mortality of about 40%. Two days after the first TAA injection, mice that received a daily dose of 10^9 CFU of NCIMB8826 strain exhibited a significant decrease of their blood ammonia levels ($p < 0.01$) (Fig. 4). Similarly, treatment with lactulose used as control also decreased blood ammonia levels ($P < 0.05$) (Fig. 4).

Table 1. Blood Ammonia and ALT Serum Levels in TAA-Induced Acute Liver Failure

	Day 0	Day 1	Day 2
Blood ammonia ($\mu\text{g/dL}$)	74 ± 10	135 ± 23	$199 \pm 26^*$
ALT serum levels (IU/L)	40 ± 4	$1009 \pm 259^{***}$	$4088 \pm 762^{***}$

TAA is intraperitoneally injected in C57BL/6 mice ($n = 8$) at 250 mg/kg on day 0 and day 1. Blood parameters were measured at baseline, day 1, and day 2. Results are expressed as means \pm SEM. $*P < 0.05$, $***P < 0.001$.

Dose Effect of *L. plantarum* Strains on Blood Ammonia. To understand the mechanisms underlying the effect of *L. plantarum* on HA, different genetically engineered mutant strains affected in ammonia metabolism were constructed (see Materials and Methods for details and Fig. 1). These strains were administered for 3 days at doses of 10^7 , 10^8 , and 10^9 CFU per oral and rectal administrations and were evaluated in a TAA-induced liver failure model.

Administration of 10^9 CFU of NCIMB8826, EV101 (AlaD⁺, ammonia hyperconsumer), and EV102 (AlaD⁻, control strain of EV101) significantly decreased blood ammonia (Fig. 5A,B,D), whereas no difference was observed in ammonia levels for strain DP103 (AmtB⁻), which is unable to metabolize extracellular ammonia (Fig. 5C). A slight improvement ($P < 0.05$) was observed at a dose of 10^8 CFU per administration only with the hyperconsuming strain EV101 (AlaD⁺) (Fig. 5B). Ammonia hyperconsuming strain EV101 (AlaD⁺) was also tested in the chronic rat model of liver insufficiency and showed a

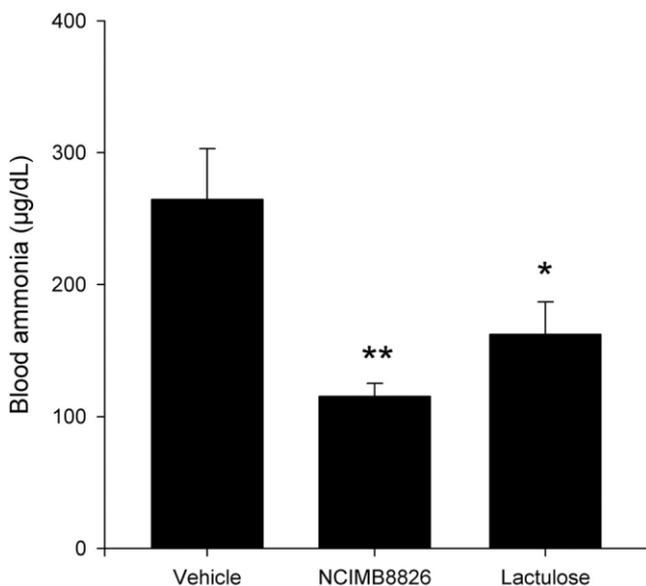


Fig. 4. Blood ammonia in TAA-injected C57BL/6 mice on day 2 after pretreatment with vehicle ($n = 7$), 10^9 CFU of NCIMB8826 ($n = 7$), or lactulose ($n = 11$). Results are expressed as means \pm SEM. $*P < 0.05$, $**P < 0.01$

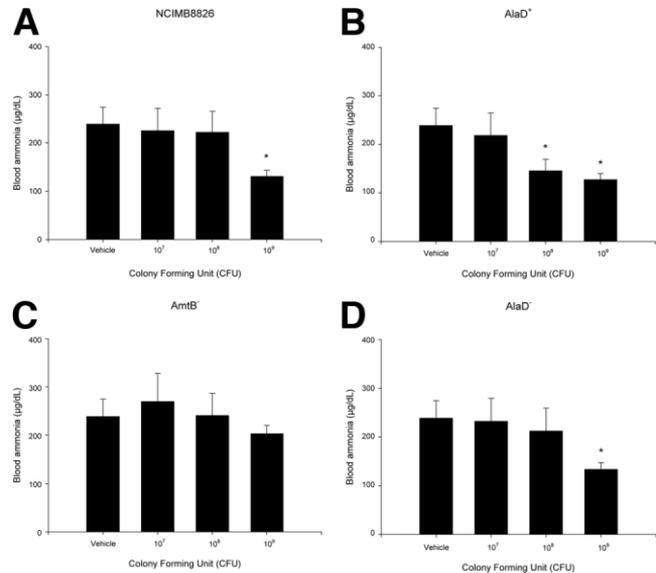


Fig. 5. Blood ammonia in TAA-injected C57BL/6 mice treated with 10^7 , 10^8 , or 10^9 CFU of (A) NCIMB8826, (B) EV101 (LDH⁻/AlaD⁺), (C) DP103 (AmtB⁻), or (D) EV102 (LDH⁻/AlaD⁻). Results are expressed in micrograms per deciliter as means \pm SEM from $n = 7$, $n = 8$, $n = 6$, and $n = 6$ animals, respectively. Mice were administered during 3 days with bacteria, followed by daily injections of TAA. Blood ammonia was measured on day 2. $*P < 0.05$.

similar effect as its wild-type counterpart NCIMB8826 at doses of 10^{10} CFU (data not shown).

Effect of *L. plantarum* Strains on Mice Mortality and Liver Injury in TAA-Induced HA. Animal survival rate, liver injury, and ALT serum levels were compared in mice treated with genetically engineered strains, lactulose, and vehicle to assess the specific effect of the administration of *L. plantarum* strains. Surprisingly, after two injections of TAA, the survival rate reached about 80% in mice pretreated with 10^9 CFU of NCIMB8826 and EV101 (AlaD⁺) ($P < 0.05$) whereas it reached only 47.5% in vehicle-pretreated mice (Fig. 6). Neither lactulose nor strain DP103 (AmtB⁻) showed any significant effect on animal survival. On day 2, histological assessment of liver injury and ALT serum levels remained unchanged between the vehicle and the different *L. plantarum* strains (Table 2).

Effect of *L. plantarum* Strains on Astrocyte Swelling in TAA-Induced HA. Central nervous system alterations and cerebral edema with astrocyte swelling have been reported in human and animals with acute liver failure.³² Given the increased survival observed in mice receiving NCIMB8826 and EV101 (AlaD⁺) strains, we performed an immunohistological analysis of astrocyte morphology from brain frontal cortex areas, using GFAP and hematoxylin staining. Obvious astrocyte swelling was detected in the gray matter of the brain cortex in TAA-injected mice receiving vehicle solution (Fig. 7B,C) and

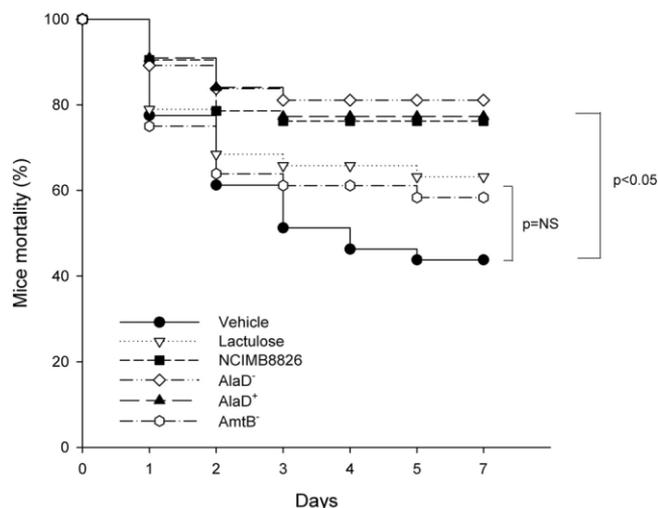


Fig. 6. Survival rate of TAA-injected C57BL/6 mice ($n = 10$ in each group) treated with vehicle (●), NCIMB8826 (■), EV101 (LDH⁻/AlaD⁺) (▲), EV102 (LDH⁻/AlaD⁻) (◇), DP103 (AmtB⁻) (○), or lactulose (▽). Mice were administered with treatment during 3 days, followed by daily injection of TAA for 2 days. Survival was controlled until day 7 after first TAA injection. * $P < 0.05$.

not in control mice (Fig. 7A). This swelling was not observed in TAA-injected mice receiving NCIMB8826 (Fig. 7D) or ammonia hyperconsuming strain EV101 (AlaD⁺) (Fig. 7F). In contrast, lactulose-administered mice still exhibited a few swollen astrocytes (Fig. 7E).

Survival of *L. plantarum* Strains in the Colon and Effect on Bacterial Flora in TAA-Induced HA. To exclude that the observed effects were due to a difference in the survival of bacteria in the colon, counts of *L. plantarum* strains were evaluated after 3 days of administration in untreated wild-type mice. Strains EV101 (AlaD⁺), EV102 (AlaD⁻), and DP103 (AmtB⁻) could be found up to 2 days after the end of bacterial administration (Table 3). On day 1 and day 2, only EV102 (AlaD⁻) showed a significantly decreased survival ($P < 0.05$). Analysis of gut content revealed no significant changes in viable counts of *E. coli*, *Streptococcus* sp., *Staphylococcus* sp., *Klebsiella* sp., *Citrobacter* sp., and *Proteus* sp. (data not shown).

Effect of *L. plantarum* Strains on Fecal Ammonia Levels in TAA-Induced HA. As observed for blood am-

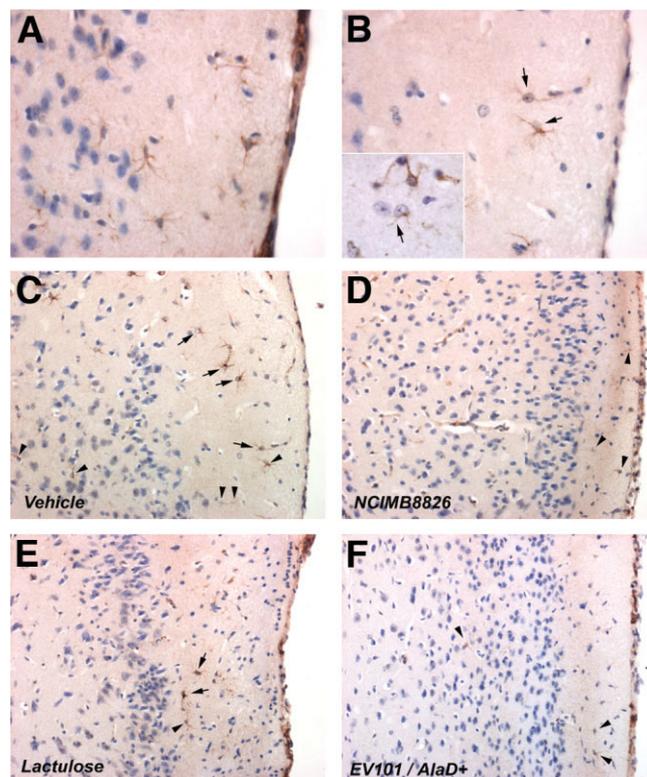


Fig. 7. Histopathological changes observed in brain cortex of TAA-injected C57BL/6 mice. Immunohistochemistry for glial fibrillary acidic protein (GFAP) on cortical brain sections with hematoxylin counterstaining. Astrocyte in brain cortex from (A) healthy control mouse and from (B) a mouse after two injections of TAA showing astrocyte ballooning (original magnification $\times 400$). Inset: Astrocyte ballooning and twinning (original magnification $\times 1000$). Normal astrocytes (arrowheads) and astrocyte ballooning (arrows) in brain cortex of mice treated with (C) vehicle, (D) 10^9 CFU of NCIMB8826, (E) lactulose, or (F) 10^9 CFU of EV101 (AlaD⁺). Illustrations are representative of three independent mice in each group (original magnification $\times 200$).

monia, administration of 10^9 CFU of NCIMB8826, EV101 (AlaD⁺), and EV102 (AlaD⁻) induced a striking reduction in intraluminal fecal ammonia concentrations (Fig. 8A,B,D). Interestingly, the lower dose of 10^8 CFU for strain EV101 (AlaD⁺) was also associated with a significant reduction of fecal ammonia as reported above for blood ammonia (Fig. 8B), whereas the other strains did not. Remarkably, DP103 (AmtB⁻) did not modify fecal ammonia concentrations of free ammonia (Fig. 8C). As shown in

Table 2. Effect of Pretreatment on Liver Injury in TAA-Induced HA

	Vehicle	NCIMB8826	AlaD ⁺	Lactulose
ALT serum levels (IU/L)	4588 \pm 732	5021 \pm 678	3895 \pm 866	5520 \pm 893
Histological score	3.6 \pm 0.2	3.4 \pm 0.2	2.8 \pm 0.6	3.5 \pm 0.3

ALT serum levels and histological score on day 2 of TAA challenge after administration of 10^9 CFU of NCIMB8826 ($n = 8$), AlaD⁺ ($n = 8$), vehicle ($n = 7$) or lactulose ($n = 5$). Results are expressed as means \pm SEM.

Table 3. Lactobacilli Quantification in Colonic Feces Expressed as the Logarithm of CFU/mL

	Day -2	Day -1	Day 0	Day 1	Day 2
AlaD ⁻	8.5 \pm 0.4	7.6 \pm 0.4	7.6 \pm 0.9	4.9 \pm 0.3*	3.1 \pm 0.7*
AlaD ⁺	8.1 \pm 0.1	8.0 \pm 0.1	8.2 \pm 0.2	7.0 \pm 0.4	5.0 \pm 0.4
AmtB ⁻	8.0 \pm 0.3	7.9 \pm 0.2	8.1 \pm 0.3	7.2 \pm 0.2	4.3 \pm 0.8

C57BL/6 mice ($n = 5$ in each group) were administered with 10^9 CFU of *Lactobacillus plantarum* from day -2 to day 0 and colonic content was quantified daily. Results are expressed as mean \pm SEM. * $P < 0.05$.

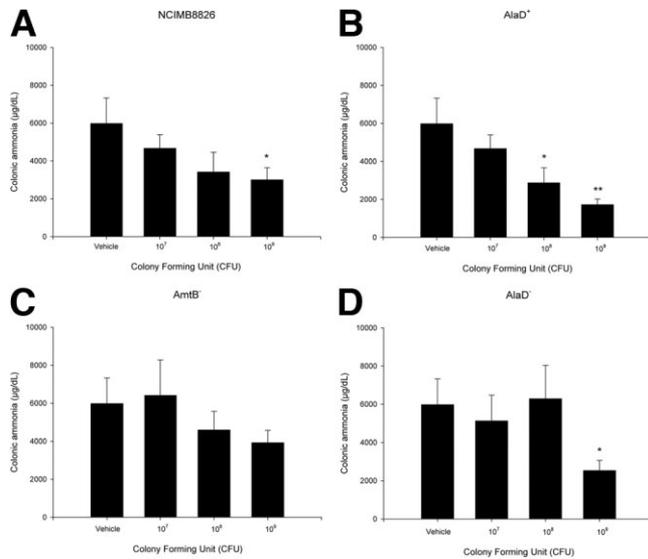


Fig. 8. Colonic ammonia in TAA-injected C57BL/6 mice treated with 10^7 , 10^8 , or 10^9 CFU of (A) NCIMB8826, (B) EV101 (LDH⁻/AlaD⁺), (C) DP103 (AmtB⁻), or (D) EV102 (LDH⁻/AlaD⁻). Results are expressed in micrograms per deciliter as means \pm SEM from $n = 7$, $n = 8$, $n = 6$, and $n = 6$ animals, respectively. Mice were administered during 3 days with bacteria, followed by daily injections of TAA. Blood ammonia was measured on day 2. * $P < 0.05$, ** $P < 0.01$

Fig. 9, a significant decrease of fecal ammonia was observed when mice were administered with 10^9 CFU of NCIMB8826 and EV101 (AlaD⁺) compared to the vehicle group, whereas treatment with lactulose did not significantly modify the amount of fecal ammonia.

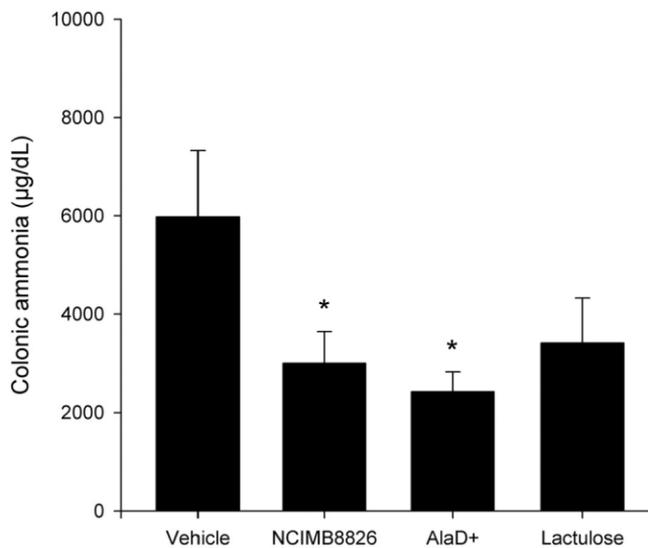


Fig. 9. Colonic ammonia in TAA-injected C57BL/6 mice treated with 10^9 CFU of NCIMB8826 ($n = 6$), EV101 (LDH⁻/AlaD⁺) ($n = 7$), lactulose ($n = 5$), or vehicle ($n = 4$). Mice were administered during 3 days with bacteria, followed by daily injections of TAA. Blood ammonia was measured on day 2. Results are expressed as means \pm SEM. * $P < 0.05$.

Table 4. Values of pH from Ileum and Colon of Mice During TAA Challenge

	Ileal pH	Colonic pH
Vehicle	7.13 \pm 0.35	7.19 \pm 0.21
NCIMB8826	6.83 \pm 0.22	6.01 \pm 0.08**
AlaD ⁺	7.34 \pm 0.4	6.41 \pm 0.29*
Lactulose	6.27 \pm 0.11* [#]	5.56 \pm 0.19*** [#]

Mice were administered orally and intrarectally for 3 days with 10^9 CFU of NCIMB8826 ($n = 8$), AlaD⁺ ($n = 8$), vehicle ($n = 7$) or lactulose ($n = 5$) and were sacrificed 2 days after first TAA injection. Results are expressed as means \pm SEM. NCIMB8826 versus vehicle or lactulose versus vehicle * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; lactulose versus NCIMB8826 [#] $P < 0.05$.

Effect of *L. plantarum* Strains on Fecal pH in TAA-Induced HA. Fecal samples from the ileum did not show any change in pH values during TAA-induced acute liver failure following treatment with all *L. plantarum* strains. Only lactulose induced a significant decrease of ileal pH (< 0.05) (Table 4).

In contrast, pH from the colonic portion of the gut was lowered after administration of NCIMB8826 ($P < 0.01$), EV101 (AlaD⁺) ($P < 0.05$), and lactulose ($P < 0.001$). Surprisingly, the colonic pH was also decreased in mice administered with DP103 (AmtB⁻) compared to vehicle (6.52 ± 0.21 versus 7.19 ± 0.19 ; $P < 0.05$).

Discussion

In the absence of liver transplantation, the mortality rate of patients with fulminant hepatic failure and HE remains high. In clinical practice, lactulose is considered the gold standard for HE treatment, but its side effects may limit its clinical use.^{5,17,18} The development of safe and well-tolerated alternative treatments is therefore justified. The use of probiotics was proposed as a potential alternative treatment for HE, based on previous experimental and clinical studies.^{21,22,33-35}

In this study, we showed that oral and rectal administration of *L. plantarum*, a species used in probiotic formulations, decreased both blood and fecal ammonia levels in rodent models of HA. In addition, we showed for the first time that this probiotic increased survival in a murine model of fulminant hepatic failure when compared to lactulose treatment. It is tempting to hypothesize that the enhanced survival might be mediated by a decrease of ammonia-induced cerebral edema, as indirectly suggested by the reduction of astrocyte swelling in brain cortex of mice treated with *L. plantarum* NCIMB8826 or its ammonia hyperconsuming strain.

To better understand the mechanisms by which these microorganisms act, an *L. plantarum* strain lacking the membrane ammonium transporter AmtB was constructed. Ammonium and ammonia metabolism is a growth-dependent

condition for bacteria.³⁶ Especially when the pH is below 7.0, AmtB could participate to the acquisition of NH_4^+ / NH_3 and could be involved in ammonia consumption in the gut.³⁷⁻³⁹ The mutant strain AmtB⁻ did not show any effect on blood ammonia, survival rate, and fecal ammonia in our hepatitis-induced HA mouse model. A similar survival of this genetically engineered *L. plantarum* strain compared to the wild-type strain was observed into the gut, showing that the difference observed after treatment with *L. plantarum* AmtB⁻ is not due to bacterial death or to a lower number of CFU. All together, these results strongly suggest a direct implication of ammonia consumption on the beneficial effects observed by *L. plantarum* administration in our animal model of HA.

In a rat model of mild HE induced by TAA, a slight improvement in ALT serum levels and in liver histopathology was shown after administration of probiotics,²² a feature we did not observe in our model of severe hepatitis. It is probable that the beneficial effect on mortality is not related to an improvement of liver function but a modulation of hyperammonemia consequences. According to this study, lactulose provoked a significant decrease of blood ammonia. In our experiments, measures of intraluminal fecal ammonia were however unchanged after treatment with lactulose. This phenomenon could be explained by the nonspecificity of the ammonia measurement technique, which is unable to differentiate between NH_3 and NH_4^+ .

To further unravel the mechanism involved in the lowering of ammonia levels, the pH was measured in different parts of the gut. Based on the same mechanism as lactulose, pH modulation was observed with all tested *L. plantarum* strains into the colon, but not into the ileum, probably due to the poor survival of *L. plantarum* during the passage through the stomach and the duodenum.⁴⁰ Experiments performed in our laboratory where treatment was administered orally, intrarectally, or by both routes showed that only the last two conditions produced a beneficial effect on blood ammonia levels (data not shown), suggesting that *in loco* colonic administration is sufficient to reduce ammonia. Enumeration of *Lactobacillus* confirmed a very low number of CFU ($<10^3$ CFU/mL) inside the stomach and the small intestine after oral administration, maybe due to strong acidic conditions and presence of pepsin and lysozyme. This suggests that, for future development, a vehicle that would protect the probiotic during its passage through the stomach and the duodenum should be considered. A decreased pH was also observed with AmtB⁻ strain, further suggesting the role of direct ammonia consumption rather than an acidification of the gut content.¹⁹

In patients with cirrhosis with minimal HE treated by synbiotic supplementation, Liu et al. showed a significant reduction in viable counts of *E. coli*, *Staphylococcus* sp., and

Fusobacterium sp. associated with a significant increase of non-urease producing *Lactobacillus* sp. in the feces.²¹ By decreasing the number of urease-positive bacteria and favoring non-urease producing bacteria, the intestinal production of ammonia is probably reduced due to a shift in these populations. Quantitative bacteriological analysis of fecal samples in our study revealed that supplementation with *L. plantarum* strains for 3 days did not modify bacterial species in the gut flora and does not seem to play a significant role in the beneficial effects of *L. plantarum* observed. Our short-term treatment of only 3 days is probably not long enough to modify the gut flora compared to the trial by Liu et al., where synbiotics were given for 30 days.

By creating new strains of bacteria, genetic engineering allows the effects of existing probiotics to be strengthened.⁴¹⁻⁴³ Using this approach, we developed a *L. plantarum* strain overproducing alanine dehydrogenase and consuming *in vitro* higher amounts of ammonia than its wild-type counterpart. When given at doses of 10^9 CFU *in vivo* to mice suffering from acute liver failure with HA, this modified strain had the same ability to decrease blood ammonia, to decrease mortality, and to consume gut ammonia as that of wild-type *L. plantarum*. Interestingly, this genetically modified strain still decreased blood and fecal ammonia at a dose 10 times lower, whereas wild-type *L. plantarum* did not show any residual effect. The better performance of this ammonia hyperconsumer strain at a lower dose strengthened the hypothesis that direct ammonia consumption plays a key role in the beneficial observed effects of *L. plantarum* in our model. The absence of stronger effect of the ammonia hyperconsumer strain at 10^9 CFU compared to wild-type *L. plantarum* may suggest a plateau effect that may be due to the survival rate of probiotics in the gut of mice.

In summary, hyperammonemia occurring constitutively or by acute or chronic induced liver failure can be efficiently decreased by the administration of *L. plantarum* with a significant effect on survival. The absence of an ammonium transporter abolishes these effects whereas a genetically modified NH_3 hyperconsuming strain exhibits significant effects on blood and fecal ammonia which are maintained at a lower dose. This also suggests the potential interest of *L. plantarum* and its genetically modified ammonia hyperconsuming strain in the treatment of HE.

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