Journal of Animal & Plant Sciences, 32(1): 2022, Page: 69-77
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2022.1.0403

SYNBIOTICS SUPPLEMENTATION AMELIORATES HIGH FAT AND SUGAR DIET-ASSOCIATED OXIDATIVE STRESS AND HISTOLOGICAL ARCHITECTURE OF INTESTINE, LIVER AND KIDNEY IN RATS

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ABSTRACT

The present study is designed to investigate therapeutic strategies to favorably change the intestinal microbiota through 'bacteriotherapy' involving the use of synbiotics, a combination of probiotics (*Lactobacilli* spp, *Bifidobacteria* spp. and *Streptococcus* spp.) and prebiotics (*Dandelion* and *Glycyrrhizin*) in high fat and high sugar (HFHS) diet-associated liver disease. The involvement of hepatic portal system with kidneys to modulate gut microbiota with synbiotics supplements is the novel strategy of the present study. The four diet treatment groups were the vehicle control (Veh), diet supplemented with synbiotics (Syn; probiotics; 2 x 10^6 CFU/rat/day + prebiotics; 300 mg/kg feed); HFHS diet group (36 + 40 %) and HFHS+Syn group. All the treatments were arranged in a completely randomized design. Synbiotics and HFHS were provided throughout the 14-week experimental period. The results showed that synbiotics supplementation significantly lowered serum cholesterol, triglyceride, aspartate aminotransferase, alkaline phosphatase, bilirubin, creatinine, uric acid, total protein, total oxidant status and malondialdehyde as compared to the HFHS+Veh group. Moreover, a significant increase in high-density lipoprotein and anti-oxidant parameters such as total anti-oxidant capacity, paraoxonase and arylesterase showed the ameliorative potential of synbiotics in the HFHS+Syn group. The histological images of the intestine, liver and kidneys in the HFHS+Veh group showed fat accumulation and cytoplasmic vacuolation whereas, synbiotics significantly improved histological architecture in the HFHS+Syn group. It was concluded that diet supplementation with synbiotics might be a potential candidate for prevention or adjuvant treatment of metabolic diseases involving oxidative stress.

Keywords: Synbiotics; Nonalcoholic fatty liver disease; Oxidative stress; Gut-Liver axis

Published first online June 14, 2021 Published final January 07, 2022.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), a broad-spectrum liver disease, starts from steatosis to nonalcoholic steatohepatitis (NASH), necrosis, fibrosis, and eventually cirrhosis (Wiest *et al*., 2017). Nutritional, environmental, and genetic factors are considered as etiological factors behind the emergence of NAFLD (Dongiovanni *et al*., 2014). Imbalanced gut microbiota "dysbiosis" is the main player in the pathophysiology of NAFLD (Le Roy *et al.,* 2013; Awaad *et al.,* 2019). The detoxification organs, liver and kidneys, through different biochemical and physiological processes are involved in reducing endogenous toxins during metabolic disorders. Gut-liver and kidney axis get predominant importance in ameliorating the metabolic diseases. Pharmacological strategies along with a healthy lifestyle including weight reduction, exercise, insulinotropic agents, balanced diet, and vitamin supplementation are adopted to alleviate the pathogenesis of NAFLD (Berná and Romero Gomez, 2020). Healthy microbiota plays important role in the efflux of excessive free fatty acids, *de novo* lipogenesis from the liver, strengthening intestinal barrier, decreased insulin resistance, and reduction in inflammatory markers (Boursier *et al*., 2016; Mehmood *et al*., 2020). In this study, dietary intervention, probiotics and prebiotics in combination "synbiotics", are introduced into the gastrointestinal tract to highlight the significance of the hepatic portal system and kidneys in combating the metabolic diseases.

High fat and high sugar (HFHS) diet and intestinal microbiota have been proposed determinants of obesity, insulin resistance, oxidative stress, NAFLD and NASH (Schnabl and Brenner, 2014). The

pathophysiological role of ethanol-producing bacteria such as *Escherichia coli* and elevated alcoholic-metabolizing enzymes; aldehyde dehydrogenase and catalase, contribute to the pathogenesis of progression from NAFLD to NASH. The specific intestinal bacteria, such as *Lactobacillus, Bifidobacterium, Prevotellaceae, Eubacteria, Blautia*, and *firmicutes/bacteridetes* ratio are well studied regarding host energy imbalance, obesity and chronic kidney diseases (Tuomisto *et al.,* 2014; Garcia *et al*., 2019). Gut dysbiosis, especially the imbalanced firmicutes / bacteroidetes ratio, causes an increase in endogenous alcohol and intestinal permeability, disrupting intestinal epithelial tight junctions that facilitate endotoxins and alcohol to induce liver injury and renal diseases (Méndez-Sánchez *et al.,* 2020).

The probiotics being "live microorganism" and prebiotics, non-digestible food ingredients, are included in microbial therapies to manipulate gut microbiota (Zhou *et al.,* 2018). The synbiotics are involved in the host gut immunity (innate and adaptive) through direct contact with intestinal epithelial cells and dendritic cells to secrete defensin and mucus, hence leading to the production of short chain fatty acids and preventing adipokines and cytokines production (Sáez-Lara *et al.,* 2016). Thus, short chain fatty acids are the primary metabolites of synbiotics playing an important role in host health beyond the energy retrieval from undigested food. For instance, butyrate maintains the integrity of the small intestine and large bowel by supplying energy to epithelial cells in the intestine. A survey of literature showed that butyrate is capable of controlling inflammation by inducing Treg differentiation and it has reduced the incidence of colon cancer in humans. It also has bactericidal activity (Ali *et al.,* 2014). The potential probiotics include *Lactobacillus* spp. and *Bifidobacteria* spp., while prebiotics include inulin and non-digestible oligosaccharides. As the synbiotics are associated with gut health, so the impact of synbiotics on the gut-liver and kidney axis is investigated that is the novel approach of the present study.

MATERIAL AND METHODS

Synbiotics: The lyophilized mixed therapeutic grade bacterial strains of *Lactobacilli* spp, *Bifidobacteria* spp. and *Streptococcus thermophilius* containing 6 x 10^6 CFU/g (Amybact) were purchased from the medicine market in Faisalabad, Pakistan. Impim composed of glycyrrhizin and total flavonoids 0.1% and 2.0% respectively, extracted from *Glycyrrhiza* and *Dandelion* fluid was procured from Keep Young Company, China. The dose of 300 mg/kg feed was calculated from the previous literature.

Experimental Design: Total twenty-four, 4 weeks old albino rats (weighing 85±5 g) were kept at the Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad according to standard conditions such as 25°C, 12h alternate light and dark cycle, *ad-libitum* assess to diet (**Table 1**) and water. After 1 week of acclimatization, rats were divided into four diet treatment groups (n=6) as vehicle control (Veh) group, *ad libitum* standard rat chow and water; Synbiotics group (Syn), rat chow with probiotics (2 x 10^6 CFU/rat/day) and prebiotics (300mg/kg feed) for 14 weeks; High-fat high sugar (HFHS) group, fed a high fat (36%) and high sugar (40%) for 14 weeks to develop NAFLD model; and HFHS+Syn group, fed as HFHS along with Synbiotics. All the treatments were arranged in a completely randomized design (CRD). The body weight of each rat was measured biweekly. Minimum pain and stress to the animals were assured during experimentation.

Table: 1 Diet Composition:

Feed Constituents	Normal Diet	High Fat and High Sugar Diet
Fat	6 %	36%
Sucrose	Nil	40%
Crude protein	20%	8.75%
Crude fiber	4.5%	1.23%
Ash	6%	0.9%
Nitrogen free extract (NFE)	63.5%	13.12%

Blood and organ collection: Blood and organ sample collection from all the experimental rats was done in the 18th experimental week. Rats were anesthetized with chloroform before sacrificing and blood of each rat was taken in a separate vacutainer for serum collection in a tube containing platelet activator gel. Serum was separated using a centrifuge machine (Centrifugal Machine, China) at 1010 x g for 15 minutes and stored in the biomedical freezer (Sanyo, Japan) at -20°C for biochemical analysis by using an automated serum analyzer (Bio-Ray 310 diagnostic). Intestine, liver and kidneys of each rat were separated and preserved in 10% neutral buffered formalin solution for histopathological analysis.

Serum Biochemical Analysis: The stored serum was thawed and analyzed for cholesterol, triglycerides, low density lipoprotein (LDL), high-density lipoprotein (HDL), aminotransferase (ALT), aspartate aminotransferase (AST), alanine alkaline phosphatase (ALP), bilirubin, blood urea, creatinine, uric acid and total protein through commercially available bio-kits (Merck, Pvt, Ltd) according to the given protocols. The oxidant and antioxidant status were assessed through measuring total oxidant status (TOS), total antioxidant capacity (TAC), malondialdehyde (MDA), superoxide

dismutase (SOD) and arylesterase levels through calorimetric method using spectrophotometer (Thermo Scientific Multiskan GO™ with SkanIt software 4.1) according to manufacturer guidelines.

Histopathology: The histopathological analysis of the intestine, liver and kidney tissue samples (four from each group randomly) was performed by preparing the slides according to protocol mentioned in the literature (Bedossa *et al.*, 2012). The histological images of intestine, liver and kidney sections were taken with the camera (TOUPCAM, ToupTek Photonics Co., Ltd; China) attached to a light microscope (Model IM-910 IRMECO GmbH & Co; Germany).

Statistical analysis: Data was assessed statistically by applying one-way ANOVA followed by DMR post hoc test. The SPSS software (version 16.0) was used with level of significant 5% ($P \leq 0.05$). All results are expressed as Mean ± SE.

RESULTS

Synbiotics supplementation restores HFHS diet-associated alterations in serum biochemical parameters: As expected**,** HFHS diet increased the levels of cholesterol, triglyceride and LDL at serum level and also affected the histological picture of intestinal tissue in HFHS+Veh group suggesting the significance of gut-liver axis. The administration of synbiotics in HFHS+Syn group significantly improved the metabolic disturbance through lowering the levels of cholesterol, triglyceride and increasing HDL. Synbiotics non-significantly improved the level of LDL in HFHS+Syn group, whereas in comparison of Cont+Veh and synbiotics groups indicated the non-significant differences in the level of cholesterol, LDL and HDL. The significant difference in triglyceride level exists in Cont+Veh and synbiotics group. The HFHS+Veh group indicated significant differences in cholesterol, triglyceride and HDL levels between Cont+Veh and synbiotics groups (**Fig. 1)**.

Synbiotics improve HFHS-induced alteration in intestinal architecture: The histopathological analysis of images of the vehicle group (Left panel) showed normal epithelial lining, villi structure, glands and intestinal mucosa. The HFHS group (Middle panel) showed fat accumulation in ileal region and damaged gut mucosa and villi. The thick epithelium showed pyknotic and eccentric nuclei. The HFHS-Synbiotics group (Right panel) showed rare cytoplasm vacuolation, normal villi and glandular epithelium suggesting ameliorative effects of synbiotics on gut health (**Fig. 2**).

Synbiotics improve HFHS diet-induced hepatic dysfunction: The metabolic disturbance generated through HFHS-diet affected the liver strongly and suggested NAFLD model as of significant rise in ALT, AST, ALP and bilirubin in HFHS+Veh group as compared to Cont+Veh and Synbiotics groups. The synbiotics significantly lowered levels of AST, ALP and bilirubin in HFHS+Syn group, suggesting therapeutic potential in ameliorating the liver disease (**Fig**. **3**). The histopathological analysis of vehicle group (Left panel) showed normal hepatocytes, hepatic triad and no fat accumulation. On the other hand, histopathological analysis of HFHS diet-treated group (Middle Panel) showed abnormal hepatic triad, cytoplasmic vacuolation, perivascular and portal cell infiltration, fat accumulation in hepatocytes, eccentric and pyknotic nuclei. The HFHS-Synbiotics group (Right panel) showed restoration of liver parenchyma suggesting ameliorative effects of synbiotics on HFHS diet-induced alterations in liver tissue (**Fig. 4**).

Synbiotics supplementation alleviates renal damage associated with HFHS-diet: HFHS-diet affected the renal system in connection with the gut-liver axis, as the serum levels of blood urea, creatinine, uric acid and total protein are raised significantly in HFHS+Veh group. The histological images of damaged nephrons in HFHS+Veh group also suggesting involvement of renal system affected by HFHS-diet. The synbiotics significantly lowered the creatinine, uric acid, and total protein levels in HFHS+Syn group. The synbiotics in HFHS+Syn non-significantly lowered the blood urea level, although significantly lowered blood urea levels were found in synbiotics group (**Fig. 5**). Histopathological analysis of the vehicle group (Left panel) showed normal Bowman's capsule and proximal convoluted tubular structure. The HFHS group (Middle panel) showed distorted glomeruli with an increased Bowman capsular space. The histological analysis of HFHS-Synbiotics group (Right panel) showed renal architecture comparable to that vehicle-treated group. Synbiotics restored serum biomarker levels in HFHS-Synbiotics group and ameliorated the overall renal architecture as observed by histological analysis (**Fig. 6**).

Synbiotics supplementation reduces oxidative stress mediated through HFHS-diet: Oxidative stress markers are thought to be indicators for assessing the homeostasis level of body. The significant rise in total oxidant status (TOS), malondialdehyde (MDA), whereas the significant decrease in total anti-oxidant capacity (TAC), paraoxonase and arylesterase showed HFHS-diet induced oxidative stress. The synbiotics in HFHS+syn group significantly lowered the TOS and MDA, whereas TAC, paraoxonase and arylesterase levels increased significantly indicating anti-oxidant potential of synbiotics (**Fig. 7**).

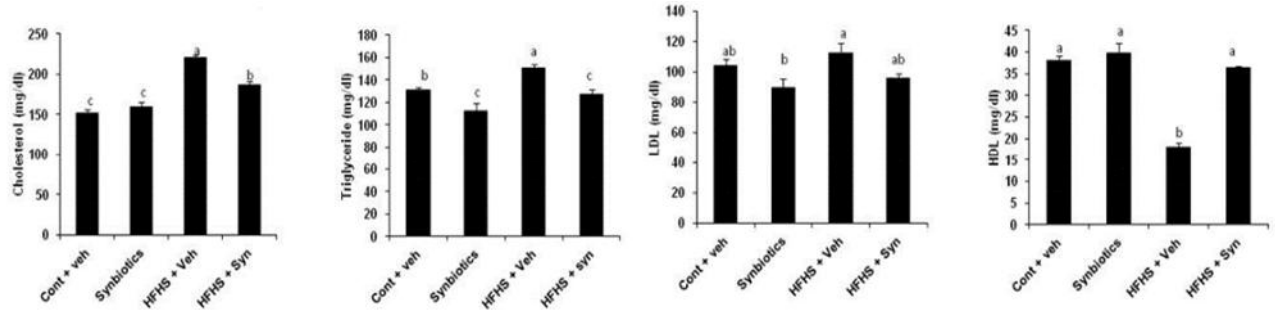

Fig. 1. Effect of HFHS-diet and synbiotics on serum lipid profile. Results are expressed as Mean ± SE. Different alphabets show statistically significant at $P < 0.05$.

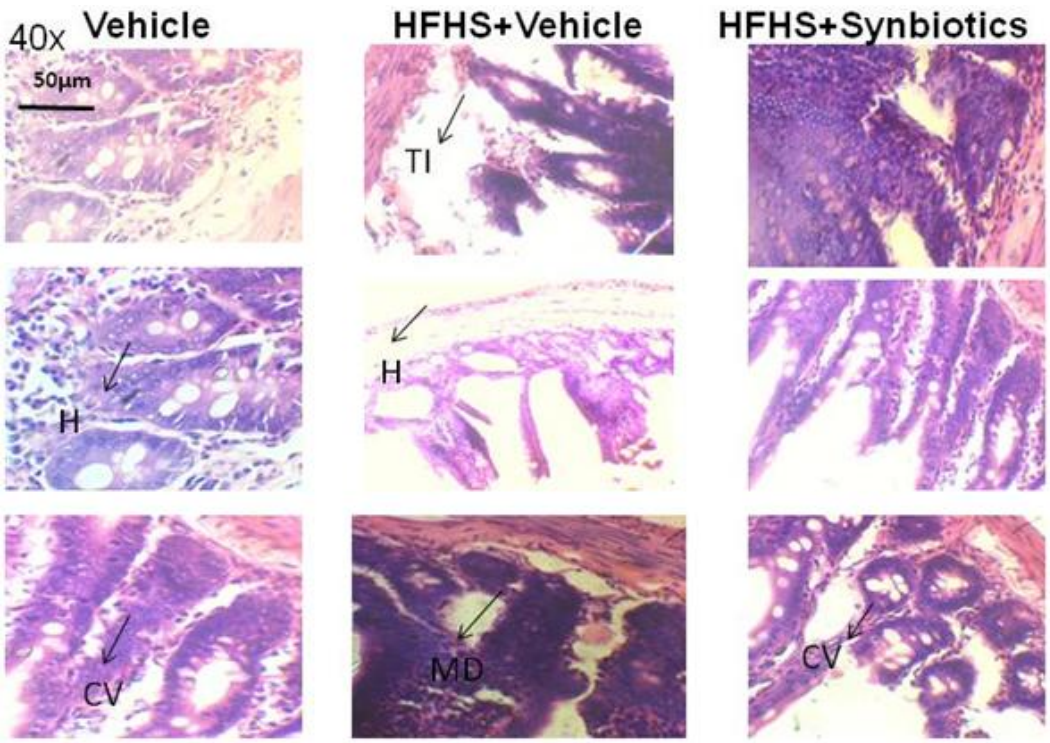

Fig. 2. Effect of HFHS-diet and synbiotics on ilium histology. Three representative images from the respective group showing different areas of ilium (hematoxylin and eosin stain, 400x). CV, cytoplasmic vacuolation; DV, damaged villi; H, hemorrhages; MD, mucosal damaged; TI, thickened intestinal muscle layer.

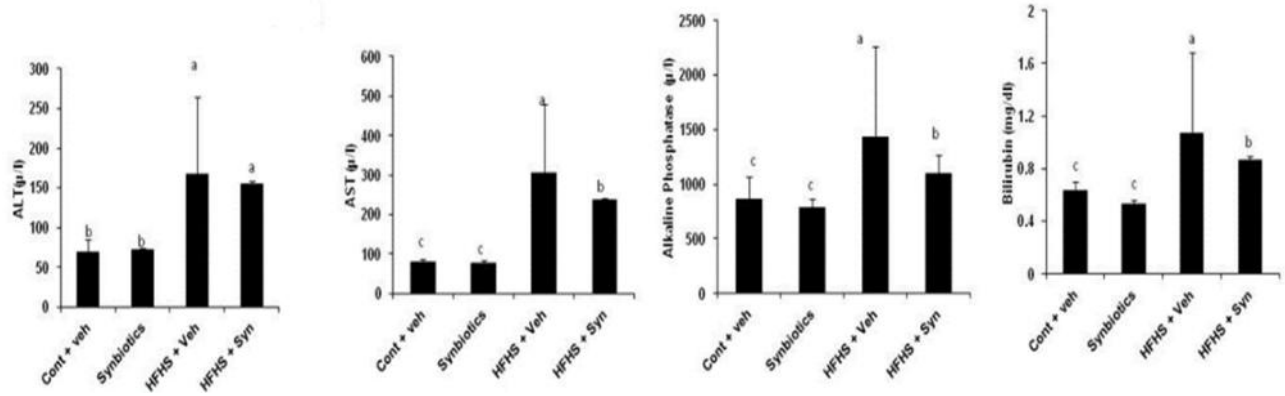

Fig. 3. **Effect of HFHS-diet and synbiotics on liver function markers.** Results shown are the Mean ± SE, while different alphabets suggest statistical significance at $P < 0.05$.

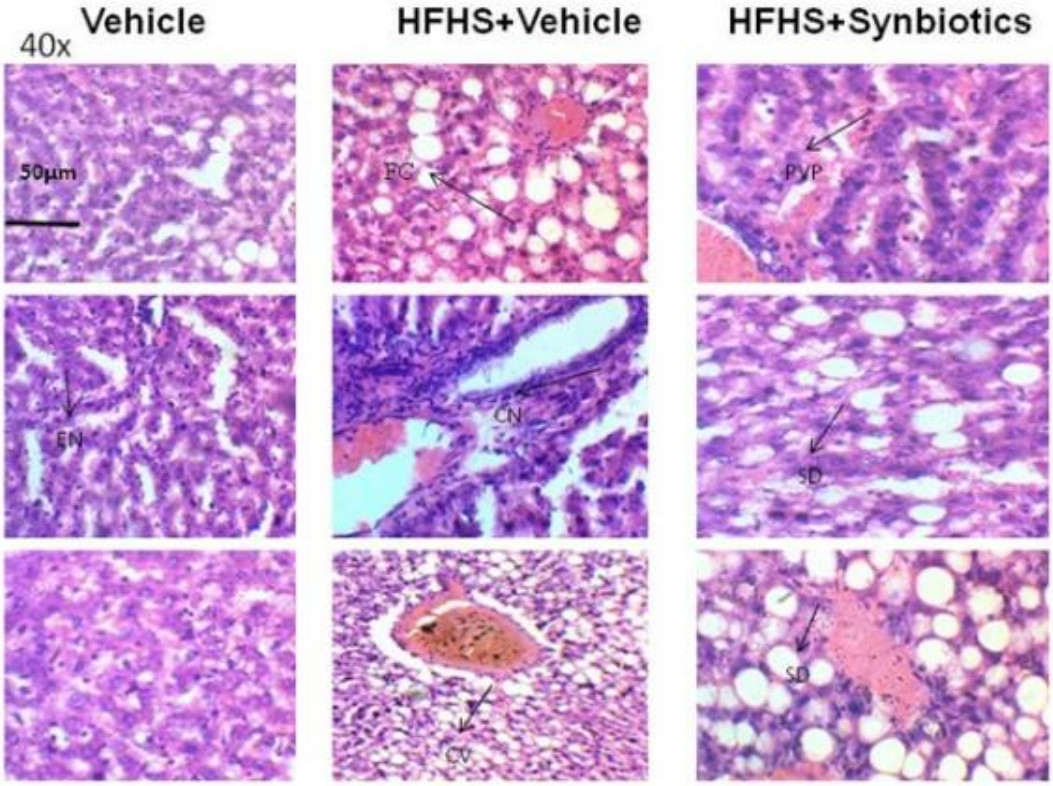

Fig. 4. **Effect of HFHS-diet and synbiotics on liver histology.** Three representative images from the respective group showing different areas of liver tissue (hematoxylin and eosin stain, 400x). CN, centrilobular necrosis; CV, cytoplasmic vacuolation; EN, eccentric nuclei; FC, focal hepatic necrosis; PVP, peri-vascular and portal cell infiltration; SD, sinusoidal dilatation.

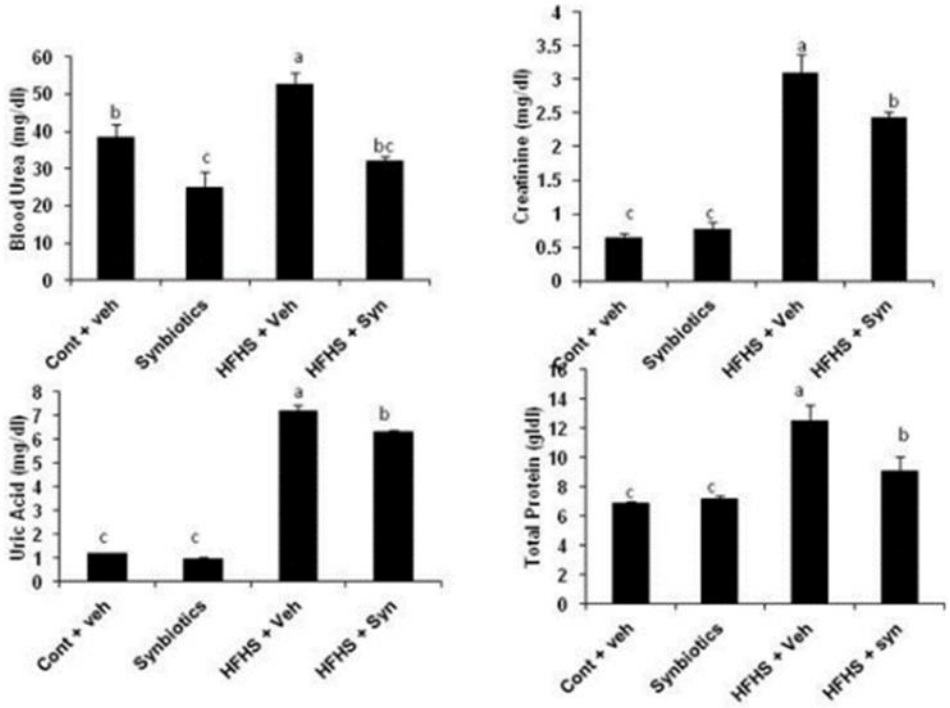

Fig. 5. Effect of HFHS-diet and synbiotics on renal function markers. Results shown are the Mean ± SE, while different alphabets suggest statistical significance at $P < 0.05$.

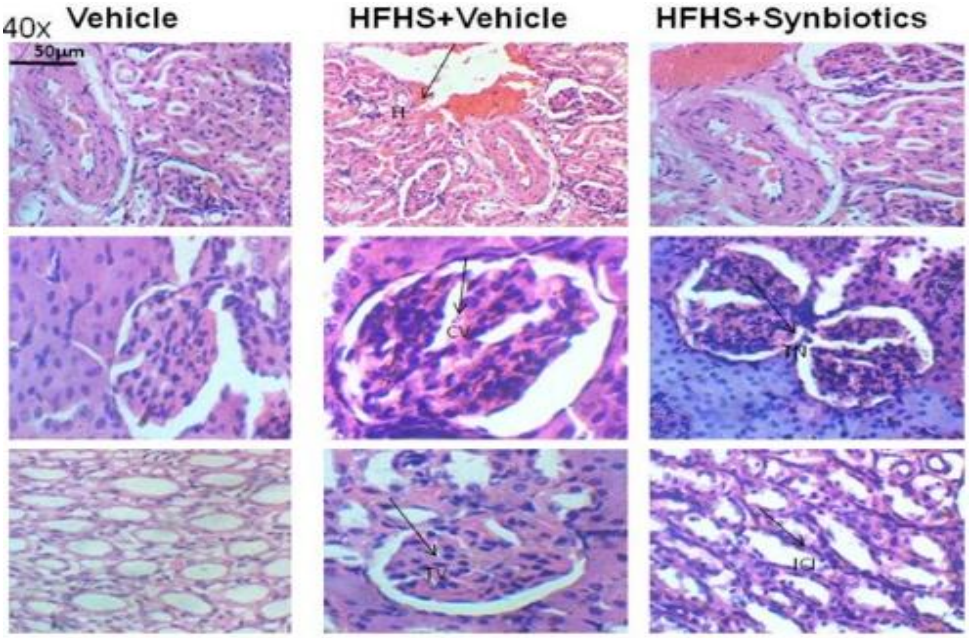

Fig. 6. **Effect of HFHS-diet and synbiotics on renal histology.** Three representative images from the respective group showing different areas of renal tissue (hematoxylin and eosin stain, 400x). CV, cytoplasmic vacuolation of tubular epithelium; ICI, interstitial cell infiltration; TN, tubular necrosis; TT, tubular thickening.

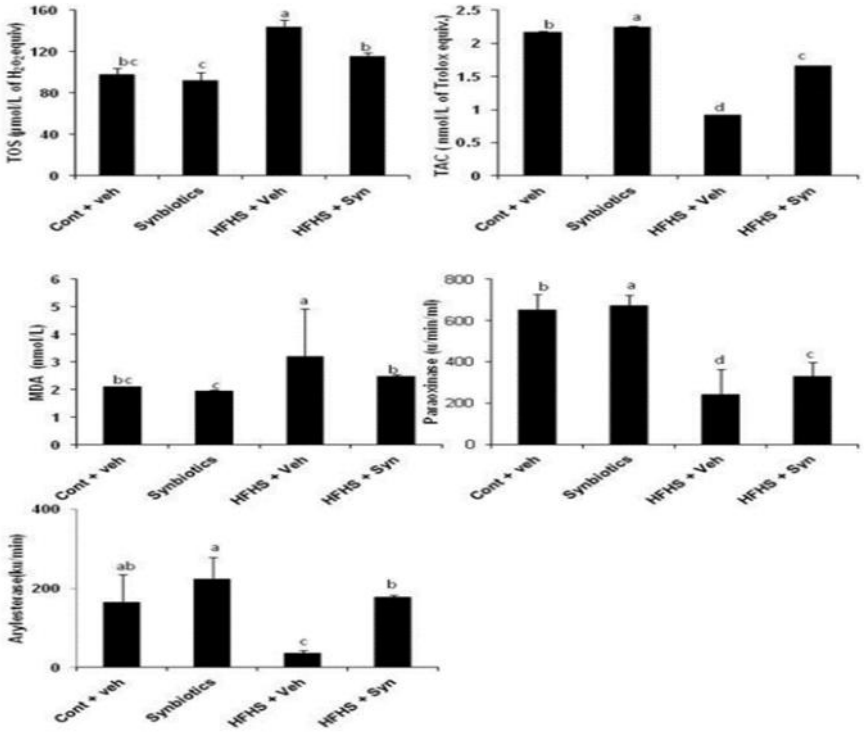

Fig. 7. Synbiotics ameliorate HFHS-diet associated oxidative stress. The oxidant and anti-oxidant status Results shown are the Mean ± SE. Different alphabets indicate statistical significance at $P < 0.05$.

DISCUSSION

The ameliorative effects of synbiotics in fatty liver diseases might be ascribed to their antilipogenic and anti-inflammatory properties. Synbiotics have played an important role in normalizing the liver enzyme levels to lessen the metabolic disease burden (Nabavi *et al.,* 2014). In liver diseases, irregularities are frequently observed in hexagonal hepatic lobule, portal triad, central vein and centric nuclei (Hussain *et al.,* 2019). The similar pathological conditions along with fat accumulation and necrosis have been observed in our study. We found that the protective effects of synbiotics supplementation against HFHS diet-induced metabolic disorder of the gut-liver and kidney axis involve antioxidant mechanism.

Lipid homeostasis is regulated through balance between lipid generation and lipid utilization. In liver diseases, dysfunction in lipolytic and lipogenic pathways occurs (Liu *et al.,* 2017). In accordance with previous literature, the increased burden of triglycerides (Nobili *et al.*, 2018), cholesterol, low density lipoprotein and drop down in high density lipoprotein were accompanied with malfunction of the liver. The results from our research model indicated successful induction of disease as observed from lipid parameters. Generally, prebiotics present in synbiotics are involved in gut-mediated peripheral and luminal metabolism through improving intestinal epithelial junctions, maintaining gut microbiota health (Rashid *et al.,* 2020), reducing bacterial derived hepatotoxins (ethanol, volatile organic acids, acetaldehyde), satiety and appetite (Parnell *et al.,* 2012). Undigested dietary fibers, oligosaccharides and resistant starch in the form of probiotics are largely fermented in distal colon and produce short chain fatty acids having anti-inflammatory effects in the gut (Menzel *et al.*, 2004).

Results from previous studies described that HFHS-diet causes gut dysbiosis, which increases LPS generation and intestinal permeability associated with metabolic disturbances (Jiang *et al.,* 2019). The elevated levels of AST, ALT and ALP have been noticed in our study in accordance with the previous studies of liver disease models (Rahmat *et al.,* 2014; Hussain *et al.,* 2018). These biochemical parameters along with bilirubin levels are considered as baseline parameters to declare hepatic impairment (Saleh Gazwi and Mahmoud, 2019; Hussain *et al.,* 2021). In our study, the elevated levels of bilirubin also validated the studied models of liver diseases.

NAFLD and NASH showed identical features to alcoholic-hepatitis even without alcoholic intake (Zhu *et al.*, 2013). Results of our study revealed that the gut-liver and kidney axis is implicated in the pathogenesis of NAFLD. Uremic toxins formed in liver diseases contribute towards renal impairment as suggested from previous studies. The impaired excretion and emulsification of fats results in ureates deposition in nephron and ultimately the renal damage (Liu *et al*., 2017). The involvement of the gut-liver and kidney axis in mitigating HFHS-induced endotoxemia is of pivotal significance (Xu *et al.,* 2019). In our study, the HFHS-diet increased endotoxin levels as noticed by significant increase in blood urea, creatinine, uric acid and total protein levels in HFHS+Veh group. Synbiotics supplementation significantly lowered the uremic toxins in HFHS+Syn group. The previous studies fully supported the therapeutic potential of synbiotics in lowering the uremic toxin generated through metabolic mediated disease (Rossi *et al.,* 2016).

Oxidative stress is associated with irregular production of adipokines, which in turn mediate metabolic syndrome (Yang *et al.,* 2019; Mehmood *et al*., 2018; Ahmed *et al*., 2021). Our study reveals that metabolic oxidative stress might be due to higher triglycerides, cholesterol and LDL oxidation. The increased levels of oxidative stress markers including TOS and MDA indicate peroxidation of unsaturated fatty acids. Antioxidative effects of synbiotics in our study might be due to reactive oxygen species scavenging properties of synbiotics by inhibition of caveolin signaling, nitric oxide production (Hafez and Gad, 2018) and modulation of TAC, paraoxonase and arylesterase activities. Overall, synbiotics supplementation increased the total antioxidant capacity and decreased oxidative stress due to HFHS diet. We suggest that synbiotics supplementation could be considered as a safe and natural tool because it had no obvious side effects on gut, liver and kidneys of treated rats. On the other hand, synbiotics supplementation is beneficial in improving the normal functioning of the gut, liver and kidneys, in addition more research is required for the identification of various transcriptional pathways involve in the pathogenesis of metabolic complications arising from HFHS diet-induced oxidative stress.

Conclusion: HFHS-diet increased lipid profile, altered intestinal architecture and severely affected the functioning of liver and kidney presumably due to increased oxidative stress, obesity and metabolic syndrome i.e. NAFLD. The synbiotics supplementation for 4 weeks improved gut histology, lipid profile, liver and renal function tests, enhanced anti-oxidant capacity suggesting therapeutic potential of synbiotics in ameliorating the HFHS-induced metabolic disease. The study of novel triad of gut-liver and kidney axis would get significant importance in handling HFHS-diet induced oxidative stress and metabolic disorders with synbiotics supplementation.

Declarations:

Ethical approval and consent to participate: This work did not involve any human data. Consent to participate is Not Applicable. The study was approved by the Institutional Bioethics Committee University of Agriculture, Faisalabad, Pakistan (Approval Number

ORIC 499/19). The animals in the present study were cared for and treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

ARRIVE guidelines: We ensure that our manuscript reporting adheres to the ARRIVE guidelines for the reporting of animal experiments (Kilkenny *et al.,* 2015).

Consent for publication: Our manuscript does not contain any individual person's information.

Availability of data and materials: All the data and materials used in our research will be available.

Competing interests: The authors declare no conflict of interest.

Funding: This research was supported by funds to J.A.K. from Higher Education Commission, Islamabad, Pakistan (project number 7538/Sindh/NRPU/R&D/HEC/2017).

Authors' Contributions: Conceptualization, H.R. and J.A.K; Formal analysis, Z. H., S.M.I. and J.A.K.; Investigation, H.R., J.A.K., A.S.Q., M.N.F., H.A., and M.R.; Methodology, H.R., M.R., A.R., M.N.F. and J.A.K.; Project administration, H.R., S.M.I. and J.A.K.; Resources, J.A.K., M. R. and H. A.; Software, H.R., A.S.Q. and M.N.F.; Supervision, J.A.K.; Validation, H.A. and S.M.I.; Visualization, Z. H., S.M.I., M. R., H.A., A.R., and J.A.K.; Writing – original draft, H.R. and J.A.K.; Writing – review & editing, H.A., M.N.F., H.R. and J.A.K.

Acknowledgments: The authors acknowledge the help of Sajid Masih, Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad, during experimental work on animals.

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List of Abbreviations: ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, ANOVA: Analysis of variance, AST: Aspartate aminotransferase, CFU: Colony Forming Unit, DMR: Duncan's new multiple range test, H and E: Hematoxylin and eosin, HDL: High-density

lipoprotein, HFHS diet: high fat high sugar diet, LDL: low-density lipoprotein, MDA: Malondialdehyde, NAFLD: Non-alcoholic fatty liver disease, SOD: Superoxide dismutase, TAC: Total antioxidant capacity, TOS: Total oxidant status.