

Progressive Metabolic Changes During High-Cholesterol Diet Feeding and Attenuation by PNBio in a Rabbit Model of MASLD

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ABSTRACT

ARTICLE DETAILS

Background: prolonged dietary cholesterol exposure can progressively disrupt lipid and glucose metabolism and promote hepatic lipid accumulation; metabolic features associated with metabolic dysfunction-associated steatotic liver disease (MASLD). The present study evaluated the preventive effect of a polyherbal nutraceutical preparation (PNBio) during ongoing exposure to a high-cholesterol diet (HCD).

Methods: Male New Zealand White rabbits were allocated to control, HCD, HCD + PNBio, and HCD + atorvastatin groups (n = 6/group) and followed for 90 days. Body weight and serum biochemical parameters, including total cholesterol, triglycerides, LDL-C, HDL-C, glucose, ALT, and AST, were monitored at baseline and monthly. At the end of the feeding period, hepatic cholesterol and triglyceride contents, ApoA-I, ApoB, ApoE levels, ApoA-I and ApoE gene expression, HMG-CoA reductase activity, and LDLR expression were assessed.

Results: Continuous HCD exposure produced progressive increases in serum total cholesterol, triglycerides, LDL-C, glucose, ALT, and AST, accompanied by reductions in HDL-C and increases in hepatic cholesterol and triglyceride accumulation. HCD also altered hepatic apolipoprotein levels and reduced HMG-CoA reductase activity and LDLR expression. PNBio attenuated these metabolic and hepatic alterations and partially restored the apolipoprotein profile, HMG-CoA reductase activity, and LDLR expression toward control levels.

Conclusion: PNBio attenuated progressive metabolic and hepatic abnormalities during sustained HCD exposure. These findings support its potential as a preventive nutritional intervention against diet-induced metabolic disturbances associated with MASLD.

KEYWORDS: High-cholesterol diet; PNBio; dyslipidemia; hepatic lipid accumulation; apolipoproteins; glucose metabolism; MASLD

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I. INTRODUCTION

Excessive dietary cholesterol can progressively disrupt lipid homeostasis, glucose metabolism, and hepatic function, leading to circulating dyslipidemia and hepatic lipid accumulation. These metabolic abnormalities may develop progressively during sustained dietary exposure rather than appearing simultaneously. In experimental rabbits, prolonged dietary lipid excess has been associated with increasing serum lipids, hepatic lipid accumulation, elevated liver enzymes, and other metabolic alterations (1). These features are relevant to metabolic dysfunction-associated steatotic liver disease (MASLD), the updated nomenclature for fatty liver disease associated with metabolic dysfunction. MASLD is characterized by hepatic steatosis occurring in the presence of metabolic risk factors and involves disturbances in lipid metabolism, glucose homeostasis, and

hepatic lipid handling (2,3). Although the present rabbit model does not establish the clinical diagnosis of MASLD, the observed metabolic and hepatic alterations provide an experimental framework for investigating diet-induced abnormalities relevant to MASLD.

Rabbits are particularly responsive to dietary cholesterol and have been widely used as experimental models of diet-induced lipid disturbances. High-cholesterol feeding produces marked alterations in circulating lipids, hepatic lipid accumulation, and hepatic lipid metabolism (4,5). These characteristics make rabbits useful for investigating progressive metabolic and hepatic changes relevant to MASLD.

Apolipoproteins play an important role in the transport and metabolism of circulating lipids and may provide additional information beyond conventional lipid measurements. ApoA-I is the major structural

apolipoprotein of HDL and is closely associated with reverse cholesterol transport, whereas ApoB is the principal structural protein of atherogenic lipoproteins, including VLDL and LDL. ApoE contributes to the hepatic uptake and clearance of remnant lipoproteins. Alterations in these apolipoproteins may therefore reflect disturbances in lipoprotein metabolism associated with sustained dietary cholesterol exposure(6,7). Hepatic cholesterol homeostasis is tightly regulated by a coordinated feedback mechanism involving HMG-CoA reductase, the rate-limiting enzyme in endogenous cholesterol biosynthesis, and the LDL receptor (LDLR), which mediates hepatic uptake and clearance of circulating LDL-cholesterol. Under conditions of excess dietary cholesterol, intracellular cholesterol accumulation suppresses SREBP-2-mediated transcription of both HMG-CoA reductase and LDLR, thereby reducing endogenous cholesterol synthesis while simultaneously impairing hepatic clearance of plasma LDL-cholesterol, a dysregulation that further exacerbates systemic hypercholesterolemia (8). Given their central role in coordinating cholesterol synthesis and uptake, hepatic HMG-CoA reductase and LDLR expression represent key molecular targets for evaluating the efficacy of cholesterol-lowering interventions.

Plant-derived bioactive compounds have attracted increasing interest as complementary approaches to managing metabolic disturbances associated with dyslipidemia and hepatic lipid accumulation (9,10). Polyherbal preparations may provide multiple bioactive components with potentially complementary effects on lipid metabolism and metabolic homeostasis. Polyherbal nutraceuticals Biologicals (PNBio), are a multi-component plant-based preparation formulated from medicinal and nutritionally relevant plants. In the present study, PNBio was evaluated as a preventive intervention dietary supplement during continued HCD exposure rather than as a treatment administered after disease establishment. Therefore, the present study investigated the progressive development of metabolic and hepatic abnormalities during 90 days of HCD exposure and evaluated whether concurrent PNBio administration could attenuate these changes. Body weight and serum biochemical parameters were monitored longitudinally, while hepatic lipid content, apolipoprotein levels and gene expression, HMG-CoA reductase activity, and LDLR expression were assessed at the end of the study.

II. MATERIAL AND METHOD

A. Chemical and Experimental Reagent

(Biochemical assay kits and reference chemicals used in the present study were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Additional analytical-grade solvents and reagents required for the experimental procedures were obtained from SRL Chemicals (Mumbai, India).

B. Plant Materials and Preparation of PNBio

PNBio was prepared from a combination of medicinal and nutritionally relevant plant materials. The formulation consisted of horse gram (*Macrotyloma uniflorum*) seeds [11], fenugreek (*Trigonella foenum-graecum*) seeds [12], green amaranth (*Amaranthus viridis*) fresh aerial parts (leaves and stems), purple amaranth (*Amaranthus tricolor*) fresh aerial parts (leaves and stems) [13], Brahmi (*Bacopa*

monnieri) whole plant [14], Njavara rice (*Oryza sativa*) grain [15], Vasa/Karunochi (*Justicia gendarussa*) leaves [16], and Rue (*Ruta graveolens*) leaves [17]. Horse gram, fenugreek, green amaranth, and purple amaranth were obtained from local sources or the local market, whereas Brahmi, Njavara rice, *Justicia gendarussa*, and *Ruta graveolens* were procured from an authorized Ayurvedic store. Each component was incorporated at 1% (w/w), except *Ruta graveolens*, which was included at 0.5% (w/w). The collected plant materials were washed, shade-dried, and pulverized separately using an electric grinder. The resulting powders were thoroughly mixed in accordance with the formulation proportions to obtain a homogeneous PNBio preparation, which was subsequently used in the animal experiments.

C. Experimental Animals

Male New Zealand White rabbits weighing 2.2–2.7 kg were housed under standard laboratory conditions at 24–26 °C with a 12-h light/dark cycle. Standard pellet feed and water were provided ad libitum. All experimental procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; Registration No. 218/CPCSEA) and were approved by the Institutional Animal Ethics Committee of the University of Kerala (IAEC-KU-31/2022-BCH-AH (46)).

D. Experimental Design

Rabbits were randomly allocated into four experimental groups (n = 6 per group) and maintained under the respective dietary and treatment conditions for 90 DAYS. The experimental groups and treatment regimen are summarized in Table 1.

TABLE 1. EXPERIMENTAL GROUPS AND TREATMENT PROTOCOL

Group	Dietary regimen	Treatment
Control	Standard diet (SD)	None
HCD	SD+1% Cholesterol, 0.5% Cholic acid	None
PNBio	SD+1% Cholesterol, 0.5% Cholic acid	PNBio, 200 mg/kg body weight/day
Atorvastatin	SD+1% Cholesterol, 0.5% Cholic acid	Atorvastatin, 5 mg/kg body weight/day

Standard pellet diet mixed with 1% Cholesterol and 0.5% Cholic acid was given to HCD group, PNBio and Atorvastatin groups for 90 days. At the end of the 90-day feeding period, animals were fasted overnight. Final body weights were recorded, and blood samples were collected by cardiac puncture under anesthesia. The animals were then euthanized, and liver tissues were excised following saline perfusion. Liver weights were recorded, and tissue samples were stored appropriately for subsequent biochemical and molecular analyses. Body weight measurement of experimental animals was recorded at baseline and weekly until euthanasia

E. Blood and Tissue Collection

Blood samples were collected from the experimental animals at baseline and at monthly intervals during the 90-day feeding period. Serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C and used for biochemical analysis. Serum total cholesterol (TC), triglycerides (TG), LDL-C, HDL-C, glucose, uric acid, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined using commercially available diagnostic reagents with an Erba EM200 fully automated biochemical analyzer according to the manufacturers' instructions. The same analytical procedures and instrument were used throughout the study to minimize analytical variation between sampling time points. At the end of the 90-day feeding period, liver tissues were collected following euthanasia and stored at -80 °C until subsequent biochemical, ELISA, and molecular analyses.

F. Hepatic Lipid and Apolipoprotein Analysis

Hepatic total cholesterol and triglyceride contents were determined using established biochemical methods. Hepatic total cholesterol was determined using the colourimetric method of Zlatkis et al. [18] with absorbance measured at 560 nm. Hepatic triglycerides were estimated using the Van Handel–Zilversmit method [19], with absorbance measured at 570 nm. Hepatic HMG-CoA reductase activity was determined colourimetrically according to Rao and Ramakrishnan [20]. Hepatic ApoA-I, ApoB, and ApoE levels were quantified by ELISA using a previously described method [21]. Briefly, 50 µL of liver homogenate was added to each well and incubated with primary antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), followed by HRP-conjugated secondary antibodies. Optical density was measured at 490 nm using a Thermo Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations in liver homogenates were determined using the Lowry method [22], and apolipoprotein levels were normalized to the corresponding tissue protein concentration.

G. Semi-Quantitative RT-PCR

Total RNA was extracted from liver tissue using TRI Reagent following the manufacturer's protocol. RNA yield and purity were evaluated spectrophotometrically by measuring absorbance at 260 and 280 nm. First-strand complementary DNA (cDNA) was synthesized from total RNA using a commercial reverse transcription kit according to the manufacturer's instructions. Semi-quantitative PCR amplification was performed using an Eppendorf Mastercycler with 35 amplification cycles. Gene expression of ApoA-I, ApoE, and LDLR was analyzed using GAPDH as the internal control. Gene-specific primers were used, and their sequences are provided in Table 2. The amplified PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized using a Bio-Rad Gel Doc imaging system. Band intensities were quantified densitometrically using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA), and the expression levels were normalized to GAPDH.

Gene	Primer Sequence (5'->3')
APOE-F	TCTTCTCCCAAGACTGACCA
APOE-R	AGCGCAGGTAATCCCAGAAG
APOA1-F	GCTGAACACCCAGTGAGGTAG
APOA1-R	TTATTCTAAGACGCCGACTGCTG
LDL-R	AGCCAGGACGGCTACACCTA
LDL-F	CGCGCATTACCGAGAGGTA
GAPDH-F	GTTTGTGATGGGCGTGAACC
GAPDH-R	GTGGAGGCAGGGATGATGTT
GAPDH-Glyceraldehyde 3-phosphate dehydrogenase	

Table 2. Gene-specific primer sequences used in this study are listed

H. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA). Two-way ANOVA followed by Tukey's multiple-comparison test was used for time-dependent measurements, while one-way ANOVA followed by Tukey's test was used for endpoint comparisons. A p-value < 0.05 was considered statistically significant.

III. RESULTS

A. Effect of PNBio on Body Weight Progression in HCD rabbits

Body weight showed distinct patterns among the groups during the 90-day feeding period (Fig. 1). The control group showed a gradual increase during the initial weeks, followed by a relatively stable pattern with minor fluctuations. The HCD group remained relatively stable during the first 6 weeks, followed by a marked increase from week 7 onward. The PNBio group showed an early increase, reaching a peak around week 4, followed by a decline and subsequent stabilization. The atorvastatin group exhibited a relatively stable pattern with a modest gradual increase throughout the study. At week 11, the HCD group showed the highest body weight among the treated groups. Overall, PNBio and atorvastatin were associated with lower body-weight progression compared with HCD.

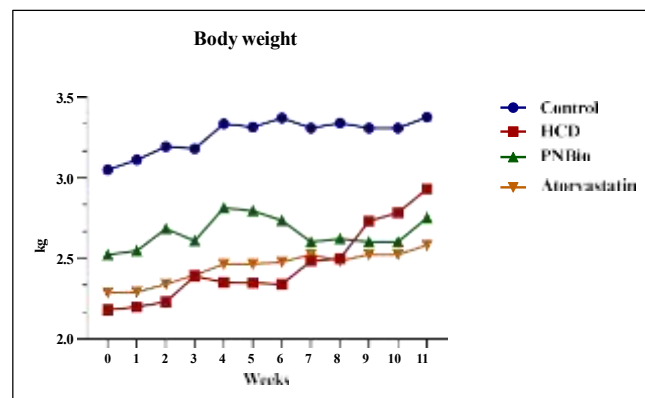


Figure 1. Effect of PNBio on body weight progression during the 90-day feeding period. Data are presented as mean ± SD (n = 6/group). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test.

B. Effect of PNBio on Serum Lipid Profile in HCD

rabbits

Serum lipid parameters showed distinct changes among the groups during the 3-month feeding period (Fig. 2). Total cholesterol (TC) remained relatively stable in the control group, whereas the HCD group showed a marked progressive increase, reaching approximately 950 mg/dL at month 3. The PNBio and atorvastatin groups showed moderate increases in TC, reaching approximately 450 mg/dL at month 3. Triglyceride (TG) levels increased progressively in the HCD group, reaching approximately 290 mg/dL at month 3, while smaller increases were observed in the PNBio and atorvastatin groups. LDL-C showed a similar marked increase in the HCD group, whereas PNBio and atorvastatin maintained substantially lower levels. In contrast, HDL-C progressively decreased in the HCD group, while the control, PNBio, and atorvastatin groups showed relatively stable or increasing HDL-C levels. Overall, PNBio reduced the magnitude of the HCD-associated alterations in the serum lipid profile, with patterns broadly comparable to those observed with atorvastatin.

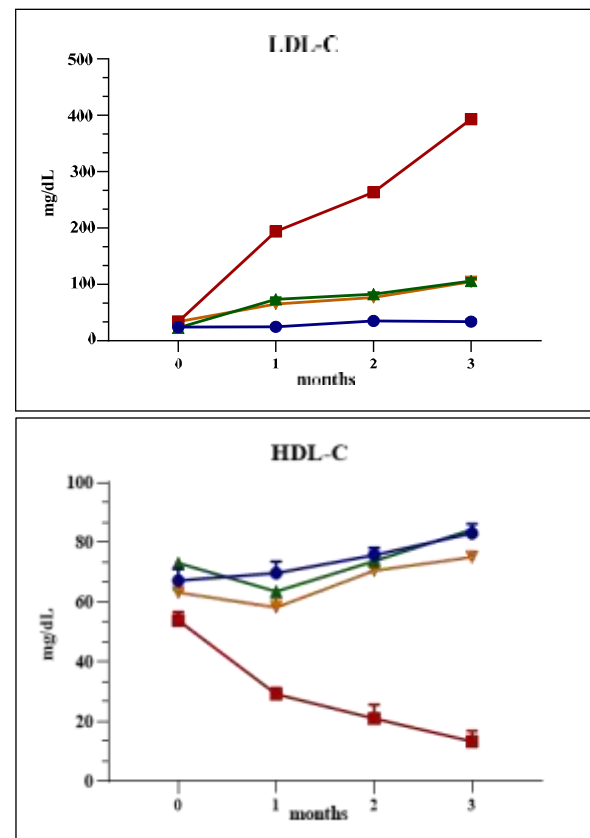
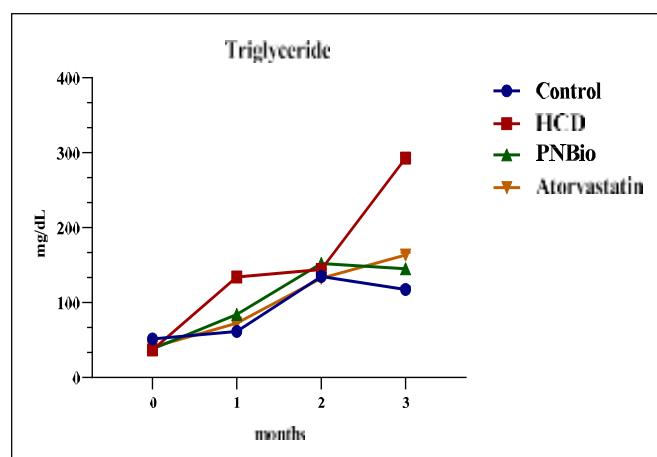
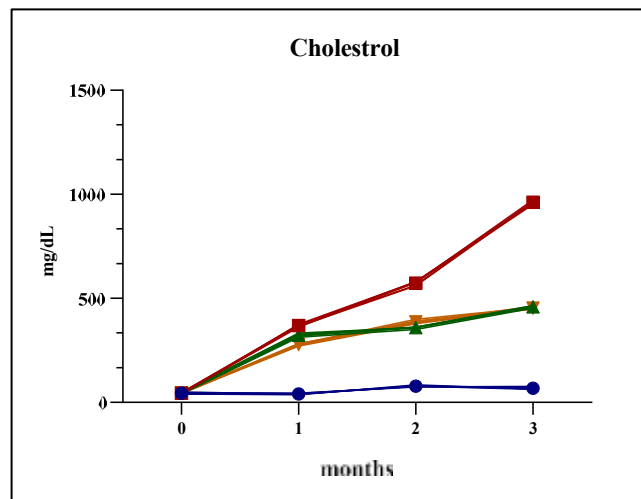


Figure 2. Effect of PNBio on serum lipid profile during the 90-day feeding period. Data are presented as mean $\pm$ SD ($n = 6/\text{group}$). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test

C. Effect of PNBio on Serum Glucose

Serum glucose levels showed distinct patterns among the experimental groups during the 3-month feeding period (Fig. 3). The control group showed relatively stable glucose levels during the first 2 months, followed by a modest increase at month 3. In contrast, the HCD group exhibited a marked progressive increase in serum glucose, reaching approximately 650 mg/dL at month 3. The PNBio group showed a gradual increase in glucose levels, reaching approximately 250 mg/dL at month 3, which remained substantially lower than that observed in the HCD group. Similarly, the atorvastatin group showed a gradual increase, reaching approximately 260 mg/dL at month 3. Generally, both PNBio and atorvastatin markedly attenuated the HCD-associated increase in serum glucose levels.

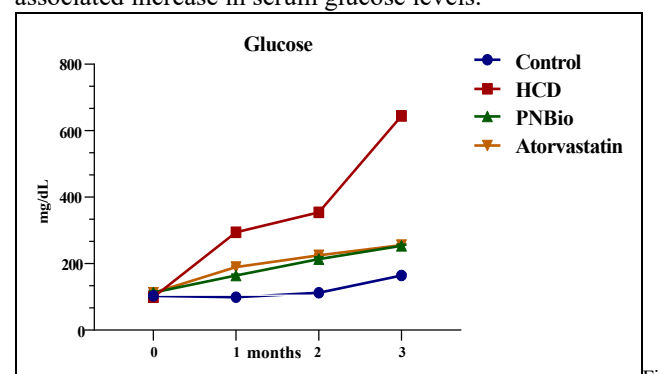


Figure 3. Effect of PNBio on serum glucose during the 90-day feeding period. Data are presented as mean $\pm$ SD ($n = 6/\text{group}$). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test.

D. Effect of PNBio on Serum ALT and AST activities

Serum ALT and AST activities showed distinct patterns among the experimental groups during the 3-month feeding period (Fig. 4). ALT activity increased markedly in the HCD group, particularly from month 1 to month 3, reaching approximately 95 U/L at month 3. In contrast, the control group remained relatively stable, while PNBio showed a moderate increase followed by a relatively stable pattern. The atorvastatin group showed a modest increase in ALT activity up to month 2, followed by a slight decline at month 3. Similarly, AST activity increased progressively in the HCD group, reaching approximately 175 U/L at month 3. PNBio showed a moderate increase in AST activity up to month 2, followed by a slight decrease at month 3, whereas atorvastatin showed a gradual increase and remained substantially lower than the HCD group. Overall, PNBio and atorvastatin attenuated the HCD-associated elevations in ALT and AST activities.

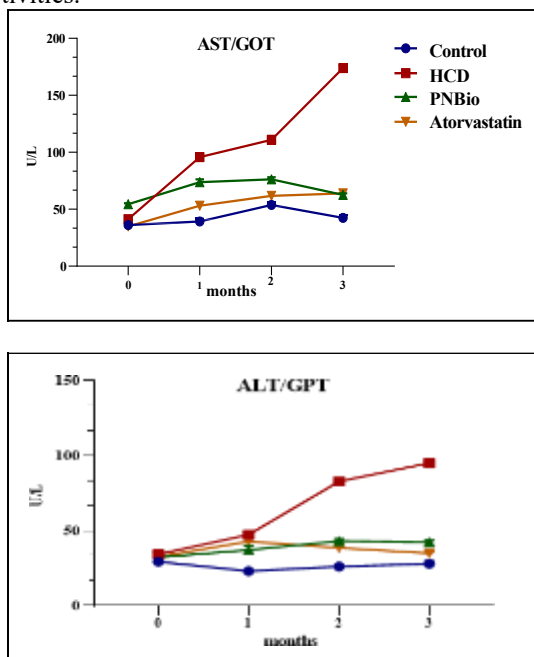


Figure 4. Effect of PNBio on serum ALT and AST during the 90-day feeding period. Data are presented as mean $\pm$ SD (n = 6/group). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test.

E. Effect of PNBio on Hepatic Cholesterol and Triglyceride Content.

Hepatic cholesterol and triglyceride contents differed among the experimental groups (Fig. 5). Hepatic cholesterol content was markedly higher in the HCD group (approximately 180 mg/dL) than in the control group (approximately 60 mg/dL). The PNBio and atorvastatin groups showed lower hepatic cholesterol contents, approximately 65 and 70 mg/dL, respectively. Similarly, hepatic triglyceride content was substantially higher in the HCD group, reaching approximately 410 mg/dL, compared with approximately 55 mg/dL in the control group. PNBio reduced hepatic triglyceride accumulation to approximately 190 mg/dL, whereas atorvastatin reduced it to approximately 140 mg/dL. Overall, both PNBio and atorvastatin attenuated hepatic lipid accumulation compared with the HCD group.

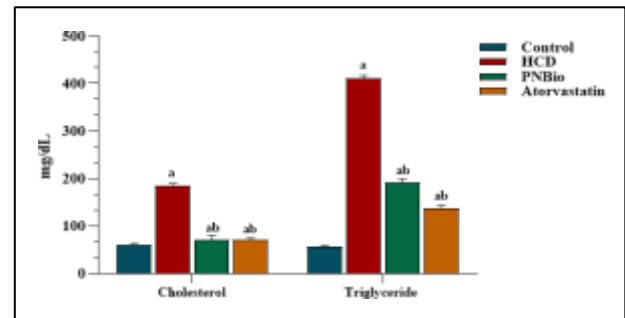


Figure 5. Effect of HCD and PNBio on hepatic cholesterol and triglyceride contents at the end of the 12-week feeding period. Data are presented as mean $\pm$ SD (n = 6/group). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. a. vs control, b. vs HCD

F. Effect of PNBio on Hepatic ApoA-I, ApoB, and ApoE Levels

Hepatic apolipoprotein levels showed distinct patterns among the experimental groups (Fig. 6). ApoA-I was highest in the control group, while the HCD group showed a lower level. PNBio increased ApoA-I compared with the HCD group, reaching a level closer to that of the control group, whereas atorvastatin showed a moderate increase. In contrast, ApoB increased in the HCD group compared with the control group, while both PNBio and atorvastatin showed lower ApoB levels. ApoE was also markedly increased in the HCD group, whereas PNBio and atorvastatin showed lower levels than HCD. Overall, PNBio attenuated the HCD-associated alterations in hepatic ApoA-I, ApoB, and ApoE levels.

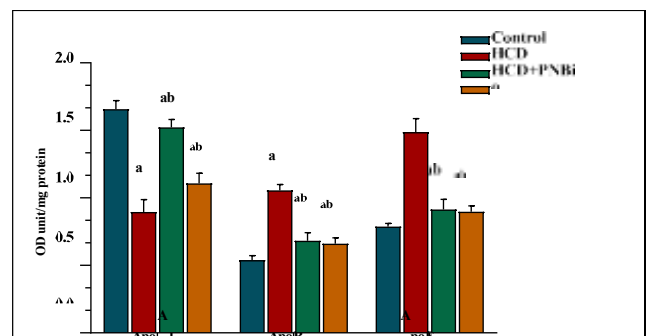


Figure 6. Hepatic ApoA-I, ApoB, and ApoE levels at the end of the 12-week feeding period. Data are presented as mean $\pm$ SD (n = 6). Different superscript letters indicate significant differences between groups according to Tukey's post hoc test (p < 0.05). a. vs control, b. vs HCD

G. Effect of PNBio on Hepatic ApoA-I and ApoE Gene Expression

Hepatic ApoA-I and ApoE expression showed distinct patterns among the experimental groups (Fig. 7). ApoA-I expression was markedly higher in the control group, whereas the HCD group showed a pronounced reduction. PNBio increased ApoA-I expression compared with the HCD group, with a level approaching that observed in the control group, while atorvastatin showed a similar pattern. In contrast, ApoE expression was higher in the HCD group than in the control group. PNBio attenuated the HCD-associated increase in ApoE expression, whereas atorvastatin reduced ApoE expression to a level comparable to that of the control group. The corresponding agarose

electrophoresis bands showed patterns consistent with the expression data, with GAPDH used as the loading control.

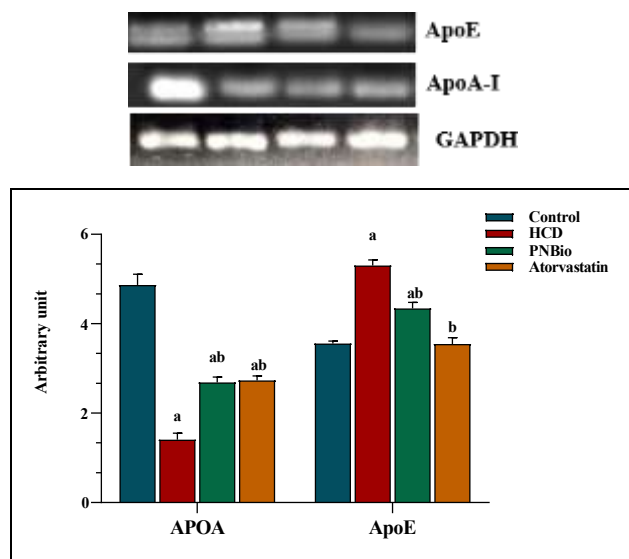


Figure 7. Effect of PNBio on hepatic ApoA-I and ApoE gene expression after 90-day of HCD feeding. Representative agarose gel electrophoresis bands for ApoA-I, ApoE, and GAPDH are shown together with densitometric analysis. Data are presented as mean $\pm$ SD (n = 6). Different superscript letters indicate significant differences between groups according to Tukey's post hoc test ($p < 0.05$). a. vs control, b. vs HCD.

H. Effect of PNBio on LDLR expression and HMG-CoA reductase activity

HMG-CoA reductase activity and LDLR expression showed distinct changes among the experimental groups (Fig. 8). HMG-CoA reductase activity was lower in the HCD group than in the control group, whereas PNBio and atorvastatin showed higher activities, approaching the control level. LDLR levels were markedly reduced in the HCD group compared with the control group. Treatment with PNBio increased LDLR levels compared with HCD, with a pattern comparable to that observed in the atorvastatin group. Consistent with these findings, the corresponding agarose electrophoresis bands analysis showed reduced LDLR expression in the HCD group, while PNBio and atorvastatin increased LDLR expression relative to HCD. GAPDH was used as the loading control.

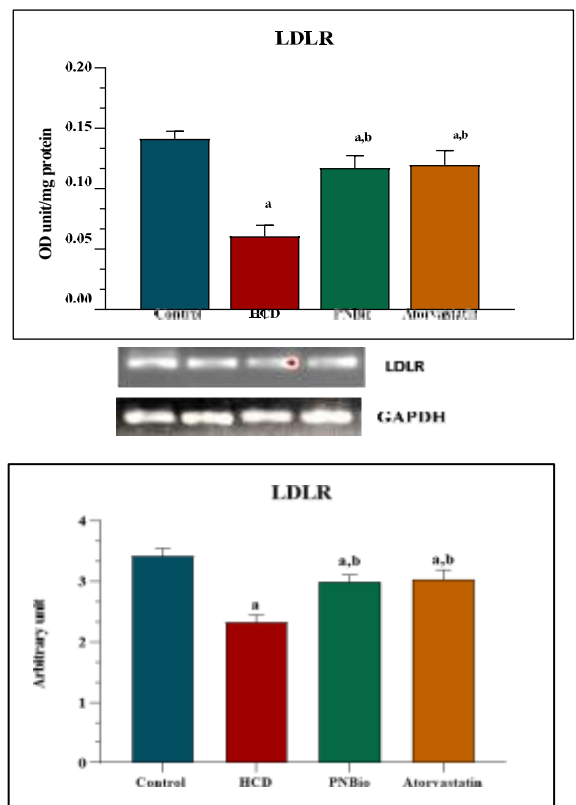
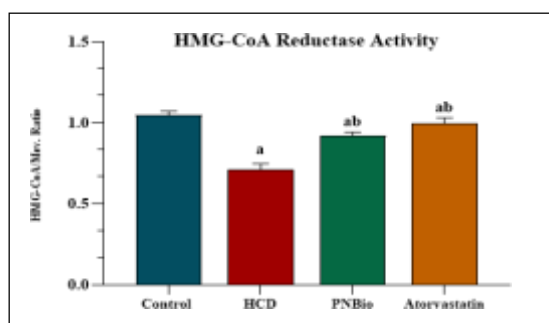


Figure 8. Effect of PNBio on HMG-CoA reductase activity and hepatic LDLR gene expression. HMG-CoA reductase activity and LDLR gene expression were evaluated in the control, HCD, PNBio, and atorvastatin groups. LDLR gene expression was analyzed by RT-PCR followed by agarose gel electrophoresis and normalized to GAPDH expression. Data are presented as mean $\pm$ SD (n = 6/group). Different superscript letters indicate significant differences among groups ($p < 0.05$). a. vs control group; b. vs HCD group

DISCUSSION

The present study demonstrates that sustained high-cholesterol diet (HCD) exposure produced progressive metabolic and hepatic abnormalities in rabbits, characterised by increased serum total cholesterol, triglycerides, LDL-C, glucose, ALT, and AST, together with reduced HDL-C and increased hepatic cholesterol and triglyceride accumulation. These alterations were accompanied by changes in hepatic apolipoprotein metabolism and cholesterol-regulatory pathways, representing metabolic and hepatic features relevant to metabolic dysfunction-associated steatotic liver disease (MASLD) (2,3). The longitudinal design showed that these abnormalities developed progressively during continued HCD exposure. Serum lipid disturbances became increasingly pronounced over the 90-day period, while hepatic lipid accumulation was evident at the endpoint. PNBio attenuated the HCD-associated changes in the circulating lipid profile and reduced hepatic cholesterol and triglyceride accumulation, indicating a beneficial effect on both systemic and hepatic lipid homeostasis.

HCD exposure also altered hepatic apolipoprotein metabolism, with reduced ApoA-I and increased ApoB and ApoE levels. These changes are consistent with disturbances in lipoprotein metabolism reported in cholesterol-fed rabbits (6,23,24).

PNBio partially reversed these alterations by increasing ApoA-I and reducing ApoB and ApoE toward control

levels. The accompanying improvement in the circulating lipid profile and hepatic lipid accumulation suggests that PNBio may help maintain lipoprotein homeostasis during continued dietary cholesterol exposure. However, cholesterol efflux and VLDL secretion were not directly assessed; therefore, the precise mechanisms underlying these effects remain uncertain.

HCD feeding also reduced hepatic HMG-CoA reductase activity and LDLR expression. Because these pathways contribute to hepatic cholesterol synthesis and LDL clearance, their alteration is consistent with disturbed hepatic cholesterol homeostasis during cholesterol overload

(8). PNBio partially restored both HMG-CoA reductase activity and LDLR expression toward control levels, suggesting a potential beneficial effect on hepatic cholesterol regulation. Nevertheless, SREBP-2 activity and other upstream regulatory mechanisms were not directly evaluated. This further indicates that prolonged HCD exposure affected metabolic pathways beyond circulating lipid metabolism. These findings are relevant to the metabolic dysfunction associated with MASLD (3,5). However, insulin resistance was not directly assessed, and histological examination of the liver was not performed. Therefore, the present findings should be interpreted as metabolic and hepatic alterations relevant to MASLD rather than definitive evidence of established MASLD.

The reduction in ALT and AST elevations by PNBio, together with improvements in serum lipids, glucose, hepatic lipid accumulation, and apolipoprotein profiles, indicates a consistent protective response across several metabolic and hepatic parameters. Importantly, PNBio was administered concurrently with HCD; therefore, these findings support a preventive effect during ongoing dietary exposure rather than a therapeutic effect after disease has been established.

Several limitations should be acknowledged. The study was limited to 90 days and did not include histological assessment, direct measures of insulin resistance, or detailed mechanistic analyses of cholesterol efflux and lipoprotein secretion. Further studies incorporating histological, metabolic, and molecular assessments are required to determine whether PNBio can prevent or limit the progression of steatotic liver disease.

CONCLUSION

The present study demonstrates that continuous high-cholesterol feeding induced progressive metabolic and hepatic disturbances in rabbits, characterized by worsening serum lipid and glucose profiles, elevated liver enzymes, and increased hepatic cholesterol and triglyceride accumulation. These changes were accompanied by alterations in hepatic apolipoprotein metabolism, including changes in ApoA-I, ApoB, and ApoE levels and altered ApoA-I and ApoE gene expression, together with reduced hepatic HMG-CoA reductase activity and LDLR expression. PNBio attenuated these abnormalities during the period of ongoing dietary cholesterol exposure, improving the circulating lipid profile, limiting hepatic lipid accumulation, restoring HMG-CoA reductase activity and LDLR expression, and shifting the apolipoprotein profile toward that of the control group. The longitudinal nature of the study further indicates that the beneficial effects of PNBio were evident during the development of HCD-induced metabolic disturbances rather than only at the final

assessment. Collectively, these findings support the potential of PNBio as a preventive nutritional intervention for limiting metabolic and hepatic alterations associated with sustained high-cholesterol exposure. Further mechanistic and longer-term studies are required to establish the pathways underlying these effects.

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