

HP inulin-MCT dietary fiber improves lipid metabolism and prevents non-alcoholic steatohepatitis in obese mice

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ABSTRACT

Introduction: Regular intake of dietary fiber (DF) can counteract the negative impacts of obesity on physical health and chronic disease risk. DF mitigates obesity by promoting fullness, reducing calories, slowing nutrient absorption, stabilizing glucose, preventing fat storage, and influencing gut microbiota. High-performance (HP) inulin, a highly fermentable water-soluble DF, shows promise, but its palatability is limited in powder form. Combining HP inulin with beneficial triglycerides like Medium Chain Triglycerides (MCT) could overcome this issue.

Aim of study: This study investigates the effects of HP inulin-MCT on lipid metabolism and immune modulation in high-fat high-sucrose (HFHS) diet-induced obese mice.

Methods: Obese mice were orally administered with 20 % (w/v) HP inulin-MCT *ad-libitum* for 30 days. We measured their peripheral blood lipid profile, including LDL, HDL, and triglycerides. Additionally, the atherogenic coefficient, a parameter for assessing cardiovascular disease risk, was determined by calculating the ratio of non-HDL-C to HDL-C. Flow cytometry was used to identify splenic regulatory CD4⁺ and CD8⁺ T cells (Tregs). The Non-Alcoholic Steatohepatitis Score was assessed through histological examination.

Results: Significant reduced body mass index, improved lipid profiles, and reduced cardiovascular disease risk were observed in the obese mice treated with HP inulin-MCT. Moreover, HP inulin-MCT appeared to ameliorate non-alcoholic steatohepatitis, a liver condition associated with obesity. These health improvements were linked to immune system modulation, associated with an increase of CD4⁺ T-regulatory cells after HP inulin-MCT supplementation.

Conclusion: HP inulin-MCT is a promising and palatable dietary intervention to combat obesity-related non-communicable diseases, offering the potential for better overall health outcomes.

1. Introduction

Non-communicable diseases (NCDs), including heart disease, stroke, cancer, diabetes, and chronic lung disease, are the leading cause of all deaths worldwide. NCDs share four major risk factors: tobacco use,

physical inactivity, the harmful use of alcohol, and unhealthy diets (Schwartz et al., 2021). It is known that dietary fiber (DF) plays an indispensable role in maintaining health and prevention of NCDs. However, DF is still inferior, especially in low- and middle-income countries (Mayén et al., 2014).

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DF exhibits a diverse range of physiochemical properties and corresponding physiological effects. However, one of the well-known DFs, Inulin, has superior physiological effects and health benefits, including anti-diabetes, anti-obesity, anti-inflammatory, cardiovascular protective and immunoregulatory (Qin et al., 2023). These health benefits are mainly based on the high anaerobic fermentation of Inulin by gut microbes leading to the production of important bioactive short fatty acids (SCFAs) (Den Besten et al., 2013). Recent studies demonstrated the importance of SCFAs on the inflammation signaling pathways, glucose and lipid metabolism (He et al., 2020). SCFA regulates blood glucose levels by increasing insulin secretion, decreasing pancreatic glucagon, and increasing leptin secretion. Similar effects could be achieved by the administration of acarbose, a drug for the treatment of adults with type 2 diabetes mellitus as an adjunct to diet (Röder et al., 2016). Acarbose is a complex oligosaccharide that can act as a reversible inhibitor of pancreatic amylase and intestinal hydrolase. By delaying the digestion of carbohydrates, acarbose decelerates glucose absorption, resulting in a reduction of postprandial glucose blood concentrations (Dalsgaard et al., 2021).

High-performance Inulin (HP inulin) is an Inulin derivative that has ten or more fructose monomers, which are considered as higher health benefits (Roshanravan et al., 2017; Wijaya et al., 2022). Despite the numerous advantages, the supplementation of HP inulin higher than 20 % in powder form alone could affect the textural and sensory characteristics of food products (Arancibia et al., 2011; González-Tomás et al., 2009). Recent studies demonstrated that MCT (Medium Chain Triglycerides) supplement could still further enhance the beneficial effects of Inulin (Wardill et al., 2023; Watanabe & Tsujino, 2022). Therefore, a good combination of HP Inulin and MCT (HP inulin-MCT), which is applicable as a food supplement with good sensory qualities and palatable taste, is eagerly awaited. This supplement mixture has not only a significant advantage by exerting a fatty-like sense, but can be formulated as non-dairy creamer applicable for many kinds of foods and beverages (Mohammadi et al., 2023). However, the physiological benefits of HP inulin-MCT as a food supplement are currently not known (de SOUZA MWS et al., 2020; Zhang et al., 2020).

Non-Alcoholic Steatohepatitis (NASH) is a progressive liver disease that represents the more severe end of the Non-Alcoholic Fatty Liver Disease (NAFLD) spectrum, characterized by inflammation and liver cell injury. Obesity is a significant risk factor for the development of NASH due to its association with chronic low-grade inflammation. This inflammatory state is primarily driven by adipose tissue expansion and dysfunction, leading to the release of pro-inflammatory cytokines such as TNF- α , IL-6, and MCP-1 (Rohm et al., 2022). An essential aspect of the immune response in obesity is the dysregulation of T-regulatory (Treg) cells. In the liver, the decreased regulatory function of Treg cells can result in heightened immune responses to lipid accumulation and oxidative stress, promoting hepatocyte injury and fibrosis (Newton et al., 2016). The chronic inflammatory environment perpetuated by impaired Treg cell function is a key driver in the transition from simple steatosis to NASH.

In this study, we tested the effect of 20 % HP inulin-MCT on metabolic and on immunomodulatory profiles in high-fat high-sucrose (HFHS)-induce obese mice. We found that HP inulin-MCT supplementation could not only reduce body mass index and improve lipid profile but also modulate the immune system mediated by Treg in our murine model. In this model, acarbose showed comparable results. This observation suggested that 20 % HP inulin-MCT, as a palatable food and beverage supplement, is beneficial to HP-inulin alone to reduce the risk of NCD in obese patients.

2. Materials and Methods

Water-soluble HP inulin-MCT (20 %) was prepared by dissolving 60.2 % inulin powder, 7.8 % accessory ingredients (4.5 % milk powder, 2 % emulsifier, 0.9 % stabilizer, and 0.4 % anti-caking agent), and 32 %

MCT, and then spray-drying the mixture. The ingredients were obtained from PT. Lautan Natural Krimerindo, Mojokerto, Indonesia. Acarbose was purchased from PT. Dexa Medica (Tangerang, Indonesia). All ingredients for the standard diet and high-fat diet (HFD) (Silva et al., 2017) were from PT Citra Ina Feedmill (Jakarta, Indonesia). The respective ingredients were weighed (Ohaus, Shanghai, China), homogenized in water (1 kg/l), dried, and cut into small pieces.

2.1. Experimental animals and design

Twenty Swiss-Webster male mice (2–3 months old; 20–25 g) were obtained from the Veterinary Farma Center, Surabaya, Indonesia. The sample size calculation was carried out based on the recommendation of the institutional animal care and use committee (IACUC) through statistical power analysis using G Power software version 3.1 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany) (Faul et al., 2009). All mice were acclimatized in a separate cage, with 12 h of light-and-dark cycles and stable ventilation at room temperature (20–24 °C) with 65 % relative humidity. The standard chow diet was given *ad libitum* for 14 days and the body weight (BW) was measured and recorded weekly. Mice with decreased BW or signs of illnesses were excluded. After acclimatization, mice were randomly divided into four cohorts (five mice in each cohort) (Fig. 1). In cohort 1, mice received a standard chow diet and water (aqua dest.) as a drink. In three HFHS (High-Fat High-Sucrose) cohorts, HFHS-1, HFHS-2 and HFHS-3, all mice were fed with high-fat diet (Silva et al., 2017) and 20 % sucrose as a drink (Gulaku; PT. Sugar Group, Jakarta, Indonesia). After the mice became obese (body mass index ≥ 310) (Finkelstein et al., 2003), all three HFHS cohorts were continuously fed with HFD food pellets and water until the end of the experiment. Obese mice in the HFHS-1 group did not receive any supplement, whereas mice in the HFHS-2 and -3 groups were treated daily with HP inulin-MCT (20 %) or acarbose solution (1 g/l), respectively. All feeds and drinks were given *ad libitum* for 30 days. The food and drink waste were documented daily. Measurements of the body weight and body length were performed once a week. This study was approved by the Health Research Ethics Committee of Widya Mandala Catholic University Surabaya (No. 142/WM12/KEPK/DOSEN/T/2021).

2.2. Blood collection, peripheral blood lipid measurement

Mice were fasted for 10–12 h before taking blood samples. The blood samples were collected using a 5 mm deep-slicing of the tail dorsal vein with the scalpel in heparin-coated capillary tubes (Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany). The total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) were measured at the beginning (day 61) and one day after euthanization (day 91) using commercial kits as recommended by the manufacturer (Stanbio laboratory, Texas, USA). LDL cholesterol (LDL-C) concentration was calculated using the Friedewald equation (Friedewald et al., 1972). The mice were euthanized at the end of the experiment with overdosed ketamine (300 mg/kg, ip) and xylazine (45 mg/kg, ip) at day 91. The liver and spleen were dissected and weighed, and the splenocytes were analyzed by flow cytometry (see below). Fluorescence labeled mAbs including R-phycoerythrin (PE)-labeled anti-CD3 (Clone 17A2; Rat IgG2b), Peridinin Chlorophyll Protein Complex/Cyanine5.5 (PerCP/Cy) labeled anti-CD4 (Clone GK 1.5; Rat IgG2b), PerCP/Cy labeled anti-CD8 (Clone 53–6.7; Rat IgG2a), fluoresceinisothiocyanate (FITC)-labeled anti-CD25 (Clone PC-61.5.3; Rat IgG1), Allophycocyanin (APC)-labeled anti-Foxp3 (Clone 3G3; Mouse IgG1) were from Elabscience Biotechnology Inc. (Houston, USA).

2.3. Atherogenic coefficient (AC) calculation

AC was calculated as (plasma total cholesterol – plasma HDLc)/HDLc according to the published formula (Li et al., 2021) and

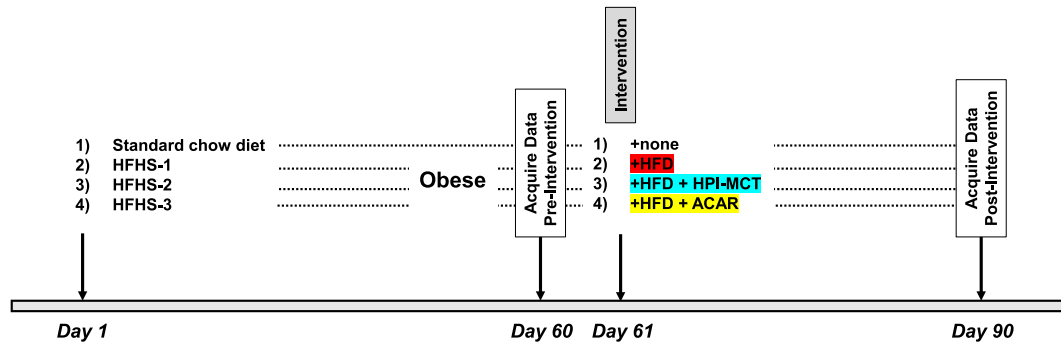


Fig. 1. Experimental design to study the effect of HP-Inulin MCT in HFD induced obese mice model. One group of mice receiving standard chow diet (control) and three groups of mice receiving High Fat High Sucrose diet (HFHS-1; HFHS-2 and HFHS-3) were established. After 60 days, intervention was started. Except the first group (+none), all other groups, HFHS groups, were fed with High Fat Diet (HFD) alone or together with HPI-MCT (HFD+HPI-MCT) or acarbose (HFD+ACAR) for 30 days as indicated. Data before intervention (pre-intervention) and after intervention (post-intervention) were collected and analyzed.

represents a ratio of non-HDL-C to HDL-C.

2.4. Splenic T-Cells analysis

Spleen (0.8 g) was treated with 1 ml of buffer containing 1000 U/ml collagenase type IV (Worthington Biochemical Corporation, New Jersey, US), 0.2 % DNase (Worthington Biochemical Corporation, New Jersey, US), and RPMI 1640 medium (Thermo Fisher Scientific, Inc., Massachusetts, US) in a 6-wells plate. After 30 min, the mixture was injected into the spleen and smashed using a syringe plunger, and the cell suspensions were filtered using a 70 μ m cell strainer (Biologix, Shandong, China). Red cells were lysed with lysis buffer containing 150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM $\text{Na}_2\text{-EDTA}$, which was added to the cell suspensions (1:5 (v/v)). After 2 min on ice, the splenocytes were centrifuged (5 min at 1500 rpm). The cell pellet was then washed with 1 ml of PBS supplemented with 0.5 % BSA (PBS-BSA; Genaxxon Bioscience GmbH, Ulm, Germany) and counted (Contacare Ophthalmics and Diagnostics, Vadodara, India) to adjust the cell concentration of 5×10^6 cells/ml. The viable splenocytes were analyzed with light microscopy (100x) and Neubauer-chamber (Assistant, Munich, Germany). The splenocytes (5×10^6 cells/ml) were stained with 10 μ g FITC-labeled anti-CD25 for 20 min and then with PE-labeled anti-CD3. Cells were then incubated with PerCP/Cy labeled anti-CD4 or PerCP/Cy labeled anti-CD8 for another 20 min in the dark and then washed (5 min at 1800 rpm). For intracellular staining, the splenocytes were resuspended in 1 ml permeabilization buffer (Thermo Fisher Scientific Inc., Massachusetts, USA) for 10 min. After washings, splenocytes were stained with 10 μ g APC-labeled anti-Foxp3, washed and fixed with fixation buffer (BD Cytotfix). T-cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, CA) using flowing software 2TM (Turku Bioscience, Finland). T-cells were first identified by forward and side scatter (FSC/SSC). The CD4^+ or CD8^+ T-lymphocytes were selected by CD3 and CD4 or CD8 markers, respectively. CD4^+ -or CD8^+ Tregs cells were determined by CD25 and Foxp3 markers. The gating strategy was performed according to previously reported experiments (Tjahjono et al., 2023).

2.5. Liver histological analysis

Liver tissues isolated from mice were immediately fixed in 10 % formalin for 24 h and embedded in paraffin. Then paraffin-embedded samples were sectioned at 5 μ m thickness and stained with hematoxylin and eosin (H&E). The Histologic Scoring System for Disease Activity (NASH) defined by the Clinical Research Network (CRN) was used for histological analysis (Liang et al., 2014). The histological sign of inflammation is the accumulation of white blood cells within connective tissue, referred to as inflammatory cells. The inflammatory cells can be manually counted by examining the intensely blue-colored and polymorphic nuclear cells.

2.6. Statistical analysis

The data were analyzed statistically with SPSS v17.0 (IBM Company, New York, the USA) using unpaired-sample T-Test methods. The data were presented graphically as the mean $\pm$ standard deviation (SD) with GraphPad PrismTM 5.0 (San Diego, USA). All results were considered significant if $p < 0.05$. The asterisks signify the significance of the data compared to respective parameters in the corresponding symbols (refer to the figure or table legends).

3. Results

Body weight, feed consumption, total caloric intake and length and body mass index from three similar HFHS (high-fat high sucrose) cohorts; HFHS-1 ($n = 5$), HFHS-2 ($n = 5$) and HFHS-3 ($n = 5$) before interventions with HFD, HFD plus HP inulin-MCT and HFD plus acarbose are shown in Table 1. There was no significant difference ($p > 0.05$) between intervention groups. After 60 days, all 15 mice in intervention groups became obese ($\text{BMI} \geq 310$). In contrast, the BMI index of the mice

Table 1

Baseline characteristics (mean $\pm$ SE) of experimental mice ($n = 5$ /cohort).

Characteristics	None	HFD	HFD+HPI-MCT	HFD+Acarbose
Initial body weight (g)	25.00 $\pm$ 2.92	22.20 $\pm$ 1.92	24.40 $\pm$ 3.36	25.00 $\pm$ 2.55
Pre-intervention body weight (g)	29.80 $\pm$ 1.92	41.20 $\pm$ 6.26	42.20 $\pm$ 3.70	40.40 $\pm$ 3.85
	$p = 0.0153^*$	$p = 0.0002^{***}$	$p < 0.0001^{****}$	$p < 0.0001^{****}$
Body length (cm)	11.86 $\pm$ 0.19	10.10 $\pm$ 0.16	10.26 $\pm$ 0.15	10.10 $\pm$ 0.17
Body mass index (Pre-intervention)	278.9014 $\pm$ 5.	331.72 $\pm$ 9.68	339.19 $\pm$ 11.70	337.88 $\pm$ 18.30
		$p < 0.0001^{****}$	$p < 0.0001^{****}$	$p = 0.0001^{***}$
Feed consumption (g/d)	2.96 $\pm$ 0.12	3.42 $\pm$ 0.25	3.06 $\pm$ 0.18	3.32 $\pm$ 0.24
Caloric intake (Kcal/d)	15.86 $\pm$ 0.65	18.32 $\pm$ 1.35	16.40 $\pm$ 0.97	17.79 $\pm$ 1.31
Total feed consumption (g)	177.60 $\pm$ 7.25	205.20 $\pm$ 15.11	183.6 $\pm$ 10.83	199.20 $\pm$ 14.62
Total caloric intake (Kcal)	951.58 $\pm$ 38.84	1099.46 $\pm$ 80.95	983.73 $\pm$ 58.04	1067.31 $\pm$ 78.35

P values: Δ compare with None; P values bodyweight: compared between initial and pre-intervention *: Significant.

Unpaired t-test, $^*(0.05 \geq p > 0.01)$; $^{**}(0.01 \geq p > 0.001)$; $^{***}(0.001 \geq p > 0.0001)$; $^{****}(p \leq 0.0001)$.

SE: Standard error; HFD: High fat diet; HPI-MCT: HP inulin-Medium Chain Triglycerides; g: gram; cm: centimetre; d: day; Kcal: Kilocalorie.

(n = 5) receiving a standard chow diet was 289.31 ± 2.54 .

3.1. HP inulin-MCT reduced the BMI of obese mice

The BMI of the mice was measured at day 60 and day 90 (pre- and post-intervention (Fig. 1). The change (Δ) of BMI (post-/pre-intervention) is shown in Fig. 2A. In comparison to the normal group (none), a significant increase of Δ BMI (39.08 ± 24.75 , $p = 0.0117$) was observed in the untreated obese mice group (HFD). When these obese mice were treated with HP inulin-MCT (HFD+HPI-MCT), significant reduction of Δ BMI (-14.63 ± 9.96 , $p = 0.002$) was observed. In the control experiment, acarbose-treated mice (HFD+ACAR) also showed strong reduction of Δ BMI (-26.21 ± 25.50 , $p = 0.0034$). However, this reduction was not significantly higher in comparison to HP inulin-MCT treated mice ($p = 0.3722$).

3.2. HP inulin-MCT improved the lipid profile and lowered risk of cardiovascular and nonalcoholic fatty liver disease in obese mice

To study the effect of HPI-MCT on the lipid profile, the concentrations of TG, TC, HDL, and LDL in the blood plasma of the mice were measured and the changes (pre- minus post-intervention) are shown in Fig. 2B and 2C. In comparison to the normal group, obese the cohort showed significant increase of Δ TG (28.20 ± 83.06 vs. 156.93 ± 13.03 ;

$p = 0.009$) and Δ HDL-C (-2.42 ± 0.76 vs. 1.69 ± 2.66 ; $p = 0.0105$), but not of Δ TC (-6.00 ± 16.61 vs. -9.60 ± 28.58 ; $p = 0.68$) and Δ LDL-C (-7.24 ± 9.88 vs. -22.13 ± 4.55 ; $p = 0.0155$) (Table 2).

Furthermore, obese mice treated with HPI-MCT revealed a significant decrease of Δ TG (156.93 ± 13.03 vs. 87.90 ± 76.04 ; $p = 0.0804$), and Δ TC (-9.60 ± 28.58 vs. 18.00 ± 12.81 ; $p = 0.0415$) but increase of Δ HDL (1.69 ± 2.66 vs. -30.39 ± 13.26 ; $p = 0.0007$). Remarkably, massive downregulation of Δ TC (-6.00 ± 16.61 vs. 18.00 ± 12.81 ; $p = 0.0337$) and upregulation of Δ HDL-C were found (-2.42 ± 0.76 vs. -30.39 ± 13.26 ; $p = 0.0015$).

This phenomenon was not observed in obese mice treated with acarbose. Although acarbose humiliated though the Δ TG (28.20 ± 83.06 vs. 95.53 ± 52.08 ; $p = 0.1631$), significant changes of Δ TC (-6.00 ± 16.61 vs. 17.20 ± 27.42 ; $p = 0.1443$), Δ HDL-C (-2.42 ± 0.76 vs. 0.19 ± 17.15 ; $p = 0.7783$) and Δ LDL-C (-7.24 ± 9.88 vs. -17.15 ± 27.37 ; $p = 0.0991$) were not detected. These results indicated that administration of HPI-MCT did not only reduce the concentration of TC, but also increased the generation of protective HDL-C. This phenomenon is reflected in the positive change of the atherogenic parameter (Δ AC) (Fig. 2D). Based on the lipid profile the calculated Δ AC of obese mice treated with HPI-MCT was significantly higher (1.96 ± 1.23 vs. 0.02 ± 0.32 ; $p = 0.0081$) than non-treated cohorts. This result indicated that administration of HPI-MCT might not only improve the lipid profile, but also lower cardiovascular risk.

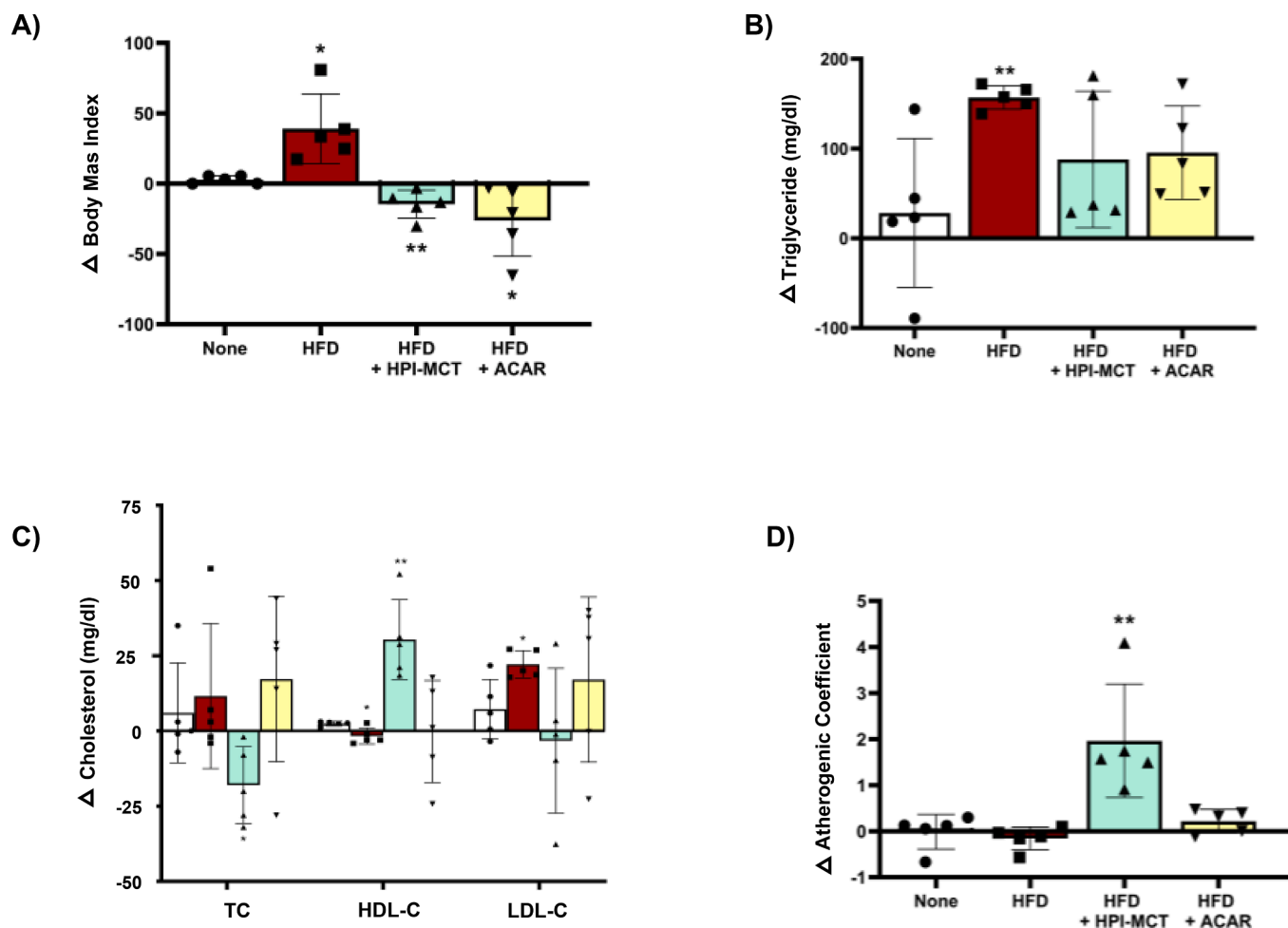


Fig. 2. Lipid profile and atherogenic coefficient of obese mice after intervention with HPI-MCT. Four groups of mice (five mice in each group) receiving standard chow diet (none), HFD diet alone (HFD) or HFD together with HPI-MCT (HFD+HPI-MCT) or acarbose (HFD+ACAR) were analyzed before and after intervention. Unless other described, the cohort description follows the color legend in Fig. 1. Delta (Δ) represents before (pre) minus after (post) intervention. (A) Delta of body mass index, (B) Delta of triglycerides, (C) Delta of cholesterol including total cholesterol (TC), HDL-C and LDL-C. (D) Atherogenic coefficient. Unpaired t -test, $^*(0.05 \geq p > 0.01)$; $^{**}(0.01 \geq p > 0.001)$.

Table 2

Lipid profile (mg/dl) of mice cohorts.

Lipid	None		HFD		HFD+HPI-MCT		HFD+Acarbose	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
TC	110.40 ± 20.94	116.40 ± 15.53	114.60 ± 13.76	123.20 ± 18.54	150.60 ± 25.18	132.60 ± 16.15	157.20 ± 31.00	17.20 ± 27.42
								(p ^Δ = 0.1443)
								(p [□] = 0.1156)
								(p [○] = 0.9543)
HDL-C	48.86 ± 3.85	51.28 ± 4.29	76.38 ± 11.44	74.69 ± 13.10	40.72 ± 8.97	71.11 ± 9.83	68.02 ± 13.37	0.19 ± 17.15
								(p ^Δ = 0.7783)
								(p [□] = 0.8105)
								(p [○] = 0.0139)*
LDL-C	26.52 ± 13.97	33.75 ± 7.07	31.69 ± 9.92	53.83 ± 8.38	26.24 ± 17.47	23.03 ± 7.59	40.94 ± 13.11	-17.15 ± 27.37
								(p ^Δ = 0.0991)
								(p [□] = 0.0135)*
								(p [○] = 0.4199)
TG	161.00 ± 73.23	132.80 ± 55.60	242.40 ± 14.79	85.47 ± 14.21	233.20 ± 63.52	145.30 ± 55.17	259.40 ± 53.96	95.53 ± 52.08
								(p ^Δ = 0.1631)
								(p [□] = 0.0338)*
								(p [○] = 0.8578)

P values: Δcompare with None; □ compare with HFD; ○ Compare with HFD+HPI-MCT; *significant.

Unpaired *t*-test, *(0.05 ≥ *p* > 0.01); ** (0.01 ≥ *p* > 0.001); *** (0.001 ≥ *p* > 0.0001); **** (*p* ≤ 0.0001).

TC: Total cholesterol; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; TG: Triglycerides.

Subsequently, we evaluated the number of monocytes (migrated into the cytoplasmic area of enlarged hepatocytes) in the liver of the mice at day 90 (Fig. 3). Compared to normal mice, a massive increased number of monocytes in the liver of obese mice (17.00 ± 19.31 vs. 386.20 ± 33.39; *p* < 0.0001) was found (Fig. 3B), indicating inflammation process took place in these mice (yellow arrow). This aggravation, however, could be reduced by supplementation with HP inulin-MCT (386.25 ± 38.55 vs. 100.00 ± 35.48; *p* = 0.0002). A similar result was obtained with acarbose (386.25 ± 38.55 vs. 121.00 ± 38.42, *p* = 0.0036).

To assess the risk of NAFLD, liver histology was analyzed according to the NASH scoring system (see *Materials and Methods*) of enlarged hepatocytes (green arrow). In the presence of HP inulin-MCT lower NASH

score was found in comparison to untreated mice (2.25 ± 0.96 vs. 7.60 ± 0.55, *p* = 0.0072) (Fig. 3C). A similar result was observed with acarbose (3.75 ± 0.50 vs. 7.60 ± 0.55, *p* = 0.0091) treated mice (Fig. 3C).

3.3. HP inulin-MCT improved the ratio of CD4⁺ Treg population in obese mice

Furthermore, we analyzed the ratio of CD4⁺, CD8⁺ T-cells as well as CD4⁺ and CD8⁺ Tregs population in the spleen of the normal group, obese, HPI inulin-MCT and acarbose-treated obese mice at day 90 (Fig. 4). Compared to the normal group, obese mice did not show

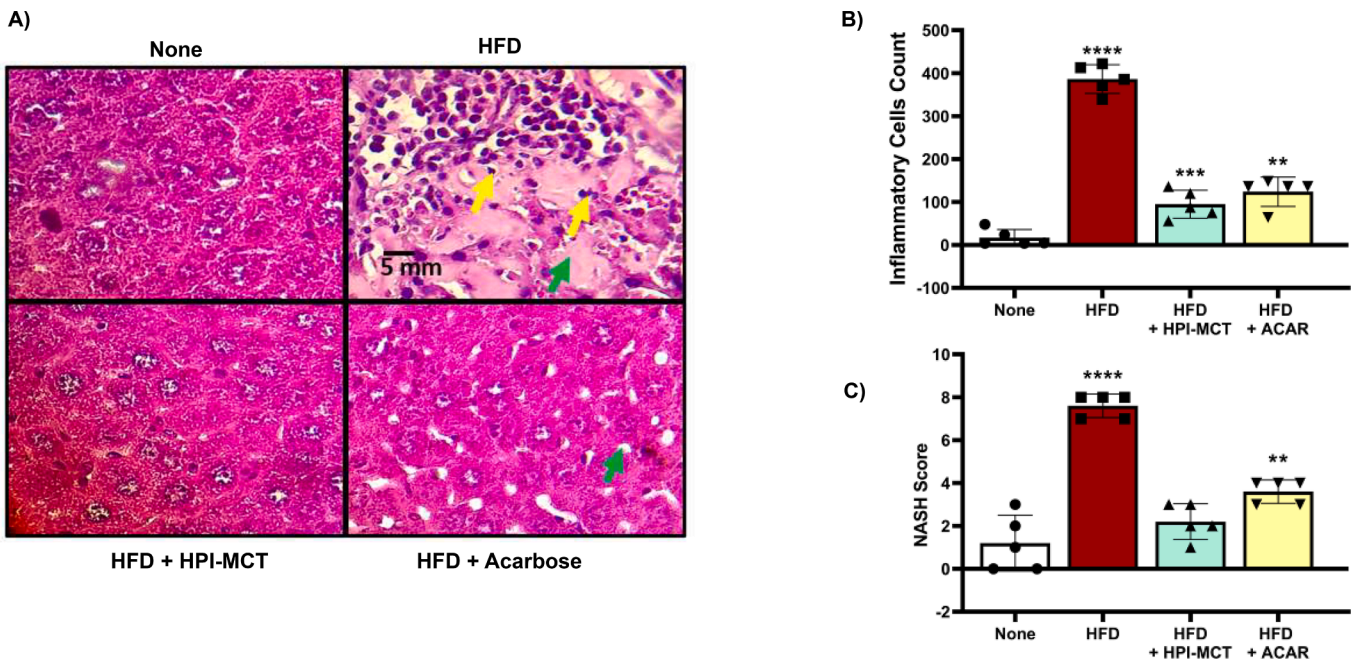


Fig. 3. Immunopathological analysis of liver from obese mice after intervention with HPI-MCT. Liver tissue of four groups of mice (five mice in each group) receiving standard chow diet (none), HFD diet alone (HFD) or HFD together with HPI-MCT (HFD+HPI-MCT) or acarbose (HFD+ACAR) were analyzed (A) representative staining result with hematoxylin and eosin. Yellow arrow represents monocytes, and green arrow represent enlarged hepatocyte (B) Number of monocytes in the liver of mice and (C) histologic scoring of non-alcoholic steatohepatitis (NASH) quantification graph. Unpaired *t*-test, *(0.05 ≥ *p* > 0.01); ** (0.01 ≥ *p* > 0.001); *** (0.001 ≥ *p* > 0.0001); **** (*p* ≤ 0.0001).

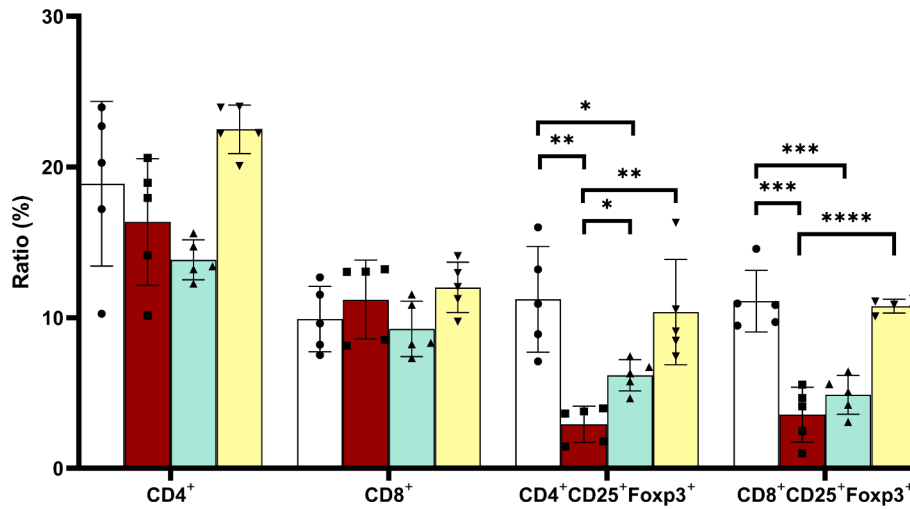


Fig. 4. The distribution of T- and Treg cells in the spleen of obese mice after intervention with HP Inulin-MCT. Lymphocytes were isolated from four groups of mice (four mice in each group) receiving standard chow diet (white columns), HFD diet alone (red columns), HFD together with HPI-MCT (green columns) and HFD together with acarbose (yellow columns). Lymphocytes were typed for CD4⁺, CD8⁺ populations as well as CD4⁺, CD25⁺, Foxp3⁺ (CD4⁺ Treg) and CD8⁺, CD25⁺, Foxp3⁺ (CD8⁺ Treg) subpopulations by flow cytometry (see Materials and Methods). Unless other described, the cohort description follows the color legend in Fig. 1. Unpaired *t*-test, * (0.05 $\geq$ *p* > 0.01); ** (0.01 $\geq$ *p* > 0.001); *** (0.001 $\geq$ *p* > 0.0001); **** (*p* $\leq$ 0.0001). The percentage of CD4 and CD8 cell populations in total T-lymphocytes (CD3⁺) as ratio (%) is presented.

significant change in the distribution of CD4⁺ and CD8⁺ T-cells (Table 3). However, significant reduction of CD4⁺ Tregs (11.22 $\pm$ 2.04 vs 3.50 $\pm$ 2.04, *p* = 0.0028) and CD8⁺ Tregs (11.10 $\pm$ 2.05 vs 3.56 $\pm$ 1.82, *p* = 0.0003) was observed. Furthermore, obese mice treated with HP inulin-MCT did not significantly alter the distribution of CD4⁺ and CD8⁺ T-cells when compared to untreated and obese mice. Interestingly, significant upregulation of CD4⁺ Tregs (3.50 $\pm$ 2.04 vs 6.18 $\pm$ 1.05, *p* = 0.0313), but not CD8⁺ Tregs (3.56 $\pm$ 1.82 vs 4.88 $\pm$ 1.29, *p* = 0.2218) was detected. However, this upregulation did not reach the original state as found in normal mice and was significantly lower when compared to acarbose-treated obese mice. Remarkably, compared with obese mice, acarbose treatment upregulated the distribution of CD4⁺ T-cells (16.36 $\pm$ 4.20 vs 22.50 $\pm$ 1.61, *p* = 0.0158) and restored the distribution of CD4⁺ Treg (3.50 $\pm$ 2.04 vs 10.37 $\pm$ 3.50, *p* = 0.0053) and CD8⁺ Treg

(3.56 $\pm$ 1.82 vs 10.76 $\pm$ 0.46, *p* = <0.0001) populations.

4. Discussion

Chronic exposure to high-fat and high-sucrose diets could seriously affect human health (Fulton et al., 2022). In this study, we demonstrated in a model of HFD-induced obese mice that the dietary fiber HP inulin-MCT could not only improve BMI and lipid profile, but also prevent cardiovascular risk and non-alcoholic steatohepatitis, most probably by modulation of the immune system.

HP inulin, engineered to minimize sensory impact, results in smoother-textured products (Qin et al., 2023). It boasts superior water solubility and enhanced stability (Mohammadi et al., 2023), making it ideal for inclusion in various food and beverage products without negatively affecting texture or consistency. In contrast, inulin can sometimes impart a gritty or chalky texture to food when used in higher concentrations (Shoaib et al., 2016). Furthermore, inulin, as a dietary fiber, has fewer prebiotic effects compared to HP inulin (Teferra, 2021), such as in reducing the post-prandial glycemic response (Wijaya et al., 2021).

Medium-chain triglycerides (MCTs) offer distinct advantages over their more common dietary counterparts, long-chain triglycerides (LCTs) (Jadhav & Annapure, 2023). MCTs are rapidly absorbed and metabolized, providing a quick energy source. They excel at inducing ketosis making them a preferred choice for adherents of ketogenic diets. Furthermore, MCTs increase satiety, promote fat oxidation, and facilitate weight management.

Remarkably, supplementation of HP-Inulin-MCT in obese mice (still receiving an HFD) for only 30 days resulted in a visible reduction in body weight and BMI. Since energy intake was consistently maintained across all cohorts, this reduction was likely determined by elevated energy consumption. This effect is most probably attributed to MCT (Wang et al., 2018). Hypercholesterolemia, characterized by increased levels of several circulating lipoproteins in the blood, is an established cardiovascular risk factor. High total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) increase the risk of acute myocardial infarction (AMI), whereas high-density lipoprotein cholesterol (HDL-C) is a strong protective factor (Brunner et al., 2019). In our murine model, HP-Inulin MCT supplementation reduces cardiovascular risk by reducing hypercholesterolemia. We observed a significant decrease in

Table 3
Lymphocyte population ratio (%) of mice cohorts.

T Cells	None	HFD	HFD+HPI-MCT	HFD-Acarbose
CD4 ⁺	18.88 $\pm$ 5.47	16.36 $\pm$ 4.20 (<i>p</i> ^A =0.4368)	13.84 $\pm$ 1.32 (<i>p</i> ^A = 0.0798) (<i>p</i> [□] = 0.2365)	22.50 $\pm$ 1.61 (<i>p</i> ^A = 0.1935) (<i>p</i> [□] = 0.0158) (<i>p</i> [○] < 0.0001) ****
CD8 ⁺	9.91 $\pm$ 2.18	11.20 $\pm$ 2.61 (<i>p</i> ^A =0.4222)	9.26 $\pm$ 1.83, (<i>p</i> ^A = 0.6212) (<i>p</i> [□] = 0.5709)	12.01 $\pm$ 1.66 (<i>p</i> ^A = 0.1241) (<i>p</i> [□] = 0.2111) (<i>p</i> [○] = 0.0374)*
CD4 + CD25 + Foxp3 ⁺	11.22 $\pm$ 3.51	3.50 $\pm$ 2.04 (<i>p</i> = 0.0028) **	6.18 $\pm$ 1.05, (<i>p</i> ^A = 0.0151)* (<i>p</i> [□] = 0.0313) *	10.37 $\pm$ 3.50 (<i>p</i> ^A = 0.7107) (<i>p</i> [□] = 0.0053) ** (<i>p</i> [○] = 0.0334)* ****
CD8 + CD25 + Foxp3 ⁺	11.10 $\pm$ 2.05	3.56 $\pm$ 1.82 (<i>p</i> ^A =0.0003) ***	4.88 $\pm$ 1.29 (<i>p</i> ^A = 0.0004) *** (<i>p</i> [□] = 0.2218)	10.76 $\pm$ 0.46 (<i>p</i> ^A = 0.7248) (<i>p</i> [□] < 0.0001) **** (<i>p</i> [○] < 0.0001) ****

P values: ^Δcompare with None; [□] compare with HFD; [○] Compare with HFD+HPI-MCT; *significant.

Unpaired *t*-test, * (0.05 $\geq$ *p* > 0.01); ** (0.01 $\geq$ *p* > 0.001); *** (0.001 $\geq$ *p* > 0.0001); **** (*p* $\leq$ 0.0001).

TC and LDL-C levels, along with a simultaneous increase in HDL-C concentrations in our obese mice after treatment with HP-Inulin MCT. This phenomenon is most probably attributed to Inulin. Inulin is important for the growth of beneficial microbiota-producing SCFA, which is known to improve the ratio of HDL cholesterol to non-HDL cholesterol (Hughes et al., 2022). Indeed, the change in the atherosclerotic index (delta AC), calculated based on the HDL cholesterol ratio to non-HDL cholesterol, improved after treating obese mice with HP-Inulin-MCT.

Lipid dysregulations due to HFD could increase the risk of NAFLD (Ragab et al., 2015). There are two types of non-alcoholic fatty liver diseases (NAFLD); non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) associated with liver inflammation which could lead to complications such as liver cirrhosis, cancer, and liver failure (Loomba et al., 2021). Furthermore, NAFLD is associated with an increased risk of developing type 2 diabetes (Loosen et al., 2023). In this study, we found an increased number of monocytes and hepatocellular ballooning (NASH) in the liver of obese mice. In addition, a high NASH score was found, indicating a progressive NASH process occurred in these mice. Supplementation with HP-Inulin MCT resulted not only in the reduction of monocytes in the liver of obese mice but also significantly lower NASH score. This observation indicated that HP-Inulin MCT could prevent the development of NASH.

Although the mechanism of NASH is complex including numerous factors, elevated uptake of fatty acid represents a key trigger for the development of hepatic steatosis due to increased lipid accumulation in liver cells (Geng et al., 2021). CD36, known as FAT (Fatty Acid Translocase) is a multifunctional receptor that plays a central role in the transport of fatty acids into various cells, including hepatocytes within the liver (Rada et al., 2020). It is known that high-fat diets could induce upregulation of CD36 expression in the liver leading to the accumulation of lipids within this tissue (Wilson et al., 2016). While the intricate relationship between HP inulin and CD36 remains partially understood, emerging research suggests that dietary fiber like inulin may potentially regulate CD36 expression and fat absorption (Khan et al., 2023). In this context, the uptake of MCTs took place via a distinct uptake mechanism (Irie et al., 2003). Exploring the intricate functions of CD36 in hepatic steatosis and its interactions with the HP Inulin-MCT represents a potential future study that holds the prospect of advancing our comprehension of NASH mechanisms and unveiling the therapeutic potential of HPI-MCT (Huang et al., 2023).

It is known that T-regulatory cells (Tregs) play an important role in reducing the high-fat diet-induced inflammation and hypercholesterolemia (Hong et al., 2019). Tregs are now highlighted as the key regulator in maintaining self-tolerance and immune cell homeostasis (Smigiel et al., 2014). There are two types of Tregs; CD4⁺ Tregs known as the immune tolerance regulator against pathogens (Traxinger et al., 2022), and CD8⁺ Tregs, known as the suppressor of CD8⁺ T-cells preventing the development of autoimmune and cancer diseases (Tang & Kumar, 2019). In this study, we found that the ratio of CD4⁺ Tregs (CD4⁺CD25⁺Foxp3⁺) and CD8⁺ Tregs (CD8⁺CD25⁺Foxp3⁺) subpopulations in obese mice was significantly decreased. However, the ratio of the total CD4⁺ and CD8⁺ T-cells was unaffected, indicating a disturbed immune regulation in these mice. It is known that prebiotics, such as inulin, can indirectly influence CD4⁺ Tregs by shaping the composition of the gut microbiota and promoting the production of metabolites like short-chain fatty acids (SCFAs) (Bassaganya-Riera et al., 2011; Chen et al., 2017; Zeng et al., 2022). Furthermore, SCFAs have been shown to enhance the expression of FOXP3 in CD4⁺ T cells, which is associated with the development of functional CD4⁺ Tregs (Amarante et al., 2018; Cai et al., 2020; Lo Conte et al., 2023). Interestingly, our result showed that HP Inulin-MCT could modulate the immune response of obese mice, particularly by increasing the number of CD4⁺ Tregs population, and thereby maintaining the balance of CD4⁺ Treg by the adaptive immune response. This response could prevent obesity-triggered chronic inflammation by producing anti-inflammatory

cytokines such as IL-10, IL-12, and TGF- β (Khan et al., 2021). Until today, little is known about the role of CD8⁺ Tregs in this respect. Here, we found no significant improvement in the CD8⁺ Tregs population after HP Inulin-MCT supplementation. Even though the function of CD8⁺ Tregs is still partially revealed (Levescot & Regulatory, 2022), the immunomodulatory action of HP Inulin-MCT might not affect the maintenance of cellular immunity.

Zhang and colleagues (2020) found that acarbose could alleviate obesity-induced chronic inflammation by increasing CD4⁺ Tregs (Zhang et al., 2020). Our results are consistent with this, showing that acarbose restored both Treg populations. Acarbose belongs to a class of drugs called alpha-glucosidase inhibitors, which slows down the digestion of carbohydrates and maintain thereby high rise of blood sugar after meal (McIver et al., 2023). However, acarbose could cause serious side effects including allergic skin reaction, liver problems and pneumatosis (Hsiao et al., 2006; Kono et al., 1999; Liao et al., 2017). Although the use of acarbose as a dietary supplement is under discussion (Meng et al., 2021); this application should be subject to careful reconsideration due to the seriousness of these potential side effects.

Taking together, in a model of HFD-induced obese mice we could demonstrate that HP Inulin-MCT supplement improved not only BMI and lipid profile, but also prevented cardiovascular risk and severe non-alcoholic liver disease. Our findings suggest a potential value of HP Inulin-MCT as an inexpensive, safe, and palatable food supplement that may contribute to preventing and managing non-communicable diseases. However, some aspects remain open for investigation, such as blood glucose and insulin levels, which are crucial for understanding insulin resistance and metabolic health among subjects. Conducted over a short experimental period, the study limits the assessment of sustained benefits and long-term effects of HP inulin-MCT supplementation. Furthermore, being conducted on mice, the applicability to human metabolic responses remains uncertain. Future studies investigating the efficacy of Inulin-MCT in human cohorts with obesity and nonalcoholic steatohepatitis could delve into personalized treatment approaches. Assessing the sustained benefits of Inulin-MCT supplementation would provide valuable insights for clinical applications.

CRediT authorship contribution statement

Yudy Tjahjono: . **Kuncoro Foe**: . **Yufita Ratnasari Wilianto**: Supervision, Investigation. **Wilson Christianto Khudrati**: Software, Formal analysis. **Senny Yesery Esar**: Supervision, Project administration, Funding acquisition. **Nico Jafet**: Supervision, Software, Methodology, Investigation. **I Made Andika Bara Kusuma**: Visualization, Software. **Lufti Ade**: . **Bernadette Dian Novita**: Writing – review & editing. **Hevi Wihadmadyatami**: Data curation. **Lidya Handayani Tjan**: Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jusak Nugraha**: Conceptualization. **Sentot Santoso**: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Hendy Wijaya**: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author contributions

YT and HW designed and carried out the experiments, analyzed the data, and prepared the manuscript. SS, KF, YRW, NJ, IMAB, BDN, HW, and JN prepared the manuscript, assisted with the experiments, and analyzed the data. SSP, SH, and LA assisted with the experiments.

Ethical Statement

This study was approved by the Health Research Ethics Committee of Widya Mandala Catholic University Surabaya (No. 142/WM12/KEPK/DOSEN/T/2021).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2024.106367>.

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