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Promoting longevity in aged liver through NLRP3 inflammasome inhibition using tauroursodeoxycholic acid (TUDCA) and SCD probiotics

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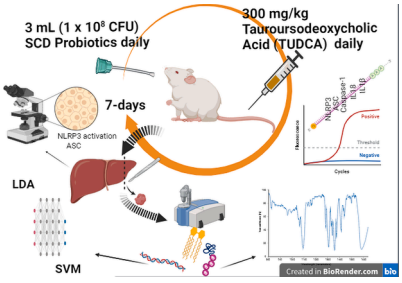
HIGHLIGHTS

- TUDCA improves lipid profiles, reducing cholesterol esters.
- SCD Probiotics alter fatty acids and protein structures.
- Both treatments reduce liver inflammation and fibrosis.
- SCD Probiotics decrease inflammasome gene expression.
- Combined therapy shows promise for aging liver health.

GRAPHICAL ABSTRACT



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ABSTRACT

This investigation explores the combined influence of SCD Probiotics and tauroursodeoxycholic acid (TUDCA) on liver health in elderly male Sprague-Dawley rats. Through the administration of intravenous TUDCA (300 mg/kg) and oral SCD Probiotics (3 mL at 1 × 10^8 CFU) daily for one week, this study evaluates the biomolecular composition, histopathological alterations, and inflammasome activity in the liver. Analytical methods encompassed ATR-FTIR spectroscopy integrated with machine learning for the assessment of biomolecular structures, RT-qPCR for quantifying inflammasome markers (NLRP3, ASC, Caspase-1, IL18, IL1β), and histological examinations to assess liver pathology. The findings reveal that TUDCA prominently enhanced lipid metabolism by reducing cholesterol esters, while SCD Probiotics modulated both lipid and protein profiles, notably affecting fatty acid chain lengths and protein configurations. Histological analysis showed significant reductions in cellular degeneration, lymphatic infiltration, and hepatic fibrosis. Furthermore, the study noted a decrease in the immunoreactivity for NLRP3 and ASC, suggesting suppressed inflammasome activity. While SCD Probiotics

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reduced the expression of certain inflammasome-related genes, they also paradoxically increased AST and LDH levels. Conversely, an exclusive elevation in albumin levels was observed in the group treated with SCD Probiotics, implying a protective role against liver damage. These results underscore the therapeutic potential of TUDCA and SCD Probiotics for managing age-associated liver disorders, illustrating their individual and synergistic effects on liver health and pathology. This study provides insights into the complex interactions of these agents, advocating for customized therapeutic approaches to combat liver fibrosis, enhance liver functionality, and decrease inflammation in aging populations.

1. Introduction

Probiotics, defined as living microorganisms, offer substantial health benefits when consumed in appropriate amounts. These benefits are particularly evident in the context of healthy aging, skin rejuvenation, and liver health, as delineated by Sivamaruthi et al. (2020). With advancing age, the human microbiome undergoes significant changes, characterized notably by a reduction in the prevalence of beneficial bacterial populations (Ceylani & Teker, 2022). This microbial imbalance is linked to the emergence of age-related pathologies, including metabolic dysfunctions, cardiovascular ailments, and systemic inflammatory responses. Probiotics are instrumental in enhancing the gut microbiota, bolstering immune capabilities, and mitigating inflammatory processes, potentially reducing the incidence of these age-associated conditions (Yoo & Kim, 2016). The liver, an essential organ responsible for metabolic regulation, detoxification, and energy storage, also experiences a decline in function with age. This decline can precipitate liver-related diseases, such as non-alcoholic fatty liver disease (NAFLD). Research indicates that probiotics facilitate liver detoxification processes and safeguard against various liver diseases, including NAFLD (Yang et al., 2021). Additionally, probiotics exhibit anti-inflammatory properties that avert hepatic inflammation (Arai et al., 2022). In a controlled clinical setting, the administration of probiotics was shown to enhance liver functionality and reduce the risk of hepatic diseases in elderly subjects (Kobyliak et al., 2018). Given their comprehensive impact on gut microbiota, immune response, liver health, and dermal rejuvenation, probiotics represent a crucial element in promoting healthy aging. Therefore, incorporating probiotic supplements into the dietary regime is essential for maintaining optimal health in the later stages of life (Suez et al., 2018; Teker et al., 2024). This enhanced understanding underscores the significant therapeutic potential of probiotics as a cornerstone in the dietary management of aging individuals, offering a multifaceted approach to health maintenance and disease prevention.

Bile acids, which are synthesized in the liver from cholesterol, possess amphipathic properties and are essential for lipid digestion. The gut microbiota plays a crucial role in regulating the synthesis and metabolic pathways of these acids (Long et al., 2017). Tauroursodeoxycholic acid (TUDCA), a conjugated bile acid derivative, is formed through the union of ursodeoxycholic acid (UDCA) and taurine. The gut microbiota produces UDCA, which is then recycled to the liver via enterohepatic circulation, where it conjugates with taurine to create TUDCA (Winston & Theriot, 2020). Recent studies have highlighted TUDCA's effectiveness in enhancing gut microbiota composition (Ceylani, 2023; Lu et al., 2021) and mitigating inflammation and intestinal barrier disruption, as demonstrated in various animal models (Song et al., 2022; Thaiss et al., 2018). TUDCA's therapeutic benefits are extensive, particularly in the context of liver health and the aging process. Its mechanisms are believed to involve the protection of hepatic cells from cytotoxic bile acids, the reduction of inflammatory responses, and the modulation of energy metabolism, all of which contribute to improved hepatic function (Hou et al., 2017). Moreover, TUDCA has been shown to enhance insulin sensitivity, a critical factor in maintaining metabolic health and preventing diabetes (Zangerolamo et al., 2022). Given its broad range of beneficial effects, TUDCA is increasingly recognized as a potential agent in anti-aging therapies, holding promise for the mitigation of age-related pathologies.

Aging is an elaborate biological process characterized by a progressive decrease in liver function, mainly due to the accumulation of reactive oxygen species, as outlined in recent studies (Yao et al., 2024). This age-related deterioration is further exacerbated by inflammation, which disrupts the dynamics within the inflammasome signaling pathways, essential for maintaining cellular integrity. Notably, the NLRP3 inflammasome, vital in the cellular response to damage and stress, may become hyperactive, resulting in heightened inflammatory responses. This phenomenon is particularly evident in the context of chronic liver conditions such as fibrosis and steatosis, which are prevalent in metabolic disorders and further intensified by the aging process (Youm et al., 2013). The interplay between the NLRP3 inflammasome complex and age-related hepatic impairment is significantly influenced by the modified regulation of the NLRP3 inflammasome components. This modification triggers the activation of inflammatory cascades within the liver, thus contributing to the pathology associated with aging. Consequently, the increased activation of inflammasomes within this molecular context is recognized as a critical catalyst in the progression of aging-related cellular and tissue deterioration (Lee et al., 2021). This heightened understanding underscores the potential for targeted interventions aimed at modulating inflammasome activity as a strategy to mitigate age-related hepatic decline and preserve organ function.

Within the realm of gerontology, the liver is subject to profound biochemical and structural transformations that predispose it to chronic pathologies. The complex interplay between the liver's biomolecular attributes and its functional capacity intimates that precise, targeted interventions could ameliorate age-related deterioration. This research posits that the concurrent application of SCD Probiotics and TUDCA might exert a synergistic effect on the liver's biomolecular framework, potentially mitigating or even reversing the age-related histological and inflammatory alterations observed. Specifically, this investigation hypothesizes that TUDCA, recognized for its choleretic and anti-apoptotic properties, in concert with SCD Probiotics, acclaimed for their advantageous impact on the gut-liver axis, could collectively augment liver functionality, diminish inflammation, and enhance histopathological outcomes in senescent hepatic tissues. This hypothesis rests on the notion that strategically modulating the hepatic microenvironment via these interventions may forge a pioneering therapeutic strategy for tackling age-associated liver ailments. This approach embodies a holistic strategy aimed at bolstering liver health among the elderly population, suggesting a comprehensive methodological framework for future investigations and clinical applications.

2. Material method

2.1. Animal studies

In this study, male Sprague-Dawley rats aged 24 months acted as the experimental model and were divided into four groups: a control group ($n = 7$), a probiotic-treated group ($n = 7$), a TUDCA-treated group ($n = 7$), and a group receiving concurrent administration of TUDCA and SCD Probiotics ($n = 7$). Each group underwent treatment for a period of seven days, during which the rats were provided with a standard rodent diet ad libitum. TUDCA was administered intravenously at a dosage of 300 mg/kg via the caudal vein (Zangerolamo et al., 2022), while the probiotic regimen comprised oral gavage of 3 mL (1×10^8 CFU) daily (Ceylani,

2023). The probiotic formulation employed in this investigation included 11 distinct microbial strains sourced from the product line of SCD Probiotics (Essential Probiotics XI - 500 ml, H.S. Code: 2206.00.7000), such as *Bacillus subtilis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Saccharomyces cerevisiae*, and *Streptococcus thermophilus*. After the treatment phase, the animals were euthanized using ether anesthesia, and their liver tissues were promptly excised, immediately flash-frozen in dry ice, and subsequently preserved at −80 °C for future analyses. The animals were housed in accordance with established care protocols for experimental animals, and the study received ethical clearance from the Ethics Committee of the Saki Yenilli Experimental Animal Production and Practice Laboratory (approval number: 2022/03), ensuring adherence to rigorous ethical standards in animal research.

2.2. Analysis of samples by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy

In the investigation, hepatic samples from all participants ((2 × 24=48 in total) were placed directly onto the Zn/Se crystal surface of the Attenuated Total Reflectance (ATR) device, an integral component of the PerkinElmer spectrometric system, without undergoing any form of pre-treatment. These samples were then analyzed using an ATR-FTIR spectrometer, also provided by PerkinElmer, configured with a spectral resolution of 4 cmˆ−1 and executed a series of 32 scans per sample. The spectral data were captured and processed utilizing the Spectrum One software by PerkinElmer, covering a comprehensive wavelength range from 4000 to 650 cm^{-1} (Teker et al., 2024).

2.3. Prediction studies with different machine learning approaches based on big spectral data

In carrying out the research, Linear Discriminant Analysis (LDA) and Support Vector Machine (SVM) were applied to differentiate between the experimental groups utilizing spectral data obtained from Fourier Transform Infrared (FTIR) spectrometers. The spectral data underwent preliminary preprocessing using The Unscrambler® X 10.3 (CAMO Software AS, Norway), which incorporated a baseline offset transformation across the 4000–650 cm-1 spectrum. Following this, the adjusted spectra were subjected to Principal Component Analysis (PCA), a technique for unsupervised pattern recognition. After normalizing the standard deviation and validating via the leveraged-correction method, the data were further assessed in specific biomolecular regions—lipids (3000–2700 cm-1), proteins (1700–1500 cm-1), nucleic acids, and polysaccharides (1200–650 cm-1)—utilizing the Singular Value Decomposition (SVD) algorithm (Gurbanov et al., 2021).

LDA, a supervised classification method, was used to project n-dimensional feature samples into an m-dimensional space. In this process, the PCA-generated data served as inputs into the LDA model within The Unscrambler® X 10.3 software. A data matrix was constructed, incorporating category variable columns, and spectra from various sample categories were compiled to form a training set. Classification was attained using the quadratic method based on projections from the top 7 PCA components, with prior probabilities derived from the training dataset. The LDA outcomes were visualized and analyzed through discrimination plots, prediction matrices, and confusion matrices (Dogan et al., 2021).

The application of the SVM technique, a robust machine learning classifier, was carried out using the same multivariate analysis software. After undergoing identical preprocessing steps, the spectra were segregated into different categories to form an additional training dataset. The SVM model utilized a nu-SVC classification type with a linear kernel method. The nu parameter was set at 0.5, and equal weights of 1.00 were assigned uniformly. The training and cross-validation accuracies were determined through a 14-segment cross-validation process. Finally, the SVM model was applied to all sample datasets to establish a comprehensive classification mode (Ceylani et al., 2022).

2.4. Quantification studies of FTIR spectral bands

The following text has been carefully selected bands with the highest absorbance within specific spectral regions. The start and end frequencies of these bands have been precisely defined. To quantify the integral areas of these predefined frequency ranges, the OPUS 5.5 software was utilized. Additionally, to assess the peak characteristics of these bands, a virtual line was projected vertically from the midpoint of each band's baseline to its peak, and the length of this line was measured using a digital ruler tool. Next, a point was marked at 0.75 of the total line length, from which a horizontal line was extended across the band to determine the bandwidth values. This method allows for an accurate determination of band characteristics, which is crucial for detailed biomolecular analysis in spectroscopic studiesd (Ardahanlı et al., 2022).

2.5. Hematoxylin & eosin (H&E) staining

Following the implementation of euthanasia, liver specimens were preserved in a 10 % neutral buffered formalin solution for a period of 48 h, adhering to established histological procedures outlined in previous research (Adomshick et al., 2020; Keskin et al., 2023). Subsequently, the liver tissues were embedded in paraffin and cut into 5 μm thick sections using a rotary microtome (Leica Biosystems, Germany). These sections were then carefully mounted onto glass slides for staining. To enhance the visualization of cellular structures and morphology, the tissue slices underwent staining with Hematoxylin and Eosin (H&E). The observations and analyses of the stained sections were conducted using an Olympus BX53 light microscope (Olympus, Tokyo, Japan).

2.6. Masson trichrome (MT) staining

After the removal of paraffin, additional sections of the liver were stained using Masson's Trichrome (MT) staining with a commercial kit (Cat No: 04–010,802, Bio-Optica, Milan, Italy) following the manufacturer's instructions. This protocol was used to evaluate the density of collagen fibers in the liver tissue, which is a critical indicator of fibrosis. The study aimed to investigate the therapeutic effects of TUDCA, SCD Probiotics, and their combined regimen on liver fibrosis. All staining procedures were performed at room temperature to ensure consistency and reliability in the histological analysis.

2.7. Quantification of histological parameters

Hematoxylin and Eosin (H&E) staining was conducted to evaluate lymphatic infiltration and micro vesicular steatosis, characterized by the presence of micro-lipid droplets, using the Image J software (Fiji version). This method was used in conjunction with Masson's Trichrome (MT) staining, as described in recent literature (Keskin et al., 2023). Microscopic examinations were performed at a magnification of 200x, allowing for detailed observation of hepatic fatty infiltration and micro vesicular steatosis in the experimental cohorts. Quantitative analyses involved measuring the stained areas under a consistent threshold. Signal intensities were recorded from five images per section for each rat within a group using advanced image processing software. Each liver section was sampled at 10–12 random areas for comprehensive histopathological evaluation (Teker et al., 2023). The microscopic analyses were performed independently by two observers who were blinded to the group allocations, ensuring impartiality in the data assessment. Microphotographs were captured and analyzed using an Olympus BX53 light microscope equipped with an Olympus DP27 camera and processed through Olympus cellSens Entry imaging software (Olympus cellSens Entry, Japan) (Livak & Schmittgen, 2001).

2.8. Immunohistochemical analysis

The procedure involved in the study utilized paraffin-embedded liver sections to investigate the effects of SCD probiotics and TUDCA on the NLRP3 inflammasome components (NLRP3 and ASC) in aged liver tissue. The liver sections underwent a series of steps, including deparaffinization and rehydration, followed by the application of a 3 % hydrogen peroxide solution for 10 min to block endogenous peroxidase activity and minimize non-specific antibody interactions. The sections were then rinsed with PBS. Subsequently, the sections were subjected to heat-induced antigen retrieval using a 10 mM sodium citrate buffer, undergoing multiple microwave exposures of 4–5 min each, followed by a cooling period at room temperature. The next phase involved overnight incubation at 4 °C with polyclonal antibodies, specifically anti-NLRP3 (Elabscience, E-AB-93,112, at a dilution of 1:200) and anti-ASC (Elabscience, E-AB-30,582, at a dilution of 1:200). The following day, the sections were processed using biotinylated antibodies (TP-125-BN, Thermo Scientific) and streptavidin peroxidase (TS-125-HR). Immunodetection was conducted using a 3–3′Diaminobenzidine (DAB substrate kit, ab64238, Abcam) as a chromogen for 3–5 min. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted for microscopic examination. The immunostained areas were imaged using an Olympus DP27 camera and Olympus cellSens Entry imaging systems under an Olympus BX53 light microscope. For quantitative analysis, the immunoprecipitation intensities of stained cells were measured in ten randomly selected fields for each group using Image J (Fiji), across three different sections per rat in each group (Ydens et al., 2015).

2.9. Biochemical analysis

Following the administration of euthanasia, blood samples were collected from all of the rats and subjected to centrifugation for 15 min at 3500 rpm in a refrigerated centrifuge set at 4 °C. This process facilitated the separation of serum from the blood samples. The serum samples were then analyzed using a Hitachi C502 automated biochemical analyzer (Roche, Germany) equipped with commercial assay kits provided by Roche Diagnostics. These assays quantitatively assessed the serum levels of liver function markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and albumin. The concentrations of these biochemical markers were reported in International Units per milliliter (IU/mL) for the enzymes and grams per liter (g/L) for albumin. All of the analytical procedures were performed with meticulous attention to detail, following the manufacturer's specified protocols (Roche Diagnostics), ensuring high fidelity in the biochemical quantification.

2.10. RNA isolation and cDNA synthesis

RNA extraction from the tissue samples was carried out using the GeneAll® Hybrid-R™ kit (Cat. No. 305–101, Korea). The NanoDrop QC SkanIt software 4.1 was employed on a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, MA, USA) to evaluate the integrity and concentration of the extracted RNA. After RNA isolation, cDNA synthesis was performed according to the protocol provided by the ProtoScript First Strand cDNA Synthesis Kit (SuScript cDNA Synthesis Kit, Catalog No: RT01A025, Türkiye). The cDNA synthesis process involved specific thermal cycling conditions: an initial denaturation step at 70 °C for 5 min, followed by a 1 h incubation period at 42 °C, and finally, enzyme inactivation at 80 °C for 5 min. The Sensoquest Thermocycler Labcycler with Thermoblock 96 Gold Plated Silver 012–103, located in Germany at Hannah-Vogt-Str.1, facilitated this thermal cycling process. Following synthesis, the cDNA was preserved at −20 °C for subsequent analyses, ensuring the precise preparation of cDNA necessary for downstream genetic analyses.

2.11. Primers and gene expression analysis

The study's primers were formulated using the Primer3 online tool. For the quantitative PCR (qPCR) assays, the Gapdh gene was chosen as the reference gene to standardize the data. The SuScript 1-Step SYBR qPCR Kit (Catalog No: RT01A046, 200Rxn, Ankara, Turkey) was employed to assess gene expression changes. The qPCR assays were executed on the Rotor-Gene Q system (QIAGEN, Inc., Hilden, Germany) with strip tubes having a capacity of 0.1 mL across four wells. Each reaction mixture for a single strip tube was carefully prepared to a total volume of 10 μL. The thermal cycling conditions comprised an initial cycle at 50 °C for 2 min, followed by 95 °C for 1 min, and then forty cycles at 95 °C for 10 s, and 60 °C for 30 s. The mRNA levels were quantified using the $2-\Delta\Delta CT$ method, a quantitative analysis technique proposed by Livak and Schmittgen (Aba et al., 2023). The primer sequences used in the qPCR assays are presented in Table 1.

2.12. Statistics

Statistical evaluations and graph plots of the results were made using GraphPad Prism 10.01 (GraphPad, USA). The data were analyzed using One-way ANOVA and/or unpaired *t*-test, and the significance levels were stated as P 0.05 *, $P \leq 0.01$ **, $P \leq 0.001$ ***, and $P \leq 0.0001$ ****. Results are presented as mean ± SEM (standard error of the mean).

3. Results

3.1. TUDCA and SDC probiotics caused significant changes in liver biomolecules

Substantial qualitative changes were detected through Linear Discriminant Analysis (LDA), yielding a precision rate of 100 % across all biomolecular compositions in liver tissues (refer to Fig. 1A, Tables S1, S2). The discrimination plots clearly positioned the data from the control group (CLI), SDC Probiotics group (PLI), TUDCA group (TLI), and the combined treatment group (PTLI) within distinct coordinates (Fig. 1A). Qualitative shifts identical to those observed in the lipid, protein, and nucleic acid profiles were achieved, with 100 % accuracy (Fig. 1B and Figs. S1, S2, Tables S3–S8). Furthermore, Support Vector Machine (SVM) classification exhibited robust effectiveness, as demonstrated by high training (95 %) and validation accuracies (92.5 %) across the biomolecular spectrum of the liver (Fig. S3).

Quantitative spectral analysis targeted specific band frequencies

Table 1
Sequences of specific primers.

Gene name	Elongation Position	Sequence (5′−3′)	Refs.
NLRP3	Forward Reverse	TGTGAGAAGCAGGTTCTACTCT GGATGCTCCTTGACCAGTTGG	(Ydens et al., 2015)
ASC	Forward Reverse	CAGCACAGGCAAGCACTCA GGTGGTCTCTGCACGAACT	(Ydens et al., 2015)
Caspase-1	Forward Reverse	GGGACCCTCAAGTTTTGCC GACGTGTACGAGTGGTTGTATT	(Ydens et al., 2015)
IL1β	Forward Reverse	GCAACTGTTCCTGAACTCAACT ATCTTTTGGGGTCCGTCAACT	(Ydens et al., 2015)
IL18	Forward Reverse	GACTCTTGCGTCAACTTCAAGG CAGGCTGTCTTTTGTCAACGA	(Ydens et al., 2015)
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	Forward Reverse	TGGACCTCATGGCCTACATG AGGGAGATGCTCAGTGTTGG	(Aba et al., 2023)

Note: Gapdh was used as a reference.

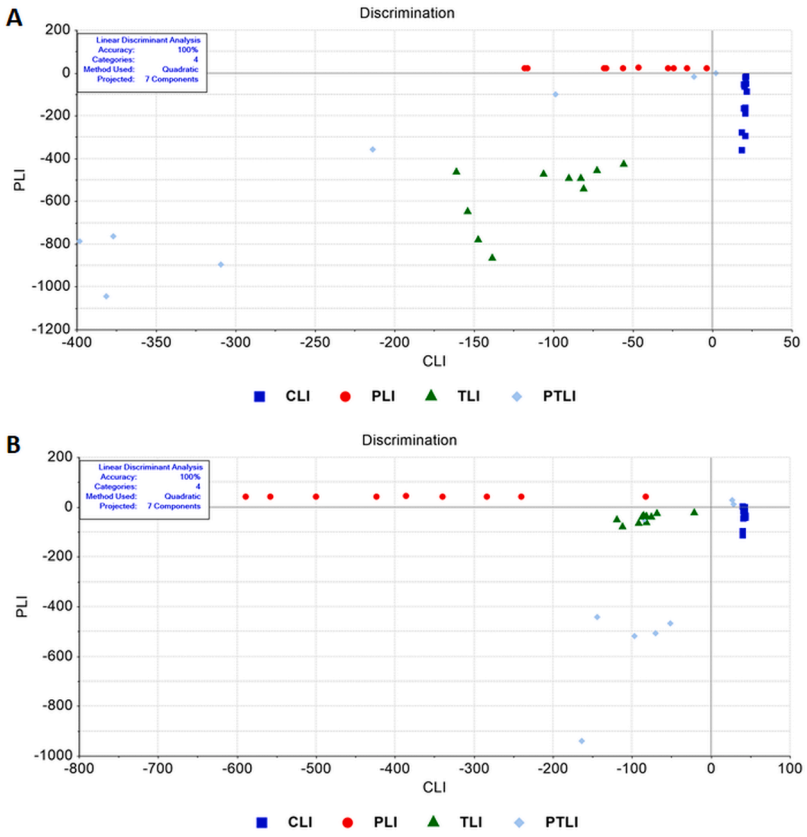

Fig. 1. LDA discrimination plot for liver samples in the full (4000–650 cm^{-1}) spectral region (A). LDA discrimination plot for liver samples in the lipid (3000–2700 cm^{-1}) spectral region (B). CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

associated with various biomolecules (Fig. 2a–f), including: CH3 antisymmetric stretching at 2955 cm^{-1}, indicative of lipids and proteins, which increased in the TUDCA group but decreased in the SCD Probiotics group. CH2 antisymmetric stretching at 2922 cm^{-1}, another lipid marker, decreased across all groups, most notably within the TUDCA group. C=O stretching at 1740 cm^{-1}, a cholesterol ester marker, which exhibited an increase in the SCD Probiotics group and a decrease in the TUDCA group. Amide I band at 1653 cm^{-1}, representing the α-helical structure of proteins, remained consistent across groups, whereas the Amide II band at 1545 cm^{-1}, indicative of β-sheet structures, showed a significant increase in the combined treatment group. PO2 antisymmetric stretching at 1239 cm^{-1} and symmetric stretching at 1080 cm^{-1} both markers of nucleic acids, decreased significantly in the TUDCA group, with the SCD Probiotics group showing a significant reduction only in the antisymmetric band.

Further analysis of the band area ratios disclosed substantial alterations: The acyl chain of fatty acids ratio (A_{2922}/A_{2955}) demonstrated an increase in the SCD Probiotics group and a decrease in the TUDCA group (see Fig. 3a). Protein phosphorylation ratios (A_{1239}/A_{2955} and A_{1080}/A_{1545}) displayed variation, with an upsurge observed in the SCD Probiotics group and a decline in the TUDCA group, except for a rise in the latter's phosphorylation ratio at (A_{1080}/A_{1545}) (see Fig. 3b, c). Protein conformation (A_{1653}/A_{1545}) notably increased in the TUDCA group (see Fig. 3d). Protein carbonylation (A_{1740}/A_{1545}) significantly decreased in all treatment groups (see Fig. 3e).

Moreover, notable changes occurred in the bandwidths of spectral parameters, such as the CH3 antisymmetric stretching at 2955 cm^{-1} and CH2 antisymmetric stretching at 2922 cm^{-1}, as well as the Amide I band at 1653 cm^{-1} and membrane dynamics (A_{2922}/A_{2955}) (see Fig. S4a-d). The CH3 antisymmetric band experienced a unique increase in the TUDCA group, while the CH2 antisymmetric, Amide I, and membrane dynamics decreased in both the SCD Probiotics and TUDCA groups, indicating significant biomolecular alterations across the experimental conditions.

3.2. *Effects of SCD probiotics, TUDCA and combined theatment on age-related liver changes*

Hematoxylin and Eosin (H&E) staining was employed to evaluate the histological changes in the livers of aged rats that were treated with SCD probiotics, TUDCA, or their combination. As depicted in Fig. 4a, the control group (CLI) exhibited several histopathological modifications, such as irregular hepatocyte arrangement, enlarged sinusoidal spaces, an increased density of bile ducts and Kupffer cells, as well as marked lymphatic and neutrophil infiltration. On the other hand, the groups treated with SCD Probiotics (PLI), TUDCA (TLI), and their combination revealed notable reductions in the intensity of lymphocytic and neutrophil infiltration and improvements in cellular degeneration associated with aging, as compared to the CLI group. The study additionally explored the effects of these treatments on microvesicular steatosis, a form of hepatic fat accumulation prevalent in aged livers. The CLI group demonstrated a significant increase in microvesicular steatosis, while the treated groups, including those receiving SCD Probiotics, TUDCA, and their combination, displayed a considerable reduction in lipid droplet density (Fig. 4b). It is worth noting that there was no discernible difference in the reduction of steatosis between the PLI and TLI groups when assessed independently. Collectively, these findings suggest that SCD Probiotics and TUDCA, either individually or in combination, hold promise for mitigating age-associated liver inflammation, bile duct proliferation, and disruptions in lipid metabolism, thereby contributing to the preservation of liver health in aged subjects.

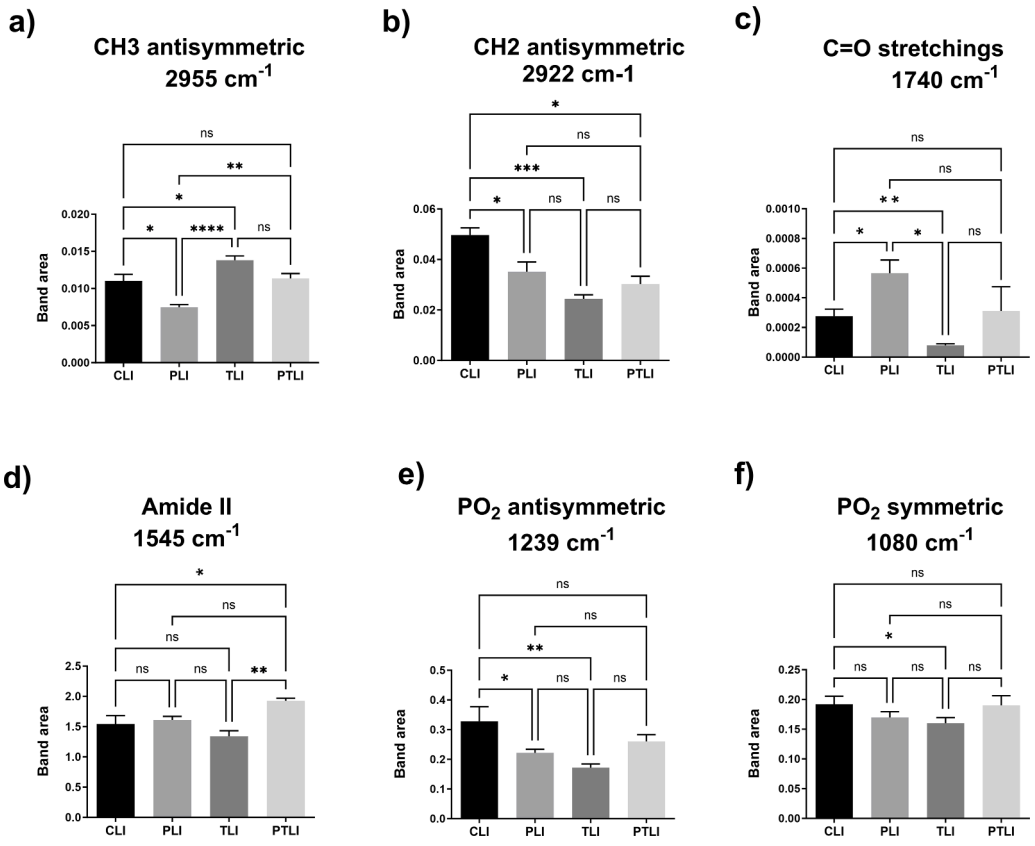

Fig. 2. The quantitative changes in liver-associated spectrochemical parameters. The band areas for (a) CH_3 antisymmetric (2955 cm^{-1}), (b) CH_2 antisymmetric (2922 cm^{-1}), (c) lipid carbonyl (C=O stretchings, 1740 cm^{-1}), (d) Amide II (1545 cm^{-1}), (e) PO_2 antisymmetric (1239 cm-1), and (f) PO_2 symmetric (1080 cm^{-1}). CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

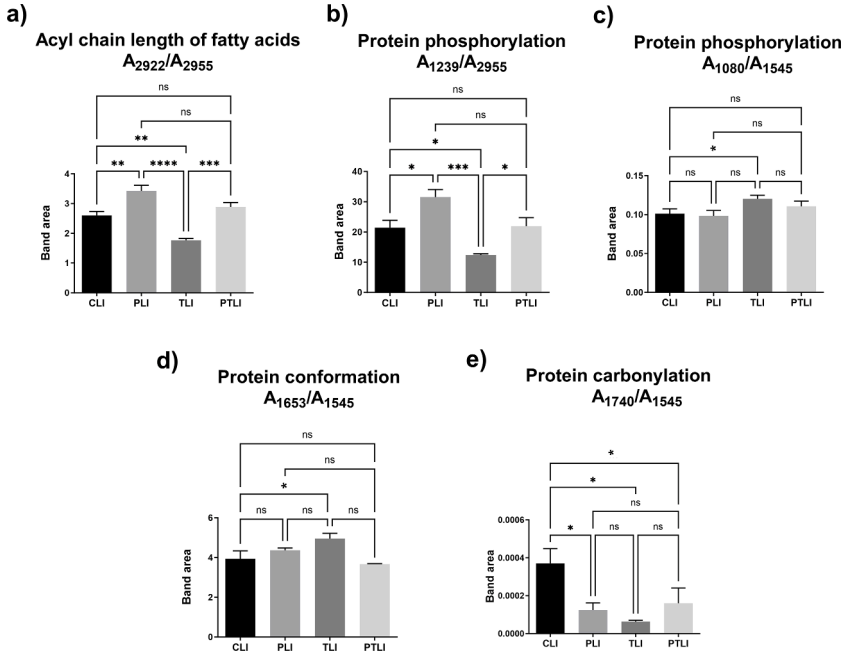

Fig. 3. The quantitative changes in liver-associated spectrochemical parameters. The band area ratios for (a) acyl chain length of fatty acids (A_{2922}/A_{2955}), (b) protein phosphorylation (A_{1239}/A_{2955}), (c) protein phosphorylation (A_{1239}/A_{2955}), (d) protein conformation (A_{1653}/A_{1545}), and (e) protein carbonylation (A_{1740}/A_{1545}). CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

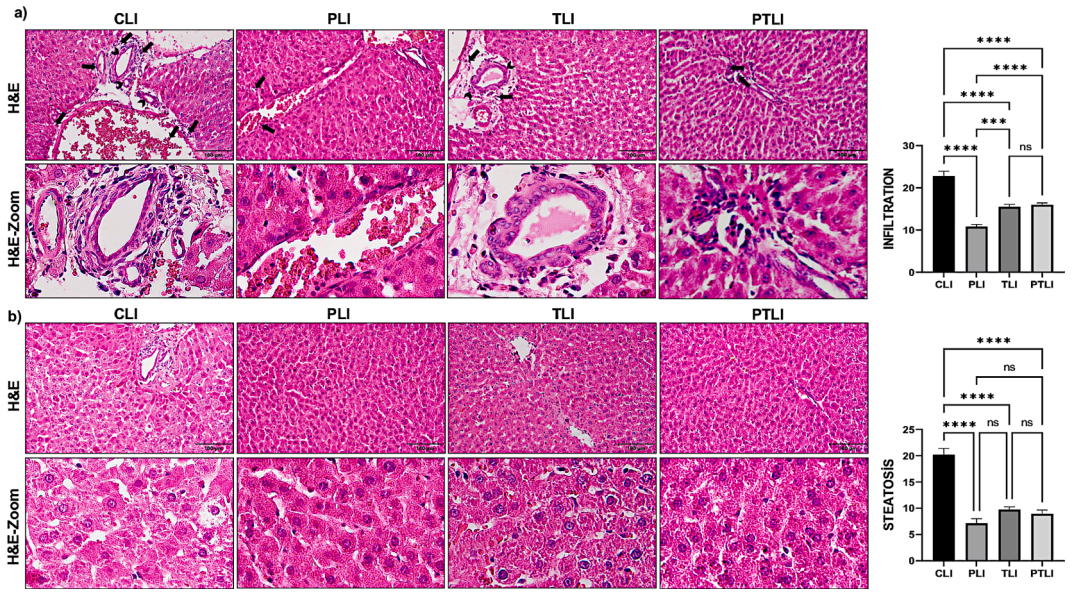

Fig. 4. Effects on liver histological architecture a. Representative images of hematoxylin and eosin (H&E) staining and quantification of infiltration b. Representative images of H&E staining and quantification of micro vesicular steatosis. Black arrows show inflammation. Black arrow's heads indicate bile ducts. The areas in the relevant microphotographs of all groups are enlarged below. Values are expressed as mean ±SEM. $p \leq 0.001$ ***, and $p \leq 0.0001$ ****, ns: not significant. Scale bar:100 μm. CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

3.3. SCD probiotics, TUDCA and combined treatments alleviates liver fibrosis in the aged liver

The evaluation of the density of collagen accumulation in liver sections stained with Masson's Trichrome (MT) using ImageJ software demonstrated the efficacy of SCD Probiotics, TUDCA, and their combination in mitigating age-related liver fibrosis. The analysis revealed that the intensity of blue-stained areas, indicative of collagen deposition in the liver tissue, was significantly reduced in the groups treated with SCD Probiotics (PLI), TUDCA (TLI), and the combined therapy (PTLI) compared to the control group (CLI), as depicted in Fig. 5. These findings suggest that SCD Probiotics, TUDCA, and their synergistic application have the potential to ameliorate age-associated liver fibrosis, highlighting their therapeutic utility in reducing pathological collagen accumulation in the liver.

3.4. SCD probiotics and TUDCA exhibited a marked reduction in inflammasome-related gene expressions

The research examines the effects of using SCD Probiotics, Tauroursodeoxycholic acid (TUDCA), and a combination of both in modifying the expression of genes related to inflammasome activity, specifically NLRP3, ASC, Caspase-1, IL18, and IL1β. The results indicate that the use of SCD Probiotics alone produced the most significant reduction in gene expression levels, as illustrated in Fig. 6. Interestingly, the concurrent use of SCD Probiotics and TUDCA showed a less pronounced impact on gene expression levels than when each was used individually. These findings were further supported by Immunohistochemistry (IHC) staining, which provided additional validation and a more in-depth understanding of the molecular mechanisms that contribute to the anti-inflammatory properties of these treatments.

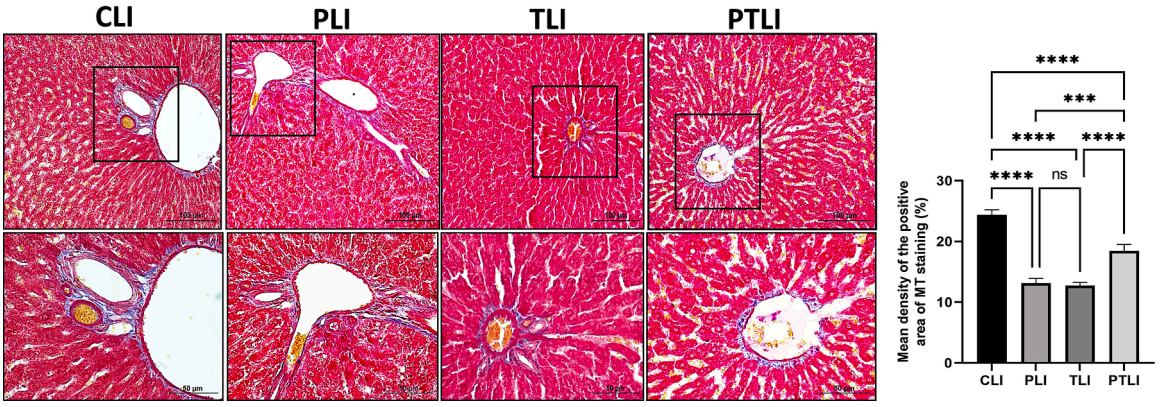

Fig. 5. Effects on liver fibrosis. Representative images of Masson trichrome (MT) staining showing collagen deposition in the aged contol rat and all treatment groups liver tissue with quantification of collagen density area fraction (%) in all groups. The areas enclosed in squares in the relevant microphotographs of all groups are enlarged below. Values are expressed as mean ±SEM. $p \leq 0.001$ ***, and $p \leq 0.0001$ ****, ns: not significant. Scale bar:50 and 100 μm. CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

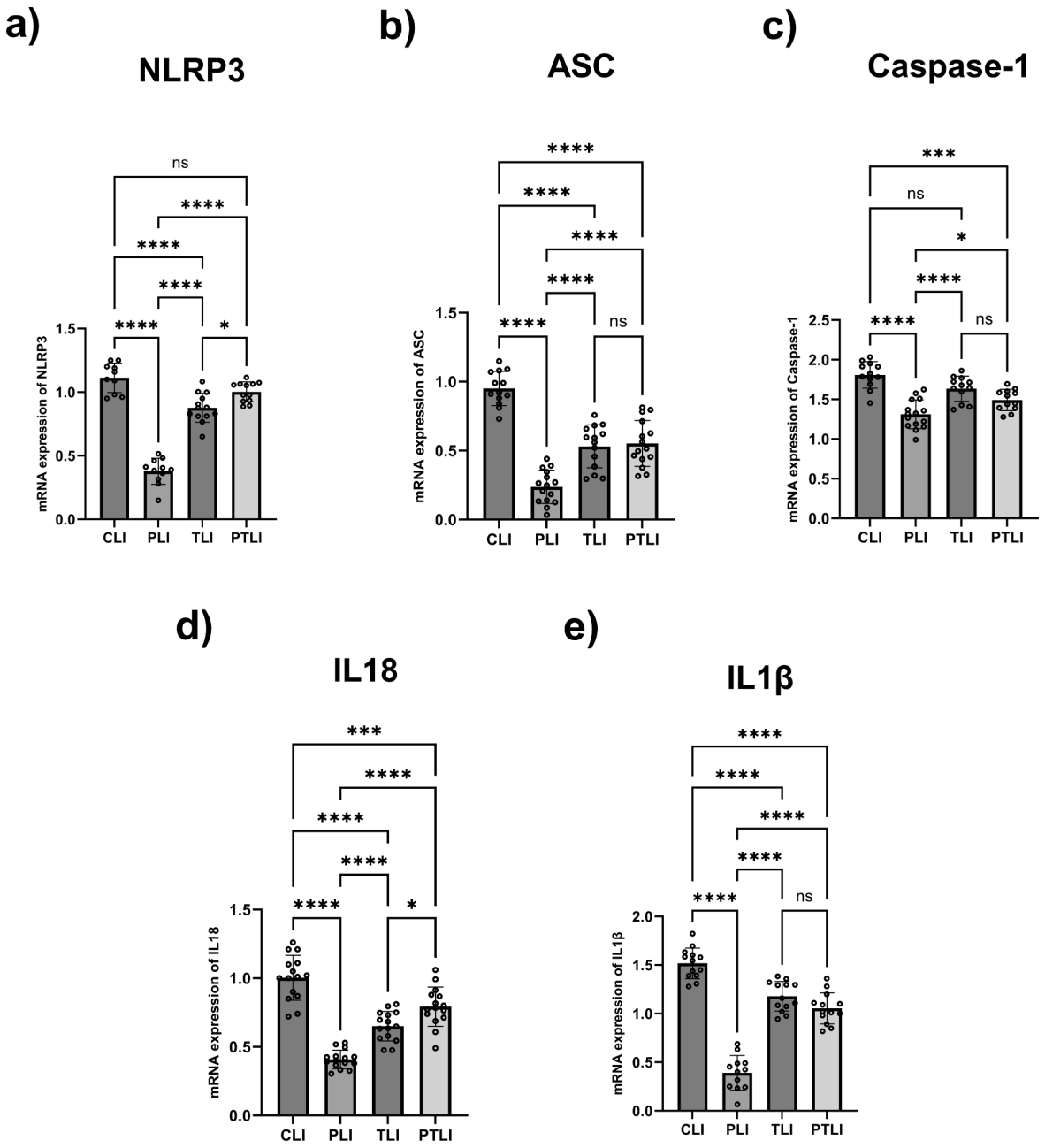

Fig. 6. The relative mRNA expression levels in inflammasome-related gene expressions. (A) mRNA expression levels of NLRP3, (B) mRNA expression levels of ASC, (C) mRNA expression levels of Caspase-1, (D) mRNA expression levels of IL18, and (E) mRNA expression levels of IL1β. All data are shown as Mean ± SEM ($n = 5$ and 3 replicates); p values are derived using Tukey's Multiple Comparisons Test followed by One-way ANOVA. Statistically significant differences are indicated in the following way: ns $p > 0.05$ (not significant); * $p < 0.05$ (significant); *** $p < 0.001$ and **** $p < 0.0001$ (extremely significant). CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

3.5. SCD probiotics and TUDCA administered to aged rats reduce inflammation by suppressing NLRP3 inflammasome activation in liver tissue

Hyperactivation of the NLRP3 inflammasome has been shown to trigger inflammatory responses and cellular damage, particularly in the aging process (Liang et al., 2024). Our histological evaluations uncovered extensive inflammation within the liver of aged specimens. In light of this, we analyzed the expression levels of NLRP3 and ASC, critical elements of the NLRP3 inflammasome complex, using immunohistochemistry (IHC)-stained liver sections. As depicted in Fig. 7, heightened expression of NLRP3 and ASC was predominantly observed in regions with severe inflammation. Compared to the control group (CLI), there was a significant reduction in the expression levels of NLRP3 and ASC in the groups treated with SCD Probiotics (PLI), TUDCA (TLI), and their combination (PTLI), as indicated by p-values less than 0.05. Moreover, the comparison between the TUDCA-only and the combined treatment groups showed no statistically significant differences in the expression levels of these markers, with p-values exceeding 0.05. Collectively, these findings demonstrate that the activation of the NLRP3 inflammasome, driven by inflammation in aged liver tissue, is markedly reduced by treatments with SCD Probiotics, TUDCA, and their combination.

3.6. Biochemical analysis of liver enzymes in blood serum

The results depict that serum AST levels were conspicuously elevated

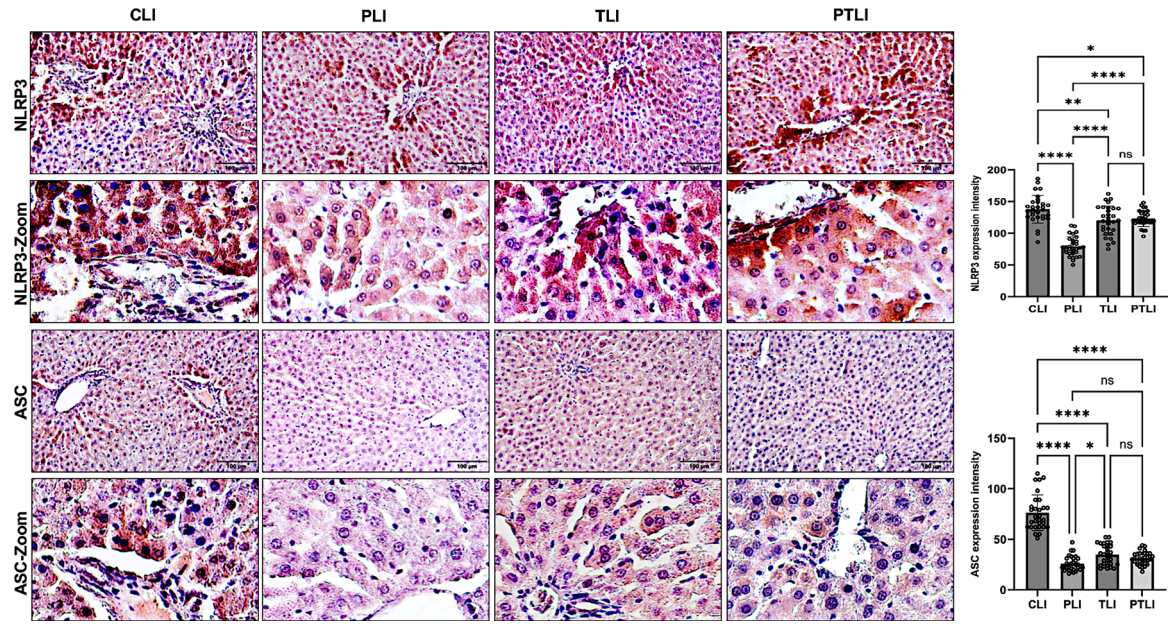

Fig.y 7. Representative photomicrographs showing NLRP3, and ASC immunostaining and respective quantifications of expression intensity in the liver. The zooms of the selected areas are shown below the respective microphotographs. Expression intensities (NLRP3 and ASC) are given in the right panel. Values are expressed as mean ± SEM. $p < 0.05$ *, $p \leq 0.01$ **, and $p \leq 0.0001$ ****, ns: not significant. Scale bar: 100 μm. CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were applied together).

in the treatment groups when compared to the control group (Fig. S5a). It is essential to acknowledge that although AST is indicative of liver function, it is not exclusive to the liver tissue; it is also present in muscle tissue, which often declines in elderly individuals due to cellular degeneration, potentially resulting in lower AST levels in aged rats. In contrast, ALT and ALP levels did not display significant variations between the groups. LDH, a vital enzyme primarily found in muscle, liver, and kidney cells, demonstrated an increase in serum levels within the treatment groups as compared to the control group (Fig. S5b). The metabolic changes that occur with aging remain inadequately understood; however, variations in albumin levels have been correlated with both acute and chronic liver conditions. Although a decline in serum albumin concentration is usually observed with aging, our study revealed that the treated groups exhibited a significant increase in serum albumin levels (Fig. S5c), suggesting a favorable response to the treatments in mitigating age-related declines in liver function.

4. Discussion

The study's results provide strong evidence that supports the initial hypothesis, demonstrating the combined effectiveness of SCD Probiotics and tauroursodeoxycholic acid (TUDCA) in addressing age-related alterations in liver tissue. The significant biomolecular changes observed, along with marked reductions in histopathological indicators of liver deterioration and inflammation, corroborate the anticipated modes of action for both TUDCA and SCD Probiotics. This concordance between predicted and observed effects not only confirms the hypothesis but also elucidates the complex biochemical pathways involved in mediating the therapeutic impacts of these treatments. The strategic integration of TUDCA's choleretic and cytoprotective properties with the gut-liver axis modulation by SCD Probiotics provides a robust approach to enhance liver function and protect against the challenges posed by aging-related diseases.

As the liver ages, it experiences significant physiological changes, including reduced blood flow and regenerative capacity, increased fat accumulation, diminished detoxification functions, and altered gene expression (Erdogan et al., 2023; Kim et al., 2015). These transformations heighten the risk of liver-related diseases and other health complications in the elderly. Furthermore, aging-related dysbiosis may result in diminished bacterial diversity, compromised gut barrier function, and various metabolic disorders (Ceylani et al., 2023; Teker & Ceylani, 2022). This study investigates the impact of a combined treatment with SCD Probiotics and TUDCA on the molecular and structural integrity of the liver in aged rats. Using machine learning techniques and spectroscopy, the research analyzed the molecular profiles and revealed the distinct effects of TUDCA and SCD Probiotics on the liver's lipid, protein, and nucleic acid content. Infrared (IR) spectroscopy provided a deeper understanding of these variations (Severcan & Haris, 2012). However, co-administration of TUDCA and SCD Probiotics produced some less pronounced or contradictory effects, which complicates drawing definitive conclusions about their individual or synergistic effects. For instance, TUDCA elevated a specific lipid marker (CH3 antisymmetric band), while SCD Probiotics decreased it, suggesting potential lipid peroxidation, which could be harmful (Valente et al., 2021). Nonetheless, it is essential to consider that free radicals, which can sometimes be increased by such biochemical changes, play vital roles in physiological signaling, and probiotics have been shown to mitigate oxidative damage in patients with liver diseases (Esposito et al., 2009; Viña et al., 2013).

Aging has a considerable impact on cholesterol metabolism in the liver, which often results in an excessive accumulation of cholesterol. This study demonstrated that TUDCA enhanced bile acid synthesis and reduced cholesterol accumulation in the serum and liver of mice, as indicated by a reduction in the C=O stretching band, which is indicative of cholesterol esters (Lu et al., 2021). In contrast, SCD Probiotics appeared to increase this band, which contradicts previous findings suggesting that probiotics, including strains such as *B. longum*, L. *acidophilus*, and L. *plantarum*, typically decrease cholesterol levels in serum and liver (Ooi & Liong, 2010). Furthermore, TUDCA was observed to decrease the acyl chain of fatty acids band. The acyl chain of fatty acids, which consists of a hydrocarbon chain terminating in a carboxyl group, plays crucial roles in membrane structure and function, energy storage, and cellular signaling (Vanni et al., 2019). Although an increase in this band can be beneficial due to the stability it provides to lipid bilayers, an excess of saturated lipid acyl chains may reduce bilayer fluidity. Therefore, a decrease might be favorable, aligning with recent research

that positions TUDCA as an effective modulator of lipid metabolism, enhancing the breakdown and synthesis of fatty acids and other lipids (Sun et al., 2020). However, further investigations are required in the cohort receiving both treatments to draw definitive conclusions, as no significant differences were observed in these parameters in the combined treatment group.

Research demonstrates that Tauroursodeoxycholic Acid (TUDCA) may alleviate endoplasmic reticulum (ER) stress, correct disruptions in the unfolded protein response, and stabilize mitochondrial function (Yoon et al., 2016). Spectrochemical protein analysis revealed significant changes in protein conformation exclusively in the TUDCA-treated group, highlighting its specific impact. Furthermore, TUDCA has demonstrated increased effectiveness in protein phosphorylation, a crucial regulatory process in cellular signaling. Protein phosphorylation, which involves the addition of a phosphate group by a kinase enzyme, modulates protein activity and influences cell growth, differentiation, apoptosis, and metabolism. This modification is essential for regulating metabolic pathways and stress responses in the liver, where dysregulated phosphorylation is associated with diseases such as NAFLD and hepatocellular carcinoma. TUDCA's impact on phosphorylation may restore signaling dynamics, improving metabolic processes and reducing inflammation through pathways like MAPK and NF-κB. These findings emphasize TUDCA's potential to enhance liver function and mitigate inflammation, underscoring the critical role of protein phosphorylation in maintaining cellular integrity and function (Lee et al., 2019). Although our current study employs ATR-FTIR spectroscopy, histological analyses, and RT-qPCR to provide significant insights into the biomolecular composition and gene expression profiles in the liver, further examination, such as proteomics of the changes in protein phosphorylation levels, is necessary to fully understand their implications. Integrating omics technologies such as transcriptomics, proteomics, and metabolomics will further enhance our understanding of the biological processes involved.

TUDCA was found to be highly effective in affecting membrane dynamics, a crucial biological parameter involving the fluidity and flexibility of cellular membranes. Membrane dynamics are essential for various cellular processes, including nutrient transport, signal transduction, and membrane fusion events. The proper functioning of membrane dynamics ensures the stability and functionality of cell membranes, which is vital for maintaining cellular homeostasis (Vanni et al., 2019). While both SCD Probiotics and TUDCA significantly reduced the spectral band associated with membrane dynamics, indicating improvements in membrane stability and functionality, the combined treatment did not display a substantial difference in this parameter. This was particularly evident in the markers evaluated for nucleic acids. This observation suggests that while both treatments are effective individually in enhancing membrane dynamics and nucleic acid markers, their combined effect does not significantly enhance these individual contributions. This highlights the potential for these treatments to act through distinct or overlapping pathways that do not exhibit additive effects when combined, underscoring the complexity of their interactions at the molecular level.

Studies have previously established that age-related hyperinsulinemia contributes to increased fat deposition, enlargement of adipocytes, and enhanced lymphatic infiltration, leading to fat accumulation in the liver and systemic insulin resistance (Kirwan et al., 2001; Templeman et al., 2017). Our research indicates that the observed increase in microvesicular steatosis and lymphatic infiltration in aged control mice may be attributed to a decline in age-associated hyperinsulinemia (Janssen, 2021; Wolden-Hanson, 2010). Notably, the elderly control group demonstrated a significant increase in both microvesicular steatosis and lymphatic infiltration compared to all treatment groups. Based on these findings, we hypothesize that the reduction in microvesicular steatosis and lymphatic infiltration observed in the groups treated with SCD Probiotics and TUDCA may be linked to mitigating age-related hyperinsulinemia, as no changes in dietary intake were reported during the one-week treatment period. Furthermore, we propose that the decreased lymphatic infiltration rates in the treatment groups may alleviate inflammation in hepatocytes associated with aging, due to reduced fat deposits in the liver. Previous research has shown that TUDCA can effectively lower body weight, reduce fat content, and decrease cellular inflammation in aged mice, as well as in diet-induced obese mice (Vettorazzi et al., 2017). Additionally, TUDCA has been demonstrated to reduce lipid accumulation in the liver of obese mice by improving insulin sensitivity (Ozcan et al., 2006). In summary, the results of our study suggest that both TUDCA and SCD Probiotics may be beneficial in preventing or mitigating the severity of tissue damage in age-related liver diseases in the aged rat liver.

Research has demonstrated a correlation between insulin resistance and the accumulation of triglycerides, as well as the buildup of fatty acid intermediate metabolites. Our findings suggest that the control group of elderly individuals exhibited an elevated concentration of shortened acyl chain fatty acids, which is indicative of insulin resistance. On the other hand, the treatment groups that received SCD Probiotics and TUDCA displayed an elongation in the length of acyl chain fatty acids, indicating a reduction in age-related hyperinsulinemia. This change in acyl chain length aligns with the observed reduction in microvesicular steatosis in the liver tissue of these treatment groups, which supports the research by Zangerolamo et al. (2022b). Furthermore, the treatment groups that received TUDCA exhibited decreased levels of hepatic triglycerides and cholesterol. This decrease may have also contributed to the reduced density of lipid droplets observed in the liver tissue of aged rats, indicating the potential of TUDCA and SCD Probiotics in mitigating lipid-related hepatic alterations in the context of aging.

Fibrosis, a hallmark of aging characterized by the excessive accumulation of extracellular matrix components such as collagen, is a prevalent degenerative phenomenon across various organs, including the liver, heart, and kidneys (Gagliano et al., 2002). In our research, Masson's Trichrome (MT) staining of liver tissues from aged rats revealed a pronounced increase in collagen density, indicative of hepatic fibrosis. In contrast, treatments with SCD Probiotics, TUDCA, and their combination resulted in a notable reduction in collagen densities. These findings led us to conclude that interventions with SCD Probiotics and TUDCA are effective in mitigating liver fibrosis associated with aging. In addition, studies have demonstrated significant improvements in serum ALT, AST, ALP, and albumin levels following TUDCA treatment, as well as a reduction in tissue fibrosis in liver biopsies from patients treated with TUDCA (Pan et al., 2013). Another study suggested that exogenous supplementation of bile components may serve as a potential therapeutic strategy to ameliorate aging-related liver fibrosis (Kusaczuk, 2019; Li et al., 2016). Consistent with these findings, our MT staining results also showed a significant decrease in collagen density in the group treated with TUDCA, further supporting the potential of this treatment approach in the clinical management of liver fibrosis in aging populations.

The results of this study convincingly reveal that the administration of TUDCA and, more specifically, SCD Probiotics, significantly restricts inflammasome activity commonly associated with the aging process. The considerable decrease in the transcription of genes, such as NLRP3, ASC, and Caspase-1, underscores the potent anti-inflammatory properties of this combined treatment. Additionally, the diminished levels of pro-inflammatory cytokines like IL18 and IL1β serve as crucial indicators of reduced hepatic inflammation. These observations strongly advocate for the potential of TUDCA and SCD Probiotics as therapeutic agents to regulate inflammasome activity in aging contexts (Kasti et al., 2021; Lebeaupin et al., 2015). The findings of this research have far-reaching implications, suggesting that the synergistic effect of these compounds may prove instrumental in mitigating age-related inflammatory processes. The reduction in gene expression, particularly prominent with SCD Probiotics, aligns well with the results of immunohistochemical analyses, thereby reinforcing the efficacy of these interventions in combating inflammatory responses during the aging

process.

Age-related liver diseases often result from hepatic inflammation, which plays a significant role in the uncontrolled immune responses observed during this proces (Youm et al., 2013). This inflammation primarily stems from the dysregulation of inflammasome functions, exacerbated by the increase of inflammatory cytokines associated with hepatic aging (Cañadas-Lozano et al., 2020). When hepatocytes are targeted by damaged cells, they contribute to the pathogenesis of age-related liver conditions (Yu et al., 2022). Such age-related damage induces inflammation, with pro-inflammatory cytokines further amplifying this inflammatory response (Lee et al., 2021). The activation of the NLRP3 inflammasome plays various roles depending on the cell type involved, particularly impacting hepatocytes (Gallego et al., 2020). Among these roles, the NLRP3 inflammasome is known to exacerbate the pathophysiology of age-related liver diseases by inducing lipid droplet formation in hepatocytes, which promotes dyslipidemia (Zilu et al., 2019). Additionally, elevated levels of NLRP3 and ASC in liver samples have been observed in previous studies, indicating their significant presence in aging livers (Adjei-Mosi et al., 2023; Yao et al., 2024). The data from this study underscore the central role of the NLRP3 inflammasome in exacerbating liver inflammation. In this research, treatments with SCD Probiotics and TUDCA were effective in regulating lipid metabolism and modulating the activation of the NLRP3 inflammasome, thereby reducing steatosis in the aged liver. The findings indicate that the NLRP3 inflammasome actively contributes to the uncontrolled activation of the age-related immune response and the natural aging process, which can intensify liver inflammation and fibrosis. Therefore, targeting the NLRP3 inflammasome with anti-inflammatory therapies offers a promising approach to alleviating chronic inflammatory hepatic pathology associated with aging and may serve as a potential therapeutic target for managing age-related liver disease.

In our research, we discovered a decline in LDH levels among the control group of elderly participants that significantly increased following the administration of SCD Probiotics, TUDCA, and their combination. This increase suggests an improvement in metabolic activity, likely influenced by these supplements. Our findings align with those of Kaczor et al. (2006), which propose that natural age-related reductions in muscle and bone mass may lead to decreased LDH levels, but interventions such as SCD Probiotics and TUDCA can mitigate this decline by stimulating metabolic processes. Additionally, our results support the notion that low ALT and AST levels in elderly individuals may indicate a reduced liver metabolic function, an idea that is consistent with research suggesting the role of these enzymes in predicting liver diseases and fibrosis during aging (Goh et al., 2015; Y. Lu et al., 2021).

5. Conclusion

In summary, this study supports the idea that SCD Probiotics and TUDCA can work together to improve the biological and histopathological features of the liver in older individuals, providing a potential treatment option for age-related liver diseases. By affecting lipid metabolism, protein conformations, and inflammasome activity, SCD Probiotics and TUDCA have been shown to significantly improve liver function and structural integrity. These results indicate the potential therapeutic benefits of SCD Probiotics and TUDCA, both separately and in combination, in reducing liver fibrosis, lowering inflammasome activity, and improving overall liver function. Further research is needed to explore the underlying mechanisms and to determine the potential of these interventions in human populations.

Data availability

All data generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Burcu Baba: Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Taha Ceylani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rafig Gurbanov:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Eda Acikgoz:** Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Data curation. **Seda Keskin:** Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Data curation. **Hüseyin Allahverdi:** Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis. **Gizem Samgane:** Visualization, Validation, Methodology, Investigation. **Huseyin Tombuloglu:** Visualization, Validation, Methodology, Investigation. **Hikmet Taner Teker:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.archger.2024.105517.

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