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Evaluation of Green Tea Yoghurt Enriched with Lacticaseibacillus paracasei E1 Microcapsules on Macrophage M1 Profile in High Fat-Fructose Diet Mice

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Abstract

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Keywords

Camellia sinensis, macrophage polarization, microencapsulation, obesity, probiotic, yoghurt

Authors

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RESEARCH PAPER

Evaluation of Green Tea Yoghurt Enriched With *Lacticaseibacillus paracasei* E1 Microcapsules on Macrophage M1 Profile in High Fat-fructose Diet Mice

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Abstract

Obesity is caused by an energy imbalance that increases chronic low-grade inflammation, including macrophage cell infiltration. Adipose tissue macrophages are polarized into pro-inflammatory macrophage type 1 (M1), secrete large amounts of pro-inflammatory cytokines, and activate transcription factors. Yoghurt with probiotics is popular at all ages for its health benefits and must be protected by microencapsulation. Green tea (*Camellia sinensis* L.) fortification provides yoghurt nutrients while improving its functional qualities and bioactivity. This study aimed to evaluate the effect of microencapsulation of *Lacticaseibacillus paracasei* E1 in green tea yoghurt (GTY) on the profile of M1 macrophages in mice fed a high-fat fructose diet (HFFD). Thirty-five male mice *Mus musculus* Balb/c (±20 g, 4 to 5-week-old) were divided into seven groups: normal; simvastatin dosage 1.3 mg/kg BW; plain yoghurt dosage 5 g/kg BW; GTY doses at 2.5, 5, and 10 g/kg BW. Mice were fed a diet for 12 weeks and treated with yoghurt daily for four weeks. Macrophages were obtained from isolated adipose cells using enzyme digestion and labeled with antibodies. The levels of $CD146^{+}NF\text{-}\kappa B^{+}$, $CD146^{+}STAT1^{+}$, $CD146^{+}TNF\text{-}\alpha^{+}$, $CD146^{+}CD11c^{+}IL\text{-}6^{+}$, and $CD146^{+}Lp\text{-}PLA_2^{+}$ were measured by flow cytometer. The data were analyzed using one-way ANOVA ($p < 0.05$). Yoghurt at all doses significantly reduced NF-κβ, STAT1, TNF-α, IL-6, and Lp-PLA_2 levels compared to the HFFD group. These findings suggest that combining green tea with probiotics in yoghurt may serve as a dietary intervention to manage obesity-induced inflammation by alleviating oxidative stress and regulating metabolism.

Keywords: *Camellia sinensis*, Macrophage polarization, Microencapsulation, Obesity, Probiotic, Yoghurt

1. Introduction

Changes in lifestyle, such as an unbalanced and controlled diet as well as a lack of physical activity, cause people to consume fast foods that are at high risk of developing obesity [1]. Obesity is caused by excessive buildup of fat in the body [2]. Obesity has a multifactorial etiology that is associated with various diseases, for instance, hypertension, hyperlipidemia, cardiovascular disease, metabolic syndrome, type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD), and some

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cancers [3,4]. Obesity is an enormous contributor to world mortality owing to many illnesses that cause untimely demise [5]. According to the World Obesity Atlas, the prevalence of obesity (BMI $\geq$30 kg/m^2) is projected to increase from 14% to 24% of the population by 2035 [6]. More than a billion people around the world are obese, including 650 million adults, 340 million adolescents, and 39 million children [7]. Obesity rates are rising in males, females, and all ages, with a higher prevalence in older people and women [3]. The global incidence percentages vary by area, country, ethnicity, and socioeconomic status. Obesity in the Americas increased from 6.8% in 1980 to 22.4% in 2019. Similarly, in Europe, the rate increased from 8.4% to 20%. In the Eastern Mediterranean region, the obesity prevalence soared from 6.4% to 17.4% in 2019, whereas in Africa, it increased from 3.8% to 10.9%. In Southeast Asia, the prevalence of obesity started at the same level as that in the Western Pacific (0.9% in 1980) and surged to 7.9% in 2019 [8]. Obesity was once considered a problem in high-income countries such as France, the United States, Australia, Japan, Norway, Sweden, and Denmark [9]. Obesity is increasing in low- and middle-income countries, especially in urban areas. Previous studies have revealed that the middle human development index is rising due to developing-country industrialization, higher salaries, low physical activity, and dietary trends toward fast food [10].

In addition, the coronavirus disease 2019 (COVID-19) lockdown and other government policies such as working from home, physical or social distancing, and returning to normal significantly impacted lifestyle changes during and after the pandemic. Lack of physical activity, poor diet quality, sleep time disruption, and heightened stress cause overeating as a response to boredom, isolation, and anxiety, which correlates with weight gain in the general population worldwide [11–13]. In Indonesia, Basic Health Research reported an increase in obesity among government institutional employees (33.7%), which is greater than in other employees. Western diet patterns and sedentary lifestyles are closely related to obesity in Indonesian urban workers [14]. Furthermore, obesity reduces societal life expectancy, making people more vulnerable to obesity-related non-communicable illnesses at a younger age [15]. Thus, positive lifestyle changes may be needed to maintain long-term health and prevent obesity. The primary reason for obesity is that the body stores more energy in the form of fat than it uses. Consumption of foods high in fat and sugar causes inflammation in peripheral tissues, including the liver, adipose tissue, skeletal muscles, and intestines [16,17]. Excess free fatty acid accumulation leads to adipose tissue hypertrophy and low-grade chronic inflammation, marked by increased innate and adaptive immune cell infiltration and activation [18].

Macrophages are the most common innate immune cells that infiltrate and concentrate in adipose tissue. In obese people, adipose tissue macrophages polarize to pro-inflammatory macrophage 1 (M1) and release a substantial number of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) [19–21]. In addition, it activates pro-inflammatory signaling pathways involving toll-like receptors (TLRs), such as c-Jun N-terminal kinase (JNK) [22]. TLR and inflammasomes modulate macrophage polarization by activating signal transducer and activator of transcription 1 (STAT1), nuclear-kappa-factor B (NF-κB), and caspase-1 to induce IL-1β production [23]. The M1 phenotype marker, cluster of differentiation-11c (CD11c), produces nitric oxide (NO), pro-inflammatory mediators TNF-α, and IL-1β, which act as signals of inflammation, disrupting adipose function and insulin sensitivity [24]. Stimulated macrophages release lipoprotein-associated phospholipase A2 (Lp-PLA$_2$), contributing to chronic inflammation in adipose tissues [25]. Lp-PLA$_2$ is a phospholipase A2 enzyme superfamily component that catalyzes phospholipid degradation in oxidative lipoproteins such as oxidized LDL (oxLDL) [26]. OxLDL promotes inflammatory mediators such as arachidonic acid, which drives the production of pro-inflammatory cytokines and lysophospholipids, which can alter the expression and activity of lipid-related enzymes such as fatty acid synthase (FAS) and lipoprotein lipase [27].

Several approaches to treat obesity use mainly drugs that are costly and contain synthetic chemicals with potential adverse effects. Therefore, the scientific community has gained interest in innovating and developing functional foods for managing obesity. Alternative probiotic-based functional food products can prevent and cure obesity while being safe and nutritious [28]. Fermented food products, such as yoghurt containing microorganisms, are growing rapidly and thoroughly approved by all consumers worldwide because of their plentiful nutritional content, distinctive flavor, long-lasting nature, and health benefits [29]. Probiotics are live microorganisms with beneficial physiological effects when administered adequately [30]. Yoghurt with probiotics provides health benefits, for which the starter culture

contains additional types of lactic acid bacteria (LABs) with probiotic properties [31]. *Lacticaseibacillus paracasei* (previously *L.casei*) is one of the most extensively utilized probiotics that may diminish the symptoms of obesity, inflammation, cholesterol-lowering, regulate the immune system, and antioxidant activity (Table 1). Previous research suggests that *L. paracasei* can control macrophage-mediated immunological responses and inhibit pro-inflammatory cytokines produced by monocyte macrophages through the TLR2-IRAK4-dependent NF-κB signaling pathway [32]. It also inhibits caspase 3, 9, and 8, pro-apoptotic Bax, NF-κBp65, pSTAT1, and iNOS, as well as pro-inflammatory macrophage gene expression and leukocyte infiltration in the liver [33,34].

LAB can enhance intestinal microbiota diversity, balance the Firmicutes/Bacteroidetes (F/B) abundance ratio and permeability of the intestine, and antioxidants and hindering NF-κB activation induced by LPS may diminish the pro-inflammatory response [44,45]. Some LAB strains produce short-chain fatty acids (SCFA) from the fermentation of several non-digestible polysaccharides [45]. Probiotics are prone to damage during this process. Thus, probiotics require protection by microencapsulation to maintain their viability [46,47]. The selection of encapsulation materials is crucial because the substances used must be food-grade, biodegradable, biocompatible, safe, serve as a physical barrier, and allow bacteria to thrive under acidic stomach conditions [48]. The modernization of the food industry has led to the addition of functional plant features to yoghurt, one of which is green tea (*Camellia sinensis* L.). Green tea boasts health benefits such as antioxidant, anti-obesity, anti-diabetes, cancer prevention, and protection against cardiovascular diseases [35,36].

Green tea is high in bioactive polyphenols, mainly epigallocatechin-3-gallate (EGCG) [49,50]. EGCG prevents TNF-α, IL-1β, and IL-6 secretion by reducing ROS-mediated NF-κB activation [51,52]. Several pharmacological effects of bioactive compounds in green tea have been widely reported (Table 1). Bioactive compounds in the form of catechin derivatives, particularly EGCG, which accounts for 50–70% of the polyphenol content of green tea, provide excellent bioactivity but have low gastrointestinal absorption [53]. During fermentation, green tea supplementation in yoghurt leads to interactions between polyphenols and probiotics [54,55]. Microorganisms release enzymes (tannase, esterase, phenolic acid decarboxylase, and glycosidase) that hydrolyze large molecular weight polyphenol compounds into small molecular weight polyphenols, such as aglycones, ellagic acid, and catechins [56]. This biotransformation enhances the amount and type of free small molecular components in fermented foods, improving bioactivity and bioavailability. According to Govindarajan et al. [57], the biotransformation of tea by tannase causes the content of ester molecules in tea to decrease, while the content of non-ester molecules and antioxidant capacity increase significantly. Polyphenols promote enzymatic production by intestinal microorganisms and selectively enhance the growth of certain beneficial bacteria in the gut (by ester bond hydrolysis and glycosidic bond breakage) while inhibiting the growth of harmful bacteria [58]. A recent study reported that the fermentation of green tea with a combination of probiotic yeast led to the creation of new compounds, such as methyl salicylate, geraniol, and 2-phenylethyl alcohol, which can enhance the aroma of tea [59]. Jin et al. (2021) found that fermenting green tea with *Levilactobacillus brevis* dramatically increased the bioactive content of γ-aminobutyric acid by 232.52% [60]. Green tea fermentation with six probiotics promoted the production of several phenolics, such as pyrogallol, coumaroylquinic acid derivatives, quercetin, and kaempferol [54]. However, studies on adding green tea to yoghurt by the encapsulation method against macrophage M1 adipose tissue are still limited and are expected to play a synergistic role as anti-obesity candidates. This study aimed to enhance the effect of microencapsulation of *L. paracasei* E1 in green tea yoghurt (GTY) on the levels of NF-κB, STAT1, TNF-α, IL-6, and Lp-PLA_2 in mice fed an HFFD.

2. Materials and methods

2.1. Bacterial preparation

The three strains of gram-positive lactic acid bacteria included *Lactobacillus bulgaricus* (FNCC 0041), *Streptococcus thermophilus* (FNCC 0040) obtained from the Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, and *L. paracasei* E1 obtained from the FMIPA Microbiology Laboratory, Brawijaya University, Malang. Bacteria were cultured in de Man Rogosa Sharpe broth (MRS) agar (110660, Merck, Darmstadt, Germany), cultivated in MRS broth (69966, Merck, Darmstadt, Germany), and incubated for 48 h [61]. Each bacterium was separated by centrifugation at 3000 rpm for 10 min at 4 °C and rinsed twice with 0.85% sterile saline solution [62,63]. The final concentration of the bacteria was 10^9 CFU/mL [64,65].

Table 1. Summary of research with green tea (C. sinensis) yoghurt and L. paracasei.

Publication year	Title	Contribution	Advantages/disadvantage	Ref. no.
2021	Functional and health properties of yoghurt supplemented with green tea or green coffee extracts and its effect on reducing obesity complications in rats	Consumption of yoghurt added to green tea reduces LDL and increases HDL, liver, and kidney function. Phenolic compounds can suppress enzyme activity in the liver, preventing damage to the liver, heart, and kidney tissue.	Adding green tea to yoghurt or coffee extract at a 1% concentration daily for up to 8 weeks restores the functional properties of obese tissue.	[28]
2023	Improving the antioxidant activity of yoghurt through black and green tea supplementation	Green tea additions improved the dry matter, ash, mineral levels, total phenolic content, and antioxidant activity.	Green tea-infused yoghurt samples showed higher pH levels and a better texture after storage.	[35]
2023	*C. sinensis*: Insights on its molecular mechanisms of action towards nutraceutical, anticancer potential, and other therapeutic applications	The main polyphenols in tea are useful as anticancer, neuroprotective, antibacterial, antiviral, antifungal, anti-obesity, antidiabetic, and antiglaucoma.	Advancements in tea catechin and nanoformulations bioavailability studies are required to increase accessibility and potential applications.	[36]
2015	Green tea yoghurt: Major phenolic compounds and microbial growth	Green tea promotes the growth of *Lactobacillus* spp. and *S. thermophilus* during milk fermentation while enhancing microbial metabolic activity by degrading phenolic compounds in green tea.	Green tea phenolic compounds composition had little effect on the antioxidant activities of green tea yoghurt after milk fermentation and during refrigerated storage.	[37]
2017	Effects of *C. sinensis* on survival of encapsulated *Lactobacillus casei* and *Bifidobacterium lactis* in ice-cream	*C. sinensis* extract can improve the survival of *L. casei* and *B. lactis* bacteria in ice cream	Application of the chitosan-alginate microencapsulation procedure and *C. sinensis* extract in the production of symbiotic ice cream with high numbers of probiotic bacteria *L. casei* and *B. lactis* after 90 days at freezing temperature.	[38]
2023	Effect of different doses of *L. paracasei* K56 on body fat and metabolic parameters in adult individuals with obesity: a Pilot study	The effect of a novel probiotic strain *L. paracasei* K56 on body fat and metabolic biomarkers in obese adults.	Supplementation with different doses of *L. paracasei* K56 reduces body fat, improves glucose metabolism, and modulates gut microbiota to favor anti-obesity characteristics, especially at a dose of 10^9 CFU/day.	[39]
2023	*L. paracasei* N1115 attenuates obesity in high-fat-diet-induced obese mice	Administration of LC-N1115 could ameliorate obesity by modulating the gut microbiota and the expression of lipid synthesis and pro-inflammatory cytokine genes.	The administration of LC-N1115 reduced weight gain, liver fat accumulation triglyceride, total cholesterol, and LDL levels and suppressed the expression of fatty acid synthase, IL-1β, and TLR4.	[40]

2023	Anti-obesity effect of *L. paracasei* LM-141 on high-fat-diet-induced rats through alleviation of inflammation and insulin resistance	LPLM141 supplementation exhibited an anti-obesity effect in HFD-fed rats by alleviating inflammation and insulin resistance.	LPLM141 significantly decreased body weight gain, liver weight, adipose tissue weight, epididymal white adipocyte size, serum lipid profile, LPS, MCP-1 levels, macrophage infiltration in adipose tissue, PPAR-γ mRNA, hepatic lipogenic gene expressions, increased serum adiponectin concentration, induced browning of epididymal white adipose tissue (eWAT), activation of interscapular brown adipose tissue (iBAT), upregulation of hepatic IRS-1 and p-Akt protein expressions.	[41]
2023	*L. paracasei* AO356 ameliorates obesity by regulating adipogenesis and thermogenesis in C57BL/6J male mice	AO356 prevents high-fat diet-induced obesity by regulating adipogenesis and thermogenesis.	Oral administration of *L. paracasei* AO356 effectively ameliorated body weight gain, abnormal body composition, obesity-induced hyperleptinemia, inflammation, downregulation of adipogenesis transcription factor, lipid metabolism-related genes, such as Srebp1c, PPARγ, FAS, C/EBPA, FABP4 and upregulation of thermogenesis-related genes, such as UCP1, Cpt1, PGC1A, Cidea, and Prdm16 in epididymal and subcutaneous fat pads.	[42]
2024	In vitro evaluation of the probiotic and antioxidant potential of *L. paracasei* ED25	Biological activities L. *paracasei* ED25 may be a probiotic agent against gastrointestinal disorder infection and oxidative stress-mediated diseases.	ED25 exhibited the maximum lactase production, cholesterol removal potential, antimicrobial and antioxidant activity, and the lowest antibiotic resistance.	[43]

2.2. Microencapsulation of probiotic

The bacteria culture (10^9 CFU/mL) was mixed with a 1.5% sodium alginate solution (71238, Sigma–Aldrich, Missouri, USA) as described by Ref. [66] in a ratio of 1:5 (v/v) using the spray-dry method. The spray-dried bacteria-alginate powder was dissolved in $CaCl_2$ (1 mol/L), added to 0.5% (w/v) chitosan (448869, Sigma–Aldrich, Missouri, USA) in 0.1% (v/v) glacial acetic acid solution for the outer microencapsulation layer and then atomized through a spray dryer.

2.3. Yoghurt preparation

A total of 4% (w/v) green tea leaves (*C. sinensis* var. *sinensis*) obtained from the Pasir Canar plantation were infused in ultra-high temperature (UHT) milk supplemented with 6% sucrose (w/v) [67], stirred, pasteurized at 85 °C for 30 min, and then stored in a refrigerator overnight [68]. The infused tea was heated to 45 °C and filtered. Then, it was added to the yoghurt starter, and 2% encapsulated *L. paracasei* E1 (w/v) was incubated at 37 °C for 30 h [69]. Plain yoghurt was prepared without the addition of green tea leaves.

2.4. Experimental design

Thirty-five male mice (20 g, 4-to 5-week-old) (*Mus musculus*) of the *Balb/C* strain were obtained from Professor Nidom Foundation, Surabaya. The inclusion criteria were healthy mice, active, with silky fur, and no abnormalities observed that could affect the research results. After arrival, the mice were kept in plastic cages under pathogen-free conditions (12/12 h light–dark cycle, controlled temperature 24 ± 1 °C, and humidity 50–60%) and given free access to water and food ad libitum for one week. Mice that died during diet or treatment were excluded from the study. The samples were calculated using Federer's formula as follows:

$$(t-1)(r-1) \geq 15 \quad (1)$$

t = number of treatment groups
r = sample size per group

After acclimatization, a diet was given to mice starting at four weeks of age. Then, mice were randomly divided into two primary groups: the normal group (n = 5) and the high fat-fructose diet (HFFD) group (n = 30). The normal group was fed a normal diet based on the modified American Institute of Nutrition 93-Growth (AIN-93G diet [70]. The HFFD group was fed a beef tallow-based high-fat diet according to the recipe from High Fat Diet-32 (HFD32) with slight modifications [71,72] and was administered 10% fructose for 12 weeks. The HFFD contained 50.05% carbohydrate, 7.15% protein, and 25.11% fat, with the remaining nutrients, including minerals, vitamins, and fiber. The HFFD group was divided into six groups: HFFD + simvastatin dosage 1.3 mg/kg BW (Sim); HFFD + plain yoghurt (Plain) dosage 5 g/kg BW; green tea yoghurt (GTY) doses at 2.5 g/kg BW (GTY1), 5 g/kg BW (GTY2), and 10 g/kg BW (GTY3). Yoghurt was administered orally every day for four weeks (Fig. 1). Body weight was measured once a week. The mice were considered obese, with a Lee index of >0.3 [73].

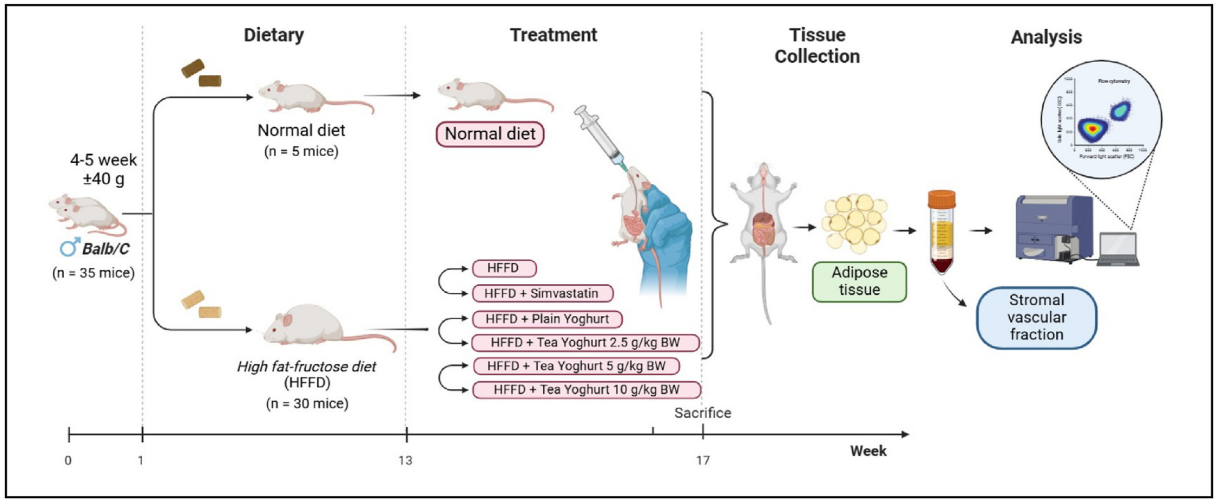

Fig. 1. Experimental design. The animal study lasted 16 weeks, with tea yoghurt or simvastatin administered in the final four weeks. The animals were sacrificed after the 16th week, and the effects were analyzed.

2.5. Adipose tissue isolation

Mice were anesthetized using 0.05 mL of ketamine-xylazine (Ket-A-Xyl®) intramuscularly, dissected to remove adipose tissue, washed using phosphate-buffered saline (PBS) (M1866, HiMedia Laboratories, USA), and then weighed. The adipose tissue isolation procedure refers to Ref. [74]. Adipose tissue was transferred into a sterile Petri dish and added to 1X Dulbecco's phosphate-buffer saline (DPBS) (without Ca and Mg) (D5773, Sigma–Aldrich, Missouri, USA) supplemented with 0.5% bovine serum albumin (BSA) (MB083, HiMedia Laboratories, USA) and cut into small pieces. Adipose tissue was transferred into conical tubes and rinsed with DPBS (0.5% BSA) and collagenase II (17101-015 Thermo Fisher Scientific, USA) digestion solution on ice. Homogenized adipose tissue was incubated in a shaker at 200 rpm for 20 min at 37 °C. The sample was then added to DPBS (0.5% BSA), homogenized using a serological pipette, filtered using a cell strainer 100 μm, transferred into a new conical tube, and centrifuged at 2500 rpm for 10 min at 4 °C. The pellet was supplemented with ammonium chloride potassium (ACK) buffer (A1049201, Thermo Fisher Scientific, USA) to lyse contaminated erythrocytes and added to fluorescence-activated cell sorting (FACS) buffer (BDB563503, Thermo Fisher Scientific, USA). The pellet obtained was the stromal vascular fraction (SVF) resuspended in FACS buffer until homogeneous.

2.6. Immunostaining and flow cytometry

The resuspension of cells was taken up to 50 μL and placed into a microtube after adding 400 μL of PBS and centrifuging at 2500 rpm at 10 °C for 5 min. The cell pellet was utilized for extracellular immunostaining using FITC anti-mouse CD146 (ME-9F1, Biolegend, San Diego, CA) and PE anti-mouse CD11c (N418, Biolegend, San Diego, CA) as an adipose tissue macrophage (ATM) M1 marker and incubated in a dark place at 10 °C for 20 min. Intracellular staining was performed using cytofix/cytoperm buffer (BD-Biosciences, Pharmingen) and rinsed with wash-perm buffer. The pellets were enriched with intracellular antibodies, namely PE-anti STAT 1 Phospho (Tyr 701) (A17012A, Biolegend, San Diego, CA), PE/Cy7 anti-mouse NF-κB (Biolegend, San Diego, CA), PE anti-mouse TNF-α (TN3-19.12, Biolegend, San Diego, CA), PE/Cy7 anti-mouse Lp-PLA_2 (Biolegend, San Diego, CA), and PE/Cy5.5-conjugated anti-mouse IL-6 (NBPI, Novus Biological, LLC, USA), then incubated for 20 min at min at 10 °C. PBS was added and measured with FACS Calibur™ (BD–Biosciences, San Jose, CA) and BD CellQuest Pro™ software. The percentage expression of STAT1, NF-κB, TNF-α, PAF-AH, and IL-6 was determined using the following formula [75]:

$$\% = UR/(UR + LR) \times 100 \quad (2)$$

LR = lower right
UR = upper right

2.7. Data analysis

Data on the relative number of cells resulting from flow cytometry were analyzed using one-way analysis of variance (ANOVA) and tested by Duncan's multiple-range test using IBM SPSS version 22.0 software for Windows. Differences between the various groups were deemed significant ($p < 0.05$) [75].

3. Results

3.1. Green tea yoghurt with probiotic microcapsule decreases NF-κβ and STAT 1 expression

The group with the addition of green tea yoghurt (GTY) was able to significantly reduce the relative number of $CD146^+NF\text{-}\kappa\beta^+$ and $CD146^+STAT1^+$ cells compared to the HFFD group without the addition of green tea yoghurt (Fig. 2). Mice administered HFFD had a 0.80% higher relative number of $CD146^+NF\text{-}\kappa\beta^+$ cells than mice fed a normal diet of 0.55%. The addition of plain yoghurt (without green tea) to mice fed an HFFD diet resulted in a 0.50% lower relative number of $CD146^+NF\text{-}\kappa\beta^+$ cells compared to the green tea yoghurt group. The results of the next study showed that mice fed HFFD alone had a relative number of $CD146^+STAT1^+$ cells of 0.75%, which was higher than that of mice fed a normal diet of 0.29%.

3.2. Green tea yoghurt with probiotic microcapsule reduces pro-inflammatory cytokines

Mice in the HFFD group had a relative number of $CD146^+CD11c^+IL\text{-}6^+$ cells of 0.29%, which was higher than that of mice fed a normal diet (0.16 %). Adding green tea yoghurt caused the relative number of $CD146^+CD11c^+IL\text{-}6^+$ cells to be lower and not significantly different compared to the HFFD group alone but not higher in the normal diet group (Fig. 3).

The results showed that mice given HFFD had a relative number of $CD146^+TNF\alpha^+$ cells of 0.81%, higher than mice fed a normal diet, 0.58%. Adding green tea yoghurt caused the relative number of

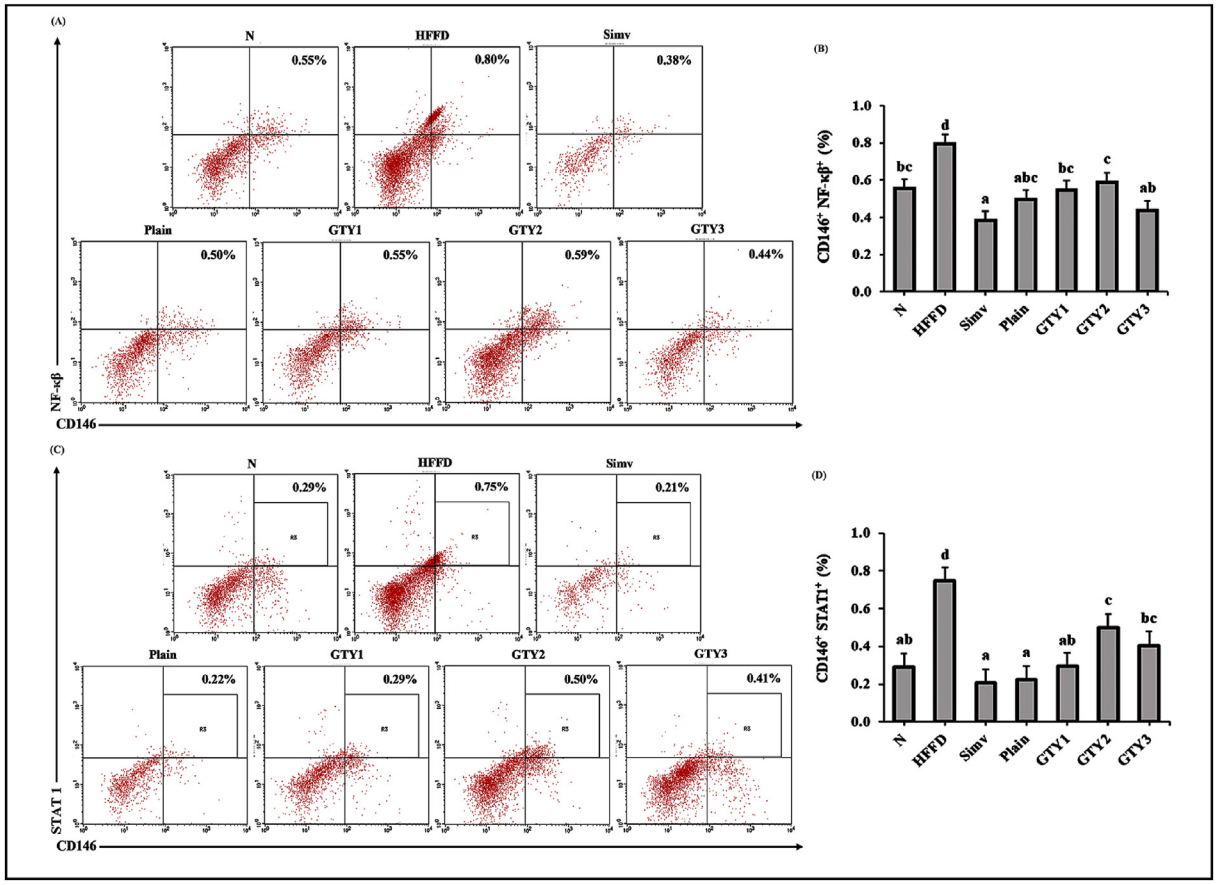

Fig. 2. Expression of transcription factors in ATMs. A-B) Dot plot analysis of CD146$^+$ NF-κβ$^+$ and CD146$^+$ STAT1$^+$ cells by flow cytometry. C-D) Percentage of NF-κβ and STAT1 was significantly increased in the HFFD induction groups. The percentage returned to normal after treatment with green tea yoghurt. The data were presented as mean ± SD and analyzed using one-way ANOVA followed by the DMRT post-hoc test.

CD146$^+$TNFα$^+$ cells to be lower than that in the HFFD group but not higher than that in the normal diet group (Fig. 3).

3.3. Green tea yoghurt with probiotic microcapsule restores Lp-PLA$_2$ levels

The results showed that mice fed HFFD alone had a relative number of CD146$^+$Lp-PLA$_2^+$ cells of 0.67%, significantly higher than that in mice fed a normal diet of 0.46%. The addition of yoghurt (plain or with green tea) to HFFD mice at all doses resulted in a relative number of CD146$^+$Lp-PLA$_2^+$ cells that were not significantly different compared to the normal group (0.46%) and the simvastatin group (0.37%) (Fig. 4).

4. Discussion

Over-dietary intake promotes an increase in free fatty acids (FFA), which are related to increased inflammatory cytokines and act as a main signal for macrophages in metabolic tissues [76]. In obese patients, adipose tissue is infiltrated by immune system cells, mostly macrophages, which mainly cause obesity-related low-grade inflammation and metabolic syndrome [77,78]. Several studies have demonstrated that consuming vegetables may assist in minimizing and avoiding obesity-associated issues [79]. Polyphenols are the most common bioactive chemicals found in plants and fruits. Green tea contains many polyphenols, the most abundant of which are catechins (Table 1). Over half of all green tea, catechins comprise EGCG, which interacts with cell surface receptors, intracellular signaling pathways, and nuclear transcription factors [80]. EGCG suppresses TLR4 signaling via the 67LR-dependent pathway, exerting anti-inflammatory activity [81]. TLR recognizes saturated fatty acids from dietary triglycerides or those produced during adipose tissue lipolysis. TLR4 polarizes macrophages into M1 phenotypes linked to classical activation and pro-inflammatory properties. M1 macrophages are attracted to visceral adipose tissue in response to obesity-induced inflammation. They produce inflammatory substances, including TNF-α,

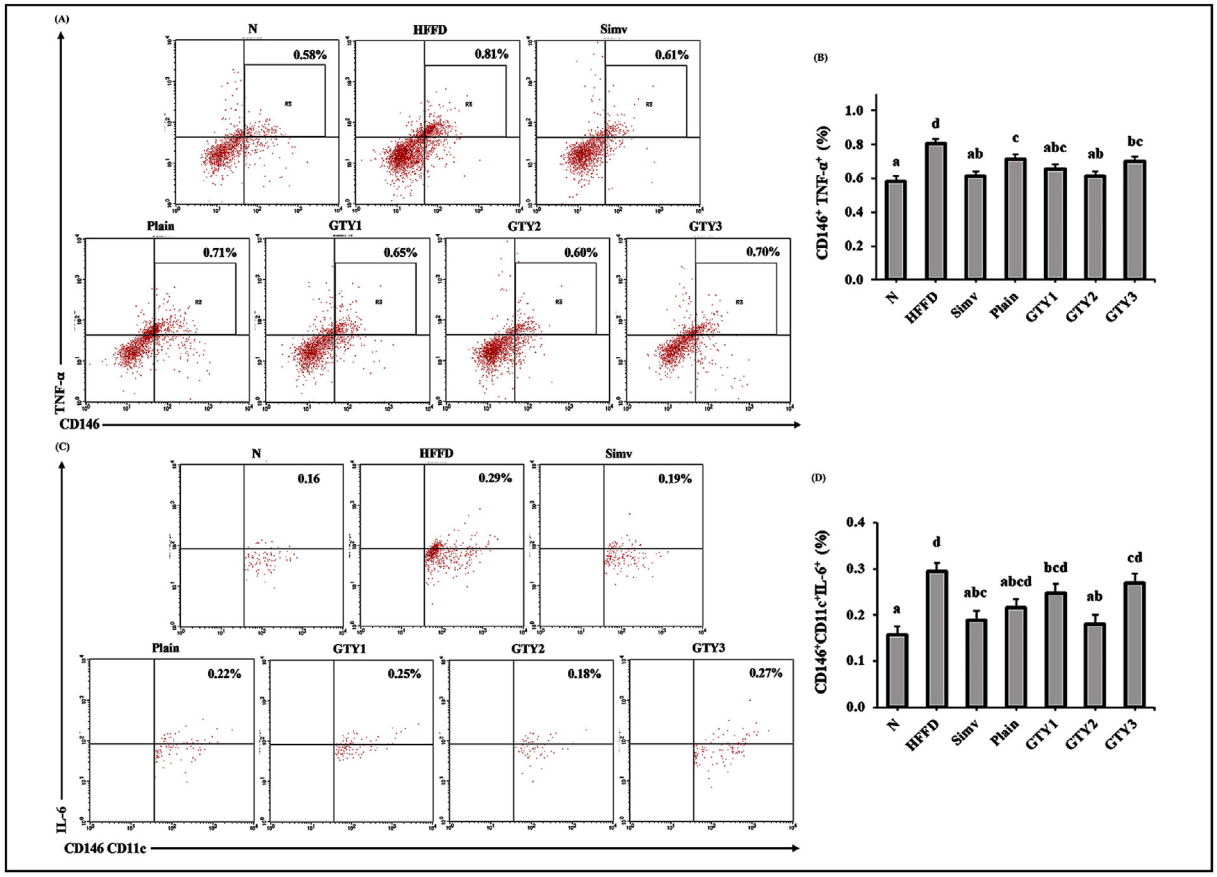

Fig. 3. Expression of pro-inflammatory cytokines in ATMs. A-B) Dot plot analysis of CD146$^+$ TNF-α$^+$ and CD146$^+$ CD11c$^+$ IL-6$^+$ cells by flow cytometry. C-D) The percentage of TNF-α and IL-6 was increased in the HFFD induction groups.

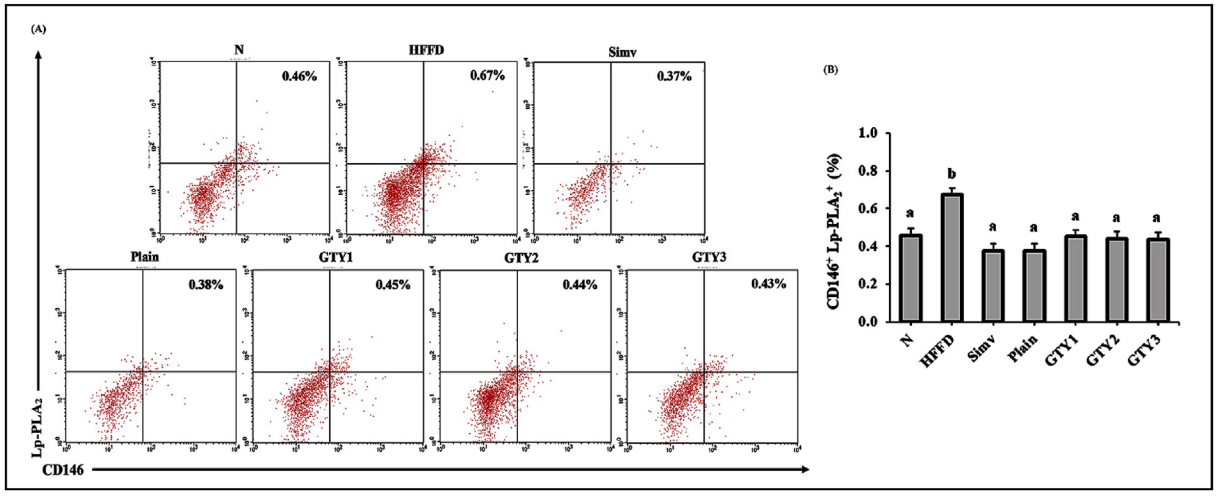

Fig. 4. Expression of Lp-PLA$_2$ in ATMs. A) Dot plot analysis of CD146$^+$ Lp-PLA$_2^+$ cells by flow cytometry. B) The percentage of Lp-PLA$_2$ was increased in the HFFD induction groups.

IL-1, IL-6, IL-12, and iNOS, as well as chemo-attractants, including MCP-1 [82]. Free fatty acids stimulate TLR4, which triggers a pathway that controls NF-κB inflammation. NF-κB is inactive in the cytoplasm due to its interaction with the inhibitor of κB (IκB), which is phosphorylated by κB kinase. IκB is ubiquitinated and eventually degraded. NF-κB translocated to the nucleus after activation. The

MAPK/ERK and NF-κB-inducing kinase/NF-κB pathways regulate the phosphorylation and activation of IκB kinase.

$CD146^+$ is a cell adhesion molecule involved in various cellular processes. Diminishing $CD146^+$ expression affects adipogenesis, adhesion, immune cell migration, and inflammatory response. NF-κB is a transcription factor that plays an important role in regulating the expression of genes involved in inflammation. $NF\text{-}κB^+$ regulates genes related to adipocytes, insulin sensitivity, and metabolism and enhances pro-inflammatory cytokines and chemokines. This affects adipose tissue metabolism, including energy balance, fat accumulation, and immune cell infiltration in obesity [83]. The addition of microencapsulated *L. paracasei* E1 to green tea yoghurt at a dose of 10 g/kg BW (GTY3) was the most effective in reducing $CD146^+NFκB^+$ expression by 0.44% in HFFD mice compared to the HFFD group (Fig. 2). In macrophages, epigallocatechin-3-gallate (EGCG) prevents IκB phosphorylation, deactivates the IKK complex, and limits excess NF-κB signaling. Previous investigations have shown that EGCG reduces LPS-induced I creases in P-IκB and P-P65 and prevents activation of the NF-κB pathway [84].

Green tea contains EGCG, which works synergistically with probiotic bacteria in yoghurt by modifying the number of gut microbiota and increasing the proportion of bacteria that produce SCFAs. In addition, green tea is an antioxidant, providing chemical protection against oxidative stress and free radicals and strengthening probiotics' effects by suppressing pathogenic bacteria's growth and supporting probiotic growth in the intestine. Probiotic L. *paracasei* produces SCFA metabolites such as acetate (C2), propionate (C3), and butyrate (C4) [85]. SCFAs bind to adipocyte and macrophage surface receptors GPR41 (FFAR3) and GPR43 (FFAR2), activating G-protein signaling and inhibiting adenylyl cyclase, thereby decreasing cAMP levels. Reduction in cAMP stimulates AMP-activated protein kinase (AMPK), which prevents IKK activation. IKK phosphorylates and degrades IκBα, thereby inhibiting NF-κB. AMPK suppresses the transcription of pro-inflammatory genes, such as TNF-α, IL-6, and IL-1β, by keeping IκBα active and preventing NF-κB from moving into the nucleus [86,87]. Activation of GPR43 reduces inflammatory signals mediated by TLR4, which recruits β-arrestins. Blocking the interaction of TLR4 with adaptor molecules such as MyD88 and TRIF reduces NF-κB activation and pro-inflammatory cytokine production. In addition, SCFAs, especially butyrate, act as HDAC inhibitors, altering histone acetylation and increasing IκBα protein [88]. This protein keeps NF-κB in the cytoplasm and prevents it from activating pro-inflammatory genes in the nucleus.

STAT1 is a transcription factor in the JAK-STAT pathway that regulates cytokine signals, especially type I interferons (IFNs) [89]. Activation of the JAK-STAT pathway involves phosphorylation of STAT1 by Janus kinase (JAK), which forms phosphorylated STAT1 dimers (p-STAT1) (Fig. 5). The p-STAT1 dimer translocates to the cell nucleus. It attaches to the interferon (IFN)-induced response element (ISRE) in gene promoters, managing the expression of immune-related genes, such as IFN-induced genes [90]. According to our findings, STAT1 expression was elevated in HFFD mice (0.75%), and $CD146^+STAT1^+$ expression declined when mice were treated with GTY (Fig. 2). GTY1 treatment at a dose of 2.5 g/kg BW was the most effective reducing $STAT1^+$ expression (0.29%) compared to other GTYtreatments. EGCG prevents STAT1 phosphorylation, binding of p-STAT1 dimers, STAT1 translocation to the nucleus, and activation of STAT1-regulated genes. EGCG may prevent STAT1 translocation to the nucleus by reducing STAT1 phosphorylation, which inhibits STAT1 from binding to DNA and activates inflammatory genes. EGCG indirectly regulates STAT1 expression in macrophages by preventing NF-κB activation and modifying the AP-1 complex. Previous research suggests that SCFAs, mainly butyrate, help prevent colorectal cancer by inhibiting HDAC and reducing IFN-γ-induced STAT1 activation [91]. Our data suggest that adding encapsulated probiotics accompanied by green tea to yoghurt may help regulate cellular immune responses by suppressing transcription factors.

In our findings, TNF-α release by $CD146^+$ macrophages differed significantly between the normal and HFFD groups ($p < 0.05$). The percentage of $CD146^+$ macrophages presenting $TNF\text{-}α^+$ was 0.55% in GTY1, 0.59% in GTY2, and 0.44 in GTY3 (Fig. 3). These findings suggest that green tea yoghurt at various dosages was effective in suppressing $TNF\text{-}α^+$ cytokine expression to close to normal levels. This was similar to the decrease in $IL\text{-}6^+$ expression after green tea yoghurt treatment compared to the HFFD group ($p < 0.05$) (Fig. 3). Activation of M1 macrophages is triggered by pro-inflammatory signals that activate the NF-κB and STAT1 transcription pathways, which work synergistically to regulate TNF-α and IL-6 levels in M1 macrophages. EGCG can prevent the activation of TNF-α receptors on the surface of macrophages by blocking the phosphorylation of adapter proteins such as TRAF2 [92]. The TNF-α receptor stimulates the MAPK and NF-κB signal transduction pathways, leading to TNF-α expression. EGCG suppresses IKK

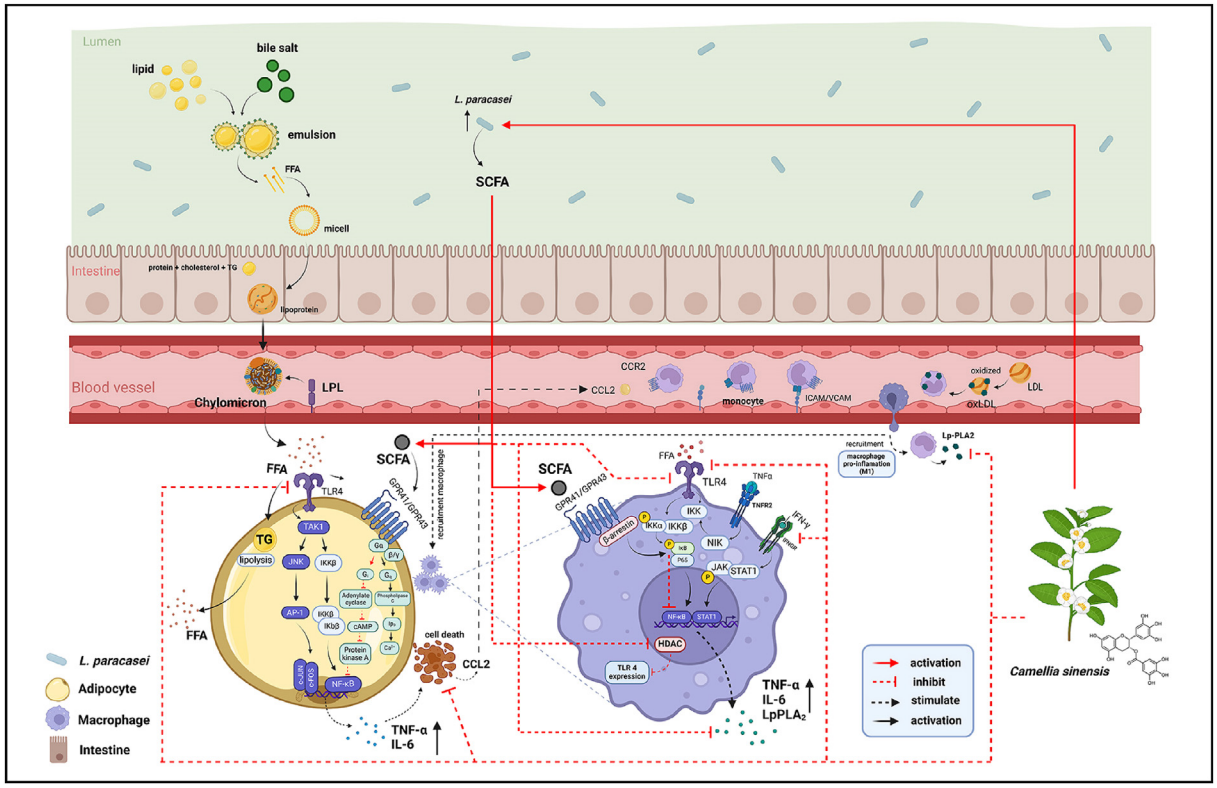

Fig. 5. Mechanism of green tea in yoghurt on the pro-inflammatory M1 macrophage signaling pathway. FFA, free fatty acid; STAT, signal transducer and activator of transcription; JAK, Janus kinase; IKK, IkappaB kinase; TLR, toll-like receptor; IFN, interferon; NF-κB, nuclear factor kappa B; $LpPLA_2$, lipoprotein-associated phospholipase A2; CCL, C-C motif chemokine ligand; ICAM, intercellular adhesion molecule; SCFA, short-chain fatty acid; LPL, lipoprotein lipase; iNOS, inducible nitric oxide synthase; oxLDL, oxidized low-density lipoprotein.

activation, IκB phosphorylation and degradation, and NF-κB activation [93–96]. NF-κB cannot bind to the κB response element (κBRE) found in the promoters of pro-inflammatory genes. Consequently, transcription and translation did not occur, and TNF-α and IL-6 were not expressed. Additionally, it affects the NF-κB and MAPK signaling pathways, which stimulate the TNF-α signaling pathway [97].

Chemokines, such as the (CCR2) CC chemokine receptor type 2, are crucial for macrophage activation and recruitment. CCR2 attracts circulating blood monocytes and transports them from the blood to inflamed tissues [98]. Earlier studies have also reported that EGCG prevents the recruitment and infiltration of adipose tissue macrophages (ATMs) by downregulating certain chemokines. The suppressive effect of EGCG on tumor development is associated with reduced ATM infiltration and downregulation of monocyte chemokines, such as CCL2 and macrophage colony-stimulating factor (CSF-1) [99]. Similarly, EGCG effectively reduced the expression of CCL2, CCL4, CCL22, CSF-1, CSF-2, IL-1β, and TNF in macrophage-like differentiated leukemia cells (transcriptional crosstalk study) [100]. Undoubtedly, EGCG exerts an anti-inflammatory effect by reducing NO and ROS production, suppressing TLR4 protein expression, and inhibiting IκB and p65 phosphorylation [84]. This suggests that EGCG has anti-inflammatory effects and can significantly suppress ATM.

Our findings revealed that GTY treatment decreased the expression level of $CD146^{+}Lp\text{-}PLA_2^{+}$ compared to HFFD treatment (Fig. 4). However, the results of GTY treatment at all doses were not significantly different: GTY1 (0.45%), GTY2 (0.44%), and GTY3 (0.43%). EGCG reduces $Lp\text{-}PLA_2$ expression in macrophages by inhibiting the NF-κB pathway [101]. The activation of NF-κB in obesity can induce $Lp\text{-}PLA_2$ gene expression. NF-κB plays a role in regulating gene transcription, including that of the $Lp\text{-}PLA_2$ enzyme. Increased NF-κB activity in obesity can increase $Lp\text{-}PLA_2$ expression [27]. Additionally, long-term inflammation caused by NF-κB activation in obesity can contribute to insulin resistance, pancreatic β-cell damage, and lipid metabolism disorders. Activation of NF-κB usually involves the binding of inhibitor of κB (IκB) and phosphorylation of IκB by the IKK complex.

EGCG inhibits IKK activation, leading to decreased IκB phosphorylation and degradation.

EGCG prevents the release of NF-κB from the IκB–NF–κB complex and terminates NF-κB activation. After IκB is degraded, the NF-κB subunit (p65) translocates to the cell nucleus and binds to DNA to regulate the expression of the target gene Lp-PLA_2 [102]. EGCG blocked p65 phosphorylation and p65/RelA complex activity and delayed the ability of NF-κB to bind to the Lp-PLA_2 gene promoter, preventing gene expression. In addition, EGCG obstructs the activation of the MAPK pathway, including ERK, JNK, and p38, which regulate Lp-PLA_2 gene expression and maintain AP-1 activity by hampering c-Jun phosphorylation [103]. EGCG may interact directly with the active site of Lp-PLA_2, causing a conformational change that reduces the enzyme's ability to bind to its substrate (oxidized phospholipid). EGCG binds to the active or allosteric site of the enzyme and may change the enzyme's structure, reducing the interaction between the enzyme and its substrate. This decreases the affinity of the enzyme for oxidized phospholipids. This enzyme can no longer bind oxidized phospholipids, thereby reducing the production of lysophosphatidylcholine and oxidative fatty acids.

The mitogen-activated protein kinase (MAPK) pathway regulates various cellular and molecular responses associated with the development of obesity by activating ERK, JNK, and p38. Most MAPKs are activated via TLR [104]. ERK (extracellular signal-regulated kinase) is activated by growth factors involved in fat cell formation or adipogenesis by regulating the expression of genes implicated in preadipocyte cell proliferation and differentiation into adipocytes. In addition, it regulates lipid metabolism by modulating lipolysis (fat breakdown) and lipogenesis (fat synthesis). Activation of JNK (c-Jun N-terminal kinase) and p38 MAPK by oxidative stress in obesity stimulates the production of pro-inflammatory cytokines, TNF-α, and IL-6 and the production of reactive oxygen species (ROS). JNK also causes insulin resistance by disrupting the normal insulin signaling pathway, possibly contributing to obesity. The p38 MAPK pathway can alter fat storage and breakdown balance, contributing to excessive fat accumulation [105]. p38 MAPK activates transcription factors, such as NF-κB or AP-1, which directly regulate gene transcription with regulatory elements in the Lp-PLA_2 gene promoter.

5. Conclusion

The administration of microencapsulated *L. paracasei* E1 in green tea yoghurt reduced the expression of the transcription factors NF-κB and STAT1, inflammatory cytokines TNFα and IL-6, and Lp-PLA_2. Interestingly, supplementation with green tea yoghurt can restore the M1 macrophage profile to approximately normal levels, thereby repairing the immune system by leveraging the synergistic effects of probiotics, anti-inflammatory compounds, and antioxidant properties to address inflammation and support metabolic health in obese individuals. Future research describing the gut microbiota population after treatment with green tea yoghurt with encapsulated probiotics through next-generation sequencing (NGS) analysis might provide useful information in pre- or clinical research. Transcriptomic and clinical trial approaches to demonstrate the effectiveness, safety, and tolerability of green tea yoghurt before its widespread use in clinical settings.

Ethical approval

The Research Ethics Commission of Brawijaya University Malang granted this study a certificate of ethical clearance with the following number 155-KEP-UB 2023, in compliance with ARRIVE guidelines [106] and associated guidelines, EU Directive 2010/63/EU for animal experiments.

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Conflict of interest

The authors declare no conflicts of interest.

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