

Daily administration of *Limosilactobacillus mucosae* DPC 6426 had negligible effects on blood lipids in mildly hypercholesterolaemic adults

Aoife N. O'Donovan · Fiona Fouhy · Ghjuvan Grimaud · Lis E. E. London · Andrea Doolan · Andrea Anesi · Fulvio Mattivi · R. Paul Ross · Noel Caplice · Catherine Stanton

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Abstract

Among the recommendations to prevent heart disease is the maintenance of normal blood cholesterol levels, through the consumption of a cardioprotective diet, and if necessary, the use of therapeutics such as statins. Alterations in the gut microbiota have previously been associated with the development of obesity, insulin resistance and increased risk of developing cardiovascular disease (CVD). Probiotics have since emerged as a potential therapeutic strategy, aimed at reversing the alterations to the gut microbiome. Indeed, we had previously found that ingestion of *Limosilactobacillus mucosae* DPC 6426 had significantly lowered both blood cholesterol and triglyceride levels in a pre-clinical apoE mouse model, as well as modulating the gut microbiota, suggesting strain-specific cardiometabolic potential. To investigate the translational relevance of these findings in humans, we conducted a randomised, double-blind, placebo-controlled clinical trial which assessed the effects of daily administration of the strain at a dose of 3.8×10^9 CFU/per day, in mildly hypercholesterolaemic adults, on blood lipid profiles and the gut microbiota composition. Results demonstrated, despite successful gastrointestinal transit and recovery of *Lactobacillus* spp., that *Limosilactobacillus mucosae* DPC 6426 was not superior to placebo in modifying blood lipid concentrations or the gut microbiota composition in healthy, mildly hypercholesterolaemic adults. Such findings highlight the complexity of translating probiotic-associated metabolic benefits from preclinical apoE mouse models to healthy human participants. Further investigations are warranted to ascertain whether the probiotic dose administered in this study was insufficient to translate from the pre-clinical apoE mouse model in such a human cohort.

Introduction

Cardiovascular disease (CVD) is the leading cause of death and morbidity worldwide, accounting for 32% of all global deaths in 2022, with 85% of these deaths attributed to heart attack and stroke^{1,2}. It has been reported that elevated low-density lipoprotein (LDL) cholesterol is a major contributor to myocardial infarction in Europe³. Those experiencing hypercholesterolemia are three times more susceptible to MI when compared with those with

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normal blood lipid profiles. Consumption of an unhealthy diet (high in fat, salt and sugar, and low in complex carbohydrates, fruits and vegetables), lack of physical activity, smoking and excessive, harmful use of alcohol are associated with the development of CVD. Other factors of relevance for the development of CVD include high blood pressure, dyslipidaemia, diabetes, obesity and older age⁴.

Among the many recommendations to avoid heart disease, is the maintenance of normal blood cholesterol levels. This can be done through the consumption of a cardioprotective diet, either as an adjunct to medication therapy, such as statins or independently in patients where medications may be poorly tolerated, too expensive or ineffective⁵. Low consumer compliance with dietary recommendations and the expense and side effects of drug therapy for many consumers, coupled with increased consumer acceptance of foods with additional health benefits have provided an opportunity for the use of functional foods in heart health.

Those with hypercholesterolemia may be able to avoid the use of cholesterol-lowering medications through implementing changes to their dietary habits or through consumption of probiotics, prebiotics or synbiotics (combination of pro- and prebiotics). Probiotics have been defined by the FAO/WHO as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host”⁶ and prebiotics have been defined by The International Scientific Association for Probiotics and Prebiotics (ISAPP) as ‘a substrate that is selectively utilised by host microorganisms conferring a health benefit’⁷.

It has previously been noted that changes in the human intestinal microbiota have been implicated in the development of obesity⁸, insulin resistance⁹ and CVD¹⁰. It has also been shown that probiotics, aside from improving gut health, demonstrate other health-promoting properties such as improving the immune system, reducing hypertension, exerting antioxidative effects, relieving symptoms of dermatitis, facilitating mineral absorption, relieving allergy symptoms¹¹, as well as being studied for their potential to reduce cholesterol^{12,13}.

Heart health protective bioactive components of food that have recognised cholesterol-lowering effects include phytochemicals and soluble dietary fibre. In a previous study, a novel, soluble dietary fibre (exopolysaccharide (EPS)) producing *Lactobacillus* strain (*Limosilactobacillus mucosae* DPC 6426), originally isolated from bovine faecal samples at Teagasc, Moorepark Food Research Centre, Fermoy, Cork, was identified by our group¹⁴. The strain when administered orally, at a concentration of 1×10^9 CFU/day for 12 weeks, caused a ~50% reduction in serum total cholesterol levels and a 15% reduction in serum triglycerides in a pro-atherogenic apoE mouse model when compared with un-supplemented control treatment. Furthermore, dietary intervention with *Lb. mucosae* DPC 6426 resulted in a significantly higher ratio of high-density lipoprotein (HDL)-cholesterol to total cholesterol in the serum and liver while significantly increased levels of faecal cholesterol excretion were associated with daily administration of *Lb. mucosae* DPC 6426 compared with un-supplemented controls¹⁵. The group has also undertaken a pilot study with 10 mildly hypercholesterolaemic (≥ 5 mmol/L and < 7.5 mmol/L) male adults, who were administered $\sim 10^{10}$ CFU viable *Lb. mucosae* DPC 6426 daily for 6 weeks in a low-fat fermented dairy beverage, which resulted in an increase ($p \leq 0.01$) in ratio of HDL to total cholesterol from start to the end of the study, providing first in man (safety and potential for efficacy) data for this strain (data unpublished).

Additionally, *Lb. mucosae* DPC 6426 was compared against plant sterol esters, oat beta-glucan and *Lb. reuteri* NCIMB 30,242 (10^9 CFU/day, CardioViva) and was found to be more effective than oat beta-glucan and *Lb. reuteri* NCIMB 30,242 (10^9 CFU/day, CardioViva) and was found to be similar to plant sterol esters in terms of its effects on serum cholesterol reduction¹⁶.

The aims of the current study were to investigate the efficacy of daily oral supplementation of *Lb. mucosae* DPC 6426 for 12 weeks on reducing plasma cholesterol levels, as well as LDL-cholesterol, HDL-cholesterol and triglyceride concentrations in healthy mildly hypercholesterolaemic (≥ 5.5 mmol/L and < 8 mmol/L) adults, compared to placebo control, with a minimum of 41 participants per group. *Lb. mucosae* DPC 6426, was given at a minimum concentration of $\sim 10^9$ CFU/day (3.8×10^9 CFU/per day), for 12 weeks, in a randomised, double-blind, placebo-controlled clinical trial. In addition, alterations in the gut microbiome were investigated and compared between the placebo and treatment groups at baseline (week 0) and end of the study (week 12). In particular, we

wanted to investigate whether the ingestion of EPS had comparable same improvements in lipid metabolism in mildly hypercholesterolaemic humans as was seen in the previous apoE mouse model.

Materials and methods

Ethical approval was obtained from the Cork University Hospital Research Ethics Committee for the Clinical Trial (Reference Number AFCRO-059/CREC Reference: ECM 4 (nn) 13/10/15). All methods were carried out in accordance with relevant guidelines and regulations, including the CONSORT statement and applicable institutional, national and international standards for clinical research. All experimental protocols were approved by The Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork. Additionally, informed consent was obtained from all participants involved in the clinical trial.

The trial is registered on the clinicaltrials.gov database (identifier: NCT07519967) and was registered on the 4th of December 2025. Eligibility criteria for inclusion in the clinical trial, the trial protocol, statistical analysis plan, and individual data and data dictionary are provided in the supplementary materials.

Inclusion and Exclusion Criteria

In order to be considered eligible for enrolment in the study, participants must have been able to give written informed consent, be between 20 and 70 years of age, have mild hypercholesterolaemia, with a total cholesterol level of between >5.5 mmol/L and <8 mmol/L, have a BMI of between 18.5 and 32 kg/m², have had a stable body weight ($\leq 5\%$) over the 3 months prior to the study commencement and be in general good health. Participants were excluded from the study if they were less than 20 or greater than 70 years of age, females who were pregnant, lactating or wishing to become pregnant during the study, a smoker, were hypersensitive to any of the components of the test product, had a significant acute or chronic, unstable and untreated disease or any condition which contradicted the entry to the study, was taking a probiotic or prebiotic product/supplement or had taken them within two weeks of the screening visit, had received a blood transfusion within the previous six months, had taken antibiotics within the previous three months, had a history of drug and/or alcohol abuse at the time of enrolment, had a Gamma-GT outside of the normal range, had an active gastrointestinal disorder or had undergone previous gastrointestinal surgery, had a condition or were taking medication that the investigator believed would interfere with the objectives of the study, pose a safety risk or confound the interpretation of the study results (including statins, cholesterol absorption inhibitors, bile acid sequestrants and non-steroidal anti-inflammatory drugs), continued/daily use of probiotics or probiotic-containing products within one month prior to the study randomisation, currently or had taken cholesterol lowering supplements in the month prior to the study commencement, had not made any major dietary changes in the 3 months prior to the study commencement, had a history of illicit drug use, individuals who, in the investigators opinion, would have been considered to be poor attendees or unlikely for any reason to be able to comply with the trial, individuals who were receiving treatment with experimental drugs, and if they were involved in a recent experimental trial, they must have been completed no less than thirty days prior to this study, and had a malignant disease or any concomitant end-stage organ disease.

Probiotic formulation, manufacture and packaging

Food grade powder containing concentrated freeze-dried *Lb. mucosae* DPC 6426 and maltodextrin, was used to produce capsules containing a minimum concentration of 10^9 CFU/g *Lb. mucosae* DPC 6426 (treatment). The placebo freeze-dried powder contained 0 CFU/g *Lb. mucosae* DPC 6426 in maltodextrin. Both products were produced by Probiotal S.p.A (Via E. Mattei, 3 28,100 Novara, Italy).

Enumeration of *Lb. mucosae* DPC 6426 in capsules

Capsules were removed from storage and weight was recorded. An adequate amount of sterile Maximum Recovery Diluent ((MRD), Sigma Aldrich, Germany) was added to reach a 1:10 dilution (i.e. to a 0.38 g capsules, MRD was added to reach 3.80 g). A dilution series, from neat to 10^{-8} was carried out and the 10^{-7} and 10^{-8} dilutions were plated in duplicate on de Man, Rogosa, and Sharpe ((MRS), Sigma Aldrich, Germany) and incubated anaerobically, in an anaerobic jar with an Oxoid AnaeroGen anaerobic gas generating sachet (ThermoFisher Scientific, Ireland), at 37 °C for 72 h. Following incubation, plates were enumerated, and CFUs were calculated and recorded.

Study design and sampling

To calculate the sample size required for this study, it was assumed that two populations would be tested for a statistically significant difference between the means of the two groups, using the one-tailed unpaired t-test. With the expected mean difference of 0.42 and standard deviation of 0.77, based on a previous pilot study (data unpublished), the level of significance of 2.5% and the power of 80, the total sample size is $n=81.81$. It was assumed for the sample size calculation that the samples are equal in size, resulting in the individual samples being about 81.8/2 or 40.9 in size, therefore resulting in a minimum of 41 participants per group. A total of 174 participants were initially screened, both male and female, aged between 20 and 70 years, with mildly hypercholesterolaemic blood lipid levels (≥ 5.5 mmol/L and < 8 mmol/L), where the inclusion and exclusion criteria were reviewed and blood pressure, body weight and body mass index (BMI) were measured. Out of those 174 screenings, 94 were found to be eligible and were randomised into the study for evaluation of cholesterol-lowering effects of the probiotic bacterial strain *Lb. mucosae* DPC 6426 compared with the placebo. Out of the 94 randomized subjects, 94 were included in the Safety population, 93 in the ITT population and 84 in the PP population. There were 7 dropouts from the study: 6 due to the Adverse Events, and 1 subject lost interest. The clinical trial was conducted at Atlantia Food CRO, Cork, Ireland. The study involved five visits over a total of 18–19 week period. Figure 1 provides a summary of the study, and a detailed summary of the assessments that were carried out at each study visit.

This study is a randomised, double-blind, placebo-controlled, parallel study. Randomisation was stratified based on the subject's gender, where 50% of the participants would be male and 50% would be female and for BMI (< 25 kg/m² and > 25 kg/m²). With respect to the blinding of the trial, all those involved, including participants, the research team, the data analysts and statistician were blinded. The random allocation sequence was generated by an independent statistician using the randomisation function in SPSS split by strata and block.

In order to determine the study sample size, two populations were tested for a statistically significant difference between the means, using the one-tailed unpaired t-test. For the sake of simplicity, it was assumed that the groups were of the same size ($n_1 = n_2$) and that the standard deviations were the same ($\sigma_1 = \sigma_2 = \sigma$). The mean difference between the two populations is taken as $\mu_1 - \mu_2$. The power is normally given as 0.8 or 80% and the level of significance is α . Leaving $n = n_1 + n_2$. The objective was to determine the desired total sample size, n . The following simplified and approximate formula was used for sample size calculation.

$$n = \left[\frac{2(z_{power} + z_{1-\alpha})}{\frac{2(\mu_1 - \mu_2)}{\sigma}} \right]^2$$

where $z_{1-\alpha}$ signifies the $1-\alpha$ quantile of the standard normal distribution. To determine the sample size for the unpaired t-test, α in this equation was simply replaced by $\alpha/2$. The above equation was used to determine the sample size for this study with an expected mean difference of 0.42 and an expected standard deviation of 0.77 (based on previous pilot study). The single tailed unpaired t-test was used, with the level of significance of 2.5% and the power of 80%. According to statistical tables, $z_{0.8} = 0.8416$ and $z_{0.975} = 1.96$. These values were inserted

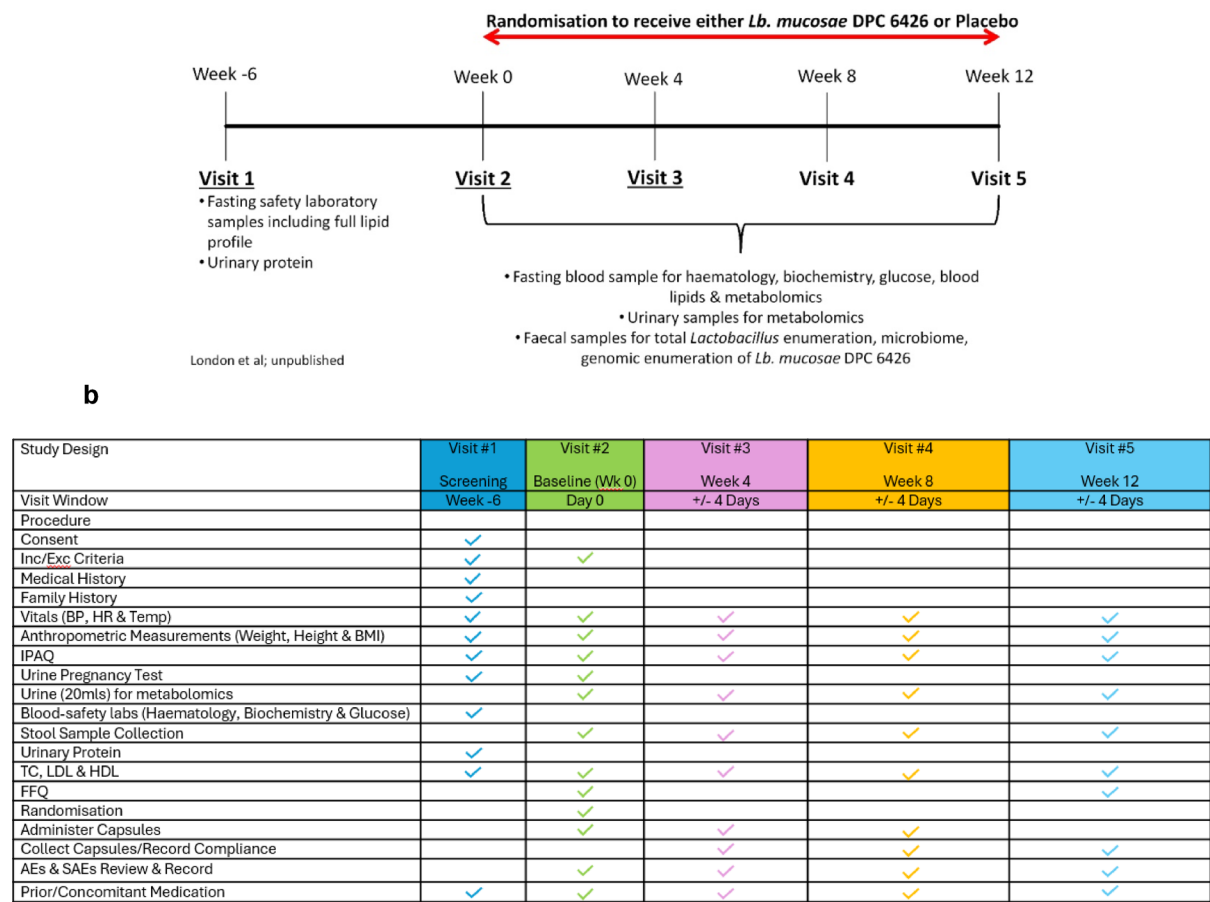


Fig. 1 (a) Summary of study design, (b) detailed summary of assessments at each study visit.

into the above equation, they gave the total sample size as follows: $n=81.81$ It has been assumed for the calculation that the samples were equal in size. The individual samples should then be about $81.8/2$ or 40.9 in size. This means that a minimum of 41 subjects were needed in each group.

Treatments administered

Participants were randomised to one of two treatment groups, *Lb. mucosae* DPC 6426 or placebo. Participants took their first dose (*Lb. mucosae* DPC 6426 or placebo) at the study site and were instructed to take one capsule daily, with a glass of water (approximately 200 ml) at the same time each day for the duration of the study. In order to conceal the interventions administered, sequentially numbered containers were used to implement the random allocation sequence. Additionally, personnel involved in the trial, including involvement in the enrolment and assigning of participants, did not have access to the random allocation sequence. With respect to the blinding of the trial, after the assignment of interventions, all those involved, including participants, the research team, the data analysts and statistician, were blinded, and this was achieved through the use of identical packaging and the products administered were similar in appearance.

In order to define any harms, such as systemic and non-systemic harms, participant vitals, safety labs and adverse events were recorded and reviewed by a clinician.

Compliance monitoring

In order to monitor compliance and non-compliance throughout the course of the study, deviations and reasons for deviations were recorded at each study visit, and a log of deviations was kept for the duration of the study (Supplementary Table 1).

Blood measurements

A fasting blood sample was collected at each visit of the study. These samples were collected following an overnight fasting period of at least 10 h, where participants were required to avoid all foods and drinks, aside from water. At the screening visit (Visit 1), 10 ml of blood were collected for haematology, and biochemistry tests (TC, LDL-cholesterol, HDL-cholesterol, triglycerides and glucose). At all following visits, 12mls of blood were collected and TC, LDL-cholesterol, HDL-cholesterol and triglycerides were measured. Analysis of blood samples was performed by Eurofins Biomnis, Ireland.

Stool collection for microbiome analysis

Stool collection and storage: Participants were provided with a stool collection kit and were instructed to collect a sample at home. Participants were instructed to urinate prior to collecting the stool sample, and in order to avoid the stool sample coming into contact with the toilet water, which would cause degradation of the sample, to remove the lid of the container and place the container into the toilet bowl. Once participants had completed their bowel movement, they were instructed to open the anaerobic sachet attached to the lid of the stool sample collection container before closing the container and to write the date and time on the container and keep the sample refrigerated. They were instructed to bring the sample to their next visit, and this was refrigerated until processing (less than 24 h).

DNA extraction

Bacterial genomic DNA was extracted from 0.25 g of faecal material using the repeated bead-beating plus column (RBB+C) protocol as described by Yu and Morrison¹⁷ in combination with the QIAamp DNA Stool Mini Kit (Qiagen, UK) described by Fouhy et al.¹⁸ with slight modifications: 1.0 mm and 2.3 mm sterile zirconia beads were used during the homogenisation step instead of 0.5 mm sterile zirconia beads to avoid sedimentation of the 0.1 mm beads and the faecal material. Cell lysis was achieved through bead-beating in conjunction with the use of 1 ml of lysis buffer containing 4% (w/v) sodium dodecyl sulphate (SDS), 500 mM NaCl and 50 mM EDTA. Samples were homogenised for 3 min at max speed (3800 strokes/min) using the Mini Beadbeater (BioSpec). Following homogenisation, samples were incubated at 70 °C for 15 min to lyse the cells. Samples were then centrifuged at 16,000 × g and the supernatant was removed, the bead beating steps were repeated with 300 µl of lysis buffer. Once supernatant was pooled, samples were treated with 10 M ammonium acetate (Sigma Aldrich, Ireland) and the DNA was pelleted, through centrifugation at 16,000 × g for 10 min. The DNA was precipitated using isopropanol (Sigma Aldrich, Ireland), centrifuged at 16,000 × g for 15 min and washed with 70% ethanol. The pellet was allowed to dry and was then re-suspended in 200 µl of TE buffer. The re-suspended DNA pellet was then treated with RNase and proteinase K, combined with 37 °C for 15 min and 70 °C for 10 min heating steps. The DNA was cleaned with Qiagen AW1 and AW2 buffers using a Qiagen column (QIAamp DNA Stool Mini Kit, Qiagen, UK). The DNA was eluted using 200 µl of Buffer AE (Qiagen, UK) and stored at −80 °C.

16S rRNA gene sequencing and analysis

The V3-V4 regions of the 16S rRNA gene were amplified through 30 PCR reactions on the template DNA using the primer pair ‘5-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGC AG-3’ and ‘5-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC-3’ according to the 16S Metagenomic Sequencing preparation protocol for the Illumina Miseq and as previously described by Fouhy et al. (2015). Indexed PCR products were visualised using gel electrophoresis and cleaned using AMPure XP magnetic beads before the DNA was quantified using the Qubit (BioSciences, Dublin, Ireland) combined with the Qubit High Sensitivity DNA Kit (Life Technologies). Samples were then pooled at equimolar concentrations, and high-throughput sequencing was performed on the Illumina MiSeq platform in the Teagasc sequencing facility using a 2 × 300 kit and following standard Illumina sequencing protocols.

Bioinformatic analysis

Raw paired-end 16S rRNA gene sequencing reads were processed using the mbtools R package (v0.0.13; Gibbons Lab, <https://github.com/Gibbons-Lab/mbtools>). Reads were quality-filtered and trimmed by removing the first 10 bases and truncating forward and reverse reads to 240 and 180 base pairs, respectively, to exclude low-quality regions. Amplicon sequence variants (ASVs) were inferred using the DADA2 algorithm as implemented within mbtools, with default parameters. A total of 25,876 ASVs were initially identified, of which 20,396 (79%) were removed as chimeras, resulting in 5,480 high-confidence ASVs for downstream analysis. Taxonomic classification was performed using the DECIPHER IdTaxa classifier with the SILVA v132 reference database, automatically retrieved by mbtools. The ASV table was converted into a phyloseq object, and taxonomic annotations and ASV abundances were exported for statistical analyses. All processing was performed in R (v4.0.3).

Differential abundance analysis was conducted using Songbird (v1.0.3), applying a multinomial regression model to ASV-level data. The model used Group2 as the covariate, with Control_V2 specified as the reference category. Training was run for 10,000 epochs with a differential prior of 0.5 and a minimum sample count of 1. Differential rankings from Songbird were visualized in R by extracting the top and bottom features associated with each treatment group relative to the Control_V2 reference. Differential coefficients were visualized as bar plots, where positive values indicate enrichment in the treatment group and negative values indicate depletion, using ggplot2.

Statistical analysis of the microbiome was carried out using GraphPad Prism (version 9.1.0), with a cut off p-value of 0.05 for significance. Where possible, statistical analysis of changes over time, resulting from diet, took the subject numbers into account, and facilitated alpha diversity linear modelling.

To assess the alpha diversity of the samples, Shannon index was calculated (Supplementary Fig. 1). Multiple beta diversity metrics were also calculated, including weighted and unweighted UniFrac PCoA plots, in which these plots were used to highlight the participants based on the treatment they received, gender and by sample time point (Supplementary Figs. 2 and 3).

Enumeration of *Lactobacillus* in stool samples

For each stool sample collected at every visit, 1 g of faecal sample, from the middle of the sample (to capture as many anaerobes as possible) was mixed with 9 ml of sterile Maximum Recovery Diluent ((MRD), Sigma Aldrich, Germany) and placed in the stomacher machine (Seward Stomacher 400 Circulator Paddle Blender) at 8 × g for 4 min. Following stomaching, a 1 in 10 dilution, with 1 ml of sample in 9 ml of MRD, was performed and 100 µl of sample was plated on *Lactobacillus* Selection (LBS) agar in duplicate from neat to 10⁻⁶ dilution. Plates were incubated anaerobically in an anaerobic jar with an Oxoid AnaeroGen anaerobic gas generating sachet, at 37 °C for 48 h and enumeration of *Lactobacillus* colonies was performed using the Stuart® Digital Colony Counter (Cole-Parmer, United Kingdom) and CFU/ml was calculated and recorded.

Plasma and urine metabolite analysis

Metabolomics analysis was performed on both plasma and urine samples from each study visit for both treatment and placebo groups as described by Anesi et al.¹⁹ and reported in Anesi et al.²⁰.

Statistical analysis

Statistical analysis was carried out by StatisticaMedica, Dublin, Ireland using statistical software Rx64 version 3.3.1. In the primary efficacy analysis, an unpaired two-tailed t-test was performed to test the difference in the total cholesterol levels from baseline to week 12 between the two treatment groups. In the secondary analysis, an unpaired two-tailed t-test was performed to test the difference between the two treatment groups for changes in the total cholesterol from baseline to weeks 4 and 8, LDL-cholesterol, HDL-cholesterol and triglycerides concentrations from baseline to weeks 4, 8 and 12. The last observation carried forward (LOCF) was applied to the Intention-to-Treat (ITT) populations and safety analysis was presented as the Clopper-Pearson Confidence Intervals. The data collected in the case report form (CRF), but was not used in the Efficacy analysis, was reported using the descriptive summaries and the frequency distributions where applicable. Scores for the Food Frequency Questionnaire (FFQ) and The International Physical Activity Questionnaire (IPAQ) were calculated using corresponding guidelines and descriptive summaries were reported (data not shown).

Results

In this study, we investigated the efficacy of daily oral supplementation of *Lb. mucosae* DPC 6426 for 12 weeks on blood cholesterol levels in healthy mildly hypercholesterolaemic (≥ 5.5 mmol/L and < 8 mmol/L) adults – a study that was prompted by very promising pre-clinical results¹⁵. The trial commenced with the first participant, first visit on the 30th of May 2016 and concluded with the last participant, last visit on the 18th of January 2017. Data of serum lipid profiles (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides) indicated no significant changes between baseline and end of the study following 12 weeks between the placebo and treatment groups. The average recovery of total *Lactobacillus* was 1.26×10^7 CFU/g faeces for the treatment group and 1.31×10^7 for the placebo group. After 12 weeks of supplementation, we found that there was no distinct separation in the gut microbiota composition, as well as no significant differences at phylum, family and genus levels between the placebo and treatment groups. Table 5 in supplementary material outlines the average CFU/g counts for total *Lactobacillus* for both the treatment and placebo at V2 (baseline) and V5 (study end), whilst Fig. 5 illustrates the average CFU/g counts for total *Lactobacillus* for both the treatment and placebo at V2 (baseline) and V5 (study end).

Treatment and control products were administered to the participants by the research team. Compliance was assessed, but was not reported in the SSR, and was recorded in the AF059 Data Entry.

Serum lipid profiles

The primary efficacy analysis was the change in total cholesterol from baseline to visit 5 (12 weeks of treatment). Table 1 shows the results of this analysis for the ITT population. Cholesterol levels at baseline were slightly higher in the placebo group compared to the treatment group, however, this difference was not statistically significant ($p=0.054$). The changes observed in the difference between the values of the placebo group and treatment group at baseline and after 12 weeks of treatment (visit 5) were 0.24 mmol/L and this was also not statistically significant ($p=0.148$). These findings are similar to those of the Per Protocol (PP) population, with the difference between groups being 0.186 mmol/L, which was also not significant ($p=0.29$). There were no statistically significant changes between Placebo and Active groups observed from baseline to Visit 5, baseline to Visit 4 or

Table 1 Primary Efficacy Analysis; Change in Total Cholesterol for ITT Population.

	N	Mean (SD)	Median	Min,Max	1Q,3Q	Missing
Baseline (V2) Placebo	46	6.42 (0.8)	6.25	4.9,8.2	5.8,7.17	NA
Active	47	6.08 (0.87)	6	3.9,8.2	5.6,6.4	NA
<i>p</i> -value=	.054					
End of Treatment Placebo	46	6.43 (0.78)	6.3	5.1,8.5	5.9,6.9	NA
(V5) Active	47	6.33 (0.74)	6.3	4.7,8.8	5.85,6.65	NA
Difference Active – Placebo	0.24					
<i>p</i> -value=	0.148					

baseline to Visit 3 for total cholesterol, LDL-cholesterol, HDL-cholesterol or total triglycerides. Results for the PP population are shown in Table 2.1 in the supplementary materials.

The results of the secondary outcomes for the change in total cholesterol concentration showed no statistically significant differences between the treatment and placebo groups from baseline to visit 3 ($p=0.197$; $p=0.297$) or baseline to visit 4 ($p=0.51$; $p=0.804$) in the ITT and PP populations, respectively. Results for the secondary outcomes for the change in total cholesterol concentration in the ITT and PP populations are shown in supplementary Table 2.2.ITT and Table 2.3.PP in the supplementary materials, respectively.

The results for the secondary outcomes for the change in LDL-cholesterol concentration showed no significant differences between the treatment and placebo groups from baseline to visit 3 ($p=0.154$; $p=0.276$), baseline to visit 4 ($p=0.495$; $p=0.808$) or baseline to visit 5 ($p=0.194$; $p=0.437$) in either the ITT or PP populations, respectively. Results for the secondary outcomes for the change in LDL-cholesterol concentration in the ITT and PP populations are shown in Table 2.4.ITT and Table 2.5.PP in the supplementary materials, respectively.

The results for the secondary outcomes for the change in HDL-cholesterol concentration showed no significant differences between the treatment and placebo groups from baseline to visit 3 ($p=0.962$; $p=0.756$), baseline to visit 4 ($p=0.769$; $p=0.782$) or baseline to visit 5 ($p=0.86$; $p=0.511$) in either the ITT or PP populations, respectively. Results for the secondary outcomes for the change in HDL-cholesterol concentration in the ITT and PP populations are shown in Table 2.6.ITT and Table 2.7.PP in the supplementary materials, respectively.

The results for the secondary outcomes for the change in triglyceride concentration showed no significant differences between the treatment and placebo groups in the change in triglyceride concentrations from baseline to visit 3 ($p=0.255$; $p=0.202$), baseline to visit 4 ($p=0.805$; $p=0.838$) or baseline to visit 5 ($p=0.818$; $p=0.797$) in either ITT or PP populations, respectively. Results for the secondary outcomes for the change in triglyceride concentrations in the ITT and PP populations are shown in Table 2.8.ITT and Table 2.9.PP in the supplementary materials, respectively.

Safety outcomes

Throughout the study, there was only one SAE reported. It was reported that subject 059–105 in the placebo group had a broken ankle, and although it did occur during the course of the study, it was labelled as unrelated to the study product and was resolved with therapy after a two week period.

Table 4 summarises a total of 61 adverse events (AEs) which were reported throughout the study, whilst Table 4 in the supplementary material outlines in more detail the AEs and subsequent actions taken. Table 3 in the supplementary material outlines the primary safety analysis, using Clopper-Pearson Confidence Intervals and Fisher's Exact/Chi-squared test, of AEs and TEAEs reported throughout the study. There were no significant differences in TEAEs reported between the Placebo and Active group ($p=0.516$). Further, there were no significant differences in intensity ($p=0.681$), relationship to product ($p=0.060$), action taken ($p=0.598$), outcomes ($p=1.000$) or serious adverse events ($p=0.491$).

Targeted quantification of 22 metabolites in plasma samples

Plasma samples were extracted according to the validated protocol by Anesi et al.^{19,20}. For each participant, the first (V2, T0) and last (V5, 12 weeks of treatment) time points were selected, however for some participants V2 time points were not available and therefore the first time point available e.g. V3 (4 weeks of treatment) was used instead. Missing values (or zero values) were replaced with an arbitrary number equal to 1/10 of the minimum value for each multivariate metabolite. Following that, data were transformed (expressed as μmol) as $\log_2$ and the difference between V5 and baseline was calculated, therefore ending up with one single point for each participant. Multivariate data analysis of both plasma and urine metabolites was performed using MetaboAnalyst 4.0 software (<https://www.metaboanalyst.ca>); no filtering, transformation and scaling were performed. Principal Component Analysis (PCA) score plots for plasma metabolites are reported in Fig. 2a. Despite the 46.3% of variation, explained by PC1 and PC2, there was no clear distinction between the treatment and placebo groups. Partial Least Square-Discriminant Analysis (PLS-DA) (Fig. 2b) was also performed, but the model was not statistically significant, ($p=0.66$ following permutation test with 2000 permutations (Fig. 2c).

Volcano plots in Fig. 2e–i visualise the statistical significance (p -value, Y axis) versus the fold change (X-axis). Figure 2e–h shows four metabolites, kynurenic acid, xanthurenic acid, kynurenine and tyrosine, which were significantly higher in the placebo (left) group compared to the treatment group (right). L-valine, highlighted

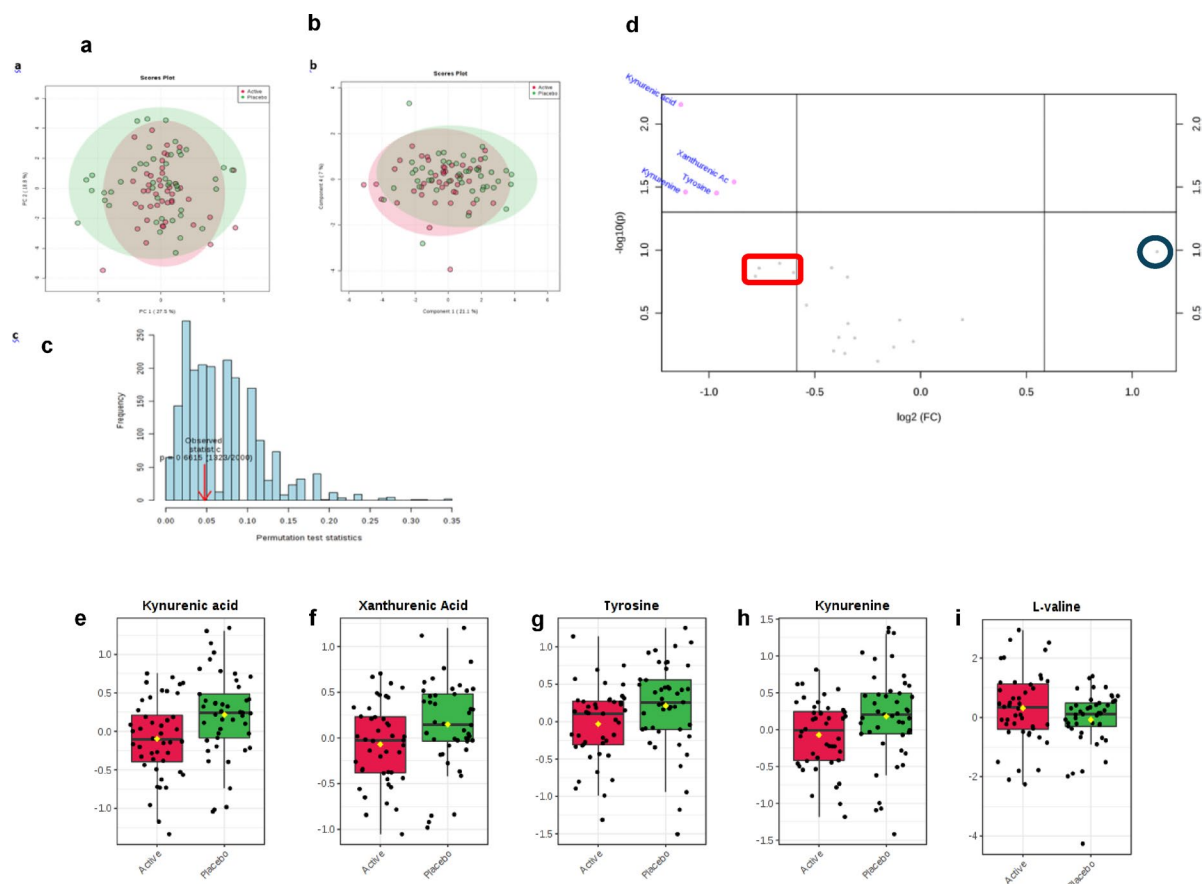


Fig. 2 Plasma metabolites analysis (a) PCA score plot, (b) PLS-DA score plot of plasma samples (active (treatment) – red, placebo – green) and (c) Permutation test statistic, (d) volcano plot of plasma sample analysis; P -value threshold: 0.05; fold change: 1.5. (e–i) box plots of selected plasma metabolites; (e–h) which were significantly higher in the placebo group (right) compared to treatment group (left), (i) Box-plot of L-valine which was > 1.5 fold higher in the active group compared to the placebo, not significant (p -value > 0.05).

by the blue circle (Fig. 2d), was >1.5 fold higher in the treatment group but was not statistically significant (p -value >0.05). Similarly, indole-3-carboxaldehyde, indole-3-lactic acid, tryptophan and L-isoleucine displayed a fold change of >1.5 in the placebo group but were not statistically significant (p -value >0.05).

Table 2 provides a summary of the plasma biomarkers detected during analysis, where data are expressed as mean $\pm$ SEM (median (IQR)). Data were analysed using linear mixed (normally distributed data) or generalised linear mixed models; p -value shown in metabolite column depicts treatment*time interaction, p -value in the same row depicts the difference between the two groups at a specific time point. * in the same column within the metabolite depicts the differences between time points within the same group. Treatment: n: baseline (V2)=47, 4-weeks (V3)=28, 8-weeks (V4)=44 and 12-weeks (V5)=42. Placebo: n: baseline (V2)=45, 4-weeks (V3)=26, 8-weeks (V4)=43 and 12-weeks (V5)=41. Graphical representations of the concentrations of plasma biomarkers are presented in supplementary material (Supplemental Fig. 4).

Targeted quantification of 30 metabolites in urine samples

Urine samples were prepared according to the validated protocol by Anesi et al.^{19,20} and data analysis was performed as described above for the targeted quantification of plasma metabolites. PCA score plot (Fig. 3a) showed no clear separation between the treatment and control groups, similar to what was described for plasma samples. Total variation, explained by PC1 and PC2, was 73%. PLS-DA (Fig. 3b) was also performed, but the model was not statistically significant, as shown by a resulting p -value of 0.385 following permutation test (Fig. 3c). Normalisation of the urine volume was not performed as the data were not available.

Volcano plots in Fig. 3d show that no metabolite was statistically different between the two groups. However, three metabolites, indole-3-acetic acid, indole-3-acetamide and 3-hydroxyanthranilic acid, highlighted by the red rectangle (Fig. 3d), were higher in the placebo group compared to the treatment group but were not statistically significant (see Fig. 3e–g. Correction for urine volume may however increase the chance of detecting differences between the groups.

Table 3 provides a summary of the urine biomarkers detected, in which data are expressed as mean $\pm$ SEM. Data were analysed as described above for plasma biomarkers. Treatment: n: baseline (V2)=45, 4-weeks (V3)=18, 8-weeks (V4)=45 and 12-weeks (V5)=44. Placebo: n: baseline (V2)=45, 4-weeks (V3)=20, 8-weeks (V4)=42 and 12-weeks (V5)=39. Graphical representations of the concentrations of urine biomarkers are presented in supplementary material (Supplemental Fig. 5).

Enumeration of faecal bacterial counts from individual participants confirmed gastrointestinal transit and recovery of total lactobacilli. Average recovery of total *Lactobacillus* throughout the trial was 1.26×10^7 CFU/g faeces for the treatment group and 1.31×10^7 for the placebo group, confirming that treatment with the probiotic did not change the average total recovery of total *Lactobacillus* between the 2 groups. However, there was a significant difference in CFU/g faeces between the baseline and visit 5 for the treatment group, with baseline producing an average recovery of total lactobacilli of 1.37×10^6 CFU/g faeces, while the average recovery for total lactobacilli was 1.26×10^7 CFU/g faeces at visit 5, i.e. following 12 weeks of dietary intervention. This difference of ~ 1 log CFU of total lactobacilli between baseline and visit 5 was a direct result of the oral supplementation with *Lactobacillus mucosae* DPC 6426 at a dose of 3.8×10^9 CFU/per day for 12 weeks..

Taxonomy-based analysis of the gut microbiome showed that at baseline (V2), the gut microbiota of participants in both placebo and treatment groups were dominated by *Firmicutes* and *Bacteroidetes* (Fig. 4a) which is consistent with previous findings^{21,22}. The most abundant families were *Lachnospiraceae*, *Streptococcaceae* and *Bacteroidaceae* (Fig. 4b); and the most abundant genera were *Bacteroides*, *Faecalibacterium*, *Agathobacter* and *Subdoligranulum* (Fig. 4c). However, daily supplementation with *Lb. mucosae* DPC 6426, at a minimum concentration of 10^9 CFU/g, (3.8×10^9 CFU/per day), did not significantly alter the relative abundances of the gut microbiota between baseline (V2) and V5 of the participants in the treatment group and appeared similar to that of the participants in the placebo group. Only one individual in the treatment group seemed to respond with a higher relative abundance of *Lactobacillus* sp. at V5 (individual T109, Wilcoxon test, $p < 0.001$). Alpha-diversity

Table 2 Concentrations of Plasma Biomarkers.

Metabolite	Treatment	Placebo	<i>P</i> -value
Methionine			0.03
Baseline (V2)	10.47 ± 0.41	10.06 ± 0.35	
4-weeks (V3)	11.44 ± 0.74	10.35 ± 0.53	
8-weeks (V4)	9.73 ± 0.41*	11.13 ± 0.44	0.02
12-weeks (V5)	11.03 ± 0.38*	11.15 ± 0.51	
L-valine			0.21
Baseline (V2)	58.54 ± 4.59	63.31 ± 3.29	
4-weeks (V3)	66.25 ± 5.81	67.72 ± 5.19	
8-weeks (V4)	56.7 ± 3.96	67.61 ± 3.28	
12-weeks (V5)	66.72 ± 4.09	62.38 ± 4.22	
Quinolinic acid			0.59
Baseline (V2)	0.47 ± 0.03	0.38 ± 0.02	
4-weeks (V3)	0.42 ± 0.03	0.37 ± 0.04	
8-weeks (V4)	0.4 ± 0.03	0.38 ± 0.04	
12-weeks (V5)	0.36 ± 0.03	0.4 ± 0.04	
Isoleucine			0.54
Baseline (V2)	26.39 ± 1.48	24.18 ± 1.1	
4-weeks (V3)	27.89 ± 2.4	27.34 ± 1.66	
8-weeks (V4)	24.15 ± 1.05	25.47 ± 1.01	
12-weeks (V5)	26.08 ± 1.21	27.61 ± 1.48	
Leucine			0.12
Baseline (V2)	52.29 ± 2.36	48.75 ± 1.68	
4-weeks (V3)	54.28 ± 3.59	52.34 ± 2.52	
8-weeks (V4)	47.95 ± 1.76	51.86 ± 1.71	
12-weeks (V5)	52.58 ± 1.97	53.23 ± 2.29	
Dopamine			0.41
Baseline (V2)	0.04 ± 0	0.04 ± 0	
4-weeks (V3)	0.04 ± 0	0.03 ± 0	
8-weeks (V4)	0.04 ± 0	0.03 ± 0	
12-weeks (V5)	0.04 ± 0	0.04 ± 0	
Serotonin			0.51
Baseline (V2)	0.05 ± 0.03	0.13 ± 0.04	
4-weeks (V3)	0.08 ± 0.05	0.02 ± 0.01	
8-weeks (V4)	0.02 ± 0	0.02 ± 0.01	
12-weeks (V5)	0.05 ± 0.02	0.04 ± 0.01	
Tyrosine			0.11
Baseline (V2)	38.05 ± 1.58	35.47 ± 1.6	
4-weeks (V3)	37.76 ± 2.74	38.38 ± 2.35	
8-weeks (V4)	35.27 ± 1.94	40.86 ± 2.22	
12-weeks (V5)	36.4 ± 1.62	40.57 ± 1.96	
Phenylalanine			0.01
Baseline (V2)	32.57 ± 1.25	29.86 ± 1.21	
4-weeks (V3)	28.45 ± 1.67	33.72 ± 1.64	0.01
8-weeks (V4)	31.42 ± 2.13	32.5 ± 1.5	
12-weeks (V5)	34.33 ± 4.11	31.32 ± 1.29	
Tyramine			0.72
Baseline (V2)	0.03 ± 0	0.03 ± 0	
4-weeks (V3)	0.03 ± 0	0.03 ± 0	
8-weeks (V4)	0.03 ± 0	0.03 ± 0	
12-weeks (V5)	0.03 ± 0	0.03 ± 0	
3,4-Dihydroxyphenylacetic acid			0.26
Baseline (V2)	0.05 ± 0	0.04 ± 0	
4-weeks (V3)	0.04 ± 0	0.05 ± 0.01	
8-weeks (V4)	0.04 ± 0	0.04 ± 0	

Table 2 (continued)

Metabolite	Treatment	Placebo	<i>P</i> -value
12-weeks (V5)	0.04±0	0.04±0.01	0.26
Kynurenic acid			
Baseline (V2)	0.07±0	0.06±0	
4-weeks (V3)	0.07±0	0.07±0	
8-weeks (V4)	0.06±0	0.07±0	
12-weeks (V5)	0.07±0	0.07±0	0.30
5-Hydroxyindole-3-acetic acid			
Baseline (V2)	0.05±0	0.04±0	
4-weeks (V3)	0.04±0	0.04±0	
8-weeks (V4)	0.05±0.01	0.05±0	
12-weeks (V5)	0.04±0	0.05±0.01	0.12
Tryptophan			
Baseline (V2)	29.89±1.11	27.6±0.92	
4-weeks (V3)	30.1±1.62	28.73±1.36	
8-weeks (V4)	27.37±0.95	30.44±1.36	
12-weeks (V5)	28.62±0.95	30.54±1.42	0.41
3-Hydroxyanthranilic acid			
Baseline (V2)	0.02±0	0.02±0	
4-weeks (V3)	0.02±0	0.02±0	
8-weeks (V4)	0.01±0	0.02±0	
12-weeks (V5)	0.02±0	0.02±0	0.49
Indole-3-Acetic acid			
Baseline (V2)	2.1±0.25	1.99±0.2	
4-weeks (V3)	2.11±0.36	2.02±0.26	
8-weeks (V4)	1.78±0.12	2.35±0.39	
12-weeks (V5)	1.84±0.18	2.21±0.25	0.61
Indole-3-Carboxaldehyde			
Baseline (V2)	0.1±0.01	0.09±0.01	
4-weeks (V3)	0.1±0.01	0.09±0.01	
8-weeks (V4)	0.09±0.01	0.09±0.01	
12-weeks (V5)	0.08±0	0.09±0.01	0.30
Indole-3-lactic acid			
Baseline (V2)	1±0.07	0.89±0.05	
4-weeks (V3)	1.08±0.13	1±0.09	
8-weeks (V4)	0.88±0.04	0.97±0.06	
12-weeks (V5)	0.91±0.05	1.01±0.07	0.45
Indole-3-Propionic acid			
Baseline (V2)	1.09±0.1	1.24±0.13	
4-weeks (V3)	1.06±0.11	1.09±0.2	
8-weeks (V4)	1.2±0.09	1.18±0.13	
12-weeks (V5)	1.12±0.12	1.35±0.17	0.07
Xanthurenic acid			
Baseline (V2)	1.02±0.04	0.91±0.03	
4-weeks (V3)	1.02±0.06	0.95±0.04	
8-weeks (V4)	0.93±0.03	1.03±0.05	
12-weeks (V5)	0.95±0.03	1.03±0.05	0.11
Kynurenine			
Baseline (V2)	1.92±0.08	1.7±0.07	
4-weeks (V3)	1.87±0.12	1.88±0.12	
8-weeks (V4)	1.76±0.08	1.97±0.11	
12-weeks (V5)	1.79±0.09	1.98±0.12	0.61
Indoxylsulfate			
Baseline (V2)	9.38±0.81	8.26±0.78	
4-weeks (V3)	7.95±0.69	8.41±0.92	

Table 2 (continued)

Metabolite	Treatment	Placebo	<i>P</i> -value
8-weeks (V4)	8.48 ± 0.66	8.58 ± 0.61	
12-weeks (V5)	9.98 ± 0.88	9.75 ± 0.97	

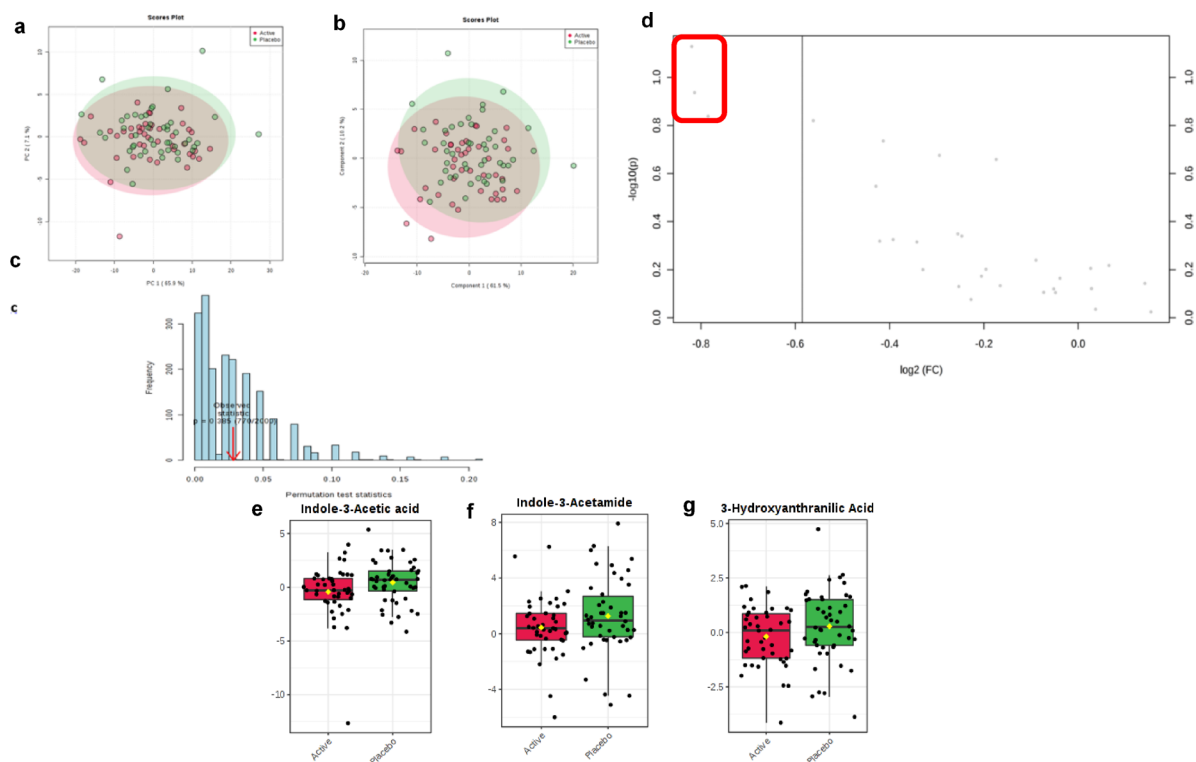


Fig. 3 Urine metabolites analysis (a) PCA score plot, (b) PLS-DA score plot of plasma samples (active (treatment) – red, placebo – green) and (c) Permutation test statistics, (d) volcano plot of urine sample analysis. Highlighted by red rectangle, three metabolites which were higher in placebo group compared to active group, (e–g) box-plots of selected urine metabolites; (e) indole-3-acetic acid, (f) indole-3-acetamide and (g) 3-hydroxyanthranilic acid, all of which were higher in placebo group (right) compared to treatment group (left), but the differences were not significant.

(Shannon Index) does not show any distinction between placebo and treatment groups and time points (Supplementary Fig. 1). Similarly, principal coordinate analysis (PCoA) generated using Bray–Curtis, unweighted UniFrac and weighted UniFrac distances showed that there was no distinct separation between the placebo and treatment groups and between time points (Supplementary Fig. 2 and 3). Nonetheless, the PCoA ordination with weighted and unweighted UniFrac distances showed a separation between the sample points in two clustered groups which do not relate to any known metadata (Supplementary Fig. 3). Differential abundance analysis using Songbird tool and when Control_V2 was used as reference *Prevotella* species, *Faecalibacterium* spp. including *Faecalibacterium prausnitzii*, *Ruminococcaceae* species and some *Bacteroides* spp. were always associated with the Treatment groups (i.e. Placebo_V5, Treatment_V2 and Treatment_V5 in this case), while *Agathobacter*, *Alistipes* spp. and some *Bacteroides* spp. were associated with the reference group (Control_V2 here) (Fig. 4d).

Table 3 Concentrations of Urine Biomarkers.

Metabolite	Treatment	Placebo	<i>p</i> -value
Methionine			0.59
Baseline	10.13±1.16	14.97±2.51	
4-weeks	9.23±2.25	10.14±2.96	
8-weeks	8.92±1.34	13.07±1.38	
12-weeks	10.36±1.48	12.41±1.49	
GABA			0.16
Baseline	0.25±0.05	0.22±0.04	
4-weeks	0.19±0.06	0.2±0.09	
8-weeks	0.22±0.04	0.31±0.06	
12-weeks	0.14±0.03	0.27±0.07	
L-valine			0.35
Baseline	35.86±3.84	51.07±7.94	
4-weeks	34.54±7.47	38.64±9.86	
8-weeks	34.58±5.15	49.4±5.6	
12-weeks	39.76±5.44	42.57±4.1	
Picolinic acid			0.77
Baseline	2.56±0.29	3.7±0.67	
4-weeks	2.91±0.66	3.28±1.09	
8-weeks	2.29±0.37	3.35±0.44	
12-weeks	2.83±0.41	3.33±0.48	
Quinolinic acid			0.33
Baseline	127.81±15.32	138.17±13.14	
4-weeks	118.58±27.19	121.98±23.13	
8-weeks	107.93±14.19	146.78±14.24	
12-weeks	113.91±12.18	140.73±13.13	
Isoleucine			0.17
Baseline	18.28±2.35	25.94±4.82	
4-weeks	18.79±5.38	18.06±4.31	
8-weeks	16.51±2.79	25.17±3.3	
12-weeks	21.8±3.59	21.76±2.82	
Leucine			0.20
Baseline	50.94±6.33	75.59±13.33	
4-weeks	56.31±17.4	53.41±12.74	
8-weeks	45.55±7.45	71.13±9.16	
12-weeks	58.03±8.91	63.32±8.45	
Dopamine			0.05
Baseline	3.13±0.37	3.98±0.53	
4-weeks	2.37±0.51	3.81±1.06	
8-weeks	2.36±0.27	4.43±0.53	0.002
12-weeks	3.26±0.45	3.35±0.37	
Serotonin			0.14
Baseline	0.89±0.09	1.3±0.17	
4-weeks	0.84±0.18	1.26±0.46	
8-weeks	0.82±0.12	1.36±0.16	
12-weeks	1.06±0.15	1.12±0.12	
Tyrosine			0.11
Baseline	186.17±20.6	296.22±48.69	
4-weeks	208.86±45.54	218.35±37.28	
8-weeks	165.05±21.49	275.64±29.77	
12-weeks	205.46±26.03	253.39±28.83	
Phenylalanine			0.04
Baseline	113.93±12.82	165.2±27.03	
4-weeks	112.76±25.18	106±16.97	
8-weeks	96.07±12.95	155.54±16.69	0.004

Table 3 (continued)

Metabolite	Treatment	Placebo	<i>p</i> -value
12-weeks	120.75 ± 15.81	139.27 ± 14.77	
Tyramine			0.43
Baseline	6.52 ± 0.67	11.5 ± 1.99	
4-weeks	7.5 ± 1.24	10.68 ± 2.88	
8-weeks	6.68 ± 0.88	10.76 ± 1.2	
12-weeks	7.85 ± 1.01	9.82 ± 1.13	
3-methoxy-p-tyramine			0.32
Baseline	0.39 ± 0.05	0.58 ± 0.09	
4-weeks	0.31 ± 0.07	0.49 ± 0.14	
8-weeks	0.35 ± 0.05	0.6 ± 0.07	
12-weeks	0.44 ± 0.07	0.51 ± 0.06	
3,4-Dihydroxyphenylacetic acid			0.06
Baseline	9.02 ± 1.12	12.7 ± 1.66	
4-weeks	8.89 ± 2.92	10.42 ± 2.22	
8-weeks	8.01 ± 1.32	15.41 ± 1.78	
12-weeks	9.13 ± 1.1	11.3 ± 1.32	
Kynurenic acid			0.71
Baseline	21.29 ± 2.56	29.12 ± 3.23	
4-weeks	21.55 ± 4.09	35.22 ± 12.96	
8-weeks	18.93 ± 2.5	30.08 ± 3.03	
12-weeks	24.04 ± 3.27	28.96 ± 2.81	
5-Hydroxyindole-3-acetic acid			0.27
Baseline	30.6 ± 3.84	46.11 ± 8.66	
4-weeks	24.55 ± 6.61	33.9 ± 9.23	
8-weeks	26.42 ± 3.48	42.85 ± 6.29	
12-weeks	29.96 ± 3.74	39.65 ± 9.12	
Tryptophan			0.09
Baseline	97.3 ± 10.02	146.53 ± 23.38	
4-weeks	100.56 ± 22.15	126.26 ± 31.95	
8-weeks	85.94 ± 11	141.81 ± 15.75	
12-weeks	110.84 ± 15.05	122.39 ± 12.88	
3-Hydroxanthranilic acid			0.94
Baseline	6.72 ± 0.92	8.07 ± 1.27	
4-weeks	4.83 ± 0.99	6.88 ± 2.06	
8-weeks	5.39 ± 0.91	7.35 ± 0.98	
12-weeks	6.06 ± 0.66	7.27 ± 0.89	
Anthranilic acid			0.55
Baseline	0.2 ± 0.03	0.2 ± 0.02	
4-weeks	0.24 ± 0.04	0.19 ± 0.03	
8-weeks	0.24 ± 0.03	0.23 ± 0.02	
12-weeks	0.31 ± 0.08	0.28 ± 0.03	
Tryptamine			0.73
Baseline	0.33 ± 0.04	0.66 ± 0.12	
4-weeks	0.38 ± 0.1	0.59 ± 0.17	
8-weeks	0.32 ± 0.04	0.62 ± 0.1	
12-weeks	0.39 ± 0.06	0.53 ± 0.09	
Indole-3-acetamide			0.04
Baseline	0.11 ± 0.03	0.11 ± 0.01*	
4-weeks	0.18 ± 0.06	0.14 ± 0.01	
8-weeks	0.14 ± 0.03	0.16 ± 0.02	
12-weeks	0.17 ± 0.03	0.27 ± 0.07*	0.05
Indole-3-Acetic acid			0.03
Baseline	53.53 ± 8.74	66.5 ± 8.38	
4-weeks	42.05 ± 6.6	32.3 ± 5*	

Table 3 (continued)

Metabolite	Treatment	Placebo	<i>p</i> -value
8-weeks	46.32±6.22	73±11.86	0.33
12-weeks	68.02±16.46	86.1±13.13*	
Indole-3-carboxylic acid			0.76
Baseline	0.41±0.07	0.66±0.16	
4-weeks	0.28±0.09	0.41±0.12	
8-weeks	0.33±0.07	0.65±0.17	
12-weeks	0.45±0.13	0.42±0.07	
Indole-3-carboxaldehyde			0.18
Baseline	0.56±0.05	0.75±0.1	
4-weeks	0.67±0.13	1.29±0.78	
8-weeks	0.58±0.09	0.78±0.1	
12-weeks	0.78±0.14	0.8±0.09	
Indole-3-lactic acid			0.63
Baseline	10.28±1.64	13.96±2.37	
4-weeks	7.85±2.45	38.41±31.06	
8-weeks	8.37±1.48	17.97±3.4	
12-weeks	16.01±4.02	16.41±4.5	
Indole-3-propionic acid			0.53
Baseline	0.08±0	0.09±0.01	
4-weeks	0.09±0.01	0.08±0.02	
8-weeks	0.08±0.01	0.1±0.01	
12-weeks	0.08±0.01	0.09±0.01	
Xanthurenic acid			0.17
Baseline	14.94±1.87	21.13±2.7	
4-weeks	14.7±2.9	33.9±19.67	
8-weeks	13.69±2.15	20.18±2.24	
12-weeks	18.21±2.74	20.88±2.49	
3-Hydroxkynurenine			0.13
Baseline	3.98±0.67	5.62±1.21	
4-weeks	3.05±0.69	5.7±1.92	
8-weeks	2.9±0.41	5.51±0.87	
12-weeks	3.64±0.5	4.41±0.47	
Kynurenine			0.42
Baseline	7.75±1.27	12.12±2.88	
4-weeks	8.95±2.82	10.22±2.42	
8-weeks	6.79±1.52	11.4±1.77	
12-weeks	7.63±1.29	10.36±1.66	
5-Hydroxytryptophan			0.10
Baseline	0.08±0.05	0.17±0.05	
4-weeks	0.11±0.06	0.19±0.07	
8-weeks	0.21±0.08	0.23±0.06	
12-weeks	0.12±0.04	0.08±0.03	
Indoxyl sulfate			0.39
Baseline	986.91±125.12	894.28±108.5	
4-weeks	884.02±236.1	745.25±161.25	
8-weeks	802.24±122.43	1035.56±114.61	
12-weeks	933.96±127.14	982.81±103.56	
Homovanillic acid			
Baseline	49.83±5.56	69.57±8.67	
4-weeks	60.81±15.38	62.44±9.34	
8-weeks	52.56±8.28	81.86±9.64	
12-weeks	61.56±7.65	76.65±9.06	

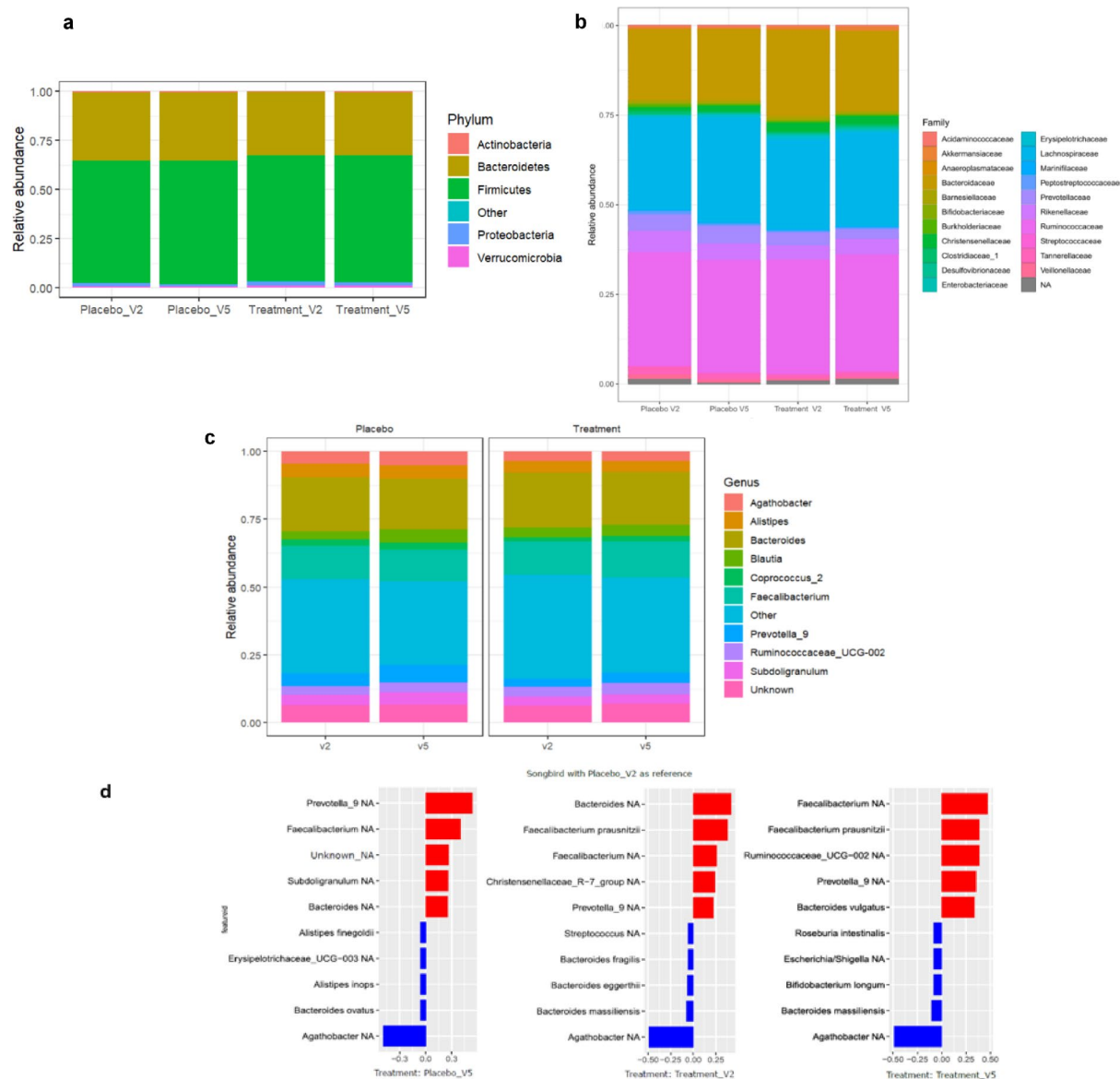


Fig. 4 (a–c) Taxonomic distribution of microbial communities in the faecal contents, expressed as a relative abundance of total population, in participants at baseline (V2) and end of the study (V5) for both placebo and treatment groups; **(a)** phylum-level analysis, **(b)** family-level analysis, **(c)** genus-level analysis; **(d)** differential abundance analysis was performed using Songbird with the formula (C(Group2, Treatment('Placebo_V2'))). Here negative (blue bars) are more abundant than reference group (Placebo_V5) where reference is compared with A. Control_V5, B. Treatment_V2 and C. Treatment_V5.

Discussion

Limosilactobacillus mucosae DPC 6426 had previously been shown to alter the composition of the bile pool and immune system tone²³ and therefore has potential as a therapeutic for CVD. In the current study, in which healthy mildly hypercholesterolaemic adults received either daily supplementation with *Lb. mucosae* DPC 6426, at a minimum concentration of 10^9 CFU/g vs. placebo, we confirmed gastrointestinal transit and recovery of the administered *Lb. mucosae* DPC 6426 strain, with an average recovery of total lactobacilli of 1.26×10^7 CFU/g faeces following 12 weeks of dietary intervention, a ~1-log increase compared to baseline. However, results of this 12-week randomised, double-blind, placebo-controlled trial, indicated no statistically significant changes

from baseline in total cholesterol, LDL-cholesterol, HDL-cholesterol and total triglyceride concentrations, or gut microbiota composition. These data are contrary to the results previously obtained in the pre-clinical apoE mouse trial¹⁵, in which the probiotic treatment caused a ~50% reduction in serum total cholesterol levels, a 15% reduction in serum triglycerides as well as a significantly higher ratio of high-density lipoprotein (HDL)-cholesterol to total cholesterol in the serum and liver compared with un-supplemented control treatment. Furthermore, in that study, dietary supplementation with *Lb. mucosae* DPC 6426 was associated with modulation of the gut microbiota, including increased relative abundance of Porphyromonadaceae and Prevotellaceae and lower proportions of Clostridiaceae, Peptococcaceae and Staphylococcaceae compared with the placebo group. However, these contradictory findings may not be surprising, given that volume of distribution and systemic effects of a similar dose of *Lb. mucosae* DPC 6426 strain are more likely to be therapeutically effective in a mouse model that is several log-fold smaller mass than a human subject. Moreover, we have shown more recently that use of a near log-fold greater dose of *Lb. mucosae* DPC 6426 strain (10^{10} CFU/day) when combined with a prebiotic can have potent cardiometabolic effects in a large animal model (pig) of human scale²⁴. The definition of probiotics states that probiotic should be taken in adequate amounts to cause the beneficial effect, but however, there is no consensus as to what the effective dose is. As reviewed by Ouwehand²⁵, there are variations among clinical trials and effective dose depends on the specific probiotic strain used, the health benefit being studied and the study population, whether healthy or diseased. (Fig. 5).

Both plasma and urine metabolites showed no clear separation between treatment groups and based on the permutation test statistic, it was also not statistically significant ($p=0.6615$ for plasma metabolites and $p=0.385$ for urine metabolites). Four plasma metabolites, kynurenic acid, xanthurenic acid, kynurenine and tyrosine were identified to be present in higher concentrations in the placebo group, and additionally, L-valine was >1.5 fold higher in the probiotic group compared to the placebo group, however this was not statistically significant ($p>0.05$). In a study by Lund et al.²⁶, it was shown that higher levels of plasma kynurenine, 3-hydroxykynurenine (HK), quinolinic acid (QA), the kynurenine-tryptophan ratio (KTR) and the ratio of HK to xanthurenic acid (HK/XA) were identified in heart failure patients compared to the control groups¹⁹. It was also shown that QA, HK, HK/XA and KTR were associated with increased mortality in patients with heart failure, whilst HK and HK/XA demonstrated weak associations with coronary artery disease¹⁹. Furthermore, Mohorko et al.²⁷ demonstrated that changes in blood concentrations of leucine, tyrosine and phenylalanine were associated with obesity and insulin resistance and have proposed the use of elevated serum levels of cysteine and tyrosine as early biomarkers in asymptomatic patients at increased risk of developing MetS. In this study, there were slight decreases observed in the concentrations of leucine and tyrosine between V2 and V5 for the probiotic group, with slight increases being observed in the placebo group. Furthermore, there was also a slight increase observed in the concentration

Fig. 5 Average CFU/g of Total Lactobacilli in faecal content.

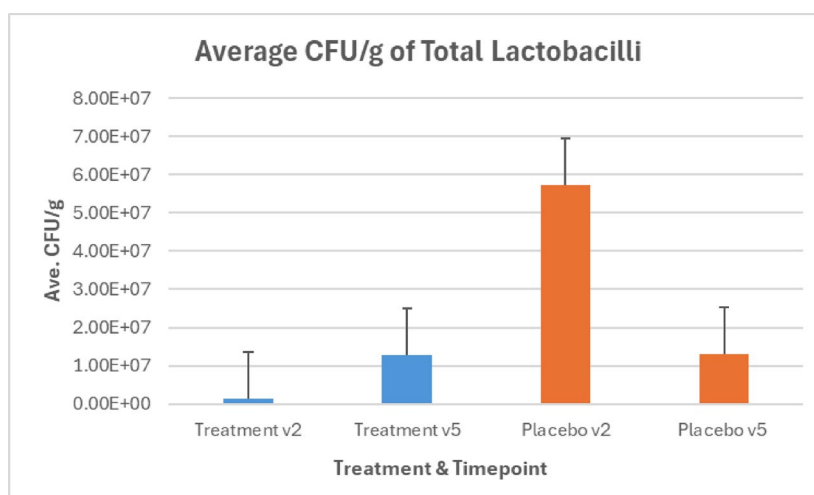


Table 4 Summary of Adverse Events Reported throughout the Study.

Group	Intensity	Adverse Event (AE)	Serious Adverse Event (SAE)	Treatment-Emergent Adverse Event (TEAE)
Placebo	25 Mild Intensity 2 Moderate Intensity	19	1	23
Treatment	26 Mild Intensity 2 Moderate Intensity	18	0	23

of phenylalanine in both the treatment and placebo groups, however this increase was not significant. Moreover, in a study by Mitrega et al.²⁸, the authors provided evidence of the anti-arrhythmic activity of L-valine in decreasing incidence of arrhythmias in a model of induced ischemia and reperfusion and have shown the potential role of L-valine as an additional therapy for cardiovascular diseases combined with an increased risk of arrhythmias. In this study, there was an increase in the concentration of L-valine between V2 and V5 in the treatment group, but it was not significant ($p>0.05$). Three urine metabolites, indole-3-acetic acid, indole-3-acetamide and 3-hydroxy-anthranilic acid, were identified to be present in higher concentrations in the placebo group compared to the active group but without statistical significance. Dou et al.²⁹ carried out a study which investigated the cardiovascular effects of indole-3-acetic acid in patients with chronic kidney disease (CKD) and found that serum indole-3-acetic acid may act as a predictor of cardiovascular events and mortality in patients with CKD. Additionally, the authors also demonstrated in vitro that indole-3-acetic acid resulted in the induction of endothelial inflammation and oxidative stress and the activation of an inflammatory pathway²⁹. Furthermore, it has been shown that 3-hydroxyanthranilic acid levels in CKD patients have been associated with the CC-chemokine family, markers of macrophage inflammation in atherosclerotic lesions³⁰.

Taken together, these findings suggest that in the current study, the dose of probiotic used may have been insufficient to exert a clinically significant effect on the lipid profile or metabolome of humans, therefore failed to yield a beneficial probiotic effect over placebo on blood lipids in healthy adults. Reasons as to why we failed to see an effect in the human trial, compared with the previous apo-E mouse animal trial¹⁵ are most likely due to the relative differences in the probiotic dose used in the human vs. mouse model. Clearly, the volume of distribution and systemic effects of a similar probiotic dose are more likely to be therapeutically effective in the mouse model that is several log-fold smaller mass than a human subject. Further evidence pointing to the dose effect being the cause of the negative effect obtained in this study, comes from a recent study in which we administered a near log fold greater dose of *Lb. mucosae* DPC 6426 strain (10^{10} CFU/day) combined with a prebiotic, that caused potent cardiometabolic effects in a pig model, i.e. a large animal model of human scale²⁴.

Studies have been conducted that investigated the dose response relation of probiotics in human interventions, as reviewed by Ouwehand²⁵, with no consensus as to what is the correct probiotic dose to elicit a probiotic effect, and indeed it likely comes down to the probiotic strain used, the health benefit being studied and the study population, whether healthy or diseased.

Limitations of the study

Whilst the phylum level taxonomy-based analysis of the apoE mouse gut microbiota and mildly hypercholesterolaemic human gut microbiota showed that they were both dominated by *Firmicutes* and *Bacteroidetes*. However, when we analysed both family and genus levels, the gut microbiota compositions were no longer comparable. This in turn suggests that the apoE mouse model may not have been a suitable model to base our experimental outcomes on (Table 4).

A possible limitation of the study was the wide range of age used for eligibility, 20–70 years, with the average age being 52 and 51 years for the placebo and treatment respectively, as with individual age, comorbidities tend to increase and there can also be shifts in the bile acid pool, both of which may influence the outcome of interest.

Following DNA extraction from fresh faecal samples, it was assumed that DNA concentration would be high and of adequate integrity to move onto PCR amplification and 16S sequencing. The authors acknowledged that disregarding this step may be a reason for the high percentage of chimeras observed in the downstream analysis.

Another limitation of the study is no other bioinformatic tools were explored beyond the use of DADA2, and this, or PCR-related issues, may account for the high percentages of chimeras reported.

As qPCR was not carried out, and only total lactobacilli was investigated, the study failed to specifically measure the administered strain and confirm gut transit and recovery from faecal samples collected. Moreover, due to the fact qPCR was not carried out, it is difficult to confidently conclude that the administered strain was responsible for the shift in lactobacilli levels in the gut.

In order to compare the enterohepatic circulation and bile acid composition differences between the previous apoE mouse model and the human participants, an investigation of the circulating bile acids could have been done to assess any effect the administered strain had on levels and whether or not the enterohepatic re-circulation was influenced. This is a limitation of the current study.

Conclusions

In this study, daily supplementation with *Limosilactobacillus mucosae* DPC 6426, a probiotic strain previously shown by us to exert a cholesterol-lowering effect in mice, did not significantly alter the blood lipid profiles or the gut microbiota of mildly hypercholesterolaemic adults. The probiotic was administered at a concentration of 3.8×10^9 CFU/per day, and it is most likely that the dose of probiotic used in the current human intervention study may have been insufficient to have a clinically significant effect and therefore failed to yield a beneficial probiotic effect over placebo on blood lipids in mildly hypercholesterolaemic adults. Results of this study warrant further human studies with increased dose of *Limosilactobacillus mucosae* DPC 6426 of 10^{10} CFU and 10^{11} CFU/day to verify efficacy in humans.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-026-66210-z>.

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Data availability The metagenomics data generated as part of this study is submitted on NCBI, with the submission ID SUB15707075, and BioProject ID PRJNA1345214, accessible at <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1345214>.

Declarations

Competing interests The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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Authors and Affiliations

Aoife N. O'Donovan^{1,2} · Fiona Fouhy¹ · Ghjuvan Grimaud^{1,5} · Lis E. E. London^{1,2} · Andrea Doolan³ · Andrea Anesi⁴ · Fulvio Mattivi⁴ · R. Paul Ross² · Noel Caplice² · Catherine Stanton^{1,2}

✉ Catherine Stanton
catherine.stanton@teagasc.ie

¹ Teagasc Moorepark, Fermoy Co., Cork, Ireland

² APC Microbiome Ireland, University College, Cork, Ireland

³ Blackpool Retail Park, Atlantia Clinical Trials, Heron House Office, Cork, Ireland

⁴ Unit of Metabolomics, Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele All'Adige, Italy

⁵ Department of Process in Life Science and Engineering, Lund University, Lund, Sweden