**Bifidobacterium longum-Derived Extracellular Vesicles Prevent Hepatocellular Carcinoma by Modulating the TGF-β1/Smad Signaling in Mice**

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Abstract

**Background:** The involvement of gut microbiota in carcinogenesis has gradually been highlighted in past decades. Bacteria could play its role by the secretion of extracellular vesicles (EVs); however, interrelationship between bacterial EVs and hepatocellular carcinoma (HCC) development has not been investigated much. **Methods:** Diethylnitrosamine (DEN) was utilized to produce HCC model in mice, of which fecal was collected for detecting Bifidobacterium longum (B. longum) with real-time polymerase chain reaction (PCR). EV isolated from B. longum (B. longum-EV) with ultracentrifugation were stained with PKH26 to investigate the cellular uptake of murine hepatocytes (AML12). After treatment with B. longum-EV, TGF-β1-induced AML12 cells were subjected to morphological observation, fibrosis- and apoptosis-related marker detection with western blot, apoptotic ratio and reactive oxygen species (ROS) level analysis with flow cytometry, and oxidative stress biomarker assessment with enzyme-linked immunosorbent assay (ELISA); meanwhile, animal studies including liver function, tumor formation rate, and histological analysis, were also performed to investigate the role of B. longum-EV in the fibrosis, apoptosis, oxidative stress, and carcinogenesis of the liver in vivo. **Results:** The levels of B. longum were significantly reduced in HCC model mice. B. longum-EV could enter AML12 cells and effectively attenuate TGF-β1-induced fibrosis, apoptosis, and oxidative stress in AML12 cells. In vivo studies showed that B. longum-EV administration alleviated DEN-induced liver fibrosis, apoptosis, and oxidative stress at the early stage. Moreover, B. longum-EV administration also effectively reduced the tumor formation rate and liver function injury in DEN-induced mice and down-regulated TGF-β1 expression and Smad3 phosphorylation of mouse liver. **Conclusions:** B. longum-EVs protect hepatocytes against fibrosis, apoptosis, and oxidative damage, which exert a potential of preventing HCC development.

**Keywords:** hepatocellular carcinoma; extracellular vesicles; Bifidobacterium longum

1. Introduction

As the predominant type of liver cancer, hepatocellular carcinoma (HCC) is responsible for a significant number of cancer-related fatalities worldwide [1], which is typically correlated with chronic viral hepatitis infections including hepatitis B or C, alcoholism, as well as metabolic syndrome [2]. In the last decade, the incidence of HCC has been on the rise every year [3]; developing diagnostic and preventive strategies for HCC therefore has been an attractive area for researchers [4]. Understanding the pathogenesis of HCC is of significance to explore novel effective strategies for HCC patients [5]. Actually, in HCC, more than 80% of cases are attributed to non-alcoholic steatohepatitis (NASH) and cirrhosis [6], which is characterized by progressive hepatic fibrosis and irreversible damage to the liver. Numerous factors, such as genetic dysregulation, abnormal metabolism, as well as gut microbiota dysbiosis, have been implicated in the progression of NASH and cirrhosis to HCC [4].

The gut microbiota comprises a diverse community of microorganisms that inhabit the gastrointestinal tract, which has been implicated in various physiological processes, encompassing digestion, metabolism, and immune modulation [7,8]. Lately, emerging evidence has suggested that the gut microbiota also plays a crucial role in hepatocarcinogenesis [9,10]. The gut–liver axis is involved in inflammation, chronic injury, and fibrosis of the liver during hepatocarcinogenesis [11,12]. For example, it has been reported that altered gut bacteria may lead to the dysregulation of bile acid metabolism, thereby contributing to the occurrence of HCC [13]. Moreover, a culture-based study revealed that Escherichia coli levels in fecal samples of patients with cirrhosis and HCC were significantly higher than those without tumors [14].

Among the gut microbes, *Bifidobacterium longum* (B. longum), a naturally probiotic bacterium that resides in the human gut, is capable of promoting gut health by modulating the immune system and improving intestinal barrier...
function, which has been reported to suppress the progression of various types of cancer [15, 16]. More importantly, recent studies indicated the potential of *B. longum* as an anti-tumor choice in HCC development [9, 17], but its specific role in hepatocarcinogenesis has not been investigated much. Recent research has unveiled that gut microbes can communicate with host cells through the release of extracellular vesicles (EVs) [18]. EVs are greatly released by various cells in a constitutive or inducible manner. They can modulate many biological processes, which have evolved from being perceived as mere cellular waste disposal systems to a novel mechanism of intercellular communication since 1983 [19]. Increasing evidence supports that EVs play a crucial role in mediating the progression of liver disease and therefore can be considered a potential therapy for HCC [20]. A recent study reported that HEK293-derived exosomes loading miR-365a-3p may act as an effective therapeutic strategy against HCC [21]. Bacterial EVs comprise diverse bioactive molecules including proteins and nucleic acids, which have the capability to modulate cellular functions. Hence, we wonder whether EVs-derived from *B. longum* (*B. longum*-EV) may serve as important mediators of HCC progression. As known, the development of HCC is a complex process relating to the abnormal activation or inactivation of multiple signaling pathways. TGF-β1 signaling contributes to all stages of liver disease progression, from initial liver injury through inflammation and fibrosis, to cirrhosis and cancer [22]. TGF-β1 can promote liver differentiation during embryogenesis and physiological liver regeneration; however, chronic liver damage leads to high levels of TGF-β1, which causes the activation of stellate cells to myofibroblasts and massive hepatocyte cell death, thereby stimulating liver fibrosis and later cirrhosis [23]. The overactivation of TGF-β1 signaling had also been proven to facilitate later tumor progression during HCC [24]. Based on this, targeting the TGF-β1 signaling pathway is thought to be a promising target for counteracting liver disease progression. The present study, aimed to investigate the effects of *B. longum*-EV on the development of HCC and elucidate the underlying mechanisms, with a specific focus on the modulation of the TGF-β1 signaling pathway, which may shed light on the intricate interplay between Bacterial EVs and HCC.

2. Materials and Methods

2.1 HCC Model Establishment and Experimental Treatment

Specific pathogen-free (SPF) grade, male, C57BL/6 strain mice weighing about 22–25 g were obtained from the Laboratory Animal Center of China Medical University (Shenyang, China). All mice were housed in an SPF environment and had free access to water. All the experiments regarding animal were designed and conducted as per ethical norms approved by the Institutional Animal Ethics Committee of Jinzhou Medical University.

After two-week acclimation, to induce HCC, mice received intraperitoneal injection of diethylnitosamine (DEN; Sigma-Aldrich, St. Louis, MO, USA) for eight weeks (once a week; the dose of the first, second, and other weeks was 20, 30, and 50 mg/kg, respectively) and then left for a week rest. Next, mice received intraperitoneal injection of 300 mg/kg of thioacetamide (TAA; Sigma-Aldrich, St. Louis, MO, USA) twice a week for another eight weeks. Mice administrated with the same volume of vehicle served as the control. Finally, mice were maintained for a further six weeks before being sacrificed by cervical dislocation. Mice received a similar injection protocol with phosphate-buffered saline (PBS) were defined as the control.

2.2 Quantification of *B. longum* in the Fecal Samples

The feces of the mice were collected in a sterilized vial after finishing TAA induction (week 18) and on the day before killing the mice. The fecal counts of *B. longum* were determined by detecting the expression of its bacterial 16S rRNA genes with quantitative real-time polymerase chain reaction (qRT-PCR) (primer set: forward, 5′-GATTCTGGCTCAAGTGAACC-3′; reverse, 5′-CGGGTGGCTTCACCTTTAC-3′).

2.3 Isolation and Characterization of *B. longum*-EV

The *B. longum* strain (#15707) supplied by American Type Culture Collection (ATCC; Manassas, VA, USA) was grown in Bifido broth (HiMedia, Mumbai, India) supplemented with L-cysteine (0.05%) (HiMedia, Mumbai, India) and cultured under anaerobic conditions at 37 °C. After collecting and purifying *B. longum*-conditioned media through centrifugation and 0.22-µm membrane filter, the *B. longum*-EV was isolated through ultracentrifugation at 200,000 × g for 60 min. After finishing ultracentrifugation, the collected pellets were resuspended with phosphate-buffered saline (PBS) and filtered by a 0.22-µm syringe filter to finally obtain pure *B. longum*-EV. The protein concentration of *B. longum*-EVs fraction is determined by a Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology; Shanghai, China).

The morphology, number, and size distribution of *B. longum*-EV was performed using a transmission electron microscope (TEM, HITACHI, Tokyo, Japan) and nanoparticle tracking analysis (NTA) with Nano-ZS 90 dynamic light scattering. The remaining *B. longum*-EV were stored at −20 °C for further experiments.

2.4 Cell Culture and Treatment

Normal mouse hepatocyte cell line (AML12; #CRL-2254) was supplied by ATCC and grown in recommended media at 37 °C in a humidified incubator containing 5% CO₂. All cell lines were validated by STR profiling and tested negative for mycoplasma. AML12 cells were exposed to 2 ng/mL of TGF-β1 (R&D System Inc, Minneapolis, MN, USA) for 48 h to mimic “fibrogenesis”
and “hepatocarcinogenesis” of hepatocytes in vitro [25]. *B. longum*-EV was applied to treat AML12 cells for 48 h in the absence (control) or presence of TGF-β1. AML12 cells without any treatment were considered as the control. Forty-eight hours later, morphological examination on AML12 cells from each group was performed under a BX51 microscope (Olympus, Tokyo, Japan), and the representative images were taken using a digital camera at the same time.

2.5 Internalization of *B. longum*-EV

To track the internalization of *B. longum*-EVs by AML12 cells, *B. longum*-EVs were labeled with PKH26 using PKH26 Red Fluorescent kit (Sigma-Aldrich) while AML12 cells were labeled with DAPI. In brief, 200 µL of *B. longum*-EVs was resuspended with 400 µL of diluent C and mixed continuously with PKH26 solution (3 µL of dye dilute in 400 µL of diluent C) by gentle pipetting. After quenching by 2 mL 10% bovine serum albumin (cat. no. 0332; Amresco, LLC; Solon, OH, USA), the resuspension was subjected to ultracentrifugation at 4 °C. PKH26-labeled *B. longum*-EVs were incubated with AML12 cells at 37 °C for 2 h. After the internalization period, AML12 cells were washed twice and subsequently fixed with 150 µL of 4% paraformaldehyde for 20 min at room temperature. Cells were washed twice and subsequently fixed with 150 µL of 4% paraformaldehyde for 20 min at room temperature. After incubation with HRP-labeled secondary antibody (#A0208; Beyotime, Shanghai, China), and Smad3 (#31-155; ProSci Inc., CA, USA) p-Smad3 (#bs-19452R; Bioss, Beijing, China), and Smad3 (#31-155; ProSci Inc., CA, USA). Next, membranes were rinsed three times prior to incubation with HRP-labeled secondary antibody (#A0208; Beyotime, Shanghai, China). Finally, protein bands were visualized and quantified by using Enhanced Chemiluminescence Substrate Kit and Image J software (version 1.49, National Institute of Health; Bethesda, MD, USA), respectively.

2.7 Cell Viability Assay

Cell viability was assessed by the Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, after finishing treatment, 10 µL of CCK-8 solution was added to each well, and the plate was further incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 2 h. The absorbance of each well at 450 nm was measured using a microplate reader.

2.8 Cell Apoptosis Analysis

Cell apoptosis was evaluated using the Annexin V-fluorescein isothiocyanate or Annexin V-propidium iodide ( Annexin V-fluorescein isothiocyanate (FITC)/Annexin V-propidium iodide (PI)) apoptosis detection kit. Cells were harvested, treated with Annexin V-FITC or Annexin V-PI, and examined by flow cytometer. Cells exhibiting Annexin V (+)/PI (−) staining were considered as early apoptotic cells, while those showing Annexin V (+)/PI (+) staining were considered as late apoptotic cells.

2.9 Reactive Oxygen Species (ROS) Measurement

Intracellular ROS of AML12 cells was detected by fluorometric assay with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, 2′,7′-dichlorofluorescin diacetate fluorescent dye (Sigma-Aldrich) was added to cells that finishing the treatment at a concentration of 25 µM per well for 15 min in the absence of light. The production of ROS was analyzed by flow cytometer.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

Oxidative stress-related factor levels in cultured supernatant of AML12 cells were detected using ELISA kits specific for mouse glutathione (GSH) (#G0206W; Grace Biotechnology, Suzhou, China), superoxide dismutase (SOD) (#KGT00150-1; KeyGEN BioTECH, Nanjing, China), and malondialdehyde (MDA) (#KGT003-1; KeyGEN BioTECH) in accordance to the manufacturer’s instructions.

2.11 Animal Treatments and Processing

Fifty-four mice were randomly divided into four groups: the control (n = 12), *B. longum*-EV (n = 12), model (n = 12), and model+*B. longum*-EV (n = 18) groups. HCC model establishment was carried out as above-mentioned. After a four-week DEN injection, mice were intravenously injected with *B. longum*-EV (20 µg/mouse) [26] or saline once a week until the end of TAA induction.

At the end of the week of treatment (week 18), six mice from each group were randomly selected and sacrificed to collect the liver for ELISA, Sirius red staining, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and immunoblotting. The remaining mice were all weighed and sacrificed after maintaining for a further six weeks (week 24), of which the liver was
harvested to investigate tumor formation rate and weight. The collected liver was subjected to hematoxylin and eosin (H&E) staining and immunoblotting. Additionally, blood samples were also collected at week 10 and 24 to monitor the hepatic function of mice.

2.12 Histological and Sirius Red Staining Analysis

The liver tissues were fixed in a 4% paraformaldehyde or 4% polyvinyl alcohol solution in PBS and embedded in paraffin. Paraffin sections (4 μm) were subjected to H&E staining and Sirius red staining. For H&E staining, following dewaxing, slices were incubated with hematoxylin for a duration of 2 min and then stained with eosin. For Sirius red staining, the sections were subjected to a 5-min treatment with a 0.2% solution of phosphomolybdic acid and subsequently exposed to Picro-Sirius red for 90 min at room temperature. Following this, the sections were briefly rinsed with acidified water containing 0.01% hydrochloric acid, dehydrated, and eventually mounted.

2.13 TUNEL Staining

Serial sections of 4 μm thickness were stained with the TUNEL agent (Boehringer Mannheim, Mannheim, Germany), and investigated under the BX51 microscope (Olympus, Tokyo, Japan).

2.14 Biochemical Analysis

Blood samples were kept for 30 min at room temperature prior to centrifugation at 3000 rpm for 10 min at 4°C for harvesting serum. The levels of alanine transaminase (ALT; #MBS164085; MyBioSource) and aspartate aminotransferase (AST; #ELK1921; ELK Biotechnology, Wuhan, China) were determined as per the standard protocol.

2.15 Statistical Analysis

Unpaired Student’s t test was used when the numerical variables between two groups were compared. A two-way ANOVA test was utilized to analyze the difference among more than two groups. All statistical analyses in this study were conducted using GraphPad Prism software (version 8.2, GraphPad Software, Inc., San Diego, CA, USA). Data were considered significant when p values were less than 0.05.

3. Results

3.1 B. longum-EVs Ameliorate TGF-β1-Induced Fibrosis of Liver Cells

Initially, a DEN-induced in situ liver cancer model was established to observe the changes in the B. longum during the occurrence and development of HCC (Supplementary Fig. 1A). The gross morphology and H&E staining of the liver collected at week 24 supported a successful establishment of the HCC model (Supplementary Fig. 1B,C). At weeks 18 and 24, fecal samples from mice were harvested to detect the quantity of B. longum, which showed there was a significant decrease in the HCC mice (Supplementary Fig. 1D). These findings suggest that the B. longum may play a critical role in the occurrence and development of HCC.

Since bacterial EVs comprise diverse bioactive molecules, which have the capability to modulate cellular functions, B. longum-EVs were isolated to determine whether B. longum regulates the occurrence and development of HCC through the EVs. Both the results of TEM and NTA confirmed our successful isolation of B. longum-EVs (Fig. 1A,B). Moreover, fluorescence microscopy images demonstrated the presence of internalized B. longum-EVs (PKH26 stained red) within the AML12 cells (Fig. 1C).

Upon treatment with TGF-β1, a crucial cytokine involved in liver fibrosis, AML12 cells exhibited notable morphological changes (Fig. 1D). As observed under the microscope, the cells elongated and acquired an interstitial cell-like morphology (Fig. 1D). However, the administration of B. longum-EV partially counteracted these TGF-β1-induced effects (Fig. 1D). Meanwhile, B. longum-EV treatment resulted in a significant decrease in the expression of both collagen I and α-SMA when compared to the TGF-β1-treated group (Fig. 1E). Overall, the morphological changes observed in the cells, along with reduced expression of fibrosis-associated markers, suggested that B. longum-EV have the potential of alleviating liver fibrosis.

3.2 B. longum-EVs Mitigate TGF-β1-Induced Apoptosis and Oxidative Stress in Liver Cells

Except for fibrosis, cellular apoptosis and oxidative stress of liver cells also contribute to the malignant transformation of hepatocytes. A significant increase in apoptosis levels of AML12 cells was observed by flow cytometry following treatment with TGF-β1 (Fig. 2A), as confirmed by the detection of apoptosis-related protein (a significant decrease in the expression of Bcl-2 and an increase in Bax levels in TGF-β1 induced AML12 cells) (Fig. 2B). Surprisingly, treatment with B. longum-EVs resulted in a substantial reduction in apoptotic levels, indicating the potential of these EVs to inhibit apoptosis of hepatocytes (Fig. 2A,B). Besides, cell viability analysis showed that B. longum-EV could also relieve the impairment of AML12 cell viability induced by TGF-β1 (Fig. 2C).

ELISA revealed that TGF-β1 treatment led to an elevation in the levels of oxidative stress markers, such as glutathione (GSH) and malondialdehyde (MDA), and a decrease in the levels of superoxide dismutase (SOD), indicating increased cellular oxidative stress (Fig. 2D–F). Conversely, treatment with B. longum-EV significantly attenuated the TGF-β1-induced oxidative stress, as evidenced by decreased MDA levels and increased GSH and SOD levels (Fig. 2D–F). In addition, treatment with TGF-β1 led to a significant increase in intracellular ROS levels, as detected by the fluorescent probe, DCFH-DA (Fig. 2C). In contrast, administration of B. longum-EV resulted in a significant decrease in intracellular ROS levels compared to the TGF-β1.
**Fig. 1.** *B. longum*-EVs alleviate TGF-β1-induced fibrosis of AML12 cells. (A) The morphology of the isolated *B. longum*-EVs was observed under TEM. Scale bar = 100 nm. (B) Size distribution of *B. longum*-EVs was analyzed by NTA. (C) Internalization of PKH26-stained *B. longum*-EVs in AML12 cells. Scale bar = 20 µm. (D) The morphology of AML12 cells after treating with TGF-β1 or/and *B. longum*-EVs, the cells without any treatment serve as the control. Scale bar = 100 µm. (E) The expression of fibrosis-related proteins (Collagen I and α-SMA) in AML12 cells were assessed by immunoblotting. ns means no significance and *** means $p < 0.001$ when vs. the control group, ## means $p < 0.01$ when vs. the TGF-β1 group. EVs, extracellular vesicles; TEM, transmission electron microscope; NTA, nanoparticle tracking analysis.

treated cells (Fig. 2G). These results highlight the potential antioxidant properties of *B. longum*-EV and their ability to counteract the detrimental effects of TGF-β-induced apoptosis and oxidative stress.

### 3.3 Protective Effects of *B. longum*-EVs on Liver Fibrosis and Apoptosis in a Mouse Model of HCC

To further investigate the protective effects of *B. longum*-EV on the liver in vivo, the HCC mouse model was established and treated with *B. longum*-EVs at weeks 6, 10, 14, and 18 post-modeling (Fig. 3A). Compared to the control group, the levels of GSH and SOD in the liver of the HCC mouse model significantly decreased, while MDA levels significantly increased. However, treatment with *B. longum*-EV significantly reversed these changes, restoring GSH and SOD levels and reducing MDA levels (Fig. 3B–D). Sirius red staining revealed that the liver of the HCC mouse models exhibited significant fibrosis, char-
Fig. 2. *B. longum*-EVs attenuate TGF-β1-induced apoptosis and oxidative stress of AML12 cells. AML12 cells were treated with TGF-β1 or/and *B. longum*-EVs, the cells without any treatment serve as the control. (A) The apoptotic rate of AML12 cells from each group was analyzed by cytometry after Annexin V/PI double staining. (B) The expression of apoptosis-related protein (Bcl-2 and Bax) in AML12 cells was assessed by immunoblotting. (C) The cell viability of AML12 cells was analyzed by CCK-8 kit. (D–F) The levels of GSH, SOD, and MDA in AML12 cells were analyzed by the corresponding ELISA kit. (G) Intracellular ROS levels of AML12 cells were analyzed by cytometry after being stained with DCFH-DA probe dye. *ns* means no significance, ** means *p* < 0.01 and *** means *p* < 0.001 when vs. the control group, # means *p* < 0.05, ## means *p* < 0.01, and ### means *p* < 0.001 when vs. the TGF-β1 group. CCK-8, Cell Counting Kit-8; GSH, specific for mouse glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; ELISA, enzyme-linked immunosorbent assay; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate.

characterized by increased collagen deposition. In contrast, the group treated with *B. longum*-EV showed pronounced improvement in liver fibrosis, with reduced collagen staining (Fig. 3E). Corresponding to the liver fibrosis observations, the protein levels of α-SMA and collagen I showed a similar pattern. In the HCC mouse model group, α-SMA and collagen I protein levels were markedly elevated. Notably, treatment with *B. longum*-EV significantly decreased
Fig. 3. *B. longum*-EVs suppress hepatic oxidation, fibrosis, and apoptosis during hepatocarcinogenesis. (A) Schematic representation of the timeline for the treatment of *B. longum*-EVs on diethylnitrosamine (DEN)-induced HCC model. Mice with or without DEN induction received treatment of *B. longum*-EVs or the same volume of PBS at weeks 6, 10, 14, and 18. Six mice randomly chosen from each group were sacrificed for collecting the liver tissues. (B–D) The hepatic levels of GSH, SOD, and MDA were analyzed by the corresponding ELISA kit. (E) Hepatic fibrosis was observed by Sirius red staining. Scale bar = 100 µm. (F) The expression of fibrosis-related proteins (Collagen I and α-SMA) in the liver tissues were assessed by immunoblotting. (G) Hepatic apoptosis was observed by TUNEL staining. Scale bar = 100 µm. (H) The expression of apoptosis-related protein (Bcl-2 and Bax) in the liver tissues was assessed by immunoblotting. ns means no significance and *** means $p < 0.001$ when vs. the control group, # means $p < 0.05$ and ### means $p < 0.001$ when vs. the Model group.
the expression of α-SMA and collagen I proteins compared to the HCC model group (Fig. 3F). TUNEL assay revealed an increase in the apoptosis of the liver tissue from HCC mouse models. In contrast, the treatment group showed a significant reduction in apoptosis levels (Fig. 3G). Further examination of apoptosis-related proteins Bcl-2 and Bax by immunoblotting confirmed the TUNEL assay results. The protein level of Bcl-2 was decreased, while Bax levels were increased in the liver of the HCC model group. However, treatment with B. longum-EVs restored the balance between Bcl-2 and Bax, with an increase in Bcl-2 levels and a decrease in Bax levels (Fig. 3H).

Overall, these results demonstrate that treatment with B. longum-EVs effectively improve liver fibrosis, restore the antioxidant status, and reduce liver injury. in the HCC mouse model, which suggested the potential therapeutic application of B. longum-EV in alleviating liver damage.

### 3.4 The Development of Liver Cancer is Suppressed by B. longum-Derived Extracellular Vesicles through Inhibiting the TGF-β Signaling Pathway

Furthermore, the intended role of B. longum-EV in hepatocarcinogenesis was also clarified. The liver function of each mouse was monitored by evaluating serum levels of ALT and AST at the 10th and 24th weeks. Compared to the control group, levels of ALT and AST in the model group exhibited a significant increase (Fig. 4A,B). However, these levels decreased considerably following the administration of B. longum-EV treatment, and there was no influence of B. longum-EV treatment in the control mouse (Fig. 4B). Meanwhile, the liver index exerted a similar tendency among the four groups (Fig. 4C). Hepatic carcinogenesis was noticeably evident in the model group, as revealed by higher tumor incidence rate and tumor number (Fig. 4D,E). As expected, the tumor incidence rate and tumor number of the model mice were impressively reduced when receiving treatment with B. longum-EVs (Fig. 4D,E). The H&E staining further confirmed these results, which showed B. longum-EV significantly alleviated the pathological changes in the liver of model mice (Fig. 4F). Mechanistically, the TGF-β1 signaling pathway was considerably activated in the model group, but was suppressed following treatment with B. longum-EVs (Fig. 4G). These findings strongly indicate that B. longum-EVs have a beneficial effect on the prevention of HCC development. The inhibition of the TGF-β1 signaling pathway may contribute to the observed therapeutic effects.

### 4. Discussion

Over the last two decades, gut microbiota has been proven to modulate various cellular pathways in the host and participate in promoting or preventing the tumorigenesis of different types of cancer, such as colorectal cancer [27] and HCC [28]. Many studies on animal models supported that the gut microbiota is involved in the pathogenesis of HCC [29]. A pioneering study reported that the number and size of tumors, as well as tumor cell proliferation and liver weight of DEN-treated rats, were reduced by antibiotic treatment [30]. In most studies, the effects of gut sterilization on HCC development are preemptive. Yamada et al. [31] observed an increased abundance of Bacteroides and Clostridium cluster XVIII and a reduction in Bifidobacterium, Prevotella, and Streptococcus in an experimental model of HCC associated with NASH. Notably, they found the whole body weight was increased and the occurrence of HCC was reduced in mice receiving antibiotic treatment. Since the key role of the microbiome in promoting the development of HCC in animal studies [32], understanding the role of gut microbiota concerning cancer is an aid in exploring novel strategies for both cancer prevention and treatment. For example, B. fragilis may be involved in the development of colitis-associated colorectal cancer by inducing T helper 17 cell-dependent pathway [33]. H. pylori-derived EVs can activate the NF-κB signaling in gastric cells by transferring virulence factors, thus promoting cell proliferation in gastric neoplasm [34]. More importantly, a previous study reported that intravenous administration of E. coli-derived EVs exert an anti-tumor function on B16BL6 metastatic melanoma, 4T1 metastatic breast cancer, as well as CT26 and MC38 colorectal cancer [35]. In our present study, it was observed that the fecal counts of B. longum in DEN-induced mice were significantly higher compared with the control. This makes us wonder whether B. longum plays an anti-tumoral role during the occurrence and development of HCC. Due to the core components and capabilities of bacterial EVs, we applied the EVs derived from B. longum to explore their effects on HCC in vitro and in vivo.

As known, most HCCs arise in the situation of chronic hepatitis (like NASH) and cirrhosis [36]. The AML12 cell line was an ideal in vitro model for studying hepatitis [37]. Several studies demonstrated that TGF-β1 can drive changes in hepatocytes during the development of liver disease [38]. At first, our study, based on AML12 cells, explored the effect of B. longum-EVs on initial liver injury, which included apoptosis and fibrosis of hepatocytes. Consistent with previous studies [39,40], our data from in vitro analysis showed that the fibrosis and apoptotic rate of AML12 cells were markedly increased after TGF-β1 stimulation. Surprisingly, B. longum-EVs treatment could significantly restrain these TGF-β1 induced changes in AML12 cells.

As the major organ attacked by ROS, the liver usually occurs an imbalance between oxidant and antioxidant agents that may cause liver injury [41]. Mounting evidence has suggested the contribution of oxidative stress during the progression of hepatic diseases [42]. The excessive ROS causes lipid peroxidation and is scavenged by antioxidant enzymes including SOD, catalase, as well as GSH [43]. Accordingly, it was found that oxidative damage in the liver is related to the elevation of MDA (lipid peroxidation indicator) level and the impairment of
Fig. 4. *B. longum*-EVs inhibit the development of HCC via the TGF-β1 signaling pathway. (A,B) Serum levels of ALT and AST of mice at weeks 10 and 24. (C) Liver coefficient = liver weight/body weight. (D) Tumor incidence. (E) The mean number of tumors from each mouse. (F) Pathological changes of the liver were observed by H&E staining (top: 100× magnification, bottom: 400× magnification). Scale bar = 100 µm. (G) The expression of TGF-β1 signaling-related proteins (TGF-β1, Smad3, and p-Smad3) in the liver tissues was assessed by immunoblotting. ns means no significance, * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$. 
the antioxidant system. Similarly, our research observed the oxidation of AML12 cells with TGF-β1 stimulation, as evidenced by the elevation of MDA and the decrease of SOD and GSH. As the existence of oxidative stress in the liver was commonly observed in numerous hepatic disorders, anti-oxidative treatment has emerged as a potential choice to alleviate liver injury and prevent the disease from worsening [41]. *B. longum* has been proven to ameliorate alcoholic liver disease by enhancing hepatic antioxidant capacity [44]. Here, *B. longum*-EVs treatment could relieve the TGF-β1-induced oxidative stress in AML12 cells, suggesting that *B. longum*-EVs functioned as an exogenous antioxidant during hepatocyte injury. These pieces of *in vitro* evidence collectively indicated the protective role of *B. longum*-EVs for hepatocytes in the progression of liver diseases.

Then, we further verified the *in vitro* results based on the DEN-induced autochthonous HCC model mice. Our data revealed that exogenous supplementation of *B. longum*-EVs was well tolerated and non-toxic in the control mice, as reflected by the no significant difference between the control mice with and without *B. longum*-EVs injection throughout the experiments. Consistent with *in vitro* results, the administration of *B. longum*-EVs significantly reduced the hepatic apoptosis as well as fibrosis of DEN-induced mice, and this was accompanied by a significant reduction of hepatic ROS levels. Moreover, the DEN-impaired liver function of mice was also observed to be alleviated after *B. longum*-EVs administration. The carcinogenesis effect of DEN is attributed to the long-term ROS generation that damages DNA [45]. We next analyzed the role of *B. longum*-EVs in preventing hepatocarcinogenesis. As expected, the incidence rate of HCC and the number of tumors in DEN-induced mice with no treatment was obviously higher than those with *B. longum*-EVs. Even though antiviral treatment has become one of the most effective strategies for preventing HCC, chemoprevention measures remain an area of significant need in the case of at-risk patients with nonviral etiologies of liver disease [46]. Several drugs, such as aspirin, metformin, and statins, have been shown to modulate risk factors and carcinogenic pathways in NAFLD/NASH-associated HCC [47]. However, these medications are not currently recommended for HCC chemoprevention since they have higher potential risks of toxicity and adverse events. Therefore, our findings are anticipated to provide scientific information for developing potential chemoprevention.

Finally, we attempted to preliminarily shed light on the mechanism by which *B. longum*-EVs can protect the liver from DEN-induced damage. TGF-β1 signaling participates in all stages of progression of liver disease, from initial liver injury to HCC development [22]. DEN stimulation increased the expression of TGF-β1 and phosphorylation of Smad3 in the TGF-β1 pathway, which is consistent with previous publications [48,49]. *B. longum*-EVs strongly inhibited DEN-stimulated activation of TGF-β1 signaling. In the last few decades, multiple studies uncovered the mechanism underlying the protection of *B. longum* in liver injury. For example, Dong *et al.* [44] revealed that *B. longum* exerts its role in alcoholic liver disease via the modulation of the gut microbiota. A recent study demonstrated that *B. longum* could improve liver injury and fibrosis by regulating NF-κB and AMPK signaling pathways. Hence, the role of *B. longum*-EV in preventing HCC might also be attributed to the regulation of the gut-liver axis or other pathways. Taken together, current *in vivo* and *in vitro* evidence supported that *B. longum*-EVs alleviate the development of hepatic fibrosis and prevent HCC occurrence, and these modulatory effects are mediated, at least in part, through regulation of oxidative stress and suppression of TGF-β1 signaling. Our findings provided scientific insights regarding the implication of *B. longum*-EVs as adjuvants for HCC treatment in the future.

However, there are some limitations in the current study. First, the present study only applied a chemical carcinogen DEN-induced HCC model, the effect of *B. longum*-EVs should be further investigated in mice with HCC triggered by hepatitis viruses or oncogenes. Besides, further studies need to identify the specific molecular cargo contained within *B. longum*-EVs and to clarify a more detailed mechanism behind the role of *B. longum*-EVs in HCC.

### 5. Conclusions

In conclusion, data from our present study reveal that *B. longum*-EVs attenuate apoptosis, fibrosis, and oxidative stress stimulated by TGF-β1 in hepatocytes and prevent DEN-induced tumorigenesis in mice by relieving hepatic injury via the inactivation of TGF-β1 signaling. Our findings firstly discovered the protection of *B. longum*-EVs during hepatocarcinogenesis.

### Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Author Contributions

BL and ZL designed experiments. XCC, YH and WTW carried out experiments, analyzed experimental results. BL wrote the manuscript. ZL revised the manuscript. All authors approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

### Ethics Approval and Consent to Participate

All the experiments regarding animal were designed and conducted as per ethical norms approved by the Institutional Animal Ethics Committee of Jinzhou Medical University (No.240066).
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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2907241.

References
[28] Fox JG, Feng Y, Theve EJ, Raczynski AR, Fiala JLA, Doemte AL, et al. Gut microbes define liver cancer risk in mice exposed to...


[38] Fabregat I, Caballero-Díaz D. Transforming Growth Factor-β-Induced Cell Plasticity in Liver Fibrosis and Hepatocarcinogenesis. Frontiers in Oncology. 2018; 8: 357.


[44] Dong Y, Wu Z, Gai Z, Han M. Bifidobacterium longum subsp. longum BL21 ameliorates alcoholic liver disease in mice through enhancement of the hepatic antioxidant capacity and modulation of the gut microbiota. Journal of Applied Microbiology. 2023; 134: e115251.


[48] Mahmoud AM, Mohammed HM, Khadrawy SM, Galaly SR. Hesperidin protects against chemically induced hepatocarcinogenesis via modulation of Nrf2/ARE/HO-1, PPARγ and TGF-β1/Smad3 signaling, and amelioration of oxidative stress and inflammation. Chemico-Biological Interactions. 2017; 277: 146–158.