Enterococcus faecalis CGz3 alleviating steatosis via BSH-mediated modulation in HepG2 cell-lines

Koujalagi Koushik* and Malaviya Alok
Applied and Industrial Biotechnology Laboratory (AIBL), Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, INDIA
*koushik.koujalagi@res.christuniversity.in

Abstract

The study aimed to evaluate the therapeutic potential of bile salt hydrolase (BSH)- producing probiotic Enterococcus faecalis CGz3 in alleviating steatosis in HepG2 hepatocarcinoma cells, with non-alcoholic fatty liver disease (NAFLD) induced by cholesterol and oleic acid (OA), focusing on its effects on lipid accumulation, metabolic gene expression and, inflammatory pathways. HepG2 cells were treated with cholesterol and OA to induce lipid accumulation, mimicking non-alcoholic fatty liver disease (NAFLD) conditions. Cells were then incubated with E. faecalis CGz3 for 6 hours at 37°C and 5% CO_2. Lipid levels were quantified using Oil Red O staining and cholesterol uptake assays, while gene expression of lipogenic, inflammatory and metabolic markers was assessed via quantitative real-time polymerase chain reaction (qRT-PCR).

Treatment with E. faecalis CGz3 significantly reduced lipid accumulation from 42.96±1.35 mg/mL in NAFLD-induced cells to 29.73±1.26 mg/mL. It down regulated lipogenic genes (SREBP-1c, FAS and ACC) and inflammatory markers (TNF-α, IL-6, CRP, TLR4, TLR9, NF-κB, JNK, ERK) while upregulating PPARα and AMPK, promoting fatty acid oxidation. No significant cytotoxicity was observed at 6 hours, though prolonged exposure (12–24 hours) reduced cell viability. This study introduces E. faecalis CGz3, a novel BSH-producing probiotic isolated from chicken gizzard, as a promising candidate for NAFLD intervention. Its selective modulation of lipid metabolism and inflammation via BSH activity offers a new perspective on probiotic-based therapies for NAFLD, warranting further in vivo and clinical exploration.

Keywords: Bile salt hydrolase (BSH), Cytotoxicity, Cholesterol assimilation, HepG2-cell lines, Non-Alcoholic Fatty Liver Disease (NAFLD), qRT-PCR.

Introduction

Non-alcoholic fatty liver disease (NAFLD), affecting over 25% of adults globally, is characterized by excessive hepatic fat accumulation (>5% of hepatocytes) without significant alcohol consumption, often progressing to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC)[3]. Driven by de novo lipogenesis (DNL) through transcription factors like Sterol-regulatory-element-binding protein 1-c (SREBP-1c) and CCAAT/enhancer-binding protein-α (C/EBP-α), NAFLD promotes triglyceride (TG) synthesis, lipid peroxidation and inflammation, exacerbating its impact in obesity, type 2 diabetes and dyslipidemia[8]. Despite its growing prevalence, NAFLD lacks approved pharmacological treatments, underscoring the urgent need for innovative therapeutic strategies[27].

Probiotics targeting the gut-liver axis have gained attention for their potential to mitigate NAFLD by reducing lipid accumulation and systemic inflammation. Recent studies have explored various strains with partial success. For instance, Xin et al demonstrated that *Lactobacillus johnsonii* BS15 reduced hepatic lipid accumulation by 25% in obese mice and upregulated Peroxisome proliferator-activated receptor alpha (PPARα), but it had limited effects on inflammatory pathways like Toll-like receptor 9 (TLR9) and NOD-like receptor pyrin domain-containing 3 (NLRP3)[25]. Similarly, Huang et al reported that *Lactobacillus plantarum* AR113 achieved a 20% lipid reduction in HepG2 cells by suppressing Fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC), yet it showed weak modulation of C-reactive protein *(CRP)* and extracellular signal-regulated kinase (ERK)[1].

Kim et al found that *Bifidobacterium longum* reduced lipid content by 22% and down regulated FAS in HepG2 cells, but it failed to impact TLR9 or NLRP3[13]. Chen et al showed that *Lactobacillus rhamnosus* GG reduced lipid accumulation by 18% and reduced *FAS* and *Tumor necrosis factor-alpha (TNF-α)* in HepG2 cells, but it did not affect *CRP* or *ERK*[12,19]. These studies highlight a critical gap: no probiotic strain comprehensively addresses both lipid accumulation and key inflammatory pathways (TLR9, NLRP3, CRP, ERK), which are essential to halt NAFLD progression to NASH.

Moreover, the bile salt hydrolase (BSH) activity of probiotics, which deconjugates bile acids to reduce cholesterol absorption and modulate gut microbiota, remains underexplored despite its therapeutic potential[23]. This study introduces *Enterococcus faecalis* CGz3, a novel BSH-producing probiotic isolated from chicken gizzard, to bridge these gaps. Its high BSH activity deconjugates bile acids, disrupting cholesterol enterohepatic circulation and promoting fatty acid oxidation via AMP-activated protein kinase (AMPK) and Peroxisome Proliferator-Activated Receptor Alpha (PPARα) up-regulation, as supported by mechanisms elucidated in similar models. Additionally, *E. faecalis* CGz3 down-regulates TLR9, NLRP3, CRP and

ERK, alongside FAS, ACC, TNF-α and c-Jun N-terminal kinase (JNK), offering a broader anti-inflammatory profile than comparator strains, which fail to modulate these pathways comprehensively[7,27]. By leveraging BSH-mediated cholesterol reduction and selective pathway modulation, *E. faecalis* CGz3 addresses the inadequacies of existing probiotics, providing a more effective intervention for NAFLD. This *in vitro* study sets the stage for *in vivo* and clinical validation to confirm its therapeutic potential.

Material and Methods

Lyophilization and viability test: *Enterococcus faecalis* CGz3 grew in MRS broth for 24 hours at 37°C and 120 rpm. Pelletising cells required 8 minutes of centrifugation at 10,000 rpm after incubation. Lyophilised powder was made by washing, resuspending and freeze-drying the particle in sterile saline (0.89%). Resuspending of the lyophilised powder in sterile saline and plating it on MRS agar tested its sterility, viability and CFU count. We counted live CFU after 24 hours of 37°C incubation. The cell-line treatment concentration was based on this CFU count.

Oleic acid/BSA solution preparation: OA was dissolved in 0.1 N NaOH and heated to 70°C in a shaking water bath to make a 100 mM stock solution. BSA was dissolved in distilled water and heated to 55°C to make a 10% (w/v) solution. To produce the OA/BSA combination, 100 mM OA was diluted with 10% BSA in the culture medium to 10 mM[5]. After sterile filtration via a 0.2-µm membrane, the solution was kept at -20°C until use.

Generation of non-alcoholic fatty liver disease and cytotoxicity analysis: The HepG2 human hepatocellular carcinoma cells (ATCC HB-8065) were cultured in DMEM supplemented with 10% FBS (MP Biomedicals, Germany) at 37°C in a humidified chamber with 5% CO_2[16]. The cell density was increased to almost 80% confluency. A non-antibiotic culture medium was used to pre-incubate the cells for 6 hours before treatment. HepG2 cells were subjected to 1 mM OA and 75 µg/mL water-soluble cholesterol (Sigma-Aldrich) in FBS-free DMEM to mimic circumstances similar to NAFLD. A medium devoid of OA and cholesterol and containing 1% BSA was used to treat the control cells. Following that, 250 µL of *E. faecalis* CGz3 solution was introduced and the cells were left to incubate at 37°C with 5% CO_2 for 6, 12, or 24 hours.

Cell viability assay: The CCK-8 reagent was used to determine the cell viability. Each well was then supplemented with 200 µL of DMEM containing 10% CCK-8 reagent after the medium was extracted. For three hours, the plates were placed in an incubator with a 5% CO_2 environment at 37°C[10,16]. To measure the absorbance of each well, a microplate reader was used, with the signal wavelength set at 450 nm and the background wavelength at 630 nm. After normalising to untreated controls, cell viability percentages were computed by removing background data[10].

Oil red O staining: After incubating HepG2 cells with *E. faecalis* CGz3 for 6 hours, they were washed three times with cold PBS and then fixed with 10% formalin for 20 minutes at room temperature. The next step was to stain the cells for 15 minutes in the dark using a newly made working solution of Oil Red O[28]. Washing the item many times with cold distilled water removed the excess discoloration. An inverted microscope was used to study and to photograph the cells. The stained lipid droplets were mixed with 100% isopropanol and then agitated for 10 minutes at room temperature to facilitate measurement. With the use of a microplate reader, the optical density was determined at 510 nm.

Cholesterol uptake assay to assess the intracellular cholesterol content in the HepG2 cells: Using Gilliland's modified o-phthalaldehyde colorimetric approach, we were able to quantify the intracellular cholesterol content. After homogenising the HepG2 cells, a mixture of 0.25 mL, 1.5 mL of ethanol (95% v/v) and 1 mL of potassium hydroxide (50% w/v) was prepared. Before being heated in a water bath at 60°C for 10 minutes, the mixture was vortexed for 15 seconds. The liquid was vortexed once more after cooling and then 2.5 mL of hexane and 1.5 mL of distilled water were added. An o-phthalaldehyde reagent solution was prepared by collecting the top hexane layer, evaporating it under nitrogen gas at 60°C and then dissolving it in 0.75 mL of glacial acetic acid. The samples were left to incubate at room temperature for 10 minutes after adding 1 mL of pure H_2SO_4. At 550 nm, the absorbance was recorded. To quantify cholesterol, a standard curve was created utilising values ranging from 0-0.2 mg/mL with a correlation coefficient of 0.995[16].

Analysis of lipid-regulating genes in HepG2 cells by qRT-PCR: Tests for inflammatory and lipid-regulating gene expression were conducted using quantitative real-time polymerase chain reaction (qRT-PCR). Transcription from HepG2 RNA to cDNA was performed. Endpoint PCR and agarose gel electrophoresis were used to verify the following primers: β-actin, SREBP-1c, acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), AMPK, PPARα, TNF-α, IL-6, CRP, TLR4, TLR9, NLRP3, ERK, JNK, AKT, NF-κB and HMGCR. With the Rotor-Gene Q thermal cycler, qRT-PCR based on SYBR Green was performed as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 58°C for 15 seconds and 72°C for 20 seconds[18]. The cDNA concentrations were consistent among samples and the primer concentrations were kept at 200 nM. Using the comparative Ct ($2^{\Delta\Delta Ct}$) technique, the relative expression was computed. Analysis of the melt curve verified that the primers were specific.

Results and Discussion

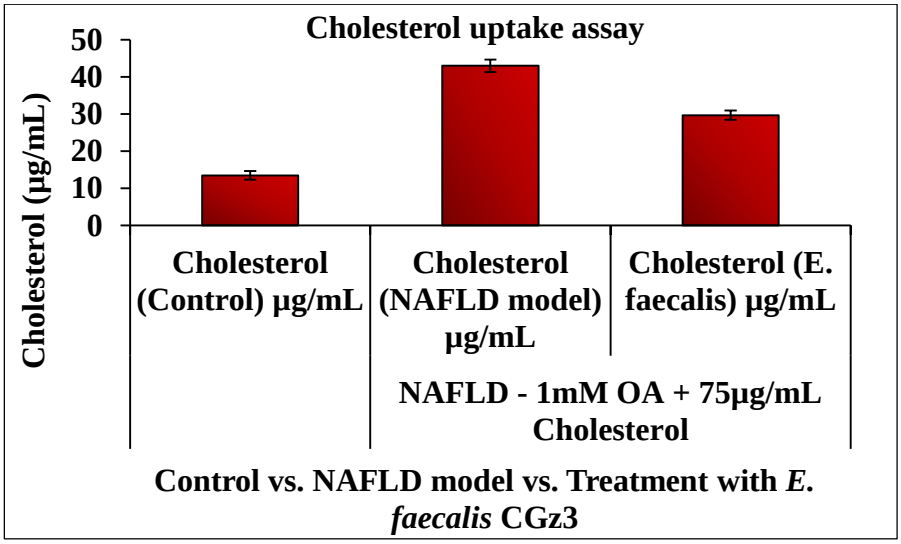
Probiotic strain's cytotoxic effects on HepG2 cells, as well as the combined effects of OA and cholesterol treatment: The cytotoxicity of *E. faecalis* CGz3 on HepG2 cells was assessed using the Cell Counting Kit-8 (CCK-8)

assay. Treatment for 6 hours showed no significant reduction in cell viability, with percentages exceeding 90% in both control and NAFLD-induced HepG2 cells. This indicates that *E. faecalis* CGz3 does not exhibit immediate cytotoxic effects at short exposure times. However, prolonged treatment for 12 or 24 hours led to a notable decrease in cell adherence and viability, suggesting potential time-dependent adverse effects. These findings stress the need for clinical treatment duration optimisation. *E. faecalis* CGz3 reduced lipid formation and intracellular lipid content in OA- and cholesterol-treated HepG2 cells in addition to cytotoxicity (Table 1).

Cholesterol uptake assay, Oil red O staining: In *E. faecalis* CGz3-treated cells, intracellular cholesterol levels were 30% lower than in untreated OA-induced cells. The impact of *enterococcus faecalis* CGz3 on lipid accumulation was tested using Oil Red O staining and cholesterol uptake.

Table 1
Cell viability assay at the 6th, 12th and 24th hour. Data represents the means ± standard error of the mean.

% Viability				
Time point	**Control HepG2**		**NAFLD - 1mM OA + 75µg/mL Cholesterol**	
	Untreated	***E. faecalis* CGz3**	**Untreated**	***E. faecalis* CGz3**
6 h	100±2.43	**90.62±2.74**	94.64±2.2	**93.04±3.0**
12 h	100±4.8	22.008±3.93	51.84±2.05	11.6±2.7
24 h	100±2.04	24.48±0.04	43.75±1.02	20.51±0.07



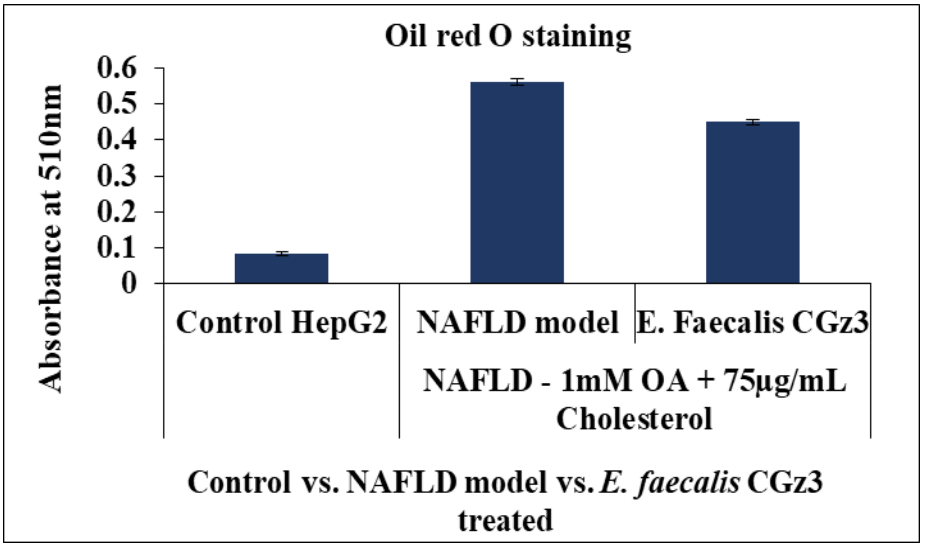


Figure 1: The effect of *E. faecalis* CGz3 on the HepG2 cells induced with 1mM OA + 75µg/mL cholesterol. Error bars represent the standard error of the mean.

Figure 1 shows that OA- and cholesterol-treated HepG2 cells displayed severe steatosis, while control cells had little staining in correlation with the cholesterol concentration of 13.49±1.17[a] mg/mL (control), 42.96±1.65[c] mg/mL (NAFLD induced HepG2 cell-lines) and 29.73±1.26[b] mg/mL (NAFLD induced HepG2 cell-lines treated with *E. faecalis* CGz3), showing a significant decrease ($p < 0.01$) of cholesterol levels. Compared to *Lactobacillus johnsonii*, which reduced hepatic fat by 25% in HepG2 cells, *E. faecalis* CGz3 achieved a 30% reduction, suggesting superior cholesterol-lowering efficacy. *Lactobacillus plantarum* AR113 reduced lipid accumulation by 20% but had limited effects on inflammatory markers [9].

Lower staining intensity showed that *E. faecalis* CGz3 decreased lipid accumulation (Figure 2B). This indicates that *E. faecalis* CGz3 effectively modulates cholesterol metabolism, reducing lipid accumulation and steatosis. These findings highlight the potential of *E. faecalis* CGz3 as a therapeutic candidate for addressing hepatic steatosis and related lipid dysregulation.

***E. faecalis* CGz3 modulates lipid-regulating genes in HepG2 cells: quantitative RT-PCR:** The molecular effects of *E. faecalis* CGz3 were evaluated by analyzing the expression of lipid-regulating and inflammatory genes using quantitative RT-PCR. Primers were validated through endpoint PCR, agarose gel electrophoresis and NanoDrop quantification (Figure 3 and Table 2). The primer sequences were determined for the genes of interest as in table 3. Treatment with *E. faecalis* CGz3 resulted in significantly reduced ($p < 0.05$) mRNA expression of lipogenesis-related genes such as acetyl-CoA carboxylase-alpha (ACC-α) and fatty acid synthase (FAS), as well as inflammatory markers including TNF-α, CRP, TLR9, NLRP3, JNK and ERK. Conversely, genes associated with fatty acid oxidation and energy regulation, such as AMPK and PPARα, were significantly ($p < 0.05$) upregulated, indicating enhanced lipid oxidation in HepG2 cells treated with *E. faecalis* CGz3.

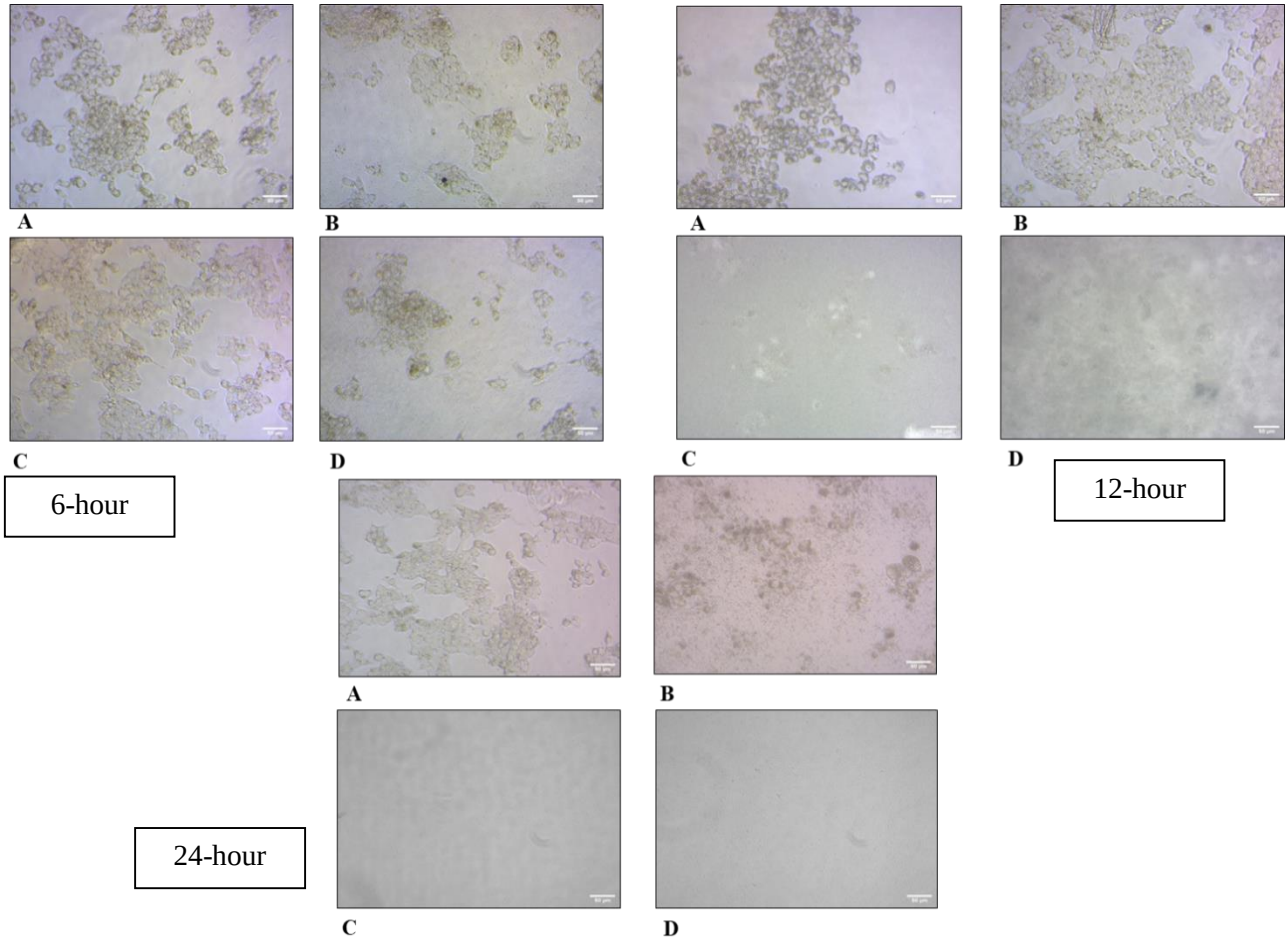


Figure 2A: Oil red O staining of the total lipid contents from control, NAFLD induced and *E. faecalis* CGz3 treated NAFLD-induced HepG2 cells. Error bars represent the standard error of the mean.
Figure 2B: 6 hour - A) Control HepG2 cells; B) HepG2+*E. faecalis* CGz3; C) HepG2+OA and cholesterol; D) HepG2+NAFLD+*E.faecalis;*
12 hour - Control HepG2 cells; B) HepG2+*E. faecalis* CGz3; C) HepG2+OA and cholesterol; D) HepG2+NAFLD+*E. faecalis* CGz3;
24 hour- A) Control HepG2 cells; B) HepG2+*E. faecalis* CGz3; C) HepG2 with OA and cholesterol (NAFLD-induced); D) HepG2+NAFLD-induced+*E. faecalis* CGz3 treated

However, no significant changes were observed in the expression of certain genes including SREBP-1c, AKT, TLR4, HMGCR, NF-κB, or IL-6 (Figure 4). This suggests that *E. faecalis* CGz3 exerts selective modulation of lipid metabolism and inflammatory pathways. These findings confirm the probiotic's ability to alleviate NAFLD-associated lipid dysregulation and inflammation, highlighting its potential as a therapeutic candidate.

PPARα is crucial for lipid metabolism, boosting fatty acid oxidation and reducing inflammation. It controls adipocyte development, insulin sensitivity and fatty acid elimination. In this work, *E. faecalis* CGz3 increased PPARα expression, supporting its involvement in decreasing steatosis and inflammation. *B. longum* down regulated FAS but showed no significant effect on NLRP3 or TLR9 [1]. *L. rhamnosus* GG reduced FAS and TNF-α but did not affect ERK or CRP [12].

L. johnsonii upregulated PPARα but had minimal impact on TLR9 or NLRP3[28]. *L. plantarum* AR113 reduced FAS and ACC but not CRP or ERK[9]. PPARα activity is linked to reduced fibrosis and stellate cell activation, suggesting anti-fibrotic actions[20]. *E. faecalis* CGz3's comprehensive down regulation of FAS, ACC, TNF-α, CRP, TLR9, NLRP3 and ERK, alongside PPARα and AMPK up regulation, underscores its unique BSH-mediated mechanism.

Table 2
Quantification of extracted RNA samples on NanoDrop

S.N.	Sample ID	NanoDrop Readings (µg/µl)	QC Status
1	Control	211.4	Pass
2	*E. Faecalis* CGz 3	198.5	Pass

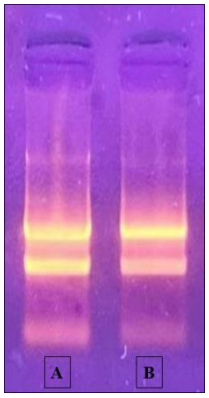


Figure 3: Agarose gel electrophoresis: (A) 100 bp primer ladder (B) NAFLD-induced model treated with *E. faecalis* CGz3 treated.

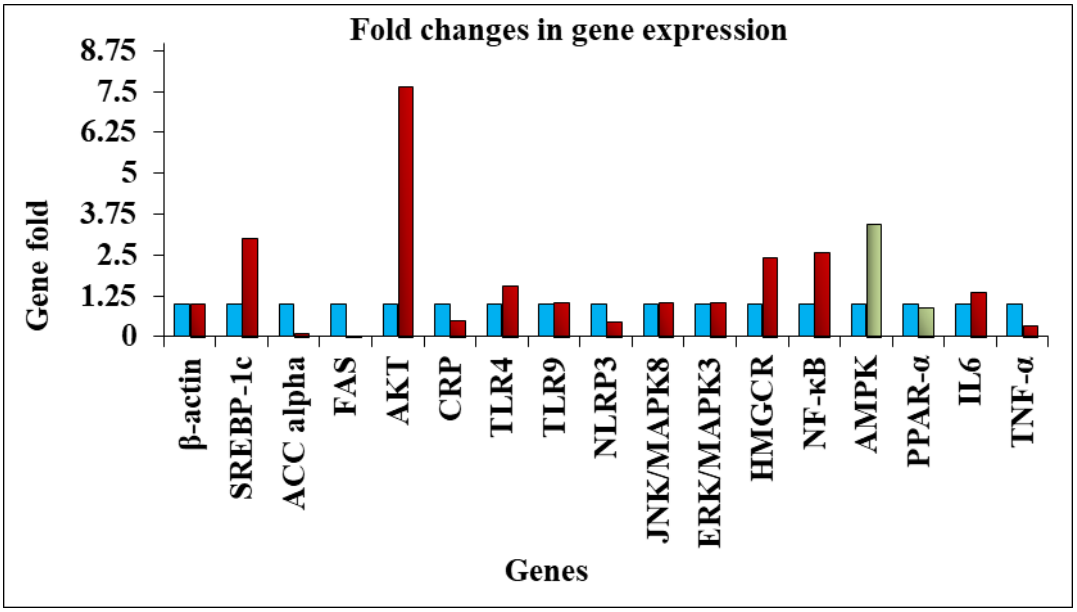


Figure 4: QRT-PCR of the genes in HepG2 cells. ACC-α, FAS, CRP, TLR9, NLRP3, JNK, ERK and TNF-α (red bars) are down-regulated. AMPK and PPARα (grey-colour bars) are upregulated.

Table 3
qRT-PCR: primer sequences of the genes involved in the non-alcoholic fatty liver disease.

Gene		primer sequence
SREBP-1C	FP	ACTTCTGGAGGCATCGCAAGCA
	RP	AGGTTCCAGAGGAGGCTACAAG
Acetyl CoA Carboxylase alpha	FP	TTCACTCCACCTTGTCAGCGGA
	RP	GTCAGAGAAGCAGCCCATCACT
Acetyl CoA Carboxylase beta	FP	GACGAGCTGATCTCCATCCTCA
	RP	ATGGACTCCACCTGGTTATGCC
fatty acid synthase	FP	TTCTACGGCTCCACGCTCTTCC
	RP	GAAGAGTCTTCGTCAGCCAGGA
AKT	FP	TGGACTACCTGCACTCGGAGAA
	RP	GTGCCGCAAAAGGTCTTCATGG
C-reactive protein	FP	TCGTGGAGTTCTGGGTAGATGG
	RP	TTCCCACCGAAGGAATCCTGCT
TLR4	FP	CCCTGAGGCATTTAGGCAGCTA
	RP	AGGTAGAGAGGTGGCTTAGGCT
TLR9	FP	TGAGCCACAACTGCATCTCGCA
	RP	CAGTCGTGGTAGCTCCGTGAAT
NLRP3	FP	GGACTGAAGCACCTGTTGTGCA
	RP	TCCTGAGTCTCCCAAGGCATTC
JNK/MAPK8	FP	GACGCCTTATGTAGTGACTCGC
	RP	TCCTGGAAAGAGGATTTTGTGGC
ERK/MAPK3	FP	TGGCAAGCACTACCTGGATCAG
	RP	GCAGAGACTGTAGGTAGTTTCGG
HMGCR	FP	GACGTGAACCTATGCTGGTCAG
	RP	GGTATCTGTTTCAGCCACTAAGG
NF k B	FP	GCAGCACTACTTCTTGACCACC
	RP	GCAGCACTACTTCTTGACCACC
Beta -ACTIN	FP	AGAGCTACGAGCTGCCTGAC
	RP	AGCACTGTGTTGGCGTACAG
AMPK	FP	AGGAAGAATCCTGTGACAAGCAC
	RP	CCGATCTCTGTGGAGTAGCAGT
PPAR-α	FP	TCGGCGAGGATAGTTCTGGAAG
	RP	GACCACAGGATAAGTCACCGAG
TNF-α	FP	ATCTGGAGGAAGCGGTAGTG
	RP	AATAGGTTTTGAGGGGCATG
IL6	FP	CATCCTCGACGGCATCTCAG
	RP	ACCAGGCAAGTCTCCTCATTG

Non-alcoholic fatty liver disease (NAFLD), a global health burden linked to obesity, dyslipidemia and type 2 diabetes, results in excessive triglyceride (TG) accumulation in hepatocytes, progressing to non-alcoholic steatohepatitis (NASH) in approximately 10% of cases, with severe risks of cirrhosis and hepatocellular carcinoma (HCC)[6]. The lack of approved pharmacological treatments underscores the urgent need for innovative interventions such as probiotics, which modulate the gut-liver axis to alleviate lipid dysregulation and inflammation [17]. This study demonstrates that *Enterococcus faecalis* CGz3, a novel bile salt hydrolase (BSH)-producing probiotic isolated from chicken gizzard, significantly reduces lipid accumulation and inflammation in an *in vitro* NAFLD model using HepG2 cells, offering a promising therapeutic candidate.

AMPK activation inhibits ACC phosphorylation, reducing malonyl-CoA production and subsequent fatty acid synthesis, while up-regulating peroxisome proliferator-activated receptor alpha PPARα enhances fatty acid oxidation, mitigating steatosis[20]. The 30% reduction in intracellular cholesterol observed with *E. faecalis* CGz3 treatment is attributed to its high BSH activity, which deconjugates bile salts, reducing cholesterol absorption and promoting its excretion[4]. *Lactobacillus rhamnosus* GG decreased lipid accumulation by 18% and down-regulated FAS and tumor necrosis factor-alpha (TNF-α) but not CRP[11] or ERK[14]. This mechanism disrupts enterohepatic circulation, lowering hepatic cholesterol levels, a critical factor in NAFLD pathogenesis.

The absence of significant sterol-regulatory-element-binding protein 1-c (SREBP-1c) down regulation suggests that *E. faecalis* CGz3 primarily inhibits lipogenesis through AMP-activated protein kinase (AMPK)-mediated suppression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS)[29]. This dual action on lipid metabolism positions *E. faecalis* CGz3 as a potent modulator of hepatic lipid homeostasis.

Compared to other probiotics, *E. faecalis* CGz3 exhibits superior efficacy. *Lactobacillus johnsonii* reduced hepatic lipid accumulation by 25% in HepG2 cells, primarily via PPARα up-regulation, but had limited effects on inflammatory pathways like toll-like receptor 9 (*TLR9*) or NOD-like receptor pyrin domain-containing 3 (NLRP3) [27]. *Lactobacillus plantarum* AR113 achieved a 20% reduction in lipid content but showed weaker suppression of CRP and ERK[9]. *Bifidobacterium longum* did not significantly affect TLR9 or NLRP3[29]. *Lactobacillus acidophilus* down regulated *FAS* and *TNF-α* but had no significant effect on NLRP3 or TLR9.

In contrast, *E. faecalis* CGz3's comprehensive down-regulation of FAS, ACC, TNF-α, CRP, TLR9, NLRP3 and ERK and up regulation of AMPK and PPARα, highlight its broader therapeutic impact, likely driven by its potent BSH activity. This shows that alternative mechanisms may be the probiotic's main impacts on lipid metabolism and underscore their unique BSH-mediated mechanism.

Inflammatory markers such as TNF-α, CRP and IL-6 are central to the pathogenesis of NAFLD[2,22,24]. TNF-α, produced by Kupffer cells and infiltrating immune cells, promotes lipogenesis through the mTOR complex-1 pathway. The inflammatory milieu in NAFLD, driven by cytokines like TNF-α, is a key contributor to disease progression via the mammalian target of the rapamycin (mTOR) pathway, promoting lipogenesis and insulin resistance[21]. *E. faecalis* CGz3 significantly reduced *TNF-α* expression, aligning with decreased NLRP3 inflammasome activity, which is critical for interleukin-1 beta (IL-1β) production and caspase-1 activation. The NLRP3 inflammasome, which is essential for IL-1β production and caspase-1 activation, was down regulated in *E. faecalis* CGz3-treated HepG2-cells.

Similarly, the marked suppression of TLR9, but not toll-like TLR4, suggests selective inhibition of innate immune responses, potentially reducing chronic inflammation without broadly disrupting immune signalling[15]. Down-regulation of c-Jun N-terminal kinase (JNK) and ERK, members of the mitogen-activated protein kinase (MAPK) family, further mitigates hepatic stress, lipid accumulation and fibrosis, as these pathways regulate myofibroblast phenotypes and diet-induced steatosis[23]. The lack of significant changes in nuclear factor-kappa B (NF-κB), protein kinase B (AKT) and interleukin-6 (IL-6) indicates that *E. faecalis* CGz3 targets specific inflammatory pathways, avoiding non-specific immune suppression, which is advantageous for therapeutic safety.

The role of BSH in *E. faecalis* CGz3's efficacy cannot be overstated. BSH deconjugates bile acids, reducing cholesterol solubility, which directly impacts hepatic lipid accumulation. This mechanism also influences gut microbiota composition, enhancing the gut-liver axis's role in systemic inflammation and metabolism. The up regulation of PPARα not only enhances fatty acid oxidation but also exerts anti-fibrotic effects by inhibiting hepatic stellate cell activation. These multifaceted effects position *E. faecalis* CGz3 as a candidate for synbiotic formulations where prebiotics could enhance BSH activity and amplify therapeutic outcomes.

The time-dependent cytotoxicity observed (viability >90% at 6 hours but reduced at 12–24 hours) suggests a need to optimize treatment duration. The lack of significant SREBP-1c modulation indicates that *E. faecalis* CGz3's effects are independent of this factor, potentially relying on post-transcriptional regulation via AMPK. The findings have significant implications for NAFLD management, targeting core pathological features and potentially halting progression to non-alcoholic steatohepatitis (NASH), fibrosis, or hepatocellular carcinoma (HCC). However, limitations of the *in vitro* model necessitate *in vivo* studies to

validate efficacy and safety. Clinical trials are critical to assess *E. faecalis* CGz3's translational potential.

Conclusion

This study establishes *Enterococcus faecalis* CGz3 as a novel, high-BSH-producing probiotic with significant potential for NAFLD management. Its novelty lies in achieving a 30% reduction in lipid accumulation in HepG2 cells, surpassing the 18–25% reductions of comparator probiotics like *Lactobacillus johnsonii*, *Lactobacillus plantarum* AR113, *Bifidobacterium longum*, *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* and in its comprehensive modulation of both lipid metabolism (via AMPK, PPARα, FAS, ACC) and inflammatory pathways (TNF-α, CRP, TLR9, NLRP3, JNK, ERK), driven by potent BSH activity that deconjugates bile acids to disrupt cholesterol enterohepatic circulation.

Unlike existing strains, which fail to address key inflammatory markers like TLR9 and NLRP3, *E. faecalis* CGz3's selective and broad-spectrum effects offer a ground-breaking approach to tackling NAFLD's multifaceted pathology. *E. faecalis* CGz3 are promising, with potential applications in synbiotic formulations combining prebiotics to enhance BSH activity and gut microbiota modulation, further amplifying its lipid-lowering and anti-inflammatory effects. Its ability to target the gut-liver axis positions it as a candidate for personalized nutrition strategies to prevent NAFLD progression to NASH, fibrosis, or HCC.

Additionally, *E. faecalis* CGz3's mechanism may extend to related metabolic disorders, such as dyslipidemia and type 2 diabetes, given its impact on cholesterol metabolism and insulin-sensitizing pathways like AMPK. Furthermore, prioritizing *in vivo* studies in animal models validates *E. faecalis* CGz3's efficacy and safety, focusing on optimal dosing and treatment duration to mitigate time-dependent cytotoxicity observed at 12–24 hours. Clinical trials are essential to assess translational potential, evaluating biomarkers like serum lipids, liver enzymes and inflammatory cytokines in NAFLD patients. Further research should explore *E. faecalis* CGz3's synergistic effects with prebiotics and its impact on gut microbiota composition to optimize therapeutic outcomes. These steps will bridge the gap between *in vitro* findings and clinical applications, positioning *E. faecalis* CGz3 as a pioneering probiotic intervention for NAFLD.

References

1. Bourgin M. et al, Bile salt hydrolases: At the crossroads of microbiota and human health, *Microorganisms*, **9(6)**, 1-12 **(2021)**

2. Braunersreuther V., Viviani G.L., Mach F. and Montecucco F., Role of cytokines and chemokines in non-alcoholic fatty liver disease, *World Journal of Gastroenterology*, **18(8)**, 727-735 **(2012)**

3. Cataldo I. et al, Pathology of non-alcoholic fatty liver disease, *Pathologica*, **113(3)**, 194-202 **(2021)**

4. Chen J. and Vitetta L., Gut microbiota metabolites in nafld pathogenesis and therapeutic implications, *International Journal of Molecular Sciences*, **21(15)**, 1-19 **(2020)**

5. Cousin S.P., Hügl S.R., Wrede C.E., Kajio H., Myers M.G.J. and Rhodes C.J., Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic beta-cell line INS-1, *Endocrinology*, **142(1)**, 229-240 **(2001)**

6. Franceschetti L., Bonomini F., Rodella L.F. and Rezzani R., Critical Role of NFκB in the Pathogenesis of Non-alcoholic Fatty Liver Disease: A Widespread Key Regulator, *Current Molecular Mkedicine*, **21(6)**, 495-505 **(2021)**

7. Fullerton M.D. et al, Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin, *Nature Medicine*, **19(12)**, 1649-1654 **(2013)**

8. Horst K.W. et al, Hepatic Insulin Resistance Is Not Pathway Selective in Humans With Nonalcoholic Fatty Liver Disease, *Diabetes Care*, **44(2)**, 489-498 **(2021)**

9. Huang W., Wang G., Xia Y., Xiong Z. and Ai L., Bile salt hydrolase-overexpressing *lactobacillus* strains can improve hepatic lipid accumulation in vitro in an nafld cell model, *Food and Nutrition Research*, **64**, 1-9 **(2020)**

10. Kang J.I. et al, Anti-Tumor Activity of Yuanhuacine by Regulating AMPK/mTOR Signaling Pathway and Actin Cytoskeleton Organization in Non-Small Cell Lung Cancer Cells, *PLoS One*, **10(12)**, 1-17 **(2015)**

11. Kasper P. et al, NAFLD and cardiovascular diseases : a clinical review, *Clinical Research in Cardiology*, **110(7)**, DOI:10.1007/ s00392-020-01709-7 **(2021)**

12. Kim B., Park K.Y., Ji Y., Park S., Holzapfel W. and Hyun C.K., Protective effects of *Lactobacillus rhamnosus* GG against dyslipidemia in high-fat diet-induced obese mice, *Biochemical and Biophysical Research Communications*, **473(2)**, 530-536 **(2016)**

13. Kim Y.T. et al, *In vivo* Trial of *Bifidobacterium longum* Revealed the Complex Network Correlations Between Gut Microbiota and Health Promotional Effects, *Frontiers in Microbiology*, **13**, https://doi.org/10.3389/fmicb.2022.886934 **(2022)**

14. Ko J.S., Yang H.R., Chang J.Y. and Seo J.K., *Lactobacillus plantarum* inhibits epithelial barrier dysfunction and interlukin-8 secretion induced by tumor necrosis factor-α, *World Journal of Gastroenterology*, **13(13)**, 1962-1965 **(2007)**

15. Kou M. and Wang L., Surface toll-like receptor 9 on immune cells and its immunomodulatory effect, *Frontiers in Immunology*, **14**, 1259989 **(2023)**

16. Kumar Vikas, Synthesis and characterization of Co (II), Ni (II) and Cu (II) complexes with unsymmetrical tetradentate Schiff base ligand, *Res. J. Chem. Environ.*, **28(2)**, 82-85 **(2024)**

17. Lee J.H., Woo K.J., Hong J., Han K.I., Kim H.S. and Kim T.J., Heat-Killed *Enterococcus faecalis* Inhibit FL83B Hepatic Lipid Accumulation and High Fat Diet-Induced Fatty Liver Damage in Rats by Activating Lipolysis through the Regulation the AMPK

Signaling Pathway, *International Journal of Molecular Sciences*, **24(5)**, 4486 **(2023)**

18. Livak K.J. and Schmittgen T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods (San Diego, Calif)*, **25(4)**, 402-408 **(2001)**

19. Min H.K. et al, Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease, *Cell Metabolism*, **15(5)**, 665-674 **(2012)**

20. Mooli R.G.R. et al, Hypoxia via ERK Signaling Inhibits Hepatic PPARα to Promote Fatty Liver, *Cellular and Molecular Gastroenterology and Hepatology*, **12(2)**, 585-597 **(2021)**

21. Thoreen C.C. et al, An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1, *The Journal of Biological Chemistry*, **284(12)**, 8023-8032 **(2009)**

22. Tilg H. and Moschen A.R., Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis, *Hepatology*, **52(5)**, 1836-1846 **(2010)**

23. Vachliotis I.D. and Polyzos S.A., The Role of Tumor Necrosis Factor-Alpha in the Pathogenesis and Treatment of Nonalcoholic Fatty Liver Disease, *Current Obesity Reports*, **12(3)**, 191-206 **(2023)**

24. Wieckowska A., Papouchado B.G., Li Z., Lopez R., Zein N.N. and Feldstein A.E., Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis, *The American Journal of Gastroenterology*, **103(6)**, 1372-1379 **(2008)**

25. Xin J. et al, Probiotic *Lactobacillus johnsonii* BS15 Promotes Growth Performance, Intestinal Immunity and Gut Microbiota in Piglets, *Probiotics and Antimicrobial Proteins*, **12(1)**, 184-193 **(2020)**

26. Xin J. et al, Preventing non-alcoholic fatty liver disease through *Lactobacillus johnsonii* BS15 by attenuating inflammation and mitochondrial injury and improving gut environment in obese mice, *Applied Microbiology and Biotechnology*, **98(15)**, 6817-6829 **(2014)**

27. Xu J. et al, *Lactobacillus johnsonii* Attenuates Liver Steatosis and Bile Acid Dysregulation in Parenteral Nutrition-Fed Rats, *Metabolites*, **13(10)**, doi:10.3390/metabo13101043 **(2023)**

28. Yao H. et al, Dioscin alleviates non-alcoholic fatty liver disease through adjusting lipid metabolism via SIRT1/AMPK signaling pathway, *Pharmacological Research*, **131**, 51-60 **(2018)**

29. Yun S.W., Shin Y.J., Ma X. and Kim D.H., *Lactobacillus plantarum* and *Bifidobacterium longum* Alleviate High-Fat Diet-Induced Obesity and Depression/Cognitive Impairment-like Behavior in Mice by Upregulating AMPK Activation and Downregulating Adipogenesis and Gut Dysbiosis, *Nutrients*, **16(22)**, 3810 **(2024)**.

(Received 22nd June 2025, accepted 25th July 2025)