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Anti-inflammatory and Anti-atherogenic Effects of *Lactobacillus plantarum* in Hypercholesterolemic Mice

Lactobacillus plantarum'un Hiperkolesterolemik Farelerde Anti-enflamatuvar ve Anti-aterojenik Etkileri

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Abstract

Introduction: Cardiovascular diseases are the most prevalent cause of human morbidity and mortality worldwide and atherosclerosis is the main underlying cause of it. Probiotics comprise live microorganisms which have been shown to have beneficial effects on the host when administered in the diet. Since probiotics are known to have immunomodulatory effects on the host immune system, these may directly (or indirectly) influence the inflammatory process by which atherosclerotic plaques grow. In this study, *Lactobacilli plantarum* is used as the probiotic of choice and its effects on T cell mediated immunity and plasma lipid profile as well as atherosclerotic plaque development are studied on an experimental animal model of the disease, the ApoE$^{-/-}$ mouse.

Material and Methods: The strains were identified by morphological, physical, enzymatic and biochemical assessment. Flow cytometry was used to study T cell subsets. IL-10 levels were determined by ELISA. The effect of *L. plantarum* on plaque grow was measured using standard histopathological techniques.

Results: The survival of *L. plantarum* along the gastrointestinal tract was confirmed after its isolation from faecal samples of treated animals. It was shown that *L. plantarum* is capable of increasing the proliferation of CD4$^+$ CD25$^+$ T cells ($p=1.4\times10^{-5}$) and the level of IL-10 ($p=0.045$) and decrease the size of atherosclerotic plaques ($p=0.019$) in the aortic sinus of the ApoE$^{-/-}$ mouse, without an improvement in cholesterol levels.

Conclusion: In conclusion, the findings of this study provide supporting data for the use of *L. plantarum* as a potential therapeutic agent against atherosclerosis.

Key words: Atherosclerosis, inflammation, *L. plantarum*, probiotic

Öz

Giriş: Kardiyovasküler hastalıklar dünya genelinde en yaygın insan morbidite ve mortalite sebebidir ve ateroskleroz bunun başlıca altta yatan nedenidir. Probiyotikler, günlük besinle alındığında konağa yararlı etkileri olduğu gösterilen canlı mikroorganizmaları içerir. Probiyotiklerin konak immün sistemi üzerinde immün düzenleyici etkileri olduğunun bilinmesi, bunların aterosklerotik plakların büyüdüğü enflamatuvar süreci doğrudan (veya dolaylı) etkileyebileceğini düşündürmektedir. Bu çalışmada, probiyotik tercihi olarak *Lactobacillus plantarum* kullanılmış, T hücre aracılı immünite ve plazma lipid profiline, ayrıca aterosklerotik plak gelişimine olan etkileri hastalığın deneysel hayvan modeli, ApoE$^{-/-}$ fare, üzerinde çalışılmıştır.

Gereç ve Yöntemler: Suşlar morfolojik, fiziksel, enzimatik ve biyokimyasal değerlendirmeler ile tanımlanmıştır. T hücre alt grupları akan hücre ölçer cihazı kullanılarak çalışılmış, IL-10 seviyeleri ELISA ile belirlenmiştir. *L. plantarum*'un plak büyümesi üzerine etkisi standart histopatolojik teknikler ile ölçülmüştür.

Bulgular: Tedavi görmüş hayvanların fekal örneklerinden izole edilmesini takiben *L. plantarum*'un sindirim kanalı boyunca hayatta kalımı doğrulanmıştır. *L. plantarum*'un CD4$^+$ CD25$^+$ T hücre proliferasyonunu ($p=1,4\times10^{-5}$) ve IL-10 seviyesini artırabildiği ($p=0,045$) ve ApoE$^{-/-}$ farelerin aortik sinüslerindeki aterosklerotik plak boyutunu kolesterol seviyelerinde bir iyileşme olmaksızın küçültebildiği ($p=0,019$) gösterilmiştir.

Sonuç: Sonuç olarak, bu çalışmanın bulguları *L. plantarum*'un ateroskleroza karşı potansiyel bir tedavi edici ajan olarak kullanımını destekler veriler sağlamıştır.

Anahtar Kelimeler: Ateroskleroz, enflamasyon, *L. plantarum*, probiyotik

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Introduction

Atherosclerosis is defined as the gradual thickening and hardening of large and medium size arteries that can eventually result in blocked arteries or a break leading to a heart attack, stroke or other dysfunction, depending on the location of the plaque.[1] It is known that as many as three quarters of all myocardial infarctions result from thrombotic blockage of the coronary artery caused by an atherosclerotic plaque rupture.[1] Several studies have shown

that inflammation is a key driving process involved in atherosclerosis.[2] Atherogenesis-associated inflammation is initiated by endothelial dysfunction which can be induced by several endogenous (heat shock protein 60, oxLDL, β2 glycoprotein-1) and/or exogenous (microbial antigens such as peptidoglycans and lipopolysaccharides) factors.[3,4] Consequently, leukocytes migrate into the inner layer of the arterial wall which is mediated by an increased expression of pro-inflammatory genes such as adhesion molecules (e.g. VCAM-1, ICAM-1), cytokines (e.g. IL-1, TNF-α) and chemokines (e.g. MCP-1).[4]

Regulatory T cells (Treg) are an important subset of T cells, which are thought to be crucial in preventing atherosclerosis, due to their anti-inflammatory nature.[5] They are identified by the high level expression of CD25, the α-subunit of IL-2 receptor. In the periphery, naive $CD4^+$ T cells (that lack CD25 expression) can be turned into $CD4^+$ $CD25^+$ Treg cells under the effect of TGF-β or IL-10 in an antigen-specific manner.[6]

Probiotics are referred to as live microorganisms, which have been shown to have beneficial effects on host organisms by promoting a favourable balance of gut flora when administered in adequate amounts in the diet.[7] They can be found as part of fermented products such as yoghurt and in diet supplementary pills. There is evidence suggesting that they may inhibit the growth of detrimental inhabitants of the gastrointestinal track by regulating the immune system against those particular microorganisms.[8,9] The potential beneficial effects attributed to probiotics are now being expanded beyond their limited use in gastrointestinal health care over the past two decades.[10] Atherosclerosis has been shown to be one of the diseases that may be attenuated by probiotic treatments.[11] Prevention of hypertension[12], modulation of immunity[13], reduction of oxidative stress[14] and improvement of serum lipid profiles[15] are four postulated applications directly or indirectly related to atherosclerosis. However, the mechanisms behind these potential benefits are still under investigation and most of these claims remain inconclusive because of the lack of sufficient *in vivo* evidence. We hypothesise that probiotics might exhibit the anti-atherogenic activity through modulation of T cell immunity, possibly by the induction of IL-10 producing anti-inflammatory Treg cell proliferation.

Certain species of bacteria and yeast have been considered as probiotics.[16] *Lactobacillus* and *Bifidobacterium* are the two groups of bacteria that come into prominence in this regard. Our choice of probiotic, *Lactobacillus plantarum,* is one of the most common microorganism used as probiotics both in the food and pharmaceutical market.[16] It is hypothesised that the regular administration of *L. plantarum* can modulate atherosclerotic plaque development in the $ApoE^{-/-}$ mouse.

Material and Methods

Animal Models

$ApoE^{-/-}$ mouse is one of the most extensively used experimental model to investigate the pathogenesis of atherosclerosis, which is unable to produce Apolipoprotein E (ApoE), a crucial protein that participates in the clearance of the excess of triglycerides from blood to the liver for processing.[17] Twelve to fourteen weeks old male $ApoE^{-/-}$, and its background strain, C57BL/6, are used in this study. Animals were housed in standard conditions: 20–22°C, 30–70% relative humidity, 12 hrs light/dark cycle and free access to food and water. Animals are divided into 4 groups of 5 animals each. Groups consisted of two untreated controls ($ApoE^{-/-}$ and C57BL/6) and two probiotic treated groups. Experiments were conducted in accordance with the local and UK Home Office animal (Scientific Procedures) Act, 1986.

Administration of *L. plantarum*

The strain was kindly provided by Prof. Roberto M. La Ragione and stored at -80°C with 30% glycerol. Before each administration, fresh inocula were prepared from the stock. *L. plantarum* is re-cultured in MRS (de Man, Rogosa and Sharpe) broth media (a selective media for Lactobacilli) and incubated 16 hrs in anaerobic conditions at 37°C, then centrifuged and re-suspended in PBS. The number of *L. plantarum* ranged from 1.1×10^9 to 4×10^9 cfu/mL in each inoculum. One hundred µL of this preparation was administered to the mice by oral gavage three times per week (Monday, Wednesday and Friday) for 16 weeks. Two control groups remained untreated.

Faeces samples were collected aseptically at 48 hr intervals during the first week of treatment, then at two weeks intervals thereafter to assess the presence of *L. plantarum*. Samples were collected after 5 hours (early sampling) and 24 hours (late sampling) following the administration of *L. plantarum*. The samples were homogenized in sterile PBS, then spreaded onto MRS agar plates. Blood samples were collected by tail-vein puncture prior to the probiotic treatment (1^{st} bleeding) and at the end of the 8^{th} week of treatment (2^{nd} bleeding). Mice were euthanized at 16^{th} week by intra-peritoneal injection with sodium pentobarbital

(100 mg/kg; VetTech, UK). Blood was collected from the abdominal aorta (3rd bleeding) and plasma was obtained by centrifugation at room temperature for 30 min at 10.000x g, and then stored at -20°C. Spleens were also collected. Animals were then perfused from the left cardiac ventricle with PBS at a constant pressure of 100 mmHg (1 mmHg=133 Pa). The hearts were dissected, then fixed in 10% (v/v) formalin in PBS for histological analyses.

Identification of strain isolates

The strains were identified according to their morphological, physical, enzymatic and biochemical properties. First of all, colonies were characterized by their appearance on the plate depending on their colour and shape. Then, gram staining was used to analyse 20 randomly selected colonies. Catalase activity of the 20 colonies was also tested by observation of air bubble formation (indicator of oxygen production) when colonies were exposed to the hydrogen peroxide. Finally, api 50 CH kit (bioMérieux, France) was used by following the manufacturer's instructions to biochemically identify the isolates. This is a standardized system based on the difference between sugar fermentation ability of different Lactobacilli strains. The isolates used for carbohydrate fermentation profiling are listed in Table 1. They are incubated in the presence of 49 different carbohydrates for 24 and 48 hours with a pH indicator to track the fermentation by colour change.

Analysis of splenic T cell subsets

Spleens were collected into RPMI 1640 (Gibco, USA) media supplemented with 5 mM EDTA, then homogenized using a 5 ml syringe plunger and a sterile petri dish. The homogenate was filtered into 50 ml falcon tubes using 40 nm cell strainers. The cell suspension was then completed to 20 ml with RPMI 1640 + 5 mM EDTA followed by a centrifugation at 400×g for 5 min at 4°C. The pellet was re-suspended in 2 mL of red blood cell lysis buffer (R&D systems, UK) and incubated for 10 min at room temperature. Erythrocyte depleted cells were then washed with 40 ml RPMI 1640 + 5 mM EDTA. About two million cells in 0.2 ml FACS buffer (BD Biosciences, USA) were incubated with 2 µl of mouse SeroBlock® (Serotec, UK) for 15 min on ice. After blocking the Fc receptors of splenocytes, fluorescently labelled rat anti-mouse monoclonal antibodies against CD3, CD4, CD8 and CD25 (all from Serotec, UK) were added to each sample tube and the corresponding control tubes. Two µl of each isotype control (all from Serotec, UK) were added to isotype control tube and all samples and controls were incubated on ice for 30 min. Then, cells were washed by centrifugation with 2 ml of FACS buffer. The cells were re-suspended in 0.3 ml of 1% (v/v) formaldehyde (Sigma, UK) in FACS buffer and kept in the dark at 4°C until flow cytometry analysis. Fifty thousand events were acquired per sample using a BD FACScan Flow Cytometer (BD Biosciences, USA).

Analysis of cytokine profile

The splenocytes ($1x10^6$ cells/mL/well) were cultured in 24-well plates in RPMI 1640 supplemented with 10% foetal calf serum, 100U/mL penicillin, 2 mM glutamine, 100 µg/mL streptomycin, 10ng/mL ionomycin calcium

Table 1. The list of isolates used for carbohydrate fermentation profiling

No	Mouse	Group	Week	Sampling	No	Mouse	Group	Week	Sampling
1	ApoE$^{-/-}$	Treatment	16	Late	16	C57BL/6	Treatment	2	Early
2	ApoE$^{-/-}$	Control	7	Late	17	C57BL/6	Treatment	16	Early
3	C57BL/6	Control	16	Early	18	ApoE$^{-/-}$	Treatment	16	Early
4	C57BL/6	Treatment	3	Late	19	C57BL/6	Treatment	16	Late
5	C57BL/6	Control	12	Late	20	ApoE$^{-/-}$	Treatment	10	Late
6	C57BL/6	Treatment	8	Late	21	C57BL/6	Control	14	Early
7	ApoE$^{-/-}$	Control	6	Late	22	ApoE$^{-/-}$	Control	14	Early
8	ApoE$^{-/-}$	Treatment	4	Early	23	ApoE$^{-/-}$	Treatment	8	Late
9	ApoE$^{-/-}$	Treatment	3	Early	24	ApoE$^{-/-}$	Treatment	5	Early
10	C57BL/6	Treatment	4	Early	25	C57BL/6	Treatment	5	Early
11	C57BL/6	Treatment	3	Early	26	C57BL/6	Treatment	6	Late
12	ApoE$^{-/-}$	Treatment	5	Early	27	C57BL/6	Control	10	Late
13	ApoE$^{-/-}$	Treatment	2	Early	28		*L. plantarum*		
14	ApoE$^{-/-}$	Treatment	2	Early	29		*L. plantarum*		
15	ApoE$^{-/-}$	Control	4	Late	30		*L. plantarum*		

salt from *Streptomyces conglobatus* (Sigma, UK) and 10ng/mL phorbol 12-myristate-13-acetate (PMA; Fluka, UK) at 37°C; 5% CO_2 for 72 hrs. Supernatants derived from the cultures and plasma samples from 3rd bleeding were used to determine the levels of IL-5, IL-10, IFN-γ and TGF-β1 using commercially available ELISA Kits, "Ready-SET-Go" (eBioscience, UK), following the manufacturer's instructions. Plasma samples were diluted 1:10 in assay diluent buffer (supplied with the kit). Supernatants were used undiluted.

Morphometric analysis of atherosclerotic plaques

The hearts were dissected, fixed in 10% (v/v) formalin in PBS, embedded in paraffin wax (Fibro-wax; BDH Ltd, UK), then the aortic sinus was sectioned into 8 μm/ sections using a Microtome 2040 Autocut (Cambridge Instrument Co., Inc., New York). 21 sequential sections were taken for morphometric analysis. They were stained with haematoxylin and eosin (H&E). The plaque lesions of 3 most representative sections were measured blinded using ImageJ software v. 1.36b (Wayne Rasband NIH, USA, freely available from http://rsb.info.nih.gov/ij/).

Determination of plasma lipid profile

The total levels of cholesterol, triacylglycerol (TAG) and high-density lipoprotein-cholesterol (HDL-C) in mouse plasma samples were determined using commercial kits from Instrumentation Laboratory UK Ltd following the instructions provided. Measurements were carried out in duplicates using an ILab-650 clinical chemistry auto analyser (Instrumentation Laboratory UK Ltd). The level of low-density lipoprotein-cholesterol (LDL-C) was calculated using the Friedewald equation.[18]

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software v. 5.04 (GraphPad Software Inc., La Jolla, CA, USA). For the *in vitro* study, each experiment was carried out at least in duplicates. Values herein are expressed as the mean ± SD (Standard Deviation). A two tailed unpaired t-test was used with Welch's correction to determine the differences between the means of the treatment groups and the control groups. To compare more than two groups of means, a one-way ANOVA was performed followed by a Bonferroni's multiple comparison test.

Results

***L. plantarum* is isolated only from the early samplings of treatment groups.** Although all isolated colonies on MRS agar plates appeared to be the same at the end of 24 hours incubation, some colonies became more distinct and attained a raised creamy shape following storage at 4°C as our probiotic strain (*L. plantarum)*, which will be referred to as creamy white colonies hereafter, while the others remained unchanged or started to disappear slowly. The creamy white colonies were found to be dominated on agar plates from early sampling treated groups, while no creamy white colony was obtained from control groups or late sampling treated groups.

All tested isolates were rod shaped and Gram positive. The absence of bubble formation was accounted for the lack of catalase activity in the isolates. These observations taken all together indicate that the isolates are *Lactobacilli* or a closely related species.

Fourteen different carbohydrate fermentation profiles were obtained from 27 isolates. Seven of them yielded exactly the same profile with *L. plantarum* at both 24 and 48 hours incubation time (Table 2).

Administration of *L. plantarum* increases the proliferation of $CD4^+CD25^+$ T cell population. The flow cytometry results showed that *L. plantarum* treatment

Table 2. The list of isolates yielding identical profile with *L. plantarum*

No	Mouse	Group	Week	Sampling	Morphology
8	ApoE-/-	Treatment	4	Early	Creamy White
9	ApoE-/-	Treatment	3	Early	Creamy White
10	C57BL/6	Treatment	4	Early	Creamy White
11	C57BL/6	Treatment	3	Early	Creamy White
12	ApoE-/-	Treatment	5	Early	Creamy White
18	ApoE-/-	Treatment	16	Early	Creamy White
24	ApoE-/-	Treatment	5	Early	Creamy White

All isolates having identical profiles with *L. plantarum* were creamy white and from the early samplings of treatment groups.

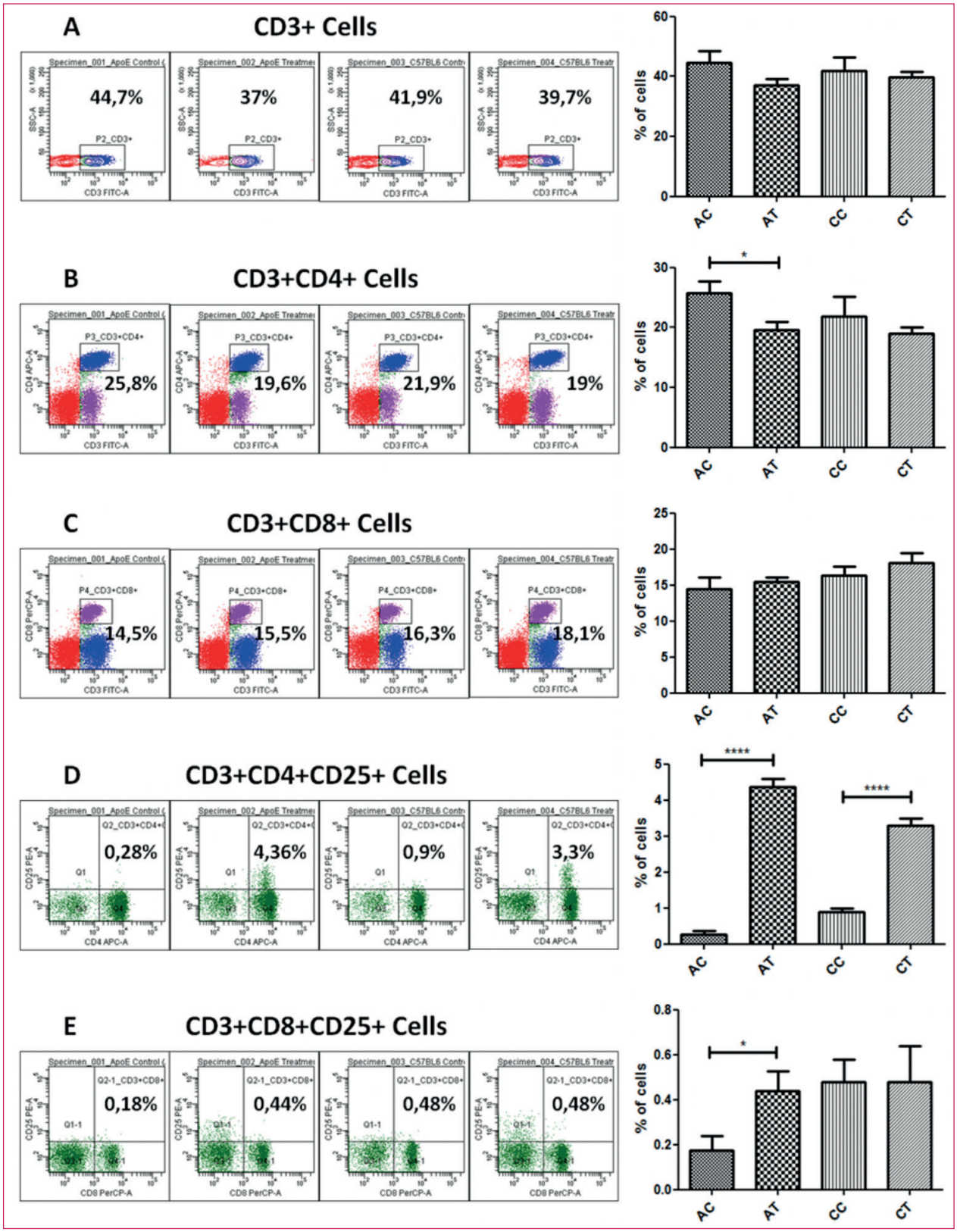

Figure 1. Effect of *L. plantarum* administration on the number of T cell populations. Representative histograms of the flow cytometry analysis are shown. The results are also displayed in bar graphs as mean ± SD (n=5; *p<0.05, ****p<0.0001). A significant increase in CD25 expression, particularly for $CD4^+$ T cells, was induced by *L. plantarum* intake, although the percentage of $CD4^+$ T Cells was reduced by the treatment in $ApoE^{-/-}$ mice (**AC**, $ApoE^{-/-}$ control group; **AT**, $ApoE^{-/-}$ treatment group; **CC**, C57BL/6 control group; **CT**, C57BL/6 treatment group).

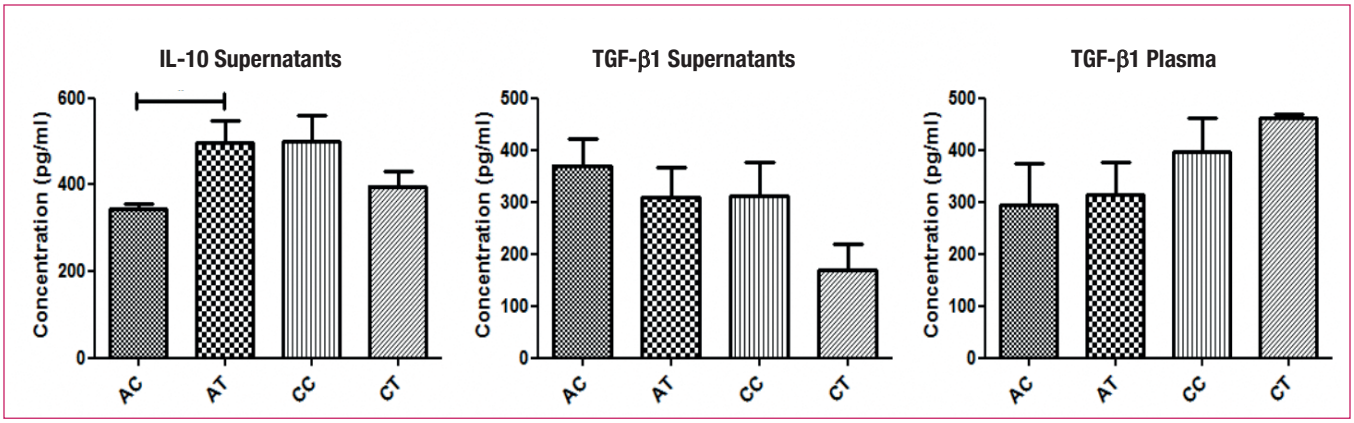

Figure 2. Effect of the *L. plantarum* intake on IL-10 and TGF-β1 levels in plasma and cell culture supernatants. ApoE$^{-/-}$ treatment group showed enhanced production of IL-10 compared to ApoE$^{-/-}$ control group (n=5; *p=0.045). No other significant changes were observed (**AC**, ApoE$^{-/-}$ control group; **AT**, ApoE$^{-/-}$ treatment group; **CC**, C57BL/6 control group; **CT**, C57BL/6 treatment group).

has no effect on total T cell ($CD3^+$; Fig. 1a) and Cytotoxic T cell ($CD3^+CD8^+$; Fig. 1c) populations. A significant increase was observed in the percentages of $CD4^+CD25^+$ T cells for both ApoE$^{-/-}$ and C57BL/6 groups (4.36±0.51% *vs* 0.28±0.17%, **** $p=1.4\times10^{-5}$ for ApoE$^{-/-}$ mice and 3.3±0.41% *vs* 0.9±0.2%, ****$p=2.2\times10^{-5}$ for C57BL/6 mice, n=5; Fig. 1d) and $CD8^+CD25^+$ T cells for ApoE$^{-/-}$ group (0.44±0.2% *vs* 0.18±0.13%, *p=0.048, n=5; Fig. 1e), although the percentage of T helper ($CD3^+CD4^+$) cells was reduced (19.6±3.1% *vs* 25.8±4.3%, *p=0.034, n=5; Fig. 1b) for ApoE$^{-/-}$ mice, but not for C57BL/6 mice.

Administration of probiotic treatment increases the level of IL-10 in ApoE$^{-/-}$ mice. The amount of IFNγ and IL-5 present in plasma and supernatant samples were below the detection limit of the assays. While the concentration of TGF-β1 displayed no significant changes between control and treatment groups neither in supernatants nor in plasma samples, a significantly increased amount of IL-10 was observed in the supernatants of ApoE$^{-/-}$ treatment group (495.8±115.7 *vs* 342.5±28.98, *p=0.045, n=5; Fig. 2). The level of IL-10 in the plasma was also below detection limit.

***L. plantarum* treatment impairs the development of atherosclerosis on ApoE$^{-/-}$ mice.** The ApoE$^{-/-}$ group treated with *L. plantarum* showed significantly less atherosclerotic plaque development compared to ApoE$^{-/-}$ control group (*p=0.019; Fig. 3), whereas the plaques were almost undetectable in 30 weeks old C57BL/6 mice as expected, so it is hard to assess any potential differences in the C57BL/6 group.

Probiotic therapy did not provide an improvement in lipid profiles. The overall result from the lipid profiling

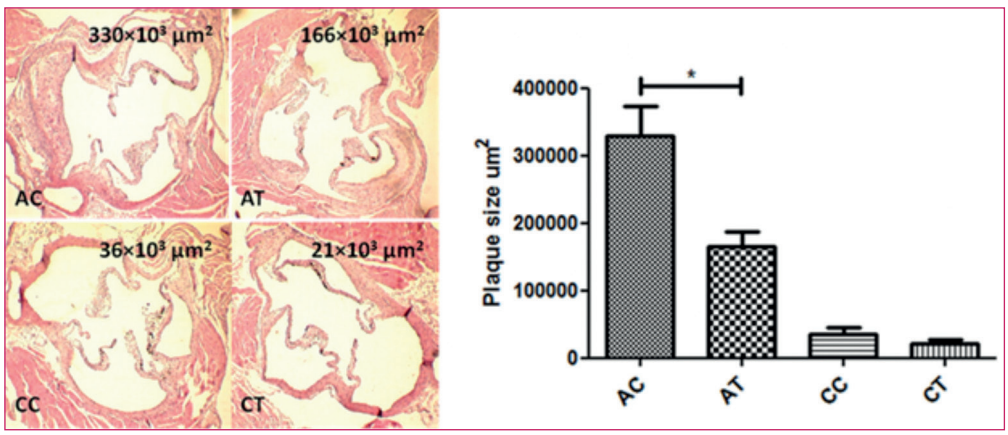

Figure 3. Effect of the *L. plantarum* treatment on atherosclerotic plaque development. The representative aortic sinus sections stained with H&E from morphometric analyses are showing the size of atherosclerotic plaques developed in the aortic sinus of ApoE$^{-/-}$and C57BL/6 mice. The results are also displayed in a bar graph as mean ± SD (*p<0.05; n=5). The ApoE$^{-/-}$group treated with *L. plantarum* showed significantly decreased level of atherosclerotic plaque development (**AC**, ApoE$^{-/-}$ control group; **AT**, ApoE$^{-/-}$ treatment group; **CC**, C57BL/6 control group, **CT**, C57BL/6 treatment group).

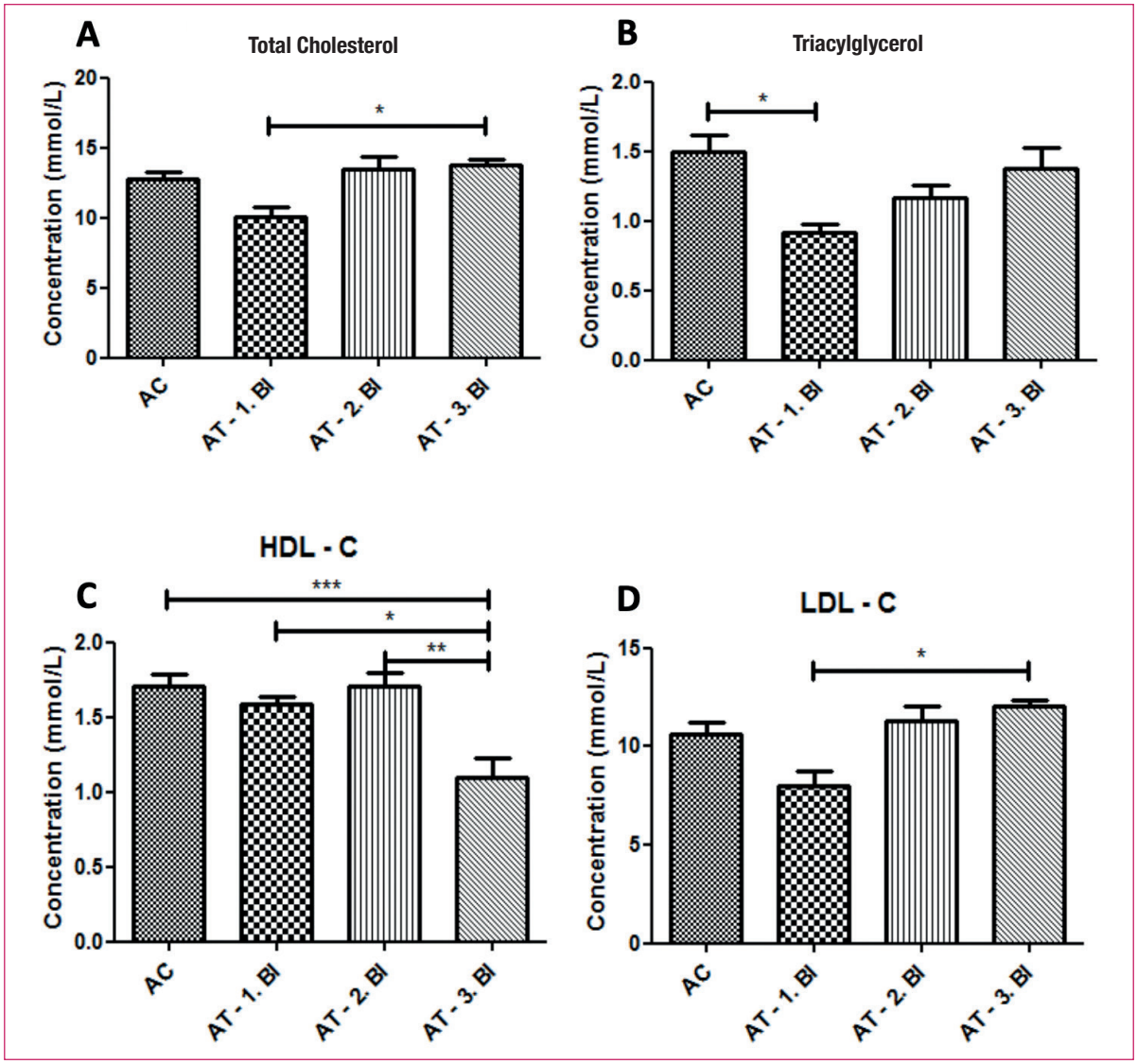

Figure 4. Effect of probiotic therapy on plasma lipid profiles of ApoE$^{-/-}$ mice. Values are presented as mean (n=5) ± SD. *p<0.05, **p<0.01 and ***p<0.001 (**AC**, ApoE$^{-/-}$ control group; **AT**, ApoE$^{-/-}$ treatment group; **BI**, bleeding).

showed an effect contrary to what was expected, especially for the ApoE-/- group. A significant increase in the amount of total cholesterol between the first and the last bleeding (10.02±1.71 *vs* 13.75±1.00, *p=0.013; Fig. 4a) was observed in ApoE$^{-/-}$ mice. A gradual increase was also observed in the levels of triacylglycerol throughout the treatment both in ApoE$^{-/-}$ (Fig. 4b) and C57BL/6 groups (data not shown), although they were not statistically significant. Levels of HDL-C were significantly lower in plasma samples collected at termination of ApoE$^{-/-}$ mice compared to first and second bleeding as well as control group (1.59±0.12; 1.71±0.18; 1.71±0.28 *vs* 1.10±0.28, *p=0.028; **p<0.0053; ***p<0.00089, respectively; Fig. 4c). Finally, an increase in LDL-C was observed in the third bleeding compared to the first bleeding in ApoE$^{-/-}$ mice (12.03±0.70 *vs* *p=0.011; Fig. 4d).

The only significant difference in C57BL/6 mice was observed in the concentration of HDL-C between the second bleeding of treatment group and the control group (2.20±0.49 *vs* 1.74±0.20, *p<0.034). No other significant changes were observed in C57BL/6 group (data not shown).

Discussion

The data produced by this study provides unique insides into the regulatory process induced by *L. plantarum* on the immune system and despite its limitations, it

offers a stepping stone to future research in the field. More and more scientists and medical professionals are looking towards treating immune-disregulated diseases by providing tailored dietary regimes as adjuvants to drug treatments.[19] Understanding and shedding light into these mechanisms represents a long and integrated process of experimental continuum to which this study has certainly contributed.

First, we examined the survival and the colonization of *L. plantarum* in the gastrointestinal tract by the identification of isolates recovered from faecal samples of treated mice. During the biochemical characterization, none of the isolates from late faeces samplings (24 hours following administration) yielded identical sugar fermentation profiles with *L. plantarum*, while all creamy white colonies isolated from early samplings yielded exactly the same profile with *L. plantarum*. Early samples were collected 5 hours after probiotic intake, which is more or less equal to gastrointestinal transit time in mice.[20] These results showed that *L. plantarum* did not succeed in colonizing the gut throughout the 16 weeks treatment period, though it is obvious that they managed to survive through the gastrointestinal track of the mice.

It is not essential that probiotic bacteria colonize the gut in order to exhibit their beneficial effects on human health.[21] Most probiotic strains do not colonize the gut and show a sharp decrease in the number of viable cells in faecal samples after interception of probiotic intake. That is why all the manufacturers of fermented products with probiotics recommend daily intake of their products. However, being transient in the gut does not prevent probiotics to improve human health as long as their consumption is continuous.[22] In our study, some immunomodulatory and anti-atherogenic effects of *L. plantarum* were observed, despite the lack of evidence of colonization.

ApoE$^{-/-}$ mice are genetically liable to enhanced pro-inflammatory responses confirmed by observation of typical high levels of circulating IFN-γ.[23] Moreover, it is reported that the number of Treg cells decreases as these animals get older, which probably results from the inhibitory function of IFN-γ on Treg cells.[23] Despite these reported interferences, our results showed a remarkable induction of CD4$^+$CD25$^+$ T cell proliferation by oral intake of *L. plantarum* on a regular basis, which may explain the potential athero-protection provided by the probiotic. Studies on Treg deficient mouse or models using CD25-neutralizing antibodies evidently demonstrated the protective role of CD4$^+$CD25$^+$ Treg cells against atherogenesis.[24] Petersen *et al*. also found that *Lactobacilli* species are capable of promoting Treg cells in CD4$^+$CD25$^-$T cell transferred mice possibly through the induction of their differentiation into CD4$^+$CD25$^+$ T cells.[25]

Treg cells can be of both CD4$^+$ or CD8$^+$ phenotypes. The CD8$^+$CD25$^+$ subset of Treg cells have recently been identified and their functions are still poorly understood. It is thought that they have a similar action as to that of CD4$^+$CD25$^+$ Treg cells, though they are less prevalent in the body.[26] We observed a significant increase in CD8$^+$CD25$^+$ T cell subset too only in ApoE$^{-/-}$ group, but to a lesser extent, which may be attributable to their low abundance.

However, CD25 is also expressed on the surface of activated T cells, but less prominently.[27] To avoid misinterpretation, further identification of FoxP3$^+$ T cells (a specific marker of Tregs) together with CD25, would strengthen this interference.[28]

The effect of *L. plantarum* treatment on regulatory immune responses was further investigated by assessing how it affects the production of regulatory cytokines, TGF-β and IL-10. TGF-β and IL-10 are specifically known as potent anti-inflammatory, thereby anti-atherogenic cytokines.[29] The increase we observed in the *in vitro* production of IL-10 by splenocytes from ApoE$^{-/-}$ group may be attributed to elevated level of IL-10 producing Treg cells, although it may also be related to other IL-10 producing cells that we did not check such as Tr1 cells. An increase in the amount of IL-10 has also been shown by other investigators as a consequence of oral administration of probiotics.[30,31] There is strong evidence supporting the protective role of IL-10 against atherosclerosis. The studies conducted on IL-10 deficient mice revealed that a lack of IL-10 is strongly associated with augmented atherosclerosis resulting from increased infiltration of inflammatory cells and elevated levels of pro-inflammatory cytokines.[32-34] The reduction we found in the plaque sizes together with an increase in the level of L-10 in ApoE$^{-/-}$ group also favor the athero-protective role of IL-10.

We also conducted a quantitative assessment to investigate the influence of probiotic treatment on the size of atherosclerotic plaques developed at the aortic sinus. The aortic sinus is one of the most susceptible segments to the atherosclerotic plaque development because of its anatomical location that is exposed to non-laminar blood

flow and shear stress.[35] Our study showed a significant reduction in the aortic sinus plaque size of the ApoE-/- treated mice. Hence, this result is a supportive evidence for the postulated protective effects of probiotics against atherosclerosis. However, the hypocholesterolemic effect of probiotics claimed by many investigators[15] was not observed for *L. plantarum* in our study. Our findings showed that *L. plantarum* seem to have a lowering effect on HDL-C levels and an increasing effect on LDL-C levels that actually contradict the expected behaviour of probiotics.

The literature investigating the hypocholesterolemic effect of probiotics accumulated to date contains controversial results.[36-39] There are other studies that have also failed to demonstrate the cholesterol lowering effect attributed to probiotics.[39,40] However, it is not possible to make a direct comparison between these studies and our study due to differences in subjects and probiotic strains, the length of the experiments, the way of administration and the experimental designs[36-40] Nevertheless, the suitability of the ApoE$^{-/-}$ mouse in lipid profile research has been questioned by several investigators due to the single mutation in the ApoE gene.[41,42] Moreover, the anti-atherogenic effects of probiotics and the way they deliver these effects may differ from strain to strain. In summary, *L. plantarum* seems to have some anti-atherogenic effects, but improvement of lipid profile may not be one of the mechanisms used by this probiotic strain to retard atherogenesis. However, further investigation using different models or human intervention studies may be required to ascertain the exact effect of *L. plantarum* on lipid profile.

In conclusion, oral administration of *L. plantarum* seemed to have a stimulatory effect on the proliferation of CD4^{+}CD25^{+} T cells and the synthesis of IL-10 in ApoE$^{-/-}$ mice, which is shown by flow cytometry and ELISA analysis. These signals are known to contribute to the reduction of inflammation and stabilization of atherosclerotic plaques, which make *L. plantarum* a potential treatment for atherosclerosis. This potential athero-protective ability of *L. plantarum* was also confirmed with the significant reduction in plaque sizes observed in the aortic sinus of ApoE$^{-/-}$ mice. Moreover, these findings provide some mechanistic information to understand how *L. plantarum* may retard atherosclerotic plaque development and also explain its role in the modulation of atherosclerosis. Nevertheless, these observations need validation by repeated experiments and direct demonstration on a reporter mouse model.

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