Journal of Functional Foods 108 (2023) 105736

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

# Probiotic cheese improves alcohol metabolism and alleviates alcohol-induced liver injury via the SIRT1/AMPK signaling pathway

Jong-Hwa Kim [a,1], Dohyun Woo [b,1], YoHan Nam [a], Jihye Baek [a], Ji-Yeon Lee [b], Wonyong Kim [a,c,*]

[a] Department of Microbiology, Chung-Ang University College of Medicine, Seoul 06974, Republic of Korea
[b] Department of Anesthesiology and Critical Care Medicine, Pain Management, Korea Cancer Center Hospital, Seoul 01812, Republic of Korea
[c] LuxBiome Co., Ltd., Seoul 06974, Republic of Korea.

ARTICLE INFO

*Keywords:*
Probiotic cheese
Alcohol
AMPK
SIRT1
NF-κB. PPARα

ABSTRACT

Excessive alcohol intake leads to alcoholic liver injury via hepatic acetaldehyde accumulation. Some probiotic bacteria, such as *Lactiplantibacillus plantarum* and *Bifidobacterium*, are known to have the ability to break down acetaldehyde; however, there is a lack of evidence on their efficacy in functional food applications. In this study, probiotic cheese containing *Lactococcus lactis* LB1022 and *Lactiplantibacillus plantarum* LB1418 was evaluated for its function in inducing alcohol metabolism and alleviating alcohol-induced hepatic injury. Probiotic cheese ameliorated alcohol metabolism induced by alcohol dehydrogenase and aldehyde dehydrogenase, enhanced the sirtuin-1 (SIRT1)/adenosine monophosphate-activated protein kinase (AMPK) signaling, and inhibited the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway. Interestingly, probiotic cheese also induced peroxisome proliferator-activated receptor α and prevented fat formation and inflammation in the liver. Taken together, probiotic cheese containing *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 could induce alcohol metabolism and alleviate alcohol-induced liver injury by regulating SIRT1 in fatty acid oxidation, AMPK in lipogenesis, and NF-κB in inflammation.

## 1. Introduction

Alcoholic liver disease (ALD) is a major health problem worldwide and refers to liver injury after years of drinking alcohol. The liver is the major site responsible for alcohol metabolism, and tissue injury could be caused by acetaldehyde converted from alcohol (Lieber, 2000). Alcohol metabolism is a two-step process: 1) alcohol is first converted to acetaldehyde by alcohol dehydrogenase (ADH), and 2) acetaldehyde is metabolized to acetic acid by aldehyde dehydrogenase (ALDH) (Mackus et al., 2020). Although several studies have reported that acetaldehyde accumulation could be reduced by improving alcohol metabolism (Je et al., 2021; Lee & Park, 2021; Pittler, Verster, & Ernst, 2005), there are no effective food products for alcohol metabolism thus far.

Continuous heavy alcohol intake results in an alcohol-associated pathology, leading to steatosis, steatohepatitis, fibrosis/cirrhosis, and hepatocellular carcinoma (Nagappan, Jung, Kim, Lee, & Jung, 2018). Sirtuin-1 (SIRT1)/adenosine monophosphate-activated protein kinase (AMPK) activation not only plays a crucial role in alcohol-associated liver disease but also induces energy metabolism following alcohol intake. SIRT1, implicated in the pathogenesis of ALD, can activate AMPK signaling, and impaired AMPK accelerates lipid accumulation and leads to alcoholic fatty liver disease development (Na et al., 2018; Ren, Wang, Wu, & Wang, 2020).

Probiotic food products are known as functional foods, which can be used to develop potential pharmaceuticals. Among them, cheese is a suitable probiotic food carrier due to its function as a buffer under highly acidic conditions and its protective effect for probiotic survival in the gastrointestinal tract (da Cruz, Buriti, de Souza, Faria, & Saad, 2009). Most probiotic cheese products are made with common starter bacteria from the *Lactococcus* or *Streptococcus* genera (Ah & Tagalpallewar,

*Abbreviations:* ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate transaminase; CC, cheese curd; FoxO1, forkhead box protein O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRAS, generally recognized as safe; H&E, hematoxylin and eosin; mRNA, messenger ribonucleic acid; NAD, nicotinamide adenine dinucleotide; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; PCC, probiotic cheese curd; SIRT1, sirtuin-1.

* Corresponding author at: Department of Microbiology, Chung-Ang University College of Medicine, 84, Heukseok-ro, Dongjak-gu, Seoul 06974, Republic of Korea.
*E-mail address:* kimwy@cau.ac.kr (W. Kim).

[1] Jong-Hwa Kim and Dohyun Woo contributed equally to this work.

https://doi.org/10.1016/j.jff.2023.105736
Received 28 May 2023; Received in revised form 11 August 2023; Accepted 16 August 2023
Available online 20 August 2023

2017). Studies have reported the association of ADH and ALDH activities with probiotics (Jung et al., 2021; Lim et al., 2021). These studies demonstrated the effectiveness of probiotics in improving humans' alcohol metabolism processes, including reducing blood alcohol levels, aldehyde decomposition capacity, and the levels of alanine aminotransferase (AST) and aspartate transaminase (ALT). However, there are presently no studies on the effect of cheese containing probiotics on alcohol metabolism and alcoholic liver injury.

In our previous studies, dairy products made with *Lactococcus chungangensis* exhibited excellent ADH and ALDH activity, which may reduce alcohol-induced hepatotoxicity (Konkit et al., 2015, 2016). However, limited studies on the mechanism of alcohol-induced liver damage and the presence of non-generally recognized as safe (GRAS) microorganism has limited its use for commertial application. Therefore, this study attempted to investigate changes caused by probiotic cheese in alcohol metabolism and alcohol-induced liver injury via the SIRT1/AMPK signaling pathway. We used probiotic cheese containing *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains that can actively promote alcohol metabolism.

## 2. Material and methods

### 2.1. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL) was purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), ADH, and ALDH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was purchased from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) and gentamycin were purchased from Gibco (Grand Island, NY, USA).

### 2.2. Culture of bacterial strains

*Lactococcus lactis* subsp. *lactis* LB1022 and *Lactobacillus plantarum* LB1418 were isolated from natural cheese and kimchi (a Korean fermented vegetable food), respectively. Both strains were cultured under aerobic conditions in tryptic soy broth (TSB; Difco, Becton Dickinson and Company, Sparks, MD, USA) at 37 °C for 24 h.

### 2.3. Assessment of cell viability

MTT assay was performed to evaluate the cytotoxic effect of *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains on the RAW 264.7 cell line. RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 0.1% gentamicin at 37 °C. The cells ($5 \times 10^5$ cells/well) were seeded in 24-well plates and cultured for 24 h. After 24 h, the medium was removed and replaced with FBS-free medium with the probiotic strains. After 24 h of incubation, the medium was removed, and the cells were treated with a medium containing 0.5 mg/mL MTT. The absorbance was measured at 590 nm using a NanoQuant spectrophotometer (Infinite M200; Tecan, Zurich, Switzerland).

### 2.4. Preparation of probiotic cheese curd

Pasteurized milk (LOTTE Confectionery, Seoul, Republic of Korea) was purchased from a local market. Pasteurized milk samples (100 mL) were kept at 32 °C with constant stirring. At that temperature, freeze-dried starter cultures (*Lc. lactis* LB1022 and *Lb. plantarum* LB1418) were added to obtain an initial inoculum of 6 log CFU/mL. After the starter cultures were inoculated in milk for 90 min, 0.01% rennet was added for coagulation at 37 °C for 1 h. The coagulum was cut into 1 cm cubes, followed by heating and stirring at 37 °C. After whey drainage, the curd was molded and stored for approximately 2 h at room temperature. The fresh cheese curd (CC) was collected and stored at 4 °C until experimentation. The CC in the comparison group was prepared in the same manner as the probiotic cheese curd (PCC); however, the milk acidification step using probiotics was replaced by the direct addition of citric acid. The PCC's starter culture was a mixture of *Lc. lactis* LB1022 and *Lb. plantarum* LB1418. The resulting curd had a uniform yellow color, semi-soft to firm texture, and a mild milky flavor.

### 2.5. In vitro assay of ADH and ALDH activities

*Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains were prepared at a concentration of $1 \times 10^8$ CFU/mL, and CC containing *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains was prepared at a concentration of 10 mg/mL. ADH activity was measured using a modified Bergmeyer's method (Bergmeyer, 1974). The reaction mixture containing distilled water, 1 M Tris-HCl (pH 8.8), 20 mM nicotinamide adenine dinucleotide (NAD), ethanol, and ADH (standard enzyme) was mixed with the samples and incubated at 30 °C for 5 min. The absorbance of generated NADH was measured at 340 nm using a NanoQuant spectrophotometer (Infinite M200; Tecan, Zurich, Switzerland). The ADH activity of the samples was quantified by determining the absorbance at the end of the reaction as a percentage of the absorbance of the control:

$$ADHactivity(\%) = \left(A_{sample}/A_{control}\right) \times 100$$

In addition, ALDH activity was measured as described previously (Koivula & Koivusalo, 1975). The reaction mixture containing distilled water, 1 M Tris-HCl (pH 8.0), 20 mM NAD, 0.1 M acetaldehyde, 3 M KCl, 0.33 M 2-mercaptoethanol, and ALDH (standard enzyme) was mixed with the samples and incubated at 30 °C for 5 min. The quantification of NADH production from NAD was performed at 340 nm using a NanoQuant spectrophotometer (Infinite M200; Tecan, Zurich, Switzerland). Similar to ALDH activity measurement, distilled water was used instead of the samples in the control group. ALDH activity was quantified by the following equation:

$$ALDHactivity(\%) = \left(A_{sample}/A_{control}\right) \times 100$$

### 2.6. Animals

Sprague-Dawley (SD) rats (7 weeks old, male) were purchased from DBL Co., Ltd. (Chungbuk, Republic of Korea). All rats were cared for in accordance with the Korean Food and Drug Administration (KFDA) guidelines and the principles and guidelines of the Chung-Ang University Institutional Animal Care and Use Committee (IACUC) (approval no. A2021032). The rats were housed with a 12 h dark: 12 h light cycle under constant conditions ($23 \pm 1$ °C temperature and $55 \pm 2$% relative humidity). In addition, the rats were provided with a normal diet (NFD; 18% crude protein and 5% crude fat). After adaptation for a week, the rats were divided into four groups ($n = 5$ per group) as follows: normal, control, PCC containing *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains (10 g/kg), and CC without probiotic culture (10 g/kg). The experiment was performed after fasting the rats for 16 h. The rats received 25% ethanol (10 mL/kg) or the equivalent amount of PBS (control group) via oral gavage 30 min after sample administration. The 25% ethanol treatment was based on a modified method from our previous study (Konkit, Kim, Kim, & Kim, 2018).

### 2.7. Assessment of serum biochemistry, blood alcohol, and acetaldehyde concentration

Blood samples were collected from the jugular vein at 0, 1, 3, 6, 12, and 24 h after ethanol administration. The collected blood samples were left at room temperature for 1 h and at 4 °C for 1 h to coagulate the blood prior to centrifugation. The serum was separated from whole blood after centrifugation at 5,000 × *g* for 30 min at 4 °C and transferred to a new tube, which was stored at − 80 °C until experimentation. To measure blood alcohol and acetaldehyde concentrations, separated serum samples were mixed with a reaction mixture containing 0.3 M potassium

phosphate buffer (pH 9.0) and 49 mM NAD. The mixture was incubated at room temperature for 5 min, and the absorbance was measured at 340 nm using a microplate spectrophotometer. In the control group, ADH (5 U/mL) and ALDH (1 U/mL) were incubated at room temperature for 5 min, and the absorbance was measured at 340 nm using a microplate spectrophotometer. In addition, serum biochemistry was performed for ALT and AST by GC Labs (Yongin, Republic of Korea) to determine the extent of liver injury following alcohol consumption.

### 2.8. Analysis of SIRT1/AMPK signaling-related mRNA expression

Total RNA from liver tissue was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the extracted RNA was stored at − 20 °C until experimentation. The RNA concentration was measured using a NanoQuant spectrophotometer, and cDNA was synthesized using the PrimeScript 1st strand cDNA synthesis kit (Takara, Kyoto, Japan). SIRT1/AMPK signaling-related mRNA expression levels were quantified by real-time PCR (7500 Fast real-time PCR system) using a SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA). The values were normalized relative to the expression of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) using the $2^{-\triangle\triangle}$ Ct method.

### 2.9. Measurement of the nuclear factor kappa light chain enhancer of activated B cells and forkhead box protein O1 activity

The extraction of the nuclear proteins nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and forkhead box protein O1 (FoxO1) was performed using NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Fischer Scientific Inc., Waltham, MA, USA), following the guidelines provided by the manufacturer. Briefly, the nuclear fraction was extracted using nuclear extraction buffer, and the protein content was quantified using a bicinchoninic acid protein assay kit (Abcam, Cambridge, UK). Subsequently, an appropriate amount of protein was separated through 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to poly (vinylidene fluoride) membranes (Immun-Blot® membrane; BioRad Laboratories, Inc., Hercules, CA, USA). The membranes were then blocked with 5% bovine serum albumin solution for 1 h at room temperature. Following the blocking step, the membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies conjugated with horseradish peroxidase for 1 h. After that, the blots were thoroughly washed with Tris-buffered saline and Tween 20 before being developed using PicoEPD western blot detection reagents (Ecocell Co., Ltd., Gyeonggi-do, Korea). Finally, the protein intensity on the blots was quantified using ImageJ software (version 1.5.3; National Institutes of Health, Bethesda, MD, USA).

### 2.10. Histopathological analysis

Liver tissue was collected after euthanization to detect alcohol-induced liver injury. Tissue samples were cut into 4–5 μm sections and fixed in 10% formalin with PBS. The tissue sections were embedded in paraffin and stained with hematoxylin and eosin (H&E). Injury in the stained liver tissue was assessed after observation by microscopy (DM 4000B; Leica Microsystems, Wetzlar, Germany) at 400 × magnification.

### 2.11. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism (v. 8.0; GraphPad Software Inc., La Jolla, CA, USA). To compare the difference of more than two groups, the results were analyzed by one-way ANOVA and adjusted by Tukey-Kramer post-hoc test. A p value of$<0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Effect of probiotic strains on cell viability

RAW 264.7 cells were treated for 24 h with 2.5, 5, 10, and 20 mg/mL PCC, and cytotoxicity was evaluated by MTT assay. No cytotoxic effect was observed up to a concentration of 10 mg/mL (Fig. 1).

### 3.2. Effect of PCC on ADH and ALDH activities in vitro

ADH and ALDH activities were examined to evaluate the effect of PCC on alcohol metabolism. The ADH activity of the PCC group was 166.77 ± 1.08%, which was significantly higher than that of the control (100.00 ± 0.06%, $p < 0.0001$) and CC (100.23 ± 0.66%, $p < 0.0001$) groups (Fig. 2a). However, there was no significant difference between the CC and control groups. In addition, the ALDH activity of the PCC group was 296.95 ± 6.91%, which was significantly higher than that of the control (100.00 ± 0.17%, $p < 0.0001$) and CC (99.27 ± 1.10%, $p < 0.0001$) groups; however, there was no significant difference between the control and CC groups (Fig. 2b).

### 3.3. Effect of PCC on ADH and ALDH activities in vivo

The effect of PCC on alcohol metabolism was investigated by measuring alcohol and acetaldehyde concentrations in the blood. Alcohol concentration was rapidly increased within 1 h after alcohol administration in all groups (Fig. 3a). However, this concentration was lower in the PCC group than in the control and CC groups. The CC group was similar to the control group. The alcohol concentration of the PCC group was 0.027 ± 0.001 mM after 1 h, which was decreased to 0.015 ± 0.001 mM after 3 h, 0.010 ± 0.002 mM after 6 h, and 0.001 ± 0.001 mM after 12 h. In comparison with the control and CC groups, the PCC group had a significantly lower alcohol concentration in the blood at 1, 3, 6, and 12 h after alcohol administration ($p < 0.0001$ for all). The acetaldehyde concentration of the PCC group was 0.005 ± 0.002 mM after 3 h, which was decreased to 0.004 ± 0.001 mM after 6 h and 0.001 ± 0.001 mM after 12 h. At 3, 6, and 12 h after alcohol administration, the acetaldehyde concentration of the PCC group was significantly lower compared with the control ($p < 0.0001$, $p < 0.0001$, and $p = 0.0285$, respectively) and the CC ($p < 0.0001$, $p = 0.0001$, and $p = 0.0285$, respectively) groups (Fig. 3b). The PCC group had significantly higher blood alcohol concentrations compared with the normal group at 1, 3, and 6 h ($p < 0.0001$, $p < 0.0001$, and $p = 0.0005$, respectively), however, there was no significant difference at 12 h. In addition, there was no significant difference in blood acetaldehyde concentrations at 3, 6,

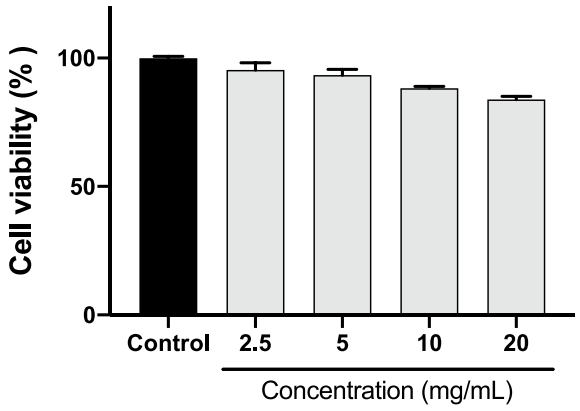


**Fig. 1.** Cytotoxic effect of probiotic cheese curd (PCC). The viability rates of RAW 264.7 cells treated with PCC at different concentrations are shown. The control group was treated with the medium only. The results of three independent experiments are presented as the mean ± SEM.

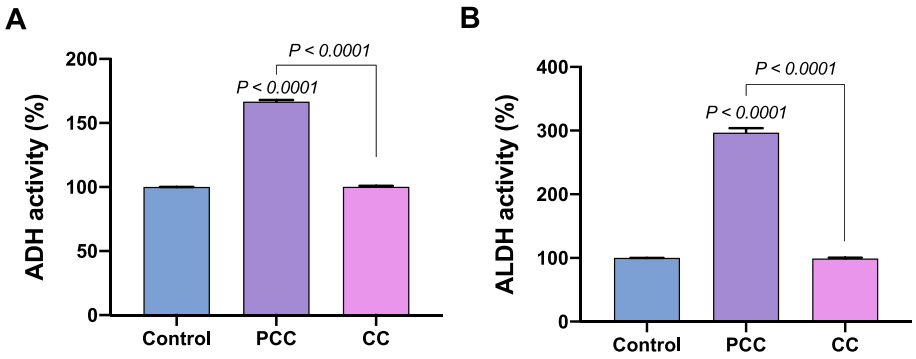


**Fig. 2.** Effect of probiotic cheese curd (PCC) on (A) alcohol dehydrogenase (ADH) and (B) aldehyde dehydrogenase (ALDH) activities. The control group was treated with ADH and ALDH. The results of three independent experiments are presented as the mean ± SEM.

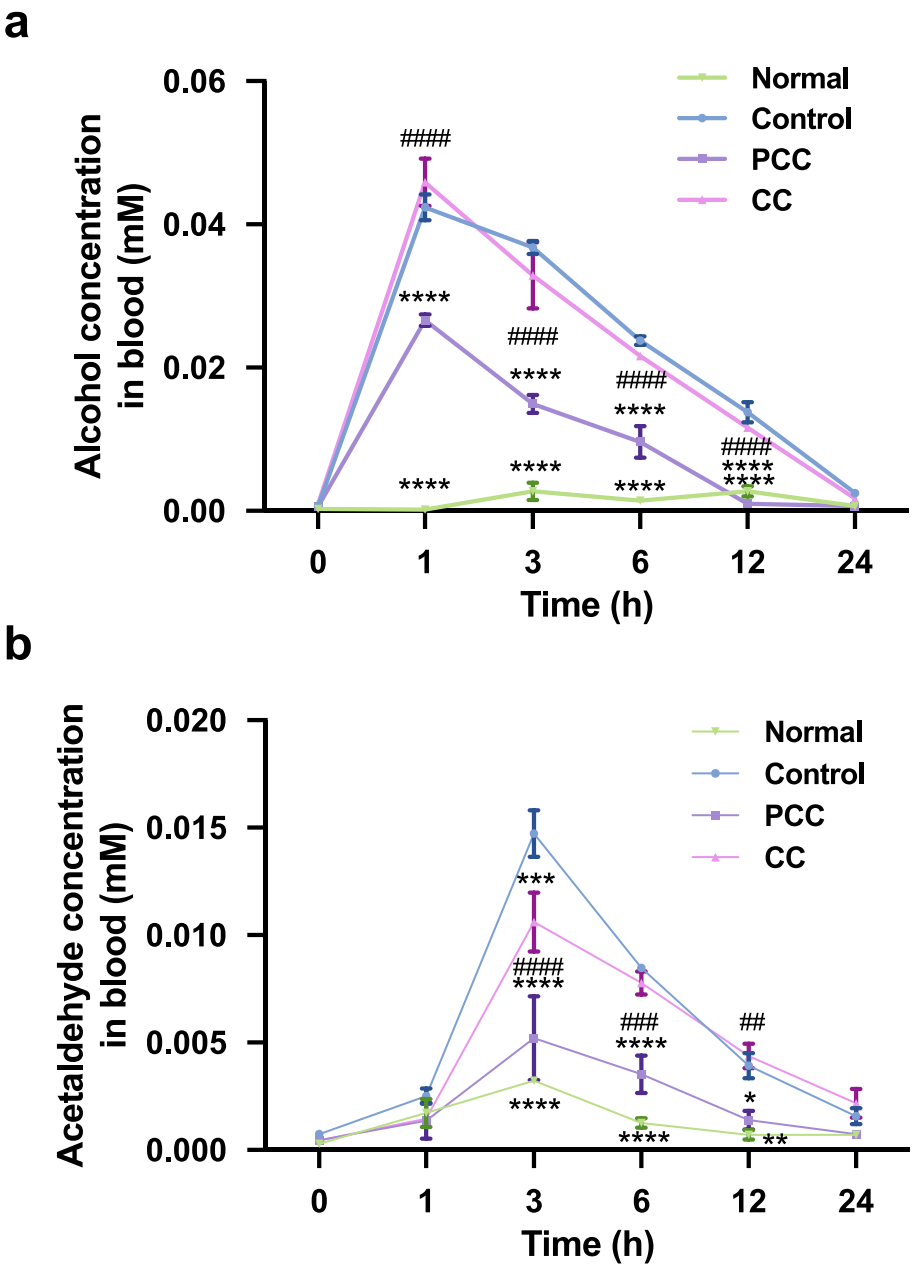


**Fig. 3.** Effect of probiotic cheese curd (PCC) on (A) alcohol and (B) acetaldehyde concentrations in the blood. The control group was treated with alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The results of three independent experiments are presented as the mean ± SEM. *$p < 0.05$; ***$p < 0.0005$; ****$p < 0.0001$. *, vs. control group, #, vs. PCC group.

and 12 h between the PCC and the normal groups.

### 3.4. Effect of PCC on liver function

Serum AST and ALT levels were measured to detect alcohol-induced liver injury. Serum AST level was significantly lower in the PCC group (189.00 ± 2.31 U/L) than in the control group (359.00 ± 41.96 U/L, p = 0.0114); however, there was no significant difference between the PCC and CC (227.67 ± 37.03 U/L, p = 0.7670) groups (Fig. 4a). In comparison with the intake of alcohol alone, the intake of PCC was found to reduce the level of AST by approximately 47%. In addition, ALT level was significantly lower in the PCC group (39.67 ± 10.68 U/L) than in the control group (82.67 ± 6.64 U/L, p = 0.0019); however, there was no significant difference between the PCC and CC (63.00 ± 12.42 U/L, p = 0.0881) groups (Fig. 4b). In comparison with the intake of alcohol alone, the intake of PCC was found to reduce the level of ALT by approximately 52%. There was no significant difference in the AST and ALT levels between the normal and PCC groups. These results indicated that the intake of PCC did not exert toxicity and could prevent liver injury.

### 3.5. Activation of SIRT1/AMPK signaling by PCC

SIRT1/AMPK activation in the liver could alleviate fatty acid oxidation and lipid accumulation following alcohol intake (Fig. 5). The intake of PCC could up-regulate SIRT1/AMPK activation. To investigate the effect of PCC intake on fatty acid oxidation mediated by the SIRT1 pathway, the mRNA expression levels of SIRT1, β-catenin, peroxisome proliferator-activated receptor α (PPARα), and PGC-1α were measured. When compared with the control group, the mRNA expression levels were significantly higher in the PCC group (177.09 ± 11.56%; p < 0.0001, 152.89 ± 0.88%; p = 0.0441, 175.41 ± 5.48%; p = 0.0022, and 185.65 ± 4.70%; p = 0.0007, respectively). However, in the CC group, only the mRNA expression level of SIRT1 showed a significant increase (56.02 ± 1.60%; p = 0.0034). When comparing the normal to the PCC group, the mRNA expression level of PGC-1 (302.40 ± 42.57%; p = 0.0277) showed a significant difference. In contrast, the differences in the mRNA expression levels of SIRT1, β-catenin, and PPARα were not statistically significant.

In addition, to investigate the effect of PCC intake on lipogenesis mediated by the AMPK pathway, the mRNA expression levels of AMPK and ACC were measured. The levels of AMPK and ACC were significantly higher in the PCC compared with the control group (143.48 ± 2.82%; p = 0.0002 and 55.23 ± 1.27%; p = 0.0004, respectively). However, only the AMPK levels showed a statistically significant increase in the PCC group (p < 0.0001), while the increase in the ACC level was not significant. Furthermore, the intake of PCC significantly inhibited alcohol-induced inflammation.

The mRNA expression levels of the NF-κB and FoxO1 were significantly decreased in the PCC compared with the control group (45.62 ± 4.16%; p = 0.0002 and 32.96 ± 3.36%; p < 0.0001, respectively). However, when comparing the normal with the PCC group, these mRNA levels of the NF-κB and FoxO1 were not statistically significant. In particular, the western blot results showed that NF-κB and FoxO1 signaling was activated in the control group; in contrast, the PCC group had significantly decreased protein levels (Fig. 6). These results indicated that the intake of PCC alleviated alcohol-induced fatty acid oxidation, lipid accumulation, and alcohol-induced inflammation in the liver by regulating mRNA expression in the SIRT1/AMPK pathway.

### 3.6. Effect of PCC on histopathological changes in alcohol-induced liver injury

Fig. 7 shows that the PCC group's histopathological changes were improved compared with those in the control and CC groups. In the control group, fat vacuoles, inflammatory cell infiltration, and an irregular hepatocyte arrangement were observed. However, the intake of PCC prevented the formation of fat vacuoles, inflammatory cell infiltration, and an irregular hepatocyte arrangement. The liver appeared almost similar to a normal liver.

## 4. Discussion

Excessive alcohol intake leads to pathological changes causing ALD, which is associated with liver inflammation and injury. Although ALD pathogenesis is still not fully understood, it seems to be related to complex interactions between alcohol and acetaldehyde (Stickel, Datz, Hampe, & Bataller, 2017). In this study, the PCC-treated group had significantly lower alcohol and acetaldehyde concentrations in the blood. In ALD, acetaldehyde, the main intermediate in alcohol metabolism, has been suggested to play an important role in hangover after drinking too much alcohol (Wiese, Shlipak, & Browner, 2000; Zakhari, 2006). In addition, we found that PCC administration significantly inhibited NF-κB and increased SIRT1/AMPK-related mRNA expression levels. In immune/inflammatory responses, SIRT-NF-κB demonstrates an inhibitory relationship; NF-κB can be suppressed through an increase in SIRT (de Gregorio, Colell, Morales, & Mari, 2020; Wu et al., 2021). In addition, the anti-inflammatory mediator AMPK indirectly inhibits NF-κB through downstream mediators such as SIRT1 (Salminen, Hyttinen, & Kaarniranta, 2011). Therefore, SIRT and AMPK are considered as major partners with similar functions in inflammation associated with NF-κB signaling. The alcohol-induced impairment of the hepatic SIRT1 signaling pathway contributes to the development of alcoholic fatty liver and inflammation. Furthermore, chronic exposure to ethanol has been found to inhibit SIRT1 and AMPK activities and induce NF-κB due to the presence of acetaldehyde, which may be associated with lipid accumulation, inflammation, and fibrosis in the liver (Bai et al., 2016; Hyun, Han, Lee, Yoon, & Jung, 2021). Nevertheless, the role of SIRT1 in ALD requires further investigation. The results revealed that PCC may play an important role in anti-inflammation and anti-lipogenesis through the

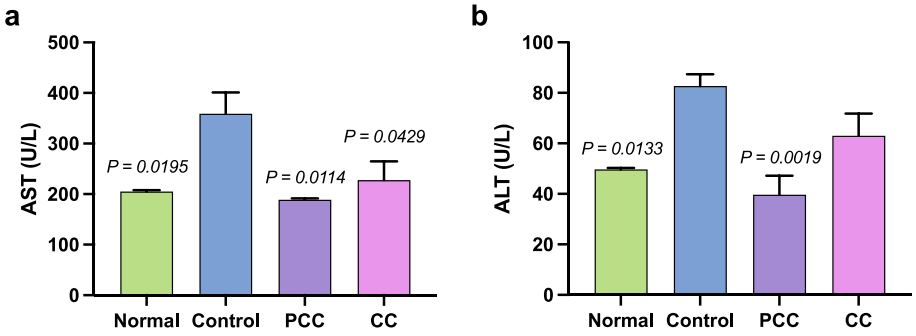


**Fig. 4.** Effect of probiotic cheese curd (PCC) on (A) aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) in the blood. The control group was treated with alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The results of three independent experiments are presented as the mean ± SEM.

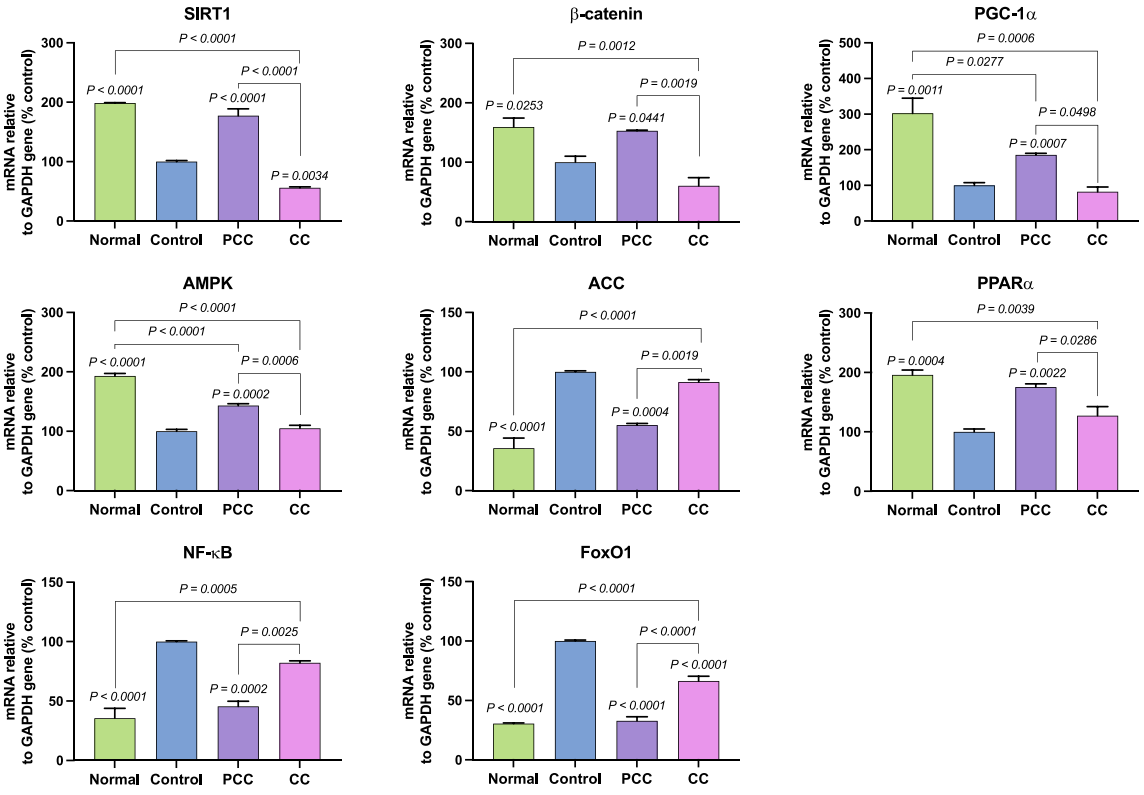


**Fig. 5.** Effect of probiotic cheese curd (PCC) on the mRNA expression levels of SIRT1, β-catenin, PGC-1α, AMPK, ACC, PPARα, NF-κB, and FoxO1 in alcohol-induced liver injury measured by real-time PCR. The results of three independent experiments are presented as the mean ± SEM.

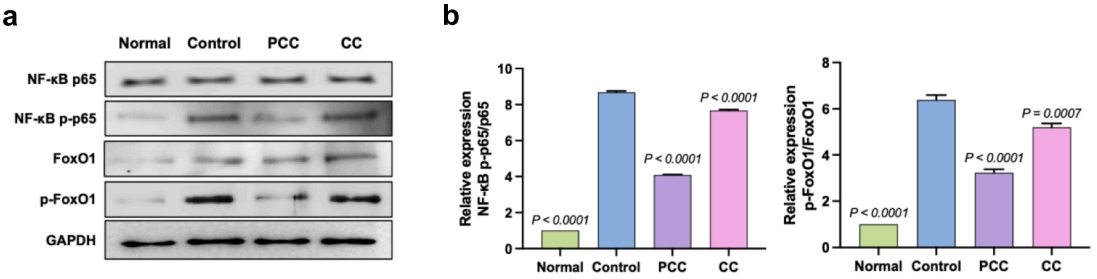


**Fig. 6.** Effect of probiotic cheese curd (PCC) on nuclear localization and activation of NF-κB and FoxO1 in alcohol-induced liver injury. NF-κB and FoxO1 protein localization from the nuclear fraction were investigated by (A) western blot analysis. (B) The relative protein expressions of NF-κB and FoxO1 were measured and normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH). The results of three independent experiments are presented as the mean ± SEM.

inhibition of NF-κB by enhancing the expression of SIRT1/AMPK.

In this study, the intake of PCC significantly reduced AST and ALT levels in the mouse serum and alleviated infiltrated inflammation in liver tissue. ALT and AST are the most common markers for liver injury (Kew, 2000). Recently, suppressed PPARα expression has been found to be associated with hepatic inflammatory injury (Jiao et al., 2014). However, it is still difficult to determine the functional role of PPARα in the pathogenesis of ALD. In our study, PCC administration induced PPARα. Studies have found that acetaldehyde can directly impair PPARα, which is highly expressed in the liver and regulates fatty acid oxidation, lipid metabolism, and inflammatory responses (Kersten, 2014; Varga, Czimmerer, & Nagy, 2011; Yue et al., 2021). PPARα exerts anti-inflammatory activity and immune-suppressive effects by inhibiting NF-κB (Sun et al., 2018). The findings suggest that PCC may exert a protective effect against alcohol-induced liver injury by regulating PPARα.

Cheese is widely applied in the food industry and is considered valuable due to its high nutritional value and wide range of uses (Mohd Shukri, Alias, Murad, Yen, & Cheng, 2022). There is a known association between cheese consumption and disease risk, which could have important implications for human health given the widespread consumption of cheese products (Tong et al., 2017). Some studies have reported that probiotic cheese could have an immunomodulatory effect (containing *Lb. acidophilus*, *B. bifidum*, and *Lb. paracasei*) (Medici, Vinderola, & Perdigon, 2004), reduce the risk of dental caries (containing *Lb. rhamnosus*) (Ahola et al., 2002), and reduce hepatic triglyceride and cholesterol levels in high-fat diet rats (containing *Lb. helveticus*) (Higurashi, Ogawa, Nara, Kato, & Kadooka, 2016). In the present study, we produced PCC using *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains that can actively promote alcohol metabolism, which alleviated alcohol-induced liver injury.

Interestingly, in our study, there was no difference in the expression of factors related to the SIRT1/AMPK signaling pathway except for four factors (β-catenin, ACC, NF-κB, and FoxO1) when administering CC without probiotics compared with alcohol alone. Moreover, the intake of CC without probiotics did not affect alcohol and acetaldehyde

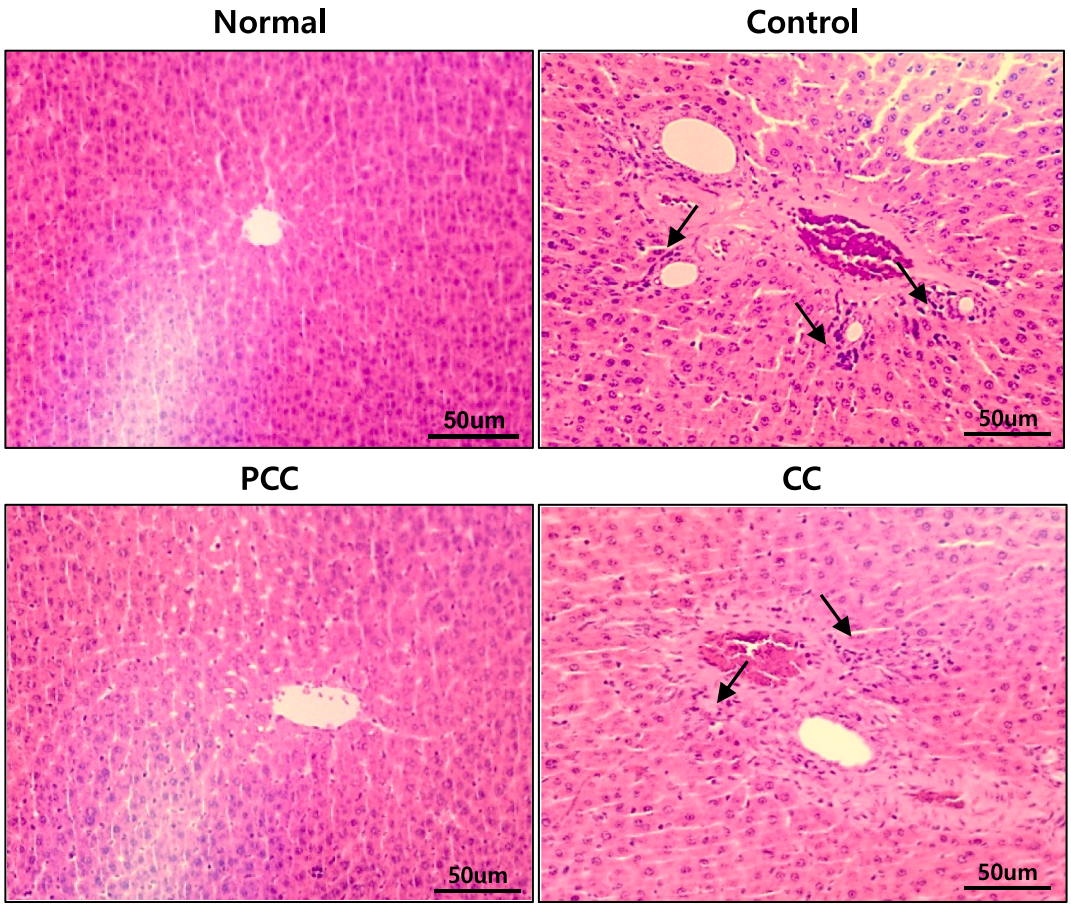


**Fig. 7.** Effect of probiotic cheese curd (PCC) on histopathological changes and in alcohol-induced liver injury. Black arrows indicate infiltrated inflammatory cells. Scale bars = 50 μm.

concentrations in the blood, AST and ALT levels, and histological changes in the liver. Therefore, the *Lc. lactis* LB1022 and *Lb. plantarum* LB1418 strains in probiotic cheese may play a substantial role in regulating alcohol metabolism, lipogenesis, and fatty acid oxidation associated with alcoholic liver injury. These results are consistent with those of another study indicating that the type of probiotic strain is important in cheese making (Hammam & Ahmed, 2019) and that the variety of cheese is determined by the fresh curd preparation method and probiotics in curd products, which may be associated with the characteristics attained during cheese production and ripening (Zheng, Shi, & Wang, 2021).

This study had the following limitations. First, although the PCC's mechanisms of alleviating alcohol-induced liver injury via the SIRT1/AMPK and NF-κB signaling pathways were proposed and explored in an animal model, safety assessment is required before considering clinical application. In addition, further clinical trials are needed to clarify PCC's mechanisms that promote alcohol metabolism and alleviate ALD. Furthermore, the ability of the starter cultures used in the cheese production to survive the aging process may impact their health-promoting effects. Nevertheless, this study's findings supported that PCC can effectively alleviate alcohol-induced liver injury.

## 5. Conclusions

In summary, we demonstrated that PCC could alleviate hangovers by reducing alcohol and acetaldehyde concentrations in the blood. In addition, PCC was found to regulate hepatic fatty acid oxidation, lipogenesis, and inflammation in alcohol-induced liver injury via the SIRT1/AMPK and NF-κB signaling pathways. Therefore, PCC may be an effective functional dairy food for the prevention of alcoholic liver injury.

### Ethical statement

Animals' experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines and approved by the Chung-Ang University Institutional Animal Care and Use Committee (IACUC) (approval no. A2021032).

### CRediT authorship contribution statement

**Jong-Hwa Kim:** Investigation, Writing - original draft. **Dohyun Woo:** Methodology, Writing - original draft. **YoHan Nam:** Investigation. **Jihye Baek:** Visualization. **Ji-Yeon Lee:** Validation. **Wonyong Kim:** Project administration, Conceptualization, Writing - review & editing.

### 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.

### Data availability

No data was used for the research described in the article.

### Acknowledgments

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2021R1C1C2003223 and NRF-2022R1A2C2012209).

## References


Ah, J., & Tagalpallewar, G. P. (2017). Functional properties of mozzarella cheese for its end use application. *Journal of Food Science and Technology, 54*(12), 3766–3778. https://doi.org/10.1007/s13197-017-2886-z

Ahola, A. J., Yli-Knuuttila, H., Suomalainen, T., Poussa, T., Ahlstrom, A., Meurman, J. H., & Korpela, R. (2002). Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. *Archives of Oral Biology, 47*(11), 799–804. 10.1016/s0003-9969(02)00112-7.

Bai, T., Yang, Y., Yao, Y. L., Sun, P., Lian, L. H., Wu, Y. L., & Nan, J. X. (2016). Betulin alleviated ethanol-induced alcoholic liver injury via SIRT1/AMPK signaling pathway. *Pharmacological Research, 105*, 1–12. https://doi.org/10.1016/j.phrs.2015.12.022

Bergmeyer, H. (1974). Lactate dehydrogenase. *Methods of Enzymatic Analysis*, 574–579.

da Cruz, A. G., Buriti, F. C. A., de Souza, C. H. B., Faria, J. A. F., & Saad, S. M. I. (2009). Probiotic cheese: Health benefits, technological and stability aspects. *Trends in Food Science & Technology, 20*(8), 344–354. https://doi.org/10.1016/j.tifs.2009.05.001

de Gregorio, E., Colell, A., Morales, A., & Marí, M. (2020). Relevance of SIRT1-NF-κB axis as therapeutic target to ameliorate inflammation in liver disease. *International Journal of Molecular Sciences, 21*(11), 3858. https://doi.org/10.3390/ijms21113858

Hammam, A. R., & Ahmed, M. S. (2019). Technological aspects, health benefits, and sensory properties of probiotic cheese. *SN Applied Sciences, 1*, 1–9. https://doi.org/10.1007/s42452-019-1154-4

Higurashi, S., Ogawa, A., Nara, T. Y., Kato, K., & Kadooka, Y. (2016). Cheese consumption prevents fat accumulation in the liver and improves serum lipid parameters in rats fed a high-fat diet. *Dairy Science & Technology, 96*, 539–549. https://doi.org/10.1007/s13594-016-0288-z

Hyun, J., Han, J., Lee, C., Yoon, M., & Jung, Y. (2021). Pathophysiological aspects of alcohol metabolism in the liver. *International Journal of Molecular Sciences, 22*(11), 5717. https://doi.org/10.3390/ijms22115717

Je, J., Song, M., Baek, J. H., Kang, J. S., Chung, H. J., Lee, K., … Kim, H. J. (2021). Combined water extracts from oxidation-treated leaves and branches of *Hovenia dulcis* has anti-hangover and liver protective effects in binge alcohol intake of male mice. *Nutrients, 13*(12), 4404. https://doi.org/10.3390/nu13124404

Jiao, M., Ren, F., Zhou, L., Zhang, X., Zhang, L., Wen, T., … Duan, Z. (2014). Peroxisome proliferator-activated receptor α activation attenuates the inflammatory response to protect the liver from acute failure by promoting the autophagy pathway. *Cell Death & Disease, 5*(8), e1397.

Jung, S. J., Hwang, J. H., Park, E. O., Lee, S. O., Chung, Y. J., Chung, M. J., … Chae, S. W. (2021). Regulation of alcohol and acetaldehyde metabolism by a mixture of *Lactobacillus* and *Bifidobacterium* species in human. *Nutrients, 13*(6), 1875. https://doi.org/10.3390/nu13061875

Kersten, S. (2014). Integrated physiology and systems biology of PPARα. *Molecular Metabolism, 3*(4), 354–371. https://doi.org/10.1016/j.molmet.2014.02.002

Kew, M. C. (2000). Serum aminotransferase concentration as evidence of hepatocellular damage. *The Lancet, 355*(9204), 591–592. https://doi.org/10.1016/S0140-6736(99)00219-6

Koivula, T., & Koivusalo, M. (1975). Different forms of rat liver aldehyde dehydrogenase and their subcellular distribution. *Biochimica et Biophysica Acta (BBA)-Enzymology, 397*(1), 9–23. https://doi.org/10.1016/0005-2744(75)90174-6

Konkit, M., Choi, W. J., & Kim, W. (2015). Alcohol dehydrogenase activity in *Lactococcus chungangensis*: Application in cream cheese to moderate alcohol uptake. *Journal of Dairy Science, 98*(9), 5974–5982. https://doi.org/10.3168/jds.2015-9697

Konkit, M., Choi, W. J., & Kim, W. (2016). Aldehyde dehydrogenase activity in *Lactococcus chungangensis*: Application in cream cheese to reduce aldehyde in alcohol metabolism. *Journal of Dairy Science, 99*(3), 1755–1761. https://doi.org/10.3168/jds.2015-10549

Konkit, M., Kim, K., Kim, J. H., & Kim, W. (2018). Protective effects of *Lactococcus chungangensis* CAU 28 on alcohol-metabolizing enzyme activity in rats. *Journal of Dairy Science, 101*(7), 5713–5723. https://doi.org/10.3168/jds.2017-13992

Lee, S., & Park, Y. S. (2021). Effect of water-soluble propolis administration on the ethanol-induced hangover in rats. *Food Science and Biotechnology, 30*(3), 455–463. https://doi.org/10.1007/s10068-020-00869-6

Lieber, C. S. (2000). Alcoholic liver disease: New insights in pathogenesis lead to new treatments. *Journal of Hepatology, 32*(1 Suppl), 113–128. https://doi.org/10.1016/s0168-8278(00)80420-1

Lim, T. J., Lim, S., Yoon, J. H., & Chung, M. J. (2021). Effects of multi-species probiotic supplementation on alcohol metabolism in rats. *Journal of Microbiology, 59*(4), 417–425. https://doi.org/10.1007/s12275-021-0573-2

Mackus, M., Loo, A. J. V., Garssen, J., Kraneveld, A. D., Scholey, A., & Verster, J. C. (2020). The role of alcohol metabolism in the pathology of alcohol hangover. *Journal of Clinical Medicine, 9*(11), 3421. https://doi.org/10.3390/jcm9113421

Medici, M., Vinderola, C. G., & Perdigon, G. (2004). Gut mucosal immunomodulation by probiotic fresh cheese. *International Dairy Journal, 14*(7), 611–618. https://doi.org/10.1016/j.idairyj.2003.10.011

Mohd Shukri, A., Alias, A. K., Murad, M., Yen, K. S., & Cheng, L. H. (2022). A review of natural cheese and imitation cheese. *Journal of Food Processing and Preservation, 46*(1), e16112.

Na, A. Y., Yang, E. J., Jeon, J. M., Ki, S. H., Song, K. S., & Lee, S. (2018). Protective effect of isoliquiritigenin against ethanol-induced hepatic steatosis by regulating the SIRT1-AMPK pathway. *Toxicological Research, 34*(1), 23–29. https://doi.org/10.5487/TR.2018.34.1.023

Nagappan, A., Jung, D. Y., Kim, J.-H., Lee, H., & Jung, M. H. (2018). Gomisin N alleviates ethanol-induced liver injury through ameliorating lipid metabolism and oxidative stress. *International Journal of Molecular Sciences, 19*(9), 2601. https://doi.org/10.3390/ijms19092601

Pittler, M. H., Verster, J. C., & Ernst, E. (2005). Interventions for preventing or treating alcohol hangover: Systematic review of randomised controlled trials. *BMJ, 331*(7531), 1515–1518. https://doi.org/10.1136/bmj.331.7531.1515

Ren, R., Wang, Z., Wu, M., & Wang, H. (2020). Emerging roles of SIRT1 in alcoholic liver disease. *International Journal of Biological Sciences, 16*(16), 3174–3183. https://doi.org/10.7150/ijbs.49535

Salminen, A., Hyttinen, J. M., & Kaarniranta, K. (2011). AMP-activated protein kinase inhibits NF-κB signaling and inflammation: Impact on healthspan and lifespan. *Journal of Molecular Medicine, 89*(7), 667–676. https://doi.org/10.1007/s00109-011-0748-0

Stickel, F., Datz, C., Hampe, J., & Bataller, R. (2017). Pathophysiology and management of alcoholic liver disease: Update 2016. *Gut Liver, 11*(2), 173–188. https://doi.org/10.5009/gnl16477

Sun, X., Wang, P., Yao, L. P., Wang, W., Gao, Y. M., Zhang, J., & Fu, Y. J. (2018). Paeonol alleviated acute alcohol-induced liver injury via SIRT1/Nrf2/NF-κB signaling pathway. *Environmental Toxicology and Pharmacology, 60*, 110–117. https://doi.org/10.1016/j.etap.2018.04.016

Tong, X., Chen, G. C., Zhang, Z., Wei, Y. L., Xu, J. Y., & Qin, L. Q. (2017). Cheese consumption and risk of all-cause mortality: A meta-analysis of prospective studies. *Nutrients, 9*(1), 63. https://doi.org/10.3390/nu9010063

Varga, T., Czimmerer, Z., & Nagy, L. (2011). PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1812*(8), 1007–1022. https://doi.org/10.1016/j.bbadis.2011.02.014

Wiese, J. G., Shlipak, M. G., & Browner, W. S. (2000). The alcohol hangover. *Annals of Internal Medicine, 132*(11), 897–902. https://doi.org/10.7326/0003-4819-132-11-200006060-00008

Wu, J., Xue, X., Fan, G., Gu, Y., Zhou, F., Zheng, Q., Liu, R., Li, Y., Ma, B., Li, S., Huang, G., Ma, L., & Li, X. (2021). Ferulic acid ameliorates hepatic inflammation and fibrotic liver injury by inhibiting PTP1B activity and subsequent promoting AMPK phosphorylation. *Frontiers in Pharmacology, 12*, Article 754976. https://doi.org/10.3389/fphar.2021.754976

Yue, R., Chen, G. Y., Xie, G., Hao, L., Guo, W., Sun, X., … Zhong, W. (2021). Activation of PPARα-catalase pathway reverses alcoholic liver injury via upregulating NAD synthesis and accelerating alcohol clearance. *Free Radical Biology and Medicine, 174*, 249–263. https://doi.org/10.1016/j.freeradbiomed.2021.08.005

Zakhari, S. (2006). Overview: How is alcohol metabolized by the body? *Alcohol Research & Health, 29*(4), 245.

Zheng, X., Shi, X., & Wang, B. (2021). A review on the general cheese processing technology, flavor biochemical pathways and the influence of yeasts in cheese. *Frontiers in Microbiology, 12*, Article 703284. https://doi.org/10.3389/fmicb.2021.703284