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ORIGINAL ARTICLE

Cholesterol lowering and inhibition of sterol absorption by *Lactobacillus reuteri* NCIMB 30242: a randomized controlled trial

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BACKGROUND/OBJECTIVES: The percentage of hypercholesterolemic individuals not reaching their LDL-cholesterol (LDL-C) goal remains high and additional therapeutic strategies should be evaluated. The objective of this study was to evaluate the cholesterol-lowering efficacy and mechanism of action of bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 capsules in hypercholesterolemic adults.

SUBJECTS/METHODS: A total of 127 subjects completed a randomized, double-blind, placebo-controlled, parallel-arm, multicenter study. Subjects were randomized to consume *L. reuteri* NCIMB 30242 capsules or placebo capsules over a 9-week intervention period. The primary outcome was LDL-C relative to placebo at the study end point.

RESULTS: *L. reuteri* NCIMB 30242 capsules reduced LDL-C by 11.64% ($P<0.001$), total cholesterol by 9.14%, ($P<0.001$), non-HDL-cholesterol (non-HDL-C) by 11.30% ($P<0.001$) and apoB-100 by 8.41% ($P=0.002$) relative to placebo. The ratios of LDL-C/HDL-cholesterol (HDL-C) and apoB-100/apoA-1 were reduced by 13.39% ($P=0.006$) and 9.00% ($P=0.026$), respectively, relative to placebo. Triglycerides and HDL-C were unchanged. High-sensitivity C-reactive protein and fibrinogen were reduced by 1.05 mg/l ($P=0.005$) and 14.25% ($P=0.004$) relative to placebo, respectively. Mean plasma deconjugated bile acids were increased by 1.00 μmol/l ($P=0.025$) relative to placebo, whereas plasma campesterol, sitosterol and stigmasterol were decreased by 41.5%, 34.2% and 40.7%, respectively.

CONCLUSIONS: The present results suggest that the deconjugation of intraluminal bile acids results in reduced absorption of non-cholesterol sterols and indicate that *L. reuteri* NCIMB 30242 capsules may be useful as an adjunctive therapy for treating hypercholesterolemia.

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INTRODUCTION

Several decades of evidence have confirmed a log-linear relationship between increasing LDL-cholesterol (LDL-C) and the relative risk of coronary artery disease (CAD).[1,2] The National Cholesterol Education Program—Adult Treatment Program III has outlined target LDL-C concentrations for the reduction of relative risk,[3] with lower targets recommended for patients with CAD, patients with very high CAD-risk equivalents and asymptomatic primary prevention patients with multiple risk factors.[4,5] Therapeutic strategies aimed at reducing LDL-C focus initially on dietary recommendations and interventions.

Probiotic bacteria are defined by the WHO *as live microorganisms which when administered in adequate amounts confer a health benefit on the host* and are being examined for their efficacy in lowering total cholesterol (TC) and LDL-C in humans.[6] It was first observed in the 1960s that germ-free animals accumulate cholesterol in greater quantities and at a faster rate than their conventionally raised counterparts.[7] The proposed explanation was that germ-free animals catabolize cholesterol at a slower rate and that the intestinal microflora was responsible for accelerating cholesterol catabolism through an increase in the elimination of bile acids.[8] This was substantiated by showing that germ-free animals have elevated levels of conjugated bile acids throughout the intestine, significantly reduced fecal biliary excretion and three times the bile-acid concentration in bile.[9–11] Further, it was shown that mice treated with oral antibiotics for 3 days increased biliary bile-acid output threefold, whereas fecal output decreased by 70%.[12] In addition, porcine studies have demonstrated that increasing the bile salt hydrolase (BSH) activity of the intestinal microflora results in a significant increase in the deconjugated bile-acid pool[13] and a reduction in circulating cholesterol.[14] Despite preclinical evidence that increased intraluminal deconjugation of bile acids may lead to increased hepatic cholesterol catabolism and reduced cholesterol absorption, evidence of a mechanism of cholesterol reduction in humans is not yet available.

Several groups have reported significant reductions in LDL-C after daily consumption of probiotic-enriched yogurt in randomized controlled trials.[15–19] The majority of randomized controlled trials, however, have reported no significant effect,[20–25] and no clinical trial has reported significant LDL-C reductions of a probiotic supplement formulation unless delivered as a synbiotic.[26] Previously, we have reported on the cholesterol-lowering efficacy of a microencapsulated BSH-active *Lactobacillus*

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reuteri NCIMB 30242 yogurt formulation.[18] Furthermore, the clinical safety and tolerability of *L. reuteri* NCIMB 30242 has been confirmed in yogurt[27] and capsule formulations.[28] However, it was not known if similar efficacy would be seen with a capsule format, if additional efficacy would be seen over a longer interventional period, or if delivery of the BSH-active strain would result in increased circulating deconjugated bile acids or reduced absorption of non-cholesterol sterols. Accordingly, the main objective of the present study was to assess the cholesterol-lowering clinical efficacy and to investigate the mechanism of action of BSH-active *L. reuteri* NCIMB 30242 supplement capsules over a 9-week intervention period in a randomized controlled trial.

SUBJECTS AND METHODS

Subjects

The present study was conducted according to the principles of the Declaration of Helsinki. Otherwise healthy hypercholesterolemic adults were recruited from six centers in Prague, CZ. The protocol was approved by the Ethics Committee for Multi-Centric Clinical Trials of the University Hospital Motol, CZ (FDA/OHRP IORG registration no. IORG0000612). The trial was registered on www.clinicaltrials.gov under study number NCT01341613.

Otherwise healthy hypercholesterolemic subjects between 20 and 75 years (inclusive) with LDL-C >3.4 mmol/l ($<15\%$ variation between successive screening visits), triglycerides (TGs) <4.0 mmol/l, body mass index of 22–32 kg/m^2, not receiving or receiving a stable dose of statin monotherapy ($\geqslant 3$ months) and at least 80% compliant with product consumption (checked after the run-in period) were included. Diabetics, subjects receiving cholesterol-lowering non-prescription supplements or prescription drugs other than statin monotherapy within the last 3 or 6 months, respectively, and subjects having experienced any cardiovascular event in the last 6 months were excluded. Additional exclusion criteria were history of chronic use of alcohol; current intake of systemic antibodies, corticosteroids, androgens or phenytoin; current involvement in a clinical trial or in an exclusion period following a clinical trial; history of angina, congestive heart failure, inflammatory bowel disease, pancreatitis, gastrointestinal, renal, pulmonary, hepatic or biliary disease, or cancer; chronic use of probiotics or fiber laxative, or stimulant laxatives; and history of eating disorders. Furthermore, subjects exercising >15 miles per week or 16 744 kJ per week or who were pregnant, breast feeding or intended to get pregnant were excluded. Subjects were permitted to take stable doses of thyroid hormone and antihypertensive agents, as long as they were continued equivalently throughout the duration of study. The study protocol was carefully explained and all subjects provided a written informed consent prior to inclusion.

Preparation of supplement and placebo capsules

L. reuteri NCIMB 30242 (cardioviva) was propagated in a 5000-l fermenter, concentrated and lyophilized in compliance with the standard operating and quality-control procedures at Probiotical S.p.A (Novara, Italy). Placebo and supplement capsules (opaque white size '0' DRcaps (Capsugel, Colmar, France)) were prepared by Probiotical S.p.A. Microbiological analyses and bacterial culture purity were confirmed following production. Placebo capsules contained 0 mg lyophilized bacteria and 300 mg maltodextrin/silicon dioxide excipient and supplement capsules contained 130 mg lyophilized bacteria and 170 mg maltodextrin/silicon dioxide excipient. Supplement capsules contained 2.9×10^9 CFU per capsule *L. reuteri* NCIMB 30242 at the study baseline and 2.0×10^9 CFU per capsule *L. reuteri* NCIMB 30242 at the study end point as measured by Exova (Portland, OR, USA). Placebo and supplement capsules were identical in taste and appearance and bottled in identical sealed high-density polyethylene bottles with desiccant.

Study design

The study design was multicenter, randomized, double-blind, placebo-controlled, parallel-arm, lasting a total of 13 weeks. This included a 2-week wash-out period, a 2-week run-in period in which subjects consumed placebo capsules twice daily at breakfast and dinner and a 9-week intervention period in which subjects consumed either placebo or supplement capsules twice daily at breakfast and dinner. The National Cholesterol Education Program—Adult Treatment Program III dietary guidelines were provided for the entire 13-week period. Wash-out provided an additional 2-week period for subjects to acclimatize to lifestyle recommendations, whereas run-in provided a 2-week period for assessment of screening eligibility and compliance to product consumption. Subjects met with the investigational team at seven time points: Visit V0 (week -4), V1 (week -2), V2-1 (week 0–1 day), V2-2 (week 0, randomization and intervention baseline), V3 (week 3), V4 (week 6) and V5 (week 9, intervention end point). Dietary intake of over 30 micronutrients at baseline and end point was evaluated by the NutriDan software program (Danone Institute, Prague, Czech Republic) for total energy (kJ), total lipids (%), total proteins (%) and total carbohydrates (%).

Sample analyses

Twelve-hour fasting blood samples were obtained by venipuncture at visits V0, V1, V2-1, V3, V4 and V5. Serum and plasma were transported immediately from each center to a central laboratory (Prevedig s.r.o., Prague, Czech Republic) for analysis. Serum LDL-C, TC, HDL-C, TG, apoB-100 and apoA-1 were analyzed on a Dimension RxL biochemistry analyzer using reagent kits (Dade Behring, Siemens, Munich, Germany). Non-HDL-cholesterol (non-HDL-C) was calculated by subtracting HDL-C from TC, and LDL-C/HDL-C and apoB-100/apoA-1 ratios were calculated. Serum high-sensitivity C-reactive protein (hs-CRP) and plasma fibrinogen were assessed at baseline and end point using a highly sensitive immunoassay and a standard functional coagulative assay, respectively. Plasma deconjugated and conjugated bile acids and plant sterols (campesterol, sitosterol and stigmasterol) were assessed at baseline and end point via liquid chromatography–tandem mass spectrometry or gas chromatography–tandem mass spectrometry as described by Scherer *et al.*[29]

Statistical analysis

The number of subjects was calculated by taking into account a difference in LDL-C of 0.34 (0.64) mmol/l between the supplement and placebo groups with $\alpha = 5\%$ and a power of 80%. Given these constraints, 57 evaluable subjects per group or 114 in total were required. To take into account possible premature withdrawal, 131 subjects were included for random assignment. A blinded statistician prepared 16 unique randomization lists using the completely randomized design generated by SAS software package version 9 procedure PLAN (SAS Institute, Cary, NC, USA). The capsule producer chose one list and prepared the capsule bottles accordingly. Records of randomization number, corresponding to placebo or supplement, were only accessible to the capsule producer until database lock.

The primary null hypothesis was that a supplement capsule is not more effective than a placebo capsule in reducing serum LDL-C after 9 weeks. The primary analysis was performed according to the intention-to-treat principle. Descriptive statistics are presented as mean (s.d.) or as geometric means and interquartile ranges for continuous variables or as a percentage for qualitative variables. The Shapiro–Wilk test was used to determine if variables were parametrically distributed. Differences between groups for baseline characteristics were analyzed using a one-way analysis of variance or a nonparametric Mann–Whitney Wilcoxon test for continuous variables, or Fisher's exact test for categorical variables. For lipids, apolipoproteins and fibrinogen multiple-linear regression was used to identify variables systematically contributing to any changes from baseline. To test the differences between treatment groups, analysis of covariance was performed to adjust for any systematic contribution to the changes from baseline using covariates identified by multiple-linear regression. Parameters not accepting parametric description were analyzed by means of Mann–Whitney Wilcoxon tests. Analysis of hs-CRP was performed on logarithmic transformed data by using a two-factor repeated measures analysis of variance with time and intervention as the two factors. Differences between groups in dietary intake of macronutrients, bile acids and plant sterols were assessed by using a two-factor repeated measures analysis of variance with time and intervention as the two factors. Plant sterols were logarithmically transformed prior to statistical analysis. A Spearman's rank correlation was used to assess the association between changes in plasma deconjugated bile acids and changes in serum LDL-C over the intervention period. Lipids, apolipoproteins, fibrinogen and dietary intake analyses were performed using SAS software package version 9 (SAS Institute). All other data analyses were performed *post-hoc* using SPSS software package version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Study parameters

A total of 131 subjects were randomized and 127 subjects completed the study as part of the intention-to-treat population. Four subjects, three in the placebo group and one in the supplement group, were excluded as they did not meet the study criteria. In all, 116 subjects completed the study as per the protocol (Supplementary Figure 1). All subjects were considered hypercholesterolemic and at risk of developing heart disease at baseline according to the National Cholesterol Education Program—Adult Treatment Program III guidelines.[3]

Baseline characteristics of subjects

The baseline characteristics of the intention-to-treat population were evaluated and are presented in Table 1. The two groups produced by randomization were largely homogeneous in terms of demographic and clinical characteristics. Male and female subjects were equally distributed with 44:56% and 42:58% males:females in the placebo and supplement groups, respectively. There were no significant differences between groups in age, body weight, body mass index, systolic, diastolic and mean blood pressure, and pulse. Serum LDL-C, TC, non-HDL-C and apoB-100 were significantly different between placebo and supplement groups at baseline ($P<0.01$). There were no other significant differences between placebo and supplement groups at baseline for continuous or categorical variables ($P>0.05$).

Table 1. Demographic and clinical characteristics at baseline

	Placebo (n = *61*) *Mean (s.d.)*	L. reuteri (n = *66*) *Mean (s.d.)*	P
Caucasian (%)	100%	100%	1.00
Male (%)	44%	42%	0.859
Age (years)	47.59 (12.88)	50.48 (14.03)	0.230
Body weight (kg)	81.66 (12.41)	78.55 (11.38)	0.145
BMI (kg/m^2)	27.62 (2.81)	26.83 (3.05)	0.133
Systolic BP (mm Hg)	131.56 (11.58)	130.12 (11.22)	0.359
Diastolic BP (mm Hg)	77.61 (6.85)	78.48 (5.35)	0.296
Pulse (bpm)	72.59 (6.06)	73.30 (7.12)	0.614
Statin intake (%)	14.8%	7.6%	0.260
TC (mmol/l)	5.89 (0.59)	6.36 (0.79)	0.001
LDL-C (mmol/l)	4.14 (0.49)	4.53 (0.65)	<0.001
HDL-C (mmol/l)	1.24 (0.30)	1.33 (0.46)	0.545
Non-HDL-C (mmol/l)	4.64 (0.55)	5.03 (0.65)	<0.001
TG (mmol/l)	1.50 (0.73)	1.50 (0.79)	0.837
ApoB-100 (g/l)	1.20 (0.14)	1.28 (0.17)	0.005
ApoA-1 (g/l)	1.61 (0.27)	1.66 (0.38)	0.394
LDL-C/HDL-C	3.51 (0.92)	3.77 (1.21)	0.227
ApoB-100/apoA-1	0.77 (0.18)	0.81 (0.22)	0.286
Fibrinogen (g/l)	2.50 (0.66)	2.63 (0.87)	0.482
hs-CRP (mg/l)[a]	1.63 (0.90–3.25)	2.00 (0.80–4.43)	0.161
Campesterol (ng/ml)	2840.8 (1471.0)	3064.2 (1860.9)	0.668
Sitosterol (ng/ml)	1368.9 (682.1)	1520.1 (891.1)	0.408
Stigmasterol (ng/ml)	68.3 (51.3)	68.4 (49.4)	0.855
Total PS (ng/ml)	4278.0 (2156.1)	4652.7 (2756.9)	0.559

Abbreviations: apoA-1, apolipoprotein A-1; apoB-100, apolipoprotein B-100; BP, blood pressure; bpm, beats per min; BMI, body mass index; HDL-C, HDL-cholesterol; hs-CRP, high-sensitivity C-reactive protein; LDL-C, LDL-cholesterol; non-HDL-C, non-HDL-cholesterol; PS, plant sterol; TC, total cholesterol; TG, Triacylglycerol. [a]Geometric mean (interquartile range).

Dietary assessment

A dietary assessment of macronutrients was performed at baseline and end point. There were no significant differences between placebo and supplement groups over the intervention period in dietary intake of total energy, lipids, proteins or carbohydrates ($P>0.05$) (Table 2).

Serum lipid profile

Serum was assessed at baseline, week 3, week 6 and end point for LDL-C, TC, HDL-C, non-HDL-C, TG, apoB-100 and apoA-1. The mean absolute and relative changes of TC, LDL-C, HDL-C, non-HDL-C, TG, apoB-100 and apoA-1 from baseline to week 3 were not statistically significant ($P>0.05$) (data not shown). The mean absolute and relative changes of TC, LDL-C, HDL-C, non-HDL-C, TG, apoB-100 and apoA-1 from baseline to week 6 and end point are summarized in Table 3. Serum LDL-C at week 6 and end point were changed from baseline by −0.37 (0.51) and −0.30 (0.57) mmol/l, which was a significant mean change relative to placebo of −11.56% ($P<0.001$) and −11.64% ($P=0.001$), respectively. Serum TC at week 6 and end point were changed from baseline by −0.36 (0.62) and −0.42 (0.63) mmol/l, which was a significant mean change relative to placebo of −9.13% ($P<0.001$) and −9.14% ($P=0.001$), respectively. Serum non-HDL-C at week 6 and end point were changed from baseline by −0.33 (0.63) and −0.42 (0.64) mmol/l, which was a significant mean change relative to placebo of −10.41% ($P<0.001$) and −11.30% ($P<0.001$), respectively. Serum apoB-100 at week 6 and end point were changed from baseline by −0.13 (0.16) and −0.14 (0.18) mmol/l, which was a significant mean change relative to placebo of −10.34% ($P<0.001$) and −8.41% ($P=0.002$), respectively. Serum TG and HDL-C were unchanged over the course of the study.

Lipid and apolipoprotein ratios and cardiovascular risk parameters

Mean absolute and relative change in fibrinogen, LDL-C/HDL-C ratio and apoB-100/apoA-1 ratio, as well as the geometric mean and interquartile range of hs-CRP, are summarized in Table 4. The LDL-C/HDL-C ratio at end point was changed from baseline by −0.22 (0.88), which was a significant mean change relative to placebo of −13.39% ($P=0.006$). The apoB-100/apoA-1 ratio at end point was changed from baseline by −0.09 (0.20), which was a significant mean change relative to placebo of −9.00% ($P=0.026$). Plasma fibrinogen at end point was changed from baseline by −0.09 (0.96) g/l, which was a significant mean change

Table 2. Dietary total energy and macronutrient intake

	Placebo (n = *61*) *Mean (s.d.)*	L. reuteri (n = *66*) *Mean (s.d.)*	P[a]
Energy (kJ)			
Week 0	8 510.6 (2 689.5)	8 685.1 (2 888.3)	0.85
Week 9	8 370.7 (2 599.5)	8 475.0 (2 455.1)	
Lipids (%)			
Week 0	34.5 (6.2)	36.2 (6.7)	0.09
Week 9	36.6 (6.4)	36.1 (5.7)	
Proteins (%)			
Week 0	17.34 (3.54)	17.27 (3.2)	0.74
Week 9	17.59 (4.73)	17.3 (3.5)	
Carbohydrates (%)			
Week 0	48.1 (7.2)	46.5 (8.2)	0.11
Week 9	45.8 (8.4)	46.6 (7.1)	

[a]Two-factor repeated measures analysis of variance.

Table 3. Absolute and relative mean changes in lipid and apolipoprotein parameters at weeks 6 and 9

	Week 6			*Week 9*		
	Placebo (n = *61*) *Mean (s.d.)*	L. reuteri (n = *66*) *Mean (s.d.)*	P	*Placebo* (n = *61*) *Mean (s.d.)*	L. reuteri (n = *66*) *Mean (s.d.)*	P
TC						
Baseline	5.89 (0.59)	6.36 (0.79)		5.89 (0.59)	6.36 (0.79)	
AbsΔ (mmol/l)	0.21 (0.70)	− 0.36 (0.62)	<0.001[a]	0.16 (0.67)	− 0.42 (0.63)	<0.001[a]
RelΔ (%)	3.82 (12.47)	− 5.31 (10.04)	<0.001[b]	2.76 (11.76)	− 6.38 (10.28)	<0.001[a]
LDL-C						
Baseline	4.14 (0.49)	4.53 (0.65)		4.14 (0.49)	4.53 (0.65)	
AbsΔ (mmol/l)	0.14 (0.54)	− 0.37 (0.51)	<0.001[a]	0.21 (0.63)	− 0.30 (0.57)	<0.001[a]
RelΔ (%)	3.61 (12.74)	− 7.95 (11.46)	<0.001[a]	5.30 (15.57)	− 6.33 (13.15)	0.001[a]
HDL-C						
Baseline	1.24 (0.30)	1.33 (0.46)		1.24 (0.30)	1.33 (0.46)	
AbsΔ (mmol/l)	0.03 (0.29)	− 0.03 (0.33)	0.350[b]	0.01 (0.29)	0.00 (0.24)	0.753[b]
RelΔ (%)	3.81 (27.23)	0.44 (23.53)	0.367[b]	2.55 (27.15)	1.42 (19.63)	0.583[b]
Non-HDL-C						
Baseline	4.64 (0.55)	5.03 (0.65)		4.64 (0.55)	5.03 (0.65)	
AbsΔ (mmol/l)	0.18 (0.57)	− 0.33 (0.63)	<0.001[a]	0.15 (0.63)	− 0.42 (0.64)	<0.001[a]
RelΔ (%)	4.16 (12.36)	− 6.25 (12.41)	<0.001[a]	3.43 (14.07)	− 7.87 (12.87)	<0.001[a]
TG						
Baseline	1.50 (0.73)	1.50 (0.79)		1.50 (0.73)	1.50 (0.79)	
AbsΔ (mmol/l)	0.06 (0.69)	0.19 (0.89)	0.579[b]	0.13 (1.04)	− 0.02 (0.71)	0.635[b]
RelΔ (%)	15.95 (51.61)	24.00 (68.71)	0.710[b]	19.67 (73.81)	7.01 (42.65)	0.498[b]
apoB-100						
Baseline	1.20 (0.14)	1.28 (0.17)		1.20 (0.14)	1.28 (0.17)	
AbsΔ (g/l)	0.01 (0.14)	− 0.13 (0.16)	<0.001[a]	− 0.03 (0.15)	− 0.14 (0.18)	0.001[a]
RelΔ (%)	0.96 (11.70)	− 9.38 (11.68)	<0.001[a]	− 1.87 (12.63)	− 10.28 (13.46)	0.002[a]
apoA-1						
Baseline	1.61 (0.27)	1.66 (0.38)		1.61 (0.27)	1.66 (0.38)	
AbsΔ (g/l)	0.04 (0.31)	0.02 (0.26)	0.499[a]	0.02 (0.29)	0.00 (0.22)	0.870[b]
RelΔ (%)	4.48 (21.65)	3.12 (17.02)	0.534[b]	2.39 (20.12)	1.63 (16.09)	0.979[b]

Abbreviations: AbsΔ, absolute change; apoA-1, apolipoproteinA-1; apoB-100, apolipoproteinB-100; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; Non-HDL-C, Non-HDL-cholesterol; RelΔ, relative change; TC, Total cholesterol; TG, Triacylglycerol. [a]Analysis of covariance. [b]Mann–Whitney Wilcoxon test.

relative to placebo of − 14.25% ($P = 0.004$). Finally, there was a significant effect of *L. reuteri* NCIMB 30242 supplementation in serum hs-CRP ($P = 0.005$), as the geometric mean was changed by − 0.13 mg/l from baseline to end point and by − 1.05 mg/l from baseline to end point relative to placebo. Among subjects with hs-CRP in average (1–3 mg/l) or high (>3 mg/l) relative risk categories at baseline, 27.1% of subjects receiving *L. reuteri* NCIMB 30242 reduced their risk category by one or more risk groups as compared with 1.7% of subjects receiving placebo (data not shown).

Plasma bile acid and plant sterol profile

The mean concentrations of conjugated and deconjugated bile acids at baseline and end point are summarized in Table 5. There was a significant effect of *L. reuteri* NCIMB 30242 supplementation in plasma deconjugated bile acids over the course of the study ($P = 0.025$), as total deconjugated bile acids was increased by 0.80 μmol/l from baseline and by 1.00 μmol/l from baseline relative to placebo. As shown in Figure 1, significant association was observed in subjects taking *L. reuteri* NCIMB 30242 ($r = -0.369$, $P = 0.003$), whereas no association was observed in subjects taking placebo ($r = 0.086$, $P = 0.516$). The regression coefficients of the *L. reuteri* NCIMB 30 242 and placebo groups were significantly different ($P = 0.012$).

The mean concentrations of sitosterol, campesterol and stigmasterol at baseline and end point are summarized in Table 6, both in absolute terms and as a ratio to TC concentrations. There was a significant effect of *L. reuteri* NCIMB 30 242 supplementation in the absolute concentrations of plasma campesterol ($P = 0.025$), sitosterol ($P = 0.031$), stigmasterol ($P = 0.042$) and total plant sterols ($P = 0.027$) over the course of the study, as campesterol, sitosterol, stigmasterol and total plant sterols were decreased by 41.5%, 34.2%, 40.7% and 38.9%, respectively, from baseline to end point relative to placebo.

DISCUSSION

Although we have previously shown that microencapsulated *L. reuteri* NCIMB 30242 reduces LDL-C in hypercholesterolemic adults when delivered in a yogurt formulation,[18] it was not known if similar effects would be seen using a supplement capsule format, if additional efficacy would be observed over a longer interventional period, or if BSH-active *L. reuteri* NCIMB 30242 would result in increased circulating deconjugated bile acids or reduced absorption of non-cholesterol sterols. Thus, the present study was undertaken to assess the cholesterol-lowering efficacy of *L. reuteri* NCIMB 30242 capsules in hypercholesterolemic adults over 9 weeks and to investigate the mechanism of action. Results show that subjects consuming *L. reuteri* NCIMB 30242 capsules attained significant reductions in LDL-C of 11.64%, TC of 9.14%, non-HDL-C of 11.30%, apoB-100 of 8.41%, LDL-C/HDL-C of 13.39% and apoB-100/apoA-1 of 10.34% relative to placebo at the study end point.

Table 4. Changes in lipid and apolipoprotein ratios and cardiovascular risk parameters

	Placebo (n = 61) Mean (s.d.)	L. reuteri *(n = 66) Mean (s.d.)*	P
LDL-C/HDL-C			
Baseline	3.51 (0.92)	3.77 (1.21)	
AbsΔ	0.21 (1.16)	−0.22 (0.88)	0.005[a]
RelΔ (%)	8.91 (33.37)	−4.48 (21.24)	0.006[a]
apoB-100/apoA-1			
Baseline	0.77 (0.18)	0.81 (0.22)	
AbsΔ	−0.02 (0.19)	−0.09 (0.20)	0.034[a]
RelΔ (%)	−0.74 (22.82)	−9.74 (17.75)	0.026[b]
Fibrinogen			
Baseline	2.50 (0.66)	2.63 (0.87)	
AbsΔ (g/l)	0.48 (0.87)	−0.09 (0.96)	0.004[a]
RelΔ (%)	17.19 (35.67)	2.95 (26.71)	0.004[a]
hs-CRP (mg/l)[c]			
Week 0	1.63 (0.90–3.25)	2.00 (0.80–4.43)	0.005[d]
Week 9	2.55 (1.50–5.40)	1.87 (0.75–5.10)	

Abbreviations: AbsΔ, absolute change; apoA-1, apolipoproteinA-1; apoB-100, apolipoproteinB-100; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; RelΔ, Relative change. [a]Mann–Whitney Wilcoxon test. [b]Analysis of covariance. [c]Geometric mean (interquartile range). [d]Two-factor repeated measures analysis of variance.

Table 5. Conjugated and deconjugated bile acid concentrations at baseline and end point

	Placebo (n = 61) Mean (s.d.)	L. reuteri *(n = 66) Mean (s.d.)*	P[a]
Conjugated bile acids (μmol/l)			
Week 0	2.33 (3.05)	2.43 (2.85)	0.738
Week 9	2.22 (2.08)	2.49 (3.20)	
Deconjugated bile acids (μmol/l)			
Week 0	1.81 (2.79)	1.82 (2.48)	0.025
Week 9	1.62 (2.02)	2.64 (3.64)	

[a]Two-factor repeated measures analysis of variance.

A recent meta-analysis by Guo *et al.*,[30] including 13 lipid-lowering probiotic clinical studies, demonstrates a LDL-C mean net change of −0.12 mmol/l (~3%). The sample size of the studies included in the meta-analysis range from 14 to 78 and none of the included studies investigated the species *L. reuteri* or strain *L. reuteri* NCIMB 30242. Furthermore, no previous clinical trial has reported a significant LDL-C reduction by a probiotic supplement formulation unless delivered as a synbiotic.[26] It has been previously postulated that some probiotic lipid-lowering clinical trials lack appropriate strain selection, method of delivery or clinical design.[31] For the current study, a systematic approach was applied, including identifying a strain, *L. reuteri* NCIMB 30242, with the appropriate phenotypic characteristics including elevated natural BSH expression, ensuring the safety of the strain through phenotypic and genotypic characterization[32] and optimizing the production parameters of the strain.

Over the intervention period, subjects consuming BSH-active *L. reuteri* NCIMB 30242 capsules showed an increase in plasma deconjugated bile acids as compared with placebo, purported to be the result of increased BSH activity and intraluminal bile salt deconjugation. On a subject to subject basis, it was confirmed that LDL-C reduction over the course of the study was significantly correlated with increased plasma deconjugated bile acids in subjects consuming BSH-active *L. reuteri* NCIMB 30242 capsules. Furthermore, absolute concentrations of plasma plant sterols, surrogate markers of cholesterol absorption,[33,34] were significantly

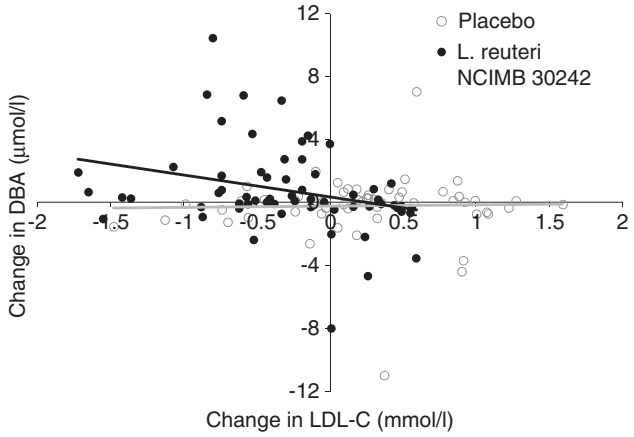

Figure 1. Individual changes in plasma deconjugated bile acids (DBAs) and associated changes in serum LDL-C over the intervention period. A significant association was observed in subjects taking *L. reuteri* NCIMB 30242 ($r = -0.369$, $P = 0.003$), whereas no association was observed in subjects taking placebo ($r = 0.086$, $P = 0.516$). The regression coefficients of the *L. reuteri* NCIMB 30242 and placebo groups were significantly different ($P = 0.012$).

Table 6. Plant sterol concentrations at baseline and end point

	Placebo (n = 61) Mean (s.d.)	L. reuteri *(n = 66) Mean (s.d.)*	P[a]
Campesterol (ng/ml)			
Week 0	2840.8 (1471.0)	3064.2 (1860.9)	0.025
Week 9	3438.3 (2041.2)	2805.0 (1649.8)	
Sitosterol (ng/ml)			
Week 0	1368.9 (682.1)	1520.1 (891.1)	0.031
Week 9	1636.8 (945.3)	1428.9 (868.5)	
Stigmasterol (ng/ml)			
Week 0	68.3 (51.3)	68.4 (49.4)	0.042
Week 9	81.2 (78.7)	62.0 (54.2)	
Total PS (ng/ml)			
Week 0	4278.0 (2156.1)	4652.7 (2756.9)	0.027
Week 9	5156.4 (3008.7)	4295.9 (2524.6)	
Campesterol/TC ratio (ng/μg)			
Week 0	1.261 (0.672)	1.262 (0.811)	0.237
Week 9	1.477 (0.854)	1.227 (0.735)	
Sitosterol/TC ratio (ng/μg)			
Week 0	0.606 (0.303)	0.626 (0.384)	0.287
Week 9	0.702 (0.397)	0.624 (0.381)	
Stigmasterol/TC ratio (ng/μg)			
Week 0	0.030 (0.024)	0.028 (0.022)	0.255
Week 9	0.035 (0.033)	0.028 (0.026)	
Total PS/TC ratio (ng/μg)			
Week 0	1.897 (0.978)	1.916 (1.198)	0.235
Week 9	2.214 (1.259)	1.879 (1.119)	

Abbreviations: PS, plant sterol; TC, total cholesterol. [a]Two-factor repeated measures analysis of variance on logarithmically transformed values.

reduced in subjects consuming BSH-active *L. reuteri* NCIMB 30242 capsules, indicating a reduced total inward transport of non-cholesterol sterols and suggesting a reduction in the absorption of dietary and biliary cholesterol.[35] As discussed by Jakulj *et al.*[36], plant sterol concentration may be presented both as absolute values and as ratios adjusted to TC. The latter is generally encouraged to eliminate the influence of high lipoprotein concentrations, such as in individuals with familial hypercholesterolemia.[37] In contrast, absolute values of plant sterols have been advocated in instances where statin-induced changes in markers of cholesterol absorption are concerned.[38]

Previously, it has been shown that germ-free rats tend to accumulate more cholesterol than their conventionally raised counterparts, and in the absence of gut microbiota, biliary bile acids and cholesterol absorption are increased by 300% and 25%, respectively.[7,8,11] Follow-up studies confirmed that germ-free animals have elevated conjugated bile acids throughout the intestine with no deconjugation and strongly decreased fecal excretion.[9] It has been hypothesized that increases in deconjugated bile acids may result in reduced farnesoid X receptor activation, increased cholesterol catabolism, reduced inhibition of liver X receptor (LXR) and upregulation of adenosine triphosphate-binding cassette (ABC)G5/G8 transporters. These transporters efflux cholesterol from hepatocytes and enterocytes[18] and are stimulated in the presence of deconjugated bile acids. In support of this, it has been shown that oral feeding of *Lactobacillus plantarum* KCTC3928 to mice resulted in significantly decreased LDL-C and TG, increased fecal bile-acid excretion, increased hepatic bile-acid synthesis, and increased expression of 7-alpha-hydroxylase (CYP7A1), the key enzyme in catabolism of cholesterol and bile-acid synthesis.[39] Such changes in lipid homeostasis and gene expression are consistent with what is known regarding the action of bile acids on farnesoid X receptor and LXR, including the findings that farnesoid X receptor activation by bile acids causes induction of small heterodimer partner and feedback inhibition of bile-acid synthesis by reducing expression of 7-alpha-hydroxylase (CYP7A1).[40,41] In turn, reduced small heterodimer partner inhibition of LXR results in reduced triglyceride and cholesterol biosynthesis[42] and stimulation of cholesterol export from cells through expression of ABCG5/G8 transporters.[43] Furthermore, it has been shown that overexpression of ABCG5 and ABCG8 in transgenic mice limits sterol absorption and promotes neutral sterol excretion[44] and that deconjugated bile acids preferentially increase ATPase activity of ABCG5/G8 transporters found on the apical membranes of enterocytes and hepatocytes, limiting the accumulation of cholesterol by transporting it into the intestinal lumen and bile.[45]

The present results indicate that increased intraluminal BSH activity, in response to *L. reuteri* NCIMB 30242 supplementation, leads to an increase in deconjugated bile acids, a reduction in non-cholesterol sterol absorption and serum cholesterol, which is consistent with much of these hypotheses. The possibility, however, that the observed changes in sterol absorption may be a result of Niemann–Pick C1-like 1 transporter inhibition or a combination of cholesterol-reducing mechanisms should be considered. Yoon *et al.*[46] reported that BSH-active *Lactobacillus* acting on Caco-2 cells resulted in the upregulation of LXR together with elevated expression of ABCG5/G8 transporters and increased cholesterol efflux without noticeable effects on Niemann–Pick C1-like 1 expression. In contrast, a study by Huang and Zheng[47] showed that the probiotic strain *L. acidophilus* ATCC 4356 reduced Niemann–Pick C1-like 1 gene expression and inhibited the cellular uptake of micellar cholesterol in Caco-2 cells, an effect at least partly mediated by LXR.

Plasma fibrinogen was significantly reduced by 14.25% relative to placebo in response to *L. reuteri* NCIMB 30242 supplementation over the intervention period. Previously, Naruszewicz *et al.*[48] showed that administration of a rose-hip drink containing *L. plantarum* 299v reduced plasma IL-6 and fibrinogen concentrations in smokers. Fibrinogen is the major coagulation protein in blood by mass, contributes to blood viscosity,[49] is an inflammatory acute-phase protein and has been independently associated with cardiovascular disease outcomes.[50] The present results also show a significant reduction in hs-CRP in response to *L. reuteri* NCIMB 30242 supplementation, both in geometric mean values as well as in subjects reducing relative risk categories. This is in contrast to a recent study by Leber *et al.*[51] showing that *Lactobacillus casei* Shirota significantly increased hs-CRP in patients with metabolic syndrome. Elevated hs-CRP is independently associated with increased risk of CAD,[52] diabetes mellitus,[53] hypertension,[54] stroke[55] and mortality[56] and is closely associated with elevated fibrinogen. Fibrinogen as well as hs-CRP is regulated by IL-6 in hepatocytes.[57,58] Furthermore, pharmacological agents currently prescribed to treat CAD, such as statins,[59] aspirin[55] and β-blockers,[60] have been reported to reduce hs-CRP, suggesting that reduced inflammation contributes to the beneficial effects of these medications.

In summary, this study demonstrates that *L. reuteri* NCIMB 30242 capsules significantly decrease LDL-C by 11.64% over 9 weeks. Furthermore, there were significant decreases in secondary lipid end points at 6 and 9 weeks, including LDL-C, TC, apoB-100 and non-HDL-C. Significant reductions in end points considered to be cardiovascular risk factors, including fibrinogen and hs-CRP, were seen at the 9-week end point. Increased plasma deconjugated bile acids and reduced plasma non-cholesterol sterols campesterol, sitosterol and stigmasterol suggest an effect on the absorption of these compounds and a novel cholesterol-reducing mechanism of action. These results show that *L. reuteri* NCIMB 30242 can be used to reduce serum LDL-C, likely by its effect on cholesterol absorption, and indicate its potential as an adjunctive therapy for the treatment of hypercholesterolemia.

CONFLICT OF INTEREST

MLJ and SP acknowledge a conflict of interest as they are cofounders and shareholders of Micropharma. CJM is employed by and is a shareholder of Micropharma.

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