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The cholesterol lowering efficacy of *Lactobacillus plantarum* ECGC 13110402 in hypercholesterolemic adults: a double-blind, randomized, placebo controlled, pilot human intervention study

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ABSTRACT

Lactobacillus plantarum ECGC13110402 is a probiotic, selected for its high bile salt hydrolase and cholesterol reducing activity. This parallel, double-blind, placebo-controlled, randomized pilot study, investigated the cholesterol reducing capacity of *L. plantarum* ECGC13110402 in 16 hypercholesterolemic adults. Participants ingested 4 $\times 10^9$ CFU encapsulated ECGC13110402 (active; n=8) or placebo (n=8), once daily, over 6 weeks, followed by a 3-week washout. Fasting blood samples were collected for blood lipid, liver function, and vitamin D analysis. After 6 weeks of *L. plantarum* ECGC 13110402 daily intake, biologically and statistically significant reductions were noted in TC by an average 34.6% (p=0.001), LDL-C by 28.4% (p=0.03), non-HDL-C by 17.6% (p=0.001) and apoB by 28.6% (p=0.008) compared to the placebo. No changes were observed in liver function biomarkers and vitamin D and no adverse effects were noted throughout the study. The findings of this study suggest that *L. plantarum* ECGC 13110402 can safely improve lipid profiles in dyslipidaemic individuals.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide. The World Health Organization (WHO) estimated that a total of 17.9 million people died from CVDs in 2019, representing 32% of global deaths (World Health Organization, 2021). Coronary heart disease (CHD), the most common type of CVD, has shown the largest increase in deaths since 2000 and is responsible for 16% of the world's total deaths (World Health Organization, 2020). Epidemiological studies have suggested a positive correlation between elevated total serum cholesterol levels and CHD risk (World Health Organization, 2003), while numerous epidemiological, clinical and genetic studies, have provided consistent evidence, unequivocally establishing a causal role for LDL-C and other apoB containing lipoproteins in CHD development (Ference et al., 2017). It is estimated that with each 1.0 mmol/L reduction in LDL-C, the annual rate of major vascular events can be reduced by just over a fifth (Ference et al., 2017), which further demonstrates the importance in managing low density lipoproteins to reduce the risk of cardiac events.

Dietary and behavioural change strategies are the first line for CHD prevention and are based on life-long adherence to low cholesterol/low saturated fat, high dietary fibre diets, stopping tobacco smoking and excess alcohol intake, together with adopting an active lifestyle (Arnett et al., 2019; Visseren et al., 2021). These strategies can be very effective but are not easily sustainable. Several well-established pharmacological approaches can provide additional support in effectively managing cholesterol, such as statins, fibrates, selective cholesterol absorption inhibitors and bile acid sequestrants (Arnett et al., 2019). However, adherence to medication can range from 50% for primary CVD prevention and 66% for secondary prevention (Arnett et al., 2019), mainly as a result of polypharmacy, side effects, and even more so, beliefs about side-effects and medication consequences. Consequently, there is a gap between patient requirements and clinical practice, with limitations of current approaches increasing the interest in non-drug therapies to supplement current strategies for improving blood cholesterol profiles and supporting cardiovascular health.

The human gut harbours a highly metabolically diverse microbiome (Claassen et al., 2013), with several bacterial groups possessing mechanisms that can impact on the metabolism of lipids, including cholesterol adsorption to cellular surfaces, assimilation into cell membranes, cholesterol esterase activity, reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase expression and the deconjugation

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of bile acids through bile salt hydrolase (BSH) activity (Klaver & Van der Meer, 1993). Several gut bacterial groups carry BSH activity including *Lactobacillus*, *Bifidobacterium, Enterococcus, Clostridium* and *Bacteroides* (Begley et al., 2006; Jones et al., 2008). BSHs play a role in enhancing bacterial survival within the gastrointestinal tract as they can minimise the deleterious impact of bile. Whilst the hydrolysis of bile benefits the cell itself, it also increases intraluminal bile salt deconjugation, disrupting enterohepatic recirculation. Once deconjugated, bile acids are less soluble and are absorbed in the intestine to be excreted in faeces. Cholesterol is then used for *de novo* bile acid synthesis in a homeostatic response that indirectly reduces serum cholesterol (Begley et al., 2006; Lew et al., 2018).

A number of human intervention studies have highlighted *Lactobacillus plantarum* strains as a promising probiotic strategy for improving hypercholesterolemia (Cairella & Marchini, 1995; Costabile et al., 2017; Fuentes et al., 2013). *L. plantarum* ECGC 13110402 is a probiotic strain, selected for its high bile salt hydrolase *in vitro,* and *in vivo* cholesterol reduction activity. The authors reported that intake of 2×10^9 CFU encapsulated *L. plantarum* ECGC 13110402 twice daily, significantly reduced LDL-C (13.9%), total cholesterol (TC) (37.6%), TG (53.9%), and significantly increased HDL-C (14.7%; in subjects >60 years of age; 6–12 weeks) in normal to mildly hypercholesterolaemic subjects (Costabile et al., 2017).

This pilot study aimed to assess the cholesterol reducing efficacy of *L. plantarum* ECGC 13110402 in 16 hypercholesterolaemic individuals. Primary efficacy outcomes included impact on blood lipids, with focus on LDL-C, apoB and non-HDL-C. Secondary study outcomes included impact on gastrointestibal (GI) symptoms and mood parameters. Liver function biomarkers and blood pressure were also determined to monitor volunteer health status. Vitamin D levels were analysed to determine potential impact on fat soluble vitamins.

2. Materials and methods

2.1. Study design, setting and experimental design

This was a double-blind, randomized, placebo-controlled, parallel-group, study, conducted in the University of Roehampton Sport and Exercise Science Research Laboratory, School of Life & Health Science, Whitelands College, UK. The study was registered at www.clinicaltrials.gov (NCT03540108) and all research procedures complied with the Declaration of Helsinki and the Guidelines of Good Clinical Practice as well as the CONSORT checklist (Table S1) and SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) guidelines. The study protocol was approved by the University of Roehampton Research Ethics Committee (ref.: LSC18/241) and the NHS Research Ethics Committee (IRAS project ID: 259363). Written informed consent was obtained from all prospective subjects, following reading the participant information sheet and prior to completion of the medical screening questionnaire, evaluating their eligibility to participate in the study.

Study participants were recruited during September 2019 through February 2021 and followed through May 2021 via adverts distributed in the local area of the University of Roehampton, in local GP surgeries and through social media platforms. Potential participants, who provided signed informed consent, and met all inclusion criteria (Table 1), attended a screening session during which measures of height and weight, sitting blood pressure and a fasting blood sample were taken. Blood samples were analysed for blood lipids, glucose and full blood count. Suitable individuals that fulfilled all selection criteria (n=16) were invited to participate in the study (Figure 1). Participants and researchers administering interventions and assessing outcomes were blinded to the intervention groups and corresponding treatment. An independent researcher generated the random allocation to treatment sequence with the use of a random number generator (GraphPad QuickCalcs, San Diego, CA, USA), for the purpose of assigning a specific number to each volunteer and to ensure that group allocation information remained concealed. Samples were un-blinded upon the completion of primary outcome analysis for all study participants. Study participants were stratified according to gender and were randomly allocated to one of the two treatment groups using a 1:1 ratio (Figure 1).

Table 1
Main inclusion and exclusion criteria.

Inclusion criteria	• Males and females • Age: 35-70 years • Body mass index (BMI): 18.5-29.9 kg/m^2 • Total cholesterol (TC):≥6 mmol/L
Exclusion criteria	• Those on medication affecting primary and secondary study outcomes including ACE inhibitors, PCSK9 inhibitors, bile acid sequestrants, cholesterol absorption inhibitors, nicotinic acid agents, fibrates, proton pump inhibitors, thyroid hormone replacement, steroids, vitamin D supplements, pre/pro and synbiotics, herbal /natural supplements/foods such as plant sterols and stanols claimed to lower blood lipids • Antibiotic intake (any) in the last 6 months • Any other medication is permitted as long it was started at least three months before inclusion to the study and there was no dose change during the three months • Individuals with requirement to take long-term medication active on the gastrointestinal tract (medication active on gastrointestinal motility including laxatives), treatment of cardio-vascular disease, or any other long-term medication • Those using cholesterol lowering medication (6 months prior to the study) • Those with history of drug or alcohol misuse or alcohol consumption exceeding 14 and 21 units/week for females and males respectively • Smokers and those having quit smoking in the 12 months preceding the study • Those suffering with any allergies to medication or food • Individuals on weight-reducing diets • Females planning pregnancy within six months from the start of the study, pregnant, lactating, or have given birth within the preceding six months • Use of antibiotics within six months preceding the study, participation in any probiotic, prebiotic or laxative study or intake of an experimental drug four weeks prior to the study start • Sufferers of familial hypercholesterolemia • Individuals with a clinically significant renal, hepatic, endocrine (including diabetes mellitus), pulmonary, pancreatic, neurologic, urogenital/rectal, or lymphatic disorders • Any major cardiovascular condition not mentioned above

2.2. Intervention and compliance

The study consisted of two phases: a treatment period (6 weeks), with either the active or the equivalent placebo and a wash-out period (3 weeks). Following the screening visit to ensure adherence to the inclusion criteria, the study included a baseline, midpoint, endpoint (week 3 and week 6, respectively) and washout visit (week 9). Compliance to the dietary supplement (active or placebo) was assessed in daily diaries, along with recording gastrointestinal symptoms and mood (Table S2).

Participants were asked to consume one capsule of either active or placebo, once a day after lunch, over the treatment period. The active treatment comprised of 4×10^9 CFU *L. plantarum* ECGC 13110402 (50mg) in capsular format (DR1 vegetable capsule) with the addition of filling carrier (188.6 mg corn starch, 3.2 mg magnesium stearate and 3.2 mg silicon dioxide). The placebo was an identically looking capsule, containing 238.6 mg corn starch, 3.2 mg magnesium stearate and 3.2 mg silicon dioxide. Active and placebo formulae had similar taste, appearance and were blended, encapsulated and blind packaged in blister packs, and placed in opaque containers of identical colour and size by Nutrilinea (Milan, Italy). Participants were advised on storage conditions, to ensure product consistency throughout the study. Volunteers were asked not to alter their usual dietary habits and physical activity patterns during the trial period (treatment and washout), not to consume prebiotic supplements, probiotics, drugs active on the primary

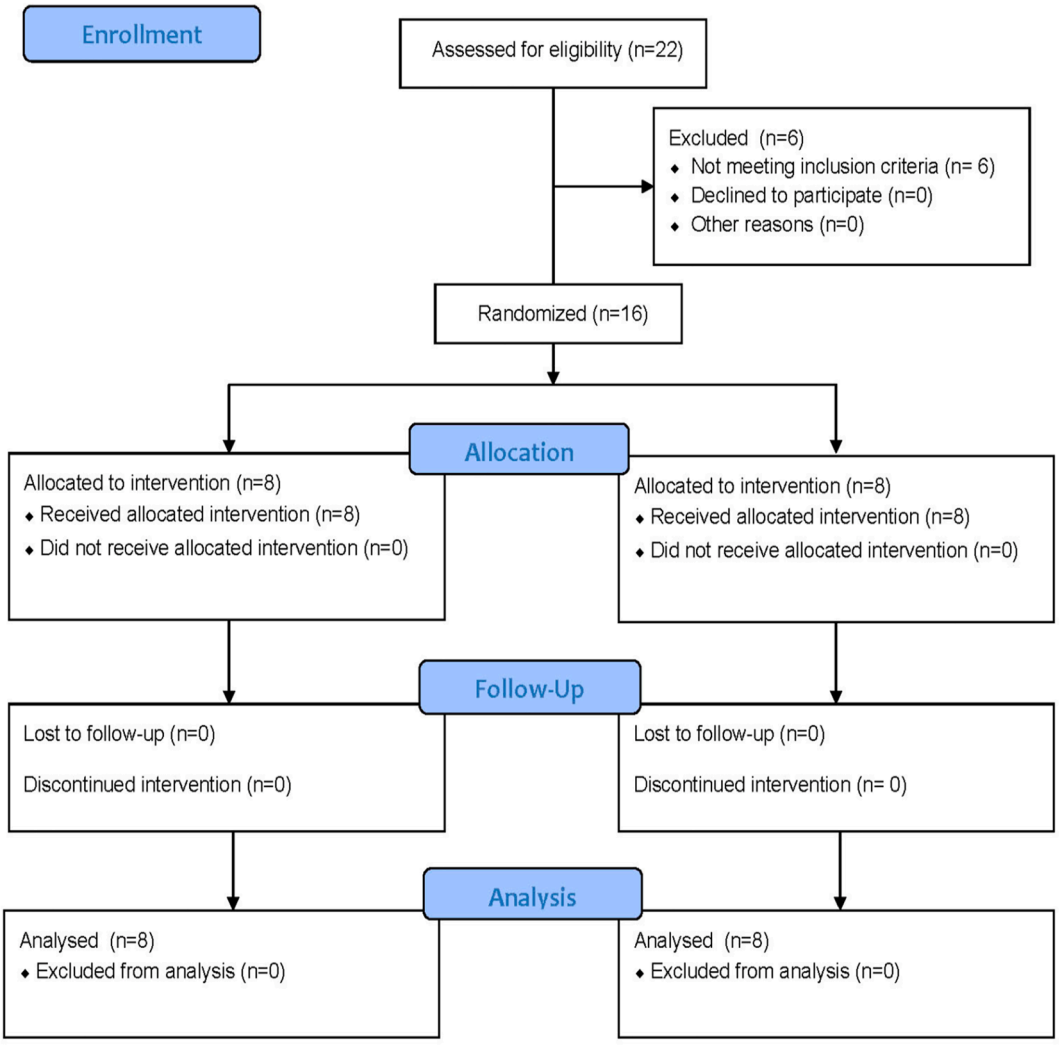

Fig. 1. Flow of study participants through the intervention.

parameters and drugs active on gastrointestinal motility as reported in Table 1. Volunteers that received antibiotics in the 6 months preceding the study were excluded and any medication taken was recorded in daily diaries.

2.3. Anthropometric measurements, body composition assessment and blood pressure

Anthropometric measurements including weight, height, waist, hip circumference, and blood pressure were measured during all visits as per standardized techniques (Lohman et al., 1988). Body composition was assessed by bioelectrical impedance analysis (BIA) using Tanita BC-418 MA Segmental Body Composition Analyzer (Tanita Corporation, Tokyo, Japan). Study participants were asked to follow the pre-testing guidelines in preparation for each visit (Table S3).

2.4. Blood collection and determination of blood chemistry

Fasting blood samples were collected from the participant's antecubital vein according to a standardized protocol (World Health Organization, 2010), using a 23G butterfly needle (Greiner Bio-One GmbH, Kremsmünster, Austria) into one 9 ml K2EDTA tube (Vacuette®; Greiner Bio-One GmbH, Kremsmünster, Austria) and into one 9 ml Lithium Heparin tube (Vacuette®; Greiner Bio-One GmbH, Kremsmünster, Austria) for fasting TC, LDL-C, HDL-C, TG, ALT, AST, ALP, apoB, GGT, albumin, total bilirubin, total protein, and Vitamin D (25-hydroxycholecalciferol). All samples were kept on ice until centrifugation. Plasma samples were recovered by centrifugation at 1000 g for 15 minutes, dispensed into 1.5 ml microcentrifuge tubes and frozen at -20°C within 1 h from collection. Sample analysis was performed by Affinity Biomarker Labs (Imperial College London, W12 0BZ) using Siemens ADVIA 1800 chemistry analyser (Siemens Healthcare Diagnostics Inc., Germany) and Siemens ADVIA Centaur XP immunochemistry analyser (Siemens Healthcare Diagnostics Inc., Germany).

2.5. Gastrointestinal and mood parameters

Changes in gastrointestinal and mood parameters were recorded during the treatment and washout periods in the gastrointestinal and mood change questionnaire. The following parameters were recorded daily: number of stools; stool consistency as per Bristol chart (Lewis & Heaton, 1997); abdominal pain, stomach or intestinal bloating and flatulence occurrence and severity (none, mild, moderate and severe). Changes in mood (happy, alert, energetic, stressed) were recorded as: less than normal, normal, more than normal.

2.6. Power calculation and statistical analysis

The study was powered to provide 80% statistical power, (MGH Biostatistics Hedwig Software) based on an average (±SD) log

change:1.2 for total cholesterol change on the basis of findings from previous human intervention studies conducted on blood lipids (Cho & Kim, 2015; Dixon et al., 2020; Tamayo, 2008). Given these calculations, 16 participants (to allow for 15% attrition) were required to detect a treatment difference at a two-sided 0.05 significance level.

The first set of statistical analyses compared the baseline characteristics of the two study groups. The continuous nature and normal distribution of all data outcomes were established, prior to unpaired t-test analysis. The Mann-Whitney U test was used to compare continuous variables that were not normally distributed. Subsequently, the outcomes at the post-baseline time-points were examined, with time-point analysed in separate analyses. The analyses were performed using analysis of covariance (ANCOVA), with the outcome at the start of the period considered as a covariate. Analysis were performed on the following study periods: week 3 – adjusted for baseline, week 6 – adjusted for baseline, washout period – adjusted for baseline, washout period – adjusted for week 6. Certain gastrointestinal variables (abdominal pain and bloating) exhibited a positively skewed distribution. In order to meet the assumptions of the analysis methods, values were analysed on the log scale. A small constant was added to all values before transformation to allow for zero values.

Statistical analyses were performed using Stata, Version 15.1 (Stata Corp., College Station, TX, USA) and GraphPad Prism Version 9.0.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significance at the level of $p < 0.05$.

3. Results

3.1. Baseline demographic variables

No statistically significant differences were found between the active and placebo groups at baseline in the anthropometric parameters measured, except for diastolic blood pressure which was significantly lower in treatment group compared to placebo ($p = 0.003$; Table S4).

Subsequent analyses compared differences between treatment groups in terms of changes in outcomes at the post-baseline time-points.

3.2. Anthropometric data, body composition and blood pressure

L. plantarum ECGC 13110402 intake resulted in significant reductions at week 3 in various anthropometric and body composition measurements including, waist circumference ($p=0.005$), hip circumference ($p=0.002$) and fat mass ($p=0.03$) compared to the placebo. The effect was transient, and no significant differences were observed at weeks 6 or 9 (Table S5) apart from a change in hip circumference, which was significantly lower in the active group compared to placebo at the end of washout (week 9) ($p=0.04$).

3.3. Lipid parameters

The impact of the active and placebo treatments on blood lipid concentrations is shown in Table 2. TC concentrations were significantly

Table 2
Blood lipid concentrations (mean ± standard deviation) in the active (n=8) and placebo (n=8) groups at baseline, week 3, 6 (end of treatment) and 9 (washout).

Outcome	Treatment	BaselineMean ± SD	Week 3Mean ± SD	Group Difference from baseline toWeek 3 (†)Mean (95% CI)	P-value	Week 6Mean ± SD	Group Difference baseline toWeek 6(†)Mean (95% CI)	P-value	Week 9Mean ± SD	Group Difference baseline toWeek 9 (†)Mean (95% CI)	P-value
TC (mmol/L)	Active	6.39 ± 0.58	5.07 ± 1.21*	0	**0.001**	5.00 ± 1.54*	0	**0.001**	5.85 ± 1.18	0	0.75
	Placebo	6.10 ± 0.11	6.39 ± 0.63	1.73 (0.82, 2.64)		6.61 ± 0.86	2.16 (1.00, 3.32)		5.95 ± 1.35	0.25 (-1.45, 1.95)	
TG (mmol/L)	Active	1.49 ± 0.88	1.28 ± 0.63	0	0.68	1.34 ± 0.71	0	0.28	1.49 ± 0.62	0	0.46
	Placebo	1.13 ± 0.46	1.15 ± 0.63	0.10 (-0.39, 0.59)		1.29 ± 0.67	0.23 (-0.21, 0.68)		1.40 ± 0.80	0.23 (-0.45, 0.91)	
HDL (mmol/L)	Active	1.46 ± 0.41	1.31 ± 0.40	0	0.11	1.24 ± 0.48*	0	**0.03**	1.42 ± 0.35	0	0.90
	Placebo	1.81 ± 0.56	1.80 ± 0.61	0.14 (-0.03, 0.32)		1.79 ± 0.44	0.23 (0.03, 0.43)		1.61 ± 0.59	-0.02 (-0.37, 0.33)	
LDL (mmol/L)	Active	4.55 ± 1.10	4.03 ± 1.13*	0	**0.01**	4.00 ± 1.34*	0	**0.001**	4.64 ± 1.03	0	0.81
	Placebo	4.10 ± 0.92	4.39 ± 1.44	0.88 (0.21, 1.54)		4.60 ± 1.69	1.23 (0.57, 1.89)		4.66 ± 1.40	0.13 (-1.05, 1.31)	
Non-HDL-C (mmol/L)	Active	4.85 ± 1.13	4.29 ± 1.18*	0	**0.02**	4.21 ± 1.35*	0	**0.001**	4.81 ± 1.13	0	0.99
	Placebo	4.58 ± 1.32	4.59 ± 1.49	0.59 (0.09, 1.08)		4.70 ± 1.77	0.83 (0.41, 1.24)		4.93 ± 1.51	0.00 (-1.17, 1.17)	
TC/HDL	Active	4.59 ± 0.89	4.00 ± 0.74	0	0.06	4.15 ± 0.85	0	0.14	4.21 ± 0.78	0	0.57
	Placebo	3.61 ± 0.91	3.84 ± 1.09	0.67 (-0.04, 1.37)		3.90 ± 1.01	0.55 (-0.21, 1.31)		3.90 ± 0.94	0.23 (-0.64, 1.11)	
apoB (g/L)	Active	0.98 ± 0.19	0.90 ± 0.20*	0	**0.005**	0.90 ± 0.24*	0	**0.008**	1.03 ± 0.26	0	0.83
	Placebo	0.91 ± 0.15	1.01 ± 0.25	0.20 (0.07, 0.33)		1.07 ± 0.32	0.27 (0.09,0.46)		1.03 ± 0.26	0.03 (-0.26, 0.32)	

TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein, Non-HDL-C: non-high-density lipoprotein cholesterol, TC/HDL ratio: total cholesterol to high density lipoprotein cholesterol ratio; apoB: apolipoprotein B.
(†) Calculated from ANCOVA analysis, adjusting for baseline value.
* Denotes statistical significance at $p < 0.05$.

lower in the active group compared to the placebo in the baseline to 3 weeks (p=0.001) and the baseline to 6 weeks of treatment period (p=0.001). No significant difference was observed in TC between the active and placebo groups at the baseline to week 9 period (washout).

LDL-C concentrations were statistically significantly lower in the active group compared to the placebo in the baseline to week 3 (p=0.01) and the baseline to week 6 period of treatment (p=0.001). No significant difference was observed between the active and placebo groups at the baseline to week 9 period (washout).

No statistically significant changes were observed in HDL-C concentrations between the two treatment groups in the baseline to 3-week period. A statistically significant reduction in HDL-C was observed in the baseline to 6-week period for the active group (p=0.03). Both active and placebo groups showed trends for reduction in this biomarker during this period. Overall, HDL-C was higher in the placebo group by an average 0.23mM over the baseline to 6-week period.

Similar to TC and LDL-C, non-HDL-C concentrations were statistically significantly lower for the active group compared to the placebo, during both the baseline to week 3 (p=0.02) and the baseline to week 6 period (p=0.001). No significant difference was observed between the active and placebo groups at the baseline to week 9 period (washout).

ApoB concentrations, were statistically significantly lower in the active group compared to the placebo, over both the baseline to week 3 (p=0.005) and week 6 treatment period (p=0.008). Similarly, to the other blood lipid biomarkers, no significant differences were observed between treatment groups for the baseline to week 9 period (washout).

No statistically significant changes were noted in the TC/HDL ratio or TG concentrations for either group over the study period.

Figure 2 shows the evolution in the concentrations of blood lipid biomarkers from baseline to 9 weeks for the active and placebo groups. TC (A), LDL-C (B), non-HDL-C (C) and apoB (D) show decreasing trends over the 6 weeks of active treatment, an effect not seen in the placebo group.

3.4. Liver function biomarkers and Vitamin D

No statistically significant changes in liver function tests (total protein, albumin, ALT, AST, GGT, bilirubin) were noted over the treatment period. A statistically significant reduction was observed in globulins for the active group at the end of the washout period compared to placebo (p=0.03). No statistically significant changes were noted in vitamin D levels throughout the study (Table 3 and Table S6).

3.5. Gastrointestinal (GI) symptoms and mood

No statistically significant changes were observed in stool frequency or form in the either study group. Similarly, no statistically significant changes were noted in abdominal pain, bloating or flatulence

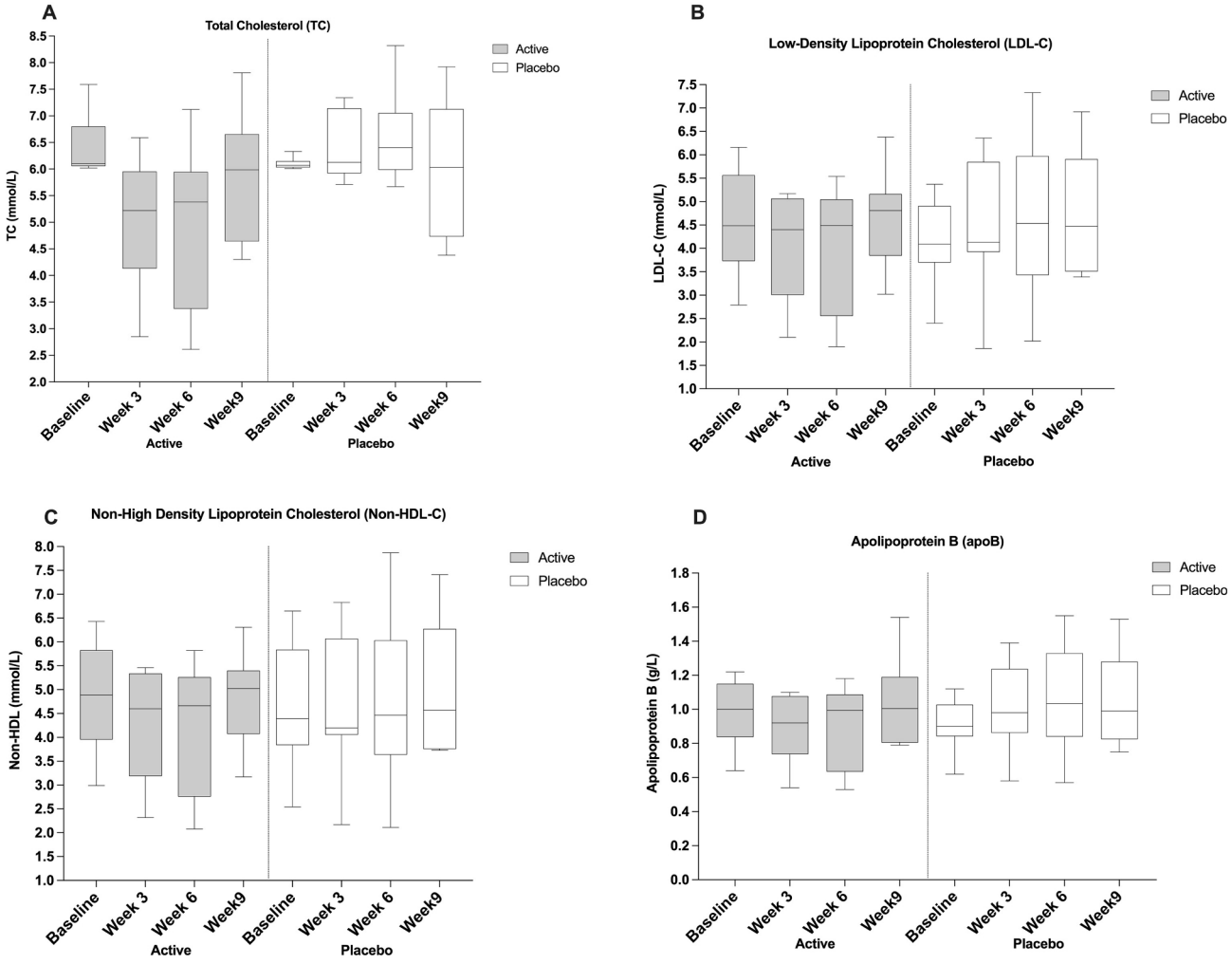

Fig. 2. **A** Total cholesterol (TC); **B** Low-density lipoprotein cholesterol (LDL-C); **C** Non-high density lipoprotein cholesterol (Non- HDL-C); **D** Apolipoprotein B (apoB) blood lipid concentrations (mmol/L) concentrations at 3 and 6 weeks (end of the treatment) and 9 weeks (washout) for the active and placebo group. Box and whisker plots represent individual data points with minimum to maximum distribution. Bold line represents the median value, the lower and upper column the 25th and 75th percentile, and the lower and upper bars the minimum and maximum value.

Table 3
Liver function biomarker and vitamin D concentrations (mean ± standard deviation) in the active (n=8) and placebo (n=8) groups at baseline, week 3, 6 (end of treatment) and 9 (washout).

Outcome	Treatment	BaselineMean ± SD	Week 3Mean ± SD	P-value	Week 6Mean ± SD	P-value	Week 9Mean ± SD	P-value
Total protein (g/L)	Active	67.0 ± 4.9	60.8 ± 8.7	0.42	58.8 ± 6.7	0.40	65.1 ± 5.8	0.32
	Placebo	63.6 ± 5.8	63.5 ± 6.0		62.6 ± 5.1		66.6 ± 4.8	
Albumin (g/L)	Active	44.6 ± 2.9	40.8 ± 5.3	0.32	39.5 ± 3.6	0.12	43.5 ± 4.9	0.42
	Placebo	42.8 ± 3.7	43.2 ± 3.8		42.7 ± 2.7		40.5 ± 1.6	
Globulins (g/L)	Active	22.4 ± 3.0	20.0 ± 4.0	0.35	19.4 ± 4.9	0.69	21.5 ± 3.5	0.03*
	Placebo	20.8 ± 3.9	20.3 ± 4.4		19.9 ± 4.1		26.1 ± 5.0	
ALT (IU/L)	Active	41.5 ± 19.2	33.1 ± 16.0	0.25	32.2 ± 13.4	0.98	37.8 ± 17.3	0.72
	Placebo	24.7 ± 9.4	22.5 ± 12.8		21.8 ± 8.2		27.5 ± 18.8	
AST (IU/L)	Active	62.1 ± 9.4	49.1 ± 13.4	0.69	44.1 ± 11.3	0.82	41.9 ± 9.9	0.64
	Placebo	45.2 ± 11.7	36.8 ± 12.5		34.3 ± 13.4		34.6 ± 13.8	
Bilirubin (umol/L)	Active	11.3 ± 3.5	11.5 ± 4.1	0.29	11.5 ± 6.2	0.68	13.0 ± 5.4	0.81
	Placebo	11.1 ± 5.1	13.3 ± 6.3		12.2 ± 4.2		12.2 ± 6.8	
GGT (IU/L)	Active	34.9 ± 17.5	28.7 ± 18.1	0.73	27.6 ± 18.3	0.82	31.1 ± 16.9	0.63
	Placebo	25.2 ± 6.0	21.2 ± 9.0		19.4 ± 7.5		21.3 ± 6.4	
ALP (IU/L)	Active	24.2 ± 10.2	21.7 ± 8.1	0.05	28.2 ± 20.7	0.37	40.2 ± 18.4	0.71
	Placebo	26.6 ± 8.3	35.5 ± 15.5		38.2 ± 20.3		40.5 ± 22.4	
Vitamin D (nmol/L)	Active	32.6 ± 12.1	38.1 ± 16.9	0.37	32.1 ± 15.4	0.71	27.5 ± 12.1	0.82
	Placebo	29.7 ± 12.3	31.6 ± 10.7		28.5 ± 8.1		26.5 ± 9.6	

ALT: aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyl transferase; ALP: alkaline phosphatase; apoB: apolipoprotein B.
(†) Calculated from ANCOVA analysis, adjusting for baseline value.
* Denotes statistical significance at $p < 0.05$.

throughout the study and none of the individual daily values reported GI symptoms above 1 (present but well tolerated). No statistically significant changes were seen in happiness, alertness, energy and stress levels throughout the study (Table S7 – S8).

4. Discussion

This double-blind, placebo-controlled study showed that the *L. plantarum* ECGC 13110402 strain is able to safely improve lipid profiles in dyslipidaemic individuals. Study findings suggested improvements in CHD parameters, including total cholesterol, LDL-C, non-HDL-C and apoB after supplementation with *L. plantarum* ECGC 13110402. These findings may be due to increased bile acid deconjugation by this *L. plantarum* strain. Additionally, supplementation did not alter tolerability, mood, vitamin D levels or hepatic parameters. Thus, this study suggests that *L. plantarum* ECGC 13110402 supplementation may be an effective and safe strategy to improve the lipid profiles of dyslipidaemic patients.

Looking at the group difference between the active and placebo groups, adjusting for the baseline for each of the study points, statistically and biologically significant reductions were observed in lipid profiles as early as 3 weeks, and remained statistically significantly reduced in the active group at the end of the intervention (6 weeks). The reductions in TC, LDL-C, apoB and non-HDL-C occurred only in the active group and were directly relevant to the ingestion of *L. plantarum* ECGC 13110402, as no significant differences were noted in lipid parameters between the active and placebo groups once the treatment was stopped (washout). Probiotics rarely colonize the intestine, however this does not impair their effectiveness, they can grow and be metabolically active during intestinal transit (Sanders et al., 2019). As participants were instructed not to change their dietary habits and activity levels during the study, the findings suggest that the impact on lipids was relevant to *L. plantarum* ECGC 13110402 intake, highlighting its potential to enhance the functionality of dietary strategies in supporting CHD risk management.

Several studies have investigated the cholesterol reducing potential of probiotic strains with variable success (Costabile et al., 2017; Fuentes et al., 2013; Jones et al., 2012). Fuentes and co-authors, reported on the cholesterol lowering efficacy of a triple *L. plantarum* formulation in hypercholesterolemic adults and noted reductions of 17.4 and 17.6% for TC and LDL-C respectively (Fuentes et al., 2013). Jones and co-authors, investigated the cholesterol lowering efficacy of *Lactobacillus reuteri* NCIMB 30242, in hypercholesterolemic adults and showed statistically significant reductions of 9.14% in TC, 11.64% in LDL-C, 11.30% in non-HDL-C and 13.39% in apoB relative to placebo (Jones et al., 2012). Most of the studies to date only focused on TC and LDL-C and reported only mild reductions. It was previously reported that *L. plantarum* ECGC 13110402 significantly improved lipid profiles in normal to mildly hypercholesterolemic adults, with the hypercholesterolemic subgroup reporting statistically significant reductions in TC of 36.7% (P=0.0045). However the impact on additional CHD biomarkers was not investigated (Costabile et al., 2017). The findings of this pilot, follow up study show highly statistically significant reductions in multiple CHD risk biomarkers, not previously reported to the author's knowledge at this magnitude of change, for the ingestion of a probiotic.

Reducing cholesterol, specifically LDL-C, is the cornerstone of CHD management worldwide. The use of pharmacological strategies targeting LDL-C has reduced CVD deaths over the past 30 years, however this is not reflected in CHD related mortality. An increasing number of patients are not achieving their LDL-C targets, partly due to a lack of high intensity statin prescriptions, or poor adherence to the prescribed treatment (Caesar et al., 2016; Le Roy et al., 2019). A US-based study, looking at statin use and impact on attaining LDL-C goals, reported that of the 27.4 million adults with elevated LDL-C, more than 60% were not able to attain their LDL-C target (Weng et al., 2010). These findings suggest that there is an unmet need for well tolerated, approaches for cholesterol management, effective on multiple CHD risk biomarkers, to support already existing lifestyle, dietary and pharmaceutical strategies (Hosono, 1999; Lye, Rahmat-Ali et al., 2010).

Blood lipid profiles can be affected both by dietary and lifestyle factors and by lipid metabolism factors of the host, with hyperlipidaemias occurring where there is an imbalance between cholesterol intake and output. It is important to regulate the dietary intake of cholesterol, however, it may not be sufficient to balance the endogenous synthesis of cholesterol in the liver, which significantly contributes to cholesterol input. Cholesterol output takes place through its bioconversion into bile acids in the liver, into insoluble bacterial metabolites in the intestine, but also through the utilisation for cell renewal and steroid hormone and vitamin D synthesis. Over the past decade, the importance of the human gut microbiome in regulating several host metabolic processes that connect the gut with multiple organs including the liver, brain and muscle has been recognized (Kenny et al., 2020; Lew et al., 2018). Recent studies suggest a role for the gut microbiome in host cholesterol homeostasis, through the gut-liver axis, highlighting its

potential as a therapeutic target (Caesar et al., 2016; Le Roy et al., 2019).

Several mechanisms have been described by which gut microbes, including lactobacilli, may interact with the cholesterol cycle in the body. Studies have shown that lactobacilli can incorporate cholesterol into their cell membrane during growth, a process that increases membrane strength and enhances resistance of the cell to lysis (Lye, Rusul et al., 2010). Cholesterol particles can also adsorb onto the cell membrane of some *Lactobacillus* strains, which is then passively removed from the body (Hosono, 1999). However, the mechanisms that are most likely to mediate changes of biological significance to the human host are those that involve bacterial enzymatic processes interacting with host metabolism. Certain gut microbes, including *Lactobacillus* strains, have been reported to express cholesterol esterases, which catalyse the conversion of cholesterol into coprostanol. Coprostanol unlike cholesterol, is poorly absorbed by the human intestine and is then excreted into faeces (Aicha et al., 2019; Kenny et al., 2020). Recent studies indicate a more direct involvement of lactobacilli in host metabolic pathways by influencing expression of genes implicated in cholesterol metabolism in the liver, such as HMG-CoA reductase (Lew et al., 2018), and by interfering in bile acid homeostasis through BSH activity.

BSHs catalyse the hydrolysis of the C24-acyle amide bond of conjugated bile acids, removing them from the enterohepatic circulation, which then forces cholesterol utilization in the liver for *de novo* bile acid synthesis to maintain their levels constant (Begley et al., 2006). Probiotics carrying BSH activity, have been shown to increase intraluminal bile salt deconjugation. Once deconjugated, bile acids are less soluble and they are absorbed in the intestine to be excreted in faeces, or to be further bio-transformed into secondary bile acid metabolites by the gut microbiome (Gérard, 2013; Joyce et al., 2014; Ridlon et al., 2006). *L. plantarum* ECGC 13110402, was selected for demonstrating multiple mechanisms of action for cholesterol reduction *in vitro*, including high BSH activity, high cholesterol assimilation and cell membrane adsorption (Costabile et al., 2017). It can be speculated that the magnitude of cholesterol reduction observed in this study is mainly due to BSH activity and cholesterol assimilation, rather than passive adsorption onto its cell membrane, which is not likely to have comparable biological significance. Through its interaction with host lipid metabolism within the gut-liver axis, *L. plantarum* ECGC 13110402 shows promise in enhancing the functionality of dietary approaches designed to deliver improvements in blood lipid profiles and to support existing strategies for cholesterol management.

Interrupting bile acid recirculation to reduce cholesterol is not a new concept. Bile acid sequestrants, such as cholestyramine, colestipol and colesevelam, have similarities with the bacterial BSH mechanism of cholesterol reduction, as by binding the acids, the drugs prevent their reabsorption in the liver, increasing hepatic demand for cholesterol. Using the maximum daily dose of 24g for cholestyramine, 20g of colestipol or 4.5g of colesevelam, the reduction in LDL-C can range from 18 to 25% (Mazidi et al., 2017). However, GI side effects, even at low doses, and major drug interactions with commonly prescribed drugs, limits their use in clinical practice. The most effective agents to manage LDL-C currently are statins. Moderate statin treatment results in approximately 30% reduction in LDL-C, while high treatment doses can bring about reductions of over 50% (Visseren et al., 2021). Despite the clear benefits, adherence to treatment is poor, partly due to muscle related side effects, but also due to negative coverage by the media (Matthews et al., 2016; Nielsen & Nordestgaard, 2016). A recent UK based study reported that after 6 years, adherence to high intensity statin treatment was down to 72% and for those on low intensity to just 48% (Khunti et al., 2018). Increasing awareness of the limitations of current pharmacological treatments, has led to growing interest in non-drug approaches to improve blood lipid profiles. Following an extensive review of over 80 studies on plant sterols and stanols, the European Food Standards Agency concluded that daily intake of 2.4g can result to a reduction in LDL-C between 7 and 10.5%. The evaluating panel deemed that a reduction of this magnitude in LDL-C is of biological significance, in the context of reducing coronary heart disease risk (Laitinen & Gylling, 2012). The findings of this study suggest that daily intake of *L. plantarum* ECGC 13110402 can result in statistically and biologically significant reductions of 28.4% in LDL-C at the end of the 6-week treatment, in the absence of side effects.

Whilst biologically and statistically significant effects were observed in multiple coronary heart disease markers in this study, it has certain limitations. It was designed as a 12-week interventions, with sampling points at 0, 6, 9 and 12 weeks followed by a 3-week washout. COVID-19 restrictions limited the duration of the study to 6-week treatment followed by a 3-week washout. A longer study, with more sampling points, as initially planned, could have given an indication as to whether effects were cumulative over a longer treatment period, or a peak was reached at 6 weeks. Similarly, COVID-19 restrictions resulted in a reduction in the original volunteer sample size from 50 to 16. Whilst results show statistical significance with a treatment difference at a two-sided 0.05 significance, a larger sample size may be more representative of a hypercholesterolaemic population. Finally, study volunteers were asked not to alter their dietary habits and physical activity patterns during the trial period or to consume prebiotic or probiotic supplements. Whilst this was necessary for study purposes it may not reflect the real-world situation.

In conclusion, the findings of this study suggest that daily intake of *L. plantarum* ECGC 13110402 maybe a well-tolerated, safe and effective means for improving lipid profiles in hypercholesterolaemic adults.

Statement of authorship

A.C., E.K., S.K., designed the study; A.C., E.K., conducted clinical trial; A.C., E.K., conducted the biochemical analyses; E.K. conducted the statistical analysis; E.K. drafted the manuscript; S. K. provided intellectual input. All authors revised and approved the final manuscript.

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Role of the funding sources

OptiBiotix Health provided the active and placebo treatment and was consulted in the initial study design. They were not involved in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Ethics and Dissemination

This study has been granted ethical approval by the University of Roehampton Research Ethics Committee (Reference number: LSC 18/241; approved September 11, 2019) and by the National Health Service Health Research Authority (IRAS project ID: 259363, REC reference: 19/LO/0724, approved August 12, 2019).

Trial Registration Number

This study has been registered with ClinicalTrials.gov Identifier: NCT03540108.

Data availability

Data are available upon reasonable request to the corresponding author.

Declaration of Competing Interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.104939.

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