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RESEARCH ARTICLE

Anticancer and antioxidant properties of a novel probiotic *Lactobacillus casei* FG12 against 7,12-Dimethylbenz [a]anthracene and Trichloroethylene-induced breast and liver cancer in rats

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ABSTRACT

Cancer exerts a significant impact on animal health and remains a substantial challenge in veterinary medicine, particularly in cases involving aggressive tumors. This study investigates the potential of a newly identified probiotic strain, *Lactobacillus casei* FG12 (*L. casei* FG12), as a natural therapeutic agent for breast and liver cancers induced in rats. To establish the cancer models, 7,12-dimethylbenz[a]anthracene (DMBA) and trichloroethylene (TCE) were employed to induce breast and liver tumors, respectively. The probiotic strain was confirmed via MALDI-TOF MS. FG12 demonstrated robust antioxidant capacity, scavenging 89% of DPPH radicals, alongside broad-spectrum antibacterial activity. In *vitro* analyses revealed that the strain decreased the viability of HepG2 and MCF-7 cancer cells by 81% and 85%, respectively. A total of ninety rats were allocated into nine treatment groups, including healthy controls, *L. casei* FG12-treated, Fluorouracil-treated, and cancer-induced groups. This research demonstrated in *vivo* that *L. casei* FG12 diminished early cancer indicators such as cellular degeneration, necrosis, and atypia in hepatic tissue, as well as hyperplastic alveolar nodules in mammary glands. The probiotic suppressed the expression of pro-apoptotic genes BCL-2 and HIF-1α, elevated the expression of Caspase-3, and restored oxidative balance. Histological examinations indicated that tissue structures resembled normal tissue, suggesting that *L. casei* FG12 provided significant protection against carcinogen-induced tissue damage. The findings concluded that *L. casei* FG12 exhibits strong multi-target potential as a probiotic for cancer prevention and support in veterinary therapeutic practices.

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INTRODUCTION

Contemporary research underscores the significance of gut microbiota as a fundamental determinant of animal health, influencing critical functions such as metabolism, immunity, and disease susceptibility, including cancer. Conversely, the gut is not merely a digestive organ but serves as a dynamic immunological and metabolic hub that interacts reciprocally with nearly all organ systems. These interactions have been identified as essential in maintaining health and conferring disease resistance, as evidenced by veterinary and biomedical research (Xu *et al.,* 2024). The equilibrium of gut microbiota is readily disrupted by infectious diseases or exposure to environmental toxins. Such disturbances can lead to chronic inflammation, immune dysregulation, and oxidative stress, all of which are strongly associated with carcinogenesis. Therefore, probiotics have been adopted as a supportive measure to address cancer-related agents in animals and to enhance their overall health and resilience (Puebla-Barragan *et al.,* 2021; Kunst *et al.,* 2023).

Probiotics are living microorganisms that, when administered in appropriate quantities, can contribute to enhancing health in various ways. They are increasingly recognized for their role in balancing the immune system, reducing inflammation, and combating cancer. Rodent studies have demonstrated that certain strains, including *Lactobacillus acidophilus, Lactobacillus rhamnosus*, and *Bifidobacterium bifidum*, may be utilized to assist in the treatment of chemical-induced tumors. The benefits appear to be linked to parameters such as augmenting the body's antioxidant defenses, decreasing tumor-promoting substances, and modifying the host's processing and exposure to toxins (Kassayova *et al.,* 2014; Wang *et al.,*

2025). These effects are mechanistically associated with the inhibition of NF-kB and COX-2 pathways, enhancement of the intestinal barrier, and the production of short-chain fatty acids (SCFAs), notably butyrate, which exhibit antiproliferative and pro-apoptotic effects against cancer cells (Molska and Regula, 2019; Liu *et al.,* 2025).

Lactobacillus casei is a probiotic that has garnered considerable interest due to its extensive biological effects and its capacity to prevent tumor development in various organs. Research has demonstrated that *L. casei* can dose-dependently delay the progression of cancerous cell populations, induce programmed cell death via *TRAIL*-mediated pathways, and also reduce tumor size in vivo (Tiptiri-Kourpeti *et al.,* 2016). Furthermore, *L. casei* BL23 has been found to stimulate immune mediators, including *IL-2* and *IFN-ɣ*, which facilitate the recruitment of cytotoxic T lymphocytes and enhance natural killer (NK) cell functions in targeting tumors (Toth, 2023). Additionally, it has been hypothesized by other researchers that extracts of *L. casei* may inhibit pathways such as NF-kB or PI3K/Akt/mTOR, which are involved in inducing apoptosis in gastric and colon cancer cells (Hwang *et al.,* 2013). Collectively, these studies highlight the capacity of *L. casei* to directly target tumor cells and indirectly act as an immune stimulant, which is particularly relevant to veterinary oncology, where immunomodulation presents an effective therapeutic strategy (Nisar *et al.,* 2025).

Notwithstanding these advancements, significant gaps remain in scientific understanding. The majority of prior research has focused on models of colorectal and gastric cancers, with limited investigations into the application of probiotics for mitigating the effects of industrial carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA) and trichloroethylene (TCE). These environmental pollutants are well-documented as potent inducers of mammary and hepatic tumors in laboratory animals. DMBA activates cytochrome P450 enzymes, resulting in the formation of DNA adducts and mutagenesis, while TCE produces hepatotoxic metabolites such as chloral hydrate, trichloroacetic acid, and dichloroacetic acid that impair mitochondrial function and elevate oxidative stress (Oliveira and Faustino-Rocha, 2023; Liu *et al.,* 2025). Existing research on probiotics has not fully elucidated the mechanisms through which certain microbial metabolites counteract chemical carcinogens or the specific molecular signatures involved in probiotic-mediated detoxification and antioxidant responses. This study emphasizes *Lactobacillus casei*, a probiotic shown to possess chemopreventive properties against carcinogenesis induced by DMBA and TCE in rat models. Instead of relying solely on traditional tumor monitoring or immune response assessments, the research employs chromatographic and metabolomic techniques to identify bioactive compounds produced by *L. casei* and correlate them with their biological functions. It investigates antioxidant and cytotoxic activities through laboratory analyses and evaluates biochemical, immune, and tissue-level modifications in *vivo*, providing comprehensive evidence of its protective effects. Furthermore, the study explores the influence of *L. casei* metabolites on oxidative stress, xenobiotic metabolism, and inflammation during chemical-induced carcinogenesis, thereby illuminating interactions between probiotics and their hosts. This investigation affirms *L. casei* as a valuable agent in reducing chemically induced and microbiota-associated cancer risks in animals by elucidating strain-specific metabolites and mechanisms that inhibit tumor development. This holistic approach paves the way for future probiotic-based therapies to enhance animal health.

MATERIALS AND METHODS

Isolation of LAB from fermented food: Culture broth was inoculated onto De Man, Rogosa, and Sharpe (MRS) agar plates (Lab M, Heywood, UK) and incubated anaerobically at 37°C under 5% CO_2 for 72 hours according to Kanklai *et al.* (2020). Colonies displaying clear inhibition zones were provisionally classified as lactic acid bacteria (LAB) as described by Kim and Kim (2012) and Phuengjayaem *et al.* (2021), and retained for subsequent identification. Presumptive LAB isolates were confirmed via Gram staining and catalase testing following the methodology of Wu *et al.* (2018).

Sifting of g-Aminobutyric acid GABA)-forming lactic acid bacteria (LAB): GABA production was screened on isolated gram-positive and catalase-negative bacteria (Park *et al.*, 2014; Lim *et al.*, 2018).GABA production was preliminarily evaluated by thin-layer chromatography (TLC) analysis (Silica gel 60 TLC F254 (Merck, Germany) (Qiu *et al.*, 2010). One microliter (1 µl) of supernatant from each isolate was spotted on a silica TLC plate with 20 × 20 cm dimensions. Then the thin layer chromatography was developed at 30°C with the developing solvent system n-butanol–acetic acid–water (5:3:2, v/v/v) (ascending technique). The TLC chamber was filled to 1/4 with the solvent system, and the lid was closed.

Screening of probiotic activities of selected isolates

Low PH tolerance and bile salt resistance: Acid resistance was determined following Sahi *et al.* (2022). Overnight cultures were diluted 1:10 into LB broth (pH 2.5) and incubated at 37°C for 3 hours. Optical density at 650 nm (OD_{650}) was measured spectrophotometrically, and cultures were normalized to absorbance (A_{650}) of 0.08 ± 0.05 to standardize bacterial cell concentrations. Bile salt tolerance testing was performed as described by Kiani *et al.* (2021). Normalized bacterial suspensions (100 µL) were inoculated into LB broth containing 0.3% w/v bile salts (Sigma-Aldrich, St. Louis, USA) and incubated at 37°C. Viability was evaluated at sequential time points (0, 1, 2, 3, and 4 hours) by spreading 100 µL of bacterial culture onto LB agar plates. Isolates exhibiting colony formation were classified as bile-tolerant; those without visible growth were classified as bile-sensitive.

$$\text{Survival rate (\%)} = \frac{\text{OD after treatment}}{\text{OD before treatment}} \text{X100}$$

Identification of GABA-producing LAB isolates using MALDI-TOF: A 110 bacterial isolates were initially collected and subjected to comprehensive phenotypic and biochemical characterization following Bergey's Manual of Determinative Bacteriology (Wu *et al.,* 2018). Selection criteria included: (1) Gram-positive staining reaction, (2) characteristic cell morphology visualized by light

microscopy, (3) typical colony morphology on primary culture media, (4) acid production capacity, and (5) negative reactions for both catalase and oxidase enzymes. Forty-two isolates satisfying all selection criteria were preserved at −20°C in Brain–Heart Infusion (BHI) broth (Merck, Germany) supplemented with 50% (v/v) glycerol for extended storage (Lim *et al.,* 2018).

Species-level identification was performed using Analytical Profile Index (API) systems (bioMérieux, Marcy l'Etoile, France) according to manufacturer specifications. Gram-positive cocci were identified using the API 20 Strep system (*Streptococci* and *Enterococci*), while Lactobacillus species were characterized using the API 50 CH kit with accompanying 50 CHL liquid medium. Anaerobic isolates underwent identification using a multi-system approach employing API 50 CH, API 20A, and API ZYM systems. All generated profiles were analyzed using Apiweb software and cross-referenced with strain-specific analytical profile indices to confirm species-level identifications (Phuengjayaem *et al.,* 2021). Prior to probiotic property assessment, bacterial isolates were cultured overnight in Luria-Bertani (LB) broth and standardized to 0.5 McFarland turbidity (equivalent to 1.5 × 10^8 CFU/mL) to ensure equivalent bacterial concentrations across experimental replicates. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was employed to identify the lactic acid bacterial isolate demonstrating the highest capacity for gamma-aminobutyric acid (GABA) biosynthesis (Aioub *et al.*, 2022).

Probiotic characteristics

Hemolytic and proteolytic activity: The hemolytic activity was investigated by streaking 18-hour-old *Lactobacillus* cultures onto the MRS agar plates with 5% (v/v) defibrinated blood. All these plates were incubated anaerobically at 37°C for 48 to 72 hours. Following incubation, the plates were observed to have clear areas around the colonies, which showed hemolytic activity (Shekh *et al.*, 2016). The well diffusion method was used to determine proteolytic activity of the selected isolates (Moslehishad *et al.*, 2013). The *Lactobacillus* broth cultures, 18 hours old, were streaked on the MRS agar plate containing 5 percent blood and incubated at 37°C for 48 hours in triplicate. The presence of a clear zone around the well was considered a positive proteolytic activity (Khanlari *et al.*, 2021). *Lactobacillus* broth culture was incubated at 37°C in triplicate for 48 hours. The clear zone surrounding the well indicates strong proteolytic activity.

Analysis by gas chromatography-mass spectrometry (GC-MS): The production of GABA was detected utilizing gas chromatography-mass spectrometry (GC-MS) (Lim et al., 2017). An integrated TSQ Quantum triple quadrupole GC-MS/MS system, coupled with the Thermo Scientific TRACE 1300 gas chromatograph (Thermo Scientific, Austin, TX, USA), was employed for analytical purposes. Samples were introduced via a Thermo Scientific™ AS3000 autosampler, and chromatographic separation was achieved using a Thermo Scientific™ Trace GOLD TG-5MS capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness). The oven temperature was programmed starting at 50°C, maintained for 1 minute, then increased at a rate of 4°C/min to 250°C, where it was held for an additional 5 minutes. The ion source and transfer line were maintained at temperatures of 250°C and 270°C, respectively. Helium was used as the carrier gas at a flow rate of 0.7 mL/min under split injection mode. The mass spectrometer operated in electron impact ionization/selective ion monitoring (EI/SIM) mode at 70 eV. The identification of 4-Aminobutanoic acid (GABA) was performed by monitoring characteristic mass fragments at m/z 304 and 174 from the 3TMS derivative. GABA's molecular formula is $C_4H_9NO_2$, with a molecular weight of 103. The compound's identity was confirmed through the diagnostic ions observed in the mass spectral data.

Evaluation of antioxidant activity by the DPPH radical scavenging method: The free radical scavenging activity of bacterial suspensions was measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Leaves and Leaves, 2014). A 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was freshly prepared in ethanol. For the antioxidant assessment, 1 ml of the DPPH solution was mixed with 3 ml of bacterial suspensions in ethanol at varying concentrations (10, 15, 30, 60, and 120 µg/ml). The mixtures were shaken thoroughly and kept in the dark at room temperature for 30 minutes to prevent any light-related breakdown of DPPH. Absorbance was subsequently measured at 517 nm utilizing a UV-VIS Milton Roy spectrophotometer, with ascorbic acid (Sigma-Aldrich, St. Louis, USA) employed as the standard antioxidant control.

Antibacterial activity of selected isolates: The antibacterial activity was investigated using agar well diffusion assay following (Al-Mohammadi *et al.,* 2021; Falah *et al.,* 2024). Antimicrobial efficacy was evaluated against ten pathogenic reference strains: *Bacillus subtilis* (MW391723), *Frendinandcohnia humi* (MW391719), *Sternotrophomonas maltophilia* (MW390826), *Bacillus anthracis* (MW390831), *Bacillus stratosphericus* (MW391714), *Enterobacter cancerogenus* (MW390637), Staphylococcus aureus (MW390870), *Pseudomonas aeruginosa* (MW390638), *Klebsiella pneumoniae* (MW390509), and *Escherichia coli*. Reference strains were cultured aerobically in Luria–Bertani broth at 37°C for 24 hours. Bioactive supernatants were prepared by culturing isolates overnight in de Man, Rogosa, and Sharpe (MRS) broth at 37°C, followed by centrifugation (8,000 × g, 15 min) and filter sterilization (0.22 µm; Millex-GP, Merck, Darmstadt, Germany). Cell-free supernatants were lyophilized (Labconco, Kansas City, Missouri, USA), reconstituted in 4 mL sterile distilled water, and subjected to antimicrobial assessment via agar-well diffusion assay (Falah *et al.*, 2024).

Cytotoxicity activity determination using MTT assay: The cytotoxicity of the suspension from bacterial strains against animal breast and liver cancer cell lines was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Van de Loosdrecht *et al.*, 1994). A sterile 96-well tissue culture polystyrene plate (flat-bottomed wells) was inoculated with 100 µL of cell suspension (cell concentration standardized to 1 × 10^5 cells/mL) per well. Plates were incubated at 37°C

in a humidified incubator containing 5% CO_2 for 24 hours to allow complete cell attachment and formation of a confluent monolayer. Confluency was verified microscopically prior to proceeding with subsequent steps. Upon achievement of monolayer confluence, the culture medium was carefully aspirated from each well using a vacuum aspirator or multichannel pipette. Adherent cells were washed twice with sterile phosphate-buffered saline (PBS; pH 7.4, Gibco/Thermo Fisher Scientific) using 100 µL per well to thoroughly eliminate residual growth medium and serum components that could interfere with assay readings.

The test sample was subjected to serial two-fold dilutions using Roswell Park Memorial Institute (RPMI) medium (Gibco/Thermo Fisher Scientific) supplemented with 2% (v/v) fetal bovine serum (FBS), designated as the maintenance medium for this assay. Dilution series were prepared to achieve a range of test concentrations spanning the anticipated dose-response spectrum. Aliquots of 100 µL corresponding to each concentration were distributed into designated treatment wells in triplicate or according to experimental design specifications. Three replicate wells designated as negative controls received 100 µL of maintenance medium only, without test sample addition. The plate was incubated at 37°C in 5% CO_2 for the duration specified by the experimental protocol (typically 24–72 hours depending on endpoint assessment requirements).

Following the designated incubation period, cellular viability was quantified using the MTT colorimetric viability assay. MTT stock solution (5 mg/mL in PBS) was diluted to working concentration, and 10 µL per well was added to all wells containing cells. Plates were incubated at 37°C for 4 hours in the dark to allow viable mitochondrial dehydrogenases to reduce MTT to purple formazan crystals. Following incubation, 100 µL of dimethyl sulfoxide (DMSO; molecular biology grade) was added to each well to solubilize formazan precipitate, and plates were incubated at room temperature (21–25°C) for 10 minutes to ensure complete crystal dissolution. Optical density (OD) was measured at 570 nm using a microplate spectrophotometer (reference wavelength 630 nm for background absorbance correction). Data were expressed as percentage viability relative to untreated control wells, with viability calculated as: $(OD_{570}$ sample $- OD_{630}$ sample) / $(OD_{570}$ control $- OD_{630}$ control) × 100%.

Animal design model: A total of 360 male Sprague-Dawley albino rats, with an average weight of 150 ± 10 g, were used in this study. Prior to the experiment, the rats were acclimated for at least 7 days in cages maintained at a controlled temperature of 22–24°C with a 12-hour light/dark cycle. The animals were then divided into nine groups, each containing 10 rats. Each group was assigned a specific treatment for 28 days as follows: Group C served as the control and received a standard basal diet. Group T1 was given the diet and injected intraperitoneally with 120 mg/kg body weight of DMBA for 7 days. Group T2 received the diet and was injected intraperitoneally with 120 mg/kg body weight of TCE for 7 days. Group T3 was fed a basal diet supplemented with 120 mg/kg body weight of *Lactobacillus casei* FG12. Group T4 consisted of rats treated with Fluorouracil. Groups T5 and T6 included mammary and liver cancer-induced rats, respectively, both treated with *Lactobacillus casei* FG12. Groups T7 and T8 were mammary and liver cancer-induced rats treated with Fluorouracil. All groups received similarly analyzed diets with carbohydrate, protein, fat, moisture, fiber, and ash contents determined by specified methodologies (AOAC, 2012). All procedures complied with internationally recognized principles and institutional standards governing the ethical care and use of laboratory animals.

Gene expression of proinflammatory and precancerous genes: RNA was extracted from rat liver tissue, and the RNA pellets obtained were dissolved in diethylpyrocarbonate (DEPC)-treated water. The concentration of RNA was determined by spectrophotometric measurement, specifically by assessing the absorbance ratio at optical densities 260/280 nm (Saif and Khan, 2022). The semi-quantitative reverse transcription PCR (RT-PCR) was performed using 3 µg of RNA. The reaction plate was first denatured in a Bio-Rad T100™ PCR thermocycler at 70 °C for 5 minutes. An amount of 0.5 ng of oligo(dT) primers was added to the RNA. For complementary DNA (cDNA) synthesis, the mixture included 2 µl of 10X reverse transcription (RT) buffer, 2 µl of 10 mM dNTPs, and 1 µl of 100 U reverse transcriptase. The reaction mixture was first incubated at 80 ºC for 5 minutes to deactivate the enzyme, followed by an incubation at 40 ºC for 1 hour to allow cDNA synthesis.

Gene expression levels were quantified through densitometry, utilizing β-actin mRNA as the internal control. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted employing the 2-ΔΔCT method for relative quantification, with actin serving as the endogenous control. Variations in gene expression were assessed by comparing cycle threshold (CT) values via this comparative approach.

Liver and mammary gland histopathology: The liver and mammary gland tissues were immediately fixed after excision by immersing them in 10% neutral buffered formalin (NBF). Following fixation, the tissues underwent standard histological processing and analysis (Chen *et al.*, 2022).

Statistical analysis: A one-way ANOVA was conducted to assess the significant impact of variations in antioxidant and anticancer activities of probiotic bacterial isolates. Tukey's test was subsequently applied to compare the means ($P<0.05$). All mean values and standard deviations were derived from a minimum of three independent replicates (n=3).

RESULTS

Isolation, purification, and evaluation of lactic acid bacteria (LAB) for their selection as probiotics: In this study, 110 lactic acid bacteria were isolated from Rumi cheese (45 isolates), Mish cheese (30 isolates), and pickled green olives (35 isolates). These were plated on MRS agar supplemented with 2% $CaCO_3$. The main characteristics of the bacterial isolates showed that all were gram-positive and catalase-negative.

Screening of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB): GABA production was

confirmed through the observation of red spots on Thin Layer Chromatography (TLC) plates. The mobility of these red spots from the culture supernatant was consistent with that of the GABA standard, as evidenced by the retention factor (Rf) value of 0.75 cm, which matched the GABA standard at detectable concentrations. Only strains exhibiting an Rf value corresponding to that of the GABA standard (0.75 cm) were selected for GABA quantification; consequently, only 42 isolates were considered to produce GABA producers.

Screening of probiotic activities of isolated bacteria *In vitro*

Tolerance against low pH and bile salts: Selected LAB 42 isolates, including 15 from Rumi cheese, 11 from Mish cheese, and 16 from pickled green olives, were tested for their ability to tolerate acidic conditions at pH 2.5. The results showed that isolates FG12, FG30, FG38, and FG41 could withstand pH 2.5 for 3 hours, with survival rates of 99%, 94%, 95%, and 96%, respectively, as shown in Fig. 1A. These isolates also survived in 0.3% bile, with survival rates of 94%, 90%, 92%, and 92%, respectively, as indicated in Fig. 1B. Therefore, the results suggest that these three strains possess probiotic features, demonstrated by high survival rates at low pH (2.5) and bile concentration (0.3%).

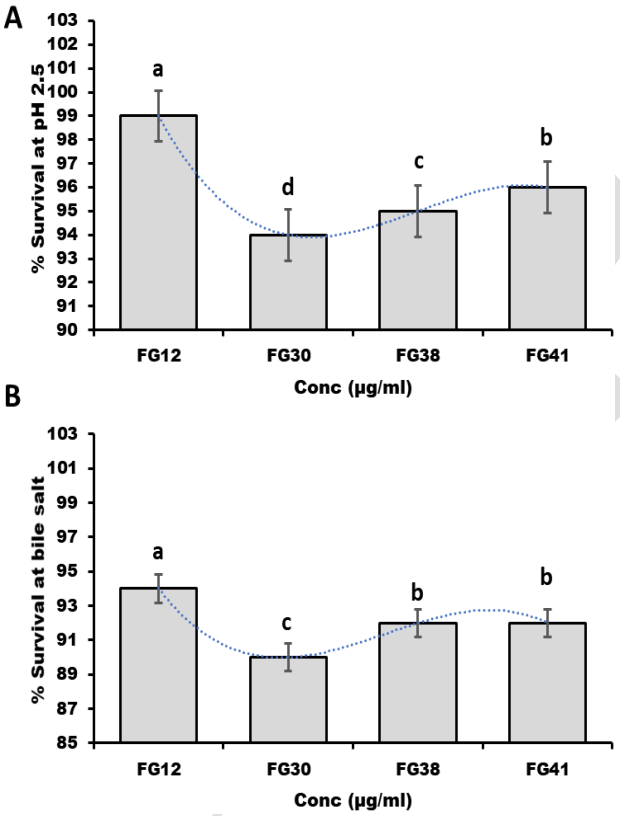

Fig. 1: The percentage of survival rate of LAB isolate against the lowest pH (A) and bile salt (B). Lowercase letters above columns indicate significant differences at P<0.05.

Gas Chromatography–Mass Spectrometry (GC–MS) analysis: Table 1 shows that GABA production by LAB isolates (FG12, FG30, FG38, and FG41) was confirmed through GC–MS analysis. The four isolates could convert monosodium glutamate into GABA. LAB FG12 isolate was the highest GABA producer at 11.23 mg/ml and was selected for further experiments, followed by LAB FG38 (10.16 mg/ml), while LAB FG30 and LAB FG41 showed lower GABA levels (8.23 and 9.56 mg/ml).

Table 1: Concentration of γ-aminobutyric acid (GABA) of *LAB isolates* analyzed by GC-MS to determine the GABA concentration

LAB isolates	Source	Concentration (mg/ml)
FG12	Rumi cheese	11.23±1.5a
FG30	Pickled green olive	8.23±0.9c
FG38	Pickled green olive	10.16±0.7ab
FG41	Rumi cheese	9.65±0.3b

Data are presented mean±SE. Differenet letters indicate significant differences at P<0.05.

MALDI-TOF identification of the selected GABA-producing LAB isolates: The isolate with the highest GABA content, as detected with GC, was identified using MALDI-TOF. The MALDI-TOF spectroscopy indicated a maximum similarity of 99% between the obtained isolate (*Lactobacillus casei* FG12) and *Lactobacillus casei* DSM 28872.

Probiotic characteristics

Hemolytic activity: The absence of hemolytic activity is considered a safety prerequisite for the selected probiotic strain, *Lactobacillus casei* FG12, which showed no α- or β-hemolysis when grown on blood agar.

Proteolytic activity: The proteolytic activity of the selected strain (*Lactobacillus casei* FG12) was measured by the diameter of the halo zone on skimmed milk agar. The strain exhibited a (28 mm) inhibition zone.

Biological activities

Antioxidant activity: Fig. 2 displays the scavenging activity of *Lactobacillus casei* FG12 against DPPH free radicals, where *Lactobacillus casei* FG12 (120 µg/ml) scavenged 89 % of free radicals, compared to ascorbic acid (91 %). The IC50 of *Lactobacillus casei* FG12 was 15 µg/ml against DPPH free radicals.

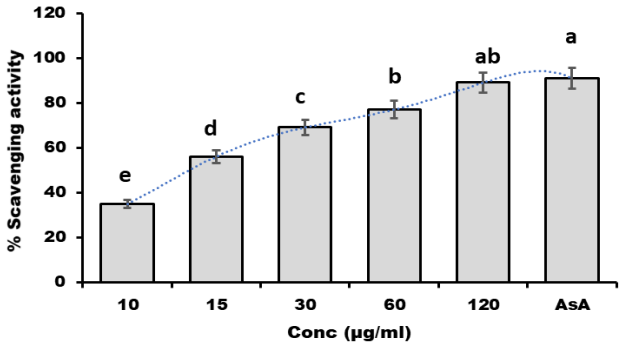

Fig. 2: The percentage of antioxidant activity against DPPH free radicals. Lowercase letters above columns indicate significant differences at P<0.05.

Anticancer efficacy: The histological examination presented in Figs. 3A-F demonstrates a clear dose-dependent reduction in cell density and viability in both breast and liver cancer cell lines following treatment with L. casei FG12. In the breast cancer cell line series (A-C), Fig. 3A depicts densely packed, viable cells exhibiting normal morphology and high cell density under the control condition. Conversely, Fig. 3B illustrates moderate cytotoxicity characterized by decreased cell density and emerging cellular damage. The most pronounced cytotoxic effects are observed in Fig. 3C, indicating extensive cell

death, a significant reduction in cell count, and visible cellular debris. Similar cytotoxicity is evidenced in the liver cancer cell lines, as shown in Fig. 3D (control) with typical hepatocellular architecture and organized cellular patterns, Fig. 3E (intermediate cytotoxicity) with preserved cellular organization but reduced viability, and Fig. 3F (severe cytotoxicity) with few viable cells remaining, widespread cell death, and loss of normal tissue architecture. Fig. 3G quantitatively demonstrates that viability data reflect a dose-related cytotoxic potential of *L. casei* FG12 on both the MCF-7 (breast cancer) and HepG2 (liver cancer) cell lines. Cell survival at 0 mg/ml remains full, declining progressively as the concentration of *L. casei* FG12 increases. Significant reductions are observed at 10 µg/ml (approximately 20% decrease), with further reductions to 60% viability at 15 µg/ml, 35% at 30 µg/ml, and a plateau between 60 and 120 µg/ml where approximately 15–20% of cells remain viable. Notably, *L. casei* FG12 exhibits cytotoxic potency comparable to doxorubicin (DOX), a well-established chemotherapeutic agent, with both treatments inducing similar levels of cell death. However, residual cell viability is slightly higher for *L. casei* FG12 (20%) compared to doxorubicin (18%).

Antibacterial activity: The three selected strains demonstrated significant antimicrobial activity against the test pathogenic panel based on inhibition zone diameter measurements. *Lactobacillus casei* FG12 exhibited broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens. Gram-positive test organisms included *Bacillus anthracis* (MW390831), Bacillus subtilis (MW391723), Fredinandcohnia humi (MW391719), *Bacillus stratosphericus* (MW391714), and *Staphylococcus aureus* (MW390870). Gram-negative test organisms comprised *Stenotrophomonas maltophilia* (MW390827), *Enterobacter cancerogenus* (MW390637), *Pseudomonas aeruginosa* (MW390638), *Klebsiella pneumoniae* (MW390509), and *Escherichia coli*.

Lactobacillus casei FG12 cell-free supernatant demonstrated potent broad-spectrum antimicrobial activity against all tested pathogens, with inhibition zone diameters ranging from 18 to 41 mm. These results were comparable to the positive control antibiotic streptomycin (19–42 mm), with no statistically significant difference observed between the two treatments (Table 2).

Table 2: Antibacterial activity of *Lactobacillus casei* DSM 28872 against the pathogenic bacteria expressed as inhibition zone diameters (mm) compared to Streptomycin

Bacterial strain	Concentration (µg/ml)/ IZDs (mm)					
	10	15	30	60	120	Antibiotic
BA	10±0.1bc	12±0.5cd	17±0.2c	21±0.3d	27±0.5e	29±0.9e
BaS	11±0.2b	15±0.2b	21±0.2ab	26±0.1b	35±0.3b	36±0.5b
FH	10±0.3bc	14±0.1bc	19±0.1b	25±0.5bc	31±0.4d	30±0.2d
BS	10±0.2bc	13±0.3c	16±0.2cd	25±0.4bc	33±0.2c	33±0.3c
SA	15±0.1a	17±0.1a	22±0.5a	29±0.2a	41±0.3a	42±0.5a
SM	9±0.0c	15±0.2b	18±0.6bc	23±0.1c	30±0.9de	31±0.9d
EnC	9±0.2c	13±0.3c	19±0.7b	23±0.3c	25±0.8f	26±0.2f
PA	8±0.3d	11±0.5d	13±0.5d	17±0.2e	21±0.2g	23±0.3g
KP	–	9±0.4e	11±0.2e	15±0.5f	18±0.3h	19±0.5i
EC	–	10±0.2de	12±0.2de	15±0.3f	19±0.2h	21±0.7h

Bacillus subtilis (BS), *Frendinandcohnia humi* (FH), *SternotroPhomonas maltoPhilia* (SM), *Bacillus* anthracis (BA), *Bacillus stratosPhericus* (BaS), *Enterobacter cancerogenus* (EnC), *StaPhylococcus aureus* (SA), P*seudomonas aeruginosa* (PA), *Klebsiella* P*neumoniae* (KP) and *E. coli* (EC). Antibiotic, Streptomycin (120 mg/L). Data are presented as mean±SE. lowercase letters indicate significant differences P<0.05

Effects of *Lactobacillus casei* FG12 on gene expression toward mammary and liver cancer: Fig. 4 illustrates the various effects of *L. casei* FG12 treatments on the expression of key apoptosis-related genes in rats subjected to carcinogenic agents DMBA and TCE. The analysis of BCL2 expression (Fig. 4A) indicates that both T1 and T2 treatments (*L. casei* FG12 alone) significantly elevate this anti-apoptotic gene by approximately 100% and 133%, respectively, in comparison to the control group (30 ng/ml), reaching peak levels of roughly 60 ng/ml and 70 ng/ml. This suggests a cellular protective response. Conversely, when combined with carcinogenic challenges, the patterns of *BCL2* expression become more intricate, with T3 (DMBA-induced breast cancer with *L. casei* FG12 treatment) exhibiting a 67% reduction in expression relative to T2; meanwhile, T4 through T8 treatments sustain moderate BCL2 levels ranging from 30 to 45 ng/ml (0 to 50% above control). As demonstrated in Figure 4B, *HIF1*-a expression is markedly elevated in groups treated with T1 and T2, with an average of approximately 110 ng/ml and 145 ng/ml, representing an increase of 83% and 142%, respectively, relative to the control level of 60 ng/ml. This indicates an enhanced cellular hypoxic resistance. Notably, the levels of *HIF1*-a are considerably lower in combination treatments (T3–T8), ranging between 50 and 80 ng/ml, which is 45–65% below the maximum levels observed. This suggests that *L. casei* FG12 can be employed to regulate hypoxia-related gene expression in cells experiencing oncogenic stress.

Similarly, Fig. 4C demonstrates that *BAX* expression, a crucial apoptotic marker, is highest in the T1 and T2 groups (28 ng/ml and 32 ng/ml), representing 180% and 220% above the control value (10 ng/ml). Under combined treatments, the levels decrease significantly (by 50-75%) (T3-T8), with values ranging from 12 to 22 ng/ml, indicating potential compensatory mechanisms induced by the probiotic during cancer-associated stress. This trend is corroborated by caspase-3 expression (Fig. 4D): The peaks in T1 and T2 are 30 ng/ml and 35 ng/ml, respectively, corresponding to 200% and 250% increase over the control, followed by substantial declines in groups receiving combination therapies, with final values between 10 and 20 ng/ml, reflecting reductions of 43-71%. Statistical analysis confirms that these gene expression changes are biologically significant (P<0.05). These findings suggest that *L. casei* FG12 induces both apoptotic and anti-apoptotic gene pathways independently and may enhance cellular defense mechanisms. Additionally, the probiotic appears to modulate these molecular signals more selectively in the presence of carcinogenic exposure, reducing peak expression by 40-75%, which may improve the balance between cell survival and cell death during cancer development and treatment.

Histological analysis of mammary and liver cancer of rats: Fig. 5 depicts the histopathological details of the protective and therapeutic effects of *L. casei* FG12 against chemically induced carcinogenesis in mammary and liver tissues. The mammary gland series (A-F) reveals that morphological changes vary with each treatment. Fig. 5A

illustrates a normal mammary gland with well-developed ducts, an intact epithelial lining, and normal supportive tissue. Fig. 5B contrasts this with severe damage induced by DMBA, characterized by disorganized ductal structures, irregular cell shapes (pleomorphism), enlarged nuclei relative to cytoplasm, and loss of normal tissue architecture, indicative of malignant transformation. The protective effect of *L. casei* FG12 pretreatment is observed in Fig. 5C, where the mammary tissue remains predominantly intact in morphology despite DMBA administration, with preserved ducts and minimal abnormal cellular changes. The therapeutic response to 5-fluorouracil treatment is demonstrated in Fig. 5D, showing significant tumor shrinkage, areas of necrosis, and a reduced number of cells, although some malignant features persist. Figs. 5E and 5F illustrate combined therapies involving *L. casei* FG12 following mammary cancer induction and subsequent fluorouracil administration; both exhibit improved tissue organization relative to untreated cancer, with varying degrees of cellular restoration and normalization. These findings are complemented by liver histopathology images (Figs. 5G-L), which reflect organ-specific responses. Fig. 5G shows normal liver tissue featuring hepatocyte cords, well-preserved vascular sinusoids, and portal triads. Fig. 5H depicts liver damage induced by TCE, including disrupted hepatocyte arrangement, cellular swelling, inflammation, and early signs of malignancy such as atypical nuclei and disorganized tissue. Fig. 5I presents the protective effect of *L. casei* FG12 against TCE-induced liver injury, with hepatocyte structures largely preserved and minimal inflammation. Fig. 5J demonstrates tissue recovery following treatment with *L. casei* FG12 immediately after liver cancer induction, with improved cellular arrangement and decreased inflammation compared to untreated cancer. Figures 5K and 5L depict the effects of fluorouracil therapy following cancer induction, illustrating sections of the liver returning to normal histological architecture, while certain regions continue to exhibit cancer-related abnormalities. All images include arrows indicating key features such as tissue destruction, inflammation, repair processes, and structural normalization.

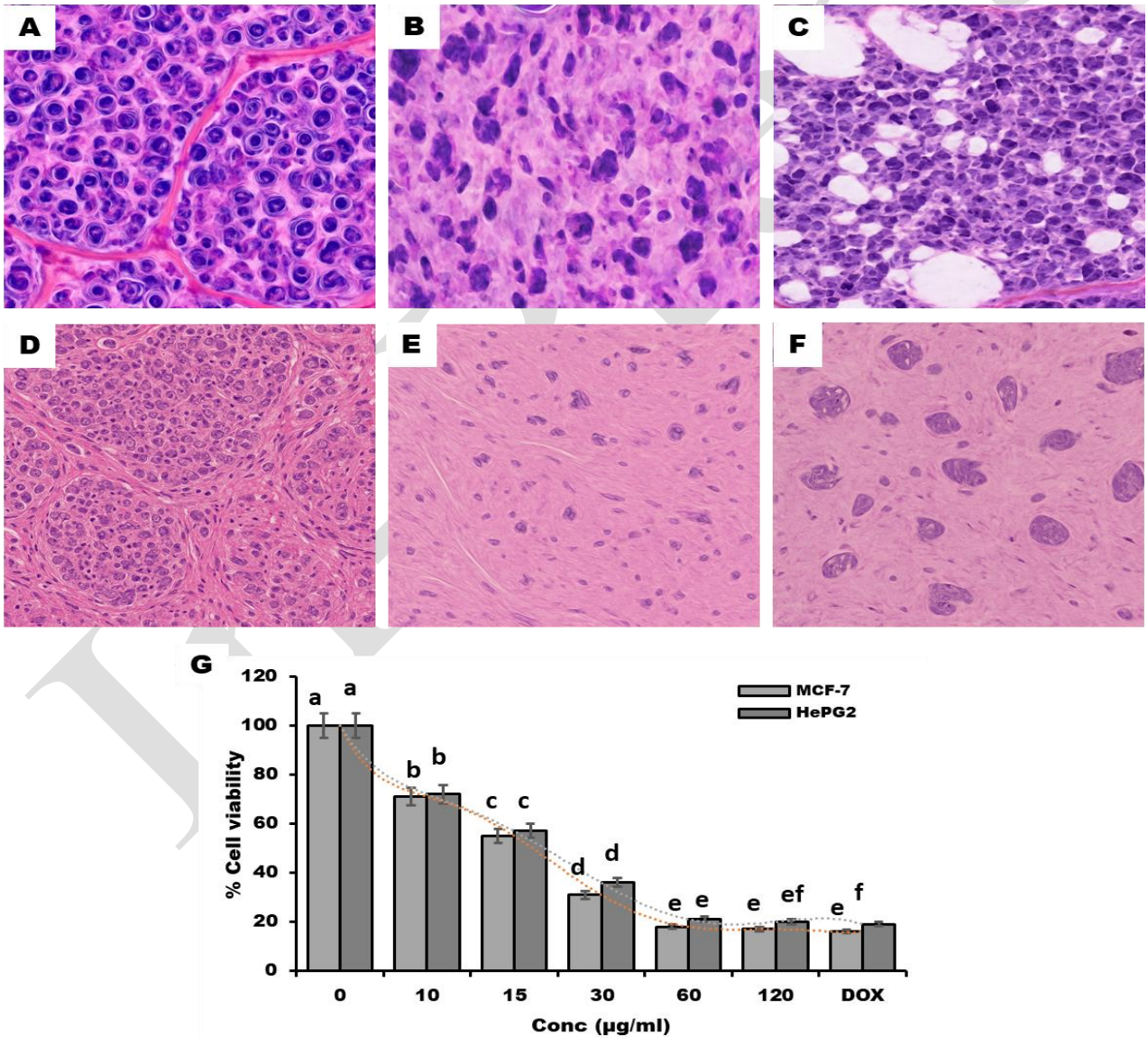

Fig. 3: Cytotoxicity of *L. casei* FG12 against breast cancer cell lines (A-C) and liver cancer cell lines (D-F) compared to Doxorubcin (DOX). (G) Histogram of Cancer cell viability response to L casei FG12 treatments against breast and liver cancer cell lines. Lowercase letters above columnsindicate significant differences at P<0.05.

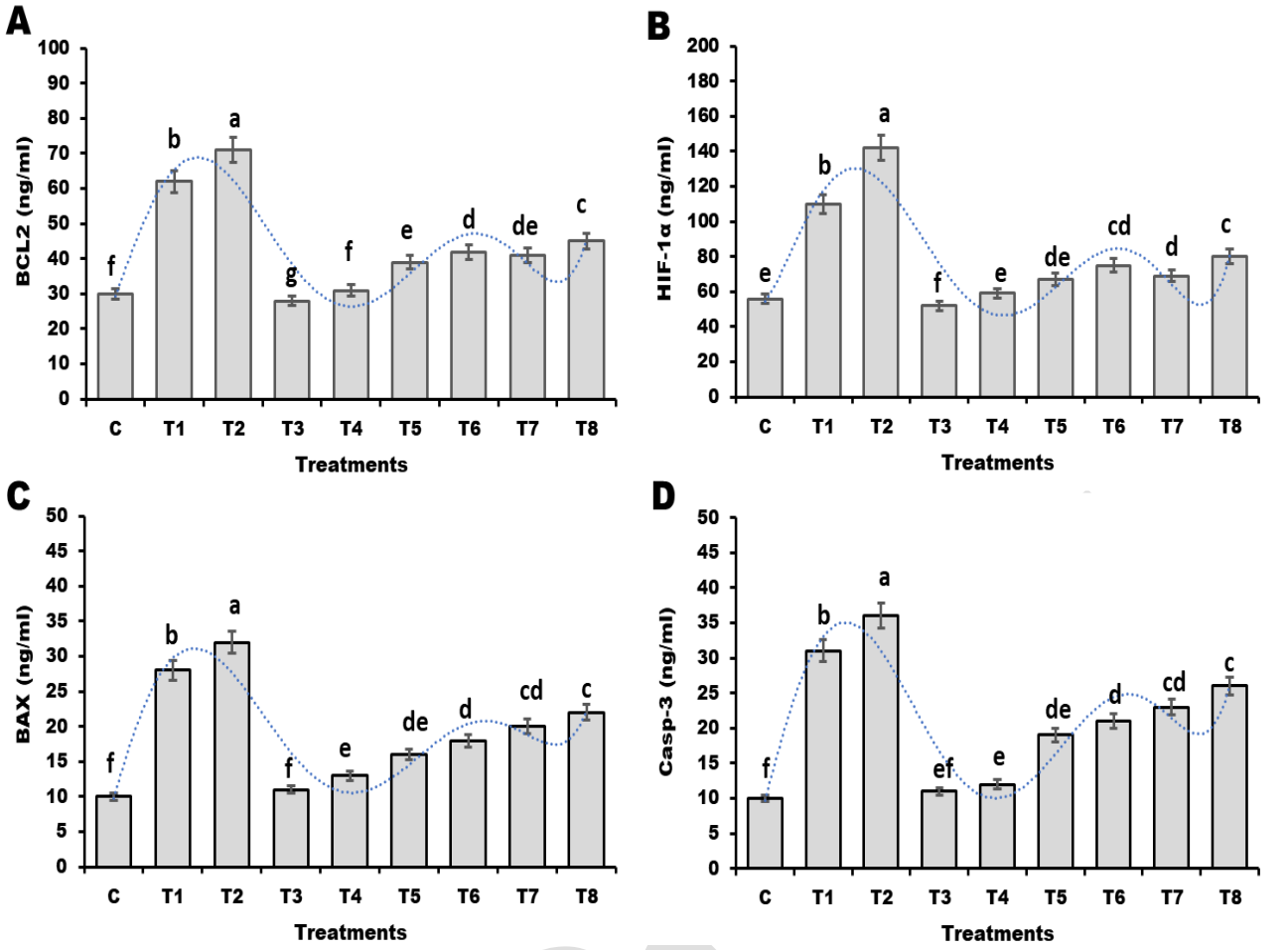

Fig. 4: The effect of *L. casei* FG12 treatments on gene expression of BCL2 (A), HIF1-α (B), BAX (C), and Casp-3 (D) against 7,12-dimethylbenz[a]anthracene (DMBA) and Trichloroethylene (TCE), inducing breast and liver cancer in rats. Data were expressed as mean ± SE (n = 5 for each group). Different letters indicate significant differences at P< 0.05. C, control rats not infected, T1, DMBA-induced breast cancer, T2, TCE-induced liver cancer, T3, *L. casei* FG12-treated rats, T4, Fluorouracil-treated rats, T5, mammary cancer induced rats and treated with *L. casei* FG12, T6 liver cancer induced rats and treated with *L. casei* FG12, T7 mammary cancer induction and treated with Fluorouracil, T8 liver cancer rats and treated with Fluorouracil.

Markers of oxidative stress in liver tissues and mammary tissues: Table 3 summarizes the biochemical and oxidative stress indicators, delineating the protective effects of *Lactobacillus casei* FG 12 against chemically induced carcinogenesis in rats (see Table 3). Notably, glucose levels were significantly disrupted in cases of DMBA-induced mammary cancer (T1: 1.47 mg/dL) and TCE-induced liver cancer (T2: 2.37 mg/dL) when compared to controls (4.04 mg/dL), indicating underlying metabolic imbalances associated with carcinogenesis. Partial metabolic recovery was observed following probiotic intervention (T3), with glucose levels approaching normal values (2.96 mg/dL). Similarly, lactate dehydrogenase (LDH) activity, which was markedly reduced in the cancer groups (T1: 1096 U/L, T2: 1100 U/L) in comparison to controls (1801 U/L), demonstrated significant improvement after probiotic treatment (T3-T6), with enzyme activity ranging from 1476 to 1603 U/L, suggesting enhanced cellular integrity. Markers of oxidative stress showed a decline in total antioxidant capacity (TAC) within the cancer groups (T1: 0.73 mmol/L, T2: 0.92 mmol/L) relative to the control (1.73 mmol/L); however, TAC was notably increased following probiotic administration (1.14-1.89 mmol/L). Further evidence of antioxidant protection conferred by *Lactobacillus casei* FG 12 against DMBA- and TCE-induced carcinogenesis was demonstrated through the activities of catalase (CAT), peroxidase (POD), and the levels of lipid peroxidation (LPO). Collectively, these data underscore the probiotic's capacity to mitigate metabolic disturbances, tissue damage, and oxidative stress associated with chemical-induced carcinogenesis, thereby affirming its protective role.

DISCUSSION

This approach to cancer causation in animal models is of vital importance in understanding the initiation and progression of tumours, and this can be applied in veterinary medicine. These models are used to model the complicated interactions of genetic, environmental and chemical elements that lead to cancer formation. The information obtained through these studies can not only advance the diagnosis and treatment of cancer in veterinary practice but also contribute to the comparative study of cancer in other species for a deeper understanding of oncogenesis (Zhang *et al.,* 2024). DMBA and TCE are two of the most prevalent carcinogens used to induce mammary and liver cancers in rodents respectively (Adewale *et al.,* 2024).

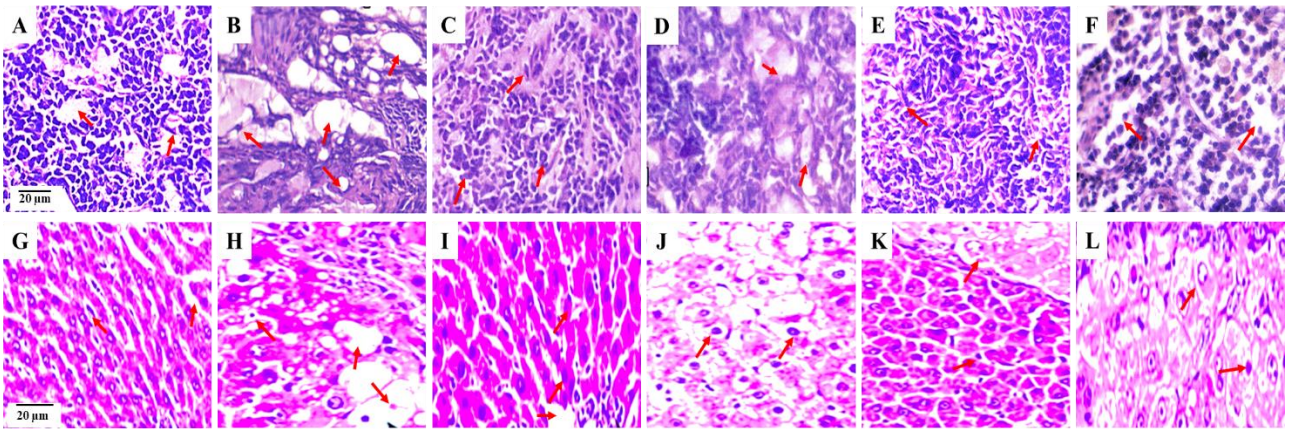

Fig. 5: Histopathological analysis demonstrated the protective effects of *L. casei* FG12 against DMBA-induced breast cancer, and TCE-induced liver cancer in rats (H&E, 100x). A) control breast tissues; (B) DMBA-induced mammary cancer; (C) *L. casei* FG12-treated group, (D) 5-fluorouracil-treated group; (E) *L. casei* FG12-treated group after mammary cancer infection, (F) Fluorouracil-treated group after mammary cancer infection, (G), control breast tissues; (H) TCE-induced liver cancer; (I) *L. casei* FG12-treated group, (J) 5-fluorouracil-treated group; (K) *L. casei* FG12-treated group after liver cancer infection, (L) Fluorouracil-treated group after liver cancer infection. Scale bar, 20µm.

Table 3: Impact of *Lactobacillus casei* DSM 28872 on cellular components and oxidative stress markers in DMBA-induced mammary cancer, and TCE-induced liver cancer in rats

Markers	C	T1	T2	T3	T4	T5	T6	T7	T8
Glucose	4.04±0.3	1.47±0.5	2.37±0.6	2.96±0.5	3.2±0.2	3.3±0.6	3.6±0.3	3.5±0.5	4.2±0.7
LDH	1801±3.5	1096±4.6	1100±6.2	1476±10.3	1488±9.2	1503±11.2	1603±10.1	1533±8.8	1625±7.5
Ca	0.21±0.01	0.22±0.01	0.29±0.00	0.2±0.0	0.26±0.02	0.15±0.01	0.19±0.01	0.25±0.04	0.33±0.01
TAC	1.73±0.5	0.73±0.02	0.92±0.05	1.14±0.2	1.22±0.3	1.35±0.3	1.89±0.2	1.46±0.4	2.2±0.2
CAT	45.05±0.6	12.05±0.6	13.2±0.6	43.84±0.9	45.33±0.8	44.1±0.8	44.6±0.6	47.33±0.5	47.33±0.9
POD	21.21±0.8	11.73±0.3	12.1±0.9	20.78±0.4	21.33±0.5	21.25±0.7	21.64±0.8	23.41±0.7	23.96±0.5
LPO	44.62±0.6	145.95±3.6	155.3±5.6	56.65±0.9	58.33±0.9	51.22±0.9	52.41±0.8	53.66±0.9	54.21±0.8
TPC	11.69±0.8	19.69±0.6	21.3±0.8	11.02±0.3	11.89±0.5	11.23±0.3	11.55±0.4	12.11±0.5	12.36±0.7

Data were expressed as mean ± SE (n = 5 for each group). Different letters indicate significant differences at P< 0.05. C, control rats not infected, T1, DMBA-induced breast cancer, T2, TCE-induced liver cancer, T3, *L casei* FG12-treated rats, T4, Fluorouracil-treated rats, T5, mammary cancer induced rats and treated with *L casei* FG12, T6 liver cancer induced rats and treated with *L casei* FG12, T7 mammary cancer induction and treated with Fluorouracil, T8 liver cancer rats and treated with Fluorouracil.

The typical metabolic activation of DMBA occurs via the enzyme cytochrome P450, with CYP1B1 being the predominant enzyme. This process converts DMBA into highly reactive epoxide species that can bind to DNA, forming damaging DNA adducts and inducing oxidative stress. These molecular events activate oncogenes such as HER-2 and c-myc, thereby promoting genomic instability that facilitates cancer initiation and progression (Currier *et al.,* 2005; Ma *et al.,* 2018).

Strains of *Lactobacillus casei* play a significant role in the prevention and treatment of DMBA-induced mammary tumors through the regulation of apoptotic pathways. They achieve this by augmenting the expression of pro-apoptotic genes such as *BAX* and *caspase*-3, as well as modulating the anti-apoptotic gene *BCL-2*, thereby leading to the elimination of damaged cells and the inhibition of tumor growth (Tiptiri-Kourpeti *et al.,* 2016; Sankarapandian *et al.,* 2022). Probiotics enhance the immune system by increasing the synthesis of *IL-2*, a crucial signaling molecule that initiates the activation of natural killer cells and T-cells. Additionally, they facilitate a shift in cytokine profiles towards *Th17*-type responses, which are vital for tumor surveillance and the eradication of tumor cells, thus supporting improved immune monitoring and response (Jacouton *et al.,* 2019; Abd El-Hack *et al.,* 2022; Sankarapandian *et al.,* 2022; Umair *et al.,* 2022).

Oncogenes such as survivin and c-myc are suppressed, and beneficial bioactive compounds including short-chain fatty acids, conjugated linoleic acids, and antimicrobial agents are produced by *Lactobacillus casei* strains. These substances play a role in preventing tumor development and maintaining the healthy functioning of tissues (Molska and Regula, 2019; Nakkarach *et al.,* 2021; George and Ghosh, 2025).

Trichloroethylene (TCE) is among the most prevalent environmental pollutants, metabolized in the liver via CYP2E1 enzyme, and is a primary causative agent of liver cancer. This metabolic process generates reactive intermediates that induce oxidative stress, mitochondrial damage, inflammation, and DNA damage, all of which contribute to the development of liver tumors (Blossom *et al.*, 2017; Ilieva *et al.*, 2022; Lou *et al.*, 2024). TCE suppresses hepatic function by inducing the depletion of glutathione, a vital antioxidant, and augmenting lipid peroxidation along with ineffective antioxidant defense mechanisms. This particularly disrupts the redox couple of cysteine/cystine, which plays a crucial role in safeguarding cells against oxidative stress. Furthermore, TCE adversely impacts gut microbiota by reducing beneficial bacterial populations and promoting dysbiosis, thereby contributing to its toxicity (Blossom et al., 2017; Ilieva et al., 2022). Additionally, it activates inflammatory pathways involving TNF-α, IL-6, and IFN-γ, which facilitate chronic inflammation and carcinogenesis, while concurrently impairing metabolic functions.

Probiotics such as *Lactobacillus casei* safeguard the liver against damage induced by TCE by reversing the effects of antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase. They also promote increased production of glutathione and

reduce lipid peroxidation, thereby maintaining oxidative balance and preserving liver tissue integrity (Jantararussamee *et al.,* 2021; Guo et al., 2023). Lactobacillus casei mitigates inflammation by decreasing pro-inflammatory cytokines and elevating the anti-inflammatory cytokine *IL*-10. Furthermore, it positively influences gut health by encouraging the proliferation of beneficial bacteria that secrete protective substances to support a balanced and healthy microbiome (Aghamohammad *et al.,* 2022; Bamola *et al.,* 2022). The anti-cancer properties of probiotics such as Lactobacillus casei are evidenced by their ability to silence oncogenic signaling pathways such as *mTOR*, Wnt/β-catenin, and *NF-κB*. They also enhance the activity of tumor suppressor genes and produce metabolites that facilitate detoxification processes by binding harmful metals, neutralizing free radicals, and upregulating phase II detoxification enzymes, thereby contributing to cancer prevention and treatment (Garavaglia *et al.,* 2025).

It is also probable that the carcinogenicity of both DMBA and TCE is attributable to the oxidative stress they induce. Consequently, antioxidant defense mechanisms play a central role in probiotic protection. The beneficial effects of probiotics encompass the production of antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase; the synthesis of antioxidants such as glutathione, vitamins, and phenolic compounds; and the direct neutralization of free radicals to mitigate oxidative damage caused by environmental carcinogens. This comprehensive antioxidant activity is advantageous for cellular health and aids in reducing the adverse effects of chemical-induced carcinogenesis (Salem *et al.,* 2022). The extent of this effect varies depending on the probiotic strain and can be augmented through genetic modification or the co-expression of antioxidant genes (Melnyk *et al.,* 2017; Gu *et al.,* 2024). Probiotics stimulate the antioxidant response via the Nrf2 pathway, a crucial regulator of cellular resistance to oxidative stress induced by ultraviolet radiation. Upon activation, Nrf2 translocates to the nucleus and binds to antioxidant response elements (AREs), thereby initiating the expression of numerous genes involved in the biosynthesis and detoxification of antioxidant enzymes. This overall activation facilitates the cellular neutralization of free radicals and toxins, thereby enhancing cellular health and resilience against environmental stressors (Melnyk *et al.,* 2017; El-Saadony *et al.,* 2021, 2022).

The significance of probiotics resides in their immunomodulatory effects, which encompass the mobilization of immune cells such as dendritic cells, macrophages, natural killer (NK) cells, and tumor-specific T-cells. They also augment the production of immunoglobulins, collectively establishing a robust immune surveillance system that detects and eradicates precancerous cells, thereby contributing to cancer prevention and management (Jacouton *et al.,* 2019; Yousefi *et al.,* 2019). These immunological responses are carefully regulated to maintain a balance between the activation of potent antitumor immunity and the inhibition of inflammation, which could otherwise facilitate tumor growth. This equilibrium is essential to prevent cancers induced by chronic inflammation, ensuring that the immune response targets malignant cells while sparing normal tissue (Farhadi Rad et al., 2024; Garavaggi *et al.,* 2025).

The influence of probiotics on enhancing anticancer effects operates through the production of short-chain fatty acids, such as butyrate. These compounds provide energy to healthy cells, lower the gut pH to suppress harmful microbes, and regulate gene activity by inhibiting histone deacetylases. Additionally, they contribute to the formation of conjugated linoleic acids, which modify fat metabolism and membrane characteristics. These systemic modifications facilitate improvements in lipid profiles, insulin sensitivity, and nutrient utilization, thereby reducing cancer risk through hormone regulation and diminished inflammation (Molska and Regula, 2019; Alqahtani *et al.,* 2024; Beyari *et al.,* 2024).

Extensive experimental literature indicates that probiotic supplementation is a promising approach for mitigating the adverse effects of environmental carcinogens and supporting conventional cancer therapies. Given their safety profile and multiple protective benefits, probiotics warrant further research and development in the realm of cancer prevention. Future research should focus on optimizing specific probiotic strains and formulations tailored to various cancer types, developing targeted delivery methods, and rigorously confirming the efficacy and appropriate dosage of probiotics through well-designed clinical trials. The potential of probiotics to improve treatment outcomes and minimize side effects when combined with chemotherapy or radiotherapy is significant, owing to their immune-enhancing and anti-inflammatory properties. Studies involving DMBA-induced and TCE-induced carcinogenesis in animal models demonstrate that *Lactobacillus casei* strains exert a comprehensive anticancer effect by modulating antioxidant defenses, immune responses, and metabolic processes. These findings underscore the potential role of probiotics both in clinical cancer prevention and as supportive agents in cancer treatment.

Conclusions: The anti-cancer effects of probiotics against chemical-induced cancer, such as DMBA and TCE, demonstrate the promising nature of microbiome-based strategies in cancer treatment in veterinary care. These advantageous bacteria act in several ways, with such pathways as antioxidant activity, immune system regulation, induction of programmed cell death, and metabolic changes, to address different cancer stages. The deeper we learn about such mechanisms, the more probiotics are likely to become a promising method of decreased cancer incidence and enhanced treatment outcomes. The integration of probiotics into conventional prevention and curative measures of cancer provides a new way of achieving improved health and reduced cancer incidence in the world.

Competing interests: The authors declare that they have no competing interests.

Authors contribution statement: Conceptualization, MGA, and HA, formal analysis, MGA, and HA, investigation, MGA, and HA, data curation, MGA, and HA, writing original draft preparation, MGA, and HA, writing final manuscript and editing, MGA, and HA,

visualization and methodology, MGA, and HA. All authors have read and agreed to the published version of the manuscript.

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