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Anti-Atherogenic Activities of Exopolysaccharides and Their Producing Strain *Limosilactobacillus fermentum* MC1 in Mice

Nada Oršolić [1,*], Barbara Toljanić [1], Dyana Odeh [1], Nina Čuljak [2], Kate Šešelja [3], Mirela Baus Lončar [3], Domagoj Đikić [1], Andreja Leboš Pavunc [2] and Blaženka Kos [2]

[1] Division of Animal Physiology, Faculty of Science, University of Zagreb, Horvatovac 102a, 10 000 Zagreb, Croatia; barbara.toljanic2@gmail.com (B.T.); domagoj.djikic@biol.pmf.hr (D.Đ.)
[2] Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10 000 Zagreb, Croatia; nina.culjak@pbf.unizg.hr (N.Č.); andreja.lebos.pavunc@pbf.unizg.hr (A.L.P.); blazenka.kos@pbf.unizg.hr (B.K.)
[3] Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10 000 Zagreb, Croatia; kate.seselja@irb.hr (K.Š.); mbaus@irb.hr (M.B.L.)
* Correspondence: nada.orsolic@biol.pmf.hr; Tel.: +385-1-4877-735

Abstract

Atherosclerosis, the leading cause of death worldwide, is a chronic inflammatory disease leading to the accumulation of lipid-rich plaques within the artery wall. Accumulating evidence indicates that intestinal microbiota plays an important regulatory role in atherosclerosis at all stages of the disease. Through numerous metabolites, the intestinal microbiota can regulate immune and inflammatory cells and their mediators, as well as lipid metabolism, thereby contributing to the development and progression of atherosclerosis. With these assumptions in mind, we investigated the possibility of using *Limosilactobacillus fermentum* MC1 (*L. fermentum* MC1) and its exopolysaccharides (EPSs) in the reduction of lipid and atherogenic parameters as a preventive strategy in preventing the occurrence of cardiovascular diseases (CVD). We investigated the effect of *L. fermentum* MC1 and its EPSs on the health status of mice by monitoring the following parameters: body weight, colon length and weight, relative weight of organs, hematological (Hgb, WBC, number of erythrocytes, MCHC, MCV, MCH), and biochemical blood parameters including glucose, serum enzymes (ALT, ALP, amylase), urea, creatinine and lipid profile (total cholesterol, triglycerides, HDL, VLDL, LDL), different atherogenic parameters, blood biomarkers such as lymphocyte-to-monocyte (LMR) and neutrophil-to-lymphocyte (NLR) ratios, molecular inflammatory markers (IL1β, IL6, MCP1, IL1α, TLR4, TNFα, CD68, TGFβ), apoptosis markers (BCL2, AIFM1, IGF-1R), and endoplasmic reticulum stress markers (CHOP and GRP94) as well as oxidative stress (NOX2) markers in the colon. Furthermore, the level of lipid peroxidation, nitric oxide and glutathione concentrations in the liver, kidneys and spleen were measured. *L. fermentum* MC1 and its EPSs may prevent the development of atherosclerosis and the progression of CVD through antioxidant, anti-inflammatory, immunomodulatory activities, and regulation of the gut microbiome and lipid metabolism. The observed reduction in lipid and atherogenic determinants suggests that *L. fermentum* MC1 and its EPSs may contribute to atheroprotection and confer multiple health benefits.

Keywords: *L. fermentum* MC1; exopolysaccharides; lipid profile; atherogenic parameters; molecular inflammatory markers; oxidative stress

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1. Introduction

Cardiovascular diseases (CVD) comprise a group of disorders affecting the heart and blood vessels and represent one of the leading causes of mortality worldwide. The increase is due to increasing risk factors for CVD such as dyslipidemia, hypertension, obesity, physical inactivity, psychological stress, poor sleep quality, unhealthy diet and smoking [1–4]. Atherosclerosis, characterized by plaque formation, vascular calcification, and a tendency toward thrombosis, represents the underlying pathological basis of most CVD. Although traditional lipid-related risk factors are well established and widely described in current guidelines, growing evidence suggests that up to 90% of CVD cases may be preventable. Importantly, the development and progression of atherosclerosis are influenced by multiple modifiable factors, including the activity of the intestinal microbiota [1–5]. Numerous studies have demonstrated differences in the fecal microbiota between individuals with symptomatic atherosclerotic disease and healthy controls. The differences include an increased abundance of bacterial genera such as *Klebsiella*, *Haemophilus*, *Veilonella*, *Collinsella*, certain species of the genera *Clostridium*, *Enterobacteriaceae*, and *Streptococcus*, along with a decreased abundance of *Eubacterium*, *Roseburia*, and *Faecalibacterium* [1,2,6–8]. Bacterial genera enriched in symptomatic atherosclerosis are often pathogenic or opportunistic, contributing to the inflammatory processes that is one of the etiopathogenetic mechanisms of atherosclerotic plaque formation. In contrast, genera that are less abundant have the ability to produce short-chain fatty acids (SCFA), highlighting the extensive metabolic role of these intestinal bacterial metabolites. In a mouse model, the transfer of pro-inflammatory microbiota accelerated atherosclerosis progression and was accompanied by reduced SCFA production [9]. Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming, anaerobic or microaerophilic microorganisms that ferment carbohydrates to produce lactic acid. They are widely distributed in humans and animals, particularly in the gastrointestinal and urogenital tracts, and are also common in fermented foods, where they play a central role in fermentation processes. LAB are catalase-negative bacteria with low G + C DNA content and high tolerance to acidic environments. The group includes several genera, such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, and *Leuconostoc* [10].

Certain strains, especially within the genus *Lactobacillus*, are widely used as probiotics due to their beneficial effects on host health. They contribute to the maintenance of intestinal microbiota balance, enhance digestion and nutrient absorption, modulate immune responses, and exhibit antioxidant and cholesterol-lowering activities [1,5–9]. In the food industry, LAB are commonly used as starter cultures, improving the nutritional value, texture, and sensory properties of fermented products.

Some LAB strains produce exopolysaccharides (EPSs), structural polysaccharides that may remain attached to the cell surface or be secreted into the surrounding environment. EPSs are classified as homopolysaccharides or heteropolysaccharides, depending on their monosaccharide composition. These compounds play important roles in bacterial adhesion, interaction with the host epithelium, and the modulation of immune responses. Furthermore, EPSs can act as prebiotic substrates, promoting the production of short-chain fatty acids that support intestinal barrier integrity, inhibit pathogen adhesion, and contribute to metabolic and antioxidant functions [10–13]. It has been confirmed that increased concentrations of lipopolysaccharides are associated with dysbiosis of the natural microbiota and inflammatory diseases, including the development of asthma, allergies, central nervous system disorders, metabolic syndrome, type 2 diabetes, obesity, inflammatory bowel disease, cancer and CVDs. Therefore, probiotic supplementation may serve as a preventive strategy to reduce the risk of CVD in individuals at risk of type 2 diabetes or those affected by chronic inflammatory conditions such as inflammatory bowel disease, rheumatoid

arthritis, and non-alcoholic fatty liver disease, psoriasis, systemic lupus erythematosus, chronic kidney disease, and periodontitis, which are all associated with increased systemic inflammation and accelerated atherosclerosis [1–5,7,11]. Lactic acid bacteria (LAB) play an important role in lowering serum cholesterol levels, which can prevent atherosclerosis and other CVDs, and they also exhibit antioxidant properties [1–3,5]. Fermented dairy products, such as yogurt, additionally enriched with probiotic bacteria including *Bifidobacterium*, *Lactobacillus acidophilus*, and *Lacticaseibacillus rhamnosus*, provide multiple health benefits such as cholesterol reduction, improved metabolism, and enhanced immune response [11].

In this study, we used *Limosilactobacillus fermentum* MC1 (*L. fermentum* MC1), a Gram-positive, catalase-negative, facultatively anaerobic, heterofermentative bacterium belonging to the family *Lactobacillaceae* and reclassified into the genus *Limosilactobacillus* based on phylogenetic analyses. The strain is capable of metabolizing various carbohydrates, including galactose, maltose, cellobiose, and arabinose, and produces exopolysaccharides (EPSs). It demonstrates high tolerance to gastrointestinal conditions, including low pH and elevated bile salt concentrations, supporting its survival and functional activity in the gut [12,13].

Previous studies have reported that the administration of *L. fermentum* strains, such as ACA-DC 179, attenuates atherosclerosis development in ApoE−/−mice, indicating potential cardioprotective effects [14]. *L. fermentum* is recognized as safe by food safety authorities in Europe, the United States, and China, and is widely used in commercial probiotic formulations [15]. This strain exhibits strong hydrophobicity, autoaggregation capacity, and adhesion to intestinal epithelial cells via surface-associated molecules, including adhesive proteins, lipoteichoic acid, and EPSs, thereby contributing to intestinal barrier integrity and microbiota homeostasis. Notably, *L. fermentum* MC1 produces three distinct EPSs containing galactofuranose and glucopyranose, adheres to Caco-2 cells, and demonstrates antimicrobial activity against *Salmonella enterica* serovar Typhimurium FP1 [16]. We therefore hypothesized that preventive administration of *L. fermentum* MC1 and its EPSs may exert beneficial effects on the cardiovascular system by modulating the gut microbiota and host metabolism. These effects could improve weight regulation, antioxidant status, immune function, and lipid profiles, thereby reducing atherogenic risk factors and representing a potential strategy for cardiovascular disease prevention.

2. Results

2.1. Effect of L. fermentum MC1 and Its EPSs on Body Weight and Relative Weight of Organs

Our recent study showed that *L. fermentum* has a positive effect on the composition of the gut microbiome and that feeding mice with strain MC1 and its EPSs increased the relative abundance of the phylum *Verrucomicrobia*, *Bacteroidota* and *Bacillota* in the feces of healthy mice and decreased *Candidatus saccharibacteria* [17]. In addition, *L. fermentum* MC1 and its EPSs can have a positive effect on reducing the consequences of dextran sulfate sodium (DSS)-induced colitis in mice. They effectively preserve intestinal mucosal integrity and promote regeneration by decreasing the disease activity index (DAI), improving the murine histological colitis index (MCHI), reducing the expression of inflammation-related genes and bleeding, and promoting the polarization of M1 macrophages toward an M2-like phenotype [17,18]. Here, we investigated the possibility of their preventive effect on health in healthy mice through their anti-inflammatory, anti-atherogenic, and immunomodulatory properties.

Interestingly, *L. fermentum* MC1 and its EPSs resulted in significantly lower weight gain compared with the control group at day 15. The percentage change in body weight after treatment with EPS or MC1 was 4.57% or 4.39%, respectively, compared with 7.01% in the control group (Figure 1).

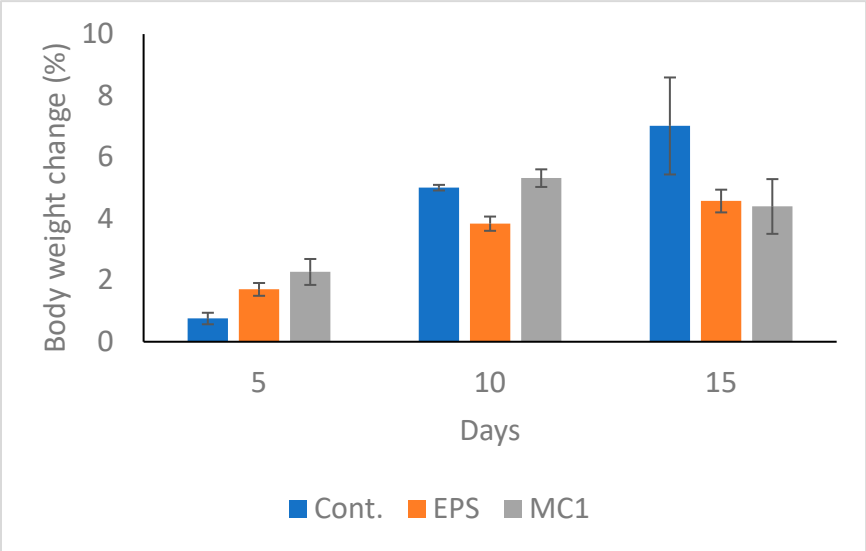

Figure 1. Change in body weight of C57BL/6 mice treated with probiotic strain *Limosilactobacillus fermentum* MC1 (*L. fermentum* MC1) and its exopolysaccharides (EPSs). Mice (*N* = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. Abbreviations: *ig*, intragastric; Cont, healthy mice (control group); EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; SE, standard error.

Table 1 confirms the effect of EPSs and MC1 on colon length and weight changes, as well as on the relative weights of the colon, liver, spleen, and kidney in C57BL/6 mice. EPSs and MC1 increased the length of the colons by more than 20.41 or 17.74% ($p < 0.05$; $p < 0.05$), the relative weight of the colons by 15.49 or 30.99 ($p < 0.05$; $p < 0.05$) and the relative weight of the spleen by 28.13% ($p < 0.01$; $p < 0.01$), compared to the control group.

Table 1. Colon length, colon weight and relative organ weight in C57BL/6 mice treated with probiotic strain *Limosilactobacillus fermentum* MC1 (*L. fermentum* MC1) and its exopolysaccharides (EPSs).

Exp. Group [a]	Length of the Colon (cm)	Weight of Colon (g)	Rel. Weight of Colon (%)	Rel. Weight of Liver (%)	Rel. Weight of Spleen (%)	Rel. Weight of Kidney L (%)	Rel. Weight of Kidney D (%)
Control	6.37 ± 0.40	0.16 ± 0.01	0.71 ± 0.04	5.21 ± 0.15	0.32 ± 0.02	0.56 ± 0.01	0.47 ± 0.03
EPS	7.67 ± 0.21 *	0.17 ± 0.01	0.82 ± 0.02 *	5.04 ± 0.14	0.41 ± 0.02 **	0.59 ± 0.02	0.65 ± 0.05 *
MC1	7.50 ± 0.23 *	0.18 ± 0.01	0.93 ± 0.06 *	4.53 ± 0.23 *	0.41 ± 0.02 **	0.53 ± 0.03	0.57 ± 0.03 *

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$). Abbreviations: *ig*, intragastric; Control, healthy mice (control group); EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; SE, standard error.

2.2. Effect of *L. fermentum* MC1 and Its EPSs on Hematological and Biochemical Parameters

At the end of the experiment, blood was taken from the mice to assess pro-inflammatory/anti-inflammatory, immunomodulatory and anti-atherogenic activity. Hematological changes are shown in Table 2. The group of mice treated with MC1 showed a statistically significant increase in the number of erythrocytes ($p < 0.01$), hemoglobin ($p < 0.01$), hematocrit ($p < 0.01$), MCHC ($p < 0.05$) and leukocytes ($p < 0.001$), and a reduced number of platelets ($p < 0.01$) compared to the control group. Significant changes in hematological parameters between MC1 and EPSs are visible in the increased number of erythrocytes, hemoglobin hematocrit ($p < 0.05$; $p < 0.01$; $p < 0.05$), and a decreased number of platelets ($p < 0.05$).

Table 2. Hematological parameters of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs.

♀Hematological Parameters [a]	Control	EPS	MC1
E (10^{12}/L)	5.2 ± 0.85	6.52 ± 0.17	8.46 ± 0.10 $^{**\Delta}$
L (10^{9}/L)	3.15 ± 0.25	4.55 ± 0.12	5.01 ± 0.27 ***
Hgb (g/L)	88.66 ± 1.28	98.00 ± 2.21	128.33 ± 1.47 $^{**\Delta\Delta}$
Hct (%)	0.36 ± 0.04	0.40 ± 0.01	0.51 ± 0.01 $^{**\Delta}$
MCV (fL)	53.31 ± 0.32	52.50 ± 0.22	53.08 ± 0.32
MCH (pg)	15.23 ± 0.09	15.00 ± 0.06	15.16 ± 0.09
MCHC (g/L)	238.33 ± 7.52	241.00 ± 1.31	249.66 ± 1.38 *
RDW (%)	47.71 ± 1.08	46.31 ± 0.57	45.03 ± 0.26 *
Plt (10^{9}/L)	13.63 ± 0.31	13.23 ± 0.16	12.86 ± 0.07 $^{**\Delta}$
MPV (fL)	5.40 ± 1.74	7.23 ± 0.09	6.76 ± 0.07

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^{9} cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). $^{\Delta}$ Statistically significantly different compared to EPS ($^{\Delta}$ $p < 0.05$; $^{\Delta\Delta}$ $p < 0.01$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; E, erythrocytes; L, leukocytes; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; Plt, platelets; MPV, mean platelet volume; SE, standard error.

Biochemical changes in enzymes and proteins are shown in Table 3 after treatment of mice with MC1 and EPSs. Treatment of mice with EPSs shows greater changes in measured indicators than MC1. Table 3 shows a statistically significant decrease in the concentration of albumin ($p < 0.001$), ALP ($p < 0.001$), amylase ($p < 0.01$) and total protein ($p < 0.001$) compared to the control group. Treatment of mice with MC1 significantly reduced the concentrations of ALP ($p < 0.01$) and amylase ($p < 0.05$) compared to the control group, while the concentrations of ALT ($p < 0.05$) and globulin ($p < 0.01$) were significantly increased compared to the group treated with EPSs (Table 3). In addition, MC1 significantly increases the level of TBIL compared to control and K^+ ions compared to EPSs (Table 3).

Table 3. Biochemical parameters in serum of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs.

♀Biochemical Parameters [a]	Control	EPS	MC1
ALB (g/L)	36.42 ± 1.71	17.85 ± 1.28 ***	27.14 ± 1.92
ALP (U/L)	32.78 ± 6.06	15.29 ± 1.41 ***	18.00 ± 1.35 **
ALT (U/L)	7.11 ± 1.25	4.00 ± 0.00	6.50 ± 0.68 $^{\Delta}$
AMY (U/L)	573.56 ± 51.29	340.60 ± 12.79 **	390.75 ± 55.38 *
TP (g/L)	38.85 ± 1.81	20.83 ± 1.01 ***	31.00 ± 1.58
GLOB (g/L)	2.43 ± 0.36	1.00 ± 0.40	3.85 ± 0.50 $^{\Delta\Delta}$
TBIL (μmol/L)	4.00 ± 0.00	4.29 ± 0.18	4.75 ± 0.16 *
BUN (mmol/L)	7.69 ± 1.07	5.36 ± 0.45	5.80 ± 0.60
PHOS (mmol/L)	1.97 ± 0.23	1.66 ± 0.11	1.70 ± 0.18
CRE (μmol/L)	21.00 ± 1.32	25.43 ± 4.55	25.37 ± 2.48
GLU (mmol/L)	10.24 ± 1.04	9.94 ± 0.99	12.57 ± 0.41
Na^+ (mmol/L)	154.11 ± 3.14	157.00 ± 0.75	153.63 ± 2.26
K^+ (mmol/L)	3.43 ± 0.27	2.71 ± 0.21	3. 60 ± 0.11 $^{\Delta}$

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^{9} cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). $^{\Delta}$ Statistically significantly different compared to EPS ($^{\Delta}$ $p < 0.05$; $^{\Delta\Delta}$ $p < 0.01$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; TP, total protein; GLOB, globulin; TBIL, total bilirubin; BUN, blood urea nitrogen; PHOS, phosphorus; CRE, creatinine; GLU, blood glucose; Na^+, sodium ions; K^+, potassium ions; SE, standard error.

2.3. Effect of L. fermentum MC1 and Its EPSs on Lipid and Atherogenic Parameters

Numerous studies support a symbiotic relationship between the intestinal microbiota and the development of atherosclerosis, indicating that microbiota can help prevent, alleviate, or reduce CVDs. Modifying the intestinal microbiota by using probiotics, such as *L. fermentum* MC1, and its EPSs could be an effective approach in preventing the development of atherosclerosis [4–10].

EPSs administration leads to a significant decrease in TC ($p < 0.001$), TG ($p < 0.01$) and HDL ($p < 0.01$) compared to the control group while MC1 leads to a decrease in TC ($p < 0.001$), HDL ($p < 0.05$) and LDL ($p < 0.05$) (Figure 2). The observed changes following MC1 administration led to a significant reduction in atherogenic determinants. Compared with the control group, MC1 significantly decreased the AIP ($p < 0.05$) and Castelli Risk Index II ($p < 0.05$), while significantly increasing the CPI ($p < 0.01$) (Table 4). When calculating the percentage of atherogenic protection of the EPS or MC1, MC1 showed a better protective effect on the vascular system than EPS (22.40% vs. 17.92%).

Table 4. Atherogenic parameters in serum of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs.

♀Parameters	Treatment [a]		
	Control	EPS	MC1
AC = [(TC − HDL-C)/HDL-C]	0.86 ± 0.11	0.71 ± 0.11	0.67 ± 0.08
AIP = [log (TG/HDL-C)]	−0.14 ± 0.03	−0.14 ± 0.02	−0.07 ± 0.01 *$^{\Delta}$
CRI-II= [LDL-C/HDL-C]	0.49 ± 0.07	0.54 ± 0.07	0.35 ± 0.03 *
CRI-I = [TC/HDL-C]	1.86 ± 0.11	1.71 ± 0.11	1.67 ±0.08
CPI = HDL-C/LDL-C	2.02 ± 0.09	1.85 ± 0.11	2.87 ± 0.07 **
IR = TG/HDL-C	0.72 ± 0.06	0.73 ± 0.04	0.84 ± 0.03

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$). $^{\Delta}$ Statistically significantly different compared to EPS ($^{\Delta}$ $p < 0.05$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; very-low-density lipoprotein cholesterol; AC, atherogenic coefficient; AIP, atherogenic index of plasma; CRI-I, Castelli Risk Index I; CRI-II, Castelli Risk Index II; IR, insulin resistance.

2.4. Effect of L. fermentum MC1 and Its EPSs on Inflammatory and Molecular Markers

The neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) are blood-based markers that can be used as indicators of systemic inflammation and the host's immune response. Table 5 shows that EPS significantly increased the numbers of neutrophils and lymphocytes ($p < 0.01$ and $p < 0.001$, respectively), whereas MC1 led to a significant increase in lymphocyte counts ($p < 0.0001$) compared with the control group. The NLR index is lower in the MC1 group (0.09) compared to the control and EPS-treated group (0.10; 0.11), while the LMR index is 1.5× higher compared to the control and EPS group (26.99 vs. 16.03 or 26.99 vs. 17.42). These data show that the increase in lymphocytes indicates an immunomodulatory effect of MC1.

The effect of *L. fermentum* MC1 and its EPSs on molecular inflammatory markers (IL-1β, IL-6, MCP-1, IL-1α, TLR4, TNF-α, CD68, TGF-β), apoptosis markers (BCL2, AIFM1, IGF1R), and endoplasmic reticulum stress markers (CHOP and GRP94), as well as the oxidative stress marker (NOX2), were further investigated in the intestine.

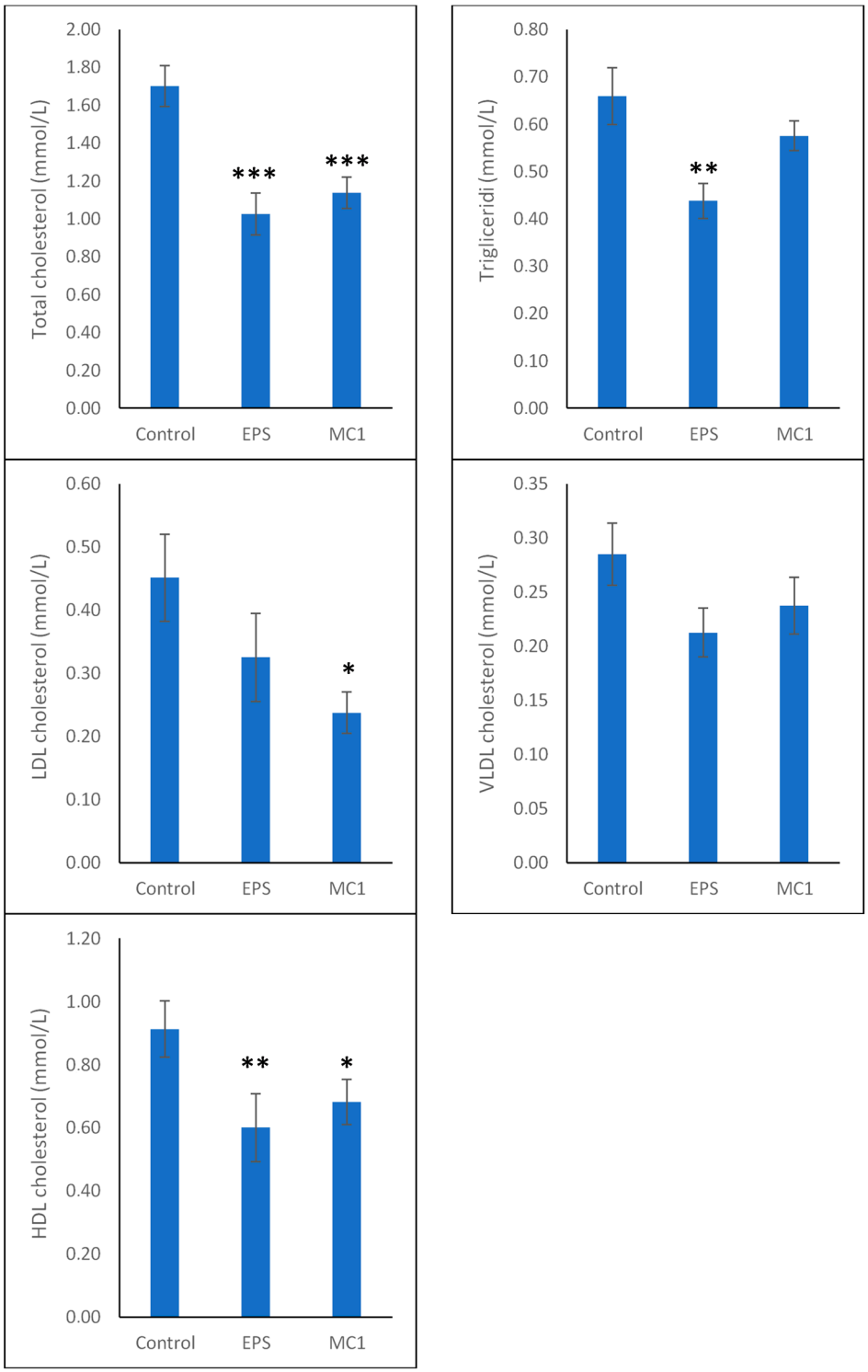

Figure 2. Lipid and atherogenic parameters in serum of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs. Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, Lactobacillus fermentum MC1-treated mice; HDL cholesterol, high-density lipoprotein cholesterol; LDL cholesterol, low-density lipoprotein cholesterol; VLDL cholesterol, very-low-density lipoprotein cholesterol; SE, standard error.

Table 5. Absolute number of differential blood count and blood-based markers of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs.

Exp. Group [a]	♀Absolute Number of Differential Blood Count			NLR	LMR
	Neutrophils ($\times 10^9$)	Lymphocytes ($\times 10^9$)	Macrophages ($\times 10^9$)		
Cont.	0.22 ± 0.04	2.17 ± 0.22	0.14 ± 0.02	0.10	16.03
EPS	0.41 ± 0.03 **	3.86 ± 0.14 ***	0.22 ± 0.03 *	0.11	17.42
MC1	0.39 ± 0.09	4.41 ± 0.051 ****	0.16 ± 0.04	0.09	26.99

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Abbreviations: *ig*, intragastric; Cont, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; SE, standard error.

The molecular inflammatory markers (IL1β, IL6, MCP1, IL1α, TLR4, TNFα, CD68 TGFβ) were measured by quantitative polymerase chain reaction (qPCR). EPS showed an increase in the concentration of inflammatory parameters IL1β, MCP1, IL1α, TLR4 ($p < 0.01$; $p < 0.01$; $p < 0.01$; $p < 0.01$), and a reduction in TGFβ ($p < 0.05$) compared to the control (Table 6). In contrast to EPS, MC1 treatment of mice increased the concentration of MCP1 ($p < 0.01$) and significantly reduced CD68 and TGFβ ($p < 0.05$; $p < 0.0001$) in relation to the control group. EPS compared to MC1 has higher levels of gene expression for IL1β, TLR4, CD68, and TGFβ ($p < 0.01$; $p < 0.05$; $p < 0.05$, $p < 0.001$), while the level of IL-6 is reduced ($p < 0.01$) (Table 6).

Table 6. Expression of genes related to inflammation of C57BL/6 mice treated with the probiotic strain *L. fermentum* MC1 and its EPSs.

Exp. Group [a]	♀Molecular Inflammatory Markers							
	IL-1β	IL-6	MCP-1	IL-1α	TLR4	TNF-α	CD68	TGF-β
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
EPS	1.54 ± 0.11 **	0.74 ± 0.22	2.09 ± 0.30 **	2.29 ± 0.28 **	1.29 ± 0.08 **	1.45 ± 0.40	1.12 ± 0.13	0.91 ± 0.03 *
MC1	0.85 ± 0.16 $^{\Delta\Delta}$	1.79 ± 0.16 **$^{\Delta\Delta}$	1.87 ± 0.19 **	1.51 ± 0.23	0.94 ± 0.10 $^{\Delta}$	1.45 ± 0.47	0.69 ± 0.09 *$^{\Delta}$	0.64 ± 0.04 ****$^{\Delta\Delta\Delta}$

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$). $^{\Delta}$ Statistically significantly different compared to EPS ($^{\Delta}$ $p < 0.05$; $^{\Delta\Delta}$ $p < 0.01$; $^{\Delta\Delta\Delta}$ $p < 0.001$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; IL-1α, interleukin 1α; IL-1β, interleukin 1β; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; CD68, cluster of differentiation 68; TGF-β, transforming growth factor β; SE, standard error.

In contrast to EPS, MC1 significantly affected molecular markers of apoptosis, endoplasmic reticulum stress, and oxidative stress (Table 7), notably leading to a marked reduction in insulin-like growth factor 1 receptor (IGF1R) and CHOP levels (both $p < 0.0001$).

Table 7. Expression of genes related to apoptosis, endoplasmic reticulum and oxidative stress of C57BL/6 mice treated with the probiotic strain *L. fermentum* MC1 and its EPSs.

Exp. Group [a]	♀Molecular Markers of Apoptosis, Endoplasmic and Oxidative Stress					
	IGF-1R	GRP94	NOX2	CHOP	AIFM	BCL2
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
EPS	0.89 ± 0.10	0.95 ± 0.09	1.38 ± 0.25	0.95 ± 0.13	0.88 ± 0.12	0.90 ± 0.13
MC1	0.68 ± 0.03 ****	0.93 ± 0.15	0.93 ± 0.21	0.72 ± 0.03 ****	0.70 ± 0.06	0.80 ± 0.09

[a] Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (**** $p < 0.0001$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, exopolysaccharides-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; IGF-1R, insulin-like growth factor 1 receptor; GRP94, glucose-regulated protein 94; NOX2, NADPH oxidase 2; CHOP, C/EBP homologous protein; AIFM1, apoptosis-inducing factor, mitochondria associated 1; BCL2, B-cell lymphoma 2; SE, standard error.

2.5. Effect of *L. fermentum* MC1 and Its EPSs on Oxidative Stress Markers

Figure 3 shows the results of the end product of lipid peroxidation-malondialdehyde, the concentration of total glutathione and the activity of nitric oxide (NO) in mice treated with the probiotic suspension MC1 and its EPSs. Treatment of mice with EPS statistically significantly reduces the level of malondialdehyde (MDA) in the kidney and liver ($p < 0.01$; $p < 0.01$) compared to the control. MC1 reduces the level of MDA ($p < 0.05$) and increases the level of glutathione (GSH) in the kidney and spleen ($p < 0.01$; $p < 0.01$). Both EPS and MC1 treatments significantly reduced nitric oxide (NO) levels in the liver compared with the control group ($p < 0.05$ and $p < 0.001$, respectively). In the spleen, MC1 treatment led to an increase in NO levels compared with EPS ($p < 0.05$).

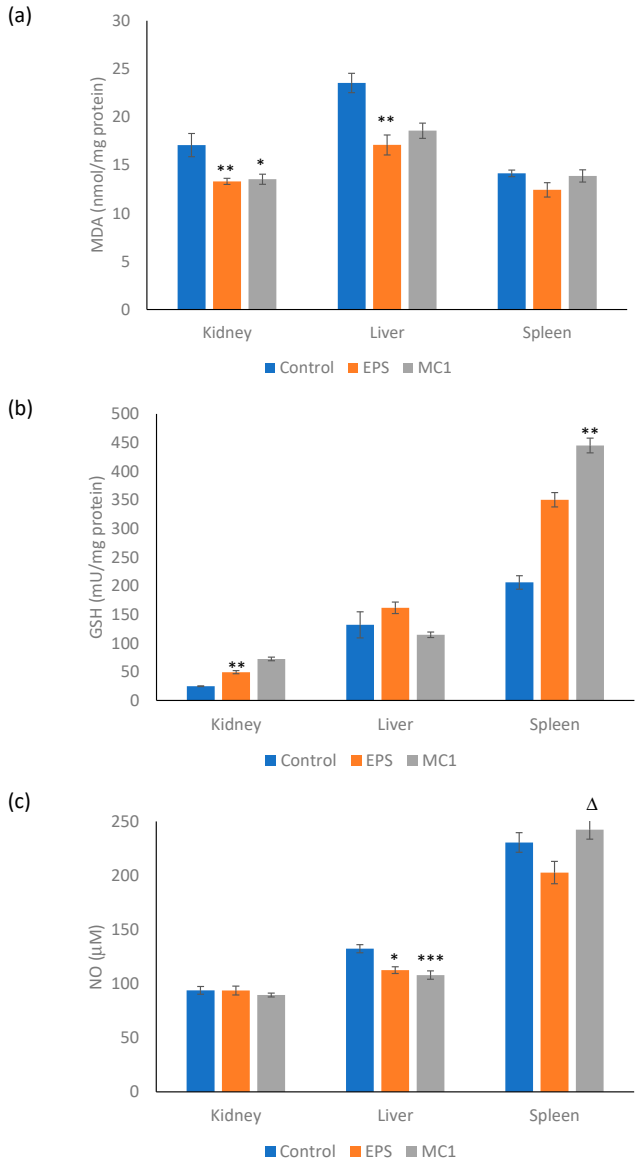

Figure 3. Oxidative stress markers in kidney, liver and spleen tissues homogenates of C57BL/6 mice treated with probiotic strain *L. fermentum* MC1 and its EPSs. Oxidative-reduction changes were determined by measuring the levels of (**a**) MDA, (**b**) GSH and (**c**) NO. Mice (N = 6, ♀) were treated *ig* with 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL or with its EPSs at a concentration of 1 mg/mL for 5 consecutive days and sacrificed on the 15th day of the experiment. Results are shown as Mean ± SE. * Statistically significantly different compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). $^{\Delta}$ Statistically significantly different compared to EPS ($^{\Delta}$ $p < 0.05$). Abbreviations: *ig*, intragastric; Control, healthy mice; EPS, EPS-treated mice; MC1, *Lactobacillus fermentum* MC1-treated mice; MDA, malondialdehyde; GSH, total glutathione; NO, nitric oxide; SE, standard error.

3. Discussion

Probiotics are currently recognized as an important category of functional foods with substantial health benefits. The functional food market, particularly products containing probiotic bacteria which account for approximately 60% of all functional foods, continues to expand steadily. Probiotics are defined as beneficial microorganisms that confer health benefits to the host by improving the balance and function of the intestinal microbiota. They contribute to gastrointestinal health, immune modulation, mental and psychiatric well-being, cardiovascular and hepatic function, and body weight regulation. Proposed mechanisms underlying their beneficial effects involve multiple mechanisms. They include modulation of intestinal microbiota composition and metabolic activity, inactivation or binding of carcinogenic compounds, and alteration of physicochemical conditions in the colon. Additionally, probiotics can degrade potential carcinogens and induce quantitative and qualitative changes in microbial populations associated with carcinogenesis (e.g., bile acid–metabolizing bacteria). They also promote the production of antitumorigenic and antimutagenic metabolites, competitively exclude pathogenic and putrefactive microorganisms, and enhance host immune responses. Other mechanisms include regulation of host physiological processes, antiproliferative effects mediated via apoptosis and cell differentiation pathways, fermentation of undigested dietary components, and inhibition of tyrosine kinase signaling pathways [11,19–22]. The gastrointestinal microbiota constitutes a complex ecosystem essential for maintaining enterocyte integrity, facilitating enzymatic digestion and metabolism of dietary substrates, modulating host metabolic processes (including steroid transformation and conversion of unconjugated bilirubin into more water-soluble urobilinogen), and protecting the intestinal epithelium from colonization by toxin-producing or invasive pathogens and foodborne contaminants such as mycotoxins [22].

Given their important regulatory role in health, this study evaluates the preventive effects of *Lactobacillus fermentum* MC1 and its exopolysaccharides (EPS) intake on body weight control and organ indices as well as on biochemical, hematological, antilipidemic, and antioxidant parameters. Furthermore, the study investigates their impact on atherogenic markers and the cardiovascular system.

3.1. Lactobacillus fermentum and Its Exopolysaccharides (EPS) Exert Beneficial Effects on Murine Health

Body weight and relative organ indices in mice may serve as relevant translational indicators of the biological and physiological effects of *L. fermentum* MC1 and its EPS in humans, given the recognized physiological similarities between the two species [3–5,22]. The findings demonstrate that administration of *L. fermentum* MC1 and its EPS is associated with increased colon length and elevated relative weights of the colon and spleen compared with the control group, suggesting potential modulatory effects on gastrointestinal morphology and immune-related organs (Figure 1, Table 1).

Furthermore, organ-associated changes are assessed through biochemical analysis of serum, whereas hematological parameters may serve as indicators for the evaluation of inflammation, infection, or alterations in hematopoietic regulation [3–5]. The analysis of blood biochemical parameters indicates that the probiotic strain MC1 and its EPSs significantly affect the metabolism of animals. Serum biochemical analysis showed statistically significant differences in enzymes and proteins, as well as metabolites and substrates. The administration of EPSs (Table 3) resulted in a significant decrease in albumin, alkaline phosphatase, total protein and amylase compared to the control group. It seems that EPSs inhibit the synthesis of key enzymes and proteins or leads to their accelerated degradation. The probiotic strain MC1 also showed a significant decrease in the levels of the enzymes

alkaline phosphatase and amylase compared to the control group, which indicates possible changes in the absorption and metabolism of nutrients such as carbohydrates and phosphates. Such changes in metabolism are probably associated with the results of body weight loss (Figure 1). In contrast, animals treated with MC1 exhibited significantly higher ALT and globulin levels compared with the EPS group (Table 3), suggesting an adaptive immune response to the probiotic. This was accompanied by increased splenic activity, indicative of B-cell proliferation, differentiation, and activation of antibody-mediated defense mechanisms [6]. Metabolite analysis showed a significant increase in bilirubin in healthy animals treated with MC1 probiotic strain (Table 3) compared to the control group, which indicates changes in liver function and metabolism with increased breakdown of erythrocytes and hemoglobin. A significant increase in potassium was also observed in animals treated with MC1 compared to the EPS group, which may indicate changes in the intestinal microbiota that enhance potassium reabsorption from the intestinal lumen. Furthermore, the increase in bilirubin in the MC1 group may be associated with a mild antioxidant and anti-inflammatory effect, since bilirubin is a known non-enzymatic antioxidant [23]. Moderate levels of bilirubin have been associated with a reduced risk of conditions linked to oxidative stress and inflammation, including CVD. Treatment with MC1 positively influenced all hematological parameters, suggesting that MC1 promotes hematopoiesis and erythropoiesis (Table 2). These findings are consistent with those of Lee et al. [24], who reported that feeding lactic acid bacteria (LAB) accelerated the self-renewal of hematopoietic stem cells (HSCs). According to Lee et al. [24], microbiota-derived lactate stimulates the secretion of stem cell factor (SCF), thereby activating hematopoiesis and erythropoiesis.

3.2. Anti-Atherosclerotic and Cardioprotective Effects of Lactobacillus fermentum MC1 and Its Exopolysaccharides

L. fermentum is a well-known probiotic that protects the intestinal mucosa, prevents dysbiosis and epithelial dysfunction. It competes with pathogenic microorganisms for nutrients and habitat, synthesizes antimicrobial components (bacteriocins) that inhibit the growth and reproduction of pathogens, and modulates the immune response through the regulation of the transcription of various pro-inflammatory and anti-inflammatory cytokines [20]. Also, its EPSs show potential in protecting cells from oxidative stress, immunoregulation, cholesterol reduction, and regulation of the intestinal microbiota, which could play an important role in the treatment of different diseases, including cardiovascular, metabolic, neurodegenerative, autoimmune and gastrointestinal diseases such as ulcerative colitis [20].

Lipid profile assessment is a standard test for determining the risk of CVD. However, the presence of a normal lipid profile cannot exclude CVD [25]. As an alternative in assessing CVD risk, the following test can be used: atherogenic index of plasma (AIP) = log [TG/HDL], Castelli Risk Index I (CRI-I) = TC/HDL, Castelli Risk Index II (CRI-II) = LDL/HDL, and triglyceride index (TG-index) = TG/HDL. The atherogenic index of plasma (AIP) and the LDL/HDL ratio are considered potential indicators of ischemic heart disease risk [26–28]. In contrast, the ratios of TC/HDL and TG/HDL, as well as low LDL levels, are associated with metabolic disorders and insulin resistance [5,29], and may serve as markers for CVD. Oxidized LDL particles are recognized by scavenger receptors, leading to foam cell formation, a key feature of atherosclerosis.

Both EPS and MC1 reduced lipid parameters compared with the control group, with total cholesterol (TC) levels decreasing by 39.71% and 33.09%, respectively. MC1 also had a pronounced effect on LDL-C, reducing it by 47.37% (Figure 2). Interestingly, HDL-C levels were slightly reduced, potentially as a consequence of weight loss (Figure 1). This observation aligns with Dong et al. [29], who reported that low-carbohydrate diets can

transiently decrease HDL-C in some individuals, especially in the short term. *L. fermentum* MC1 appears to exert cardioprotective effects, increasing the CPI by 42%, reducing the atherogenic coefficient by 22.12%, Castelli Risk Index I (CRR) by 10.23%, and Castelli Risk Index II by 29%. The TG-index remained unchanged compared with the control and EPS groups. Reduced platelet counts further contribute to the anti-atherogenic effects of MC1 (Table 2). EPS treatment showed a positive effect on the AC and Castelli Risk Index I, decreasing them by 17.64% and 8.2%, respectively, compared with the control, while other atherogenic parameters were not significantly altered. Overall, the estimated percentage of atherogenic protection was higher for MC1 than EPS (22.40% vs. 17.92%) (Table 4).

3.3. Regulation of Oxidative Stress and Inflammation by Lactobacillus fermentum and Its Exopolysaccharides Contributes to Cardioprotective Effects

Oxidative stress (OS) is a known factor in the development and progression of numerous age-related diseases, mainly those affecting the cardiovascular and nervous systems. OS is crucial in the development of diabetes, cancer, the aging process, autoimmune diseases, and CVDs, where atherosclerosis is a primary underlying cause [1–5,30]. Atherosclerosis is disorder characterized by the accumulation of lipids within blood vessel walls and an aberrant immune response, including the buildup of cholesterol-rich macrophages in the arterial intima. Therefore, the reduction in oxidative stress is crucial for understanding the therapeutic potential of the probiotic strain MC1 and its EPSs in CVD. Oxidative stress disrupts the balance between oxidants and antioxidants, leading to damage of proteins, lipids, and DNA. It also causes endothelial cell degeneration and necrosis, which promotes the recruitment and activation of immune cells, particularly phagocytes, thereby increasing the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [12].

Our results demonstrate that EPSs significantly reduced MDA levels in both the kidney and liver, whereas MC1 not only decreased MDA but also increased glutathione (GSH) levels, the key intracellular antioxidant in the kidney and spleen (Figure 3a,b). Both EPSs and MC1 treatments led to a significant reduction in NO levels in the liver compared with the control group. In contrast, MC1 treatment increased NO levels in the spleen relative to EPS treatment (Figure 3c). These differences in redox status likely reflect the distinct metabolic activities of the organs. The liver and kidneys exhibit high metabolic activity due to their roles in detoxification and elimination of metabolic products, which involves active redox processes. By comparison, the spleen primarily functions in immunity, where activated immune cells generate reactive radicals (oxidative bursts) to combat infections. Under normal conditions, NO functions as a vasodilator and protects the endothelium. However, in the presence of elevated superoxide levels, NO can react to form peroxynitrite ($ONOO^-$), a powerful oxidant that exacerbates cellular damage. Literature data indicate that *L. fermentum* possesses a complete glutathione system, encompassing glutathione synthesis, transfer, and utilization, which enables the reduction of superoxide into harmless molecules [31,32]. Additionally, *L. fermentum* can activate the Nrf2-ARE signaling pathway, promoting the synthesis of antioxidant enzymes such as GSH and SOD, consistent with the observed high concentrations of GSH in target organs [33]. According to Guo et al. [34] and Pan et al. [35], *L. fermentum* can scavenge and neutralize ROS/RNS and inhibit the Fenton reaction, which can otherwise amplify ROS levels up to 1000-fold. The antioxidant activity of the probiotic strain MC1 likely contributes to the observed reductions in atherogenic indices and enhanced vascular protection (Figure 2, Table 4). It is known that cell damage triggers the release of pro-inflammatory mediators such as cytokines, chemokines and enzymes, which activate the immune system. Activated immune cells stimulate the production of reactive oxygen species (ROS), contributing to the development of oxidative stress (OS). Cells exposed to injury, particularly neutrophils and endothelial cells, secrete large amounts of enzymes, among which NADPH oxidase and

myeloperoxidase play an important role in OS development. NADPH oxidase catalyzes the conversion of molecular oxygen to O_2^-, which can subsequently be transformed into other reactive species, such as H_2O_2 or •OH. Neutrophils migrating to the site of injury further enhance OS by releasing myeloperoxidase from azurophilic granules. In the presence of H_2O_2, myeloperoxidase generates hypochlorous acid (HOCl), a very strong oxidant that can damage DNA, proteins, and lipids.

Our results show that MC1 reduces the level of NLR while increasing LMR (Table 5). A reduced NLR is indicative of an anti-inflammatory effect, whereas an increased LMR reflects immunostimulatory and regulatory activity. This is further supported by decreased expression of CD68, a protein primarily found on the surface of macrophages, a reduction in the immunosuppressive cytokine TGFβ, and an increase in IL-6 levels (Table 6). IL-6 plays a key role in the activation, proliferation and survival of lymphocytes during active immune responses. An increase in IL-6, together with a decrease in IGF-1R and CHOP protein, is apparently crucial for reducing aging-induced diseases and promoting survival (Table 7). Reduced signaling through the insulin-like growth factor IGF type 1 (IGF-1) receptor has been shown to increase lifespan in multiple invertebrate organisms [36]. In addition, some studies using mouse models with reduced neuroendocrine signaling have generally supported the concept that the IGF type 1 (IGF-1) pathway is a primary modulator of longevity. IGF-1 also exerts protective effects on the brain and cardiovascular system. It attenuates inflammatory processes associated with atherosclerosis, and cardiac-specific expression of IGF-1 protects against age-related changes in cardiac function, thereby contributing to increased lifespan [37–40]. Thus, IGF can affect age-related changes in specific tissues by maintaining their functional integrity. Our data (Tables 5 and 6) are consistent with the findings of Rodríguez-Sojo et al. [41], who demonstrated that *L. fermentum* promotes the activation of NK and T cells, modulates Treg cell activity, and enhances immune responses without causing excessive inflammation. In contrast, EPSs led to increased expression of inflammatory parameters, including IL1β, MCP1, IL1α, and TLR4 (Table 6). Application of EPSs increases the number of monocytes/macrophages, neutrophils and lymphocytes, which, through interaction with TLRs, leads to increased production of inflammatory cytokines and modulation of the immune response. As metabolic products, EPSs exert immunomodulatory effects without triggering excessive immune activation, since, unlike *L. fermentum*, they do not directly interact with immune cells, thereby avoiding potential adverse effects. However, some studies show inhibitory effects of EPSs through indirect interaction with TLR receptors and reduction of pro-inflammatory cytokines, while other studies show that other types of EPSs contain structural features that, by recognizing TLRs, can directly stimulate the immune response as shown by our results (Tables 5 and 6). TLRs are primarily found on immune cells (macrophages, dendritic cells, B cells, and certain T cells), epithelial cells, and stromal cells. They recognize a wide variety of ligands, including nucleic acids, polysaccharides, proteins, and lipids, and induce diverse biological responses [42]. EPSs present on the bacterial cell wall may have beneficial effects and may protect "good" bacteria from immune response, opsonization, and phagocytosis [43]. Thus, during inflammatory processes, EPSs can promote changes in the intestinal microbiota, increasing the abundance of short-chain fatty acid-producing bacteria, such as *Veillonellaceae*, *Lactobacillaceae*, *Lachnospiraceae* and *Rhodospirillales*. Interactions between LABs and TLRs confer health-promoting effects, including the enhancement of host defenses against pathogenic infections, reinforcement of gut barrier functions, and modulation of immune responses [44]. These interactions are crucial for intestinal mucosa repair and the maintenance of epithelial barrier integrity, and they also contribute to the regulation of energy balance, improving nutrient utilization. SCFAs, such as acetate, butyrate and propionate, produced by intestinal bacteria during fiber fermentation, have

been shown to exert anti-atherogenic effects. However, it has been confirmed that SCFAs display both pro-inflammatory and anti-inflammatory effects on vascular health. Probiotic bacterial strains that produce high amounts of EPSs have significant antioxidant activity (Figure 3). Isolated EPS polymers also demonstrate strong free radical scavenging, metal-chelating, and antioxidant activities in vitro [21]. In addition to their antioxidant effects, EPSs contribute to reductions in reactive radicals, total cholesterol (39.71%), triglycerides (33.59%), LDL-C (27.38%), and VLDL-C (25.44%) (Figure 2). These anti-atherogenic effects are somewhat less pronounced than those observed with MC1 treatment. Furthermore, alterations in the intestinal microbiota can influence lipid metabolism by regulating the absorption and processing of dietary fats and cholesterol.

A similar effect has been reported previously, where EPSs, in combination with the probiotic strain *L. fermentum* Lf2, reduced the levels of pro-inflammatory cytokines, including IFNγ, IL12, and IL2, in the colon, while increasing IgA production in the small intestine [45,46]. Additionally, they enhanced the activity of antioxidant enzymes such as CAT and SOD, and influenced the composition of the gut microbiota by promoting the growth of SCFA-producing bacteria, including *Veillonellaceae*, *Lactobacillaceae*, *Lachnospiraceae* and *Rhodospirillales*. Our data, together with findings from other studies [45,46], confirm that *L. fermentum* and its EPS can reduce oxidative stress, lower cholesterol levels, and regulate anti-inflammatory cytokines, which may be important for maintaining cardiovascular health and preventing the development of CVDs.

4. Materials and Methods

4.1. Probiotic Suspensions of L. fermentum MC1 and Its EPSs

Probiotic suspensions of *L. fermentum* MC1 and its EPSs were prepared at the Laboratory for Antibiotic, Enzyme, Probiotic and Starter Cultures Technology, Department of Biochemical Engineering, University of Zagreb, Faculty of Food and Biotechnology. The *L. fermentum* MC1, an EPS producer isolated from breast milk, is part of the Microorganism Collection of the aforementioned laboratory, which is stored at −80 °C in MRS broth (Biolife Italiana srl, Monza, Italy) with 15% (*v*/*v*) glycerol (Sigma-Aldrich, St. Louis, MO, USA). A suspension of *L. fermentum* MC1 cells (50 mL) at a concentration of 10^9 CFU/mL was prepared by propagating the overnight culture of the aforementioned strain to 300 mL and centrifuging for 10 min at 4200 rpm, and the resulting cell pellet was washed twice with saline. EPS suspension at a concentration of 1 mg/mL was prepared by resuspending 50 mg of lyophilized EPSs in 50 mL of sterile distilled water.

4.2. Experimental Animals and Ethics Statement

Highly inbred C57BL/6J mice from the Department of Animal Physiology, Faculty of Science, University of Zagreb, were used as experimental animals. During the study, mice, aged 2–3 months, weighing 20–30 g, were kept in cages under standard conditions, including a daily rhythm of 12 h of light and 12 h of darkness, a temperature of 25 °C and controlled air humidity. They were fed standard food for laboratory animals (Standard Diet GLP, 4RF21, Mucedola, Settimo Milanese, MI, Italy) with food and water ad libitum. The study was conducted on a total of 18 female mice that were divided into 3 groups according to the treatment method. The research was approved by the Committee for Bioethics and Animal Welfare of the Faculty of Science, University of Zagreb (approval number: 251-58-10617-19-285, approval date: 12 April 2019) and was conducted in accordance with the ethical directives valid in the Republic of Croatia (Animal Protection Act, NN 102/17, 32/19; Ordinance on the Protection of Animals Used for Scientific Purposes, NN 55/13, 37/17, 116/19) as well as in accordance with the Guide for the Care and Use of Laboratory Animals, DHHS (NIH) Publ. # 86–23, National Research Council, 2011.

4.3. Experimental Design and Animal Treatment

The animals were randomly assigned to three groups based on the treatment received:

1. Control group: mice received 0.5 mL sterile distilled water intragastrically.
2. EPS group: mice received 0.5 mL of an aqueous suspension of EPSs at a concentration of 1 mg/mL, administered intragastrically.
3. MC1 group: mice received 0.5 mL of an aqueous suspension of the probiotic strain *L. fermentum* MC1 at a concentration of 10^9 cells/mL, administered intragastrically.

The animals received intragastric administrations of *L. fermentum* MC1 and its EPSs for five consecutive days and were sacrificed on day 15 of the experiment for collection of blood and target organs. The animals were adequately anesthetized and analgesized by intraperitoneal administration of a combination of ketamine (Narketana®, Bioveta, Inc., Ivanovice na Hané, Czech Republic) at a dose of 75 mg/kg with xylazine (Xylapana®, Bioveta, Inc., Ivanovice na Hané, Czech Republic) at a dose of 10 mg/kg. Blood samples were obtained by cardiac puncture for biochemical and hematological analyses. The collected organs (spleen, liver, kidney) were stored at −80 °C until further analysis.

4.4. Body and Organ Weight Change in Experimental Animals

Animals were marked and weighed before the start of the experiment, during treatment with *L. fermentum* MC1 or EPSs, and on the day of sacrifice (on days 0, 5, 10, and 15). A digital scale (Analytical balance ABS 220–4, Kern & Sohn GmbH, Balingen, Germany) was used to measure body weight. The body weight change for each individual mouse was calculated according to the following formula:

$$\% \text{ change in weight} = [(\text{weight on the first day} - \text{weight during the experiment})/(\text{weight on the first day})] \times 100$$

Kidneys, liver, and spleen were collected and individually weighed to assess the possible toxic, inflammatory/anti-inflammatory effect of *L. fermentum* MC1 or EPSs. Relative organ weight was calculated according to the following formula:

$$\text{Relative organ weight (g/100 g)} = (\text{total organ weight/final body weight}) \times 100$$

4.5. Blood Sampling

After anesthetizing the animals, blood was obtained by cardiac puncture for biochemical and hematological analyses, and the animals were then sacrificed by cervical dislocation. Blood was collected in accordance with the guidelines of the Clinical Laboratory Standards Institute [47] and the World Health Organization [48]. Depending on the needs of the individual analysis, blood was collected in vacutainers without coagulant and with EDTA. Serum was separated by centrifugation at 3000× *g* for 10 min at 4 °C and stored in 500 µL aliquots at −80 °C until further use.

4.5.1. Analysis of Biochemical Parameters in Blood

Blood samples were collected in vacuum tubes without anticoagulant for biochemical analysis. For the analysis of biochemical parameters, the VetScan® kit (Comprehensive Diagnostic Profile reagent rotor) was used on the VetScan® VS2 device (Abaxis, Union City, CA, USA). A 100 µL aliquot of serum was taken from each blood sample for the determination of the following biochemical parameters: albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total protein (TP), globulin (GLOB), total bilirubin (TBIL), blood urea nitrogen (BUN), phosphates (PHOS), creatinine (CRE), blood glucose, sodium and potassium ions.

4.5.2. Analysis of Hematological Blood Parameters

Blood samples, previously collected in vacutainers with EDTA, were stored at 4 °C for 2 h until the determination of hematological parameters. The following indicators were determined on the Horiba ABX169 electronic counter (ABX Micros ESV 60, Montpellier, France): total number of erythrocytes, leukocytes and platelets, hematocrit, hemoglobin, erythrocyte volume (MCV), average hemoglobin concentration in erythrocytes (MCHC), average hemoglobin content in erythrocytes (MCH), erythrocyte size distribution (RDW) and differential blood count (DBC).

4.5.3. Lipid Parameters and Atherogenic Risk Predictor Indices (ARPI) Calculation

The unhemolyzed serum were used for the analysis of total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentration. All lipid parameters were analyzed using enzymatic methods as described previously [3–5,30]. Briefly, triglycerides were measured photometrically following hydrolysis by lipoprotein lipase, which rapidly and completely converts triglycerides into glycerol. Glycerol is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminophenone and 4-chlorophenol in the presence of peroxidase to form a red-colored dye. Cholesterol concentration was measured at 512 nm following the formation of a quinone-imine red dye. Briefly, cholesterol esters were hydrolyzed by cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzed the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide subsequently participates in the oxidative coupling of phenol and 4-aminoantipyrine, producing a characteristic red dye. The analysis of HDL-C involved a two-step procedure. First, in the presence of magnesium ions, dextran sulfate selectively forms water-soluble complexes with LDL-C, very-low-density lipoprotein cholesterol (VLDL-C), and chylomicrons, rendering them resistant to polyethylene glycol–modified enzymes. In the second step, cholesterol esters are quantitatively hydrolyzed into free cholesterol and fatty acids by cholesterol esterase. In the presence of peroxidase, the generated hydrogen peroxide reacts with 4-aminoantipyrine to form a purple–bluish dye, the intensity of which is directly proportional to the cholesterol concentration. Direct LDL-C and VLDL-C levels were measured using a Hitachi 717 automatic analyzer (Hitachi, Ltd. Tokyo, Japan). After determining the concentrations of TC, TG, HDL-C, and LDL-C in mmol/L, the atherogenic risk index (ARI), percentage protection, atherogenic risk predictor indices (ARPI), cardioprotective index (CPI), and the indicator of insulin resistance (TG/HDL-C ratio) were calculated using the formulas presented in Table 8. A detailed description of these methods is provided in previous studies [3,5,30].

Table 8. Atherogenic parameters.

Abbreviations	Full Name	Formula	Note/Synonyms
AC	Atherogenic coefficient	[(TC − HDL-C)/HDL-C]	Atherogenic risk index (ARI)
AIP	Atherogenic index of plasma	TG/HDL-C ratio	ARPI-1
CRI-II	Castelli Risk Index II	LDL-C/HDL-C	ARPI-2
CRI-I	Castelli Risk Index I	TC/HDL-C	ARP3 or Cardiac Risk Ratio (CRR)
CPI	Cardioprotective Index	HDL-C/LDL-C	-
TG-index	TG/HDL-C ratio	TG/HDL-C	Indicator of insulin resistance (IR)

The percentage of protection was calculated using the following formula:

$$\% \text{ protection} = [(ARI_{\text{Negative control group}} - ARI_{\text{Treated group}})/ARI_{\text{Negative control group}}] \times 100$$

where

Negative control group = untreated animals.
Treated group = animals treated with *L. fermentum* or its EPSs.

4.6. Tissue Preparations for Quantitative Analysis of Oxidative Stress Biomarkers

Liver, kidney, and spleen samples were prepared as described in paper [5]. Homogenates were centrifuged by Micro 200R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) for 15 min at a speed of 10,000 rpm at +4 °C. The supernatant was used for the measurements of lipid peroxidation (MDA) and glutathione (GSH) levels.

4.6.1. Malondialdehyde (MDA) Levels

Malondialdehyde (MDA), a key biomarker of oxidative stress, is one of the main products of lipid peroxidation. Reactive products in the body are formed due to the degradation of polyunsaturated fatty acids by reactive oxygen radicals. The level of MDA was determined in tissue homogenates (liver, kidney and spleen) using the method described in previous studies [4,5], which is based on the derivatization of MDA with 2-thiobarbituric acid (TBA). The reaction takes place under acidic conditions at high temperature (95 °C), where MDA reacts with two equivalents of TBA, forming a red fluorescent complex MDA-(TBA)2 whose concentration was measured spectrophotometrically (Libra S22, Biochrom, Cambridge, UK) at a wavelength of 532 nm. The MDA levels were determined using the molar absorption coefficient for the malondialdehyde–thiobarbiturate (MDA–thiobarbituric acid, TBA) complex, which is 1.56×10^5 M^{-1} cm^{-1}. The results of the measurements were expressed in nmol/mg of protein.

4.6.2. The Total Glutathione (GSH) Levels

Total glutathione (tGSH) levels (μmol/mg proteins) were measured spectrophotometrically at 440 nm by mixing 40 μL of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) with 20 μL of tissue supernatant pretreated with 40 μL of 0.035 M HCl and incubated for 10 min. DTNB reacts with GSH to form the chromophore 2-nitro-5-thiobenzoic acid (NTB) and a small amount of glutathione disulfide (GSSG). Absorbance was measured at 440 nm using a Tecan Infinite 200 microplate reader (Tecan Austria GmbH, Grödig, Austria), and tGSH concentration was calculated using the molar absorption coefficient ε(DTNB) = 8.22 M^{-1} cm^{-1}. The concentration of tGSH was expressed as mU/mg of protein.

4.6.3. Detection of Nitric Oxide (NO) via the Griess Reaction

Nitric (II) oxide concentrations were measured in homogenized liver, kidney and spleen samples [49]. NO levels were determined using the Griess reaction, which quantifies nitrate and nitrite concentrations, employing a commercial nitrate/nitrite assay kit (Griess Reagent System, Promega, Madison, WI, USA). The Griess reaction is based on the chemical reaction between sulfanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions, resulting in the formation of azo-colored compounds. Absorbance was measured at 540 nm using a microplate spectrophotometer (Ao Absorbance Microplate Reader, Azure Biosystems, Dublin, CA, USA). A sodium nitrite (0.1 M) standard curve was prepared using concentrations ranging from 0 to 100 μM. Sample absorbance values were averaged and converted to nitrite concentrations (μM) based on the standard curve. The assay detection limit was 2.5 μM.

4.7. Quantitative Polymerase Chain Reaction (qPCR) Method

4.7.1. Tissue Homogenization and RNA Isolation

Homogenization and isolation of RNA from the colon tissue were performed according to the manufacturer's instructions using the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany). RNA purity and concentration were assessed using a spectrophotometer (NanoPhotometer® N60, Implen GmbH, München, Germany), and samples were diluted to a final RNA concentration of 1 µg per reaction.

4.7.2. Synthesis of cDNA by Reverse Transcription

Reverse transcription of RNA into cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions.

4.7.3. Amplification of cDNA Using the qPCR Method

The synthesized cDNA was used for quantitative polymerase chain reaction (qPCR) analysis using a SYBR Green–based method (Thermo Fisher Scientific, Waltham, MA, USA), as described by Šešelja et al. [50]. For normalization, two reference genes, β-actin (ACTβ) and β2-microglobulin (B2M), were used. The expression of the following genes were analyzed: GRP94—glucose-regulated protein 94, IGF-1R—insulin-like growth factor 1 receptor, IL1α—interleukin 1α, IL1β—interleukin 1β, IL6—interleukin 6, MCP1—monocyte chemoattractant protein 1, NOX2—NADPH oxidase 2, TLR4—Toll-like receptor 4 receptor, TNFα—tumor necrosis factor α, TGFβ—transforming growth factor β, CD68 antigen, CHOP—protein homologous to CCAAT-enhancer-binding protein, BCL2—B-cell lymphoma protein 2, and AIFM1—apoptosis-inducing factor mitochondria associated 1. Specific primer sequences and qPCR conditions are presented in Table 9.

Table 9. Primers used for qPCR analysis with optimized conditions.

Target Gene	Primer Sequence (5′-3′)	qPCR Conditions *
GRP94	AAGAATGAAGGAAAAACAGGACA AAACAAATGGAGAAGATTCCGCC	58 °C, 3 mM
IGF-1R	CCAAGTTACCAGAAGGGCTAAT GGTAGGGACAATACAGGCTAAAT	59 °C, 3 mM
IL1α	CCTTACACCTACCAGAGTGA AACCAAGTGGTGCTGAGATA	65 °C, 3 mM
IL1β	ATGGGCAACCACTTACCTATTT GTTCTAGAGAGTGCTGCCTAATG	64 °C, 3 mM
IL6	GATAAGCTGGAGTCACAGAAGG TTGCCGAGTAGATCTCAAAGTG	59 °C, 3.5 mM
MCP1	CCTGGATCGGAACCAAATGA CGGGTCAACTTCACATTCAAAG	62 °C, 3 mM
NOX2	ACTCCTTGGGTCAGCACTGG GTTCCTGTCCAGTTGTCTTCG	62 °C, 3 mM
TLR4	TGGTCAGTGTGATTGTGGTATC GCTTTCTCCTCTGCTGTACTT	58 °C, 3 mM
TNFα	GTCTCAGAATGAGGCTGGATAAG CATTGCACCTCAGGGAAGAA	63 °C, 2.5 mM
TGFβ	CTTTAGGAAGGACCTGGGTTG GTGTGTCCAGGCTCCAAATA	66 °C, 3 mM
CD68	CTCTTGCTGCCTCTCATCATT CTGGTAGGTTGATTGTCGTCTG	58 °C, 2.5 mM
CHOP	TTGAGCCTAACACGTCGATTAT CACTTCCTTCTGGAACACTCTC	58 °C, 3 mM
BCL2	TCCAGCCACCTTCTTTCTATG GTTTCACCATGTTGCCTCTTAC	61 °C, 3 mM
AIFM1	CGGGAGGTCAAGTCAATTACAG GGAACAGCTGGATCACTTCTATG	61 °C, 3.5 mM

* Primer annealing temperature and MgCl2 concentration.

The amplification results of target genes obtained by qPCR were analyzed using the REST© (Relative Expression Software Tool, version 2.0.13) program and the ΔΔCt method. Values were normalized to the reference genes β-actin (Actβ) and β2-microglobulin (β2m). Statistical significance was determined at the level of $p < 0.05$. Relative gene expression (R) was calculated using the following equation:

$$R = \frac{E\ \text{target gene}^{\Delta Ct\ \text{target gene}\ (A\ \text{control} - A\ \text{sample})}}{E\ \text{ref.gene}^{\Delta Ct\ \text{ref.gene}\ (A\ \text{control} - A\ \text{sample})}}$$

4.8. Statistical Analysis

Statistical analyses were performed using the STATISTICA 14 program (StatSoft, Tulsa, OK, USA). Results are presented as mean ± standard error of the mean (Mean ± SE). Data were analyzed using the Kruskal–Wallis ANOVA test, followed by multiple comparisons of mean values across all groups. For qPCR data, statistical significance was assessed using Student's *t*-test.

5. Conclusions

These results suggest that dietary intake of *L. fermentum* and its EPSs may help prevent the onset and progression of atherosclerosis and CVD through their antioxidant, anti-inflammatory, immunomodulatory activities, as well as through regulation of the gut microbiome and lipid metabolism. Furthermore, the observed reduction in lipid and atherogenic determinants indicates that *L. fermentum* and its EPSs may contribute to atheroprotection and provide multiple health benefits.

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Abbreviations

The following abbreviations are used in this manuscript:

AC	Atherogenic Coefficient
AIFM1	Apoptosis-Inducing Factor Mitochondria Associated 1
AIP	Atherogenic Index Of Plasma
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AMY	Amylase
BCL2	B-cell Lymphoma Protein 2
BUN	Blood Urea Nitrogen
CD68	Cluster of differentiation 68
CHOP	C/EBP Homologous Protein
CPI	Cardioprotective Index
CRI-I	Castelli Risk Index II
CRI-II	Castelli Risk Index I
CRE	Creatinine
CVD	Cardiovascular Diseases
DAI	Disease Activity Index
DSS	Dextran Sulfate Sodium
EPS	Exopolysaccharide
GLOB	Globulin
GLU	Blood Glucose
GRP94	Glucose-regulated Protein 94
GSH	Total Glutathione
Hct	Hct-Hematocrit
HDL-C	HDL-C–High-density Lipoprotein Cholesterol
HGB	Hgb-Hemoglobin
Ig	Intragastric
IGF-1R	Insulin-like Growth Factor 1 Receptor
IL1α	Interleukin 1α
IL1β	Interleukin 1 Beta
IL6	Interleukin 6
IR	Insulin Resistance
K^+	Potassium ions
LAB	Lactic Acid Bacteria
LDL-C	Low-density Lipoprotein Cholesterol
LMR	Lymphocyte-to-monocyte Ratio
MCH	Hemoglobin Content In Erythrocytes
MCHI	Histological colitis index
MCHC	Hemoglobin Concentration In Erythrocytes
MCP1	Monocyte Chemoattractant Protein-1
MCV	Mean Corpuscular Volume
MDA	Malondialdehyde
MPV	Average Platelet Volume Distribution
Na^+	Sodium ions
NLR	Neutrophil-to-lymphocyte Ratio
NO	Nitric Oxide
NOX2	NADPH Oxidase 2
OS	Oxidative Stress
PHOS	Phosphorus
Plt	Platelets

qPCR	Quantitative Polymerase Chain Reaction
RDW	Erythrocyte Size Distribution
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SCFA	Short-chain Fatty Acids
SE	Standard Error
SOD	Superoxide Dismutase
TBIL	Total Bilirubin
TC	Total Cholesterol
TG	Total Triglycerides
TGFβ	Transforming Growth Factor Beta
TLR	Toll-like receptor
TLR4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor Alpha
TP	Total Protein
VLDL-C	Very-low-density Lipoprotein Cholesterol
WBC	White Blood Cell

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