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Lactobacillus bulgaricus ATCC 11842 Exopolysaccharides Suppress Hepatic Inflammatory Signaling and Tumorigenesis in a DEN-Induced Rat Model via TLR2/STAT3/P38-MAPK Inhibition

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ABSTRACT
Growing evidence supports probiotics as a beneficial, economical, and non-toxic strategy for the control and prevention of hepatocellular carcinoma (HCC). Exopolysaccharides synthesized by specific lactic acid bacteria demonstrate significant health-promoting effects, such as anticancer and immune-regulating properties.

This research focused on assessing the immune-regulating capabilities of exopolysaccharides (EPSs) obtained from Lactobacillus bulgaricus ATCC 11842 in male rats with hepatocellular carcinoma (HCC), experimentally induced by diethylnitrosamine (DEN) and gamma irradiation (IR), aiming to determine their preventive or therapeutic value. Elevations in hepatic markers including MDA, IL-17, TGF-β1, STAT3, and p38MAPK were observed in biochemical assessments, along with raised serum levels of ALT and γ-GT.
Moreover, a notable increase in the hepatic expression of the TLR2 gene was detected. EPS administration, either as a preventive or therapeutic approach, led to significant improvements in the majority of the assessed biochemical and molecular markers. The liver tissues' histological findings matched the biochemical results that had been recovered. In conclusion, exopolysaccharides derived from Lactobacillus bulgaricus ATCC 11842 effectively suppress hepatocellular carcinoma by modulating the pro-inflammatory TLR2/STAT3/P38-MAPK signaling pathway. Therefore, in individuals at risk, our novel EPSs from Lactobacillus bulgaricus ATCC 4356 may serve as a safe, effective, and promising probiotic candidate for the prevention of hepatocarcinogenesis.

Keywords: Exopolysaccharides (EPSs)**,** Hepatocellular Carcinoma (HCC)**,** Inflammatory Signaling Pathways**,** TLR2/STAT3/p38 MA PK Axis**,** Lactic Acid Bacteria

1. INTRODUCTION

Among adults, hepatocellular carcinoma (HCC) represents the most frequently occurring primary liver malignancy and is considered one of the deadliest types of solid tumors. Over the past two decades, its global incidence has been steadily rising and is projected to reach nearly one million new cases annually. Key etiological factors contributing to chronic liver inflammation and the subsequent progression to fibrosis, cirrhosis, Continuous alcohol misuse and recurring hep B & hep C virus infections are among the

reasons of hepatic cell carcinoma, ingestion of aflatoxin-contaminated food, and various metabolic syndromes (1,2) . Furthermore, tumor cells are known to secrete chemokines that modulate immune responses within the tumor microenvironment (3). The effectiveness of immune checkpoint blockade (ICB) therapy and the increase in intratumoral $CD8^+$ T-cell responses have been found to depend on IFNγ-inducible chemokines (4,5) . Therefore, promoting the transformation of tumor tissues into "hot" tumors—characterized by an inflammatory, An IFNγ-enriched tumor microenvironment is considered a potential strategy for improving the therapeutic outcomes of immune checkpoint blockade (ICB). One of the key factors that can either increase or decrease the effectiveness of ICB treatments is the host's gut flora (6–9) . It was suggested that metabolites produced by bacteria or elements of their cell walls, like short-chain fatty acids, were responsible for these effects (10), inosine(11), and peptidoglycan (12)(16According to reports, commensal bacteria altered the microenvironment around tumors. (13) additionally that IFNγ-producing CD8+ T cells generated by intestinal commensal bacteria enhanced antitumor responses in ICB treatments (14). In addition, it was recently shown that inosine-produced Bifidobacterium pseudolongum triggered Th1 cells and had anticancer effects when IFNγ or other co-stimulating agents were present (15) The precise mechanisms underlying the enhanced anticancer effects of immune checkpoint blockade (ICB), immune function of CD8 T lymphocytes linked to the gut in the tumor microenvironment, remain insufficiently understood. Live bacteria known as probiotics can improve health when taken in the right amounts (16) , believed to have an impact on the biological responses and functioning of the host. Probiotics have a number of useful compounds and mechanisms that have been documented before (17,18), Additionally, metabolites from probiotics are appealing agents to increase the effectiveness of immune treatments, especially against cancer(19,20). Microorganisms release sugar residues into the environment as high-molecular-weight polymers called exopolysaccharides (EPS). In our investigation into the biological roles of Lactobacillus-produced EPS, we discovered that these EPS caused splenocytes to release IFNγ (21). It has been demonstrated, for instance, exopolysaccharide-containing fermented skim milk (EPS-R1) is produced by Lactobacillus delbrueckii subsp. bulgaricus OLL1073R-1 (L. bulgaricus R1), which significantly increases the production of IFNγ (24). Our investigation into the effects of oral EPS-R1 administration demonstrated that Peyer's patches activated $CCR6^+$ $CD8^+$ T cells, which subsequently migrated into tumor tissues expressing CCL20, as a result, it enhances the therapeutic impact of ICB treatment in experimental models of tumors in rats. Common cancer treatments include radiation and chemotherapy. Unfortunately, the adverse side effects associated with radiation and chemotherapy significantly hinder their clinical applicability and greatly diminish the quality of life in cancer patients. A wide range of natural products, including herbal extracts, purified compounds, and traditional formulations, have shown efficacy in cancer prevention and therapy. Importantly, their use has also been linked to a reduction in treatment-associated toxicities—such as damage to the oral mucosa, gastrointestinal tract, liver, kidneys, heart, nervous system, and hematological functions—commonly observed with chemotherapy and radiotherapy (22). It was hypothesized that probiotics would consistently help treat HCC (23). The production of exopolysaccharides (EPSs) by lactic acid bacteria (LAB), prominent probiotic organisms, involves their initial assembly at the bacterial cell surface followed by secretion into the extracellular medium (24). Beyond enhancing immune function and providing antioxidant effects, specific EPSs isolated from LAB have demonstrated significant potential as anticancer agents, contributing to a broad range of health-promoting activities. These qualities have drawn special attention from numerous research teams(25) . For example, Seo et al.(26) EPSs from Lactobacillus plantarum that were isolated and purified and have strong antioxidant properties. It has previously been demonstrated that L. acidophilus EPSs have immunomodulatory and anticancer properties against colon cancer. The EPSs from L. acidophilus stimulated the immune system and directly killed the tumor's cells through apoptotic processes (24). Th17/IL-17+ cells may be crucial to the angiogenesis and progression of HCC because it is a highly vascularized tumor. Factors that promote inflammation and angiogenesis, including IL-17, may therefore be helpful in regulating the formation of HCC (27). Hepatocellular carcinoma (HCC) arises and advances due to the combined influence of multiple interacting factors (27). Transforming growth factor-β1 (TGF-β1), produced by hepatic stellate cells following liver damage, is a major contributor to liver fibrosis and the progression of

hepatocarcinogenesis (28) . Furthermore, HCC cells and tissues demonstrate constitutive activation of STAT3, and the use of STAT3 decoy oligodeoxynucleotides has been shown to selectively inhibit STAT3 signaling, thereby suppressing tumor cell proliferation and promoting apoptosis (29). A new investigation into the immune-modulating impact of probiotics on extra-intestinal cancers, particularly hepatocellular carcinoma (HCC), has been published in the Proceedings of the National Academy of Sciences. Although earlier studies have predominantly highlighted the role of probiotics in mitigating gastrointestinal inflammation and preventing colorectal cancer, the current research broadens their therapeutic scope to include liver cancers. Therefore, this study aimed to explore the immunomodulatory properties of exopolysaccharides (EPSs) extracted from Lactobacillus bulgaricus ATCC 11842 in a male rat model of hepatocellular carcinoma (HCC), induced by diethylnitrosamine (DEN) and promoted through gamma irradiation (IR), assessing their efficacy as both a preventive and therapeutic strategy.

2 MATERIALS AND METHOD

2.1 Animals

This study employed thirty mature male Swiss albino rats that weighed 180–220 g. The animals were procured from the Departmental Animal Facility, TMCOP. Housing conditions included standard plastic cages in a well-aerated room, with a controlled environment of 12-hour light/dark cycles, temperature at 27 ± 2 °C, and $60 \pm 5\%$ humidity. Throughout the study, the rats had free access to clean water and a standard pellet diet. All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985), maintaining high ethical standards.

2.2 Induction of Hepatocellular Carcinoma

The source of diethylnitrosamine (DEN) was BioRad in Gujarat, India. A fresh solution of DEN was prepared daily by dissolving it in 0.9% sodium chloride (NaCl). To induce hepatocellular carcinoma (HCC), DEN was administered orally to the rats at a daily dosage of 20 mg per kg of body weight (30) eight weeks of constitutive time.

2.3 Extraction Procedure for EPS Derived from Lactobacillus bulgaricus ATCC 11842

The EPSs derived from Lactobacillus bulgaricus ATCC 11842 were obtained from the Microbiological Resources Center, Cairo, Egypt, they were later cultivated in De Man, Rogosa, and Sharpe (MRS) broth or on MRS agar plates, follow guidelines provided in the ATCC product sheet. To inactivate endogenous enzymes that could degrade the polysaccharides, for fifteen minutes, the bacterial cultures were heated to 100°C. Following 30 minutes of incubation at 4°C, the cells were harvested by centrifugation at 12,785 × g. The pH 7.2 of the resultant supernatant of the culture was adjusted, the mixture was incubated at 4 °C overnight after adding three volumes of pre-cooled 95% ethanol to precipitate the exopolysaccharides. Centrifugation at 11,325 × g for 20 minutes was used to collect the precipitated EPS. Following a 24-hour dialysis session against deionized water at 4°C, the pellet was redissolved in the latter. To further purify the EPS, it was dissolved in 10% trichloroacetic acid to remove protein contaminants and lyophilized. The resulting solution was again dialyzed against deionized water at 4 °C for five days to ensure thorough removal of residual impurities (26).

2.4 Characterization of EPSs

FTIR spectrometry was employed to characterize the structural features of the extracted exopolysaccharides (EPSs). Prior research on colon cancer established the extracted EPSs' half-inhibitory concentration (IC 50) (31).

2.5 Radiation Exposure

A Canadian Gamma Cell-40 (137Cs) source was used to irradiate rats' entire bodies at Teerthanker Mahaveer Medical College & Research Centre (TMMCRC). In accordance with TMMCRC protocols, a total of 1 Gy of gamma radiation (γ-r) was administered

2.6 Experimental Protocol

The study used 30 male Swiss albino rats, which were randomly divided into five groups of six rats each (n = 6). The grouping and treatments were as follows:

Group 1 – Normal Control: The animals were fed a regular diet for eight weeks in a row and given 1 mL of physiological saline orally every day.

Group 2 – DEN (Positive Control): Diethylnitrosamine (DEN) was diluted in 0.9% saline and given orally to rats at a dosage of 20 mg/kg body weight, via gastric gavage for eight consecutive weeks, following a modified protocol by Darwish and El-Boghdady (32). Additionally, the animals were exposed to whole-body gamma radiation (^137Cs, 1 Gy/week for three weeks, totaling 3 Gy) at a dose rate of 0.423 Gy/min, as described in prior studies (33).

Group 3 – EPSs Only: Rats received exopolysaccharides (EPSs) derived from Lactobacillus bulgaricus ATCC 11842 at a dose of 100 mg/kg body weight daily by oral gavage for eight weeks (31).
Group 4 EPSs + DEN (prevention): Similar to group 2, the animals received DEN (20 mg/kg b.w.) for eight weeks, along with 100 mg EPSs/kg b.w. administered daily by gastric gavage.
Group 5 DEN + EPSs(treatment): The animals were subjected to γ-radiation as in group 2 after receiving DEN (20 mg/kg b.w.) for eight consecutive weeks. For the next eight weeks, they were given EPSs (100 mg EPSs/kg b.w.) every day.
After the experiment was over, all of rats were euthanized & samples of their blood and liver were taken for histopathological, molecular, and biochemical analyses.

2.7 Biochemical Assays

2.7.1 Liver Enzymes

Colorimetric analysis was used to evaluate the alanine transaminase (ALT) activity in serum (34), utilizing a kit that was provided by the Biodiagnostic Company in Egypt. Using colorimetric analysis, the activity of total proteins in serum was assessed (35) . As directed by the manufacturer, a standard enzymatic test kit was used to colorimetrically assess the activity of gamma-glutamyl transferase (γ-GT) in serum samples (36).

2.7.2 Oxidant/Antioxidant Status

To measure the several enzymatic antioxidant characteristics, including CAT, liver homogenates were used(37) SOD(37) (GPx) (38) No enzymatic antioxidant parameters, such as glutathione (GSH), were assessed or included in the analysis (39,40), Vitamin C (41) and (G6PD) (42) by commercially available kits. As previously mentioned, the amount of TBARS in liver homogenate was measured(43) In contrast, a pink color was produced at 532 nm when one MDA molecule reacted with two thiobarbituric acid molecules.

Liver tissue levels of reduced glutathione (GSH) were measured according to the method outlined by Beutler et al.(44) Malondialdehyde (MDA) levels were measured to assess lipid peroxidation in liver tissue, following the method established by Yoshioka et al.(45).

2.7.3 Inflammatory Biomarkers

Levels of interleukin-17 (IL-17), interleukin-10 (IL-10), and transforming growth factor beta-1 (TGF-β1) in liver tissue were quantified using ELISA kits supplied by Mybiosource (San Diego, CA, USA). Every assay was conducted using the sandwich-ELISA principle. Liver tissues were first homogenized in lysis buffer to extract the proteins, and the resulting homogenates were then used for cytokine analysis. The sandwich-ELISA technique involved the specific binding of target cytokines to capture antibodies pre-coated on the wells, followed by detection using enzyme-linked secondary antibodies, allowing for the quantitative measurement of IL-17, IL-10, and TGF-β1 levels in the liver samples.

2.7.4 Expression Profiling of p-STAT3 and p-p38 MAPK via Western Blot

Liver proteins such as p-STAT3 and p-p38 MAPK were extracted using TRIzol reagent (Invitrogen), based on the method described by Chomczynski (46) . Levels of p-STAT3 and p-p38 MAPK proteins were measured through the Bradford protein assay (47). A total of 20 µg of protein per sample was loaded onto a 10% SDS-PAGE gel, followed by transfer onto PVDF membranes. To block non-specific binding, membranes were incubated for two hours at room temperature in a 5% non-fat dried milk solution made with TBS-T buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20). Following the blocking step, membranes were incubated with rabbit primary antibodies targeting phosphorylated STAT3 (p-STAT3), phosphorylated p38 MAPK (p-p38 MAPK), and β-actin as a loading control.

Following primary antibody incubation, the membranes were washed three times with TBS-T buffer and then exposed to HRP-linked goat anti-rabbit IgG secondary antibodies for two hours at room temperature. Subsequently, four additional washes with TBS-T were performed to eliminate any unbound secondary antibodies.

According to the manufacturer's protocol, protein bands were visualized using the Amersham chemiluminescence detection kit and detected by exposing the membranes to X-ray film for signal development. A laser scanning densitometer was employed to quantify the expression of p-STAT3 and p-p38 MAPK proteins.

2.8 Evaluation of Toll-like Receptor-2 Expression in Hepatic Tissue

2.8.1 Isolation of RNA and Synthesis of cDNA from Liver Tissue

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen), following Chomczynski's protocol, to assess the mRNA expression of the Toll-like receptor-2 (TLR-2) gene (46). According to the manufacturer's instructions, 1 µg of total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using Invitrogen reverse transcriptase.

2.8.2 Quantitative Real-Time PCR (qPCR) Analysis

Real-time PCR was performed using the Step One Plus thermal cycler (Applied Biosystems, USA), with Sequence Detection Software (PE Biosystems, CA) employed for data acquisition and analysis. The specific oligonucleotide primers used in the study are listed in Table 1. Each 25 µL reaction mixture contained 2 µL of synthesized cDNA, 900 nM of each forward and reverse primer, SYBR Green PCR Master Mix. 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds comprised the amplification technique, which started with an initial denaturation at 95°C for 5 minutes. The comparative Ct (ΔΔCt) approach, as outlined by Pfaffl, was used to determine the relative expression of TLR-2 mRNA. (48) .

2.9 Histopathological Examination

Rat liver tissues from each group were taken at sacrifice and preserved for 48 hours in 10% formalin. Following fixation and dehydration through a graded series of alcohols, after being cleaned with xylene,

the tissue samples were embedded in paraffin wax at 60°C. Using a sliding microtome, a section with a thickness of five microns was cut. Tissue sections mounted on glass slides were stained with Hematoxylin and Eosin (H&E) for histopathological examination (49).

2.10 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical comparisons among groups were conducted using one-way ANOVA, followed by the Least Significant Difference (LSD) post hoc test to assess significance. Statistical significance was considered at $p < 0.05$. All analyses were performed using SPSS software, version 13.0 for Windows.

3. RESULTS

3.1 Characterization of Exopolysaccharides (EPSs)

The FTIR analysis of exopolysaccharides (EPSs) derived from Lactobacillus bulgaricus displayed multiple bonds stretching vibrations at distinct peaks, as illustrated in Figure 1. The spectra exhibited both prominent and subtle absorption bands. A strong absorption at 3280.17 cm^{-1} corresponded to hydroxyl (–OH) group vibrations, while a distinct peak at 1636.94 cm^{-1} indicated the presence of carboxylate (COO^-) groups. Additionally, a pronounced peak at 608.81 cm^{-1} suggested the existence of C–H and C–N bonds.

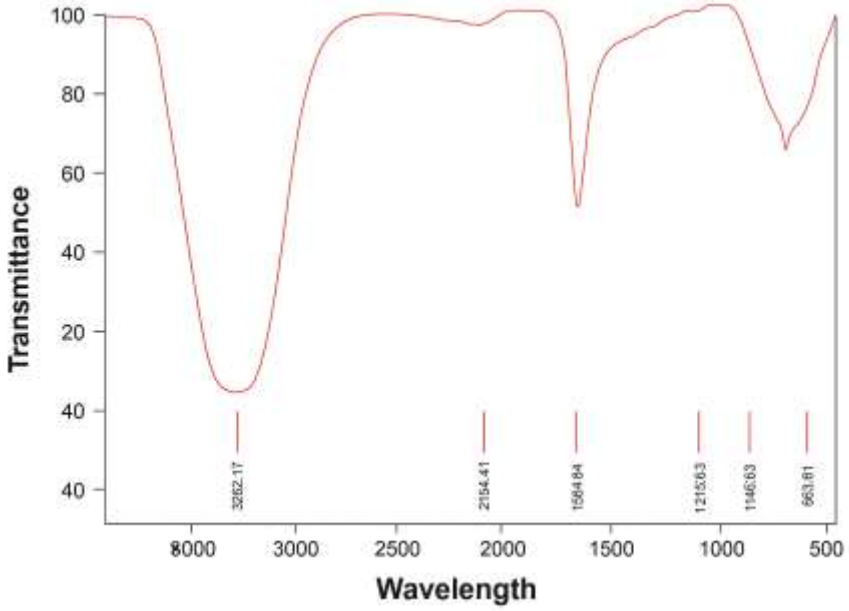


Figure 1 FTIR spectrum of EPSs

3.2 Impact of EPSs on Liver Injury and Inflammatory Markers in DEN-Treated Rats

DEN-treated rats exhibited a marked decrease in antioxidant biomarkers, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), reduced glutathione (GSH), glucose-6-phosphate dehydrogenase (G6PD), and vitamin C. Treatment with EPSs significantly restored these parameters in a dose-dependent manner. In comparison to the normal control group, DEN exposure caused a substantial decline in hepatic GSH and IL-10 levels, as well as in total serum protein. Conversely, there was a pronounced increase in serum levels of alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), malondialdehyde (MDA), and the pro-inflammatory cytokines IL-17 and TGF-β1, as illustrated in , Figure 2 and Figure 3. Administred of exopolysaccharides, whether used therapeutically or prophylactically, resulted in significant improvements in these biochemical and inflammatory parameters relative to the DEN group. Notably, prophylactic treatment with EPSs was more effective in alleviating liver toxicity and correcting the disturbed markers.

Table 1 Primer sequence for TLR-2 gene (50).

Gene	Primer	Sequence (5′→3′)	Tm (°C)	Amplicon Size (bp)	Accession Number
TLR-2	Forward	CTTCCTGTGAACTCCTGTCCTT	60.2	144	XM_008761104.3
	Reverse	AGCCTGTCTGGCCAGTCAAC	61.5		

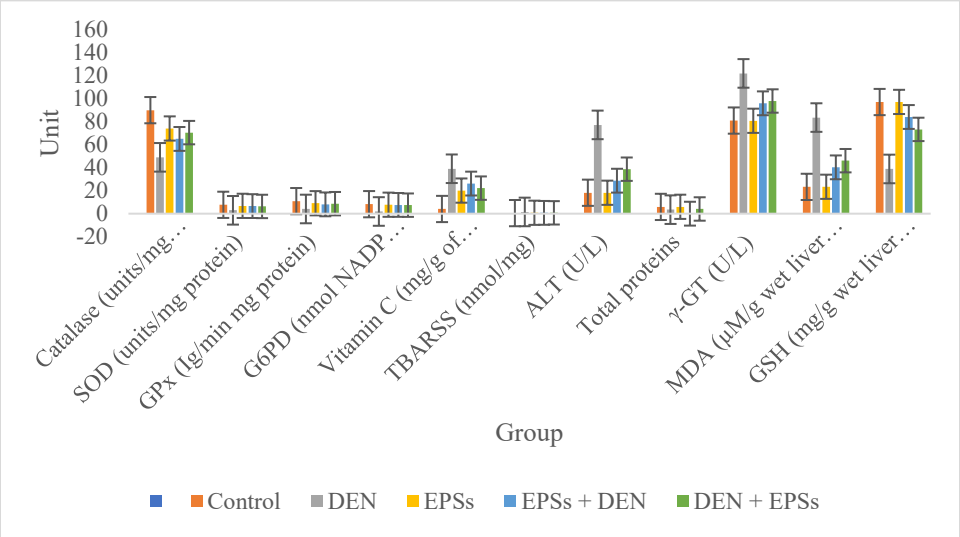


Figure 2 Impact of EPSs on Liver Injury and Inflammatory Markers in DEN-Treated Rats

DEN: Diethylnitrosamine; EPSs: Exopolysaccharides.
Each value is expressed as the mean ± standard error (SE) based on eight observations ($P \leq 0.01$).
a Indicates significant difference compared to the control (C) group.
b Indicates significant difference compared to the DEN-treated group.
c Indicates significant difference compared to the EPSs-treated group.

3.5 Alteration of TLR-2 Gene Expression by EPSs in DEN-Induced

The TLR-2 gene's expression level was assessed through the use of RT-PCR. "TLR-2 transcript levels were significantly elevated in the DEN-treated group compared to the control group, indicating an upregulation of inflammatory signaling pathways associated with hepatocarcinogenesis. Notably, administrated of EPSs either prevented or markedly suppressed the DEN-induced increase in TLR-2 gene expression (Figure 3).

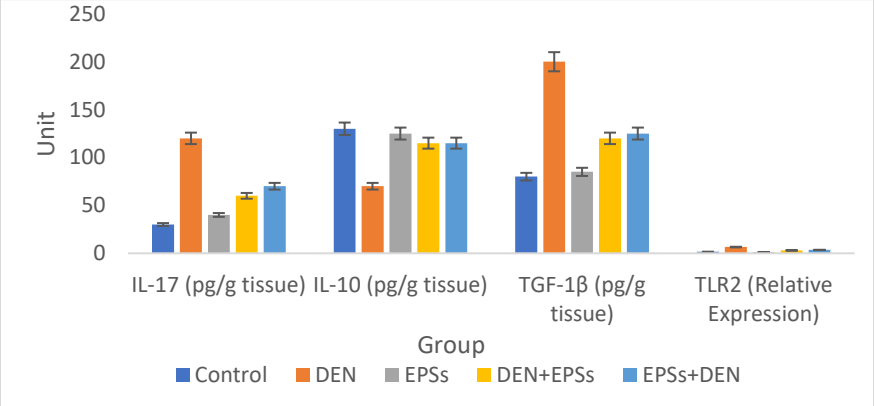
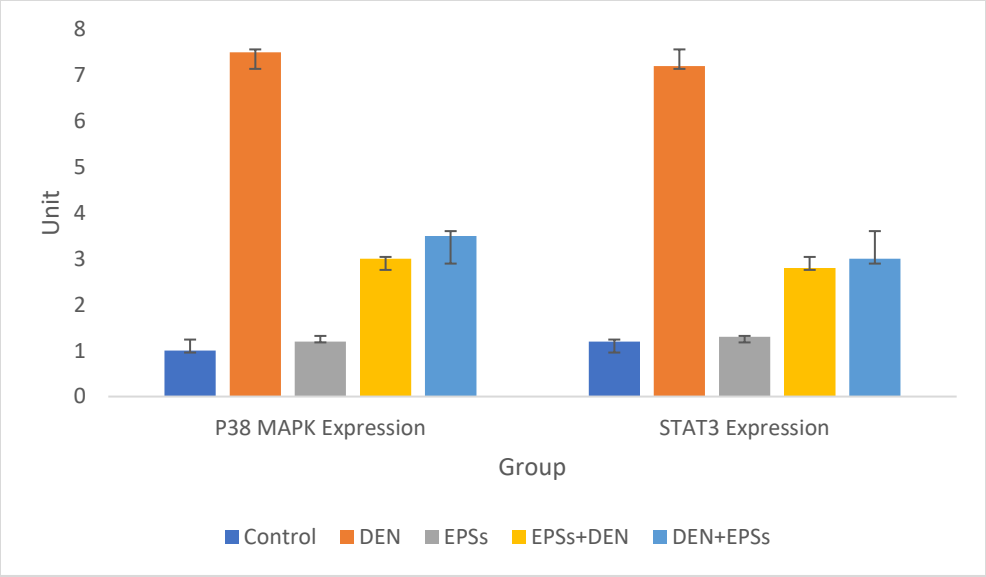
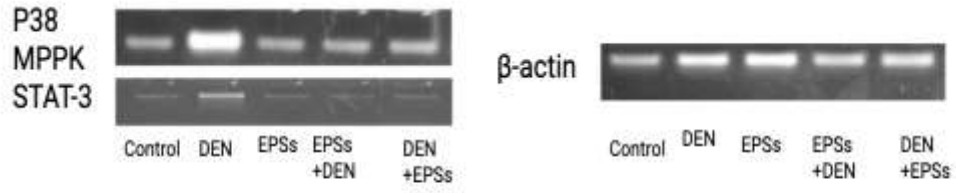


Figure 3 Average amounts of inflammatory biomarkers in liver tissue from various experimental groups (IL-17, IL-10, TGF-β1, and TLR-2). Each bar represents the mean ± standard error (SE) for animals in each group. Statistical significance is indicated as follows:
a $P < 0.05$ vs. Control group;
b $P < 0.05$ vs. DEN group;
c $P < 0.05$ vs EPSs group

3.6 Impact of EPSs on the Activation of STAT3 and p38 MAPK Signaling pathway

DEN administration led to a marked increase in the expression of signaling molecules p38 MAPK and STAT3. While both therapeutic and preventive administration of EPSs significantly reduced these elevated levels compared to the DEN group, the preventive approach proved to be more effective. As shown in Figure 4, these results strongly suggest that DEN-induced hepatocarcinogenesis is closely associated with the activated of the STAT3 & p38 MAPK signal route.





a: Significant vs. Control group
b: Significant vs. DEN group
c: Significant vs. EPSs group

Figure 4 Western blot analysis of signaling proteins p38 MAPK and STAT3 in liver tissues of experimental rat groups. Protein expression levels were assessed to evaluate the impact of treatments on DEN-induced hepatocarcinogenesis. β-actin was used as the loading control.

4. HISTOPATHOLOGICAL FINDINGS

Rats treated with EPSs Figure 5 (C)) and control (A)) showed a typical hepatic architecture, including normal vascular and parenchymal architecture, as well as normal stroma devoid of degenerative alterations and inflammatory infiltration or the portal tracts' fibrosis. Liver tissues from rats treated with DEN exhibited marked cellular and nuclear pleomorphism, coarse chromatin, nuclear inclusions, accompanied by infiltration of inflammatory cells in the portal region (Figure 5 B). In contrast, liver sections from rats that received EPSs as a preventive measure following DEN exposure (Figure 5 D) Demonstrated significant vascular dilation and congestion, with extravasation of erythrocytes and accumulation of hemosiderin. Additionally, a moderate inflammatory response was observed, along with focal areas of tumor tissue degeneration accompanied by dense inflammatory cell infiltration. When EPSs-treated rats were intoxicated with DEN, the liver sections that were analyzed revealed tumor tissue with apoptotic bodies and noticeable degenerative alterations (Figure 5 (E)).

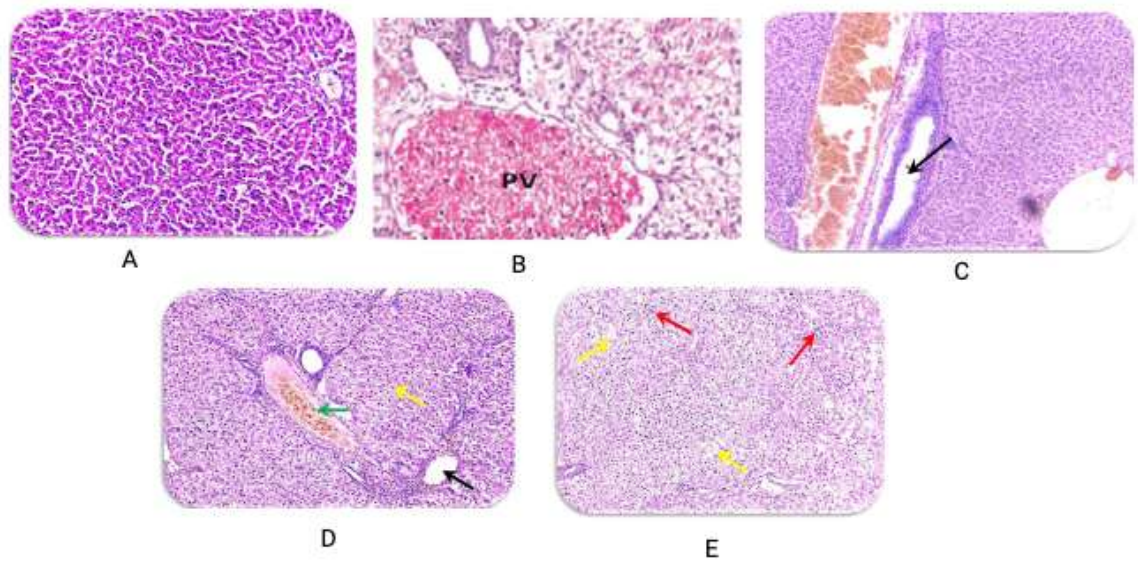


Figure 5 .Liver specimens photographed under a hematoxylin and eosin stain. Control animals' normal hepatic architecture (A). (B) Liver samples treated with DEN. EPSs treated rats (C). D) The liver slices from EPSs that were analyzed refrained rats. The liver sections of rats treated with EPSs were analyzed (E).

5. DISCUSSION

Probiotics have been shown in earlier research to be a natural anticancer drug that prevents extra-intestinal cancers, particularly in HCC (26). EPSs' anti-inflammatory properties have been documented in numerous studies in the past (51,52). Accordingly, the present research was conducted to investigate how lactic acid bacteria-derived exopolysaccharides (EPSs) modulate immune responses associated with hepatocellular carcinoma (HCC).

The physicochemical properties of the isolated EPSs were analyzed using FTIR spectroscopy, which confirmed the presence of hydroxyl and carboxyl functional groups. The results given above coincide with Palaniyandi et al.'s earlier FTIR (53).They revealed that the carboxyl and hydroxyl

groups present in the EPSs' FTIR spectra are crucial for the flocculation process and for binding divalent cations. Renowned for altering the enzymes needed for DNA repair and replication, DEN is a strong hepatocarcinogenic nitrosamine that is frequently used as a carcinogen to induce liver carcinogenesis (54) .In the current investigation, an abnormal rise in serum ALT and γ-GT levels in addition

Rats intoxicated with DEN showed lower serum total proteins, but EPS therapy improved serum ALT and γ-GT activity in addition to serum total proteins. These results aligned with earlier research (55,56). Based on these findings, hepatocellular injury in the DEN-treated group resulted in elevated serum ALT and γGT activities, indicating impaired liver function. Additionally, the formation of hepatic lesions appeared to disrupt protein synthesis, leading to a reduction in total serum protein levels (55) . Although EPSs administration enhanced liver function as indicated by blood total proteins, long-term probiotic therapy slowed the development of fibrosis in rats. Treatment with EPSs of certain lactobacilli also significantly reduced serum ALT and GGT activities, guarding against liver disease and low-grade inflammation (57,58) . In contrast, investigations showed that DEN raised the level of lipid peroxidation when compared to the normal control group. The development of hepatocarcinogenesis is known to be significantly influenced by oxidative stress. Initiating and promoting hepatic carcinogenesis might be significantly aided by reactive oxygen species (ROS). Antioxidants guard against carcinogens, toxins, and ROS (59). DEN-intoxicated rats in our investigation showed an abnormal rise in MDA levels and a decrease in glutathione content, whereas EPS therapy improved both MDA levels and glutathione content. According to these findings, DEN increases oxidative damage by raising hepatic MDA and decreasing GSH antioxidant indicators(60). According to earlier reports, EPSs' strong antioxidant activity may be the reason for their ability to reduce oxidative stress and liver toxicity (58). Probiotics have been shown to increase glutathione biosynthesis, which lowers oxidative stress and, consequently, inflammation, while probiotic bacteria that produce EPSs have been shown to dramatically reduce oxidative stress (61) . Additionally, supplementing with EPSs may suppress excessive ROS generation and lower MDA levels. Through the reduction of oxidative stress, the restoration of GSH levels improves liver damage. As previously mentioned, EPSs' antioxidative properties are essential to their hepatoprotective effects (6 2) . The progression of hepatocellular carcinoma (HCC) is largely fueled by the dysregulated activation of several key molecules within signaling pathways that control cellular processes such as proliferation, differentiation, survival, apoptosis, and cell cycle regulation. Persistent activation of STAT3 has been closely associated with more aggressive tumor phenotypes, while the MAPK signaling cascade is recognized as a pivotal contributor to HCC development. It is well documented that a range of cytokines and growth factors can initiate the activation of the STAT signaling pathway.(63). In this study, DEN administration resulted in a marked elevation of hepatic IL-17, TGF-β1, STAT3, and p38 MAPK levels. Additionally, there was a significant upregulation of TLR-2 gene expression in the liver tissue. Conversely, IL-10 levels in the liver were markedly reduced compared to the normal control group. Crucially, IL-17 might have encouraged the growth of tumor angiogenesis and HCC cell induction. Many inflammatory and tumor cells use different pathways, including the p38 MAPK and STAT-3 pathways, for IL-17 to mediate signaling (60). It was observed that IL-17 could cause the hepatic tissue of HCC patients to undergo an epithelial-mesenchymal transition. It is crucial to stress that practically all liver resident cells expressed more proinflammatory factors including TGF-β1 after being exposed to IL-17 in the hepatic tissue(64). Given that TLR signaling has been linked to malignancies associated with inflammation. According to the increase of TLR signaling in HCC, this signaling is crucial for the prognosis of inflammatory and chronic illnesses that eventually lead to HCC (65). The current study demonstrated the immunomodulatory potential of EPSs supplementation through a significant reduction in liver tissue levels of IL-17, TGF-β1, STAT3, and p38 MAPK, along with a downregulation of TLR-2 gene expression. In contrast to the untreated group, the liver tissue's IL-10 content significantly increased. A prior study shown how probiotic bacteria significantly inhibit the growth of HCC in mice by interacting with Th17 cells (66). Since Th17 cells are considered the principal source of IL-17 in the tumor microenvironment—and IL-17 is

known to promote hepatocellular carcinoma (HCC) progression and angiogenesis—its reduction in tumor tissue may be attributed to diminished immune cell trafficking or recruitment from the gut. Therefore, probiotics' anti-tumor effect was probably linked to gut microbiota regulation via promoting the release of the anti-inflammatory cytokine IL-10 and inhibiting the development of Th17 cells in the gut. Reduced intestinal Th17 cell recruitment and the IL-17 they released impaired liver tumor angiogenesis, which in turn inhibited tumor growth(67). A study conducted in 2018 reported that Lactobacillus species were capable of enhancing IL-10 production while simultaneously suppressing STAT3 activation, a pathway closely linked to the reduction of Th17 cells and IL-17 expression (68). "TGF-β1 levels in liver tissue were significantly reduced following EPS treatment, suggesting a potential anti-inflammatory and anti-fibrotic effect exerted by the exopolysaccharides, suggesting that they have a protective impact against the spread of tissue damage (2 8) . As previously reported, EPS treatment significantly increased the expression of the p38MAPK protein. Furthermore, STAT3 overexpression and constitutive activation have been shown to have the carcinogenic potential in a range of human cancers in both experimental and clinical trials. As a transcriptional activator of VEGF signaling, it plays a crucial part in angiogenesis. According to a published study, Lactobacillus bulgaricus has the capacity to activate TLR2 signaling, which subsequently stimulates cytokine release through the p38 MAPK pathway. This suggests that L. bulgaricus may play a crucial role in modulating host immune responses by activating key transcriptional factors involved in the production of beneficial immune-related cytokines (69). Similarly, strains of Lactobacillus were reported to be able to inhibit TLR2 signaling activity, which in turn affects TLR2 expression. Inflammatory illnesses can also be prevented and/or treated using L. bulgaricus (25).

Histopathological analysis of the liver tissues from rats given DEN intoxication revealed a notable infiltration of inflammatory cells in the portal region; this result is in line with another study(70). The present study showed that hepatocyte structure in liver tissues improved significantly in both the EPSs preventive and treatment groups following supplementation. This aligns with previous research demonstrating that EPSs exert hepatoprotective and anti-inflammatory effects in HCC-induced liver injury, as evidenced by reduced necrotic damage and decreased inflammatory cell infiltration. These results further support the previously proposed protective role of EPSs supplementation in preventing liver damage (7 0) .

6. CONCLUSION

EPS supplementation seems to inhibit DEN-induced hepatocarcinogenesis, at least in part, by downregulating the IL-17/TLR2/p38 MAPK/STAT3 signaling cascade. Given the observed downregulation of TLR2, p38 MAPK, and STAT3 expression, it can be inferred that EPSs reduce IL-17 levels, which subsequently leads to decreased TGF-β1 expression in liver tissue through these signaling mechanisms. This study suggests that EPSs hold promise as a potential therapeutic agent for inflammation-associated hepatocarcinogenesis. However, further investigation is necessary to fully understand their mechanism of action and to support their translation into clinical applications.

7. FUTURE PROSPECTS

Clinical Translation – Evaluate safety, pharmacokinetics, and dosing of EPSs in HCC patients. Gut–Liver Axis – Study microbiota and intestinal immune modulation in EPS-mediated anti-tumor effects. Synergistic Therapy – Combine EPSs with chemotherapy or immunotherapy for enhanced efficacy and reduced toxicity. Structural Optimization – Modify EPSs to improve stability, bioactivity, and target specificity. Functional Foods – Develop EPS-based supplements for liver health and cancer prevention. Targeted Delivery – Use nanoparticles/microencapsulation to boost bioavailability and liver targeting. Broader Cancer Use – Test EPSs against other inflammation-driven cancers (colorectal, gastric, pancreatic). Genetic/Epigenetic Effects – Explore EPS influence on gene regulation, fibrosis, and tumorigenesis. Long-Term Safety – Conduct chronic toxicity and genotoxicity studies for regulatory approval. Personalized Therapy – Tailor probiotic/EPS treatment to individual microbiome profiles.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or other-wise.

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