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Genetically engineered *E. coli* Nissle attenuates hyperammonemia and prevents memory impairment in bile-duct ligated rats

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ABBREVIATIONS. HE, hepatic encephalopathy; CLD, chronic liver disease; BDL, bile-duct ligation; EcN, *Escherichia coli* Nissle 1917 bacterium; TAA, thioacetamide, OTC, ornithine-transcarbamylase; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TNF-α, tumor necrosis factor alpha; IL, interleukin; IFNγ, interferon-gamma; IP-10, IFNγ-induced protein 10 kDa; MIP-2, macrophage inflammatory

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protein-2; MCP-1, monocyte chemoattractant protein-1; STM, short-term memory; LTM, long-term memory; TGF-β, transforming growth factor; α-SMA, alpha-smooth muscle actin; Bcl-2, B-cell lymphoma-2; TJ, tight junction; BDNF, brain-derived neurotrophic factor.

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ABSTRACT

Hyperammonemia associated with chronic liver disease (CLD) is implicated in the pathogenesis of hepatic encephalopathy (HE). The gut is a major source of ammonia production that contributes to hyperammonemia in CLD and HE and remains the primary therapeutic target for lowering hyperammonemia. As an ammonia-lowering strategy, *Escherichia coli* Nissle 1917 bacterium was genetically modified to consume and convert ammonia to arginine (S-ARG). S-ARG was further modified to additionally synthesize butyrate (S-ARG+BUT). Both strains were evaluated in bile-duct ligated (BDL) rats; experimental model of CLD and HE. **Methods:** One-week post-surgery, BDLs received non-modified EcN (EcN), S-ARG, S-ARG+BUT ($3x10^{11}$ CFU/day) or vehicle until sacrifice at 3- or 5-weeks. Plasma (ammonia/pro-inflammatory/liver-function), liver fibrosis (hydroxyproline), liver mRNA (pro-inflammatory/fibrogenic/anti-apoptotic) and colon mRNA (pro-inflammatory) biomarkers were measured post-sacrifice. Memory, motor-coordination, muscle-strength, and locomotion were assessed at 5-weeks. **Results:** In BDL-Veh rats, hyperammonemia developed at 3- and further increased at 5-weeks. This rise was prevented by S-ARG and S-ARG+BUT, whereas EcN was ineffective. Memory impairment was prevented only in S-ARG+BUT vs BDL-Veh. Systemic inflammation (IL-10/MCP-1/endotoxin) increased at 3- and 5-weeks in BDL-Veh. S-ARG+BUT attenuated inflammation at both timepoints (except 5-week endotoxin) vs BDL-Veh, whereas S-ARG only attenuated IP-10 and MCP-1 at 3-weeks. Circulating (ALT/AST/ALP/GGT/albumin/bilirubin) and gene expression liver-function markers (IL-10/IL-6/IL-1β/TGF-β/α-SMA/collagen-1α1/Bcl-2) were not normalized by either strain. Colonic mRNA (TNF-α/IL-1β/occludin) markers were attenuated by synthetic strains at both timepoints vs BDL-Veh. **Conclusion:** S-ARG and S-ARG+BUT attenuated hyperammonemia, with S-ARG+BUT additional memory protection likely due

to greater anti-inflammatory effect. These innovative strategies, particularly S-ARG+BUT, have potential to prevent HE.

KEYWORDS

Hepatic encephalopathy; probiotics; ammonia; cirrhosis; memory; *E. coli* Nissle; arginine; butyrate; bile-duct ligature; inflammation.

1 INTRODUCTION

Hepatic encephalopathy (HE) is a frequent neuropsychiatric complication which arises in patients with either acute liver failure or chronic liver disease (CLD). It is reported that as many as 80% of patients with CLD develop some degree of HE during the course of the disease.1 HE is characterized by neurological alterations including personality changes, poor memory, sleep problems, decreased concentration, reduced speed of information processing and motor-incoordination that significantly affect the quality of life of the patient. In addition, HE can progress to lethargy, gross disorientation, asterixis and coma, which lead to a poor prognosis.2

Although the mechanisms that lead to HE are not fully understood, hyperammonemia is accepted to play an important role in the pathogenesis of HE. Hyperammonemia occurs since the capacity to remove ammonia is significantly reduced during liver injury. Subsequently, an increase in blood derived ammonia enters the brain causing deleterious effects including alterations in brain chemical homeostasis and neurotransmission.3

The gut is the major source of systemic ammonia due to dietary protein metabolism and by urease containing bacteria which metabolize urea to ammonia.3 Accordingly, gut-derived ammonia remains a primary therapeutic target to reduce ammonia absorption, alleviating hyperammonemia.4 The non-absorbable disaccharide lactulose is widely used to treat hyperammonemic patients. Lactulose accelerates fecal transit-time and reduces urea production rate, thereby reducing entry of ammonia into circulation.5 Also, the non-absorbable antibiotic rifaximin, which inhibits the activity of urease-producing bacteria, and alters the gut microbiota.5,6 Nevertheless, the therapeutic response rate to these treatments is not uniform and other therapies are limited. Moreover, HE patients often show poor compliance and adherence to these treatments.5,7,8 Therefore, developing novel

therapeutic strategies with better compliance, adherence, safety and improved effectiveness to prevent HE are highly needed.

Engineered bacteria have been developed as a new approach to prevent disease by either scavenging toxic molecules and/or delivering beneficial ones. Recently, as a novel therapy to prevent and reduce hyperammonemia *Escherichia coli* Nissle 1917 bacterium (EcN), a well characterized and safe non-pathogenic Gram-negative strain, was genetically modified to consume ammonia via the generation of L-arginine (S-ARG or SYNB1020 strain).9 In addition, the S-ARG strain was further modified to synthesize butyrate (S-ARG+BUT), a short-chain fatty acid with anti-inflammatory/antioxidant properties that could protect the gut-barrier and brain function.10 Both strains, when exposed to ammonia, consume higher amounts of ammonia (>85%) and produce >10 fold more arginine versus unmodified-EcN.9 However, only S-ARG+BUT produces butyrate (Fig.S1). In healthy volunteers, S-ARG treatment increased plasma and urine nitrates (arginine metabolites), indicating synthetic bacteria are active in the human GI tract. In animal models of hyperammonemia such as thioacetamide (TAA)-induced liver injury rat model and ornithine-transcarbamylase deficiency (OTC *spf-ash*) mice (urea cycle disorder model), S-ARG significantly reduced hyperammonemia compared to unmodified-EcN, which did not lower ammonia.9 Nevertheless, the beneficial effect of S-ARG (or S-ARG+BUT) on HE has never been tested. Thus, we investigated the therapeutic effects of these strains in rats with bile-duct ligation, an animal model of CLD with HE.11 The aims were to explore the potential beneficial effects of these strains on (i) reducing hyperammonemia and protecting cognitive function, (ii) reducing systemic, hepatic and colon inflammation; and (iii) alleviating hepatic fibrosis, hepatic apoptosis, and bacterial translocation.

2 METHODS

2.1 Animal model of CLD and HE. Bile-duct ligation (BDL) was performed to induce CLD in male Sprague-Dawley rats (200-210g) (Charles-River, Canada), and Sham-operated rats were the controls, as reported previously.11 Rats were housed in pairs (12h-12h light-dark cycle) with free access to food and water.

2.2 Treatments: Non-genetically modified (EcN), and genetically modified EcN (S-ARG and S-ARG+BUT) strains, were generated by Synlogic Inc. One week post-surgery, BDL rats were gavaged with EcN, S-ARG, S-ARG+BUT ($3x10^{11}$ CFU/day, BID) or vehicle (100mM sodium-bicarbonate) until they were sacrificed along with respective Shams (vehicle). Dose was established based on preliminary studies in BDL rats (Suppl.1).

2.3 Experimental groups. Rats were divided into 5 groups (20 rats/group); Sham, BDL-Veh, BDL-EcN, BDL-S-ARG, and BDL-S-ARG+BUT. In order to explore the temporal effects (early/late) of synthetic bacteria, half of the rats were sacrificed at 3 weeks and half at 5 weeks.

2.4 Body weight, food-intake and body composition. Body weight and food-intake were monitored daily. Body composition, lean and fat mass, was estimated at 5 weeks in non-sedated rats by nuclear magnetic resonance-EchoMRI-700® (EchoMRi LLC, USA).

2.5 Blood and tissue samples. At sacrifice, arterial plasma (heart), liver and colon (3-cm section proximal to rectum) were snap frozen and stored (-80˚C). Additionally, liver samples were placed in RNA stabilization solution (RNAlater, Invitrogen).

2.6 Plasma measurements (3- and 5-weeks post-surgery).

2.6.1 Plasma ammonia and liver function/injury markers. Ammonia and liver markers were measured in arterial plasma using Cobas-c111 analyzer (Roche). Liver markers analyses included alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin and albumin.

2.6.2 Plasma inflammation markers. Cytokines tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interferon-gamma (IFNγ), IFNγ-induced protein 10 kDa (IP-10, CXCL10), macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) were measured using Multiplex immunoassay using MILLIPLEX-MAP Rat Cytokine/Chemokine magnetic panel (EMD Millipore, #RECYTMAG-65). Out of the six markers analyzed, only IP-10 and MCP-1 were detected, reported, and discussed.

2.6.3 Plasma endotoxin. Endotoxin assay kid for plasma was used (Pierce™ Chromogenic Endotoxin Quant Kit A39552S).

2.7 Behavioral assessments (only in 5-week groups)

2.7.1 Short-term (STM) and long-term (LTM) recognition memory. Novel object recognition test was conducted 5 days before sacrifice in a quiet and dim-lit room. Memory test was divided in four 5-minute phases as follow: During habituation; rats were placed individually in an open-field arena (60x45x33 cm). Familiarization/acquisition; 10 min later rats were placed in the arena containing two identical objects (A+A). STM, 1h later rats returned to the arena containing a familiar and a novel object (A+B). LTM, 24h later rats returned to the arena containing a familiar and a new object (A+C). STM and LTM were video-recorded for offline-blind analysis. Time spent exploring, sniffing/touching objects (not standing close or leaning on the object) were considered as exploration.

2.7.2 Locomotor and rearing activity during the 12h dark/active phase. 4 days before sacrifice rats were individually placed in an open-field (plexiglass chamber, 42x42x22 cm) equipped with infrared sensors to monitor horizontal and vertical (rearing) movements (Omnitech-electronic Inc). Rats were placed 2h before the dark phase for habituation. Access to food and water was *ad libitum*. Total distance traveled and rearing activity (time and frequency) were monitored during the dark phase.

2.7.3 Motor coordination, and motor-skill learning. RotaRod test (AccuRotor-EzRod, Omnitech) was conducted 3 days before sacrifice for three consecutive days as follow: Habituation (day 1), rats were placed on the horizontal and non-moving cylinder (7-cm diameter) positioned 40 cm from the apparatus floor for 5 min. Testing-training phase, after habituation and without removing the rat from apparatus, the cylinder moved from 0 to 40 rpm within 5 min, so rats are forced to walk the cylinder to avoid falling. 3 trials per rat were obtained with an inter-trial resting time of 5 min. Latency to fall (s) was automatically recorded and the best performance time was used for analysis. The same protocol was repeated 24h (day 2) and 48h (day 3) after to explore motor-skill learning.

2.7.4 Forelimb and hind-limb skeletal muscle grip strength. One day before sacrifice, a grip strength digital meter (Chatillon®, USA) was used to measure forelimb and hind-limb grip strength (Suppl.1).

2.8 Liver and colon measurements (3- and 5-weeks post-surgery)

2.8.1 Liver-hydroxyproline content. Hydroxyproline content was assessed as an index of tissue collagen and fibrosis in liver tissue lysates by Hydroxyproline Assay Kit (Colorimetric/Abcam, ab222941).

2.8.2 Liver and colon gene expression. The presence of the mRNA levels was detected by quantitative real-time polymerase chain reaction qPCR. The hepatic genes analyzed were TGF-β, α-SMA, Collagen-1α1, IL-10, IL-6, IL-1β, and Bcl-2. The colon genes analyzed were TNF-α, IL-1β, and occludin (Suppl.1).

2.9 Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). One-way or two-way ANOVA analyses with Bonferroni post-hoc test were performed. All tests were run using GraphPad-Prism4. P-values<0.05 were considered statistically significant.

3 RESULTS

3.1 Body weight, food-intake, and body composition in cirrhotic-BDL rats treated with S-ARG and S-ARG+BUT. Body weight of BDL-Veh, BDL-EcN, and S-ARG+BUT were significantly lower compared to control-Shams from week 2 to week 5 post-surgery ($p<0.05$). However, in the BDL-S-ARG group such effect was solely observed at week 5 ($p<0.05$). In addition, S-ARG treatment lead to a significantly higher body weight at 3 weeks compared to BDL-Veh, BDL-EcN, and S-ARG+BUT groups ($p<0.05$). Food-intake was lower in all BDL groups during the first and fifth week post-surgery compared to Shams ($p<0.05$). No significant differences in food-intake were detected between BDL groups (Fig.1A). At 5 weeks, lean and fat mass were reduced in all BDL groups (untreated and treated) compared to Shams ($p<0.001$) (Fig.1B).

3.2 S-ARG and S-ARG+BUT attenuate hyperammonemia in cirrhotic-BDL rats. At 3 weeks, plasma ammonia was significantly higher in BDL-Veh compared to Shams ($p<0.01$). Similar findings were found in EcN and S-ARG groups ($p<0.05$) but this difference was lost in the S-ARG+BUT treatment group. At 5 weeks, plasma ammonia was significantly higher in all BDL groups (treated and non-treated) compared to Shams ($p<0.05$). Nevertheless, at 5 weeks, S-ARG and S-ARG+BUT lead to a significant reduction of ammonemia compared to both BDL-Veh and BDL-EcN groups ($p<0.05$). Moreover, the longitudinal analysis shows that in BDL-Veh ammonia is further increased from 3 to 5 weeks ($p<0.05$), while this significance is abolished in treated BDLs (Fig.2).

3.3 Enhanced beneficial effect of S-ARG+BUT vs S-ARG treatment in preventing some but not all neurological deficits in BDL rats. ***Recognition memory.*** STM and LTM were significantly impaired in control BDL-Veh rats vs Shams ($p<0.05$), which was not found in S-ARG and S-ARG-BUT groups. EcN-treatment did not prevent memory impairment. Moreover, STM impairments were significantly attenuated in S-ARG+BUT group when compared to BDL-Veh or BDL-EcN group ($p<0.05$), while LTM was improved only when compared to BDL-Veh (Fig.3A). ***Locomotion and rearing activity.*** Total distance traveled and rearing activity (time and frequency) during the 12h active/dark phase were significantly reduced in all BDL groups (untreated and treated) compared to Shams ($p<0.001$). No effect of either treatment was found (Fig.3B). ***Muscle strength.*** Forelimb and hind-limb grip strength were significantly reduced in all BDL groups (untreated and treated, $p<0.05$) except for S-ARG+BUT where hind-limb strength was not significantly decreased vs Shams (Fig.3C). ***Motor-coordination, and motor-skill learning.*** The latency to fall was significantly shortened in all BDL groups (untreated and treated) regardless of the session day (day 1, 2 or 3) when compared to Shams ($p<0.05$). The longitudinal comparison in motor-skill learning showed significantly longer latency to fall in all groups at day 3 when compared to previous performance at day 1 within the same group ($p<0.05$) (Fig.3D).

3.4 Enhanced beneficial effect of S-ARG+BUT vs S-ARG treatment in decreasing systemic inflammation in BDL rats. At 3 weeks, the plasma IP-10 inflammatory marker was significantly increased in BDL-Veh rats ($p<0.05$), which was not found in S-ARG and S-ARG+BUT, when compared to Shams. At 5 weeks, plasma IP-10 was significantly elevated in all BDL groups

(untreated and treated) vs Shams ($p<0.01$). Nonetheless, S-ARG+BUT treatment demonstrated a significant decrease in IP-10 when compared to BDL-Veh ($p<0.05$) or BDL-S-ARG group ($p<0.001$). MCP-1 was increased in BDL-Veh vs Shams at both 3 and 5 weeks ($p<0.05$). At 3 weeks such effect was canceled by S-ARG and S-ARG+BUT whereas at 5 weeks, S-ARG+BUT significantly lowered MCP-1 vs BDL-Veh ($p<0.05$), while S-ARG showed a tendency to significance vs BDL-Veh ($p=0.2$) (Fig.4).

3.5 S-ARG and S-ARG+BUT treatments do not impact systemic markers of liver injury/function in cirrhotic-BDL rats. At 3 weeks, AST, GGT, and bilirubin were significantly increased in all BDL groups compared to Shams ($p<0.001$). At 5 weeks, ALT, AST, ALP, GGT and bilirubin were significantly increased in all BDL groups vs Shams ($p<0.001$). Albumin was decreased in all BDL groups at both timepoints ($p<0.001$). S-ARG+BUT treatment lead to a significant lowering of ALP as compared with BDL-Veh ($p<0.01$) at 5 weeks, and prevented a further increase in ALP and AST levels from 3 to 5 weeks as observed in BDL-Veh and S-ARG ($p<0.05$) (Fig.5A).

3.6 Liver histopathological changes and collagen deposition were attenuated by S-ARG treatment in cirrhotic-BDL rats. At 3 weeks, hydroxyproline content in liver tissue was significantly increased in BDL-Veh and S-ARG+BUT groups ($p<0.01$) but not in S-ARG group when compared to Shams. In fact, hydroxyproline levels in S-ARG treated rats were significantly lower than BDL-Veh rats ($p<0.01$). At 5 weeks, hydroxyproline was increased in all BDL groups ($p<0.001$) with no protective effect from either of the treatments (Fig.5B).

3.7 S-ARG or S-ARG+BUT treatments do not impact liver mRNA expression of pro-inflammatory, fibrogenic and anti-apoptotic markers in cirrhotic-BDL rats. RNA gene expression of hepatic anti-inflammatory marker IL-10 was decreased in all BDL groups compared to Shams at both timepoints ($p<0.001$). IL-6 was significantly increased in all BDL groups at 5 weeks ($p<0.001$) but not at 3 weeks. IL-1β was significantly reduced in all BDL groups vs Shams at 5 weeks ($p<0.001$), while IL-1β was increased in S-ARG+BUT at 3 weeks vs all groups ($p<0.001$). In addition, levels of IL-10, IL-6 and IL-1β significantly lower or increased from 3 to 5 weeks in all BDL groups ($p<0.05$) (Fig.6A). Results with pro-fibrogenic hepatic markers showed that TGF-β was significantly increased in all BDL groups at both timepoints ($p<0.05$), except for S-ARG that did not

achieved significant increase at 3 weeks ($p>0.05$). At 5 weeks, α-SMA was significantly increased in all BDL groups vs Shams ($p<0.001$). Collagen-1α1 was increased in all BDL groups compared to Shams at both timepoints ($p<0.01$) (Fig.6B). Anti-apoptotic marker Bcl-2 was increased in all BDL groups compared to Shams at both timepoints ($p<0.001$). Moreover, levels of Bcl-2 were lower at 5 vs 3 weeks in BDL-Veh group ($p<0.05$) (Fig.6C).

3.8 S-ARG and S-ARG+BUT attenuate mRNA expression of pro-inflammatory markers in colon tissue from BDL rats. No significant differences were detected for these markers between groups at either 3 or 5 weeks. However, the longitudinal comparison showed TNF-α and IL-1β to be significantly higher in BDL-Veh at 5 vs 3 weeks, while such effect was not significant in BDL treated groups (Fig.7A).

3.9 S-ARG and S-ARG+BUT protect colon tight junction (TJ) and intestinal integrity in BDL rats. The relative mRNA expression of occludin, which is associated with TJ integrity and intestinal permeability, was significantly reduced in BDL-Veh but not in treated BDL rats at 5 weeks vs Shams ($p<0.01$) or compared to 3 weeks ($p<0.05$) (Fig.7B). S-ARG+BUT showed a strong trend towards protecting against decreased occludin expression (vs BDL-Veh at 5 weeks, $p=0.06$).

3.10 S-ARG+BUT and not S-ARG treatment, prevents endotoxemia in cirrhotic-BDL rats. Plasma endotoxin was significantly increased in BDL-Veh rats at both 3 and 5 weeks vs Shams ($p<0.01$). S-ARG+BUT significantly reduced endotoxin at 3 weeks ($p<0.05$) with a tendency to reduce endotoxin at 5 weeks ($p=0.07$) vs BDL-Veh. S-ARG did not significantly reduce endotoxin at 3 or 5 weeks (Fig.7C).

4 DISCUSSION

The current study investigated the therapeutic impact of two genetically modified bacteria; both capable of metabolizing ammonia (S-ARG) whereas only one was engineered to additionally synthesize butyrate (S-ARG+BUT).9 The S-ARG strain demonstrated positive benefits on attenuating hyperammonemia which lead to a lessening in memory impairment in BDL rats. However, the S-ARG+BUT strain which demonstrated a similar attenuation in hyperammonemia, demonstrated additional positive effects on systemic inflammation and endotoxemia which lead to full protection against memory deterioration. The non-genetically modified bacteria (EcN) did not have a beneficial effect on blood ammonia or memory in BDL rats which confirms ammonia consumption and butyrate production in the gut by synthetic bacteria are decisive in preventing HE. The additional anti-inflammatory benefit of S-ARG+BUT which lead to a further neurological improvement represents a more effective approach than S-ARG in attenuating both hyperammonemia and systemic inflammation in CLD and HE.

Blood ammonia levels were increased at 3 and 5 weeks post-surgery in BDL-Veh rats compared to sham-operated controls, which is characteristic of the BDL model.11,12 At 5 weeks, S-ARG and S-ARG+BUT lead to a significant attenuation in hyperammonemia, supporting results from previous studies where S-ARG demonstrated to reduce hyperammonemia in TAA and OTC deficient mice, alternative models of hyperammonemia.9 Furthermore, the significant progression of hyperammonemia levels from 3 to 5 weeks in BDL-Veh rats did not develop in S-ARG and S-ARG+BUT groups. Interestingly, EcN also prevented this progressive increase in ammonia from 3 to 5 weeks, nevertheless this effect is due to non-significant (tendency) higher levels of blood ammonia at 3 weeks vs BDL-Veh group. The ammonia-lowering capabilities of the genetically modified strains were not due to improvement in liver function since liver enzymes ALT, AST, GGT, ALP, bilirubin as well as albumin and hydroxyproline (collagen marker) did not improve in comparison to BDL-Veh.

Liver mRNA expression of pro-inflammatory, fibrogenic and anti-apoptotic markers were investigated in BDL rats. TGF-β1, a cytokine that promotes hepatic fibrosis by the activation of hepatic satellite cells (HSC)13 as well as α-SMA and collagen14 markers of fibrosis were found

increased in BDL rats. The genetically modified strains in BDL rats were not effective at preventing fibrosis. Furthermore, hepatic pro-inflammatory cytokines IL-6, and IL-1β were increased in BDL rats and were not significantly reduced by S-ARG or S-ARG+BUT groups. The anti-inflammatory IL-10 decreased progressively in all BDL groups and was unaffected in treatment groups. Furthermore, hepatic Bcl-2 (anti-apoptotic), involved in the regulation of apoptosis and fibrosis in the liver,15,16 was increased in all BDL groups with no beneficial effect of treatments. Overall, the negative impact on the liver by the genetically modified strains confirms their benefits on the brain are not due to liver improvement.

S-ARG+BUT attenuated systemic pro-inflammatory markers MCP-1 and IP-10 at 5 weeks, suggesting anti-inflammatory properties. It has been shown that gut butyrate production provides benefit associated with reducing inflammation, stimulating antioxidant production, and improving gut-barrier integrity.10 *In vitro* and *in vivo* studies have showed that the anti-inflammatory properties of butyrate are associated with the inhibition of NF-κB pathway (nuclear factor kappa B), which regulates the expression of genes encoding pro-inflammatory cytokines.17 Butyrate supplementation reduces systemic inflammation in patients with metabolic syndrome.18 Recently, a qualitative model of microbial-derived butyrate-inflammation interplay in human gut proposed to treat inflammation with butyrate enemas or dietary interventions such as butyrate-promoting dietary fibers for complications associated with low-grade inflammation.17-19 Therefore, S-ARG+BUT represents a good approach to not only reduce hyperammonemia but also systemic inflammation in CLD.

Hyperammonemia in BDL rats is accompanied by neurological deficits such as motor-incoordination, muscle weakness, hypolocomotion, and poor memory, as previously reported.11,12,20,21 Furthermore, we demonstrated for the first time in the BDL model, a remarkable reduction in night-rearing activity which suggests a motivational state deterioration. Moreover, we demonstrated that motor-coordination is impaired in BDLs, while the acquisition of motor-skill learning remains intact. We also report that both STM and LTM are impaired in BDLs. Interestingly, Leke *et al* found LTM to be intact in BDL rats, nevertheless discrepancies are likely due to difference in strain (Wistar rat).20 For instance, the progression of liver disease in Wistar rats is slower with very good survival up until 8 weeks post-BDL,22 vs 6 weeks in Sprague-Dawley rats.11

S-ARG positively impacted memory but S-ARG+BUT fully prevented memory impairment in BDL rats, suggesting the concomitant attenuation of hyperammonemia and systemic inflammation is a superior treatment strategy. Although no previous reports have explored whether ammonia-scavenging drugs prevent memory deficits in BDL rats, the exacerbation of hyperammonemia using high-ammonia diet in BDL rats aggravated spatial memory deficits,23 suggesting that memory is sensitive to ammonia levels. *In vivo* and *in vitro* studies have shown that hyperammonemia is associated with neurotransmission impairments in the hippocampus, a brain region associated primarily with memory, as well as down-regulation of brain-derived neurotrophic factors (BDNF) necessary for memory formation and learning in the hippocampus.24 In contrast, the anti-inflammatory infliximab (anti-TNF-α) reduces systemic inflammation, prevents neuroinflammation (hippocampus) and improves memory with no changes on hyperammonemia in rats with portacaval shunt, a model of hyperammonemia and HE,25 suggesting that memory is affected by inflammation. However, it has been demonstrated that chronic hyperammonemia and inflammation cooperate to induce neurological impairments in liver disease.3,26 For instance, endotoxin injection in hyperammonemic mice (OTC) lead to enhanced plasma pro-inflammatory cytokine response and worsen cognitive impairments compared to hyperammonemic control mice.27 Clinically, systemic inflammatory response aggravates neuropsychological decline induced by hyperammonemia in patients with CLD and HE.28 In addition, the combination of lactulose plus rifaximin (nonabsorbable antibiotic),5 is more effective than lactulose alone in the prevention of HE,29 supporting a synergistic role for hyperammonemia and systemic inflammation in the pathogenesis of HE. Here, the anti-hyperammonemia and anti-inflammatory properties of S-ARG+BUT represent an optimal therapeutic approach for the prevention of HE.

Interestingly, butyrate has demonstrated to protect the brain from age-related neuroinflammatory complications.30 Moreover, butyrate modulates hippocampal BDNF (up-regulation), required for memory and learning.31 Sodium butyrate treatment improves cognition in a mouse model of Alzheimer's disease, an effect linked to elevated hippocampal histone acetylation and increased expression of genes implicated in learning.32 Additionally, butyrate has been shown to recover/improve muscle mass and performance (hand-grip strength) in cirrhotic patients following liver transplantation.33 Remarkably, S-ARG+BUT prevented muscle strength weakness in BDL rats.

Nevertheless, the extra-intestinal effects of butyrate generated by S-ARG+BUT in CLD remain to be thoroughly investigated.

Evidence shows that HE patients suffer from intestinal inflammation, increased gut permeability consequently leading to endotoxemia.34,35 Particularly, pro-inflammatory TNF-α, IL-6 and IL-1β have shown to impair TJ proteins and gut barrier permeability, which allow the passage of substances throughout the membrane.36,37 In BDL rats, the reduction of bile acids in the gut facilitates endotoxin-induced bacterial translocation and mucosal permeability.38 Our findings show increased colonic mRNA expression of TNF-α and IL-1β from 3 to 5 weeks in BDL-Veh rats, with a significant difference for TNF-α at 5 weeks vs Sham. TJ integrity and permeability are also impaired in BDL-Veh rats as shown by a reduction in the relative RNA expression of occludin. Moreover, plasma endotoxin levels were increased in BDL rats. S-ARG and S-ARG+BUT attenuated the increase in colonic TNF-α and IL-1β markers and the decrease in occludin expression from 3 to 5 weeks, while S-ARG+BUT showed a strong trend in protecting against a reduced occludin expression vs BDL-Veh at 5 weeks (*p=0.06)*. S-ARG and S-ARG+BUT attenuated endotoxemia at 3 weeks, while the beneficial effects of S-ARG+BUT extended to 5 weeks. This suggest S-ARG+BUT is superior in preventing increased gut permeability, as well as endotoxemia, the latter being an important factor associated with the severity of HE.39,40

Recently, a small clinical trial (phase 1b/2a study) using SYNB1020 ($5x10^{11}$ CFU TID/6 days), a similar strain to S-ARG, demonstrated to be safe but it failed to demonstrate efficacy to treat and reduce hyperammonemia, as well as other endpoints including inflammatory markers (IL-6, TNF-α), endotoxemia, and psychometric HE score in patients with cirrhosis and hyperammonemia (n=9) vs patients on placebo (n=8) (www.synlogictx.com). Nevertheless, our study proposes that preventing hyperammonemia by genetically modified may be a better therapeutic strategy than treating and reducing hyperammonemia. Furthermore, encompassing both the ammonia-lowering and systemic anti-inflammatory capabilities, the S-ARG+BUT (as oppose to solely ammonia-lowering of S-ARG) is an optimal therapy to prevent HE. Further larger studies to evaluate the efficacy of S-ARG+BUT in HE patients are warranted.

CONFLICT OF INTEREST

C.B.K., M.P., and B.K were all employees and shareholders of Synlogic Inc. at the time these studies were conducted. Synlogic Inc. owns issued patents regarding S-ARG (SYNB1020) and S-ARG+BUT (SYNB1536).

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AUTHOR CONTRIBUTION

R.O., C.F.R., M.P., B.K and C.B.K. contributed to the design of experiments, analysis and interpretation of results. R.O and C.F.R wrote the manuscript. M.T performed ammonia/hydroxyproline analysis. R.O., G.P. and M.M.O. contributed to treat rats/behavioral experiments. C.R.B. and A.P. performed endotoxin and qPCR analyses, respectively.

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FIGURE LEGENDS

Figure 1. Bodyweight, average daily food-intake, and body composition following bile-duct ligation (BDL) and treatment with EcN, S-ARG and S-ARG+BUT. (**A**) Bodyweight and food-intake (repeated measures ANOVA). (**B**) Lean and fat mass by EchoMRI at 5 weeks post-surgery. Mean ± SEM. *$p<0.05$, ***$p<0.001$ all BDL groups vs Sham; +$p<0.05$ BDL-Veh, EcN, and S-ARG+BUT vs Sham, $^{\#}p<0.05$ S-ARG vs BDL-Veh; and $^{\&}p<0.05$ S-ARG vs S-ARG+BUT. Data from groups sacrificed at 5 weeks.

Figure 2. Plasma ammonia following bile-duct ligation (BDL) and treatment with EcN, S-ARG and S-ARG+BUT. Ammonia was significantly increased in BDL-Veh, EcN, and S-ARG, but not in S-ARG+BUT group at 3 weeks. At 5 weeks, S-ARG and S-ARG+BUT significantly reduced ammonia levels vs BDL-Veh. *$p<0.05$, ***$p<0.001$ vs Sham; and $^{+}p<0.05$, $^{++}p<0.01$ other comparisons.

Figure 3. Behavioral assessments in 5-week BDL rats treated with EcN, S-ARG and S-ARG+BUT. (**A**) Short- (STM) and long-term memory (LTM) (evaluated with novel object exploration times) were significantly reduced in BDL-Veh and EcN vs Shams. S-ARG+BUT increased/protected STM and LTM compared to BDL-Veh. (**B**) The 12h locomotion and rearing activity were significantly reduced in all BDL groups with no protective effect of treatment. (**C**) Forelimb and hind-limb muscle strength were significantly reduced in all BDL groups with no protective effect of treatment, except for hind-limb strength that was protected by S-ARG+BUT. (**D**) Motor-coordination, regardless of the testing day, was impaired in all BDL groups without protective effect of treatments, while, similar to Shams, motor-skill learning was not impaired in BDL groups (repeated measures ANOVA). Mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs Sham; and +$p<0.05$, ++$p<0.01$ other comparisons.

Figure 4. Pro-inflammatory markers following bile-duct ligation (BDL) and treatment with S-ARG and S-ARG+BUT. Plasma IP-10 and MCP-1 were significantly increased in BDL-Veh but not in S-ARG and S-ARG+BUT groups at 3 weeks. At 5 weeks, IP-10 and MCP-1 were increased in BDL-Veh and S-ARG vs Shams. S-ARG+BUT significantly reduced IP-10 and MCP-1 compared to

BDL-Veh at 5 weeks. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs Sham; and +$p<0.05$, ++$p<0.01$; +++$p<0.001$ other comparisons.

Figure 5. Biomarkers of liver function/injury in plasma and livers from BDL rats treated with S-ARG and S-ARG+BUT. (**A**) Plasma liver injury/function markers: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin and albumin were impaired in BDL groups (**B**) Hepatic fibrosis estimation by hydroxyproline content was significantly reduced in S-ARG group vs BDL-Veh at 3 weeks, while all BDL groups showed increased hydroxyproline at 5 weeks. Mean ± SEM. **$p<0.01$, ***$p<0.001$ vs Sham; and +$p<0.05$, ++$p<0.01$ other comparisons.

Figure 6. Hepatic measurements in BDL rats treated with S-ARG and S-ARG+BUT. (**A**) Anti-inflammatory interleukin-10 (IL-10) and pro-inflammatory interleukins IL-6 and IL-1β relative expression. (**B**) Hepatic pro-fibrotic markers TGF-β (transforming growth factor-β), α-SMA (alpha-smooth muscle actin) and collagen-1α1 relative expression. (**C**) Anti-apoptotic Bcl-2 protein expression. Mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs Sham; and +$p<0.05$, ++$p<0.01$, +++$p<0.001$ other comparisons.

Figure 7. Gene expression of TNF-α, IL-1β and tight junction protein occludin in the colon and gut and plasma endotoxin in BDL rats treated with S-ARG and S-ARG+BUT. (**A**) Colonic expression of pro-inflammatory TNF-α and IL-1β significantly increased at 5 weeks (vs 3 weeks) in BDL-Veh group but not in treated BDL groups. (**B**) Tight junction protein occludin was significantly decreased in BDL-Veh vs Shams at 5 weeks (as well as vs 3 weeks) but not in treated BDL groups. (**C**) Plasma endotoxin was significantly increased in BDL-Veh group at both 3 and 5 weeks. Endotoxin was significantly increased in S-ARG group vs Shams at 5 weeks but not at 3 weeks. S-ARG+BUT significantly decreased endotoxin levels compared to BDL-Veh at 3 weeks. Mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs Sham; and +$p<0.05$ other comparisons.

Figure 1

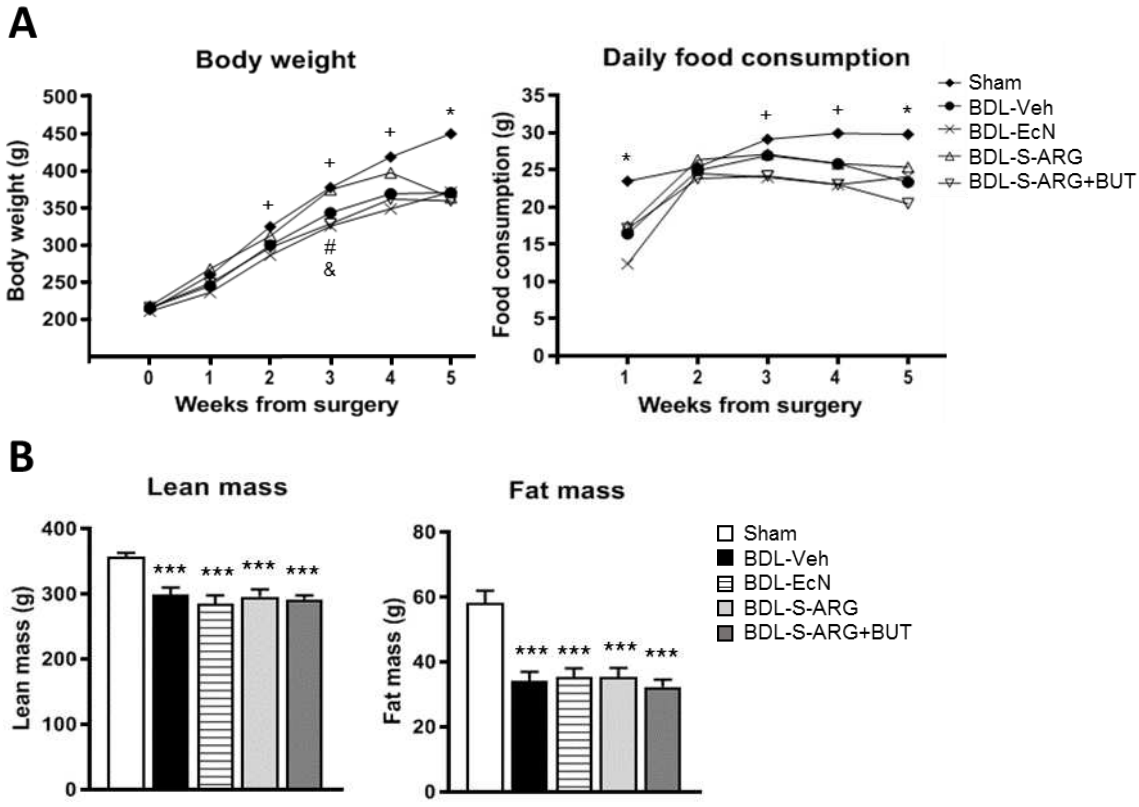
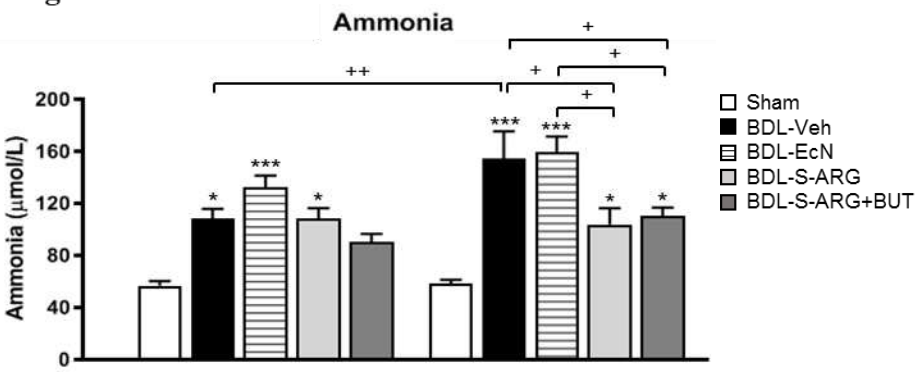


Figure 2



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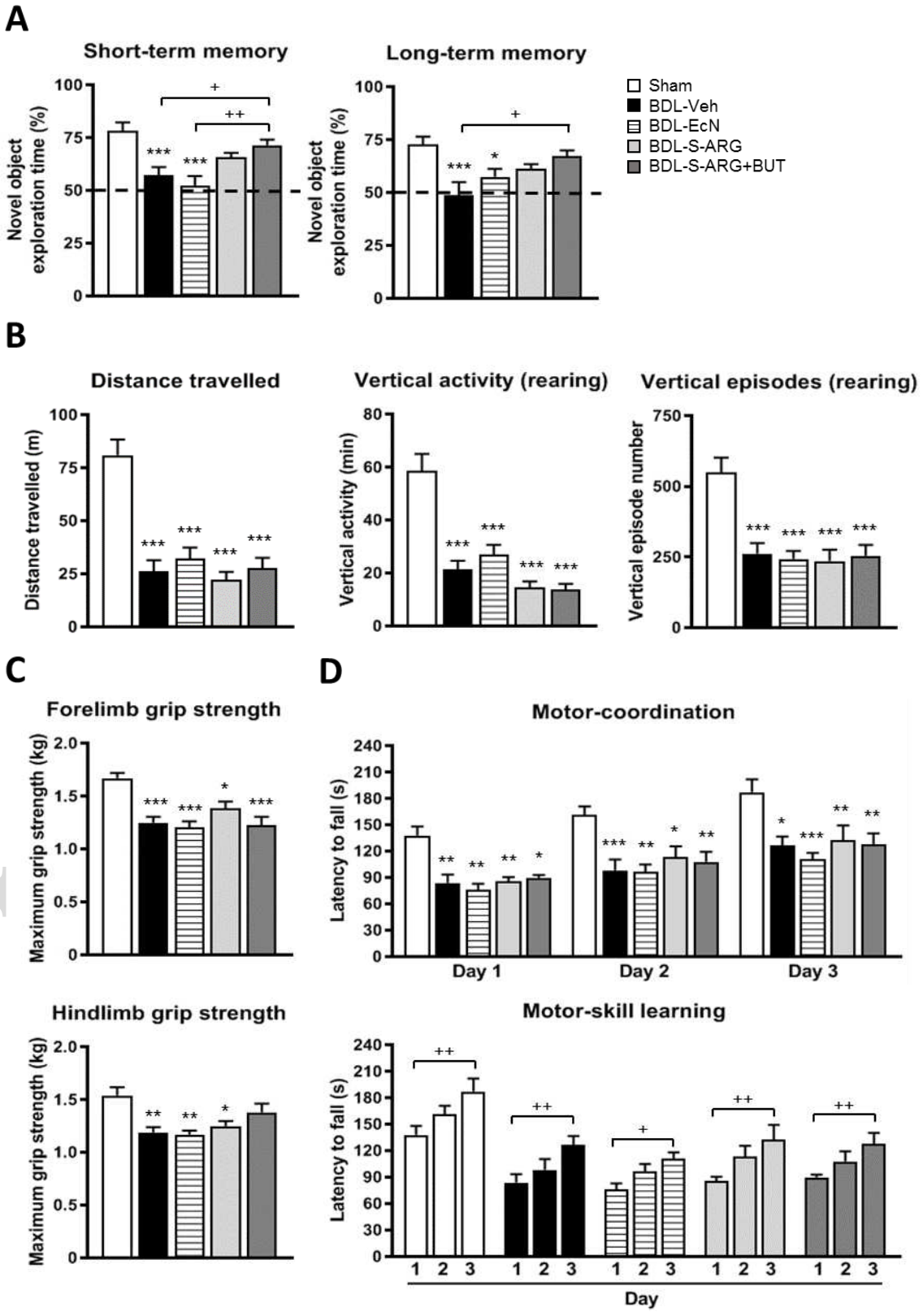

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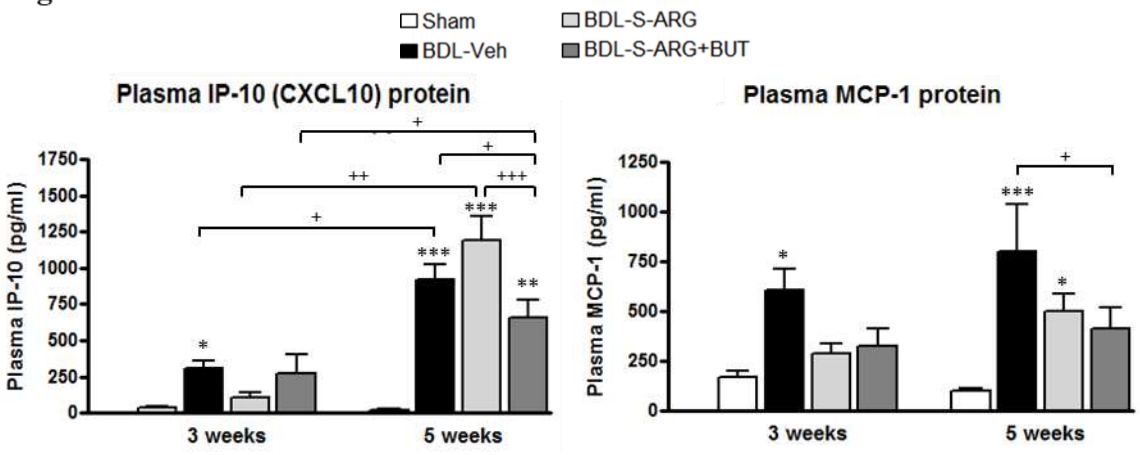

Figure 5

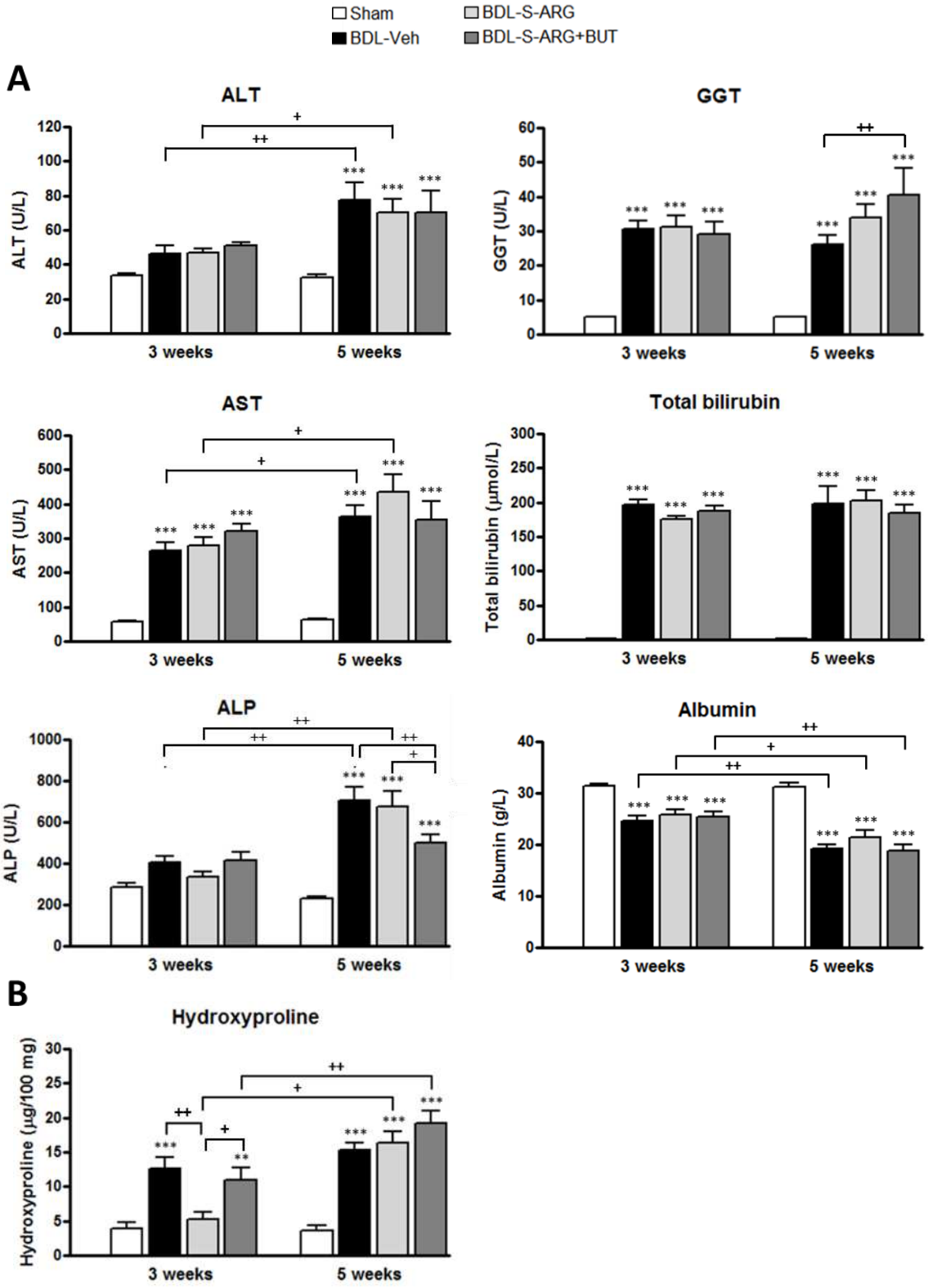

Figure 6

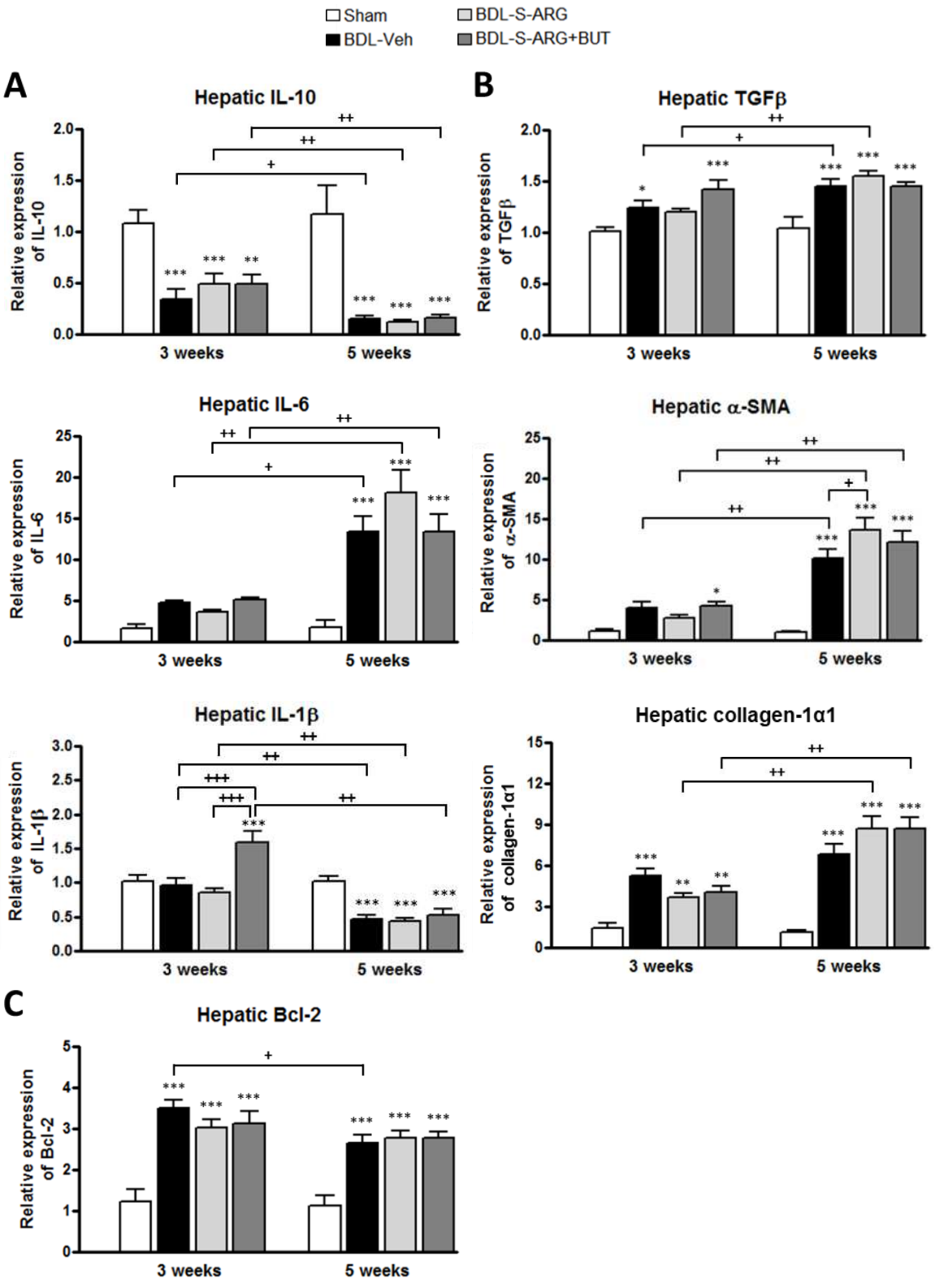

Figure 7