Beneficial Microbes, 2019; 10(5): 497-509

Wageningen Academic Publishers

Three probiotic strains exert different effects on plasma bile acid profiles in healthy obese adults: randomised, double-blind placebo-controlled crossover study

T. Culpepper[1], C.C. Rowe[1], C.T. Rusch[1], A.M. Burns[1], A.P. Federico[1], S.-A. Girard[2], T.A. Tompkins[2], C. Nieves Jr.[1], J.C. Dennis-Wall[1], M.C. Christman[3,4,5] and B. Langkamp-Henken[1*]

[1]Food Science and Human Nutrition Department, University of Florida, 572 Newell Drive, Gainesville, FL 32611, USA; [2]Lallemand Health Solutions Inc., 6100 Royalmount, Montréal, QC H4P 2R2, Canada; [3]Department of Statistics, University of Florida, 102 Griffin-Floyd Hall, Gainesville, FL 32611, USA; [4]Department of Biology, University of Florida, Bartram Hall, 876 Newell Drive, Gainesville, FL 32611, USA; [5]MCC Statistical Consulting LLC, 2219 NW 23rd Ter, Gainesville, FL 32605, USA; henken@ufl.edu

Received: 10 November 2018 / Accepted: 22 March 2019

RESEARCH ARTICLE

Abstract

Microbial metabolism in the gut may alter human bile acid metabolism in a way that beneficially affects lipid homeostasis and therefore cardiovascular disease risk. Deconjugation of bile acids by microbes is thought to be key to this mechanism but has yet to be characterised in blood and stool while observing lipid markers. The aim of this study was to determine the effect of 3 different probiotic strains on plasma and stool bile acids in the context of lipid and glucose metabolism. In this 18-week, randomised, double-blind crossover study, healthy adults (53±8 years) with a high waist circumference underwent a 1-week pre-baseline period and were then randomised to receive 1 capsule/day of *Bacillus subtilis* R0179 (2.5×10^9 cfu/capsule; n=39), *Lactobacillus plantarum* HA-119 (5×10^9 cfu/capsule; n=38), *Bifidobacterium animalis* subsp. *lactis* B94 (5×10^9 cfu/capsule; n=37) or placebo for 6 weeks. Following a 3-week washout and second pre-baseline week, participants were crossed to the other intervention for 6 weeks followed by a 1-week post-intervention period. Blood and stool samples were collected at the beginning and end of each intervention to measure bile acids, serum lipid profiles, and glucose and insulin levels. Data from the placebo intervention were combined for all participants for analyses. In obese participants, the difference (final-baseline) in the sum of deconjugated plasma bile acids was greater with consumption of *B. subtilis* (691±378 nmol/l, P=0.01) and *B. lactis* (380±165 nmol/l, P=0.04) than with placebo (98±176 nmol/l, n=57). No significant differences were observed for any probiotics for stool bile acids, serum lipids, blood glucose or insulin. These data suggest that *B. subtilis* and *B. lactis* had no effect on glucose metabolism or serum cholesterol but increased deconjugated plasma bile acids in obese individuals. Additional studies should be conducted to confirm these findings and explore potential mechanisms. This trial was registered at clinicaltrials.gov as NCT01879098.

Keywords: *Bacillus subtilis* R0179, *Lactobacillus plantarum* HA-119, *Bifidobacterium animalis* subsp. *lactis* B94, deconjugated bile acids, serum lipids

1. Introduction

Cardiovascular disease (CVD) contributes to an estimated 31% of all global deaths each year (WHO, 2018). Blood cholesterol is the one of the main modifiable risk factors of CVD. Bile acid sequestrants comprise a subset of therapeutic agents used to lower blood low-density lipoprotein (LDL) cholesterol. While these and other medications are fairly efficacious, many patients continue to seek non-pharmacological ways of modulating disease because of side effects and a general inclination towards less aggressive therapies. Well known lifestyle factors that can modulate cholesterol homeostasis include diet, weight management, and stress reduction (MedlinePlus, 2018); gut microbes may also play a role (Allayee and Hazen, 2015; Wostmann *et al.*, 1966). Although probiotics have

typically been studied for their gastrointestinal and immune benefits, certain aspects of microbial metabolism are now thought to alter human bile acid metabolism in a way that may beneficially affect lipid homeostasis and CVD risk. Evidence supports the potential efficacy of some but not all probiotic strains to lower cholesterol (Guo *et al.*, 2011).

Bile acids are important for the absorption of cholesterol, dietary fats and lipid-soluble vitamins. Primary bile acids are synthesised in the hepatocytes from cholesterol and are excreted into the lumen in a conjugated form (bound to taurine or glycine) or salt form, which is ionic and more likely to form micelles. Once in the lumen, microbes that exhibit bile salt hydrolase (BSH) activity are able to deconjugate bile acids from the taurine or glycine conjugates. Bile acids can also be modified into secondary bile acids by bacterial 7 alpha-dehydroxylase; an example of this is the production of lithocholic acid (LCA) from chenodeoxycholic acid (CDCA). It has been proposed that these bacterial modifications to bile acids may be the reason for cholesterol-lowering properties of probiotics (Ishimwe *et al.*, 2015; Pavlovic *et al.*, 2012). Probiotics have been proposed to inhibit bile acid reabsorption and recycling similarly to the actions of bile acid sequestrants, thus reducing the bile acid pool which will attenuate signalling through the bile-sensing transcription factor farnesoid X receptor (FXR). Reduced signalling will subsequently activate the hepatic enzymes for conversion of hepatic cholesterol to bile to compensate for the faecal loss of bile (Ishimwe *et al.*, 2015). However, this notion is still controversial, as probiotic interventions have resulted in an increase (Jones *et al.*, 2012b) or no change (Ooi *et al.*, 2010) to blood deconjugated bile acids in conjunction with decreases in both total and LDL cholesterol. This dissonance warrants further mechanistic investigation.

BSH activity has been commonly used to identify candidate probiotic strains that are tolerant to the harsh environment of the intestines (Begley *et al.*, 2006), and now this property is being studied in the context of blood cholesterol metabolism. *In vitro*, *Lactobacillus plantarum* HA-119 displayed positive BSH activity in glycochenodeoxycholic acid (GCDCA) and 0.025% bile salt mixture, and *Bifidobacterium animalis* subsp. *lactis* B94 displayed positive BSH activity in taurodeoxycholic acid (TDCA) but *Bacillus subtilis* R0179 did not display BSH activity with the bile salts tested (unpublished data). *B. subtilis* R0179 has, however, been shown to survive passage in the gastrointestinal tract in humans (Hanifi *et al.*, 2015), indicating that it still tolerates and may interact directly with bile acids in an *in vivo* setting. Additionally, *B. subtilis* has been shown to modulate abnormal lipid metabolism when administered to mice with a high-fat diet (Lei *et al.*, 2015). For these reasons and because of the presence of BSH genes identified via whole genome sequencing in all three strains, these three strains were identified for the current study.

While numerous studies have been conducted to assess the effect of probiotics on blood lipid profiles, few have undertaken characterisation of blood bile acid profiles (Jones *et al.*, 2012b; Ooi *et al.*, 2010) or stool bile acid profiles (Jones *et al.*, 2012a; Trautvetter *et al.*, 2012), and none have characterised both of these profiles simultaneously with blood lipid markers. This study aimed to characterise the effect of three probiotic strains (*L. plantarum* HA-119, *B. lactis* B94, and *B. subtilis* R0179) on all 15 naturally occurring bile acids in both blood and stool samples in conjunction with blood lipid profiles in healthy adults with a high waist circumference (NHLBI, 2018). It was hypothesised that supplementation with the study probiotics would increase the conversion of conjugated bile acids to their deconjugated form, resulting in increases in deconjugated bile acids in both blood and stool samples. Thus, the primary outcome of this study was the change (final-baseline) in plasma deconjugated bile acids.

2. Materials and methods

Participants

Participants were recruited via flyers, posters, post cards and newspaper and radio ads. Men and nonpregnant women were included in the study if they were between 35 and 65 years of age, had a waist circumference greater than 102 cm for males or greater than 88 cm for females, were able to complete the informed consent form in English, were willing to maintain their regular dietary intake and levels of physical activity for the 18-week study, were willing to discontinue any probiotic and prebiotic-containing foods or supplements or other immune-enhancing supplements, had not taken any statins or cholesterol-lowering medications within the previous 6 months, were not taking medications for constipation or diarrhoea on a regular basis, were not taking androgens or receiving regular immune suppressing interventions, were not taking plant sterols, n-3 fatty acids, fish oil, soy protein, soluble oat fibre, psyllium seed husk or other cholesterol-lowering supplements within the 3 months leading up to the start of the study, were not being treated for or had physician-diagnosed diseases including pancreatitis, immune modulating, kidney, pulmonary, hepatic, biliary, or gastrointestinal disease or conditions (with the exception of gastroesophageal reflux disease), did not have a central venous catheter, colostomy or ileostomy, were not taking medications for type 1 or type 2 diabetes, had not received antibiotic therapy or colonoscopy in the 2 months leading up to the start of the study, did not typically consume >20 g of fibre daily assessed by a fibre screener (Block *et al.*, 2000) and did not engage in more than 60 min per day of moderate exercise. This study was approved by the University of Florida Institutional Review Board and was carried out in accordance with the guidelines of the Declaration of Helsinki. This trial was registered at clinicaltrials.gov as NCT01879098.

Experimental design and questionnaires

Participants were enrolled in this 18-week, randomised, double-blind, placebo controlled, crossover study from August of 2013 to February 2014 (Figure 1). Participants completed a 1-week pre-baseline period during which they completed daily and weekly questionnaires. Following the pre-baseline week the participants were randomised via sealed envelope to receive a probiotic or placebo. The randomisation scheme and envelopes were prepared by an individual who did not have contact with the participants. The participants took the study supplement or placebo and completed daily and weekly questionnaires for the next 6 weeks. Participants continued to complete questionnaires during the 4 weeks after the first intervention (1-week post-intervention with 2 additional weeks of washout, and 1 pre-baseline week for the second intervention) and during the second 6-week intervention and 1-week post-intervention periods.

Each day of the study, participants received an email with a link to an online questionnaire (Qualtrics; Provo, UT, USA). Daily questionnaires asked participants to report intake of the study supplement, number of stools, adverse events, and daily alcohol consumption. Weekly questionnaires asked participants to report the symptom intensity (1 = no discomfort to 7 = very severe discomfort) for 15 gastrointestinal symptoms, which were classified into 5 syndromes: diarrhoea (diarrhoea, loose stools, and bowel movement urgency), abdominal pain (abdominal pain, hunger pains, and nausea), reflux (heartburn and acid regurgitation), indigestion (stomach rumbling, bloating, burping, and flatulence), and constipation (constipation, hard stools, and feeling of incomplete evacuation) (Revicki *et al.*, 1998). Participants completed 12 weekly unannounced Automated Self-administered 24-hour Dietary Recalls over the placebo and probiotic interventions to assess total fibre, fat, saturated fat, and cholesterol (Subar *et al.*, 2012). The International Physical Activity Questionnaire (IPAQ) was completed before and after each intervention to assess activity levels (Craig *et al.*, 2003).

Study capsule blinding and administration protocol

The blinding of the study capsules was performed by the study sponsor. A total of 12 different 4-digit codes were randomly generated and paired together (placebo and probiotic or probiotic and placebo) by the sponsor to make 6 different groups. At the study site, the groups were randomly assigned via sealed envelope. Those participants who volunteered to provide stool samples were randomised separately via sealed envelope. No one involved in the blinding or generating the randomisation envelopes had direct contact with the study participants. The study

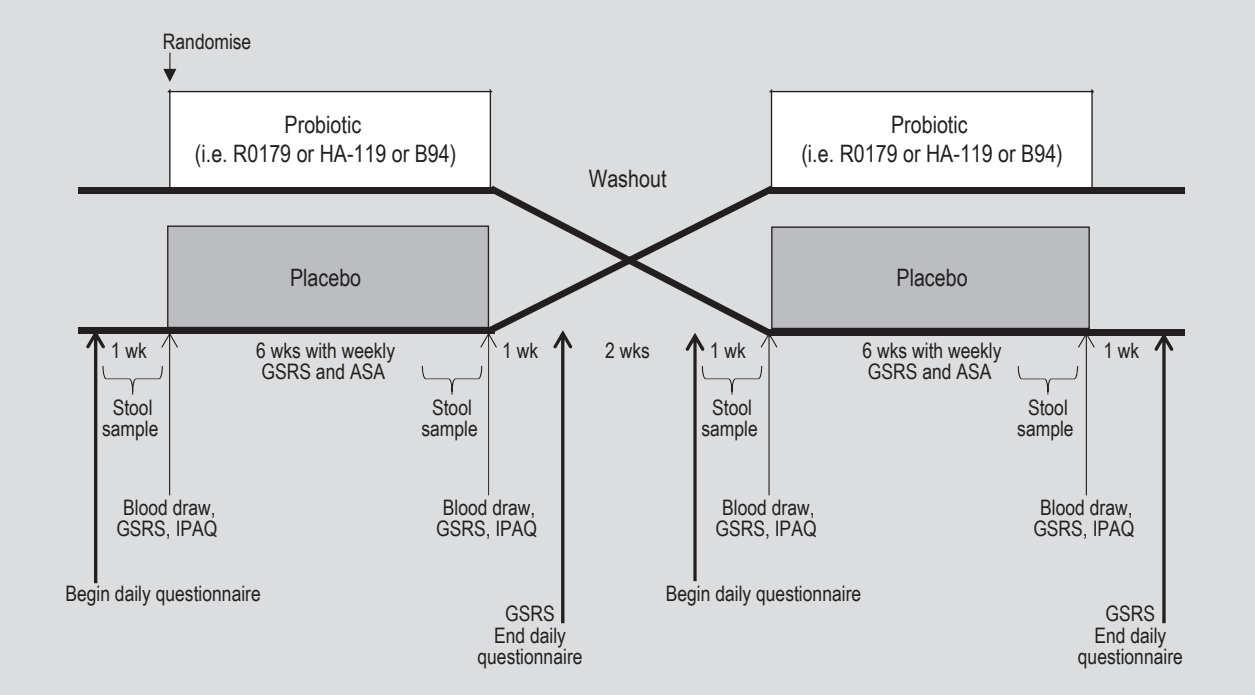

Figure 1. Study design. After a 1-week pre-baseline period participants were randomised to receive *Bacillus subtilis* R0179, *Lactobacillus plantarum* HA-119, *Bifidobacterium animalis* subsp. *lactis* B94, or placebo. Following a 6-week intervention, 3 weeks of washout, and second pre-baseline week, participants were crossed to the opposite intervention (i.e. probiotic to placebo or placebo to probiotic). ASA = Automated Self-administered 24-hour Dietary Recall; GSRS = Gastrointestinal Symptom Rating Scale; IPAQ = International Physical Activity Questionnaire.

supplements were provided in coded, sealed, plastic bottles that were similar in size and shape to commercially available multivitamin bottles. All capsules had a similar physical appearance and contained an off-white powder. 50% (n=30) of the participants who received a probiotic during the first intervention period correctly identified that they received the probiotic and 59% (n=19) correctly identified that they received the placebo during the first intervention period. This suggests successful blinding (P=0.498). Each capsule contained either 2.5×10^9 cfu (cfu expected after 2 y at room temperature) of *B. subtilis* R0179, 5×10^9 cfu of *L. plantarum* HA-119, 5×10^9 cfu of *B. lactis* B94, or a placebo (potato starch and stearate; Lallemand Health Solutions, Montreal, QC, Canada). Prior to the start of the second intervention, participants received a second bottle of capsules. Participants who received the probiotic during the first intervention were crossed over to the placebo and those who received the placebo initially were crossed over to one of the three probiotics. Participants were instructed to store the capsules in a refrigerator and to consume one capsule daily, with a meal, for the duration of the intervention.

Sample collection

Fasting blood samples were collected on the first and last day of each intervention to measure the complete blood cell count with differential (CHC Labs, Lake City, FL, USA), bile acids, and blood lipids. Blood samples from women with childbearing potential were tested for human chorionic gonadotropin (hCG) using Sure-Vue Serum/Urine hCG Test Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Stool samples were collected by participants during the two pre-baseline weeks and during the final week of each intervention using a Commode Specimen Collection System (Thermo Fisher Scientific), but only the samples from the first 10 participants in each intervention-placebo group who provided all stool and blood samples were sent for analysis for stool bile acids. Participants delivered the specimen collector to the study site within 4 h of defecation. The stool samples were homogenised by stirring in the collection kit, aliquoted into tubes, and stored at -70 °C until they were processed for bile acids.

Laboratory assays

The primary outcome of this study was the change (final-baseline) in plasma deconjugated bile acids; secondary outcomes included changes in all other bile acids and metabolic parameters mentioned below. Plasma and stool bile acids were analysed by the Southeast Center for Integrated Metabolomics at the University of Florida (Gainesville, FL, USA) using previously described methods (Bathena *et al.*, 2013). Briefly, plasma samples were prepared by adding 300 µl methanol to 100 µl of plasma. Samples were mixed via agitation, centrifuged at 2,000×*g* for 10 min, and 10 µl of supernatant was injected onto the LC-MS/MS. For each stool sample, approximately 100 mg was transferred to 15 ml conical tubes containing 1.5 ml methanol. Samples were sonicated in a room temperature water bath for 30 min. 4 ml tert-butyl methyl ether was added, and the samples were sonicated for a further 60 min. Phase separation was then induced by adding 2 ml water followed by centrifugation at 1000×*g* for 10 min. The samples were stored at -76 °C for at least 6 h before the upper organic liquid phase was collected. After thawing, the lower aqueous phases were re-extracted with 4 ml tert-butyl methyl ether and sonicated, and the organic phase was collected as before. The two organic extracts were combined and dried under a nitrogen stream at 50 °C. The dried residue was reconstituted in 3 ml tert-butyl methyl ether, to which 1.5 ml water was added, and the organic phase was collected and dried as before. The sample was reconstituted in 0.5 ml methanol containing [$^{18}O_2$] labelled bile acid standards. 10 µl of each sample was injected onto the LC-MS/MS. Samples were chromatographed on an Accucore RP-MS column (100×2 mm, Thermo Fisher Scientific) and eluted with a water-acetonitrile gradient (containing 0.1% formic acid). Tandem mass spectrometry was conducted on a TSQ Quantum Ultra (Kobis Ltd., Mlakarjeva 26, Slovenia) using heated electrospray ionisation (H-ESI). Of the bile acids quantified in the plasma and stool (Table 1), lithocholic acid was not discernible in the plasma samples. Bile acids were classified as either deconjugated or conjugated and either primary or secondary for statistical analyses (Table 1).

Blood lipids [triglycerides (TG), high-density lipoprotein (HDL) and LDL cholesterol, and non-esterified fatty acids (NEFA)] and glucose were measured directly using enzymatic colorimetric assay kits (Wako Chemicals USA, Inc., Richmond, VA, USA). Total cholesterol was calculated using the Friedewald formula: Total = LDL + HDL + (TG/5). Insulin and apolipoproteins A1 and B100 were analysed using ELISA (insulin, ALPCO, Salem, MA, USA; apolipoproteins, Abcam, Cambridge, MA, USA).

Statistical analyses

Sample size calculation

Based on a published study by Jones *et al.* (2012b) in hypercholesterolaemic adults, who demonstrated a mean difference between treatments of about 1.0, a significant difference in deconjugated bile acids was predicted to be observed with 30 subjects per probiotic/placebo cross at sigma = 3, alpha = 0.05, power >80%, an assumed relationship between measurements on the same individual of 0.75, a difference of means of at least 0.8, and a 15% attrition rate. For the three probiotic/placebo crosses, the total number of participants required was 90.

Table 1. Classification of bile acids and limit of detection within plasma and stool.

Bile acids		Abbreviation	Primary/secondary	Limit of detection	
				Plasma nmol/l	Stool arbitrary units/g
Deconjugated bile acids	Chenodeoxycholic acid	CDCA	primary	12.00	0.20
	Cholic acid	CA	primary	1.80	4.00
	Deoxycholic acid	DCA	secondary	5.00	4.00
	Lithocholic acid	LCA	secondary	[a]	0.15
	Ursodeoxycholic acid	UDCA	secondary	5.00	1.25
Conjugated bile acids	Taurochenodeoxycholic acid	TCDCA	primary	1.50	[b]
	Taurocholic	TCA	primary	0.12	[b]
	Taurodeoxycholic	TDCA	secondary	1.50	[b]
	Taurolithocholic	TLCA	secondary	0.10	[b]
	Tauroursodeoxycholic	TUDCA	secondary	0.15	[b]
	Glycochenodeoxycholic	GCDCA	primary	4.00	0.05
	Glycocholic	GCA	primary	3.00	[b]
	Glycodeoxycholic	GDCA	secondary	1.50	0.10
	Glycolithocolic	GLCA	secondary	1.00	0.20
	Glycoursodeoxycholic	GUDCA	secondary	6.00	0.50

[a] LCA limit of detection: 30 nmol/l.
[b] Below the limit of detection in arbitrary units.

Labs

The change score method was used for all bile acid and blood lipid outcomes (i.e. the difference between baseline and final was the variable analysed). Data were initially checked for extraneous effects related to the crossover nature of the study, including an effect of sequence (order of the interventions), an effect of period (to account for unexpected variations throughout the study), and an effect of group (three different start dates for participants: August, November, or January). Baseline measurements allowed for testing of carryover effects and were also used to determine order effects, if any. Data were then analysed using general or generalised linear mixed models including the above effects when appropriate and also fixed effects of intervention (either probiotic or placebo), waist circumference categories (tertiles) and/or body mass index (BMI) categories (<30 or ≥30), sex, and any relevant interactions. Effects that did not contribute significantly to the model were removed hierarchically; intervention was always retained in models regardless of contribution to significance. Models assessed probiotic strains individually by comparing only individuals on the intervention to themselves on the placebo. After unblinding, a pooled analysis was conducted in which all placebo data was combined to increase the power of the analyses; data from the individual probiotic strains were only compared to the placebo data and not between strains. Pooling of placebo data was done for outcomes not affected by the extraneous effects of the crossover study. Model assumptions were checked and transformations of the response variable were performed to correct for non-normality or heterogeneous variance when necessary. Random effects were included to account for the repeated observations on participants and testing was performed to identify the correct covariance structure to capture the correlation among repeated observations. Bile acid values that were below the limit of detection were treated as zeroes for the purpose of analyses.

Questionnaires

Intake of fibre and nutrients, including total fat, saturated fat, and cholesterol, was compared between probiotic and placebo using two-tailed paired t-tests or Wilcoxon Signed Rank Tests when the normality test failed. Data represent the mean ± standard error of the mean.

Physical activity was categorised by metabolic equivalent (MET) minutes measured by the International Physical Activity Questionnaire (IPAQ) into three groups: low (<600 MET-min/week), moderate (600 to <3,000 MET-min/week), or high (≥3,000 MET-min/week) physical activity during each intervention period.

GSRS syndrome scores were converted to a binary variable; any value greater than 1 indicated some level of syndrome score and any value equal to 1 indicated 'no discomfort at all' as is written in the validated version of the questionnaire.

This variable for each syndrome score was then analysed in a binary, logit-linked mixed model with fixed effects of intervention, sequence (order of the interventions), period (either 1 or 2, to account for unexpected variations throughout the study), and time (either pre-baseline, intervention, or post-intervention) and the interactions between these. A random effect of subject was included in the model and the variance matrix was blocked by participant in order to account for the within-subject correlation.

Safety outcomes

For hypothesis testing of all variables related to the complete blood cell count with differential, weight, blood pressure, glucose, and TG, the statistical model was a randomised block, repeated measures cross-over study with 2 arms for each investigational product. Models were considered with varying fixed effects: group (block for start month – August, November, or February), sequence (placebo then probiotic or probiotic then placebo), period (1 or 2), intervention (probiotic or placebo), time (baseline or final), and all relevant interactions with interventions. Nonsignificant effects were removed hierarchically to result in the final model. The need for a repeated measures covariance was tested using a likelihood ratio test and was found to be a statistically significant effect for all blood variables. Hence all models had a non-diagonal covariance structure to account for the repeated measurements.

All analyses were conducted while investigators were blind to interventions. After unblinding, the placebo groups from all three interventions were combined for the pooled analyses. Data were analysed on an intent-to-treat basis for all outcomes and for all eligible subjects over the two 6-week interventions. Data are reported using the model least squares means ± standard error of the mean and significance was determined using a type I error rate cut-off of 0.05, unless otherwise stated. All statistical models were analysed using statistical software [SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and Sigma Plot Version 12.5 (Systat Software Inc., San Jose, CA, USA)].

3. Results

Participants and compliance

A total of 150 participants provided written informed consent; however, 37 participants were excluded prior to randomisation because they were no longer interested in participating or did not meet inclusion criteria (Figure 2). Six participants were withdrawn for non-compliance during the pre-baseline period prior to randomisation. Following randomisation five participants assigned to the placebo intervention withdrew during or after the intervention period because they were too busy to complete study activities (n=2), were lost to follow-up (n=2), or moved away from the study location (n=1). One participant was lost to follow-up after 35 days on the *B. lactis* intervention. Two participants who were assigned to receive *B. subtilis* withdrew because of time commitments. The first withdrew prior to taking any study capsules and the second withdrew after 41 days of intervention. One participant randomised to *L. plantarum* was lost to follow-up after 29 days of intervention. Two participants were lost to follow-up after the second intervention of the study (placebo, n=1; *L. plantarum*, n=1). Ten participants from each intervention provided stool samples for bile acid analyses. The majority of study participants were white females with a BMI that categorised them as obese; on average, participants reported taking 90% of the daily study capsules (Table 2).

The difference from baseline to final in the sum of plasma deconjugated bile acids was not statistically significantly different from the placebo for *L. plantarum* or *B. subtilis*; however, a trend (P=0.0752) was observed with *B. lactis*. When data from the placebo groups were pooled, the effects of *B. lactis* and *B. subtilis* were statistically significant for those in the ≥30 BMI category (Figure 3). Data for BMI-stratified cohorts can be found in Supplementary Table S1. At baseline, the average sums of deconjugated plasma bile acids ranged from 814 to 1,100 nmol/l and were not different between any of the study probiotics compared with the pooled placebo group. The difference (final-baseline) in the sum of deconjugated plasma bile acids was positive and greater with *B. lactis* (P=0.0402) and *B. subtilis* (P=0.0104) compared with the pooled placebo group but only in participants who had a BMI ≥30 and not those who had a BMI <30. Additionally, the difference in the sum of deconjugated plasma bile acids was greater with *B. subtilis* in participants who had a BMI ≥30 compared with those who had a BMI <30 (P=0.0055).

Plasma and stool bile acids

An interaction between BMI category and *B. subtilis* (P=0.0592) and *B. lactis* (P=0.0267) was also observed for the difference in the sum of plasma secondary bile acids. This interaction was lost for *B. lactis* but remained for *B. subtilis* when compared with the pooled average for all placebo groups (Figure 4). With *B. subtilis* the difference (final-baseline) in the sum of plasma secondary bile acids was negative after the intervention in those with a BMI <30 and positive after the intervention in those with a BMI ≥30. Within the BMI ≥30 group, plasma secondary bile acids tended to be higher with *B. lactis* than with the placebo (P=0.0890). Certain individual plasma bile acids increased or decreased depending on the intervention and BMI subgroup (Supplementary Figure S1).

No significant differences were observed between the pooled placebo group and each of the three study probiotics

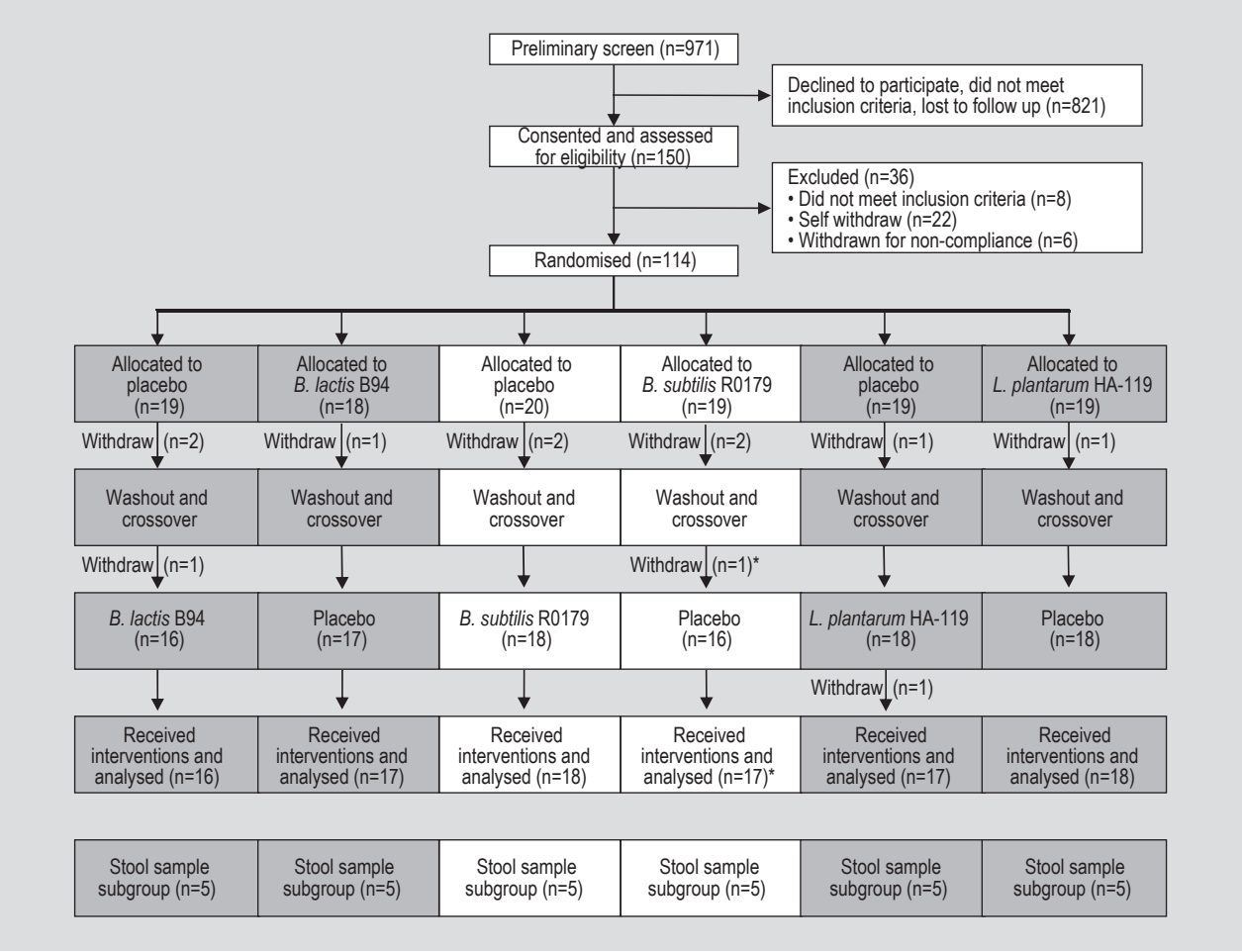

Figure 2. Participant enrolment and flow for the main study and subgroup that provided stool samples. Primary outcome – change in plasma deconjugated bile acids. * Participant was analysed for the *B. subtilis* R0179, but not the placebo period.

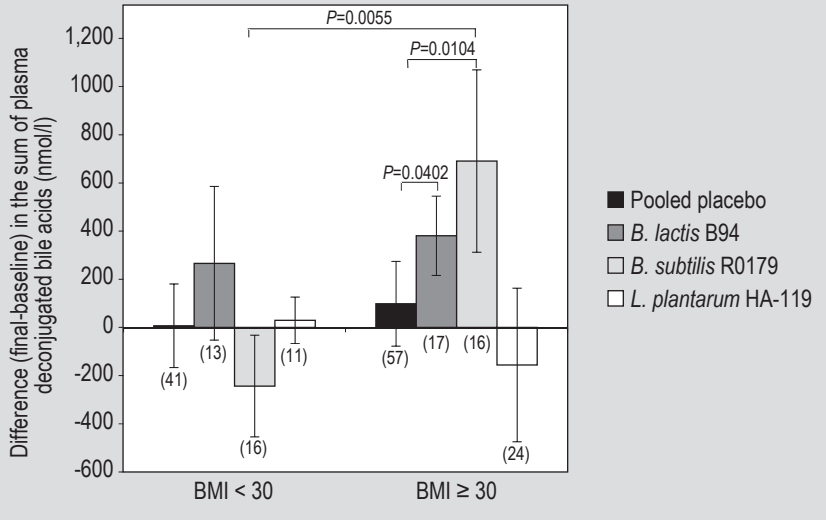

Figure 3. Plasma deconjugated bile acids. The difference (final-baseline) in the sum of plasma deconjugated bile acids in participants with a body mass index (BMI) <30 and ≥30 who received placebo and one of the three probiotics (*Bifidobacterium animalis* subsp. *lactis* B94, *Bacillus subtilis* R0179, or *Lactobacillus plantarum* HA-119). Data from the placebo intervention from each of the three crossovers were pooled and all data were log transformed before taking differences for analysis. Data represent the untransformed means ± standard error of the mean. The sample size for each bar is represented by the number in the parentheses.

Table 2. Participant characteristics at screening and study compliance.[1]

		***Bifidobacterium animalis* subsp. *lactis* B94 (n=33)**	***Bacillus subtilis* R0179 (n=35)**	***Lactobacillus plantarum* HA-119 (n=35)**
Sex, n (%)	Male	10 (30)	3 (9)	10 (29)
	Female	23 (70)	32 (91)	25 (71)
Age, years		51.2±1.4	53.2±1.4	54.3±1.1
Hispanic, n (%)	Yes	2 (6)	3 (9)	3 (9)
	No	31 (94)	32 (91)	31 (89)
Race, n (%)	White	25 (76)	28 (80)	25 (71)
	Black	7 (21)	1 (3)	8 (23)
	Other	1 (3)	6 (17)	2 (6)
Body mass index, kg/m^2, n (%)	<18.5	0 (0)	0 (0)	0 (0)
	18.5-24.9	1 (3)	2 (6)	3 (9)
	25-29.9	14 (42)	14 (40)	8 (23)
	≥30	18 (55)	19 (54)	24 (68)
Waist circumference, cm	Males	116.1±4.1	104.9±1.5	112.1±1.7
	Females	105.0±2.0	105.7±2.5	105.5±3.0
Daily capsules consumed (%)		93.1±2.2	90.0±3.3	90.1±3.4

[1] Unless stated otherwise, values indicate the mean ± standard error of the mean.

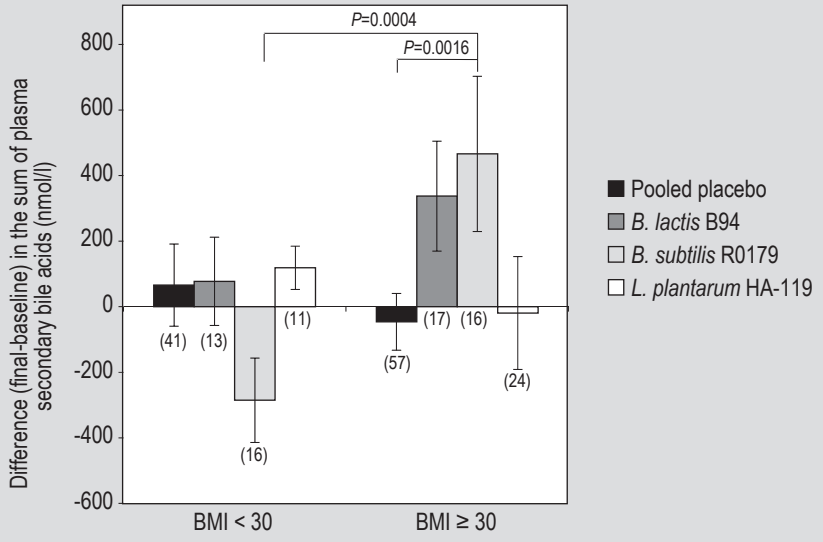

Figure 4. Plasma secondary bile acids. The difference (final-baseline) in the sum of plasma secondary bile acids in participants with a body mass index (BMI) <30 and ≥30 who received placebo and one of the three probiotics (*Bifidobacterium animalis* subsp. *lactis* B94, *Bacillus subtilis* R0179, or *Lactobacillus plantarum* HA-119). Data from the placebo intervention from each of the three crossovers were pooled and all data were log transformed before taking differences for analysis. Data represent the untransformed means ± standard error of the mean. The sample size for each bar is represented by the number in the parentheses.

for the change in the ratio of plasma deconjugated to conjugated bile acids or for the ratio of secondary to primary plasma bile acids (data not shown). For the stool bile acids, no significant differences were observed between the pooled placebo group and each of the three study probiotics for the change in the sum of deconjugated bile acids, the ratio of deconjugated to conjugated bile acids, the sum of the secondary bile acids, or the ratio of secondary to primary bile acids (data not shown).

Markers of glucose and lipid metabolism

The changes in the plasma levels of glucose, insulin, and blood lipids were not different between each of the three study probiotics and the pooled placebo; however, there was a trend (P=0.08) toward an increase in plasma HDL concentrations with *L. plantarum* and in ApoB100 with *B. subtilis* (Table 3). No interactions between BMI and the probiotics were observed for any of the markers of glucose metabolism and blood lipids. Data for BMI-stratified cohorts can be found in Supplementary Table S1.

Diet and exercise

Participants were told to maintain their typical diet and physical activity throughout the study. No differences in dietary intake of total fibre, total fat, or saturated fat were observed during the placebo and probiotic intervention periods for all three probiotics. On average participants consumed 17±1 g of fibre, 80±3 g of total fat, and 27±1 g of saturated fat per day. Dietary intake of cholesterol was significantly greater when participants consumed the placebo (227±13 mg) versus *L. plantarum* (210±15 g/d; P=0.034) but was not different between the placebo and probiotic interventions with the other two probiotics. On average all participants consumed 218±9 g/d of cholesterol. Across all probiotic/placebo groups only 14 participants reported consuming one or more alcoholic beverages per day. Based on responses to the IPAQ, there was no difference in categorical ranks of activity based on average weekly METs between the placebo and probiotic interventions (data not shown). There were no differences in baseline weights between the baseline pooled placebo and probiotic interventions, nor were there any differences in weight changes across the pooled placebo and each probiotic intervention. The median (25%, 75% percentile) baseline weight was 85.3 (75.6, 101.2) kg and the median change in weight was 0.2 (-0.7, 1.1) kg.

Gastrointestinal function

Differences in gastrointestinal symptoms measured by the GSRS or bowel movement frequency were not observed between any of the three probiotic interventions compared with the placebo groups (data not shown).

Safety outcomes

No effects of the probiotics were observed on white blood cell numbers or percentages (data not shown). On average (mean ± standard error of the mean), nausea, vomiting, or stomach upset was reported by 6.7±1.8 participants on 16.7±4.7 days when on placebo and by 6.0±1.5 participants on 16.0±2.0 days when on probiotic. Headaches were reported by 9 participants during the placebo arms of the interventions and by one participant receiving *B. subtilis*.

4. Discussion

The primary purpose of this study was to characterise the effect of three probiotic candidates on bile acid metabolism. Compared with placebo, *B. lactis* B94 produced higher plasma deconjugated bile acids in participants with a BMI ≥30, and *B. subtilis* R0179 resulted in higher plasma deconjugated and secondary bile acids in participants with a BMI ≥30. *L. plantarum* HA-119 did not affect bile acids but displayed a trend towards higher plasma HDL concentrations compared with placebo. To our knowledge, this is the first randomised, placebo-controlled trial assessing the effect of probiotics on the entire profile of bile acids in both blood and stool samples in conjunction with a comprehensive blood lipid profile. This study employed a crossover design to control for the many dietary and lifestyle-related variables that may affect bile acid and lipid metabolism. A crossover design was also deemed appropriate because the outcomes studied were predicted to be stable, assuming consistent dietary intake, which was measured during the study, and reversible during the washout period. To capture any shortcomings of the crossover design, period and sequence effects were accounted for statistically.

Deconjugated and secondary bile acids in the plasma were altered with *B. subtilis* or *B. lactis*. These two categories of bile acids are thought to be produced following interaction with gut microbes, whereas any conjugated and primary bile acids measured in blood or stools are likely in the same form in which they were synthesised. Because bile acids are excreted directly after synthesis, measurements taken in blood samples represent bile acids that have entered enterohepatic circulation and therefore reflect the profile of bile acids in the ileum. An exception to this is LCA, which is generally not reabsorbed but lost in the stool. This was evident in this study as LCA was below the limit of detection in plasma samples. Though the conjugation of bile acids with taurine or glycine increases solubility in water due to the addition of polar functional groups, the effect of bacterial deconjugation on intestinal reabsorption of bile acids is less clear and is likely dependent on other factors such as pH and transport or trafficking mechanisms. One explanation for why our results depended on BMI category is that deconjugated bile acids may be more readily reabsorbed in obese individuals than in nonobese individuals in response to certain probiotic strains (Figure 3). Participant characteristics may also explain differences seen between *in vitro* testing (unpublished data) and *in vivo* (Supplementary Figure S1) results, such as the ability of *L. plantarum* to decrease GCDCA *in vitro* but only in obese participants *in vivo*. The increase in secondary and deconjugated plasma bile acids seen in obese participants consuming *B. subtilis* (Figures 3 and 4) is perplexing but seems unrelated to BSH activity, which would be consistent with the negative results from *in vitro* testing prior to the

Table 3. The change (final-baseline) in biomarkers of glucose and lipid metabolism and plasma and stool bile acids in participants who received placebo and one of the three probiotics.[1,2]

	Biomarkers		Placebo[3] (n=98-99)	*B. lactis* (n=33)	*B. subtilis* (n=32-33)	*L. plantarum* (n=35)
Glucose metabolism (plasma)	Glucose (mmol/l)	*Baseline*[4]	5.77±0.17	6.05±0.17 *	5.83±0.17	5.77±0.17
		Change[4]	0.06±0.08	0.01±0.12	0.04±0.08	0.03±0.09
	Insulin (pmol/l)	*Baseline*	71.5±4.9	72.2±7.0	64.6±7.0	69.5±7.0
		Change	-1.4±4.2	-4.2±7.0	7.6±4.9	2.78±4.9
Blood lipids[5]	Total cholesterol (mmol/l)	*Baseline*	5.62±0.13	5.67±0.16	5.70±0.16	5.44±0.16
		Change	-0.07±0.07	-0.12±0.12	-0.14±0.13	0.15±0.11
	HDL cholesterol (mmol/l)	*Baseline*	1.52±0.03	1.50±0.04	1.51±0.04	1.48±0.04
		Change	-0.01±0.02	-0.04±0.03	-0.01±0.04	0.08±0.03[5]
	LDL cholesterol (mmol/l)	*Baseline*	3.06±0.08	3.03±0.10	3.08±0.10	2.98±0.10
		Change	-0.03±0.04	-0.02±0.09	-0.11±0.09	0.07±0.07
	Triglycerides (mmol/l)	*Baseline*	2.31±0.13	2.49±0.17	2.38±0.17	2.17±0.17
		Change	-0.08±0.09	-0.13±0.18	-0.05±0.13	0.01±0.10
	NEFA (µmol/l)	*Baseline*	492±22	510±35	469±35	529±34
		Change	-4±22	-9±40	17±43	-25±46
	ApoA1 (µmol/l)	*Baseline*	63.9±1.8	63.9±2.5	62.5±2.5	57.1±2.5 *
		Change	3.9±1.1	2.9±1.4	4.6±2.1	12.5±4.3
	ApoB100 (µmol/l)	*Baseline*	1.90±0.09	1.80±0.12	1.90±0.12	1.87±0.12
		Change	0±0.06	0.17±0.12	0.22±0.10 #	0.10±0.11
	HOMA-IR	*Baseline*	2.86±0.28	3.15±0.65	2.76±0.44	2.33±0.27
		Change	-0.08±0.23	-0.07±0.25	0.42±0.23	0.13±0.19
			(n=97)	**(n=30)**	**(n=31)**	**(n=35)**
Bile acids (plasma)[6]	Deconjugated (nmol/l)	*Baseline*	991±115	927±180	814±175	1,100±171
		Change	62±126	331±164	238±236	-97±219
	Deconjugated / conjugated (ratio)	*Baseline*	1.07±0.13	1.35±0.21	0.94±0.21	1.50±0.20 *
		Change	0.22±0.14	0.19±0.26	0.23±0.25	-0.31±0.17
	Seconday (nmol/l)	*Baseline*	949±81	881±116	902±113	912±111
		Change	-12±72	225±112	111±152	24±119
	Secondary / primary (ratio)	*Baseline*	1.05±0.09	1.05±0.12	0.67±0.12 *	1.09±0.12
		Change	-0.03±0.06	-0.06±0.09	0.03±0.12	-0.11±0.10
			(n=30)	**(n=10)**	**(n=10)**	**(n=10)**
Bile acids (stool)[7]	Deconjugated (sum)	*Baseline*	4,393±868	5,494±1,287	4,236±1,287	2,796±1,287
		Change	-570±853	286±2376	-1,815±1123	1,086±764
	Deconjugated / conjugated (ratio)	*Baseline*	602±130	1,044±223	863±223	558±223
		Change	-203±116	-396±416	-565±365	-41±340
	Secondary (sum)	*Baseline*	1,930±266	1,851±432	2,028±432	1,370±432
		Change	-460±357	-44±441	-983±416	696±606
	Secondary / primary (ratio)	*Baseline*	16.45±5.16	14.90±7.95	31.61±7.95	16.66±7.95
		Change	8.5±4.6	-0.7±6.2	-12.6±14.1	-5.4±5.0

[1] Apo = apolipoprotein; *B. lactis* = *Bifidobacterium animalis* subsp. *lactis* B94; *B. subtilis* = *Bacillus subtilis* R0179; HDL = high density lipoprotein; HOMA-IR = homeostatic model assessment for insulin resistance; *L. plantarum* = *Lactobacillus plantarum* HA-119; LDL = low density lipoprotein; NEFA = non-esterified fatty acids.

[2] # P=0.08 versus the pooled placebo control. * P<0.05 versus pooled placebo.

[3] Pooled data from the placebo intervention from each of the three crossovers.

[4] Values at baseline represent LSmeans ± standard error of the mean; whereas values for change are represented by differences between means ± standard error of the mean.

[5] Total, HDL, and LDL cholesterol and NEFA were analysed in the serum and ApoA1 and ApoB100 were analysed in the plasma.

[6] Data represent the sum of individual bile acids.

[7] Data represent arbitrary units.

study; if BSH were responsible for changes, GCDCA and TDCA would be expected to decrease. However, this was not the case in obese participants. Unelucidated bile acid reabsorption mechanisms as mentioned above could be influencing these results.

The difference observed in this study between individuals with a BMI <30 and individuals with a BMI ≥30 is an important finding. While it is expected that there are physiological differences between these two populations, not all previous studies assessing hypocholesterolaemic effects of probiotics have controlled for weight or BMI; higher BMI would likely be an overlapping characteristic in people with high cholesterol. Many other studies have confirmed differences in microbiomes between lean and obese individuals, such as a higher ratio of *Firmicutes*:*Bacteroidetes* in obese individuals (Turnbaugh *et al.*, 2009); because this study is a probiotic intervention, baseline microbial differences are of particular interest in this mechanism. Although not measured in this study, it could be speculated that obese individuals began the study with more microbes that were able to modify bile acids in conjunction with *B. subtilis*, such as other microbes with BSH and/or 7alpha-dehydroxylase activity. In the current experimental setting, it is difficult to pinpoint the strongest contributing factors to the observed bile acid profile differences between BMI categories. It should be noted that statistical power may have been reduced by conducting this subgroup analysis. Further study is warranted in order to verify the relationship between BMI and probiotic effects on bile metabolism.

While this study was specifically designed to characterise effects of probiotics on bile acid metabolism, it was of interest to measure more relevant health outcomes such as cholesterol levels. The probiotics studied herein did not significantly lower plasma total or LDL cholesterol; however, the numerical reduction in LDL cholesterol from baseline to final in the group consuming *B. subtilis* was 3.6% on average, which may improve cardiovascular health (Grundy *et al.*, 2004; Ishimwe *et al.*, 2015). A statistically insignificant reduction in total cholesterol was also seen with *B. subtilis* (2.5%) and with *B. lactis* (2.1%). Participants consuming *L. plantarum* did not follow this pattern, which may be related to the difference observed in dietary cholesterol between placebo and probiotic arms. Importantly, the placebo group also experienced a 1.0 and a 1.2% average reduction in LDL and total cholesterol, respectively. These values suggest that the current study may have been underpowered to assess effects on plasma cholesterol or that there was really no effect. Other studies that have reported a significant reduction of cholesterol from probiotics had many more participants (Jones *et al.*, 2012b) or were longer in duration (Fuentes *et al.*, 2013); it is possible that any potential cholesterol-lowering effects of the probiotics could occur in different people at varying rates and that 6 weeks was not long enough to see a group reduction in this study's sample. Additionally, average cholesterol levels were slightly lower to begin with (5.44-5.70 mmol/l, or ~210-220 mg/dl) than in other studies (Fuentes *et al.*, 2013; Jones *et al.*, 2012a,b) that based inclusion criteria on cholesterol levels (~230-260 mg/dl). This study used inclusion criteria on waist circumference rather than cholesterol levels.

The generalisability of these results depends on the characteristics of the included sample. Participants in this study were included if they had a high waist circumference. Accordingly, these participants also had an average baseline BMI of 32.2 kg/m^2, indicating that many of the participants were obese. Due to the exclusion of many potential participants based on diagnoses of various diseases which could confound these results, the generalisability of the results here is limited because many obese individuals have comorbid conditions that can affect biological processes such as bile acid and lipid metabolism. Future studies observing the effects of probiotics would need to specifically account for comorbidities in order to generalise further. Because average total cholesterol at baseline was borderline high (Mayo Clinic, 2018), bile acid and lipid metabolic pathways that were of interest in this study may be physiologically different from individuals with either normal cholesterol levels or high cholesterol levels.

Although this study was carefully designed, several limitations must be addressed. A washout period of 3 weeks was selected, and because no significant sequence effects were detected in the measured outcomes, a carryover effect was unlikely. Effects of period were also seen with some outcomes (data not shown), indicating that there may have been unmeasured extraneous variables that changed during the study period; this is one of the inherent limitations of the crossover design. Many of the stool conjugated bile acids were below the limit of detection using this HPLC-based method, but this could be expected because most conjugated bile acids (~95%) are reabsorbed. Finally, the study was not specifically powered to measure differences in cholesterol or other lipid markers, and it was not designed to assess differences based on BMI category.

In conclusion, these three strains appear to exert different *in vivo* effects on bile acid metabolism. *B. subtilis* R0179 and *B. lactis* B94 both appear to modulate plasma bile acid profiles, and the corresponding clinical relevance of these effects to BMI and cholesterol levels should continue to be explored. *B. subtilis* R0179 should continue to be studied for its potential to lower total and LDL cholesterol and *L. plantarum* HA-119 for its potential to raise HDL cholesterol. Additional benefits may be discovered with these new probiotic candidates.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2018.0151.

Table S1. The change (final-baseline) in biomarkers of glucose and lipid metabolism in participants with a body mass index <30 or ≥30 kg/M^2 who received placebo and one of the three probiotics.

Figure S1. Heatmap for plasma bile acids.

Figure S2. Heatmap for plasma and stool bile acids for the subgroup of study participants who provided both stool and plasma samples.

Acknowledgements

We wish to thank the undergraduate students from the University of Florida and the participants who helped us carry out the study.

Conflict of interest

Lallemand Health Solutions provided study supplements and funding for this project. Co-authors S-AG and TAT are employed by the study sponsor. MCC and BLH have received funding for other research projects from the study sponsor. None of the other authors has a personal or financial conflict of interest to report. Additional funding was received from the University of Florida Agriculture Experiment Station Project number FLA-FOS-005129.

References

Allayee, H. and Hazen, S.L., 2015. Contribution of gut bacteria to lipid levels: another metabolic role for microbes? Circulation Research 117: 750-754.

Bathena, S.P., Mukherjee, S., Olivera, M. and Alnouti, Y., 2013. The profile of bile acids and their sulfate metabolites in human urine and serum. Journal of Chromatography B 942-943: 53-62.

Begley, M., Hill, C. and Gahan, C.G., 2006. Bile salt hydrolase activity in probiotics. Applied and Environmental Microbiology 72: 1729-1738.

Block, G., Gillespie, C., Rosenbaum, E.H. and Jenson, C., 2000. A rapid food screener to assess fat and fruit and vegetable intake. American Journal of Preventative Medicine 18: 284-288.

Craig, C.L., Marshall, A.L., Sjostrom, M., Bauman, A.E., Booth, M.L., Ainsworth, B.E., Pratt, M., Ekelund, U., Yngve, A., Sallis, J.F. and Oja, P., 2003. International physical activity questionnaire: 12-country reliability and validity. Medicine and Science in Sports and Exercise 35: 1381-1395.

Fuentes, M.C., Lajo, T., Carrion, J.M. and Cune, J., 2013. Cholesterol-lowering efficacy of *Lactobacillus plantarum* CECT 7527, 7528 and 7529 in hypercholesterolaemic adults. British Journal of Nutrition 109: 1866-1872.

Grundy, S.M., Cleeman, J.I., Merz, C.N., Brewer Jr., H.B., Clark, L.T., Hunninghake, D.B., Pasternak, R.C., Smith Jr., S.C., Stone, N.J., the National Heart, Lung, and Blood Institute, the American College of Cardiology Foundation and the American Heart Association, 2004. Implications of recent clinical trials for the national cholesterol education program adult treatment panel III guidelines. Circulation 110: 227-239.

Guo, Z., Liu, X.M., Zhang, Q.X., Shen, Z., Tian, F.W., Zhang, H., Sun, Z.H., Zhang, H.P. and Chen, W., 2011. Influence of consumption of probiotics on the plasma lipid profile: a meta-analysis of randomised controlled trials. Nutrition, Metabolism, and Cardiovascular Diseases 21: 844-850.

Hanifi, A., Culpepper, T., Mai, V., Anand, A., Ford, A.L., Ukhanova, M., Christman, M., Tompkins, T.A. and Dahl, W.J., 2015. Evaluation of *Bacillus subtilis* R0179 on gastrointestinal viability and general wellness: a randomised, double-blind, placebo-controlled trial in healthy adults. Beneficial Microbes 6: 19-27.

Ishimwe, N., Daliri, E.B., Lee, B.H., Fang, F. and Du, G., 2015. The perspective on cholesterol-lowering mechanisms of probiotics. Molecular Nutrition and Food Research 59: 94-105.

Jones, M.L., Martoni, C.J., Parent, M. and Prakash, S., 2012a. Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. British Journal of Nutrition 107: 1505-1513.

Jones, M.L., Martoni, C.J. and Prakash, S., 2012b. Cholesterol lowering and inhibition of sterol absorption by *Lactobacillus reuteri* NCIMB 30242: a randomized controlled trial. European Journal of Clinical Nutrition 66: 1234-1241.

Lei, K., Li, Y.L., Wang, Y., Wen, J., Wu, H.Z., Yu, D.Y. and Li, W.F., 2015. Effect of dietary supplementation of *Bacillus subtilis* B10 on biochemical and molecular parameters in the serum and liver of high-fat diet-induced obese mice. Journal of Zhejiang University. Science B 16: 487-495.

Mayo Clinic, 2018. Cholesterol test. Available at: http://tinyurl.com/y8wczryp.

MedlinePlus, U.S. National Library of Medicine, 2018. How to lower cholesterol. Available at: https://medlineplus.gov/howtolowercholesterol.html.

National Heart, Lung, and Blood Institute (NHLBI), 2018. Assessing your weight and health risk. Available at: https://www.nhlbi.nih.gov/health/educational/lose_wt/risk.htm.

Ooi, L.G., Ahmad, R., Yuen, K.H. and Liong, M.T., 2010. *Lactobacillus gasseri* [corrected] CHO-220 and inulin reduced plasma total cholesterol and low-density lipoprotein cholesterol via alteration of lipid transporters. Journal of Dairy Science 93: 5048-5058.

Pavlovic, N., Stankov, K. and Mikov, M., 2012. Probiotics – interactions with bile acids and impact on cholesterol metabolism. Applied Biochemistry and Biotechnology 168: 1880-1895.

Revicki, D.A., Wood, M., Wiklund, I. and Crawley, J., 1998. Reliability and validity of the Gastrointestinal Symptom Rating Scale in patients with gastroesophageal reflux disease. Quality of Life Research 7: 75-83.

Subar, A.F., Kirkpatrick, S.I., Mittl, B., Zimmerman, T.P., Thompson, F.E., Bingley, C., Willis, G., Islam, N.G., Baranowski, T., McNutt, S. and Potischman, N., 2012. The Automated Self-Administered 24-hour dietary recall (ASA24): a resource for researchers, clinicians, and educators from the National Cancer Institute. Journal of the Academy of Nutrition and Dietetics 112: 1134-1137.

Trautvetter, U., Ditscheid, B., Kiehntopf, M. and Jahreis, G., 2012. A combination of calcium phosphate and probiotics beneficially influences intestinal lactobacilli and cholesterol metabolism in humans. Clinical Nutrition ESPEN 31: 230-237.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R. and Gordon, J.I., 2009. A core gut microbiome in obese and lean twins. Nature 457: 480-484.

World Health Organization (WHO), 2018. Cardiovascular disease. Available at: http://www.who.int/cardiovascular_diseases/en/.

Wostmann, B.S., Wiech, N.L. and Kung, E., 1966. Catabolism and elimination of cholesterol in germfree rats. Journal of Lipid Research 7: 77-82.