

1 **Promoting Longevity in Aged Liver through NLRP3 Inflammasome Inhibition Using**  
2 **Tauroursodeoxycholic Acid (TUDCA) and SCD Probiotics**

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## Abstract

25 This study investigates the combined impact of SCD Probiotics and tauroursodeoxycholic  
26 acid (TUDCA) on the biomolecular makeup, histological changes and levels of  
27 inflammasome in the liver tissue of 24-month-old male Sprague-Dawley rats. By  
28 administering TUDCA (300 mg/kg, intravenously) and SCD Probiotics (3 mL (1 x 10<sup>8</sup> CFU),  
29 orally) daily for a week, the researchers employed ATR-FTIR spectroscopy along with  
30 machine learning approaches such as Linear Discriminant Analysis (LDA) and Support  
31 Vector Machine (SVM) to analyze the biomolecular profiles. In addition, the study measured  
32 the expression levels of inflammasome markers NLRP3, ASC, Caspase-1, IL18, and IL1 $\beta$   
33 using RT-qPCR and examined liver sections for histopathological changes and NLRP3  
34 inflammasome activation. The results revealed significant differences in the levels of lipids,  
35 proteins, and nucleic acids, with TUDCA having a noteworthy impact on enhancing lipid  
36 bands and reducing cholesterol ester bands, while SCD Probiotics showed the opposite  
37 effects. Furthermore, TUDCA was found to decrease the acyl chain length of fatty acids and  
38 improve protein conformation, whereas SCD Probiotics increased both the acyl chain length  
39 and protein phosphorylation ratio, suggesting a decrease in lipid and protein dynamics from  
40 both treatments. The histological assessments showed significant reductions in cellular  
41 degeneration, lymphatic infiltration, hepatic fibrosis, and the immunoreactivity of NLRP3 and  
42 ASC in the treated groups. SCD Probiotics exhibited a marked reduction in inflammasome-  
43 related gene expressions, and the lowest gene expression levels were observed in the group  
44 receiving both treatments. Despite an increase in serum AST and LDH levels across all  
45 groups, only the SCD Probiotics group showed an increase in albumin levels. The findings  
46 suggest that SCD Probiotics, TUDCA, and their combined administration may provide a  
47 promising avenue for therapeutic interventions in age-associated liver conditions and may  
48 mitigate age-related liver fibrosis while enhancing liver functionality.

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50 **Keywords:** Keywords: TUDCA, SCD Probiotics, NLRP3 inflammasome, Inflammation,  
51 Fibrosis, Liver, ATR-FTIR

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## 55 INTRODUCTION

56 Probiotics are living microorganisms that, when consumed in adequate amounts, provide  
57 health benefits. Among their many benefits, probiotics have been shown to support healthy  
58 aging, rejuvenation, and liver health (1). Changes occur in the microbiome with aging,  
59 including a decline in the abundance of beneficial bacteria (2). This shift is associated with  
60 the onset of age-related diseases, including metabolic disorders, cardiovascular disease, and  
61 inflammation. Probiotics have been found to improve gut microbiota, promote immune  
62 function, and decrease inflammation, which can help to reduce the risk of these diseases (3).  
63 The liver is a vital organ that plays a critical role in metabolism, detoxification, and energy  
64 storage. However, liver function declines with age, which can lead to the development of liver  
65 diseases such as non-alcoholic fatty liver disease (NAFLD). Studies have shown that  
66 probiotics can aid in the removal of toxins from the liver and prevent various liver health  
67 issues, including NAFLD (4). Furthermore, probiotics' anti-inflammatory properties can  
68 prevent liver inflammation (5). In a randomized controlled trial, probiotic supplementation  
69 was found to improve liver function and reduce the risk of liver disease in elderly participants  
70 (6). The positive effects of probiotics on gut microbiota, immune function, liver health, and  
71 skin rejuvenation make them an important tool for healthy aging. Therefore, incorporating  
72 probiotics into the diet may be an essential component of maintaining good health in old age  
73 (7).

74 Bile acids are molecules that are produced in the liver from cholesterol, possessing both  
75 hydrophilic and hydrophobic properties, and serving a critical role in lipid digestion. The gut  
76 microbiota regulates the synthesis and metabolism of bile acids (8). Tauroursodeoxycholic  
77 acid (TUDCA) is a type of conjugated bile acid derivative formed by the combination of  
78 ursodeoxycholic acid (UDCA) and taurine. The gut microbiota generates UDCA, which then  
79 returns to the liver via enterohepatic circulation, where it conjugates with taurine to create

80 TUDCA (9). TUDCA has been shown to improve the gut microbiota (10) and reduce  
81 inflammation and barrier disruption in the intestines in animal studies (11,12). TUDCA has a  
82 wide range of health benefits, particularly for liver and aging health. It is believed to work by  
83 protecting liver cells from toxic bile acids, reducing inflammation, and regulating energy  
84 metabolism, which can enhance liver health (13). Additionally, TUDCA improves insulin  
85 sensitivity, which is essential for maintaining metabolic health and preventing diabetes (14).  
86 Its potential to safeguard against age-related diseases makes it a promising candidate for  
87 future anti-aging therapies.

88 Aging is a critical process characterized by the loss of hepatic function due to the  
89 accumulation of reactive oxygen species (15). Age-related inflammation disrupts interactions  
90 within the inflammasome signaling pathway, which serves a vital function within the cell. The  
91 NLRP3 inflammasome can be overactivated and produce an aggravated inflammatory process  
92 after chronic damage, such as metabolic disorders occurring during liver fibrosis and  
93 steatosis, or during a progressive deterioration process associated with aging (16). The  
94 relationship between the NLRP3 inflammasome complex and age-related liver damage is  
95 mediated by the altered expression of NLRP3 inflammasome components and the eventual  
96 activation of an inflammatory process in the liver. Therefore, the progression of severe  
97 activation of inflammasomes in this molecular framework can be defined as a crucial trigger  
98 in aging (17).

99 This study aims to investigating the synergistic effects of SCD Probiotics and TUDCA on  
100 histopathology and biomolecular profiles in aged rat liver tissue. By examining the  
101 biomolecular structures, histological alterations, gene expression and employing advanced  
102 analytical techniques, we hope to shed light on the potential benefits of these treatments in  
103 mitigating age-related liver damage. The insights gained from this study could pave the way  
104 for further investigations into the therapeutic potential of SCD Probiotics and TUDCA in

105 managing age-related liver disorders and improving the quality of life particularly for the  
106 elderly population.

## 107 **MATERIALS AND METHODS**

108 This section is presented as a supplementary file.

## 109 **RESULTS**

### 110 **TUDCA and SDC Probiotics caused significant changes in liver biomolecules**

111 Significant qualitative modifications were detected by LDA analysis with 100% accuracy for  
112 full biomolecules of the liver (**Fig. 1, Tables S1-S2**). Based on the discrimination plot, the  
113 data for the control (CLI), SDC Probiotics (PLI), TUDCA (TLI), and the applications (PTLI)  
114 in which the TUDCA and SCD Probiotics were applied together are clustered in separate  
115 coordinates (**Fig. 1**). The same qualitative changes were also seen for the lipids, proteins, and  
116 nucleic acids with 100% accuracy (**Fig. 2 and Figs. S1-S2, Tables S3-S8**). SVM  
117 classification capacity was also quite evident for the whole biomolecular content of the liver,  
118 revealing high training (95%) and validation accuracies (92.5%) (**Fig. S3**).

119 The quantitative analyses of spectral bands at  $2955\text{ cm}^{-1}$  ( $\text{CH}_3$  antisymmetric stretching: lipids  
120 and proteins),  $2922\text{ cm}^{-1}$  ( $\text{CH}_2$  antisymmetric stretching: lipids),  $1740\text{ cm}^{-1}$  ( $\text{C}=\text{O}$  stretching:  
121 cholesterol ester),  $1653\text{ cm}^{-1}$  (Amide I:  $\alpha$ -helical structure of proteins),  $1545\text{ cm}^{-1}$  (Amide II:  
122  $\beta$ -sheet structure of proteins),  $1239\text{ cm}^{-1}$  ( $\text{PO}_2$  antisymmetric stretching: nucleic acids), and  
123  $1080\text{ cm}^{-1}$  ( $\text{PO}_2$  symmetric stretching: nucleic acids) positions were performed for detailed  
124 analysis of biomolecules (**Fig. 3a-f**). The  $\text{CH}_3$  antisymmetric stretching, a commonly used  
125 lipid marker, increased in the TUDCA group but decreased in the SCD Probiotics group. The  
126  $\text{CH}_2$  antisymmetric, another lipid marker, decreased in all groups, with the greatest reduction  
127 observed in the TUDCA group. The  $\text{C}=\text{O}$  stretching, a marker of cholesterol ester, increased

128 in the SCD Probiotics group but decreased in the TUDCA group (**Fig. 3a-c**). The Amid I  
129 band, a widely used protein marker, did not show a significant difference between groups.  
130 However, in the group where both applications were used, there was a significant increase in  
131 the Amid II band, which is also considered a protein marker. (**Fig. 3d**). TUDCA caused a  
132 significant decrease in the PO2 antisymmetric and PO2 symmetric bands, which are  
133 considered nucleic acid markers, while SDC Probiotics only resulted in a significant decrease  
134 in the PO2 antisymmetric band (**Fig. 3e-f**).

135 The band area ratios for the acyl chain of fatty acids ( $A_{2922}/A_{2955}$ ), Protein phosphorylation  
136 ( $A_{1239}/A_{2955}$ ), Protein phosphorylation ( $A_{1080}/A_{1545}$ ), Protein conformation ( $A_{1653}/A_{1545}$ ) and  
137 Protein carbonylation ( $A_{1740}/A_{1545}$ ) of proteins were changed significantly (**Fig. 4a-d**). The  
138 SCD Probiotics group showed an increase in the acyl chain of fatty acids, whereas the  
139 TUDCA group showed a decrease (**Fig. 4a**). Similarly, the phosphorylation ratio ( $A_{1239}/A_{2955}$ )  
140 increased in the SCD Probiotics group but decreased in the TUDCA group. However, the  
141 phosphorylation ratio ( $A_{1080}/A_{1545}$ ) only increased in the TUDCA group (**Fig. 4b-c**). Protein  
142 conformation ( $A_{1653}/A_{1545}$ ) analysis showed a significant increase only in the TUDCA group  
143 (**Fig. 4d**). Protein carbonylation significantly decreased in all treated group (**Fig. 4e**).

144 Moreover, bandwidths of spectral parameters  $2955\text{ cm}^{-1}$  (CH3 antisymmetric stretching: lipids  
145 and proteins),  $2922\text{ cm}^{-1}$  (CH2 antisymmetric stretching: lipids),  $1653\text{ cm}^{-1}$  (Amide I:  $\alpha$ -  
146 helical structure of proteins), and membrane dynamics ( $A_{2922}/A_{2955}$ ) changed significantly  
147 (**Fig.S4a-d**). The CH3 antisymmetric band increased only in the TUDCA group, while the  
148 CH2 antisymmetric, Amide I, and membrane dynamics decreased in both the SCD Probiotics  
149 and TUDCA groups.

150

151 **Effects of SCD Probiotics, TUDCA and combined treatment on age-related liver**  
152 **changes**

153 H&E staining was performed to evaluate the histological changes in aged liver due to SCD  
154 probiotics, TUDCA, and combined treatments. As shown in **Fig.5a**, irregular hepatocyte  
155 arrangement, enlargement of sinusoidal areas, relative increase in bile duct density and  
156 Kupffer cells, and intense lymphatic and neutrophil infiltration were observed in CLI group.  
157 On the other hand, PLI, TLI and combined treatment groups showed a significant decrease in  
158 lymphocytic and neutrophil infiltration intensity and improvements in cellular degenerations  
159 developing in the aging process compared to the CLI group. From the findings obtained, it  
160 was observed that the combined treatment of SCD Probiotics and TUDCA may educe age-  
161 related inflammation and bile duct proliferation in the aged liver. However, when the effects  
162 of SCD Probiotics, TUDCA and combined treatment on microvesicular steatosis, a type of  
163 hepatic fat accumulation in aged livers were examined. It was observed that microvesicular  
164 steatosis was significantly increased in the CLI group, whereas a significant decrease in the  
165 density of lipid droplets was observed in the SCD Probiotics, TUDCA and combine treated  
166 groups compared to the CLI group (**Fig.5b**). There was no difference in the improvement rate  
167 (steatosis) between PLI only and TLI only groups. All findings indicate the potential of SCD  
168 Probiotics and TUDCA treatments to alleviate age-related liver damage, inflammation and  
169 lipid metabolism disorders.

170 **SCD Probiotics, TUDCA and combined treatments alleviates liver fibrosis in the aged**  
171 **liver**

172 Collagen accumulation density changes in MT stained liver sections were analyzed by ImageJ  
173 to investigate whether SCD Probiotics, TUDCA and combined treatments alleviate age-  
174 related liver fibrosis. In the results, the intensities of blue stained areas representing collagen

175 accumulation in the liver tissue of the PLI, TLI, and PTLI groups were significantly decreased  
176 compared to the CLI group (**Fig. 6**). These results together suggest that SCD probiotics,  
177 TUDCA, and combined treatment could reduce age-related liver fibrosis.

178 **SCD Probiotics and TUDCA exhibited a marked reduction in inflammasome-related**  
179 **gene expressions.**

180 The results of the study involving the use of SCD Probiotics, Tauroursodeoxycholic acid  
181 (TUDCA), and their combination in modulating the expression of NLRP3, ASC, Caspase-1,  
182 IL18, and IL1 $\beta$  genes related to inflammasomes are presented. The application of SCD  
183 Probiotics alone demonstrated the most significant reduction in the expression of the genes, as  
184 shown in **Figure 7**. Interestingly, the combination of SCD Probiotics and TUDCA resulted in  
185 a less pronounced effect on gene expression levels compared to when each treatment was  
186 applied independently. These observations were further supported by Immunohistochemistry  
187 (IHC) staining, which reinforced the initial findings and provided a deeper understanding of  
188 the molecular mechanisms underlying the anti-inflammatory effects of the treatments.

189 **SCD Probiotics and TUDCA administered to aged rats reduce inflammation by**  
190 **suppressing NLRP3 inflammasome activation in liver tissue**

191 Hyperactivation of the NLRP3 inflammasome contributes to inflammatory responses and  
192 cellular damage in aging (18). Our histological results revealed severe inflammation in the  
193 aged liver. Therefore, expression levels of NLRP3 and ASC associated with the NLRP3  
194 inflammasome complex were analyzed using IHC-stained liver sections. As shown in **Fig. 7**,  
195 NLRP3 and ASC expression were found to be high, particularly in areas of intense  
196 inflammation. Compared with the CLI group, a decrease in the expression levels of NLRP3  
197 and ASC was seen in the PLI, TLI, and PTLI groups ( $p < 0.05$ ). Furthermore, there was no  
198 statistical difference in NLRP3 and ASC expression levels between the TLI and combination

199 groups ( $p>0.05$ ). Together, the results obtained showed that NLRP3 inflammasome activation  
200 triggered by inflammation in the aged liver was significantly suppressed by SCD Probiotics,  
201 TUDCA and combined treatments.

## 202 **Biochemical analysis of liver enzymes in blood serum**

203 The results showed that serum AST levels significantly increased in the treatment groups  
204 compared to the control group (**Fig. 8a**), while ALT and ALP levels showed no significant  
205 differences. AST, not the only liver-specific enzyme, is also found in muscle tissue, which  
206 decreases in elderly individuals due to cellular degeneration. Consequently, AST levels may  
207 be low in aged rats. LDH, an essential enzyme found primarily in muscle, liver, and kidney  
208 cells, showed increased serum levels in the treatment groups compared to the control group  
209 (**Fig. 8b**). Metabolic adaptations associated with aging are not well understood, but changes in  
210 albumin levels have been linked to acute and chronic liver diseases. A decrease in serum  
211 albumin concentration with age has been previously reported, but this study found that treated  
212 groups had significantly increased serum albumin levels (**Fig. 8c**).

## 213 **DISCUSSION**

214 In the process of aging, the liver undergoes significant transformations, including a decline in  
215 blood flow and regenerative capacity, accumulation of fat, decreased detoxification abilities,  
216 and gene expression alterations (19,20). These shifts can boost the risk of liver disease and  
217 other health issues in seniors. The aging-related imbalance in gut microbiota, known as  
218 dysbiosis, can also lead to a decline in bacterial diversity, weakened gut barrier, and various  
219 metabolic disorders (21,22). This study explores how a combination of SCD Probiotics and  
220 TUDCA impacts the liver's molecular and tissue structure in aged rats. A machine learning  
221 approach and spectroscopy were used to examine molecular profiles, demonstrating distinct  
222 effects of TUDCA and SCD Probiotics on lipid, protein, and nucleic molecules. To better

223 understand these differences, infrared (IR) spectroscopy was used (23). Yet, when TUDCA  
224 and SCD Probiotics were used together, some effects were less pronounced or contradictory,  
225 complicating conclusions about their collective or separate benefits. For example, while  
226 TUDCA increased a lipid marker (CH<sub>3</sub> antisymmetric band), SCD Probiotics reduced it,  
227 suggesting possible lipid peroxidation in the liver, which could be negative (24). However,  
228 free radicals, sometimes increased by such changes, play important roles in physiological  
229 signaling and probiotics can reduce oxidative damage in liver disease patients (25,26).

230 Aging can impact liver's cholesterol metabolism, leading to increased accumulation. TUDCA,  
231 in this study, enhanced bile acid synthesis in the liver, and reduced cholesterol build-up in  
232 serum and liver of mice, as marked by a decrease in the C=O stretching band, a cholesterol  
233 ester marker (10). Contrarily, SCD Probiotics increased this band, contrary to previous  
234 research indicating that these probiotics, containing strains like *B. longum*, *L. acidophilus*, and  
235 *L. plantarum*, usually reduce cholesterol levels in serum and liver (27). Further, TUDCA  
236 caused a decrease, while SCD Probiotics led to an increase in the acyl chain of fatty acids  
237 band, a component of lipids involved in membrane structure and function, energy storage, and  
238 cellular signaling (28). While an increase can be seen as beneficial, saturated lipid acyl chains  
239 could create a less fluid bilayer, so a decrease might be advantageous. This decrease with  
240 TUDCA aligns with recent studies suggesting its role in modulating lipid metabolism,  
241 possibly by controlling the synthesis and breakdown of fatty acids and other lipids (29).  
242 However, in the group receiving both treatments, no significant difference was seen in these  
243 parameters, suggesting further analyses are necessary to draw clear conclusions.

244 The endoplasmic reticulum (ER) is a complex organelle that plays a crucial role in various  
245 cellular processes, including protein synthesis, folding, and modification, lipid metabolism,  
246 and calcium homeostasis. Research studies suggest that TUDCA can attenuate ER stress,  
247 prevent dysfunction in the unfolded protein response, and stabilize mitochondria (30). In

248 spectrochemical analysis of proteins, it has been observed that there is a significant difference  
249 in protein conformation only in the group treated with TUDCA, indicating its effect.  
250 Furthermore, TUDCA appears to be more effective in terms of protein phosphorylation.  
251 However, the interpretation of the decrease or increase in protein phosphorylation levels  
252 requires further analysis. Protein phosphorylation is a process where a phosphate group is  
253 added to a protein molecule by a protein kinase enzyme. This modification can regulate the  
254 activity of the protein, often by activating or deactivating it. Protein phosphorylation plays a  
255 crucial role in a variety of cellular processes, including signal transduction, gene expression,  
256 and cell cycle control. It is also involved in various diseases, such as cancer, diabetes, and  
257 neurodegenerative disorders (31). Another important parameter in which TUDCA was more  
258 effective was membrane dynamics. Interestingly, both SCD Probiotics and TUDCA caused a  
259 significant reduction in this band, but there was no significant difference when applied  
260 together as markers evaluated for nucleic acid.

261 Previous research has shown that age-related hyperinsulinemia can increase fat deposition,  
262 adipocyte size, and lymphatic infiltration, leading to fat accumulation in the liver and  
263 systemic insulin resistance (32,33). Our study suggests that the increase in microvesicular  
264 steatosis and lymphatic infiltration in control aged mice may be associated with a decrease in  
265 age-related hyperinsulinemia (34,35). In the elderly control group, we observed significantly  
266 increased microvesicular steatosis and lymphatic infiltration compared to all treatment groups.  
267 We propose that the decrease in microvesicular steatosis and lymphatic infiltration rates in  
268 treatment groups given SCD Probiotics and TUDCA could be related to age-related  
269 hyperinsulinemia, as we did not observe changes in food intake during the one-week  
270 treatment period. Furthermore, we believe that the reduction in lymphatic infiltration rates in  
271 treatment groups improves inflammation in hepatocytes due to aging, as a result of the  
272 decrease in fat deposits in the aged liver. TUDCA treatment has been previously found to

273 effectively reduce body weight, fat content, and cellular inflammation in aged mice, as well as  
274 in diet-induced obese mice (36). Additionally, TUDCA has been reported to decrease lipid  
275 accumulation in the liver of obese mice by improving insulin sensitivity (37). Together, our  
276 study's findings suggest that TUDCA and SCD Probiotics may be effective in preventing or  
277 reducing the severity of tissue damage in aging-related liver diseases in aged rat liver.

278 Studies have shown that insulin resistance is associated with triglyceride accumulation and  
279 fatty acid intermediate metabolites. In our study, the shortened acyl chain fatty acids in the  
280 elderly control group indicate the presence of insulin resistance. The increase in acyl chain  
281 fatty acid length in treatment groups given SCD Probiotics and TUDCA suggests a decrease  
282 in age-related hyperinsulinemia. This finding is closely related to the reduction of  
283 microvesicular steatosis in the liver tissue of the treatment groups and is consistent with the  
284 results of Zangerolamo et al. (2022b). Therefore, the observed reductions in hepatic  
285 triglyceride and cholesterol levels in aged rats treated with TUDCA may have contributed to a  
286 decrease in lipid droplet density in the aged liver.

287 Fibrosis is a sign of aging in various organs, including the liver, heart, and kidney. In liver  
288 fibrosis that develops with aging, there is an increase in the accumulation of collagen, one of  
289 the extracellular matrix elements (ECM) (39). In our study, we found a significant increase in  
290 collagen density in hepatic tissue in the MT staining results of liver tissues of aged rats. In  
291 contrast, there was a significant decrease in collagen densities in SCD Probiotics, TUDCA,  
292 and combined treatment groups. Based on the MT staining results, we concluded that SCD  
293 Probiotics and TUDCA applications can prevent liver fibrosis due to aging. In one study,  
294 when liver enzyme results were evaluated together with histopathological examinations, it  
295 was reported that TUDCA treatment significantly improved serum ALT, AST, ALP, and  
296 albumin concentrations. Among patients who underwent percutaneous liver biopsy, a  
297 histological relief of tissue fibrosis in the liver was observed in one patient in the TUDCA

298 group (40). In another study, it is believed that exogenous administration of bile components  
299 may be a plausible therapeutic approach to aging, improving liver fibrosis (41,42). In the MT  
300 staining results we obtained, a significant decrease in collagen density was detected in the  
301 treatment group given TUDCA. Our results are consistent with the studies mentioned above.

302 One process leading to age-related liver diseases is hepatic inflammation (16). Since  
303 inflammatory cytokines rise with hepatic aging, dysregulation of inflammasome function is a  
304 major factor in the uncontrolled immune response during inflammation (43). Hepatocytes are  
305 targeted by damaged cells, leading to the pathogenesis of age-related liver diseases (44).  
306 These age-related damages promote inflammation, and pro-inflammatory cytokines further  
307 potentiate inflammation (17). Activation of the NLRP3 inflammasome triggers different  
308 processes according to hepatocytes and other cell types (45). One of them is that the NLRP3  
309 inflammasome contributes to the pathophysiology of age-related liver diseases and induces  
310 hepatocytes to form lipid droplets, which in turn promotes dyslipidemia (46). Previous  
311 studies have reported higher NLRP3 and ASC levels in liver samples due to aging  
312 (15,45,47,48). From the data obtained, it was concluded that the NLRP3 inflammasome plays  
313 a central role in determining the severity of liver inflammation. In the present study, SCD  
314 probiotics and TUDCA treatments were shown to regulate lipid metabolism and modulate  
315 NLRP3 inflammasome activation by reducing steatosis in the aged liver. Taken together, we  
316 report that the active role of the NLRP3 inflammasome in the relationship between the  
317 uncontrolled activation of the age-related immune response and the natural aging process,  
318 which may exacerbate liver inflammation and fibrosis, alleviates age-related chronic  
319 inflammatory hepatic pathology with anti-inflammatory therapies and may therefore be a  
320 target for age-related liver disease.

321 Low alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the low  
322 physiological range are surrogate markers for decreased liver metabolic function. These

323 enzyme levels may suggest a relationship with other chronic diseases that occur during the  
324 aging process (49). In a previous study, it was reported that reductions in the aspartate-alanine  
325 aminotransferase ratio during the aging period can provide insight into its ability to predict  
326 nonalcoholic fatty liver disease and advanced fibrosis (50). In our study findings, we found  
327 that LDH levels decreased in the elderly control group and increased significantly in the  
328 groups given SCD Probiotics, TUDCA, and combined supplements. As muscle and bone  
329 mass decrease with age, this may cause a decrease in the LDH enzyme levels. Since SCD  
330 Probiotics and TUDCA supplements can accelerate metabolism, which is low in elderly  
331 individuals, increased metabolic activity may have increased the LDH enzyme level. The data  
332 we obtained are compatible with the data of Kaczor et al. (2006). Furthermore, lower LDH  
333 activity in older adults is a robust finding still present after accounting for the lower total  
334 protein content associated with aging.

## 335 **CONCLUSION**

336 This study examined the combined impact of SCD Probiotics and TUDCA on the liver tissue  
337 of older male rats, offering insights into their potential therapeutic benefits. Using ATR-FTIR  
338 spectroscopy and machine learning, it explored changes in the liver at the molecular level,  
339 indicating both TUDCA and SCD Probiotics may alleviate age-related liver fibrosis and  
340 improve liver function. Notable changes in lipid, protein, and nucleic acid bands were  
341 observed, alongside significant improvements in liver fibrosis, cellular degradation, lymphatic  
342 infiltration, and bile duct growth. Furthermore, TUDCA and SCD Probiotics reduced liver  
343 microvesicular steatosis and collagen accumulation in older rats. Although all treatment  
344 groups saw increased serum AST and LDH levels, only the SCD Probiotics group exhibited a  
345 significant increase in albumin levels. SCD Probiotics exhibited a marked reduction in  
346 inflammasome-related gene expressions, and the lowest gene expression levels were observed  
347 in the group receiving both treatments This suggests different molecular mechanisms behind

348 the positive effects of TUDCA and SCD Probiotics on liver health. The findings lay the  
349 groundwork for further research into the exact mechanisms through which these treatments  
350 benefit liver health. Understanding the effect of SCD probiotics and TUDCA supplements,  
351 NLRP3 inflammasome in ageing may pave the way to developing a targeted and adequate  
352 therapeutic strategy. Future studies should explore the long-term effects and the potential use  
353 of these treatments in clinical settings to prevent and treat age-related liver diseases.

#### 354 **Author Contributions**

355 HTT and TC contributed to all aspects of this study, from conceptualization to writing and  
356 editing. SK performed the histopathological and biochemical analysis, interpreted results, and  
357 contributed to writing and editing. BB, GS, HT were involved in the investigation,  
358 methodology, validation, and visualization. EA contributed to histopathological interpretation,  
359 visualization, and manuscript editing. RG conducted the investigation, methodology, and  
360 contributed to writing and editing. All authors have approved the final manuscript.

#### 361 **Conflicts of Interest**

362 There are no relevant financial or non-financial competing interests to report.

#### 363 **Data Availability**

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

#### 366 **Ethical Approval**

367 This study was approved by the Ethics Committee (approval number: 2022/03) of the Saki  
368 Yenilli Experimental Animal Production and Practice Laboratory.

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## 527 **FIGURE LEGENDS**

528 **Fig. 1** LDA discrimination plot for liver samples in the full (4000-650  $\text{cm}^{-1}$ ) spectral region.

529 CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the  
530 TUDCA and SCD Probiotics were applied together)

531 **Fig. 2** LDA discrimination plot for liver samples in the lipid (3000-2700  $\text{cm}^{-1}$ ) spectral region.

532 CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the  
533 TUDCA and SCD Probiotics were applied together)

534 **Fig. 3** The quantitative changes in liver-associated spectrochemical parameters. The band

535 areas for **a)**  $\text{CH}_3$  antisymmetric ( $2955 \text{ cm}^{-1}$ ), **b)**  $\text{CH}_2$  antisymmetric ( $2922 \text{ cm}^{-1}$ ), **c)** lipid

536 carbonyl ( $\text{C}=\text{O}$  stretchings,  $1740 \text{ cm}^{-1}$ ), **d)** Amide II ( $1545 \text{ cm}^{-1}$ ), **e)**  $\text{PO}_2$  antisymmetric ( $1239$

537  $\text{cm}^{-1}$ ), and **f)**  $\text{PO}_2$  symmetric ( $1080 \text{ cm}^{-1}$ ). CLI (control), TLI (TUDCA), PLI (SDC

538 Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were

539 applied together)

540 **Fig. 4** The quantitative changes in liver-associated spectrochemical parameters. The band area

541 ratios for **a)** acyl chain length of fatty acids ( $A_{2922}/A_{2955}$ ), **b)** protein phosphorylation

542 ( $A_{1239}/A_{2955}$ ), **c)** protein phosphorylation ( $A_{1239}/A_{2955}$ ), **d)** protein conformation ( $A_{1653}/A_{1545}$ ),

543 and **e)** protein carbonylation ( $A_{1740}/A_{1545}$ ). CLI (control), TLI (TUDCA), PLI (SDC

544 Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were

545 applied together)

546 **Fig 5.** Effects on liver histological architecture **a.** Representative images of hematoxylin and

547 eosin (H&E) staining and quantification of infiltration **b.** Representative images of H&E

548 staining and quantification of micro vesicular steatosis. Black arrows show inflammation.

549 Black arrow's heads indicate bile ducts. The areas in the relevant microphotographs of all

550 groups are enlarged below. Values are expressed as mean  $\pm$ SEM.  $p \leq 0.001$  \*\*\*, and  $p \leq$

551  $0.0001$  \*\*\*\*, ns: not significant. Scale bar:100  $\mu\text{m}$ . CLI (control), TLI (TUDCA), PLI (SDC

552 Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were  
553 applied together).

554 **Fig 6.** Effects on liver fibrosis. Representative images of Masson trichrome (MT) staining  
555 showing collagen deposition in the aged control rat and all treatment groups liver tissue with  
556 quantification of collagen density area fraction (%) in all groups. The areas enclosed in  
557 squares in the relevant microphotographs of all groups are enlarged below. Values are  
558 expressed as mean  $\pm$ SEM.  $p \leq 0.001$  \*\*\*, and  $p \leq 0.0001$  \*\*\*\*, ns: not significant. Scale bar:50  
559 and 100  $\mu$ m. CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications  
560 (in which the TUDCA and SCD Probiotics were applied together)

561 **Fig. 7** The relative mRNA expression levels in inflammasome-related gene expressions. (A)  
562 mRNA expression levels of NLRP3, (B) mRNA expression levels of ASC, (C) mRNA  
563 expression levels of Caspase-1, (D) mRNA expression levels of IL18, and (E) mRNA  
564 expression levels of IL1 $\beta$ . All data are shown as Mean  $\pm$  SEM (n = 5 and 3 replicates); p  
565 values are derived using Tukey's Multiple Comparisons Test followed by One-way ANOVA.  
566 Statistically significant differences are indicated in the following way: ns p > 0.05 (not  
567 significant); \* p < 0.05 (significant); \*\*\* p < 0.001 and \*\*\*\* p < 0.0001 (extremely  
568 significant). CLI (control), TLI (TUDCA), PLI (SDC Probiotics), and the PTLI applications  
569 (in which the TUDCA and SCD Probiotics were applied together)

570 **Fig 8.** Representative photomicrographs showing NLRP3, and ASC immunostaining and  
571 respective quantifications of expression intensity in the liver. The zooms of the selected areas  
572 are shown below the respective microphotographs. Expression intensities (NLRP3 and ASC)  
573 are given in the right panel. Values are expressed as mean  $\pm$  SEM.  $p < 0.05$  \*,  $p \leq 0.01$  \*\*, and  
574  $p \leq 0.0001$  \*\*\*\*, ns: not significant. Scale bar: 100  $\mu$ m. CLI (control), TLI (TUDCA), PLI

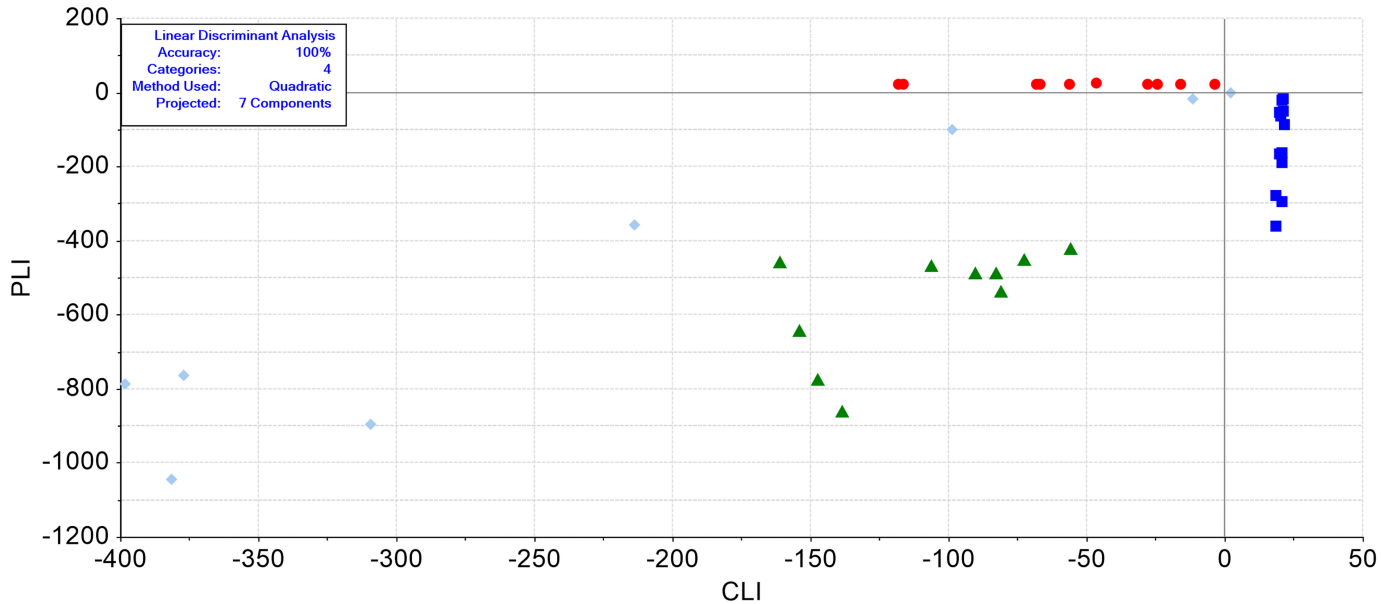
575 (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD Probiotics were  
576 applied together)

577 **Fig 9.** Effects of all treatment groups (PLI, TLI and PTLI groups) on the serum levels of **a)**  
578 AST, **b)** ALP, **c)** Albumin, and **d)** LDH for one week treatment. Values were provided as  
579 mean  $\pm$  SD, n = 7/group. \*P < 0.05 and \*\*P < 0.01 vs. control. CLI (control), TLI  
580 (TUDCA), PLI (SDC Probiotics), and the PTLI applications (in which the TUDCA and SCD  
581 Probiotics were applied together)

582

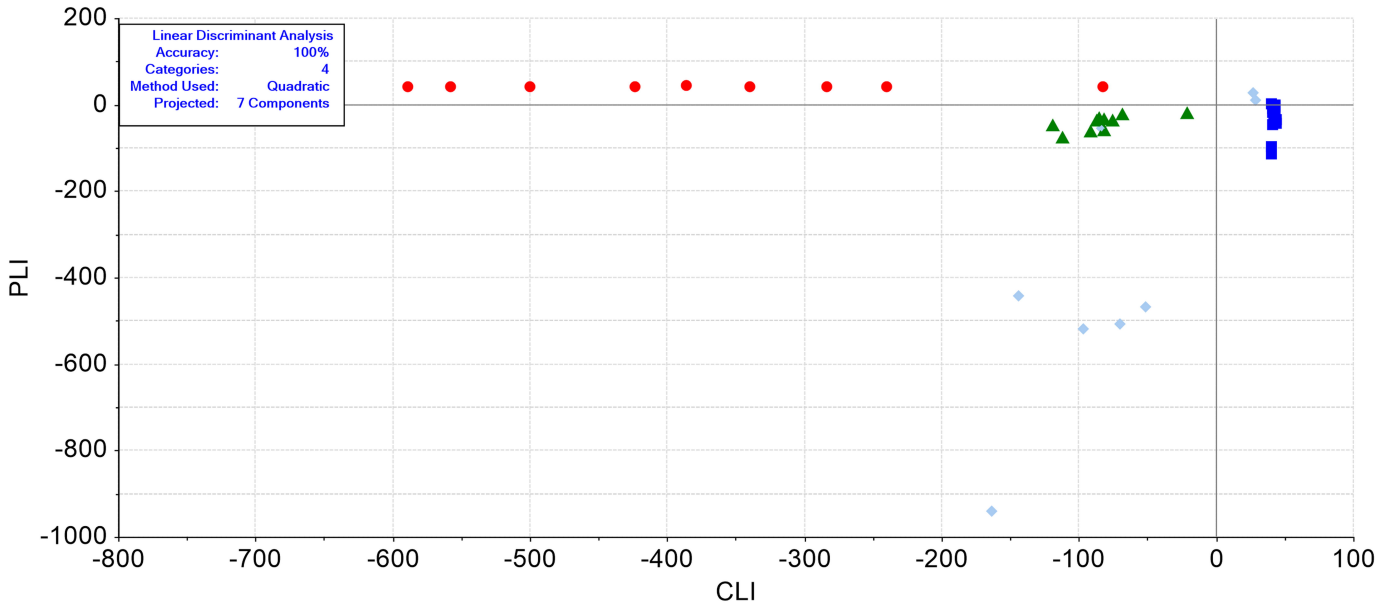
# Discrimination

Linear Discriminant Analysis  
Accuracy: 100%  
Categories: 4  
Method Used: Quadratic  
Projected: 7 Components



# Discrimination

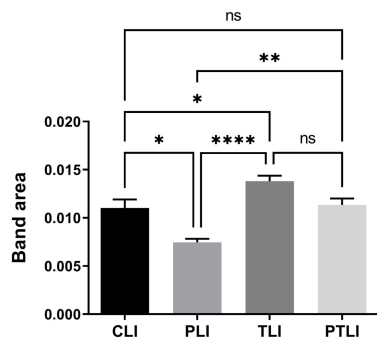
Linear Discriminant Analysis  
Accuracy: 100%  
Categories: 4  
Method Used: Quadratic  
Projected: 7 Components



■ CLI    ● PLI    ▲ TLI    ◆ PTLI

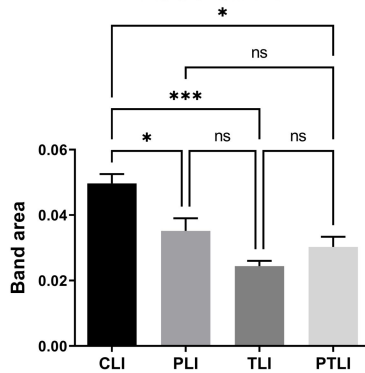
a)

**CH3 antisymmetric**  
**2955 cm<sup>-1</sup>**



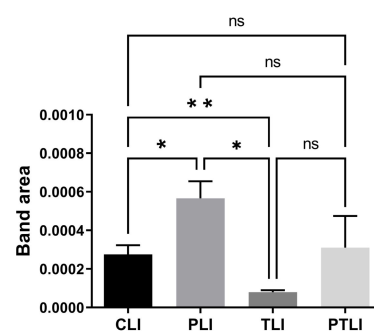
b)

**CH2 antisymmetric**  
**2955 cm<sup>-1</sup>**



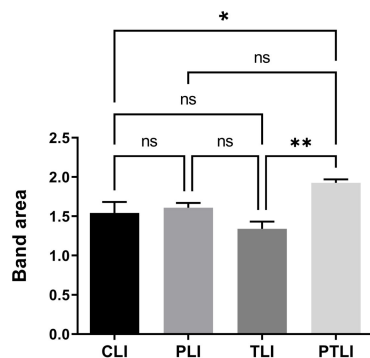
c)

**C=O stretchings**  
**1740 cm<sup>-1</sup>**



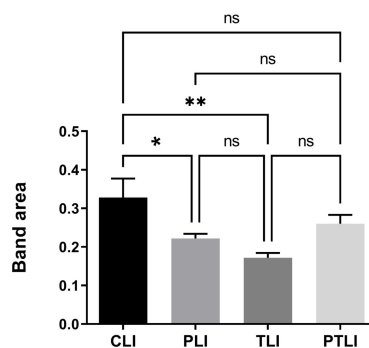
d)

**Amide II**  
**1545 cm<sup>-1</sup>**



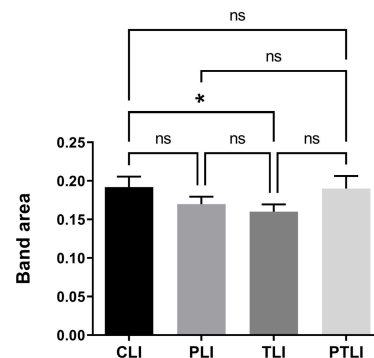
e)

**PO2 antisymmetric**  
**1239 cm<sup>-1</sup>**



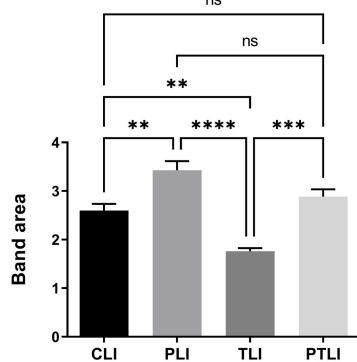
f)

**PO2 symmetric**  
**1080 cm<sup>-1</sup>**



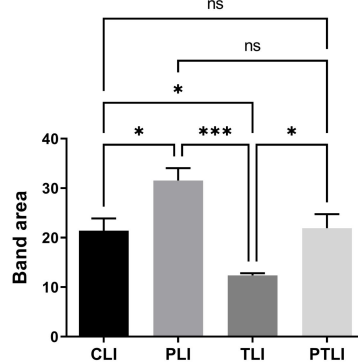
a)

## Acyl chain length of fatty acids

 $A_{2922}/A_{2955}$ 

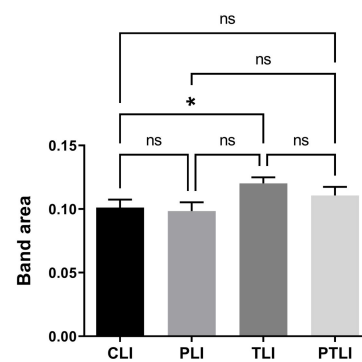
b)

## Protein phosphorylation

 $A_{1239}/A_{2955}$ 

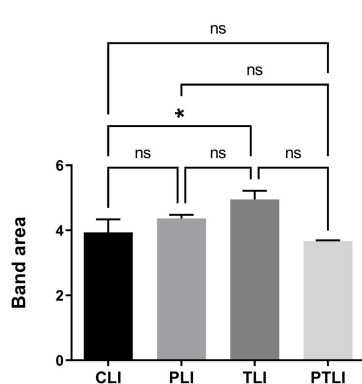
c)

## Protein phosphorylation

 $A_{1080}/A_{1545}$ 

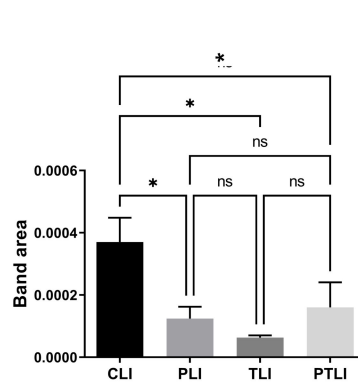
d)

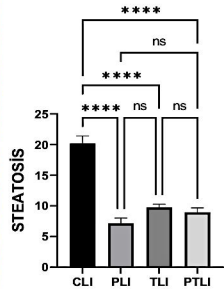
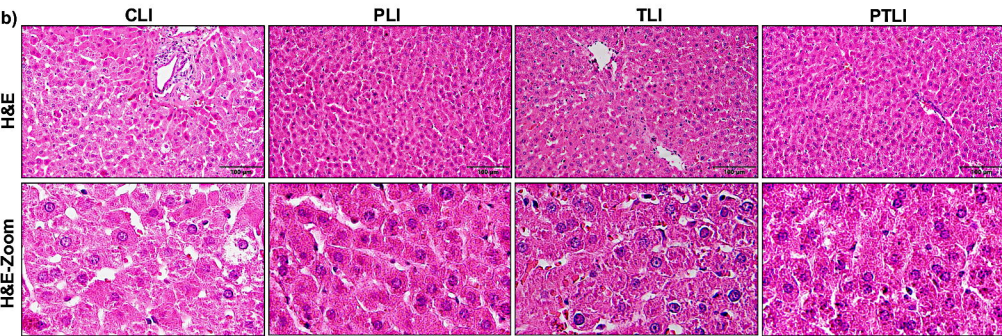
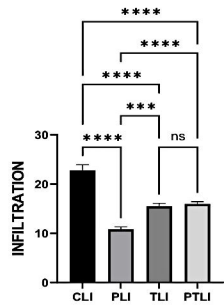
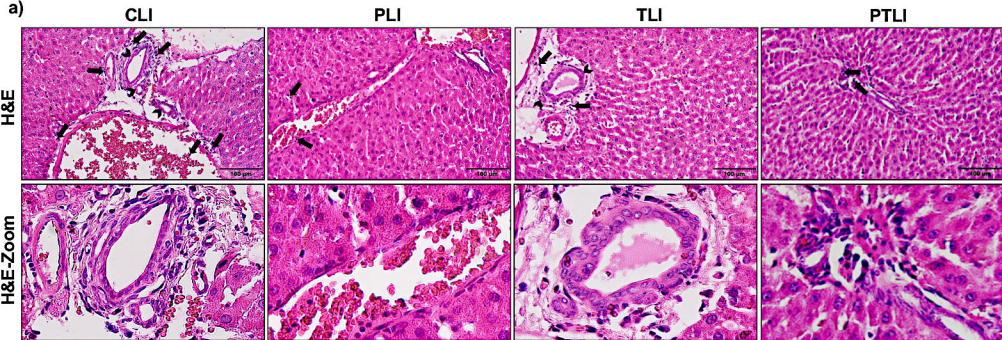
## Protein conformation

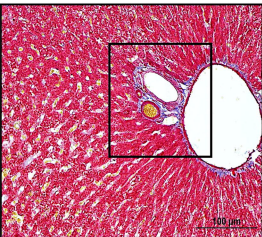
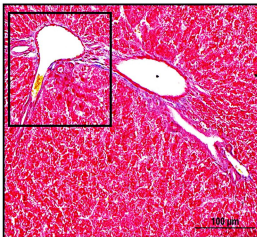
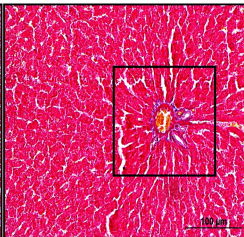
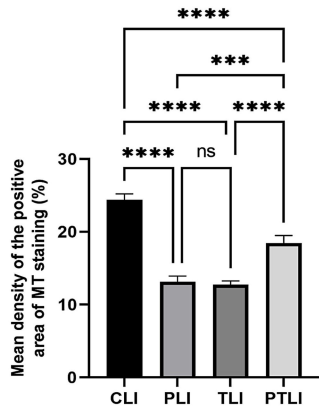
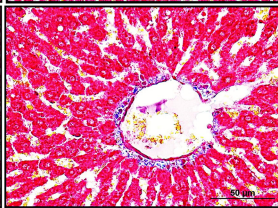
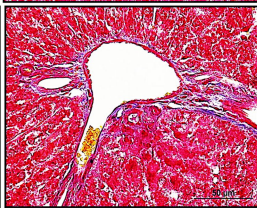
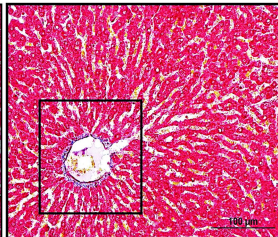
 $A_{1653}/A_{1545}$ 

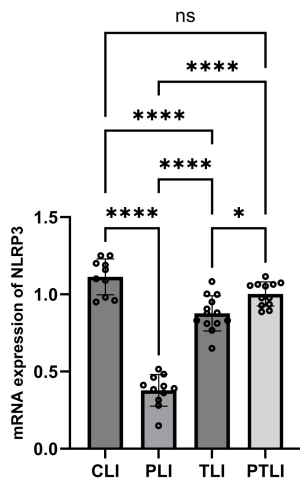
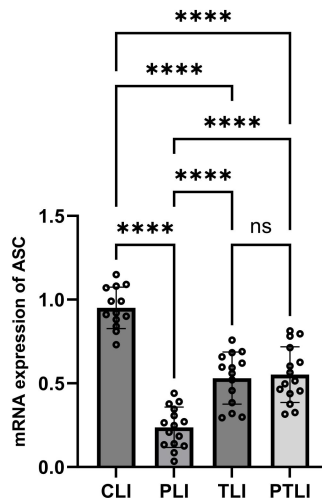
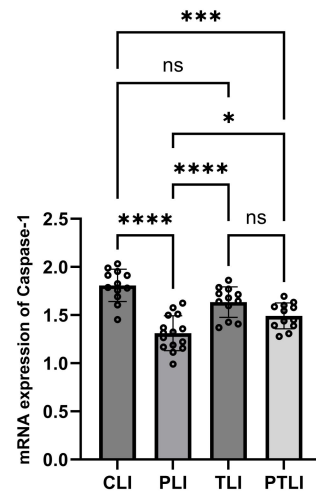
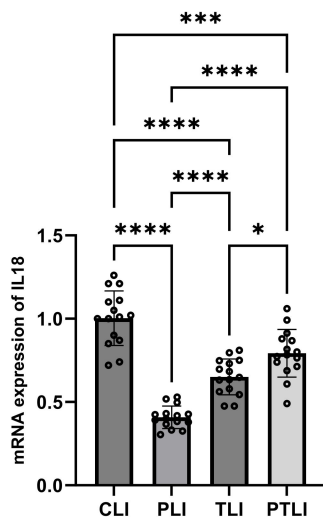
e)

## Protein carbonylation

 $A_{1740}/A_{1545}$ 



**CLI****PLI****TLI****PTLI**

**a)****NLRP3****b)****ASC****c)****Caspase-1****d)****IL18****e)****IL1 $\beta$** 