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Valorization of broiler edible byproducts: a chicken-liver hydrolysate with hepatoprotection against binge drinking

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ABSTRACT Over 10,000 metric-ton broiler livers are produced annually in Taiwan. Concerning unpleasant odor and healthy issue, broiler livers are not attractive to consumers. Although the patented chicken-liver hydrolysates (**CLHs**) through pepsin digestion possess several biofunctionalities, there is no study on hepatoprotection of CLH-based formula capsule (GBHP01) against binge drinking (Whiskey, 50% Alc./Vol.). GBHP01 led to an accelerated blood-alcohol clearance in rats, as evidenced by lowering blood-alcohol increment within 0 to 4 h, increasing blood-alcohol decrement within 4 to 8 h, and smaller blood alcohol concentration areas under the curve (**BAC AUC**) in the 8-h period ($p < 0.05$). The ameliorative effects of GBHP01 against binge drinking in rats over 6 wk were attributed to accelerated alcohol metabolism by further increasing alcohol dehydrogenase (**ADH**) and aldehyde dehydrogenase (**ALDH**) activities while downregulating cytochrome P450 2E1 (CYP2E1) protein expression, elevating antioxidant capacity, decreasing zonula occludens-1 (**ZO-1**) protein decrement and serum endotoxin, and reducing inflammation related protein levels, that is, toll-like receptor 4 (**TLR4**) and mitogen-activated protein kinase (**MAPK**), and proinflammatory cytokines. The development of CLH supplements could not only enhance the added value of broiler livers through nutraceutical development but also offer a strategy to maximize the utilization of poultry processing residues, as shown in this study.

Key words: alcohol clearance, antioxidant effects, anti-inflammatory effect, chicken-liver hydrolysate, intestinal permeability

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INTRODUCTION

According to a report by World Health Organization (WHO) (2022), excessive alcohol drinking contributes 3 million deaths per year, accounting for approximate 5.3% of all global deaths. Moreover, a clinical review article from Day et al. (2015) classified a higher drinking or binge drinking as consuming more than 6 units daily for women or more than 8 units daily for men in a regular drinking. Each unit comprises 8 g of alcohol equivalent to 10 mL of pure ethanol. Alcohol undergoes metabolic processes in livers, with alcohol dehydrogenase (**ADH**) converting it to acetaldehyde, followed by further conversion to acetic acid by aldehyde dehydrogenase (**ALDH**) (Zakhari, 2006). Meanwhile, excessive alcohol intake, surpassing the ADH ability triggers CYP2E1 leading to production both acetaldehyde and reactive oxygen species (**ROS**). Hence, during the alcohol metabolism, alcohol, acetaldehyde, and ROS result in alcoholic steatosis and steatohepatitis. Additionally, alcohol consumption also increases gut permeability, leading to bacterial translocation and Kupffer cell

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activation which play crucial roles in pathomechanism of alcoholic hepatitis (Szabo et al., 2011). Given the significant role of alcohol in social and cultural communication, there is a compelling need for the development of new functional foods as an alternative to medical interventions to protect individuals from the harmful effects of binge drinking.

Due to the escalating volume of agricultural residues generated annually, various global agricultural authorities and agencies have put forth the agricultural circular economy action plan, known as "AgroCycle". This initiative is designed to enhance sustainability through an increased recycling and valorization of agricultural residues (Troops et al., 2017). Notably, the AgroCycle innovation chain also encompasses the participation of nutraceuticals and pharmaceuticals. In comparison with parent proteins, protein hydrolysates or peptides exhibit superior absorbability and demonstrate various biofunctionalities (Kim et al., 2012; Koopman et al., 2009). A potential avenue for optimizing the utilization of animal by-products has been identified through protein hydrolyzation (Wu and Chen, 2022). In Taiwan, poultry has climbed the top one meat consumption per capita among all meat categories while approximately 263.1 million broilers were slaughtered in 2022 resulting in an annual production of over 10,000 metric tons of broiler livers were produced yearly (Ministry of Agriculture, Executive Yuan, Taiwan, 2023). Our team has recently achieved a significant milestone by successfully developing a functional chicken-liver hydrolysate (**CLH**) using a patented hydrolyzation technology (Chen et al., 2018). This innovative product showcases various biofunctionalities (Chou et al., 2014; Yang et al., 2014; Lin et al., 2017; Chen et al., 2017; Wu et al., 2020; Wu et al., 2021; Yeh et al., 2022). Based on the free amino-acid profile in our CLHs (Wu et al., 2020), several specific amino acids within CLHs have been characterized as hepatoprotective effects against alcohol consumption, i.e. leucine (Murakami et al., 2012), taurine (Fang et al., 2011), glycine (Akao and Kobashi, 1995) and more. Moreover, the hepatoprotection of our patented CLHs in chronic alcohol diet feeding mice (Lieber-DeCarli alcoholic diet, 5% Alc./Vol.) has been verified (Lin et al., 2017). The protection has been attributed to the upregulation of fatty acid β-oxidation, downregulation of fatty-acid synthesis, as well as an increase in antioxidant abilities and enhancement of ALDH abilities.

It has been estimated that approximately 13.5% of global deaths are linked to alcohol, while more than half of liver diseases worldwide are highly attributable to alcohol consumption (World Health Organization (WHO), 2022). Despite this, there is limited research on the positive impacts of natural agents in mitigating liver damages caused by binge drinking. In this study, our objective was to explore the alcohol clearance capacities of the CLH supplement following an acute binge drinking. Additionally, we aimed to elucidate the protective effects of CLH supplement on both liver function and intestinal permeability in the context of binge drinking for 6 wk.

MATERIALS AND METHODS

Chicken-Liver-Hydrolysate Supplement, BCM95 (九將軍®) Supplement, and Whiskey

A CLH-based formula capsule (GBHP01) mainly consisting of CLH and hesperidin (HAYASHIBARA HESPERIDINTM S, Nagase Taiwan Co., Ltd., Taipei, Taiwan) was generally offered by Great Billion Biotech Co., Ltd. (New Taipei City, Taiwan). The hepatoprotective effects of both CLH and GBHP01 supplements against chronic alcohol consumption have been elucidated using the Lieber-DeCarli regular EtOH liquid diet (Lin et al., 2017; Wu et al., 2024). According to the composition analysis of free amino acids and imidazole-ring dipeptides in a GBHP01 capsule (average 650 mg) from the Food Industry Research and Development Institute (FIRDI, HsinChu, Taiwan), it contains 12.50, 68.32, and 0.08 mg of free essential and non-essential amino acids and imidazole-ring dipeptides, respectively, with a total free branched chain amino acid (**BCAA**), glutamic acid, glycine, and taurine content of 5.46, 2.85, 1.09, and 54.92 mg, respectively. One GBHP01 capsule per day for a 60 kg adult is recommended as the daily intake by Great Billion Biotech. Co., Ltd. As a positive control agent, the BCM95 (九將軍®, curcumin based product) supplement (Health food No. A00378) was certified by the Taiwan Food and Drug Administration in 2019 for its proven efficacy against the Lieber-DeCarli regular EtOH liquid diet (Taiwan Food and Drug Administration, 2024) while it was procured from local pharmaceutical sources. Two BCM95 capsule per day for a 60 kg adult is a recommended daily intake. The Black Bull Whiskey (50% Alc./Vol., Duncan Taylor Whiskey Ltd., Scotland, UK) were purchased from the local winery company. According to the dose translation between rat and humans (Reagan-Shaw et al., 2008), 66.8 mg GBHP0 content/kg BW and 123 mg BCM95 (九將軍®) content/kg BW, respectively, were calculated as 1X doses each for rat per day in this study. Besides, a binge drinking was regarded as consuming about 6∼8 units daily for human equivalent to 60 to 80 mL of pure ethanol (Day et al. 2015); the 15 mL Black Bull Whiskey/kg BW rat was to mimic a binge drinking situation.

Animal Trials

There were 2 animal trials in this study: 1) to explore the alcohol clearance capacities of the CLH supplement following an acute binge drinking, and 2) to elucidate protective effects of CLH supplement on both liver function and intestinal permeability in the context of binge drinking. The animal use and protocol were reviewed and approved by the National Taiwan University Care Committee (IACUC No.: NTU-113-EL-00018).

Alcohol Clearance Capacities in an Acute Binge Drinking

Thirty male Wistar rats, aged 10 wk, were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan).

The rats were acclimatized in a controlled environment at 22±2°C and for 12/12 h light/dark cycle. They had *ad libitum* access to water and chow diet (Laboratory Rodent Diet 5001, PMI Nutrition International/Purina Mills LLC, Richmond, IN, USA). Before the commencement of the experiment, all rats (12 wk old) were randomly assigned to the following groups: 1) EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 20 EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 3) EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 4) EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 5) EtOH_GBHP01_6X: 400 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey /kg BW. The administration procedure involved oral gavage of ddH_2O or ddH_2O containing BCM95 or GBHP01 to the rats, followed by oral gavage of whiskey 30 min later. Blood samples were collected from each rat every hour until the 8th h of the experiment, allowing for the observation of potential temporal effects on blood alcohol concentration.

Liver Function and Intestinal Permeability in the Context of Binge Drinking

Another 40 male Wistar rats, aged 10 wk, were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The rats were acclimatized in a controlled environment at 22 ± 2°C and for 12/12 h light/dark cycle with 2 rats per cage and ad libitum access to water and chow diet (Laboratory Rodent Diet 5001, PMI Nutrition International/Purina Mills LLC, Richmond, IN). Before the commencement of the experiment, all rats (12 wk old) were randomly assigned to the following groups: 1) Control: isocaloric solution (15 mL ddH_2O containing 13.125 g glucose)/kg BW; 2) EtOH: 1.5 mL ddH_2O/rat +15 mL whiskey (50%, v/v)/kg BW; 3) EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 4) EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; 5) EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW. The experiment lasted for 6 wk, with average daily feed and water intakes calculated per rat daily basis, respectively. After an overnight fast (8h), rats were euthanized by CO_2 on the final day of the experiment. The initial and final body weights were recorded. Tissues (liver, heart, kidney, spleen, stomach, and visceral adipose tissues) were removed, weighed individually, and then stored at -80°C. In this animal trial, the alterations of serum biochemical vales, liver antioxidant capacities, liver inflammation related protein expressions and cytokines, liver alcohol metabolism enzymes expressions, liver histological observation/evaluation, and tight junction related protein expressions in the duodenum were investigated. Liver and duodenum homogenates (10% w/w ratio with phosphate buffer saline) were prepared, and after centrifugation (12,000 × *g*) at 4°C, the liver and duodenum filtrate was collected and stored at -80°C for further analyses. Protein levels in liver and duodenum filtrates were measured using a Bio-Rad protein assay kit (Cat. #: 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Analyzing Parameters

Blood Chemical Values Blood samples were collected by orbital sinus, placed at room temperature for clotting, and then centrifuged (3,000× *g*) at 4°C for 15 min to obtain sera. Serum triglyceride (**TG**), total cholesterol (**TC**), aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), albumin (**ALB**), and alkaline phosphatase (**ALP**) were analyzed by using commercial enzymatic kits (Randox Laboratories Ltd., Antrim, UK). Regarding serum endotoxin level, the blood was collected in hepatic portal vein. The endotoxin level was detected using the lipopolysaccharides (**LPS**) level using a commercial kit (LPS ELISA kit, Fine Test Co., Ltd, Wuhan, China). Besides, the whole blood was used to measure the alcohol concentration by using a commercial kit (Alcohol Ethanol Assay, BIOLABO S.A.S., Maizy, France).

Blood Alcohol Concentration Area Under Curve, and Increased/Decreased Rates of BAC Tendencies The blood alcohol concentration under the curve (**BCA AUC**) was calculated by the trapezoidal rule (Pikaar et al., 1988). Additionally, the increased rate of BCA was computed by analyzing the change between the initial and peak BCA per h within the 0 to 4-h timeframe following the oral administration of whiskey. Conversely, the decreased rate of BCA was determined by assessing the BCA elimination during the 4 to 8-h period post-oral gavage of whiskey.

Liver Antioxidant Capacities and Inflammatory Cytokines In accordance with the methods outlined in Wu et al. (2021), various parameters were assessed in liver tissues. Thiobarbituric acid reactive substances (**TBARS**), reduced glutathione (**GSH**) levels, and trolox equivalent antioxidant capacity (**TEAC**) were measured. Additionally, the activities of superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPx**) were determined. GPx activity was analyzed using the Ransel Glutathione Peroxidase commercial kit (Randox Laboratories Ltd., Antrim, UK). The levels of inflammatory cytokines, namely interleukin-1β (**IL-1β**), interleukin-6 (**IL-6**), and tumor necrosis factor-alpha (**TNF-α**) were qualified using enzyme-linked immunosorbent assay (**ELISA**) following the manufacturer's instructions (eBioscience Inc., San Diego, CA).

ADH and ALDH Activities in Livers The ADH and ALDH activities in liver tissues were assessed following the procedure as described by our previous publication (Wu et al., 2011). For ADH activity, the measurement involved detecting nicotinamide adenine dinucleotide with hydrogen (**NADH**) production during the conversion of ethanol to acetaldehyde. ADH activity was calculated using the extinction coefficient of NADH, set at

$66.22 \times 10^3/\mu M/cm$ at 340 nm. One unit of ADH activity was defined as the amount of enzyme that produces one mmol NADH per min at 25°C. Similarly, for ALDH activity, the assessment focused on detecting NADH production resulting from the conversion of acetaldehyde to acetic acid. ALDH activity was calculated by taking the extinction coefficient of NADH at 340 nm. One unit of ALDH was expressed as the amount of enzyme that produces one mmol NADH per min at 25°C.

Histological Examination Histopathological examinations were conducted using hematoxylin and eosin (**H&E**) staining following the protocol outlined in Chen et al. (2021). The microscopic analyses were performed using a Zeiss Axioskop 340 microscope equipped with an AxioCam ERc 5s camera system and AxioVision Release 4.8.2 (06-2010) 341 software from Carl Zeiss Microscopy, LLC, Thornwood, NY, USA. After H&E staining, liver sections were observed, and the histology activity index (**HAI**) score was employed to evaluate the inflammatory status in the liver. The HAI score included parameter such as portal inflammation, intralobular inflammation, and periportal necrosis (Brunt, 2000).

Western Blotting

The procedures of sample (liver or duodenum homogenate) preparation, SDS-PAGE, transfer, and antibody hybridization were conducted according to a previous report (Wu et al., 2024). The electrophoresis was performed by using Bio-Rad's Mini-PROTEAN Tetra Cell System (BioRad Laboratories Inc., Hercules, CA), and the proteins were transferred via the Criterion Wet Transfer Blotter. The antibodies used in this study were CYP2E1 (1:1,000 dilution, Thermo Fisher Scientific, Inc., Waltham, MA), ZO-1 (1:1,000 dilution, Thermo Fisher Scientific, Inc., Waltham, MA), TLR4 (1:1,000 dilution, Thermo Fisher Scientific, Inc., Waltham, MA), p38 MAPK (1:2,000 dilution, Bethyl laboratories, Inc., Montgomery, TX), and β-actin (1:5,000 dilution, Cell Signaling Technology, Inc., Danvers, MA). The secondary antibody for β-actin was anti-mouse IgG-horseradish peroxidase with 1:1,000 dilution. The secondary antibody for others was anti-rabbit IgG-horseradish peroxidase with 1:5000 dilutions. The protein bands were detected with the enhanced chemiluminescence (**ECL**) kit (ImmobilonTM Western, Millipore Co., Billerica, MA) under a MultiGel-21 (TOP BIO CO., New Taipei City, Taiwan) while the Image J (National Institutes of Health, Bethesda, MD, USA) was used to quantify the optical density of the protein bands by using the GAPDH band as a reference. The folds of protein expressions of other groups were relatively expressed to those of Control group.

Statistical Analysis

The experiment was conducted using a completely random design (**CRD**). Data were analyzed using analysis of variance (ANOVA). When a significant difference among all groups was statistically analyzed at 0.05 probability level, differences between treatments were further examined via the least significant difference (**LSD**) test. All data were conducted via SAS 9.4 software (SAS Institute Inc., Cary, NC, 2002).

RESULTS

Effects of Chicken-Liver Hydrolysate Supplement on Alcohol Clearance Ability in Acute Binge Drinking Rats

The blood alcohol concentrations (**BAC**) in rats were increased due to alcohol consumption (binge drinking) during the first 4 or 5 h, and then decreased (Figure 1A). Specially, the BACs were 543.72 mg/mL for EtOH group and 554.48 mg/mL for EtOH_P group at the 4th h, 534.46 mg/mL for EtOH_GBHP01_1X group and 492.71 mg/ mL for EtOH_GBHP01_3X group at the 5th h, and 474.23 mg/ mL for EtOH_GBHP01_6X group at the 4th h. However, after the period of BAC peak, a decreasing trend was observed among all groups. During the assay period, supplementing chicken-liver-hydrolysate supplement (GBHP01) and BCM95 could decrease ($p < 0.05$) the BAC, especially 6X GBHP01 supplementation after 4 h of binge drinking. Furthermore, regarding the BAC area under curve (BAC AUC) in the assay period (0−8 h) (Figure 1B), the EtOH group had the largest ($p < 0.05$) value, followed by EtOH_GBHP01_1X, EtOH_P, EtOH_GBHP01_3X, and EtOH_GBHP01_6X group. Interestingly, the EtOH_GBHP01_3X and EtOH_GBHP01_6X groups showed a significantly greater decrease ($p < 0.05$) in BAC AUC compared to the BCM95 supplemented group. In the period of 0 to 4 h (Figure 1C), supplementing GBHP01 decreased the BAU increment rate, especially with 3X and 6X GBHP01 supplementation ($p < 0.05$), while a significant BAU decrement rate ($p < 0.05$) was observed with BCM95 or 1X GBHP01 supplementation in the period of 4 to 8 h (Figure 1D).

Hepatoprotection of Chicken-Liver-Hydrolysate Supplement on Rats in the Context of Binge Drinking Rats

Growth Performance and Serum Biochemical Values in Binge Drinking Rats There were no ($p > 0.05$) differences on initial body weight among all groups (Table 1). However, the binge drinking (50%, Alc./Vol., whiskey intake) for 6 wk resulted in lower ($p < 0.05$) final body weights and weight increases (g) in rats, while the body weights of EtOH and EtOH_P groups at the end of experiment were even lower than those at the beginning of experiment. However, decreased body weights of EtOH_GBHP01_1X and EtOH_GBHP01_3X groups were lessened ($p < 0.05$) than that of EtOH group. A similar tendency was observed for food and water intakes as final body weights and weight increases. Regarding the relative sizes of organs and

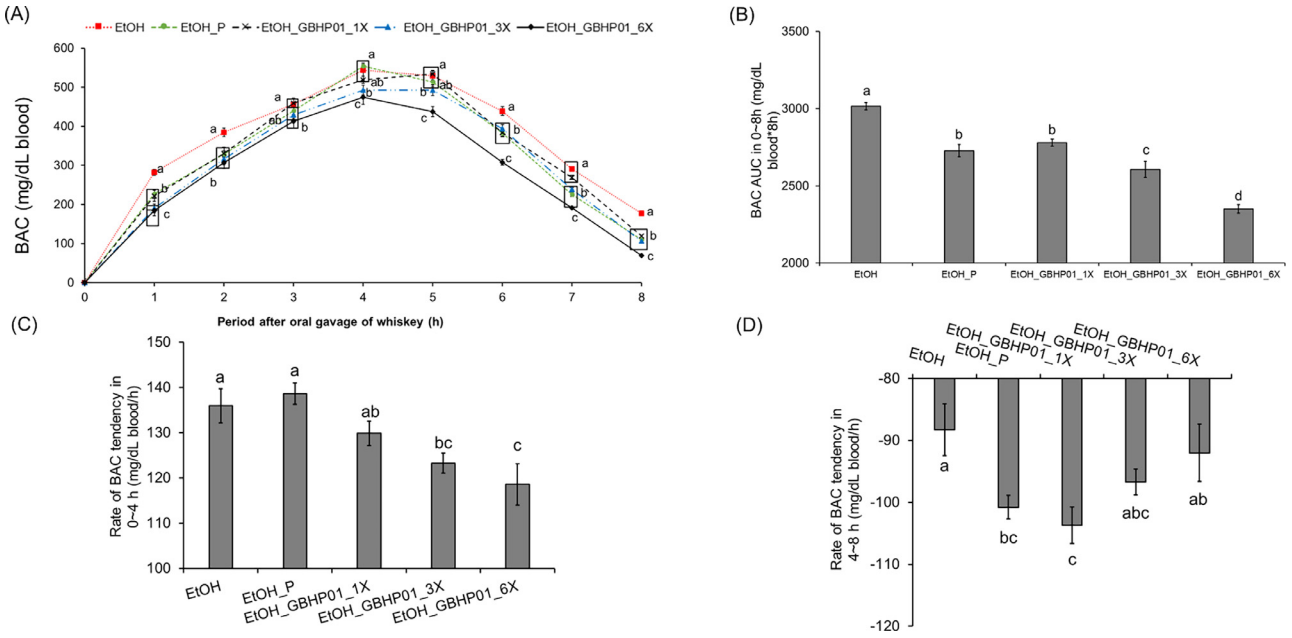

Figure 1. Effects of chicken-liver-hydrolysate supplement on (A) blood alcohol concentration (**BAC**) and (B) changes of BAC area under the curve (**BAC AUC**) during the 0 to 8 h period, as well as (C) increased and (B) decreased rates of BAC tendencies of experimental rats during the 0 to 4 and 4 to 8 h periods, respectively, after oral gavage of whiskey. * Values are given as mean±SEM (n = 6). Data points in each test period among group or data bars without a common letter indicate a significant difference ($p < 0.05$). ** EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey (50%, v/v)/kg BW; EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_6X: 400 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW.

Table 1. Effects of chicken-liver hydrolysate supplement on growth performance, relative sizes of organ and visceral adipose tissue, serum biochemical vales and blood alcohol levels, and liver antioxidant capacity and inflammatory cytokines in experimental rats.

Group	Control	EtOH	EtOH_P	EtOH_GBHP01_1X	EtOH_GBHP01_3X
Growth performance					
Initial body weight (g)	507.45±7.28a	521.00±11.13a	521.23±10.99a	520.98±10.60a	521.48±10.31a
Final body weight (g)	623.95±13.52a	478.90±9.86c	508.03±12.30bc	523.70±15.33b	527.41±6.07b
Weight increase (g)	138.86±2.64a	−41.10±8.28c	−13.20±14.73bc	2.72±19.86b	5.93±10.78b
Food intake (g/rat/day)	27.94±0.51a	13.04±0.68d	15.84±0.88c	17.85±0.89b	18.63±0.32b
Water intake (mL/rat/day)	71.27±0.80a	47.29±2.88c	49.96±1.17bc	55.03±3.80b	54.38±2.21bc
Relative size (g/100g BW)					
Liver	2.73±0.09c	3.51±0.20a	2.97±0.09bc	2.84±0.06bc	3.06±0.05b
Heart	0.26±0.01b	0.30±0.01a	0.27±0.01b	0.27±0.01b	0.26±0.01b
Kidney	0.57±0.02d	0.70±0.02a	0.59±0.02cd	0.64±0.02b	0.62±0.01bc
Spleen	0.24±0.01a	0.27±0.02a	0.26±0.02a	0.24±0.02a	0.25±0.02a
Stomach	0.47±0.02c	0.66±0.02a	0.57±0.03b	0.55±0.02b	0.53±0.02bc
Visceral adipose tissue	3.05±0.11a	2.53±0.29a	2.66±0.23a	2.50±0.08a	2.74±0.28a
Serum biochemical value and blood alcohol level					
TC (mg/dL serum)	58.70±3.21b	104.62±7.93a	73.75±5.09b	64.05±5.33b	69.40±3.85b
TG (mg/dL serum)	72.77±3.44c	143.67±8.48a	105.88±6.36b	108.46±7.30b	105.88±9.13b
AST (U/L serum)	82.26±2.56c	179.74±5.36a	138.88±6.03b	127.27±3.28b	132.23±6.63b
ALT (U/L serum)	33.64±1.23d	95.16±6.69a	74.41±2.63b	59.26±2.08c	62.15±1.47c
ALB (g/dL serum)	4.50±0.07a	3.06±0.13c	3.54±0.06b	3.42±0.05b	3.47±0.06b
ALP (U/L serum)	54.22±3.38d	142.15±6.74a	89.85±5.57c	107.33±3.01b	117.19±6.57b
Alcohol (mg/dL blood)	0.00±0.00b	8.05±1.52a	2.08±0.85b	1.63±0.60b	1.20±0.49b
Liver antioxidant capacity					
TBARS (nmole MDA eq./mg protein)	1.23±0.08cd	2.88±0.14a	2.13±0.12b	1.52±0.10c	1.18±0.09d
TEAC (μmole/mg protein)	1.32±0.03a	0.98±0.05c	1.20±0.04b	1.34±0.04a	1.19±0.03b
Reduced GSH (nmole/mg protein)	55.25±2.86bc	28.91±3.05d	47.62±4.19c	61.92±3.01ab	68.38±3.35a
SOD (unit/mg protein)	7.92±0.26b	7.62±0.39b	8.41±0.24ab	8.25±0.41ab	9.28±0.47a
CAT (unit/mg protein)	114.07±4.07b	125.81±5.60ab	131.53±6.28a	134.89±5.79a	140.22±3.73a
GPx (unit/mg protein)	6.40±0.29a	3.92±0.34b	4.64±0.33b	6.38±0.26a	6.87±0.4a
Liver inflammatory cytokine (pg/mg protein)					
IL-1β	172.56±11.61b	259.67±15.31a	158.61±12.93c	159.11±15.26c	201.78±14.96b
IL-6	116.70±9.34c	187.20±12.52a	140.10±9.82bc	149.19±10.28b	127.60±6.76bc
TNF-α	84.04±8.32b	173.87±17.18a	102.15±9.27b	101.86±14.41b	108.49±10.88b

*Data are given as means ± SEM (n = 8, except food and water intake, n = 4). Mean values without a common letter in the same test parameter indicate a significant difference ($p < 0.05$).

**Control: isocaloric solution (15 mL ddH_2O containing 13.125 g glucose)/kg BW; EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey (50%, v/v)/kg BW; EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW.

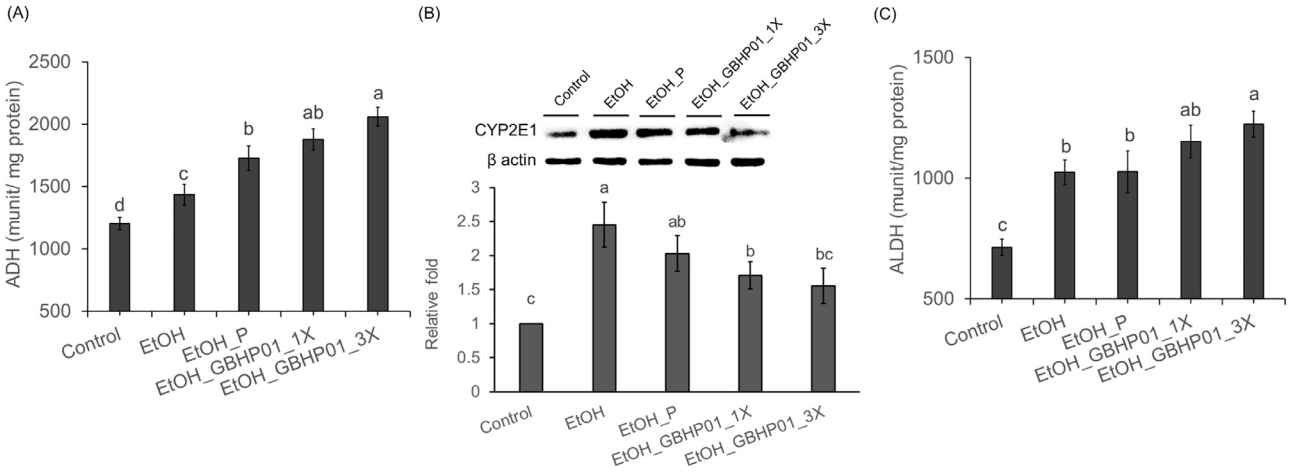

Figure 2. Effects of chicken-liver hydrolysate supplement on (A) ADH activity, (B) CYP2E1 protein levels, and (C) ALDH activity in livers of experimental rats. * Values are given as mean±SEM (n=6). Data bars without a common letter indicate a significant difference ($p < 0.05$). ** Control: isocaloric solution (15 mL ddH_2O containing 13.125 g glucose)/kg BW; EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey (50%, v/v)/kg BW; EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW.

visceral adipose tissues (Table 1), there were no ($p > 0.05$) differences on sizes of spleen and visceral adipose tissue among all groups, but EtOH group had the larger ($p < 0.05$) sizes of liver, heart, kidney, and stomach than the Control group. GBHP01 or BCM95 supplementation (EtOH_P, EtOH_GBHP01_1X, and EtOH_GBHP01_3X groups) lessened ($p < 0.05$) the increased sizes of those organs. Some binge drinking groups with supplementation even had similar ($p > 0.05$) organ sizes as the Control groups (liver: EtOH_P and EtOH_GBHP01_1X vs. Control; heart: EtOH_P, EtOH_GBHP01_1X, and EtOH_GBHP01_3X vs. Control; kidney: EtOH_P vs. Controol; stomach: EtOH_GBHP01_3X vs. Control). Regarding serum biochemical values (Table 1), higher ($p < 0.05$) TC, TG, AST, ALT, and ALP values, but lower ($p < 0.05$) ALB value in the EtOH group were measured than those in Control group. GBHP01 and BCM95 supplementation could reduce ($p < 0.05$) these elevated values and increase ($p < 0.05$) ALB value of binge drinking rats.

Expressions of Alcohol Metabolism in Binge Drinking Rats After 6 wk of experiment, all binge drinking groups showed higher ($p < 0.05$) blood alcohol levels than the Control group (Table 1). GBHP01 or BCM95 supplementation apparently decreased ($p < 0.05$) blood alcohol levels in binge drinking rats. Regarding the major liver enzyme expressions related alcohol metabolism (Figure 2), ADH activity, CYP2E1 protein expression, and ALDH activity were activated and increased by a binge drinking ($p < 0.05$). The ADH activity was further increased ($p < 0.05$) by GBHP01 or BCM95 supplementation (Figure 2A). The CYP2E1 protein expressions in binge drinking rats were decreased ($p < 0.05$) by GBHP01 supplementation (Figure 2B). There was a tendency toward higher ADLH activities in binge drinking rats supplemented with GBHP01 (EtOH_GBHP01_3X vs. EtOH, $p < 0.05$) (Figure 2C).

Liver Antioxidant Capacities and Histological Observation After 6 wk of binge drinking, EtOH group exhibited higher ($p < 0.05$) TBARS values as well as lower ($p < 0.05$) TEAC and reduced GSH levels compared to the Control group (Table 1). There were no ($p > 0.05$) differences on activities of antioxidant enzymes, such as SOD and CAT, between EtOH and Control groups, but the lower ($p < 0.05$) GPx activity in EtOH group was detected compared to that in the Control group. There was a tendency toward higher SOD and CAT activities in BCM95 or GBHP01 supplemented group, but 3X GBHP01 supplementation significantly increased ($p < 0.05$) SOD activities in binge drinking rats. Furthermore, while BCM95 supplementation did not ($p > 0.05$) had an ability to increase GPx activities in binge drinking rats, both 1X and 3X GBHP01 supplementation significantly increased ($p < 0.05$) GPx activities.

According to the hepatic histological observation, the Control group showed clear polygonal hepatocytes (H), nuclei, and narrow radiating blood sinusoids (s) around the central vein (**CV**) (Figure 3A). In contrast, livers of binge drinking rats exhibited unclear boundary hepatocytes, ballooning of hepatocytes (**B**), inflammatory cell infiltration (**IF**), Kupffer cells (**KC**), darkly stained nuclei (**DN**), and vacuolated cytoplasm (**v**) around CV. However, BCM95 or GBHP01 supplementation appeared to reduce these abnormal pathological changes and increase the number of intact polygonal hepatocytes. The interpretation of pathological slides (Figure 3B) revealed that the HAI scores (portal inflammation, intralobular inflammation, and periportal necrosis) were elevated ($p < 0.05$) by binge drinking. There was a dramatic reduction ($p < 0.05$) in these HAI scores in the BCM95 or GBHP01 supplemented groups while no ($p > 0.05$) differences on the HAI scores were evaluated between EtOH_GBHP01_3X and the Control groups.

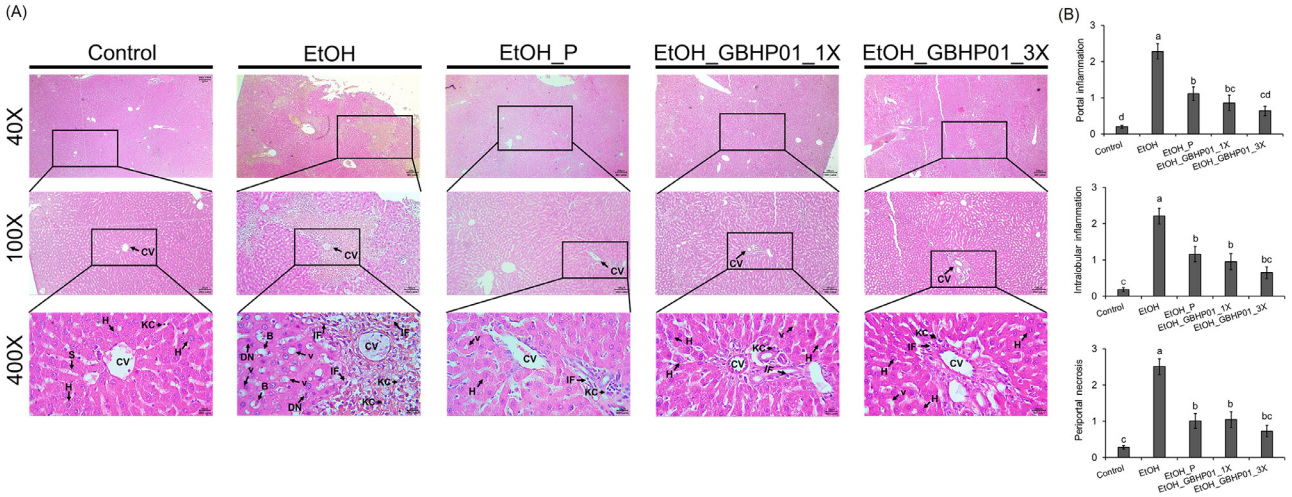

Figure 3. Effects of chicken-liver hydrolysate supplement on (A) H&E stain illustration and (B) the HAI scores of liver tissues of experimental rats. * Values are given as mean±SEM (n=8). Data bars without a common letter indicate a significant difference ($p < 0.05$). ** Polygonal hepatocytes (H), Narrow radiating blood sinusoids (**s**), central vein (**CV**), ballooning of hepatocytes (B), inflammatory cell infiltration (IF), Kupffer cell (**KC**), darkly stained nuclei (**DN**) and vacuolated cytoplasm (**v**). *** Control: isocaloric solution (15 mL ddH_2O containing 13.125 g glucose)/kg BW; EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey (50%, v/v)/kg BW; EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_1X: 66.8 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW.

Intestinal Permeability as well as Liver Inflammatory Protein and Cytokine Expressions After 6 wk of binge drinking, various parameters related to intestinal permeability and liver inflammation were assessed, including the expression of the intestinal permeability-related protein ZO-1, serum endotoxin levels, and liver inflammatory protein and cytokine expressions,

The expression of ZO-1 protein was lowered ($p < 0.05$) in binge drinking groups, but GBHP01 supplementation elevated ($p < 0.05$) ZO-1 protein expressions (Figure 4A). The EtOH group exhibited a higher serum endotoxin level (Figure 4B), with a lower tendency of serum endotoxin levels observed in BCM95 or GBHP01 supplemented groups. In terms of liver inflammatory protein expressions, the EtOH group showed a higher ($p < 0.05$) TLR4 and MAPK protein expressions than the Control group (Figures 4C and 4D). However, BCM95 or GBHP01 supplementation reversed ($p < 0.05$) TLR4 protein expressions in the livers of binging drinking rats. Although BCM95 or GBHP01 supplementation downregulated MAPK protein in livers of binging drinking rats, a significant downregulated ($p < 0.05$) MAPK protein expression was observed in the 3X GBHP01 supplemented group, with a similar ($p > 0.05$) level as that of the Control group. Additionally, the EtOH group had the higher ($p < 0.05$) IL-1β, IL-6, and TNF-α than the Control group (Table 1). However, these cytokine levels in binge drinking rats were significantly reduced ($p < 0.05$) by supplementing BCM95 or GBHP01, with levels even similar to those of the Control group.

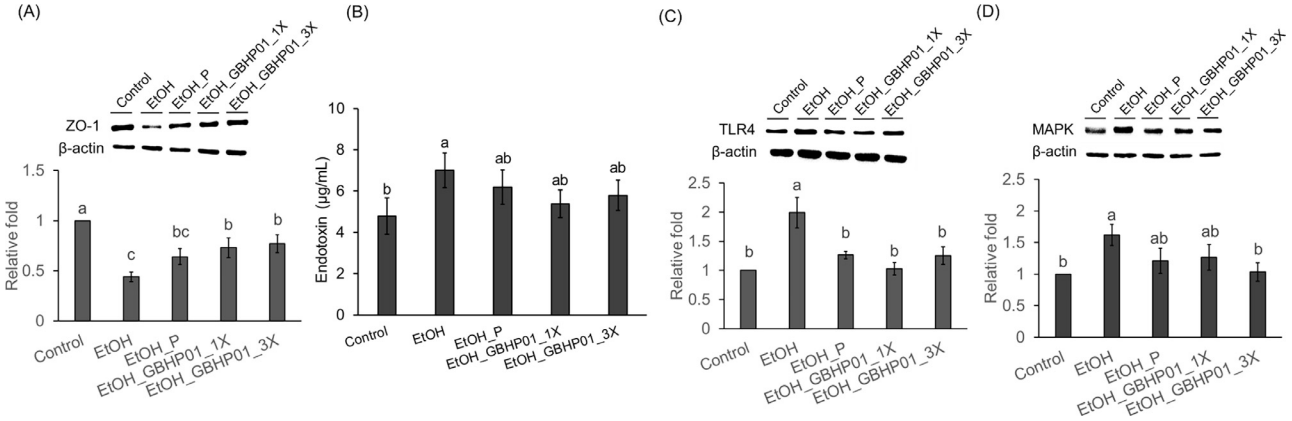

Figure 4. Effects of chicken-liver-hydrolysate supplement on tight junction related protein expressions in the duodenum, endotoxin level in sera, and inflammation related protein expressions in the livers of experimental rats: (A) ZO-1, (B) endotoxin, (C) TLR4, and (D) MAPK. * Values are given as mean±SEM (n = 8). Data bars without a common letter indicate a significant difference ($p < 0.05$). ** Control: isocaloric solution (15 mL ddH_2O containing 13.125 g glucose)/kg BW; EtOH: 1.5 mL ddH_2O/rat+15 mL whiskey (50%, v/v)/kg BW; EtOH_P: 123 mg BCM95 (九將軍®)/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_1X: 66.8 mg GBHP01kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW; EtOH_GBHP01_3X: 200 mg GBHP01/kg BW in 1.5 mL ddH_2O/rat+15 mL whiskey/kg BW.

DISCUSSION

The increasing number of agricultural residues globally has prompted agricultural authorities and agencies to propose the agricultural circular economy action plan, known as "AgroCycle", which aims at enhancing sustainability through increased recycling and valorization of agricultural residues (Troops et al., 2017). Additionally, the potential application of agricultural residues in production of nutraceuticals and pharmaceuticals is being explored within the AgroCycle innovation chain. Protein hydrolyzation presents a feasible application of animal by-products, especially in parts rich in proteins, that is, feathers, blood, viscera, and skin. Several literatures have shown that hydrolysates of animal by-products are characterized as antioxidant properties (Nikhita & Sachindra, 2021) and lipid-lowering effects (Chen et al., 2021; Wu et al., 2021). In Taiwan, the slaughter of over 263 million broilers annually results in over 10,000 metric tons of broiler livers being generated each year (Ministry of Agriculture, Executive Yuan, Taiwan, 2023). Due to the concerns related odor and health, these broiler livers are not preferred by consumers. Our team has endeavored to develop an antioxidant ingredient from broiler livers by means of enzyme selection and its optimization of the hydrolysis process (Chou et al., 2014). This innovative product showcases various biofunctionalities, as demonstrated in several studies (Chou et al., 2014; Yang et al., 2014; Chen et al., 2017; Lin et al., 2017; Wu et al., 2020; Wu et al., 2021; Yeh et al., 2022).

Alcoholic beverages have played a significant role in human history and society. Paton (2005) reported that consumed alcohol is primarily absorbed by the stomach (20%) and small intestine (80%) in the body. However, an excessive alcohol drinking (binge drinking, women: > 6 units daily and men: > 8 units daily; one unit: 8 g ethanol) contributes to 3 million deaths per year, accounting for approximate 5.3% of all global deaths (World Health Organization (WHO), 2022). Alcohol is mainly metabolized in the liver, with alcohol dehydrogenase (**ADH**) converting it to acetaldehyde, which is further converted to acetic acid by aldehyde dehydrogenase (**ALDH**) (Zakhari, 2006). The hepatoprotection of our patented chicken-liver hydrolysates (**CLHs**), containing abundant free amino acids, especially BCAAs, glycine, and taurine, as well as imidazole-ring dipeptides against a chronic alcohol consumption (Lieber-DeCarli alcohol diet, 5% Alc) (Lin et al., 2017), thioacetamide (**TAA**) induction (Chen et al., 2017), and long-term high-fat diet (Wu et al., 2021) has been verified. Specific amino acids, such as leucine (Murakami et al., 2012), taurine (Fang et al., 2011), glycine (Akao and Kobashi, 1995) within CLHs have been characterized for their hepatoprotective effects against alcohol consumption. They mentioned that leucine and taurine supplementation can elevate ADH and ALDH activities to accelerate the alcohol clearance while glycine can suppress alcohol absorption from the gastrointestinal tract by lowering the gastric emptying rate of ethanol. The composition analysis of free amino acid and imidazole-ring dipeptides in CLH supplement (GBHP01, 650 mg/capsule) revealed 5.46, 2.85, 1.09, 54.92, and 0.08 mg for total BCAAs, glycine, taurine, and imidazole-ring dipeptide content, respectively. Hence, the effects of GBHP01 on alcohol clearance capacities and intestinal permeability in a binge drinking is the major investigation in this study. Moreover, curcumin, the major ingredient in BCM95, has been shown to elevate the ADH and ALDH activity (Lee et al., 2013). These findings suggest that the lower blood alcohol concentration (**BAC**) observed in rats supplemented with GBHP01 or BCM95 after binge drinking could be attributed to these major ingredients (Figure 1A). The decreased BCA area under curve (AUC) in 0 to 8 h period (Figure 1B) and the slow rate of BAC increment in 0 to 4 h period (Figure 1C), respectively, in GBHP01 supplemented rats after binge drinking might be explained by their higher alcohol metabolism ability and lower gastric emptying rate of alcohol. Consequently, the higher rate of BCA decrement in GBHP01 or BCM95 supplemented rats could be indicative of their enhanced alcohol clearance ability (Figures 1A and 1D).

In the study by Romero-Herrera et al. (2023), binge drinking (3 g/kg/day) lead the lower body weight of Wistar rats, possibly due to lower adipose mass and higher oxidative stress in skeletal muscle, leading to an imbalance in myokines related to muscle turnover. Similar results were observed in our study, where lower final body weight/weight increase (g) and smaller visceral adipose-tissue size were noted in Table 1. BCAAs can activate mammalian target of rapamycin (**mTOR**) to stimulate the muscle growth (Lane et al., 2017), potentially explaining the heavier final body weight/weight increase in GBHP01 supplemented group compared to the BCM95 supplemented group. Additionally, alcohol consumption does not only injure liver but also other organs, such as spleen, heart, and stomach (Epstein, 1997; Wong et al., 2008; Li et al., 2021). Higher serum lipid or liver damage indices and lower ALB values were always observed in liver damages (Fang et al., 2011; Chang et al., 2013; Chen et al., 2017; Lin et al, 2023). Based on the biofunctionalities of GBHP01 (**CLHs**) and BCM95 (curcumin) (Lee et al., 2013; Chen et al., 2017; Lin et al., 2017), GBHP01 or BCM95 supplementation may assist to lessen those unnormal changes in serum lipid, liver damage indices, and ALB values. The liver plays a crucial role in metabolizing alcohol to acetic acid through enzymes, ADH, ALDH and CYP2E1. Excessive alcohol consumption (binge drinking) can lead to the production of toxic metabolic intermediates, acetaldehyde and ROS, which can damage livers (Zakhari, 2006). Our results indicate that although bring drinking activated ADH and ALDH activities, and CYP2E1 in livers, GBHP01 or BCM95 supplementation further increased ADH and ALDH activities but downregulated CYP2E1 (Figure 2). This acceleration of alcohol metabolism in supplemented groups could be attributed to their bioactive compounds, such as curcumin, BCAAs, glycine, and taurine.

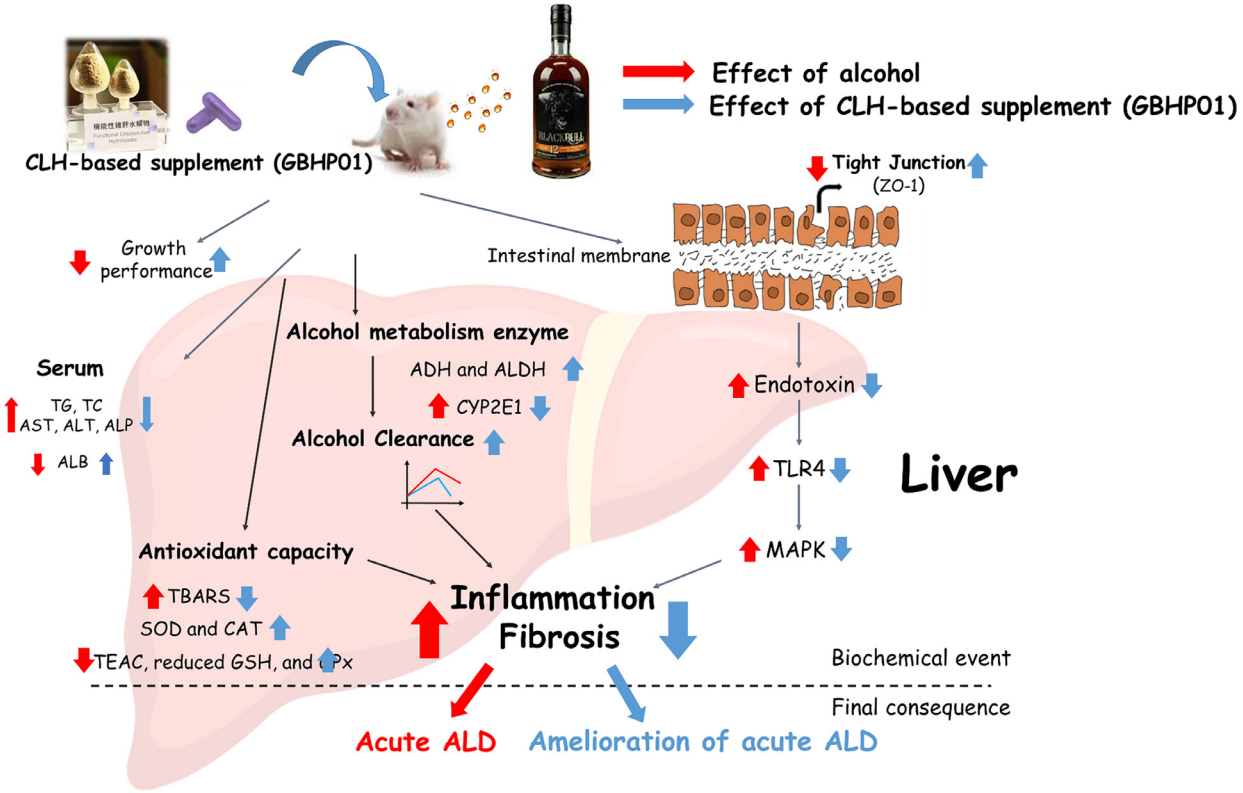

Figure 5. Hepatoprotection of chicken-liver-hydrolysate supplement against alcoholism via accelerating alcohol clearance and decreasing intestinal permeability.

Alcohol consumption can increase gut permeability, leading to bacterial translocation and Kupffer cell activation which play crucial roles in pathomechanism of alcoholic hepatitis (Szabo et al., 2011). Studies have shown that this patented CLHs can ameliorate liver fibrogenesis by TAA induction and development of alcoholic fatty liver caused by chronic alcohol consumption (Liber-DeCarli ethanol diet, 5% Alc). The beneficial effects are achieved by enhancing antioxidant capacity, and downregulating anti-inflammatory and antifibrogenic proteins, and modulating fatty acid metabolism (Chen et al., 2017; Lin et al., 2017). After metabolizing ethanol, acetaldehyde could accumulate in the intestinal wall and disrupt tight junction and adherent junction related protein, such as ZO-1 (Purohit et al., 2008). Decreased ZO-1 protein levels result in increased intestinal permeability, allowing macromolecules like endotoxin enter the bloodstream (Fasano and Nataro, 2004). Endotoxin further binds lipopolysaccharide-binding protein and recognized by toll-like receptor 4 (**TLR4**), leading to an activation of Kupffer cells and the production of ROS, nuclear factor kappa B (**NFκB**) and mitogen-activated protein kinases (**MAPKs**). This cascade promotes the secretion of proinflammatory cytokine (IL-1β, IL-6, and TNF-α) (Mencin et al., 2009). Based on the H&E stain illustration and HAI scores (Figure 3), despite less lipid accumulation was observed in livers of binge drinking rats compared to those fed with the Liber-DeCarli ethanol diet (Lin et al., 2017), it was evident that the liver injury and inflammation caused by binge drinking were mitigated by supplementing GBHP01 (chicken-liver hydrolysates) or BCM95 (curcumin). The amelioration of CLH and this GBHP01 formula against alcoholic diet (DeCarli diet, 5% Alc.) were mainly attributed to downregulated fatty acid synthesis, upregulated fatty-acid β-oxidation, and increased antioxidant capacities (Lin et al., 2017; Wu et al., 2024). Based on the data from this study (Table 1, Figures 3A and 4), the amelioration of liver injuries by supplementing GBHP01 formula against binge drinking may result from increased liver antioxidant capacities, decreased serum endotoxin and liver proinflammatory cytokine/inflammation related proteins, as well as reduced Kupffer cells in liver tissues.

CONCLUSIONS

In Taiwan, there are over 10,000 metric tons of broiler livers being generated annually. Due to the concerns regarding odor and health, these broiler livers are considered waste. Maximizing the utilization is thus crucial for government, industry and academia. Excessive alcohol consumption, such as a binge drinking habit, can increase intestinal permeability and liver oxidative stress and inflammation, thus leading to hepatitis. However, the CLH supplement (GBHP01) has shown an ameliorative effect on development of hepatitis induced by binge drinking (Figure 5). Our results demonstrated that binge drinking could increase alcohol level in sera, and elevate alcohol metabolism enzyme expressions, oxidative stress, and proinflammatory cytokines in livers

while also leading to a lower ZO-1 protein level in duodenum, which reduces tight junction. The mitigation of GBHP01 on those abnormal physiological responses to binge drinking are attributed to the following factors: 1) acceleration of alcohol clearance by further increasing ADH and ALDH activities while downregulating CYP2E1 protein expression, 2) elevation of antioxidant capacity, 3) decreases of intestinal permeability (ZO-1 protein decrement) and serum endotoxin, and 4) downregulation of inflammation related protein levels, such as TLR4 and MAPK, and proinflammatory cytokines.

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DISCLOSURES

The authors declare no conflicts of interest.

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