



OPEN LC2767 alleviates LPS-induced liver injury and fibrosis by regulating FXR expression, NF- κ B activation, and gut microbiota

Dong-Yun Lee¹, Hyo-Min Jang¹, Jung-Woo Shin¹, Yoon-Jung Shin¹, Sung Hye Kim², Seung-Won Han² & Dong-Hyun Kim^{1,3}✉

LC2767, a mix of *Lactiplantibacillus plantarum* LC27 and *Bifidobacterium longum* LC67, alleviates high-fat diet- or alcohol-induced liver injury and steatosis in mice. In the present study, we examined the action mechanism of LC2767 on lipopolysaccharide (LPS)-, LPS/D-galactosamine (LGc)-, or LPS/thioacetamide (LTc)-induced liver damage and fibrosis in mice. Short-termly or long-termly intraperitoneal injection of LPS, LGc, or LTc caused liver damage and fibrosis in mice: they increased alanine transaminase (ALT), aspartate transaminase (AST), and γ -glutamyl transpeptidase (γ -GTP) in the blood and α -smooth muscle actin (SMA) and tumor necrosis factor (TNF)- α expression and NF- κ B activation in the liver. Short-termly orally gavaged LPS also caused liver damage. However, oral administration of LC2767 significantly reduced LPS- or LGc-, or LTc-induced ALT, AST, and γ -GTP levels in the blood. LC2767 also suppressed LPS- or LGc-induced collagen-1, α -SMA, TNF- α , CYP7A1, tissue inhibitor of metalloproteinase (TIMP)-1, and matrix metalloproteinase (MMP)-14 expression, NF- κ B activation, non-alcoholic fatty liver disease activity score, and fibrosis score in the liver, and TNF- α expression and NF- κ B activation in the colon, while LPS- or LGc-suppressed farnesoid X receptor (FXR) expression was increased in the liver and colon. Furthermore, LC2767 alleviated LPS-induced gut dysbiosis: it increased *Akkermansia* population and decreased LPS-producing *Christensenellaceae*, *Porphyromonadaceae*, and *Rhodospirillaceae* populations. These findings suggest that LC2767 can alleviate liver damage and fibrosis by regulating NF- κ B activation, FXR expression, and gut microbiota.

Keywords LC2767, Liver fibrosis, NF- κ B activation, Farnesoid X receptor, Gut microbiota

Abbreviations

ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
ASBT	Apical sodium-dependent bile salt transporter
AST	Aspartate transaminase
BSEP	Bile salt export pump
CFU	Colony-forming unit
Collagen1	Collagen type 1
CTP7A1	Cholesterol 7 α -hydroxylase
ELISA	Enzyme-linked immunosorbent assay
FXR	Farnesoid X receptor
GFAP	Glial fibrillary acidic protein
γ -GTP	γ -Glutamyl transpeptidase
IL	Interleukin
LC2767	a (3:1) mix of LC27 and LC67
LDH	Lactate dehydrogenase
LGc	LPS and D-galactosamine
LPS	Lipopolysaccharide

¹Lab of Microbiology and Immunology, College of Pharmacy, Kyung Hee University, Seoul 02447, Korea. ²NVP Healthcare, Jangan-ro, 448 beon-gil, Jangan-gu, Suwon, Kyunggi-do 16209, Korea. ³PBLbiolab, Seoul 03174, Korea. ✉email: dhkim@khu.ac.kr

LTc	LPS and thioacetamide
MMP	Matrix metalloproteinase
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NC	Normal control group
RIPA	Radio-immunoprecipitation assay
SHP	Small heterodimer partner
SMA	Smooth muscle actin
TBA	Total bile acid
TCh	Total cholesterol
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Liver damage is caused by a number of factors, including viral infections, cancers, genetic predispositions, toxin exposure (e.g., alcohol), and excessive high-fat intake^{1,2}. Moreover, these factors induce endotoxin-rich gut dysbiosis. In the United States, non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder, affecting an estimated 20% to 30% of the adult population^{3–5}. Of those with NAFLD, approximately 20% can progress to non-alcoholic steatohepatitis (NASH), a more severe form of the disease affecting around 5% of American adults⁶. NASH is strongly associated with an increased risk of developing advanced fibrosis and liver cirrhosis^{5,7}.

Gut dysbiosis, particularly characterized by the overproduction of endotoxins such as lipopolysaccharide (LPS), has been identified as a key factor in the progression of liver damage and fibrosis^{3,8,9}. LPS is overproduced in response to a high-fat diet and stresses^{10–12}. Chronic exposure to LPS leads to inflammation in both the gut and liver¹³. Furthermore, gut dysbiosis contributes to the increased deconjugation of bile acids, which exacerbates conditions such as NAFLD and NASH^{14,15}.

Live biotherapeutic products including probiotics have shown potential in alleviating liver damage caused by LPS or LPS/D-galactosamine (LGc) by inhibiting NF- κ B activation and preserving the intestinal barrier^{16–18}. *Lactobacillus rhamnosus* GG, for example, improves liver injury in rats by enhancing intestinal barrier integrity¹⁹. *Lactiplantibacillus plantarum* LC27, *Bifidobacterium longum* LC67, and their mix (LC2767) reduce LPS-, high fat diet-, or alcohol-induced liver injury and steatosis by modulating NF- κ B and AMP-activated kinase (AMPK) signaling pathways^{9,20,21}. Despite these findings, the precise mechanisms of LBPs that confer protection against liver damage and fibrosis remain unclear.

Therefore, the present study aimed to investigate the action mechanism of LC2767 in mice with liver damage and fibrosis induced by LPS, LGc, or LPS/thioacetamide (LTc).

Results

Effect of LC2767 on liver damage and colitis in mice intraperitoneally injected with LPS

First, to investigate whether the mix of *L. plantarum* LC27 and *B. longum* LC67 could inhibit the secretion of alanine transaminase (ALT) and aspartate transaminase (AST) in LPS-stimulated HepG2 cells, we mixed LC27 and LC67 in ratios of 9:1, 3:1, 1:1, 1:3, and 1:9 and examined their effects in LPS-stimulated HepG2 cells (Supplement Fig. S1). They decreased AST, ALT, and lactate dehydrogenase (LDH) levels dose-dependently. Among the tested combinations, the 3:1 ratio of LC27 to LC67 (LC2767) showed the strongest effect in lowering LPS-induced AST, ALT, and LDH levels. LPS also induced NF- κ B activation (p-p65/p65) and suppressed farnesoid X receptor (FXR) expression in Caco2 cells (Supplement Fig. S2 and Table S1). LC2767 strongly increased LPS-suppressed FXR expression, while LPS-induced NF- κ B activation and TNF- α expression weakly decreased.

Therefore, we examined the effects of LC2767, *Escherichia coli*, and obeticholic acid on liver injury and colitis in mice intraperitoneally injected with LPS (Fig. 1A–E, Supplement Fig. S3A). LPS injection led to elevated levels of AST, ALT, γ -glutamyl transpeptidase (γ -GTP), total bile acid (TBA), total cholesterol (TCh), and interleukin (IL)-6 in the blood. However, oral administration of LC2767 significantly reduced LPS-induced AST, ALT, γ -GTP, TBA, TCh, and IL-6 levels. Of these, the efficacy of LC2767 at a dose of 1×10^9 CFU/mouse/day was comparable with that of obeticholic acid. Intraperitoneal injection of LPS also led to an increase in the toll-like receptor 4 (TLR4), tumor necrosis factor (TNF)- α , α -smooth muscle actin (SMA), collagen type 1 (collagen1), and cholesterol 7 α -hydroxylase (CYP7A1) expression in the liver to 204.8%, 199.2%, 276.2%, 171.7%, and 252.7% of LPS-untreated NC group, respectively (Fig. 1F–O, Supplement Fig. S3B–F and Table S2). LPS also increased NF- κ B^{F4/80+} and α -SMA^{GFAP+} cell populations in the liver. However, LPS treatment decreased FXR expression to 40.1% of NC. Concurrently, there was a decrease in small heterodimer partner (SHP) expression and AMP-activated kinase (AMPK) phosphorylation. Oral administration of LC2767 most potently reduced LPS-induced TLR4 and TNF- α expression by 62.9% and 43.4%, respectively. LC2767 also suppressed LPS-induced NF- κ B activation, and the NF- κ B^{F4/80+} cell population, followed by obeticholic acid. *E. coli* did not suppress their expressions. Both LC2767 and obeticholic acid significantly downregulated LPS-induced expression of α -SMA, collagen1, and CYP7A1, as well as the α -SMA^{GFAP+} cell population. LC2767 increased LPS-suppressed FXR expression by 320.5%. LC2767 also increased expression of SHP and bile salt export pump (BSEP), along with enhancing AMPK activation.

Intraperitoneal exposure to LPS caused colon shortening and resulted in elevated levels of TLR4 and TNF- α in the colon to 241.0% and 183.6% of NC, respectively (Fig. 2, Supplement Fig. S3G–M and Table S3). LPS also increased NF- κ B^{CD11c+} cell population. FXR expression was suppressed to 37.9% of NC by LPS treatment. Oral administration of LC2767 partially recovered LPS-induced colon shortening. LC2767 also suppressed

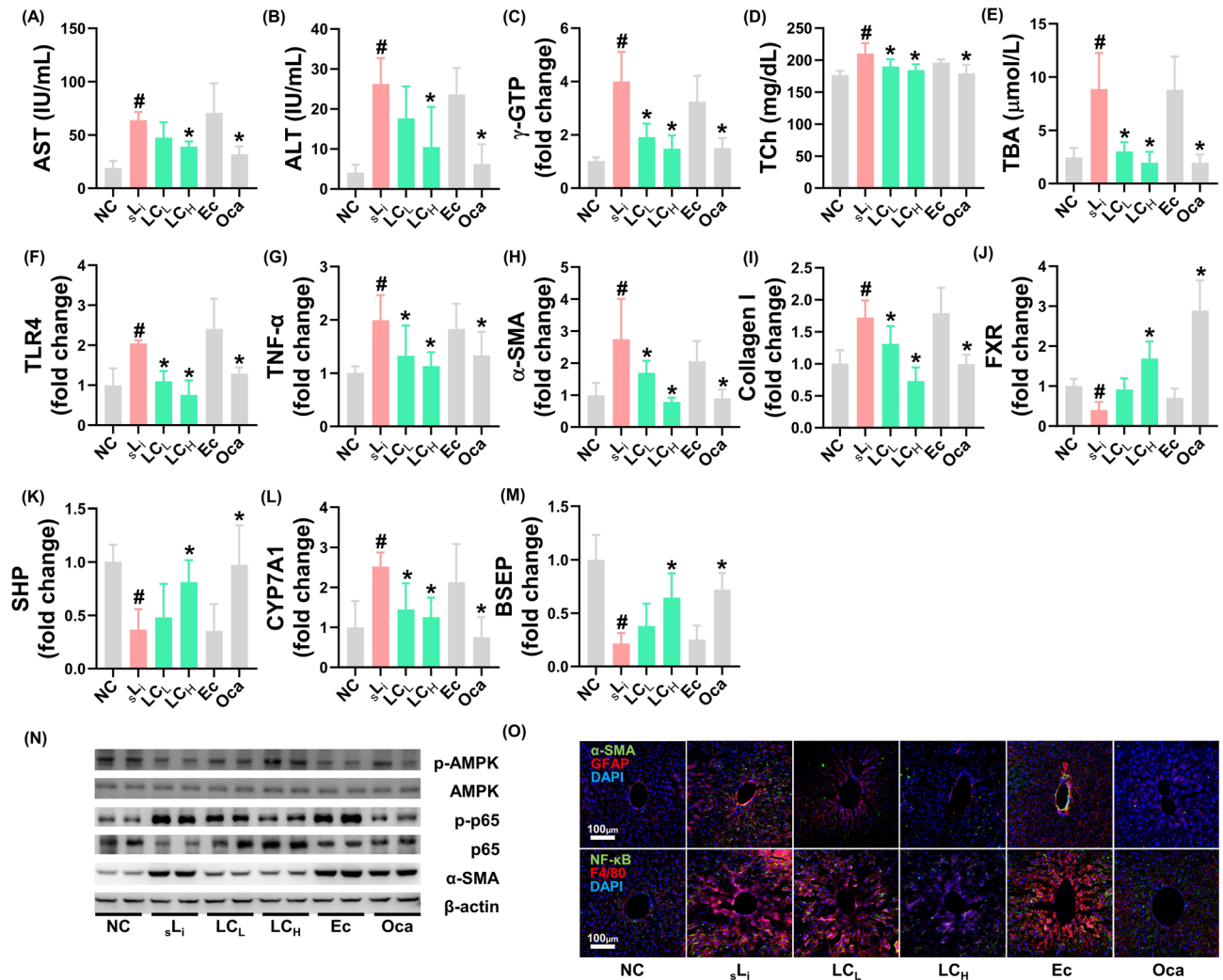


Fig. 1. Effects of LC2767 on liver damage in mice intraperitoneally injected with LPS. Effects on AST (A), ALT (B), γ -GTP (C), TCh (D), and TBA (E) levels in the blood. Effect on LPS-induced TLR4 (F), TNF- α (G), α -SMA (H), collagen1 (I), FXR (J), SHP (K), CYP7A1 (L), and BSEP (M) expression (assessed by qPCR), AMPK activation, NF- κ B activation, and α -SMA expression (N, assessed by immunoblotting), α -SMA⁺GFAP⁺ and NF- κ B⁺F4/80⁺ cell populations (O, assessed by immunofluorescence staining) in the liver. LC2767 at doses of 1×10^8 (LC_L) and 1×10^9 CFU/mouse (LC_H), *E. coli* at a dose of 1×10^9 CFU/mouse (Ec), obeticholic acid at a dose of 5 mg/kg (Oca), and saline (sLi) were orally administered once a day for 5 days to mice that were intraperitoneally injected with LPS for 5 days. NC group was treated with saline. Data are shown in mean \pm SD ($n = 8$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. sLi (LPS).

LPS-induced TLR4 and TNF- α expression in the colon by 60.8% and 48.6%, respectively, while increasing LPS-suppressed FXR expression by 217.0%. Additionally, LC2767 decreased LPS-induced NF- κ B⁺CD11c⁺ cell population. The efficacy of LC2767 at a dose of 1×10^9 was the most potent.

Effect of LC2767 on liver damage and colitis in mice orally gavaged with LPS

We next explored the effect of LC2767 on liver damage and colitis in mice orally gavaged with LPS for 5 days (Fig. 3). Orally gavaged LPS led to an increase in AST, ALT, γ -GTP, TCh, and TBA levels in the blood (Fig. 3A–E). However, oral administration of LC2767 significantly reduced these LPS-induced AST, ALT, γ -GTP, TCh, and TBA levels in the blood.

Oral exposure to LPS upregulated TLR4, TNF- α , α -SMA, and CYP7A1 expression to 184.4%, 146.4%, 143.1%, and 244.9% of NC, respectively, while FXR expression downregulated to 52.4% of NC. LPS also increased NF- κ B activation, and NF- κ B⁺F4/80⁺ and α -SMA⁺GFAP⁺ cell populations in the liver, while AMPK activation (phosphorylation) was reduced (Fig. 3F–R, Supplement Fig. S4A–E and Table S4). However, oral administration of LC2767 significantly suppressed the LPS-induced TLR4, TNF- α , α -SMA, collagen1, and CYP7A1 expression by 45.3%, 34.6%, 22.6%, and 27.6%, respectively. LC2767 also decreased LPS-induced NF- κ B activation and α -SMA⁺GFAP⁺ and NF- κ B⁺F4/80⁺ cell populations. Additionally, LC2767 increased LPS-suppressed FXR

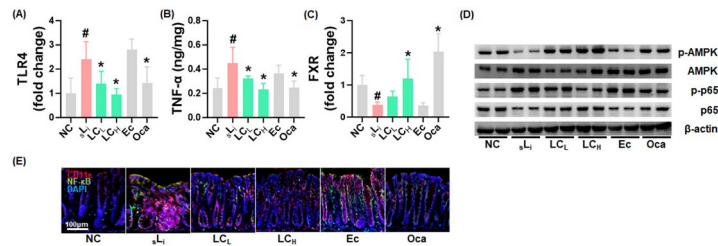


Fig. 2. Effects of LC2767 on LPS-induced colitis in mice. Effect on TLR4 (A, assessed by qPCR), TNF- α (B, assessed by ELISA), and FXR expression (C, assessed by qPCR), AMPK and NF- κ B activation (D, assessed by immunoblotting) and NF- κ B⁺CD11c⁺ cell population (E, assessed by immunofluorescence staining) in the colon. LC2767 at doses of 1×10^8 (LC_L) and 1×10^9 CFU/mouse (LC_H), *E. coli* at a dose of 1×10^9 CFU/mouse (Ec), obeticholic acid at a dose of 5 mg/kg (Oca), and saline (sLi) were orally administered once a day for 5 days to mice that were intraperitoneally injected with LPS for 5 days. NC group was treated with saline. Data are shown in mean \pm SD ($n=8$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. sLi.

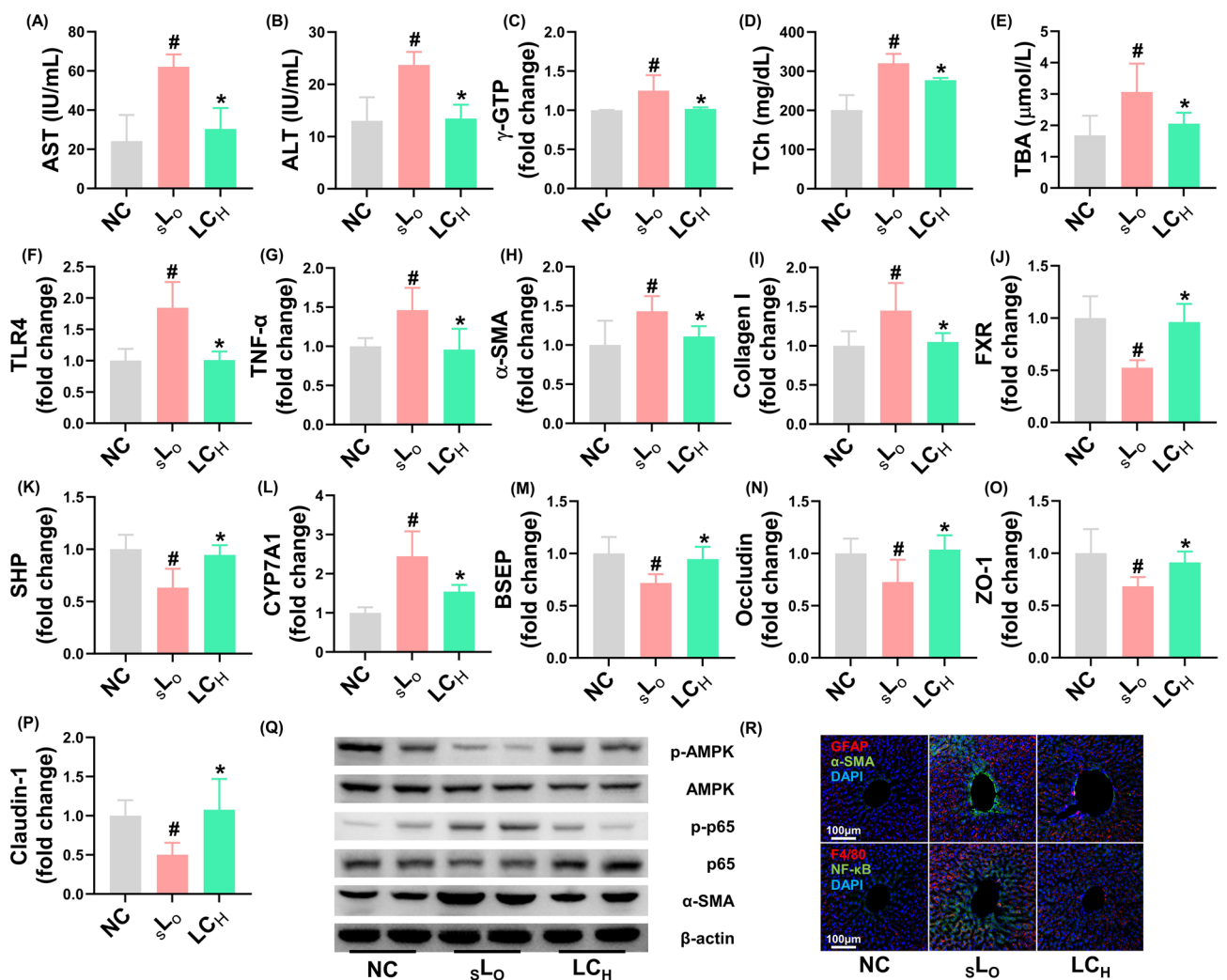


Fig. 3. Effect of LC2767 on liver damage-related biomarker levels in mice orally exposed to LPS. Effect on AST (A), ALT (B), γ -GTP (C), TCh (D), and TBA (E) in the blood. Effect on TLR4 (F), TNF- α (G), α -SMA (H), collagen1 (I), FXR (J), SHP (K), CYP7A1 (L), and BSEP (M), occludin (N), ZO-1 (O), and claudin-1 (P) expression (assessed by qPCR), AMPK α activation, NF- κ B activation and α -SMA expression (Q, assessed by immunoblotting) and α -SMA⁺GFAP⁺ and NF- κ B⁺F4/80⁺ cell populations (R) in the liver. LC2767 at a dose of 1×10^9 CFU/mouse (LC_H) and saline (sLo) were orally administered once a day for 5 days to mice that were orally gavaged with LPS for 5 days. NC group was treated with saline. Data are shown in mean \pm SD ($n=8$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. sLo.

expression by 83.4%. Furthermore, LC2767 increased LPS-suppressed SHP, BSEP, ZO-1, claudin-1, and occludin expression.

Oral exposure to LPS caused colon shortening and colitis: it increased TLR4 and TNF- α expression in the colon to 129.8% and 246.4% of NC, respectively, while FXR expression decreased to 50.2% of NC (Fig. 4, Supplement Fig. S4F–I and Table S5). LPS also increased NF- κ B⁺CD11c⁺ cell population in the colon, while AMPK activation were reduced. Oral administration of LC2767 alleviated LPS-induced colon shortening. LC2767 also suppressed LPS-induced TLR4 and TNF- α expression in the colon by 21.4% and 43.9%, respectively, while increasing LPS-suppressed FXR expression by 55.4%. Additionally, LC2767 decreased LPS-induced NF- κ B⁺CD11c⁺ cell population and increased LPS-suppressed colon length and AMPK activation.

Effect of LC2767 on liver damage and colitis in mice long-termly, intraperitoneally injected with LPS

We examined the effects of LC2767 and obeticholic acid on liver damage and colitis in mice subjected to long-term LPS exposure. Long-termly intraperitoneal exposure to LPS resulted in elevated levels of AST, ALT, γ -GTP, TBA, TCh, TNF- α , and IL-6 in the blood (Fig. 5A–E, Supplement Fig. S5A). However, oral administration of LC2767 at doses of 1×10^8 and 1×10^9 CFU/mouse significantly reduced elevated levels of these biomarkers. Similarly, obeticholic acid at a dose of 5 mg/kg significantly decreased LPS-induced levels of AST, ALT, γ -GTP, TBA, and TCh, while TNF- α and IL-6 expression were weakly reduced compared to those of LC2767. Of these, LC2767 at a dose of 1×10^9 CFU/mouse showed the strongest inhibition.

Long-term exposure to LPS strongly increased TLR4, TNF- α , α -SMA, collagen1, and CYP7A1 expression in the liver to 151.6%, 149.6%, 147.8%, 142.6%, and 325.5% of NC, respectively (Fig. 5F–X, Supplement Fig. S5B–F and Table S6). LPS also increased tissue inhibitor of metalloproteinase (TIMP)-1, matrix metalloproteinase (MMP)-9, and MMP-14 expression, NF- κ B phosphorylation, NF- κ B⁺F4/80⁺ and α -SMA⁺GFAP⁺ cell populations, α -SMA, collagen, and lipid accumulation, and NAFLD activity score (NAS) and fibrosis score in the liver. However, LPS exposure decreased FXR expression to 29.8% of NC. LPS also suppressed the expression of SHP, BSEP, MMP-10, ZO-1, claudin-1, and occludin was reduced, along with AMPK activation.

Oral administration of LC2767 significantly inhibited LPS-induced TLR4 and TNF- α expression by 56.6% and 37.6%, respectively. LC2767 also decreased LPS-induced NF- κ B activation, and the NF- κ B⁺F4/80⁺ cell population, whereas obeticholic acid weakly inhibited them compared to LC2767. LC2767 and obeticholic acid all effectively suppressed LPS-induced α -SMA (45.3% and 48.0%, respectively), collagen1 (48.9% and 35.9%, respectively), and CYP7A1 expression (53.7% and 62.2%, respectively), along with an increase in α -SMA⁺GFAP⁺ cell population and NAS and fibrosis score. LC2767 also decreased LPS-induced α -SMA, collagen, and lipid accumulation in the liver. LC2767 enhanced LPS-suppressed expression of FXR by 199.4%. LC2767 also increased LPS-suppressed SHP and BSEP expression, AMPK activation, and FXR⁺ cell population. Additionally, LC2767 and obeticholic acid reduced LPS-induced expression of MMP-9, MMP-14, and TIMP-1, while increasing LPS-suppressed MMP-10 expression. Furthermore, LC2767, but not obeticholic acid, restored LPS-suppressed expression of TIMP-1 and tight junction proteins ZO-1, occludin, and claudin-1. LC2767 at a dose of 1×10^9 CFU/mouse was the most effective.

Long-term exposure to LPS caused colon shortening and colitis: it induced TLR4 and TNF- α expression in the colon to 245.0% and 179.5% of NC, respectively, while FXR expression decreased to 17.1% of NC (Fig. 6, Supplement Fig. S5G–M and Table S7). LPS also increased NF- κ B⁺CD11c⁺ cell population in the colon, while AMPK activation were reduced. Oral administration of LC2767 partially recovered colon shortening. LC2767 also suppressed LPS-induced TLR4 and TNF- α expression in the colon by 51.8% and 50.0%, respectively, while increasing LPS-suppressed FXR expression by 322.7%. Additionally, LC2767 decreased LPS-induced NF- κ B⁺CD11c⁺ cell population, while LPS-suppressed colon length and AMPK activation increased.

Effect of LC2767 on liver damage in mice long-termly, intraperitoneally exposed to LGc

We evaluated the effects of LC2767 and obeticholic acid on liver damage in mice subjected to long-term exposure to LGc (Fig. 7A–E). Long-term exposure to LGc elevated AST, ALT, γ -GTP, TBA, and TCh levels in the blood.

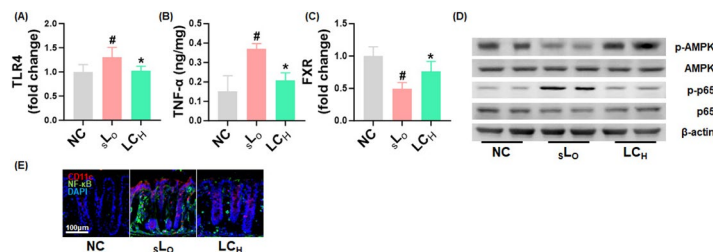


Fig. 4. Effects of LC2767 on colitis-related biomarker levels in mice orally exposed to LPS. Effects on TLR4 (A, assessed by qPCR), TNF- α (B, assessed by ELISA), and FXR expression (C, assessed by qPCR), AMPK and NF- κ B activation (D, assessed by immunoblotting) and NF- κ B⁺CD11c⁺ cell population (E, assessed by immunofluorescence staining) in the colon. LC2767 at a dose of 1×10^9 CFU/mouse (LC_H) and saline (sLo) were orally administered once a day for 5 days to mice that were orally gavaged with LPS for 5 days. NC group was treated with saline. Data are shown in mean \pm SD ($n = 8$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. sLo.

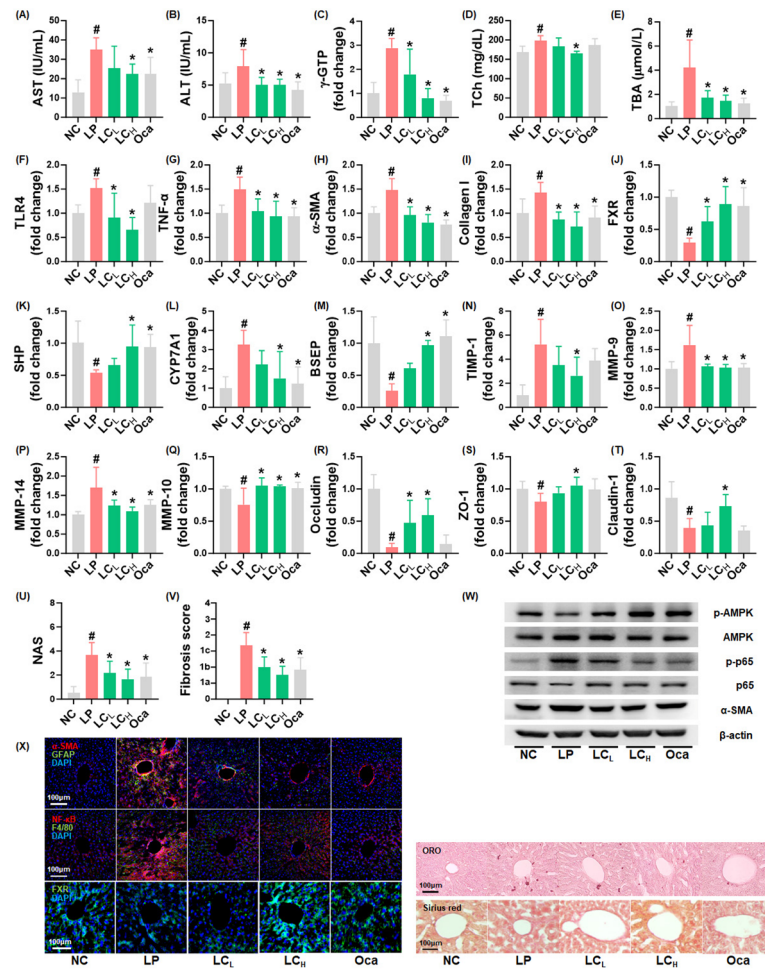


Fig. 5. Effect of LC2767 on liver injury and fibrosis-related biomarker levels in mice longtermly, intraperitoneally exposed to LPS. Effect on AST (A), ALT (B), γ -GTP (C), TCh (D) and TBA levels (E) in the blood. Effect on TLR4 (F), TNF- α (G), α -SMA (H), collagen1 (I), FXR (J), SHP (K), CYP7A1 (L), BSEP (M), TIMP-1 (N), MMP-9 (O), MMP-14 (P), MMP-10 (Q), occludin (R), ZO-1 (S), claudin-1 (T) expression (assessed by qPCR), NAS (U), fibrosis score (V), α -SMA expression and NF- κ B and AMPK activation (W, assessed by immunoblotting), α -SMA⁺GFAP⁺, NF- κ B⁺F4/80⁺, and FXR⁺ cell populations (assessed by immunofluorescence staining), Sirius red staining, and oil red staining (X) in the liver. LC2767 at doses of 1×10^8 (LC_L) and 1×10^9 CFU/mouse (LC_H), obeticholic acid 5 mg/kg/mouse (Oca), and saline (LP) were orally administered once a day for 2 weeks to mice that were intraperitoneally injected with LPS for 4 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n=6$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. LP.

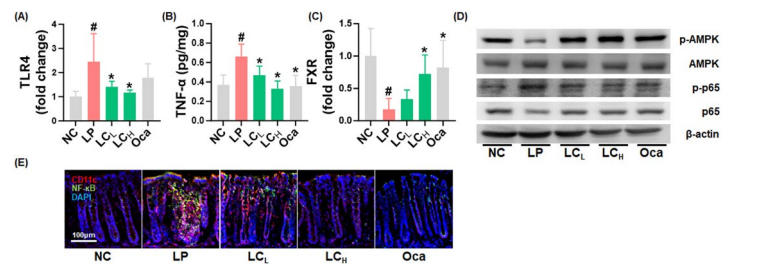


Fig. 6. Effect of LC2767 on colitis-related biomarker levels in mice longtermly, intraperitoneally exposed to LPS. Effects on TLR4 (A, assessed by qPCR), TNF- α (B, assessed by ELISA), and FXR expression (C, assessed by qPCR), AMPK and NF- κ B activation (D, assessed by immunoblotting) and NF- κ B⁺CD11c⁺ cell population (E) in the colon. LC2767 at doses of 1×10^8 (LC_L) and 1×10^9 CFU/mouse (LC_H), obeticholic acid (5 mg/kg/mouse, Oca), and saline (LP) were orally administered once a day for 2 weeks to mice that were intraperitoneally injected with LPS for 4 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n=6$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. LP.

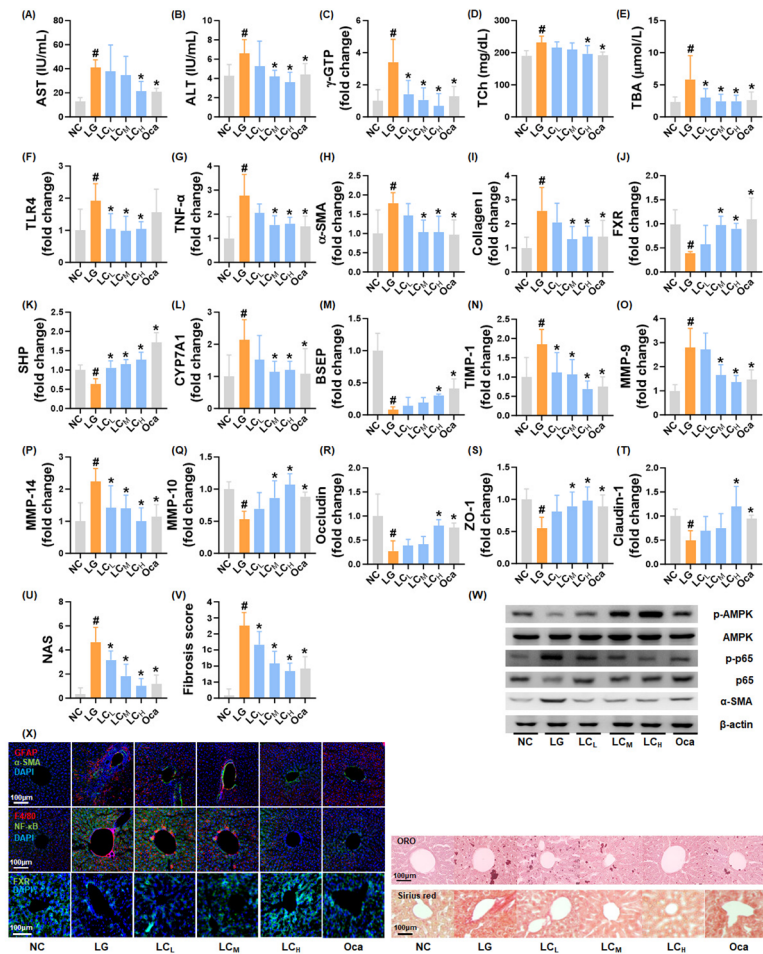


Fig. 7. Effect of LC2767 on liver injury and fibrosis-related biomarker levels in mice longtermly, intraperitoneally exposed to LGc. Effect on AST (A), ALT (B), γ -GTP (C), TCh (D), and TBA (E) in the blood. Effect on TLR4 (F), TNF- α (G), α -SMA (H), collagen1 (I), FXR (J), SHP (K), CYP7A1 (L), BSEP (M), TIMP-1 (N), MMP-9 (O), MMP-14 (P), MMP-10 (Q), occludin (R), ZO-1 (S), claudin-1 (T) expression (assessed by qPCR), α -SMA expression, NAS (U), fibrosis score (V), and NF- κ B and AMPK activation (W, assessed by immunoblotting), α -SMA⁺GFAP⁺, NF- κ B⁺F4/80⁺, and FXR⁺ cell populations (X, assessed by immunofluorescence staining), Sirius red staining, and oil red staining in the liver of mice. LC2767 (LC₁, 1×10^8 CFU/mouse; LC₂, 3×10^8 CFU/mouse; LC₃, 1×10^9 CFU/mouse), obeticholic acid 5 mg/kg/mouse (Oca), and saline (LG) were orally administered once a day for 2 weeks to mice that were intraperitoneally injected with LGc for 4 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n = 6$). * $p < 0.05$ vs. NC. * $p < 0.05$ vs. LG.

However, oral administration of LC2767 at doses of 1×10^8 , 3×10^8 , and 1×10^9 CFU/mouse or obeticholic acid at a dose of 5 mg/kg significantly reduced LGc-induced expression of these biomarkers. Of these, LC2767 at 1×10^9 CFU/mouse showed the strongest inhibitory effect on LGc-induced increases in AST, ALT, γ -GTP, TBA, and TCh levels.

Long-term exposure to LGc led to increased expression of TLR4, TNF- α , α -SMA, collagen1, and CYP7A1 in the liver by 192.0%, 277.5%, 178.8%, 253.6%, and 212.8% of NC, respectively (Fig. 7F–X, Supplement Fig. S6A–F and Table S8). LGc also induced the expression of MMP-9, MMP-14, and TIMP-1, along with the expansion of NF- κ B phosphorylation, NF- κ B⁺F4/80⁺ and α -SMA⁺GFAP⁺ cell populations, α -SMA, collagen, and lipid accumulation, NF- κ B⁺F4/80⁺ and α -SMA⁺GFAP⁺ cell populations, and NAS and fibrosis score in the liver. However, LGc suppressed the expression of FXR to 40.0% of NC. LGc also decreased the expression of SHP, BSEP, MMP10, ZO-1, claudin-1, and occludin, AMPK activation (phosphorylation), and FXR⁺ cell population.

Oral administration of LC2767 significantly inhibited LGc-induced TLR4 and TNF- α expression by 45.8% and 41.9%, respectively. LC2767 also decreased LGc-induced NF- κ B activation and NF- κ B⁺F4/80⁺ cell population. However, obeticholic acid weakly suppressed TNF- α and TLR4 expression and NF- κ B⁺F4/80⁺ cell population compared to LC2767. LC2767 and obeticholic acid markedly suppressed LGc-induced α -SMA (42.2% and 46.0%, respectively), collagen1 (42.3% and 42.5%, respectively), and CYP7A1 expression (43.9% and 49.3%, respectively). They also decreased LGc-induced α -SMA⁺GFAP⁺ cell population, NAS and fibrosis score, α -SMA, collagen, and lipid accumulation in the liver. LC2767 increased LGc-suppressed FXR expression

by 128.0%. LC2767 also induced LGc-suppressed SHP, and BSEP expression, and enhanced AMPK activation. Additionally, LC2767 and obeticholic acid reduced LGc-induced MMP-9, MMP-14, and TIMP-1 expression, while restoring LGc-suppressed MMP-10 expression. LC2767 also increased LGc-suppressed tight junction proteins ZO-1, occludin, and claudin-1 expression, AMPK phosphorylation, and FXR⁺ cell population. Of these, LC2767 at a dose of 1×10^9 CFU/mouse showed the strongest inhibitory effect.

Exposure to LGc caused colon shortening and colitis: it induced TLR4 and TNF- α expression in the colon to 256.3% and 163.6% of NC, respectively, while FXR expression decreased to 27.5% of NC (Fig. 8, Supplement Fig. S6G–L and Table S9). LPS also increased NF- κ B⁺CD11c⁺ cell population in the colon, while AMPK activation were reduced. Oral administration of LC2767 partially recovered LGc-induced colon shortening. LC2767 and obeticholic acid also suppressed LPS-induced TLR4 (69.6% and 32.3%, respectively) and TNF- α expression (33.2% and 23.2%, respectively) in the colon, while increasing LPS-suppressed FXR expression by 361.9% and 587.3%, respectively. Additionally, LC2767 decreased LPS-induced NF- κ B⁺CD11c⁺ cell population, while LPS-suppressed colon length and AMPK activation increased. However, obeticholic acid weakly increased AMPK activation alone.

Effects of LC2767 on liver damage in mice long-termly, intraperitoneally injected with LTc

Next, we examined the impact of LC2767 and obeticholic acid on liver damage in mice subjected to long-term exposure to LTc (Fig. 9). Long-term exposure to LTc increased AST, ALT, γ -GTP, TBA, and TCh levels in the blood (Fig. 9A–E). However, oral administration of LC2767 at doses of 1×10^8 and 1×10^9 CFU/mouse markedly reduced LTc-induced AST, ALT, γ -GTP, TBA, and TCh levels.

Long-term exposure to LTc elevated TLR4, TNF- α , α -SMA, collagen1, and CYP7A1 expression in the liver to 219.7%, 229.0%, 359.8%, 311.2%, and 512.0% of NC, respectively. However, LTc decreased FXR expression in the liver to 30.1% of NC. LTc also decreased SHP and BSEP expression (Fig. 9F–M). Oral administration of LC2767 significantly inhibited LTc-induced TLR4 and TNF- α expression by 49.0% and 54.0%, respectively, whereas obeticholic acid weakly decreased TLR4 and TNF- α expression. LC2767 (LC_H) and obeticholic acid significantly reduced LTc-induced expression of α -SMA (by 50.5% and 60.1%, respectively), collagen1 (by 58.4% and 32.1%, respectively), and CYP7A1 (by 57.6% and 73.7%, respectively). Furthermore, LC2767 restored LTc-suppressed FXR, SHP and BSEP expression and AMPK activation.

Effects of LC2767 on gut dysbiosis in mice long-termly exposed with LPS

Gut dysbiosis is closely associated with liver diseases²². Therefore, to understand whether the action mechanism of LC2767 for the amelioration of liver injury and fibrosis, we investigated the effect of LC2767 on the gut microbiota composition in mice long-termly (8-weeks) exposed with LPS (Fig. 10, Supplement Tables S10–S12). Furthermore, LPS exposure significantly shifted β -diversity (PCoA) in gut microbiota, while α -diversity was not affected. Exposure to LPS significantly increased the population of Proteobacteria including *Christensenellaceae*, *Porphyromonacaceae*, and *Rhodospirillaceae*, while the population of *Clostridiaceae* significantly decreased. The population of *Verrucomicrobiota* including *Akkermansiaceae* showed a weak but non-significant decrease, while the population of *Helicobacteriaceae* increased slightly without statistical significance. However, oral administration of LC2767 partially shifted LPS-induced β -diversity to those of NC. LC2767 significantly increased the population of *Verrucomicrobiota* including *Akkermansiaceae*, *Erysipelotrichaceae*, *Sutterellaceae*, and *PAC001057_f*, while *Rhodospirillaceae* population significantly decreased. *Christensenellaceae* and *Helicobacteriaceae* populations were weakly, but not significantly, reduced by treatment with LC2767. *Rhodospirillaceae*, *Helicobacteriaceae*, *Deferribacteriaceae*, and *Christensenellaceae* populations, which were positively correlated with TNF- α , α -SMA, and collagen1 expression levels in the liver and ALT, AST, and TNF- α levels in the blood. *Akkermansiaceae* population was positively correlated with FXR expression level in the liver and colon. *Coryobacteriaceae* population was negatively correlated with TNF- α , α -SMA, and collagen1 expression levels in the liver and AST and TNF- α levels in the blood.

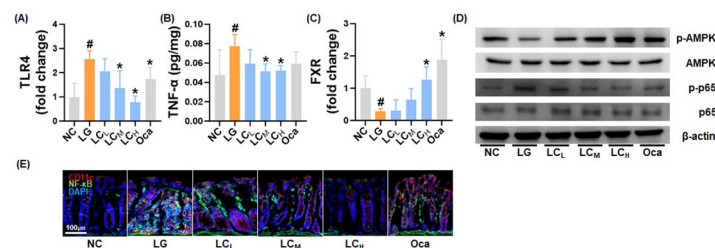


Fig. 8. Effect of LC2767 on colitis-related biomarker levels in mice longtermly, intraperitoneally exposed to LGc. Effects on TLR4 (A), TNF- α (B), and FXR expression (C, assessed by ELISA), AMPK and NF- κ B activation (D) and NF- κ B⁺CD11c⁺ cell population (E) in the colon. LC2767 (LC_L, 1×10^8 CFU/mouse; LC_M, 3×10^8 CFU/mouse; LC_H, 1×10^9 CFU/mouse), obeticholic acid (Oca, 5 mg/kg/mouse), and saline (LG) were orally administered once a day for 2 weeks to mice that were intraperitoneally injected with LGc for 4 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n = 6$). # $p < 0.05$ vs. NC. * $p < 0.05$ vs. LG.

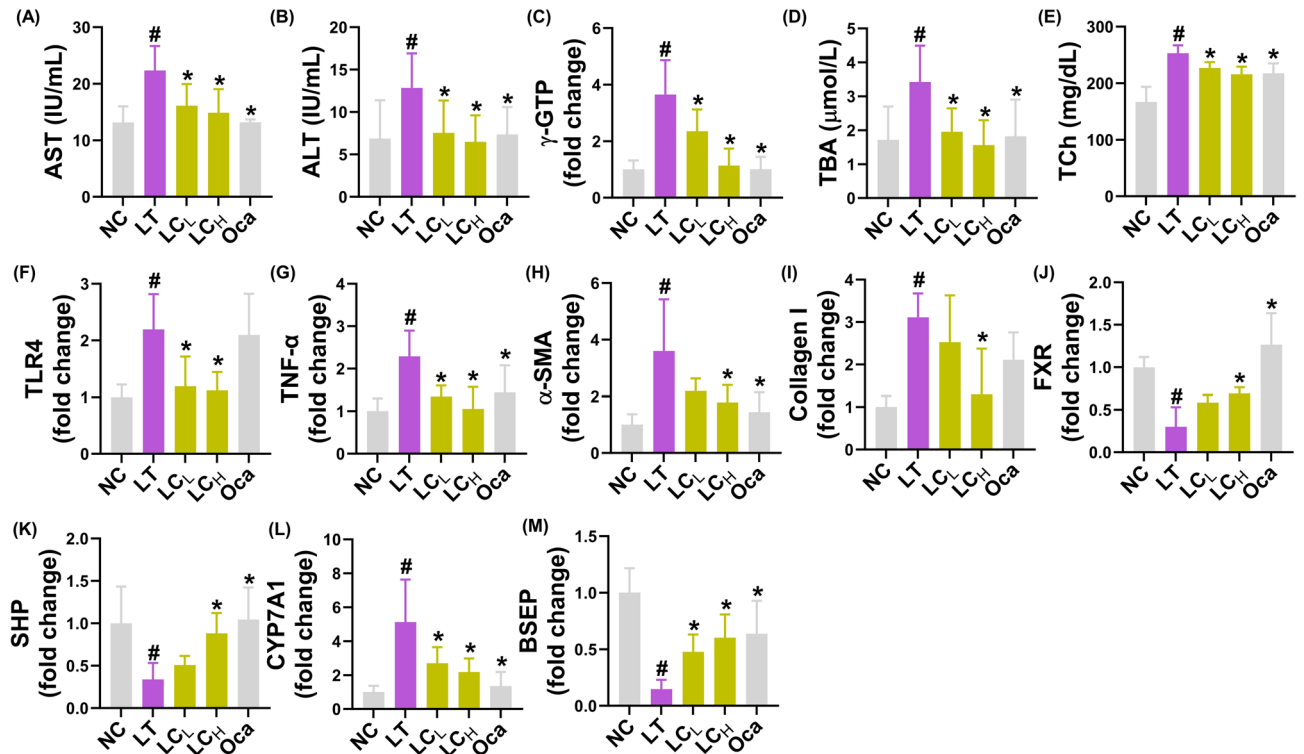


Fig. 9. Effect of LC2767 on liver injury and fibrosis-related biomarker levels in mice intraperitoneally exposed to LTc. Effect on AST (A), ALT (B), γ -GTP (C), TBA (D), and TCh (E) in the blood. Effect on TLR4 (F), TNF- α (G), α -SMA (H), collagen1 (I), FXR (J), SHP (K), CYP7A1 (L) and BSEP (M) expression (assessed by qPCR) in the liver. LC2767 (LC_L, 1×10^8 CFU/mouse; LC_H, 1×10^9 CFU/mouse), obeticholic acid (Oca, 5 mg/kg/mouse), and saline (LT) were orally administered once a day for 2 weeks to mice that were intraperitoneally injected with LTc from 8 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n = 6$). * $p < 0.05$ vs. NC. # $p < 0.05$ vs. LT.

Discussion

Acute inflammation is a natural and advantageous response to injuries and infections, while chronic inflammation can lead to various inflammatory diseases, such as gut inflammation, hepatitis, and rheumatoid arthritis^{23,24}. Gut inflammation can be activated by gut dysbiosis and gut microbiota byproducts such as LPS, peptidoglycan, and reactive oxygen species²⁴. Excessive exposure to high-fat diet, ethanol, or stressors induces the overexpression of endotoxins including LPS in the gut microbiome, which causes gut inflammation through the induction of NF- κ B activation and suppression of tight junction protein expression^{11,25,26}.

Thus, LPS causes a leaky gut via gut inflammation, which accelerates the absorption of gut bacterial LPS into the blood^{25,27}. D-galactosamine causes hepatocyte death (necrosis and apoptosis) through the inhibition of RNA synthesis¹. Thioacetamide induces hepatotoxicity through the upregulation of TNF- α expression²⁸. LPS absorbed into the liver stimulates the expression of proinflammatory cytokines including TNF- α via the activation of TLR4-mediated NF- κ B signaling, resulting in hepatitis and steatohepatitis. These results suggest that excessive exposure to LPS can cause liver injury and fibrosis through the gut inflammation and hepatotoxic ingredients including D-glucosamine and thioacetamide can accelerate LPS-inducible liver injury. However, LC2767 alleviates ethanol-, 2,4,6-trinitrobenzenesulfonic acid-induced liver injury, or high-fat diet-induced steatohepatitis with obesity in mice by the regulation of NF- κ B signaling^{9,20,21,29}. These findings suggest that suppressing gut bacterial endotoxin production and TLR4-mediated NF- κ B signaling may be important for the amelioration of liver injury and fibrosis.

In the present study, we found that shorttermly or longtermly intraperitoneal or oral exposure to LPS, LGc, or LTc increased ALT, AST, and γ -GTP levels in the blood and TLR4, TNF- α , and IL-6 expression and NF- κ B activation in the liver, while decreasing the expression of tight junction proteins ZO-1 and occludin. Although they weakly, but not significantly, increased bodyweight (Supplement Fig. S7), total cholesterol levels significantly increased in the blood. Although they did not cause significant morphological changes in the liver, NF- κ B- and α -SMA-positive cell populations and NAS and fibrosis scores increased. Thus, intraperitoneal or oral exposure to LPS caused systemic inflammation including gut inflammation and hepatitis. Exposure to LPS, LGc, or LTc increased α -SMA, and collagen1 expression in the liver. They increased TIMP-1, MMP-9, and MMP-14 expression in the liver, while MMP-10 expression decreased. They also increased ALT, AST, and γ -GTP levels in the blood and TNF- α and IL-6 expression and NF- κ B activation in the liver. In particular, LGc or LTc more potently increased the expression of inflammation-related markers in the liver than LPS alone. Moreover, LPS exposure significantly shifted β -diversity (PCoA) in gut microbiota and increased the population

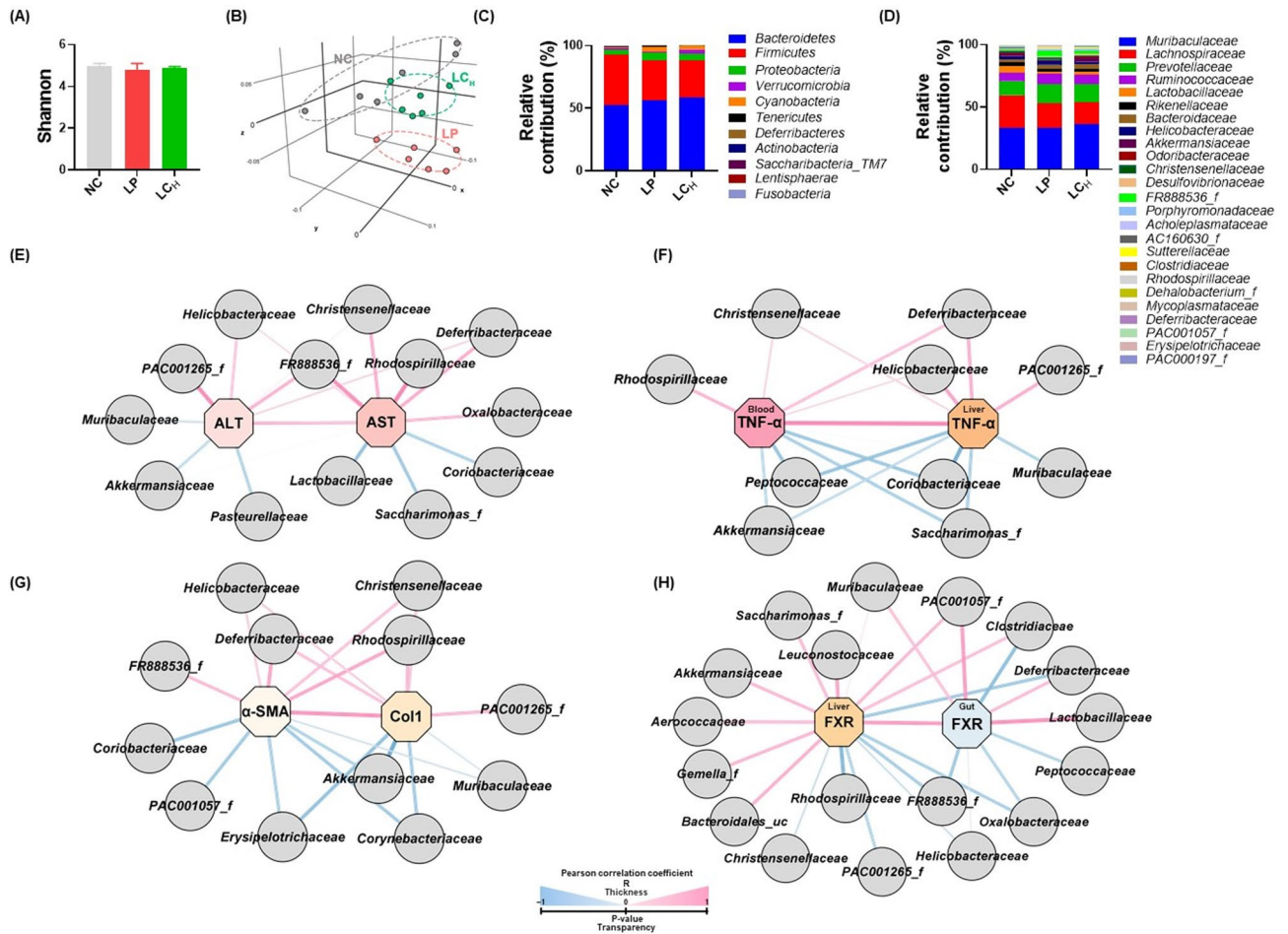


Fig. 10. Effect of LC2767 on the gut microbiota composition in mice longtermly, intraperitoneally exposed to LPS. Effect on (A) α -diversity (Shannon index), (B) β -diversity (principal coordinate analysis plot based on Bray–Curtis analysis), phylum (C) and family levels (D) and network analysis—(E) gut microbiota and ALT/AST levels in the blood, (F) gut microbiota and blood/liver TNF- α levels, (G) gut microbiota and α -SMA/collagen1 expression levels, and (H) gut microbiota and liver/gut FXR expression levels. LC2767 at a dose of 1×10^9 CFU/mouse (LC_H) and saline (LP) were orally administered one day for 2 weeks to mice that were intraperitoneally injected with LPS for 4 weeks. NC group was treated with saline. Data are shown in mean \pm SD ($n = 6$). * $p < 0.05$ vs. NC. * $p < 0.05$ vs. LP.

of LPS-producing *Proteobacteria* including *Christensenellaceae*, *Porphyromonadaceae*, and *Rhodospirillaceae*. These results suggest that excessive exposure to LPS, LGc, or LTC may cause liver injury and fibrosis through the modulation of NF- κ B-involved TIMP/MMP expression and overpopulation of LPS-producing gut microbiota.

However, LC2767 (at doses of 1×10^8 and 1×10^9 CFU/mouse) reduced blood ALT, AST, γ -GTP, TNF- α , and TCh levels in mice with LPS-, LGc-, or LTC-induced liver injury, while reduced bodyweight weakly, but not significantly, recovered. LC2767 also reduced LPS-, LGc-, or LTC-induced TNF- α and TLR4 expression, NF- κ B activation, and NF- κ B-positive cell population in the liver and TNF- α , myeloperoxidase, and TLR4 expression levels and shortening in the colon, while LPS-, LGc-, or LTC-suppressed ZO-1 and occludin expression in the liver increased. LC2767 inhibited LPS-induced cytotoxicity in vitro: it reduced ALT, AST, and LDH levels in LPS-stimulated HepG2 cells. LC2767 suppresses LPS-induced TNF- α expression and NF- κ B activation in the macrophages.

LC2767 partially shifted LPS-induced β -diversity to those of NC: it significantly decreased *Rhodospirillaceae* population and increased *Akkermansiaceae*, *Sutterellaceae*, *Erysipelotrichaceae*, *Sutterellaceae*, *PAC001057_f* populations. *Rhodospirillaceae*, *Helicobacteriaceae*, *Deferribacteriaceae*, and *Christensenellaceae* populations, which were positively correlated with TNF- α , α -SMA, and collagen1 expression levels in the liver and ALT, AST, and TNF- α levels in the blood. *Akkermansiaceae* population was positively correlated with FXR expression level in the liver and colon. *Coryobacteriaceae* population was negatively correlated with TNF- α , α -SMA, and collagen1 expression levels in the liver and AST and TNF- α levels in the blood. *Akkermansia muciniphila* is negatively correlated with the obesity and diabetes^{30,31}. LC2767 decreases blood and fecal LPS levels in mice with high diet- or 2,4,6-trinitrobenzenesulfonic acid-induced liver injury^{9,20}. These

results suggest that LC2767 may alleviate liver damage including hepatitis by suppressing TLR4-mediated NF- κ B activation and gut dysbiosis.

Although some results were confusing, most of results showed that LC2767 suppressed LPS-, LGc-, or LT-induced α -SMA, collagen1, TIMP-1, MMP-9, and MMP-14 expression in the liver, while MMP-10 expression decreased. Furthermore, LC2767 decreased LPS- or LGc- induced α -SMA, collagen, and lipid accumulation, and NAS and fibrosis score in the liver. Moreover, in gene expression microarray analysis using the Affymetrix Mouse Gene 2.0 ST Array platform, LC2767 downregulated LPS-induced TLR4, TNF- α , NF- κ B, collagenase-1, CYP7A1, TIMP-1, MMP-9, and MMP14 genes, while upregulating LPS-suppressed MMP-10 and SHP genes (Supplement Figure S8A). LC2767 also downregulated LGc-induced TLR4, TNF- α , NF- κ B, collagenase-1, CYP7A1, TIMP-1, and MMP-9 genes, while upregulating MMP-10, MMP-14, BSEP, and SHP genes (Supplement Fig. S8B). TIMP1 is upregulated during hepatic fibrogenesis³². MMP-9, which promotes the apoptosis of hepatic stellate cells, is highly expressed in liver fibrosis³³. MMP14 is highly expressed in liver fibrosis^{34,35}. Collagen1 expression is increased and collagenase-1 (MMP1) degrades extracellular matrix in hepatic fibrosis^{36–38}. α -SMA-positive myofibroblast-like cells increase their expression of fibrillar collagen and MMPs such as MMP-9 in fibrotic tissue^{36,39}. However, MMP-10 promotes hepatic repair and regeneration after partial hepatectomy and bile duct ligation⁴⁰. These results suggest that LC2767 may alleviate liver injury and fibrosis through the regulation of NF- κ B-involved TIMP/MMPs expression.

Exposure to LPS, LGc or LTc increased TC and TBA levels in the blood and CYP7A1 expression in the liver. However, they decreased FXR, SHP, and BSEP expression in the liver and FXR expression in the colon. However, LC2767 decreased LPS-, LGc-, or LTc-induced TC and TBA level in the blood and CYP7A1 expression in the liver and increased LPS-, LGc-, or LTc-suppressed FXR, SHP, and BSEP expression in the liver and FXR expression in the colon. FXR contributes directly and indirectly to the homeostasis of bile acid, hepatic triglyceride, and glucose. Activation of FXR results in the downregulation of CYP7A1: it suppresses the expression of profibrotic genes including α -SMA, collagen1, and TIMP-1^{41,42}. The overexpression of CYP7A1 activates the bile acid synthesis pathway⁴³. Blic acids are transported from hepatocytes into the bile canaliculus through the BSEP, then enter the small intestine via the common bile duct, and are absorbed in the enterocytes of terminal ileum and colon through the apical sodium-dependent bile salt transporter⁴⁴. Here, bile acids can activate the nuclear bile acid receptor, FXR, which results in the upregulation of fibroblast growth factor (FGF)19 and subsequent release of FGF19 into the portal circulation⁴⁵. These results suggest that LC2767 may alleviate liver steatosis by suppressing the biotransformation of cholesterol to bile acid metabolites through the induction of FXR expression and the suppression of cholesterol biosynthesis.

LC2767 increased AMPK activation in the liver and colon. AMPK is a cellular energy sensor that can alleviate hepatic fibrosis⁴⁶. LC2767 suppress the accumulation of fat accumulation in the liver of high-fat diet-fed mice⁹. These results suggest that LC2767 may alleviate liver steatosis and fibrosis through the suppression of gut microbiota-associated TLR4/NF- κ B activation and the induction of AMPK activation.

In the present study, we could not perform MMP activity assay and in vivo histological assessments to confirm that the observed changes in gene expression translate into functional reductions in fibrosis. Therefore, to clarify these suggestions, further studies including these aspects are necessary to clarify the active components of LC2767 against liver injury and fibrosis.

In conclusion, LC2767 was found to suppress bacterial LPS biosynthesis in the intestinal fluid and blood while inhibiting the expression of TLR4 and TNF- α , and reducing the NF- κ B-positive cell population in both the intestine and liver. In the liver, it also downregulated the expression of collagen1 and α -SMA. Additionally, LC2767 activated FXR and AMPK signaling pathways, which led to the attenuation of LPS- or LGc-induced liver inflammation (hepatitis), liver steatosis, and gut inflammation. Overall, LC2767 effectively alleviated liver damage, fibrosis, and intestinal barrier dysfunction by regulating TLR4-mediated NF- κ B activation, FXR expression, AMPK activation, and gut microbiota.

Materials and methods

Preparation of LC2767

L. plantarum LC27 and *B. longum* LC67 strains were cultivated in a modified MRS and GAM medium, as previously reported²¹. Following cultivation, the cells were harvested by centrifugation at 5000 g for 20 min at 4 °C. The harvested cells were then resuspended in a 2% trehalose solution and subjected to freeze-drying. The resulting dried cells of LC27 and LC67 were then combined in ratios of 9:1, 3:1, 1:1, 1:3, and 1:9. For in vitro experiments, the combined cells were rehydrated in phosphate buffered saline (PBS). For in vivo studies, the freeze-dried cells were also suspended in sterilized saline.

Culture of HepG2 and Caco2 cells

HepG2 cells (Korean Cell Line Bank) and Caco2 cells (Korean Cell Line Bank) were cultured in DMEM (Sigma) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum, and maintained at 37 °C. HepG2 cells were seeded at a density of 2×10^6 cells/mL and treated with or without LPS (100 ng/mL) in the absence or presence of LC2767, a (3:1) mix of LC27 and LC67, at concentrations of 1×10^2 , 1×10^3 , 1×10^4 , and 1×10^6 colony-forming unit (CFU)/mL for 20 h. The levels of ALT, AST, and LDH were then measured using their respective assay kits. Caco2 cells were seeded at a density of 1×10^6 cells/mL and treated with or without LPS (100 ng/mL) in the absence or presence of LC2767 at concentrations of 1×10^3 and 1×10^5 CFU/mL for 20 h. FXR expression level and NF- κ B activation (p-p65/p65) were assayed using quantitative real-time polymerase chain reaction (qPCR) and immunoblotting, respectively.

Animals

Male C57BL/6 mice, aged 8 weeks and weighing between 19 and 21 g, were purchased from Koatech Inc. (Korea). The mice were housed in wire cages under controlled environmental conditions (temperature: 20–22 °C; humidity: 50 ± 10%) and provided with standard laboratory chow and water ad libitum. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kyung Hee University (IACUC No: KHUASP(SE)-23087, KHUASP(SE)-23390) and were ethically conducted in accordance of the University Guideline for Laboratory Animals Care and Usage and ARRIVE guideline⁴⁷.

Preparation of mice with liver injury or fibrosis and treatment with LC2767

First, we investigated liver injury-ameliorating effect of LC2767 in mice intraperitoneally injected with LPS. In brief, mice except normal control group (NC) received intraperitoneal injection of bacterial LPS (250 µg/kg/day, isolated from *Escherichia coli*, Sigma) for 5 days. Thereafter (24 h after the final LPS treatment), test agents (sLi, saline; LC_L, 1 × 10⁸ CFU/mouse/day of LC2767; LC_H, 1 × 10⁹ CFU/mouse/day of LC2767; Ec, 1 × 10⁹ CFU/mouse/day of *Escherichia coli*; Oca, 5 mg/kg/day of obeticholic acid) was orally administered for 5 days. NC was treated with saline instead of LPS and test agents.

Second, we investigated liver injury-ameliorating effect of LC2767 in mice orally gavaged with LPS. In brief, mice except Nc received oral gavage of LPS (500 µg/kg/day) for 5 days. Thereafter, test agents (sLo, saline; LC_L, 1 × 10⁹ CFU/mouse/day of LC2767) was orally administered for 5 days. NC was treated with saline instead of LPS and test agents.

Third, we examined the effect of LC2767 on subacute LPS-induced liver injury in mice. Mice except Nc received intraperitoneal injection of bacterial LPS (10 µg/kg/day) for 4 weeks (every other day). Thereafter, test agents (LP, saline; LC_L, 1 × 10⁸ CFU/mouse/day of LC2767; LC_H, 1 × 10⁹ CFU/mouse/day of LC2767; Oca, 5 mg/kg/day of obeticholic acid) was orally given every day for 2 weeks. NC was treated with saline instead of LPS and test agents.

Fourth, we tested the effect of LC2767 on subacute LGc-induced liver injury in mice. Mice except Nc received the intraperitoneal injection of LGc (LPS [10 µg/kg/day] and D-galactosamine [50 mg/kg/day, Sigma]) every other day for 4 weeks, as previously reported¹⁶. Thereafter, test agents (LG, saline; LC_L, 1 × 10⁸ CFU/mouse/day of LC2767; LC_M, 3 × 10⁸ CFU/mouse/day of LC2767; LC_H, 1 × 10⁹ CFU/mouse/day of LC2767; Oca, 5 mg/kg/day of obeticholic acid) was orally administered every day for 2 weeks, starting 24 h after the last LGc treatment. Mice were sacrificed 20 h after the final treatment. NC was treated with saline instead of LGc and test agents.

Fifth, we tested the effect of LC2767 on subacute LTC-induced liver injury in mice. Mice except Nc received the intraperitoneal injection of LTC (LPS [10 µg/kg/day] and thioacetamide [50 mg/kg/day, Sigma]) every other day for 4 weeks. Thereafter, test agents (LT, saline; LC_L, 1 × 10⁸ CFU/mouse/day of LC2767; LC_H, 1 × 10⁹ CFU/mouse/day of LC2767; Oca, 5 mg/kg/day of obeticholic acid) was orally administered every day for 2 weeks. NC was treated with saline instead of LPS/thioacetamide and test agents.

Mice were euthanized through CO₂ exposure in a closed chamber 20 h after the final treatment with test agents, followed by cervical dislocation. The blood (collected from the carotid artery), liver, ileum, and colon were removed for the analyses of enzyme-linked immunosorbent assay (ELISA), immunoblotting, and histological exam. Sera were separated by centrifugation (4 °C, 10 min, 5000 g), as previously reported²⁹.

Determination of ALT, AST, LDH, γ-GTP, TBA, and TCh

The levels of ALT, AST, γ-GTP, TBA, TCh (Asan Pharm Co., Korea), and TBA (Elabscience, Texas) in serum and cell culture supernatant were measured using their respective assay kits.

ELISA and immunoblotting

Liver and colon tissues were homogenized on ice using radio-immunoprecipitation assay (RIPA) lysis buffer (Biosesang, Korea) that was supplemented with 1% phosphatase inhibitor cocktail and 1% protease inhibitor cocktail (Roche). The homogenates were then centrifuged (12,000 g, 15 min, 4 °C). In their supernatants, cytokine and biomarker expression levels were assayed.

For cytokine assays, the supernatants were transferred to a 96-well plate and analyzed using their ELISA kits (R&D Systems), as previously reported²⁰.

For the immunoblotting analysis, the supernatants were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes⁴⁸. Proteins were detected using specific antibodies for p-NF-κB-p65 (S536, Cell Signaling), NF-κB-p65 (D14E12, Cell Signaling), α-smooth muscle actin (α-SMA, Invitrogen), F4/80 (Invitrogen), CD11c (Invitrogen), AMP-activated protein kinase a (AMPK, Santa Cruz), p-AMPK (Santa Cruz), and β-actin (Santa Cruz) and secondary antibodies conjugated with horseradish peroxidase (HRP). Conjugated proteins were visualized using an ECL detection kit.

qPCR analysis

mRNA (2 µg) was extracted from liver and colon tissues using the Qiagen RNeasy Mini Kit (Qiagen, Germany) and reverse-transcribed into cDNA using the PrimeScript cDNA Synthesis Kit (Takara, Japan)⁴⁹. PCR amplification was carried out on the Rotor-Gene Q 5plex Platform (Qiagen) utilizing the TB Green Premix Ex Taq II (Takara). Primer sequences used for amplification are listed in Supplement Table S13.

Histopathological examination

Livers were collected, and the median lobe was fixed in 4% paraformaldehyde. Histological assessment and scoring (NAS, fibrosis score, oil red staining, and Sirius red staining) were evaluated according to the method of Kroh et al.⁵⁰.

Immunofluorescence staining

Liver and colon tissues were harvested from mice, following transcardial perfusion with 4% paraformaldehyde on ice, sectioned at a thickness of 10 μm , incubated with primary antibodies (NF- κB , F4/80, $\alpha\text{-SMA}$, glial fibrillary acidic protein [GFAP], CD11c, and/or FXR) for 12 h, and treated with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) for 2 h⁴⁸. Subsequently, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Stained sections were then visualized using a confocal microscope and intensities were determined using ImageJ, as previously described⁴⁹.

Gut microbiota analysis

Bacterial genomic DNAs were extracted from the fresh stools of mice using a QIAamp DNA stool mini kit (Qiagen) and 16 S rRNA genes were sequenced⁵¹. Amplification of the genomic DNA was performed using barcoded primers, which targeted the V4 region of the bacterial 16 S rRNA gene. Sequencing for each amplicon was performed using Illumina iSeq 100 (San Diego, CA). Predictive functional genes were analyzed using the phylogenetic investigation of communities by reconstruction of unobserved states. Sequenced reads were deposited in the short read archive of NCBI under accession number PRJNA1230391.

Statistics

Data are expressed as mean \pm SD using GraphPad Prism 10 software. The normal distribution of the data was assessed using the Shapiro–Wilk test. Statistical significance was analyzed using one-way ANOVA with Dunnett's multiple comparisons test. The correlation between gut microbiota and biomarkers (network and heatmap analyses) were analyzed using Pearson correlation coefficient. The statistic data are indicated in Supplement Table S14.

Data availability

All data supporting our findings can be found in the main paper or supplementary files. Sequencing data are available via the NCBI's short read archive PRJNA1230391. All data will be shared on request.

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Author contributions

D.-Y.L., H.-M.J., S.-W.H., and D.-H.K. wrote the main manuscript text. D.-Y.L., H.-M.J., and S.H.K. prepared Figs. 1 and 2. D.-Y.L., H.-M.J., and J.-W.S. prepared Figs. 3, 4, 5, 6, 7 and 8. D.-Y.L. prepared Fig. 9. D.-Y.L. and Y.-J.S. prepared Fig. 10. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval and consent to participate

Animal experiments were approved by the Committee for the Care and Use of Laboratory Animals in the Kyung Hee University (IACUC No.: KHUASP(SE)-23087, KHUASP(SE)-23390) and conducted in accordance with the university's guidelines for the care and use of laboratory animals.

Additional information

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Correspondence and requests for materials should be addressed to D.-H.K.

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