

Hyperammonemia induces gut microbiota dysbiosis and motor coordination disturbances in mice: new insight into gut-brain axis involvement in hepatic encephalopathy

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Hepatic encephalopathy (HE) is a neuropsychiatric hepatic-induced syndrome in which several factors are involved in promoting brain perturbations, with ammonia being the primary factor. Motor impairment, incoordination, and gut dysbiosis are some of the well-known symptoms of HE. Nevertheless, the link between the direct effect of hyperammonemia and associated gut dysbiosis in the pathogenesis of HE is not well established. Thus, this work aimed to assess motor function in hyperammonemia and gut dysbiosis in mice. Twenty-eight Swiss mice were distributed into three groups: two-week and four-week hyperammonemia groups were fed with an ammonia-rich diet (20% w/w), and the control group was pair-fed with a standard diet. Motor performance in the three groups was measured through a battery of motor tests, namely the rotarod, parallel bars, beam walk, and static bars. Microbial analysis was then carried out on the intestine of the studied mice. The result showed motor impairments in both hyperammonemia groups. Qualitative and quantitative microbiological analysis revealed decreased bacterial load, diversity, and ratios of both aerobic and facultative anaerobic bacteria, following two and four weeks of ammonia supplementation. Moreover, the Shannon diversity index revealed a time-dependent cutback of gut bacterial diversity in a treatment-time-dependent manner, with the presence of only *Enterobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae* at four weeks. The data showed that ammonia-induced motor coordination deficits may develop through direct and indirect pathways acting on the gut-brain axis.

Key words: hepatic encephalopathy, gut dysbiosis, gut-liver brain axis, hyperammonemia, motor coordination

INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric complication associated with chronic and acute liver failure or without liver tissue injury in the case of portocaval anastomosis. HE is characterized by a spectrum of neurological complications marked by several symptoms, including motor deficits (Borkowska et al., 2000; El Khat et al., 2022). Several classification grades have been distinguished for HE that interestingly reveal the gradual development of motor symptoms (Butz et al., 2010). A slight impairment in fine motor control has been reported in patients with covert (previously named subclinical) HE even though they lack the apparent motor symptoms (Hazell and Butterworth, 1999; Joebges et al., 2003; Mechtcheriakov et al., 2006). However, in overt HE, as described through the four grades defined in the West Heaven criteria, motor symptoms appear from the first grade with an apparent mini-asterixis relevant during a maintained posture. With the advancing severity of HE in grades 2 and 3, more striking motor symptoms manifest as asterixis (Timmermann et al., 2002; 2005; 2008). Moreover, motor impairment in HE has been demonstrated in various experimental animal models of HE (El Hiba et al., 2012; Gonzalez-Usano et al., 2014; Hernandez-Rabaza et al., 2016; Cabrera-Pastor et al., 2018). The various motor deficits reported in HE are the result of pathological synchronization of oscillatory cerebral activity (Timmermann et al., 2008).

While several factors are considered to promote brain perturbation in HE, ammonia is believed to be the primary compound implicated in its pathophysiology (Azorín et al., 1989; Hernández-Rabaza et al., 2016; Alshhar and Rahimi, 2019). In fact, in such pathological cases, ammonia does not efficiently enter the urea cycle in the liver, which leads to an increase in its systemic circulation and a diminished blood-brain barrier, facilitating the translocation of ammonia into the central nervous system (CNS) (Mouri et al., 2017). Astrocytes are the main cell type responsible for decreasing the amount of ammonia in the brain. In fact, at the astrocytic level, glutamate synthase (GS), an astrocyte-confined enzyme, catalyzes the turnover of glutamine into glutamate through the integration of excessive NH_3 (Butterworth, 2019). Revealing the potential links between hyperammonemia and the severity of HE, early clinical reports stated the existence of a positive correlation between the two factors (Ong et al., 2003a). Though the reliability of such a correlation has recently been doubted due to several issues, hyperammonemia on its own may promote important neurological difficulties, including motor impairment, which, once reduced, is reflected

through the deterioration of different neuronal areas (Ferenci et al., 2002; Ong et al., 2003b; Bajaj et al., 2011; Ferenci, 2017).

Inflammation is another factor, after hyperammonemia, implicated in the pathophysiology of HE (Jiang et al., 2015; García-Lezana et al., 2017; Trebicka et al., 2021). The mechanism by which hyperammonemia drives inflammation has been the focus of several recent research studies. Additionally, evidence of a link has been postulated regarding the interaction between gut dysbiosis, hyperammonemia, and inflammation (Kang et al., 2016; Rogers et al., 2016; Trebicka et al., 2021). Accordingly, targeting the intestinal microflora and inflammation in the treatment of HE has been a clinical focus of the last decade (Hatton and Shawcross, 2019).

Patients with gut dysbiosis are diagnosed with abnormalities of the CNS (Rogers et al., 2016), and a relationship between gut dysbiosis, taxonomic dysbiosis, bacterial dislocation, and translocation, and the precipitation of HE has been established (Chen et al., 2015; Rai et al., 2015). Clinical studies in patients with liver cirrhosis showed a significant decrease in microbial communities with a predominance of *Fusobacteria* and *Proteobacteria*; faced with a reduced proportion of *Bacteroidetes* phylum, the overgrown bacterial types are typically pathogenic. Among these, from patients' stool samples, the family *Enterobacteriaceae* was found to positively correlate with end model-stage liver disease, and *Streptococcaceae* correlates positively with the severity of HE and ascites according to Child-Turcotte-Pugh scores (Chen et al., 2015). In addition to the possible effects of the bacterial endotoxins produced, urease enzymes, especially from gram-negative *Enterobacteriaceae*, significantly generate ammonia in the colon (Hansen and Vilstrup, 1985; Romero-Gómez and Jover, 2009). Thus, the focus has recently shifted to the gut, which is considered the main source of hyperammonemia (Hatton and Shawcross, 2019). The link between gut dysbiosis, hyperammonemia, and HE is indeed self-evident. However, the link between gut dysbiosis in hyperammonemia and motor coordination in HE has not been considered. Therefore, the present study was designed to shed light on motor function in hyperammonemic conditions, together with the resulting gut microbiota dysbiosis in mice.

METHODS

Chemicals

Ammonia acetate was purchased from Sigma-Aldrich (Netherlands, BCCD1670).

Animals

Adult (2 months of age) Swiss adult mice (n=28) weighing 28±2 g were used in our experiments. Mice were housed in plexiglass cages with free access to water and food ad-libitum at the animal facilities of the Cadi Ayyad University, with room temperature maintained constantly at 25°C and a 12-h dark-light cycle. The experimental protocol, including treatments and the number of animals used, was approved by the Ethics Committee of the Moroccan Society of Ethics and Animal Research (MoSEAR).

Induction of hyperammonemia *in vivo*

Mice were made hyperammonemic by feeding them an ammonia-containing diet as described by Felipoet al. (1988). Briefly, mice were fed an ammonia acetate-supplemented diet (20% w/w) as approved by the 2021 ISHEN guidelines on HE models (DeMorrow et al., 2021). Mice were divided into three groups: Group 1=2 weeks (2W), mice (n=9, 28.6±0.41 g) were fed with an ammonia-rich diet for two consecutive weeks; Group 2=4 weeks (4W), mice (n=9, 28.39±0.55 g) fed similarly for four consecutive weeks; and Group 3=control (CTR), mice (n=10, 28.68±0.72 g) were pair-fed to avoid significant differences in body weight between the controls and hyperammonemic mice.

Blood samples were collected from the jugular vein of mice under anesthesia with urethane (1g/kg i.p.) from each group at the end of the treatment and centrifuged (3000×g, 4°C, 20 min) to collect serum. Samples were then subjected to biochemical analysis for ammonemia and urea in the laboratory of Biochemistry at the Faculty of Medicine and Pharmacy, Cadi Ayyad University, Marrakech.

Behavioral study

Before each behavioral test, mice of all groups were handled during the preceding week for at least 10 min per day by the same manipulator who conducted the behavioral test.

Assessment of motor coordination: To assess for potential deterioration in motor coordination in the hyperammonemic mice, each animal of each group was subjected to motor coordination assessment using the rotarod, the triple horizontal bars, the static rods, and the parallel bars tasks.

Rotarod test: The rotarod test detects the motor coordination deficits in mice placed on a horizontal bar of 3 cm in diameter and 30 cm in high, rotating at a fixed speed (12 RPM) about its long axis, where the

animals are forced to walk forward to stay upright and avoid falling off. Animals were first briefly pre-trained a day before during a maximum period of 5 min; then, after 24 h, mice were allowed three test trials of 300 s each, separated by a maximum of 20 min period of rest (Monville et al., 2006).

Triple horizontal bars: This test consisted of 3 bars of the same length (38 cm) with different diameters (2, 4, and 6 mm), fixed at both ends on a wooden support at the height of 49 cm. The mice were tested first on the 2 mm bar, as the standard bar. However, since most of the animals often reach maximum scores on the 2 mm bar, more precision is needed in the measurements, and two successive tests on the 4 mm and then 6 mm bars were performed (Deacon, 2013).

Mice were placed in the middle, perpendicular to the axis of the bar. The orientation and latency times were then noted within the cut-off time of 30 s. The times noted on each bar were then converted into scores as described by Deacon (2013).

Static rods: Five wooden rods of different diameters (35, 28, 22, 15, and 9 mm), each 60 cm long, were fixed by a clamp to a shelf 30 cm high, such that the rods extended 50 cm from the edge. The mouse being tested was placed at the end of the widest rod (35 cm), with the muzzle facing toward the edge of the bar. The orientation time (the time needed to orient 180° from the starting position) and the latency time (the time needed to traverse the bar to its opposite side) were recorded. The maximum time allowed was 120 s. The mice were then returned to the cage before repeating the same procedure on the other bars in descending order of the bars' diameters (Deacon, 2013).

Parallel bars: The test was conducted on a device consisting of two parallel bars, each 1 m long and 4 mm in diameter, separated by a distance of 30 mm and placed on a support at the height of 60 cm from the ground. The mouse being tested was placed in the centers of the two bars with its longitudinal axis perpendicular to that of the bars, the two front legs on one bar and the two hind legs on the other bar.

The time to orient to 90° from the starting position and the latency to move to one of the two end supports of the parallel ends were measured, with a 120 s maximum time limit for each mouse (Deacon, 2013).

Microbiologic analysis

Sample collection: To assess microbiologic gut disturbance related to the enriched ammonia diet, mice were anesthetized, and then subsequently, dissection was performed under septic conditions. The intestine

was completely detached from the duodenum to the rectum, and samples of duodenum, jejunum, ileum, and rectum were taken and cut into small pieces, which were then vortexed in 10 ml of physiological water for two minutes to achieve a homogeneous stock solution.

Isolation of aerobic bacteria of the gut: The isolation and purification of aerobic bacteria in the gut were carried out by a repeated streaking and dilution plating technique (10⁻¹ to 10⁻⁵), using plate count agar (PCA) medium (Atlas and Snyder, 2013). The plates were incubated at 37°C, 5% CO₂ for 24 h. Afterward, all isolates were maintained in glycerol 20% at -20°C.

Morphological, physiological, and biochemical characterization of aerobic bacteria

The morphological, physiological, and biochemical characteristics of the selected isolates were evaluated using specific media, following the manufacturers' protocols: Man, Rogosa, and Sharpe agar (MRS) for lactobacillus (De Man et al., 1960); Slanetz and Bartley agar (SB) for enterococci (Slanetz and Bartley, 1957); MacConkey agar for gram-negative lactose fermenting bacteria (Mossel et al., 1962); Cystine Lactose Electrolyte Deficient agar (CLED) for urinary microorganisms (Murray et al., 1995); Kenner Fecal (KF) Streptococcal agar for fecal streptococci (Kenner et al., 1961); and, finally, Violet Red Bile Glucose Agar (VRBL) for enterobacteria (Mossel et al. 1978).

The bacterial colonies' macroscopic and microscopic appearance was analyzed after Gram staining. Then, biochemical identifications were made using both catalase and oxidase tests; the catalase test for gram-positive bacteria followed the drop method as described by Duke and Jarvis (1972), and the oxidase test for gram-negative bacteria followed the filter paper method of Kovacs (1956). A sub-culture was made afterwards for the identification of enterobacteria using Uriselect agar and staphylococci by Chapman-Mannitol Salt Agar, following the manufacturers' guidance. The identification of isolated bacterial species was carried out using the API20E test system (Analytical Profile Index, Bio-Rad).

Statistical analysis

Differences between study groups were assessed by one-way analysis of variance (ANOVA), and *post hoc* analysis was performed based on the Tukey method using Sigmaplot V12.5. A p-value of less than 0.05 was considered significant. Shannon diversity index (H'log₂) values of each sample were calculated on Excel

software, and then an ANOVA test was performed using Sigmaplot V12.5 to investigate whether the Shannon index differed between study groups.

RESULTS

Ammonemia level assessments

In the mice fed with an ammonia-enriched diet, blood ammonia levels showed a significant linear increase following two (251,9%; $P=0,012$) and four (259,2% and $P=0,027$) weeks of feeding as compared with control mice (Fig. 1).

Motor incoordination assessment

The rotarod test showed a significant decrease in the latency time in the 2-week-treated group ($P=0.001$). Though a slight recovery of motor coordination performance was noticed in the 4-week-treated mice, there was a significant difference compared with controls ($P=3.02$) (Fig. 2).

Using the parallel bars test, a time-dependent decrease in motor coordination was observed in the hyperammonemic mice compared to the control group. Indeed, hyperammonemic mice exhibited a significantly delayed rotation reflex time following four weeks of ammonia treatment ($P=0.029$) (Fig. 3A).

The orientation time for all groups increased with decreasing diameter of the sticks. Thus, the differences between the groups seem to appear to start from the two smallest rods of 15 and 9 cm, where the orientation time increases significantly compared to control for both the 2W group ($P<0.001$ for the 15 cm rod; $P=0.007$ for the 9 cm rod) and the 4W group ($P=0.014$ for the 15 cm rod; $P=0.014$ for the 9 cm rod) (Fig. 3B).

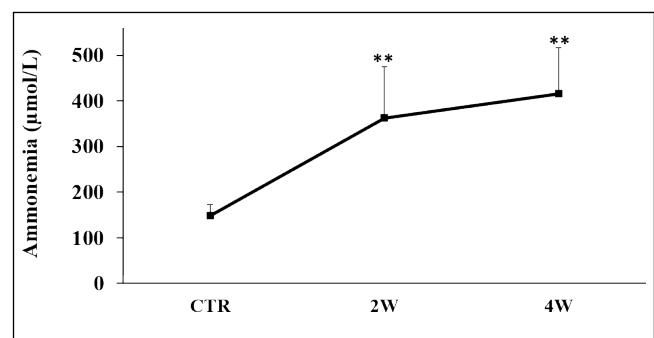


Fig. 1. Ammonia concentration in systemic blood of 2-week-treated (2W), 4-week-treated (4W), and control (CTR) mice (means \pm SEM). ** $P<0.01$ vs. control.

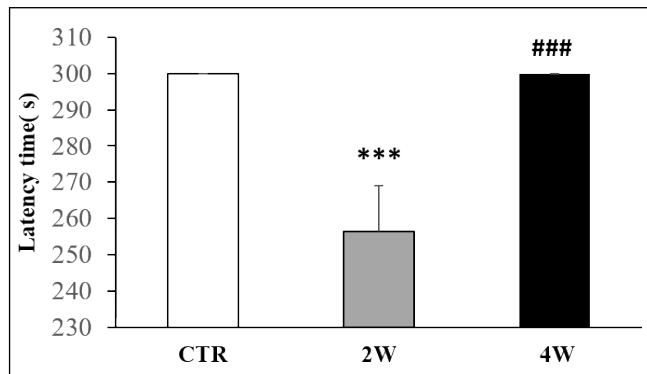


Fig. 2. Latency time (s) for the 2-week-ammonia-treated (2W), 4-week-ammonia-treated (4W), and control (CTR) mouse groups on the rotarod test (means ± SEM). ****P*<0.001 vs. control and ###*P*<0.001 vs. 2W group.

In the parallel bars test, both the 2W and 4W groups demonstrated a significantly higher latency time (*P*<0.001), while the HA mice exhibited an increased latency time on the static rods, with the difference being particularly significant for the 4W-treated mice compared to CTR. The difference between the CTR and 4W groups was significant, especially on the 35 cm (*P*=0.004), 28 cm (*P*<0.001), 15 cm (*P*=0.015), and 9 cm (*P*=0.003) rods (Fig. 4).

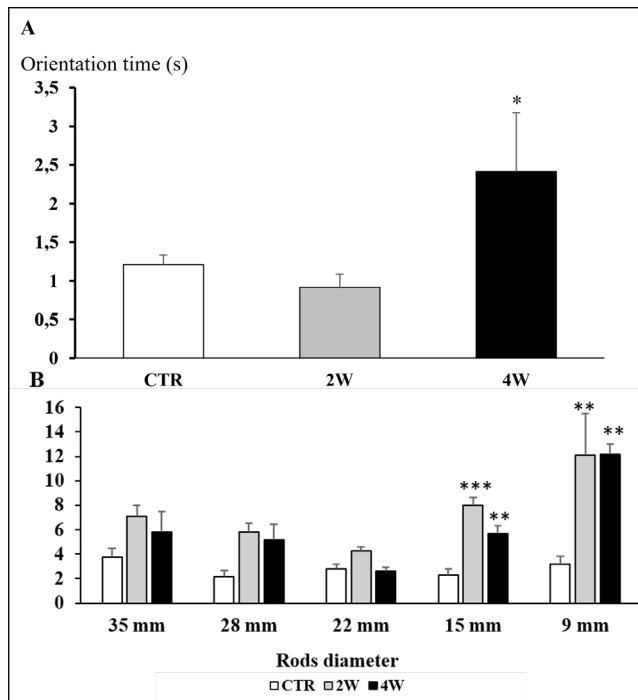


Fig. 3. Orientation time of 2-week-ammonia-treated (2W), 4-week-ammonia-treated (4W), and control (CTR) mouse groups on the parallel bars (A) and static rods (B) test (means ± SEM). **P*<0.05 vs. control, ***P*<0.01 vs. control and ****P*<0.001 vs. control.

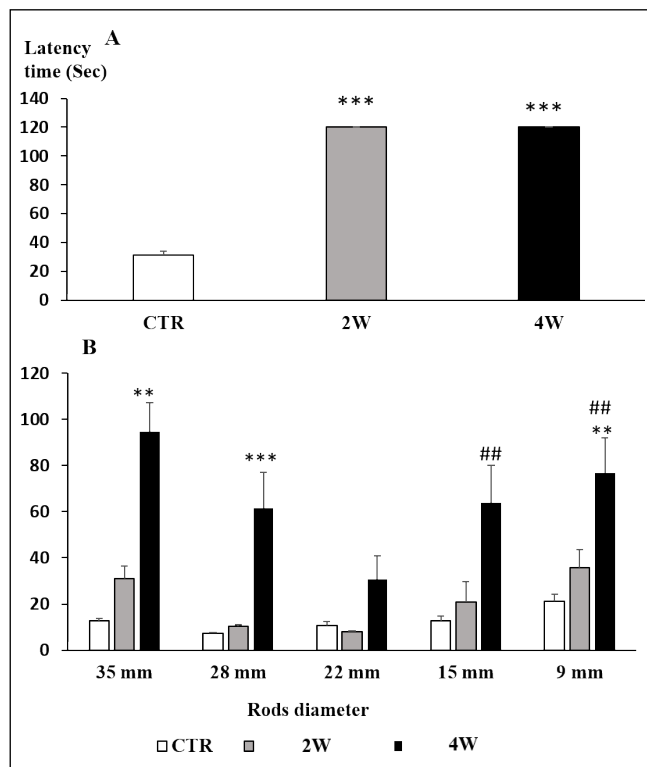


Fig. 4. Latency time for the 2-week-ammonia-treated (2W), 4-week-ammonia-treated (4W), and control (CTR) mouse groups on the parallel bars (A) and static rods (B) tests (means ± SEM). **P*<0.05 vs. control, ****P*<0.001 vs. control, ***P*<0.01 vs. control, and ##*P*<0.01 vs. 2W.

The horizontal bar results further confirmed the findings from the static and parallel bars, showing decreased coordination for the hyperammonemic groups. Indeed, HA mice scored significantly less in comparison with the CTR for latency time at both two weeks (*P*=0.001) and four weeks (*P*=0.001) of ammonia treatment (Fig. 5).

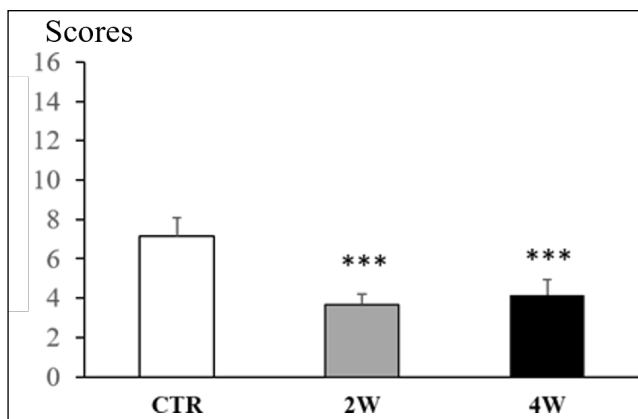


Fig. 5. Latency time for 2-week-ammonia-treated (2W), 4-week-ammonia-treated (4W), and control (CTR) mouse groups on the triple horizontal bars test (means ± SEM). ****P*<0.001 vs. control.

However, no significant difference was found between the two HA groups for the triple horizontal bars or for the other motor coordination tests.

Gut dysbiosis assessment

On the PCA agar, a difference in the number of colonies was obvious between the three groups, which became more clear at the 10^{-2} dilution; a downward tendency between the control and the 2W group and then the 4W group was observed, indicating a genuine decrease in the bacterial load for the two treated groups, in a treatment-dependent manner (Fig. 6).

Thus, colony counts from the three groups showed a significant decrease between the control and 2W ($P < 0.001$) groups. This tendency of a constant decrease continued in the 4W group, resulting in a significantly lower bacterial load compared to both the CTR ($P < 0.001$) and 2W ($P = 0.004$) groups.

Averaged across intestinal samples of each group, measures of bacterial diversity showed that *Escherichia coli* and Group D *Streptococcus sp.* remained the most abundant throughout the treatment period but with clear differences in their ratios. In fact, from the CTR to 4W group, a downward tendency in *Escherichia coli* abundance was obvious, with a decrease from $38.78 \pm 2.17\%$ in the CTR group to $24.33 \pm 0.72\%$ in the 2W group and reaching $23.35 \pm 2.36\%$ in the 4W group. However, the ratio of Group D *Streptococcus sp.* increased significantly ($P < 0.001$), a 1.5-fold increase after two weeks and a 2.5-fold increase after 4W. Additionally, *Enterococcus faecalis*, with a $4.47 \pm 0.29\%$ initial ratio, remained constant during the first two weeks but showed a significant 3-fold increase ($P < 0.001$) after four weeks. *En-*

terobactercloacae also showed a significant 2-fold increase in the ratio for the 2W group but disappeared completely from the cultures in the 4W group. Notably, initially less abundant, at $6.26 \pm 0.28\%$ for *Acinetobacter sp.* and $4.21 \pm 0.31\%$ for *Micrococcus sp.*, the bacteria decreased 1.3-fold and then disappeared at four weeks. However, all the phyla with a less than 2 percent ratio before treatment (*Klebsiella sp.*, *Enterococcus faecalis*, Group F *Streptococcus sp.*, *Moraxella sp.*, *Proteus sp.*, *Citrobacter freundii*, and *Pseudomonas sp.*) showed the highest vulnerability to intestinal hyperammonemia, disappearing during the first two weeks (Table 1).

Concomitantly, the decreasing Shannon diversity index ($H' \log_2$) was notably dependent on the period of treatment with an ammonia-rich diet, with the 2W group being significantly ($P < 0.001$) lower than the CTR group. Most notably, the 4W mouse group showed a diversity index that was significantly ($P < 0.001$), 41% lower than the CTR group and 35% lower than the 2W group (Fig. 7).

DISCUSSION

Through different behavioral tools, we have been able to screen the motor coordination and subsequent gut microbiota dysbiosis in mice under hyperammonemic conditions. Our data showed a significant impairment in motor coordination following two and four weeks of ammonia overload, which appears to corroborate previous reports in rats with chronic moderate hyperammonemia. These studies emphasized altered motor patterns, demonstrated through various behavioral tasks: in the beam walking test, reduced motor coordination with increased foot faults

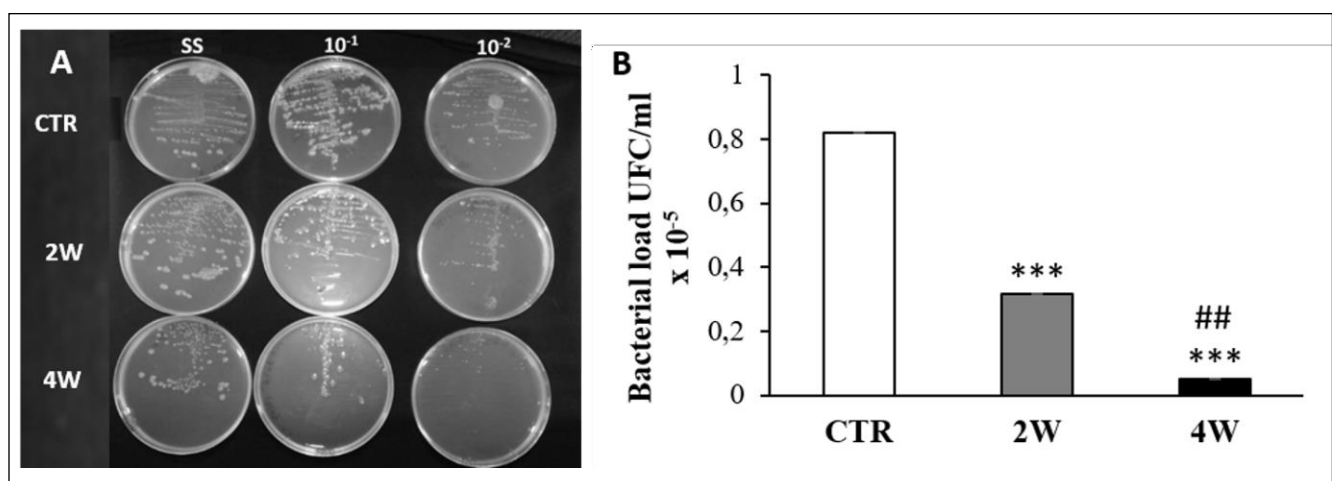


Fig. 6. (A) Photographic image of PCA agar from the three study groups (CTR, 2W, and 4W), SS: stock solution. (B) Intestinal load of the bacterial flora for the treated groups (CTR, 2W, and 4W). *** $P < 0.001$ vs. control and ## $P < 0.01$ vs. 2W.

was reported in hyperammonemic rats, with a 2- to 3-fold increase in systemic ammonia and nearly 40 % elevation in the brain (Gonzalez-Usano et al., 2014; Johansson et al., 2015). Not only was motor coordination altered, but spontaneous locomotor activity decreased with time in young rats within five days of a postnatal ammonia-diet supplementation (Aguilar and Min, 2000). Similarly, studies on other models of surgically-induced HE, such as portacaval shunts (type B HE) and bile duct ligation (type C HE), known to exhibit hyperammonemia, showed reduced motor activity and coordination four weeks after surgery

(Cauli et al., 2014; El Hiba et al., 2016). Even in acute models of HE, where ammonemia was shown to be significantly increased 12 h following an initial thioacetamide (TAA) administration, locomotor activity decreased simultaneously in the open field and rotarod tasks (El Khat et al., 2022). Further evidence of ammonia-induced motor disturbances was provided by clinical observations reporting asterixis, ataxia, and motor impairment in patients with decompensated liver cirrhosis with minimal HE and hyperammonemia (Shawcross et al., 2004; Butz et al., 2010; Hassan et al., 2019). Deficits in motor coordination

Table 1. Identification of the gut strains present in the intestine of CTR, 2W, and 4W mice at the end of treatment.

Strains	Medium	GRAM and shape	Biochemical identification	Family	Type of identified bacteria	CTR	2 W	4 W
1	VRBG	(-) coccobacilli	Oxidase (-);		<i>Escherichia coli</i>	38.78±2.17	24.33±0.72***	23.35±2.36***
2	VRBG	(-) bacilli	Oxidase (-);		<i>Enterobacter cloacae</i>	10.31±0.64	19.47±0.62**	*** ###
3	CLED	(-) bacilli	Oxidase (-)	<i>Enterobacteriaceae</i>	<i>Proteus sp.</i>	0.34±0.01	***	***
4	VRBG	(-) sticks	Oxidase (-);		<i>Klebsiella sp.</i>	0.34±0.01	***	***
5	CLED	(-) bacilli	Catalase (+); Oxidase (+)		<i>Citrobacter freundii</i>	1.83±0.09	***	***
6	SB	(+) cocci	Blackening on bile-esculin agar + antibiogram	<i>Enterococcaceae</i>	<i>Enterococcus faecalis</i>	4.47±0.29	4.68±0.17***	13.43±0.76***
7	SB	(+) cocci	Blackening on bile-esculin agar + antibiogram		<i>Enterococcus faecium</i>	1.43±0.18	***	***
8	KF	(+) cocci	Catalase (-)	<i>Streptococcaceae</i>	Group D <i>Streptococcus sp.</i>	22.45±0.90	34.35±0.75**	54.94±3.87*** ##
9	KF	(+) cocci	Catalase (-)		Group F <i>Streptococcus sp.</i>	2.56±0.13	***	***
10	CLED	(-) cocci	Oxidase (-)	<i>Moraxellaceae</i>	<i>Acinetobacter sp.</i>	6.26±0.28	4.71±0.19***	*** ##
11	CLED	(-) coccobacilli	Oxidase (+)		<i>Moraxella sp.</i>	0.18±0.01	***	***
12	MRS	(+) bacilli	Catalase (-)	<i>Lactobacillaceae</i>	<i>Lactobacillus sp.</i>	6.68±0.18	8.79±0.17***	8.28±1.26*** ###
13	CLED	(-) bacilli	Oxidase (+)	<i>Pseudomonadaceae</i>	<i>Pseudomonas sp.</i>	0.15±0.02	***	***
14	CLED	(+) cocci	Catalase (+)	<i>Micrococcaceae</i>	<i>Micrococcus sp.</i>	4.21±0.31	3.67±0.14***	*** ###

****P*<0.001 vs. CTR, ***P*<0.01 vs. CTR, ****P*<0.001 vs. 2W, and ***P*<0.01 vs. 2W.

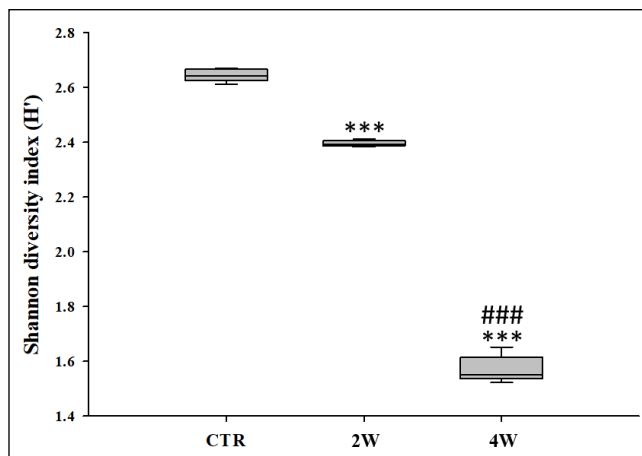


Fig. 7. Boxplots depicting the distribution of Shannon diversity (H') values of the bacterial communities in the 2-week-ammonia-treated (2W), 4-week-ammonia-treated (4W), and control (CTR) mouse groups. *** $P < 0.001$ vs. control and ### $P < 0.001$ vs. 2W.

were reported as being due to increased ammonemia and neuroinflammation, both of which are associated with increased GABAergic tone, particularly in cerebellar Purkinje cells. Indeed, the involvement of the cerebellum in the observed motor impairments has been described in several previous studies, where the activation of Purkinje cell terminals provoked inhibitory postsynaptic potentials in the lateral nucleus (Sastry et al., 1997). Moreover, extracellular GABA in the cerebellum has been correlated with motor coordination deficit severity (Boix et al., 2010). A direct interaction between ammonia and the GABA_A receptor complex has long been believed to occur, leading to enhanced GABAergic transmission (Takahashi et al., 1993; Basile and Jones, 1997; Jones and Basile, 1998). Such an interaction was first demonstrated through *in vitro* or *in vivo* on isolated neurons and cultured astrocytes. The first postulate by Takahashi et al. (1993) reported an extracellular concentration-dependent increase of GABA-induced chloride current after ammonia supplementation (0.2-0.5 mM) *in vitro*. Furthermore, ammonia may act synergistically with benzodiazepine receptor ligands, which potentiate GABA agonists, leading to an intensified ability of GABA to induce neuronal depression (Ha and Basile, 1996). Not only did they enhance its release, but a recent study on cultured astrocytes has reported that treatment with ammonia contributes further to GABAergic abnormalities by inhibiting GABA uptake by 50-60%, leading to an increased GABA concentration in the synaptic cleft and potentiating GABA_A receptor activation (Bender and Norenberg, 2000). The described effects of ammonia in cerebellar astrocytes and neurons compromise an influence on Purkinje

cells. An interesting and well-designed study showed that ammonia acetate combined with lipopolysaccharides induced neurodegeneration in Purkinje cells in a model of episodic HE, providing evidence that the resulting neuronal death may lead to transitory motor abnormalities similar to those observed clinically (García-Lezana et al., 2017). Such a loss of Purkinje cells was shown to occur along with morphological changes in the cerebellum manifested by swelling of astrocytes and Bergmann glia, activation of microglia, cytotoxic edema, and inflammasomes in a model of portocaval anastomosis (López-Cervantes et al., 2021). The described multi-cerebellar effects of ammonia reported *in vivo* and *in vitro* are congruent with reduced motor performance observed in animal models and patients with HE. Indeed, an imbalance in CNS inhibitory signals in HE has long been reported to evoke motor impairment, of which a potential link with altered microbiota is yet to be established.

Our study has provided new evidence of gut microbiota dysbiosis in hyperammonemic mice, using a microbiological approach based on traditional microbiological techniques of growing cultures in targeted media for a preliminary study on potentially induced changes in gut microbiota. Indeed, the qualitative and quantitative microbiological analyses revealed decreased bacterial load, diversity, and ratios of both aerobic and facultative anaerobic bacteria following two and four weeks of ammonia supplementation, with several bacterial phyla disappearing at four weeks and only *Enterobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae* families surviving. In fact, despite the lack of data regarding gut microbiota responsiveness in hyperammonemic conditions in rodents, few studies have experimentally addressed such relationships. A study carried out in poultry showed that exposure to atmospheric ammonia in laying ducks (35 ppm) over 4-6 weeks, and broiler chickens (75 ppm) over ten days, triggered an intestinal microbiota dysbiosis with clearly decreased diversity and an induced inflammatory response (Tao et al., 2019; Zhou et al., 2021). Early clinical studies consistently suggested that reducing protein, which is indeed turned over into ammonia by gut bacteria, intake may control periods of overt HE among cirrhotic patients (Riordan and Williams, 1997). Furthermore, patients with cirrhosis and HE showed gut dysbiosis and gut septicemia or bacterial peritonitis (Bleichner et al., 1986; Trebicka et al., 2021). Hence, hyperammonemia may exert an effect on gut bacteria, as multiple studies have suggested an interplay between the two. Nevertheless, to date, no experimental or clinical study focused on the effects of ingested ammonia on intestinal microbiota.

Additionally, the metabolic process of ammonia generation may also regenerate with altered bacterial functionalities, especially with the reduction of their diversity. It was previously shown that such a supply may simulate increased degradation of amino acids and glutaminase activity, exacerbating hyperammonemia (Kang et al., 2016). Indeed, bacterial characterizations of patients' stools have revealed a positive correlation between the severity of cirrhosis and HE and increased non-autochthonous bacteria, mainly *Enterobacteriaceae* and *Streptococcaceae* (Bajaj et al., 2012b; Bajaj, 2014a). A relative abundance of *Streptococcaceae* in saliva from patients with prior HE was also reported (Bajaj et al., 2015) and was responsible for increased ammonia production in the intestine (Zhang et al., 2013). *Enterobacteriaceae* and *Enterococcaceae* were detected in abundance within the salivary and stool microbiomes of patients with prior HE and cirrhosis, and the amount of such pathogenic micro-

bial communities in the stool was highly correlated with systemic inflammation (Bajaj et al., 2015). The inflammatory response associated with dysbiosis may result from increased histamine related to pathogenic *Escherichia coli* and endotoxins (Zhang et al., 2013). The induced systemic inflammation has been strongly linked to a defective microbial intestinal barrier with increased intestinal permeability, overgrown intestinal bacteria, and delayed gastrointestinal motility, which promote infections and intensify symptoms of HE involving neuroinflammation (Trebicka et al., 2021), a state commonly observed in patients presenting with hyperammonemia (Bajaj, 2014a; Chen et al., 2015; Rai et al., 2015).

A strong association between peripheral and central inflammation has been demonstrated in several animal models, including models of HE. Indeed, suppression of peripheral inflammation by ibuprofen in hyperammonemic rats was associated with significant

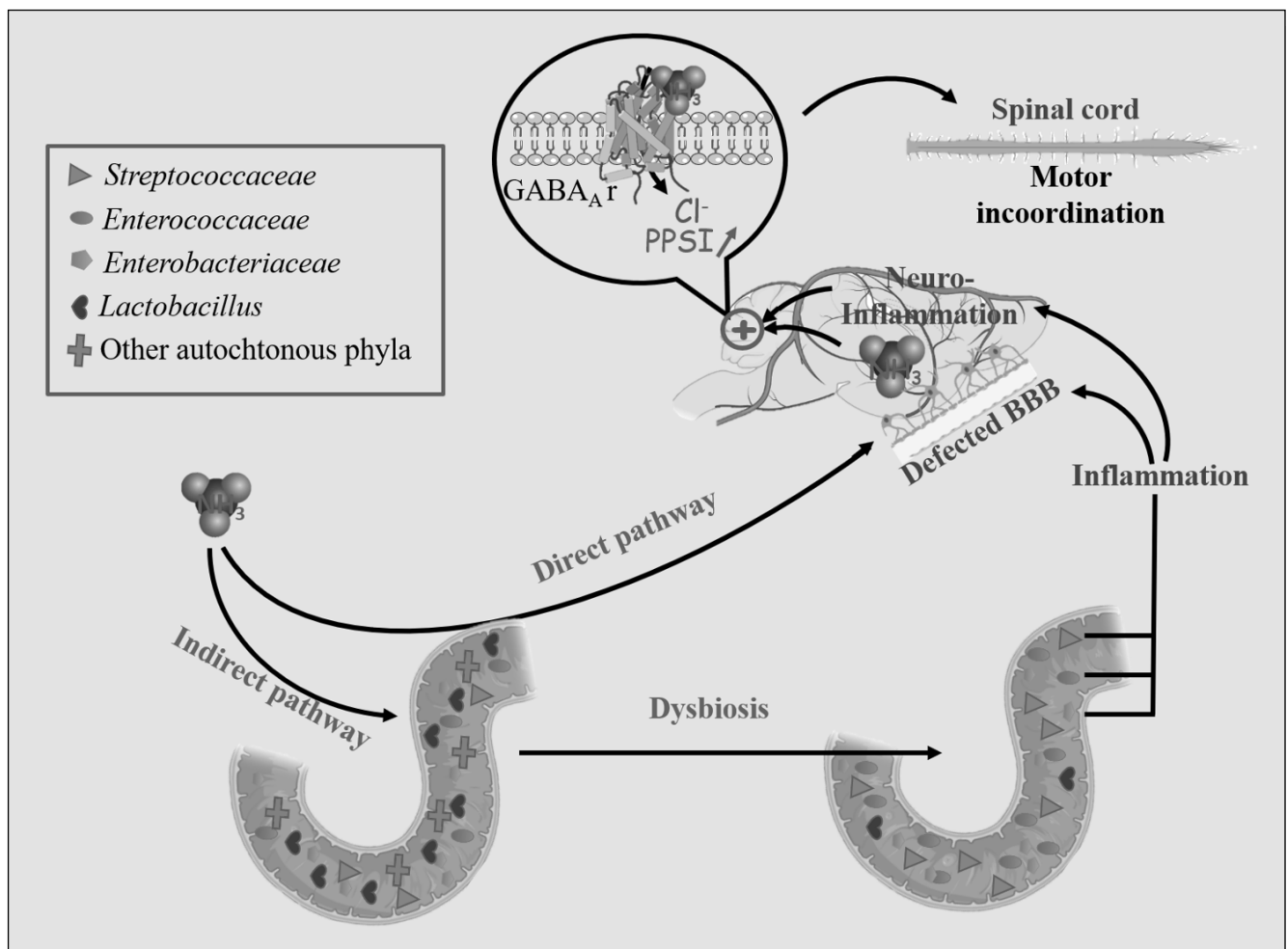


Fig. 8. Graphical abstract: a possible mechanism revealed in the study by which hyperammonemia and gut dysbiosis may act through a direct or indirect pathway to set off HE.

alleviation of neuroinflammation. Accordingly, central inflammation, especially neuroinflammation in the cerebellum, is driven by systemic inflammation associated with an altered immune system (Cabrerá-Pastor et al., 2019). Additionally, gut dysbiosis is believed to elicit systemic, cerebellar, and cortical inflammation, including glial and microglial activations (Butterworth, 2011; Kang et al., 2016), along with an increased concentration of systemic and central ammonia, causing astrocyte swelling (Felipo and Butterworth, 2002; Chatauret et al., 2006). Yet, induced systemic inflammation may weaken the integrity of the blood-brain barrier in the case of hyperammonemia and increase its permeability, facilitating the flow of ammonia to the CNS (Skowrońska and Albrecht, 2012). A mechanism proposed by Cabrera-Pastor et al. (2018) stated that hyperammonemia might set off cerebellar neuroinflammation and glial activation, which elicits increased GABA transmission and alters extracellular GABAergic receptors responsible for reduced motor coordination.

However, through this study, we have revealed a direct effect of hyperammonemia and associated gut dysbiosis on the pathogenesis of HE. Until now, it was clear that ammonia supplementation, *per se*, could trigger intestinal dysbiosis, as similarly reported in several studies in patients with cirrhosis, decompensated HE, and prior HE (Shawcross et al., 2008; Bajaj, 2014a; Bajaj et al., 2015; Trebicka et al., 2021). At the central level and related to the increased flow of ammonia, the cerebellar cortex, which is involved in motor synchronization through Purkinje cells and granule cells, showed impaired activity due to astrocytic swelling as well as impulses from glutamatergic and GABAergic neurons (Cauli et al., 2014).

Accordingly, the neurotoxic effect opposing the astroglial response in the midbrain, ventral tegmental area, and dorsal striatum could be responsible for reduced sensorimotor coordination (El Hiba et al., 2016), linked essentially to increased ammonia (Jover et al., 2006). Seen from another angle, the intestinal microbiota is believed to have a mainstay role in balancing functions of the CNS. Interestingly, the functioning neurotransmitters in the CNS are also produced by intestinal bacteria, which are proposed to have a possible distal effect. Hence, a change in human microbial composition has been associated with the changed fecal concentration of neurotransmitters (Altaib et al., 2021). GABA is produced by several bacterial species, such as those belonging to *Streptococcaceae* and *Enterococcaceae* (Cui et al., 2020), and reaches the CNS through vagal or systemic circulation pathways of the gut-brain axis (Mazzoli and Pessione, 2016). In a previous study by Alba et al., chronic treatment of rats with ammonia

was reported to induce altered GABAergic flux in the cerebellum associated with obvious deficits in motor coordination (Gonzalez-Usano et al., 2014), seemingly induced by the prevalence of GABA-producing bacteria in the intestine.

However, bacterial neurochemical activity has been detected in *Enterobacteriaceae* (Jiang et al., 2015) without a direct effect on motor coordination. Nevertheless, *Enterobacteriaceae* have been shown to generate urease activity, exacerbating hyperammonemia (Guarner, 2003), and promote an inflammatory response through histamines and endotoxins, such as occurs with pathogenic *Escherichia coli* (Zhang et al., 2013). In a study established by Bajaj et al., patients with HE showed mucosal microbiome dysbiosis with a particular abundance of *Enterococcaceae*; associated with their dysbiosis, these patients presented with inflammatory symptoms and cognitive issues (2012a). Moreover, the enterococci, *Group D Streptococcus*, including *Enterococcus faecalis* in the case of our study, can decarboxylate amino acids freely available from a standard diet, allowing for the production of biogenic amines (Giraffa, 2014). Among these, tryptamine, tyramine, and phenylethylamine are responsible for several physiological and neuro-modulatory complications, as well as toxicological effects when translocated into the bloodstream (Tofalo et al., 2015). Biogenic amines have also been associated with, among other possible complications, Parkinson's disease. Tyramine is particularly associated with adverse reactions to monoamine oxidase inhibitors and increases in catecholamine production in the sympathetic nervous system (Tofalo et al., 2015), which may also explain the reduced motor coordination in our mice. Even though an association may be suggested between the predominance of enterococci and deficits in motor coordination, this postulate needs further study to be confirmed.

Unlike the negative effects of abundant *Enterobacteriaceae* and *Streptococcaceae*, *Lactobacillus* are characterized by their antimicrobial properties beneficial for the intestinal microbiota; for this reason, they are the most used in prebiotics as a treatment for correcting dysbiosis, notably in the case of cirrhosis and HE (Bajaj, 2014b). Nevertheless, they represent barely 8% of the total bacterium in our hyperammonemic mice, with a moderate increase in their ratio compared with the control group. That may underly the slight but not generalized improvement in motor coordination, observed particularly in the rotarod test, taking into consideration the evidence that *Lactobacillus* significantly contributes to the systemic alleviation of endotoxemia and associated inflammation (Liu et al., 2004), with the latter known to be involved in ammonia-induced neurotoxicity (Shawcross, 2015).

CONCLUSION

Our study has demonstrated increased deficits in motor coordination in mice with hyperammonemia concomitant with intestinal dysbiosis characterized by an abundance of *Enterobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae*. Such data provide a new piece of the puzzle in the understanding of ammonia-induced motor impairment through possible direct and indirect mechanisms acting on the gut-brain axis (neurochemical imbalances, inflammation, and neuro-inflammation generation). However, to elucidate the mechanisms underlying the role each bacterial species separately plays in the pathogenesis of the motor syndrome in hyperammonemia and HE, further studies, including a metagenomic analysis of the gut microbiota, are necessary.

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