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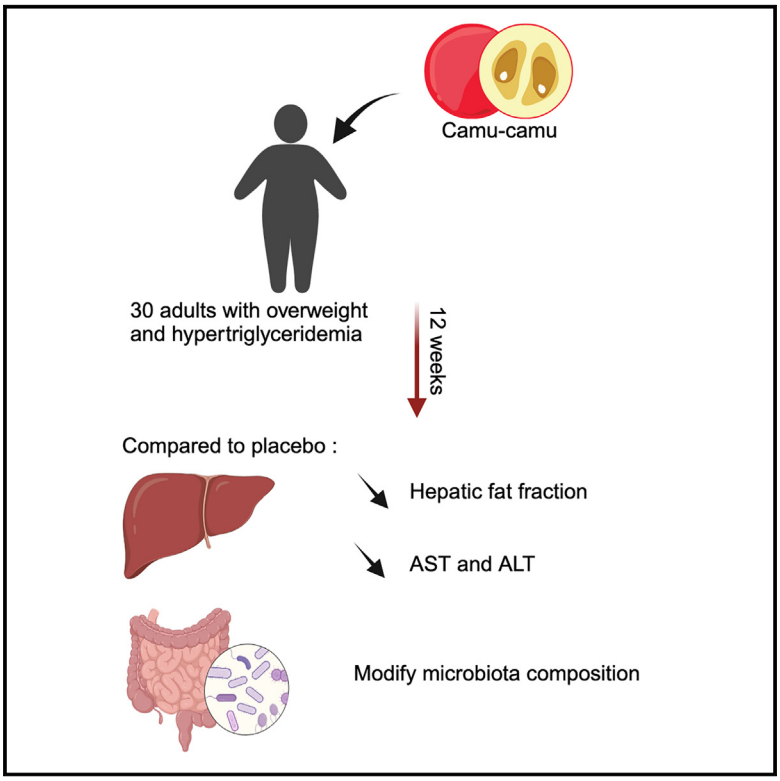
Camu-camu decreases hepatic steatosis and liver injury markers in overweight, hypertriglyceridemic individuals: A randomized crossover trial

Graphical abstract



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In brief

Agrinier et al. demonstrate that camu-camu (CC) supplementation reduces liver fat and improves liver injury markers in individuals with overweight and hypertriglyceridemia. CC also alters gut microbiota composition, supporting its potential as a polyphenol-rich prebiotic supplement for reducing non-alcoholic fatty liver disease risk.

Highlights

- Camu-camu reduces hepatic steatosis in adults with overweight and hypertriglyceridemia
- Camu-camu supplementation decreases plasma AST and ALT levels
- Camu-camu supplementation alters gut microbiota composition

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Camu-camu decreases hepatic steatosis and liver injury markers in overweight, hypertriglyceridemic individuals: A randomized crossover trial

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SUMMARY

Non-alcoholic fatty liver disease (NAFLD), recently referred to as "metabolic dysfunction-associated steatotic liver disease" (MASLD), affects 25% of the adult population with no effective drug treatments available. Previous animal studies reported that a polyphenol-rich extract from the Amazonian berry camu-camu (CC) prevented hepatic steatosis in a mouse model of diet-induced obesity.

This study aims to determine the impact of CC on hepatic steatosis (primary outcome) and evaluate changes in metabolic and gut microbiota profiles (exploratory outcomes). A randomized, double-blind, placebo-controlled crossover trial is conducted on 30 adults with overweight and hypertriglyceridemia, who consume 1.5 g of CC capsules or placebo daily for 12 weeks. CC treatment decreases liver fat by 7.43%, while it increases by 8.42% during the placebo intervention, showing a significant difference of 15.85%. CC decreases plasma aspartate and alanine aminotransferases levels and promotes changes in gut microbiota composition. These findings support that polyphenol-rich prebiotic may reduce liver fat in adults with overweight, reducing the risk of developing NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), recently referred to as "metabolic dysfunction-associated steatotic liver disease" (MASLD), is the most prevalent liver disease worldwide, affecting approximately 25% of the adult population.[1] NAFLD is defined by an increased hepatic fat content, mainly in the form of neutral lipids within intracellular lipid droplets, exceeding 5% of the liver mass.[2] It is a complex disease encompassing a range of liver pathologies, including simple steatosis (non-alcoholic fatty liver), non-alcoholic steatohepatitis (NASH) (recently referred to as "metabolic-dysfunction associated steatohepatitis" [MASH]), and fibrosis. NAFLD is also strongly linked to elevated risks of developing liver cirrhosis and hepatocellular carcinoma.[2] NAFLD is usually asymptomatic until it progresses to cirrhosis, highlighting the importance of prevention. NAFLD is accompanied by obesity, metabolic dysfunctions, and type 2 diabetes (T2D) mellitus in >90% of patients.[3]

Lifestyle behavior changes and weight loss are the first therapeutic approaches to prevent the development and progression of NAFLD.[4,5] As of today, only a few pharmacological agents have reached phase 3 clinical trials, and full regulatory approval may still be years away.[6] Natural bioactive compounds, namely found in fruits and vegetables, have been identified as promising agents capable of reversing NAFLD.[7,8] Camu-camu (CC) is an Amazonian fruit particularly rich in polyphenols, such as proanthocyanidins (PAC) and ellagitannins, and is well documented for its antioxidant and anti-inflammatory activities.[9–12] Our group has previously demonstrated that CC prevented weight gain and hepatic steatosis in diet-induced obese mice and shifted the fecal gut microbiota composition toward a leaner phenotype.[9] In line with this, an important body of literature has reported that dietary polyphenols influence the gut microbiota composition and provide cardiometabolic health benefits such as reduction of blood pressure and levels of low-density lipoprotein cholesterol.[13] In addition, it was demonstrated that polyphenols found in CC have an antitumorigenic activity.[14]

Growing preclinical and clinical evidence suggests that the gut-liver axis plays a central role in NAFLD pathophysiology.[15] Indeed, the liver is exposed to substantial amounts of

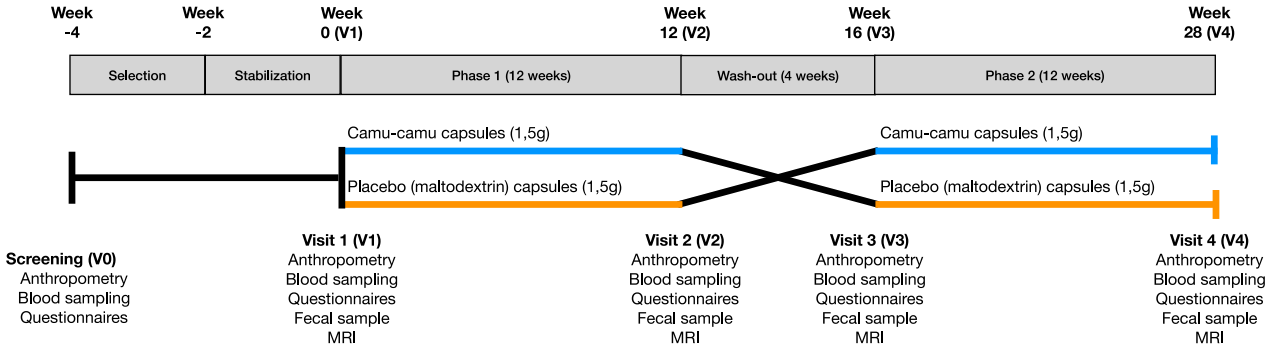


Figure 1. Graphical representation of the study protocol
See also Figure S1.

dietary factors, bacterial components, and metabolites derived from the intestine.[16,17] Hence, CC could represent a potential microbiome-based therapeutic strategy against obesity and its associated metabolic comorbidities, such as fat deposit in liver. Nonetheless, up to now, no clinical trial has thoroughly investigated its potential.

Accordingly, this 12-week randomized, double-blind, placebo-controlled crossover trial (Figure 1) aimed to determine the impact of 1.5 g/day CC supplementation in subjects with overweight and hypertriglyceridemia on hepatic steatosis as the primary outcome, and on gut microbiota and metabolic syndrome parameters such as anthropometric measurements, indices of glucose homeostasis, plasma lipids, and blood pressure as secondary outcomes. It was hypothesized that a 12-week daily treatment with CC reduces hepatic fat content and adiposity and improves glucose homeostasis in association with changes in gut microbiota composition and function as compared to the placebo.

RESULTS

Trial flow, baseline characteristics, and compliance

Overall, a total of 257 individuals contacted the clinical study coordinator, and 91 individuals met pre-screening criteria by email or phone and were therefore scheduled for a screening visit at the clinical investigation unit of Institute of Nutrition and Functional Foods (Figure S1). Of the 91 participants screened, 54 did not meet the inclusion criteria, and 2 participants withdrew from the study during the stabilization period, leaving 35 participants that were included in this randomized, double-blind, placebo-controlled crossover study. Respectively, one and two participants withdrew for personal reasons during phase 1 during the washout period. Finally, two participants withdrew from the second phase for personal reasons and possible side effects (minor digestive discomfort possibly associated with the capsule intake). Analyses were conducted on a final sample of 30 participants who completed the two intervention phases.

Baseline characteristics for age, gender, and anthropometric and metabolic variables are presented in Table 1. These variables were not significantly different between the CC and placebo groups at baseline except for free fatty acids (FFA), which was higher in the placebo group than in the CC group. For the two interventions, adherence to the study protocol according to the daily log was respectively of 98% in the first phase, and of 99% in the second phase. Based on the number of capsules returned following the interventions, compliance was 95% in the first phase and 98% in the second phase.

Dietary intake

Dietary intake was assessed before and after each intervention period. Changes in the intake of calories, alcohol, fibers, and macronutrients were similar between the groups during the study (Table 2).

Hepatic and cardiometabolic outcomes

CC treatment significantly changed the primary outcome, as revealed by a statistically significant decrease ($p = 0.003$) in hepatic fat fraction (HFF) measured by MRI, expressed as the mean percentage of liver fat derived from 5 regions of interest per participant compared to the placebo (Figure 2A). No statistically significant changes were observed in MRI-measured volumes of subcutaneous or visceral adipose tissues (Figures 2B and 2C; Table S1) or in total body, trunk, android, and gynoid fat depots as well as lean mass by dual energy X-ray absorptiometry (DEXA) (Table S2).

At baseline, HFF between the CC and placebo groups was similar at 9.1% and 10.0%, respectively ($p = 0.60$). The relative changes from baseline in MRI-measured liver fat for each individual enrolled in the CC and placebo arms are shown in Table S3. Most participants had decreased percentage of HFF values in the CC-treated arm as compared to baseline (mean relative change of −7.43%), whereas most values in the placebo arm increased compared to baseline (mean relative change of +8.42%). This corresponds to a relative difference of 15.85% between the CC and placebo groups. The individual changes in HFF percentages pre- vs. post-treatment are also depicted in Figure 2D and further illustrates that CC treatment is promoting more consistent reduction in liver fat as compared to placebo.

Supplementation with 1.5 g of CC/day over a 12-week period in participants with metabolic alterations significantly decreased the circulating levels of aspartate aminotransferase (AST) ($p = 0.04$) and alanine aminotransferase (ALT) ($p = 0.0006$) relative to placebo (Figures 3A and 3B). Further analyses confirmed that the differences in HFF and ALT levels were attributable to the

Table 1. Baseline characteristics of study participants

	V1 (n = 30)		
Variable	CC	Placebo	p value
Age (y)	56.5 ± 12.3	58.4 ± 10.6	0.66
Gender (% female [n])	69.2 (9)	64.7 (11)	0.79
Weight (kg)	86.3 ± 19.3	84.5 ± 14.73	0.78
Height (cm)	1.68 ± 0.10	1.67 ± 0.11	0.82
BMI (kg/m^2)	30.6 ± 5.7	30.3 ± 4.1	0.88
Waist circ. (cm)	103.2 ± 11.4	104.3 ± 9.4	0.77
SBP (mmHg)	121.9 ± 19.1	124.0 ± 9.9	0.69
DBP (mmHg)	75.5 ± 11.3	76.5 ± 8.3	0.77
Heart rate (bpm)	71.0 ± 6.9	68.5 ± 9.3	0.43
TG (mmol/L)	1.58 ± 0.74	1.83 ± 0.73	0.22
Total-C (mmol/L)	5.41 ± 1.09	6.06 ± 0.94	0.09
HDL-C (mmol/L)	1.36 ± 0.38	1.48 ± 0.4	0.41
LDL-C (mmol/L)	3.32 ± 0.88	3.74 ± 0.95	0.23
Total-C/HDL-C	4.18 ± 1.14	4.4 ± 1.52	0.66
AST (U/L)	19.5 ± 5.76	19.13 ± 7.6	0.89
ALT (U/L)	31.69 ± 11.92	31.47 ± 10.67	0.96
Free fatty acids (μM/L)	0.52 ± 0.12	0.61 ± 0.11	0.03*
HbA1c (%)	0.055 ± 0.003	0.056 ± 0.002	0.49
ApoB (mg/L)	1.02 ± 0.23	1.14 ± 0.26	0.20
C-reactive protein	2.49 ± 2.33	2.82 ± 2.8	0.65

BMI, body mass index; Waist circ., waist circumference; Hip circ., hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; ApoB, apolipoprotein B; Total-C, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; total-C/HDL-C, total cholesterol/high-density lipoprotein -cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HbA1C, glycated hemoglobin. Results are presented as raw means ± SD. Comparisons were made with the general linear model (GLM) procedure with SAS Studio v.3.8. GLM was performed with log10 transformed values for TG and C-reactive protein.
*p value < 0.05.

effect of CC treatment per se even if changes were also observed in the placebo group, whereas the difference in the AST levels was mainly caused by an increase in values during the placebo phase. No carryover effect on the AST, ALT, and HFF variables was observed (Table S4). No other statistically significant change in indices of cardiometabolic health or glucose homeostasis indices was observed (Table 3; Table S5).

Gut microbiota and short-chain fatty acids

CC supplementation did not alter α-diversity measured by the Shannon and Simpson indices (Figures 4A and 4B). No carryover effect on gut microbiota composition was observed (Figure S2). Moreover, no drastic changes in the community structure of the gut microbiota were observed, as evidenced by the principal component analysis (Figure 4C). However, when calculating the linear discriminant analysis effect size following 12-week CC and placebo supplementation (post-placebo vs. post-CC), significant changes in the gut microbiota composition were observed. *Adlercreutzia* and *Erysipelatoclostridium* were overrepresented in the placebo group, and CC supplementation induced a bloom of *Enterococcus*, *Lactobacillus*, and *Lactococcus* genera (Figure 4D). *Intestinibacter*, *Shuttleworthia*, *Adlercreutzia*, and *Erysipelatoclostridium* were less abundant following CC supplementation (Figure 4E). When comparing the baseline fecal microbiota composition of participants in the placebo group to its final composition, no statistically significant changes were detected, suggesting that the placebo did not alter the gut microbiota composition.

To comprehensively document taxonomic and functional changes in the gut microbiota of individuals who underwent CC supplementation, we conducted complementary shotgun metagenomic analyses of the fecal microbiota before and after the CC intervention (pre-CC vs. post-CC).

The DESeq2 analysis of the shotgun metagenomics sequencing showed notably a significant increase in the abundance of *Pediococcus pentosaceus*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus* following the CC consumption (Figure 4F). Furthermore, in concordance with the 16S rRNA data, a decreased abundance of *Adlercreutzia equolifaciens* and *A. hattorii* was observed after CC consumption (Figure 4F).

Shotgun sequencing also revealed alterations in bacterial metabolic pathways before and after CC intake. On one hand, the pathways related to L-tyrosine biosynthesis, *Bifidobacterium* shunt, and myo-inositol degradation were downregulated following CC consumption (Figure 4G). On the other hand, pathways associated with lactate fermentation to propanoate, acetate, and hydrogen, degradation of 3-phenylpropanoate and 3-(3-hydroxyphenyl)propanoate, glycolysis, pyruvate dehydrogenase, tricarboxylic acid and glyoxylate bypass, 2-methylcitrate cycle II, biosynthesis of mycolate, metabolism of L-threonine, and mitochondrial NADPH production were upregulated following the CC intervention (Figure 4G). Gut microbiota-derived short-chain fatty acids in the feces did not change significantly after CC supplementation (Table S6). We have also performed correlation analysis between bacteria genera and the primary outcome (hepatic fat) but found no significant correlation, which may be attributable to the relatively small number of subjects for which we had baseline and post-treatment MRI-based analysis of liver fat for both CC and placebo intervention arms.

DISCUSSION

In this randomized, double-blind, placebo-controlled crossover clinical trial, we have assessed the impact of 1.5 g/day supplementation of CC on 30 participants with overweight and hypertriglyceridemia on hepatic fat (primary outcome) and a number of well-known anthropometric and cardiometabolic risk factors (secondary outcomes). The results show that CC supplementation for only 12 weeks decreased hepatic steatosis by 15.85% compared to placebo and improved plasma levels of AST and ALT despite neither affecting body weight nor adiposity.

Accumulation of liver fat, which is commonly observed in cases of obesity and T2D, is an important early step in the pathogenesis of NAFLD.[15] It is interesting to mention that about half of the subjects on CC treatment had a relative reduction in liver fat of >20%, while most subjects on placebo actually gained more liver fat. This compares well with a very recent study using resmetirom, the first Food and Drug Administration-approved drug for NAFLD, which showed relative reductions of 34%–

Table 2. Changes in daily dietary intakes of all participants

Variable	CC		Placebo		Delta change (*n* = 29)		Adjusted *p* value
	Pre (*n* = 29)	Post (*n* = 29)	Pre (*n* = 29)	Post (*n* = 29)	CC	Placebo	
Calories (kcal)	2,060.16 ± 707.89	1,852.48 ± 561.77	1,909.72 ± 645.69	1,884.1 ± 634.52	−207.68 ± 459.25	−25.62 ± 394.66	0.13
Alcohol (g)	5.95 ± 5.68	4.24 ± 3.78	5.39 ± 5	4.17 ± 3.41	−1.72 ± 4.77	−1.22 ± 3.12	0.86
Total fats (g)	91.28 ± 39	78.88 ± 29.66	79.52 ± 31.81	79.05 ± 31.42	−12.4 ± 27.37	−0.47 ± 19.89	0.10
Total saturated fatty acid (g)	31.4 ± 15.23	26.47 ± 10.63	26.29 ± 11.61	26.97 ± 12.4	−4.93 ± 11.12	0.68 ± 6.91	0.09
Total trans fatty acids (g)	3.0 ± 1.15	2.93 ± 1.4	2.56 ± 1.07	2.69 ± 1.15	−0.07 ± 0.77	0.13 ± 0.86	0.63
Proteins (g)	86.27 ± 37.45	79.12 ± 27.18	79.74 ± 29.14	80.45 ± 30.57	−7.15 ± 24.87	0.71 ± 16.69	0.19
Carbohydrates (g)	227.8 ± 72.42	212.81 ± 66.1	224.78 ± 81.02	219.66 ± 78.64	−14.99 ± 48.45	−5.12 ± 55.27	0.39
Total fibers (g)	23.31 ± 9.16	22.24 ± 8.19	24.32 ± 10.73	21.75 ± 8.55	−1.06 ± 7.46	−2.58 ± 7.1	0.67

Results are presented as raw means ± SD. Comparisons were made with the MIXED procedure with SAS Studio v.3.8 adjusted for age, gender, BMI, baseline, sequence, and interaction between treatment and sequence.

38% (vs. a 6% reduction for placebo) after a 16-week treatment of subjects with NAFLD.[18] Furthermore, the observation of a 1.6% absolute reduction in liver fat with only 12 weeks of CC treatment vs. placebo compares favorably with the 4% absolute reduction in liver fat with a 20-week treatment with the sodium-glucose transport protein 2 (SGLT-2) inhibitor empagliflozin vs. control (e.g., standard treatment for T2D).[19] It is also important to mention that the effect of this polyphenol-rich CC supplement on liver fat was observed after only 12 weeks of treatment vs. 16–20 weeks for two of the most potent pharmaceutical drugs recently used to treat NAFLD. The CC supplement is the only prebiotic known to have such an effect on liver fat (and on plasma AST and ALT levels) in humans, as demonstrated in the present randomized, double-blind, placebo-controlled trial.

The effect of CC supplementation on human liver fat is most likely explained by its high content in polyphenols such as ellagitannins and PAC. Indeed, we have previously reported that mice gavaged with 200 mg/kg/day of polyphenol-rich CC crude extract for 8 weeks were protected from hepatic steatosis.[9] A dose-ranging study in diet-induced obese mice also showed that administering 62.5 mg/kg/day of CC extract for 5 weeks decreased non-high-density lipoprotein cholesterol and free fatty acids, while 200 mg/kg/day prevented hepatic steatosis.[20] Our group also reported that a PAC-rich cranberry extract (200 mg/kg/day) reversed hepatic steatosis in mice and that this was linked to an upregulation of genes involved in lipid catabolism.[21] Based on the human equivalent dose calculation, 200 mg/kg/day in mice is equivalent to 16 mg/kg in humans, which represents 1.36 g/day in an 85 kg individual, which is in the same range as the dose (1.5 g/day) of CC used in this clinical study.

While placebo-controlled randomized clinical interventions with polyphenol-rich supplements are still very limited, there is emerging evidence supporting the potential benefits of polyphenol supplementation in humans. In a parallel-arm clinical study on patients with NAFLD, hesperidin, a flavonoid, given in combination with healthy lifestyle habits (dietary and physical activity) resulted in a decrease in ALT concentration and a decrease in steatosis scores assessed by FibroScan after 8 weeks of intervention.[22] Another parallel-arm clinical trial investigated the effect of a calorie-deficit Mediterranean diet and a calorie-deficit Mediterranean diet enriched in polyphenols, notably epigallocatechin gallate and flavonoid, in combination with physical activity on participants with abdominal obesity/dyslipidemia. After 18 months of intervention, the Mediterranean diet enriched in polyphenols showed superior results compared to the Mediterranean diet alone, resulting in a decrease in ALT and a median reduction of 2% in liver fat assessed by MRI after 18 months of intervention.[23] In comparison, in the present study, the median absolute decrease in liver fat by MRI was 1.6% after only 12 weeks of intervention with CC, which neither included a reduction in caloric intake nor changes in physical activity habits, suggesting that CC supplementation is a clinically relevant approach to reduce liver fat and help prevent NAFLD in overweight hypertriglyceridemic subjects. Another study also demonstrated that castalagin, an ellagitannin found in CC, exerted an antitumoral activity and reestablished the efficacy of immunotherapy against cancer.[14] Whether this ellagitannin-type polyphenol also contributes to the metabolic benefits of CC on human NAFLD remains to be determined.

Another important step in the progression from steatosis to NAFLD and NASH is lipotoxicity, which leads to inflammation and hepatocyte damage.[2] Whereas steatosis can be diagnosed by imaging, NASH can only be diagnosed by liver biopsy, which is an invasive procedure.[2] Therefore, levels of AST and ALT were measured to indirectly assess liver injury.[24] We observed that CC treatment decreased circulating levels of AST and ALT, further suggesting that the polyphenol-rich CC had beneficial effects on liver health. In line with this finding, a clinical study demonstrated that CC decreased circulating and urinary markers of inflammation and oxidative stress.[25] These data suggest that CC may protect against NAFLD progression by limiting liver injury, possibly through an anti-inflammatory action.

Although our findings showed that hepatic health markers improved after CC supplementation, it did not alter body weight or adiposity. In line with this, other clinical studies have reported that liver fat can be significantly decreased independently of body weight changes.[26,27] We also investigated the impact of CC treatment on glucose homeostasis, as the fruit extract was

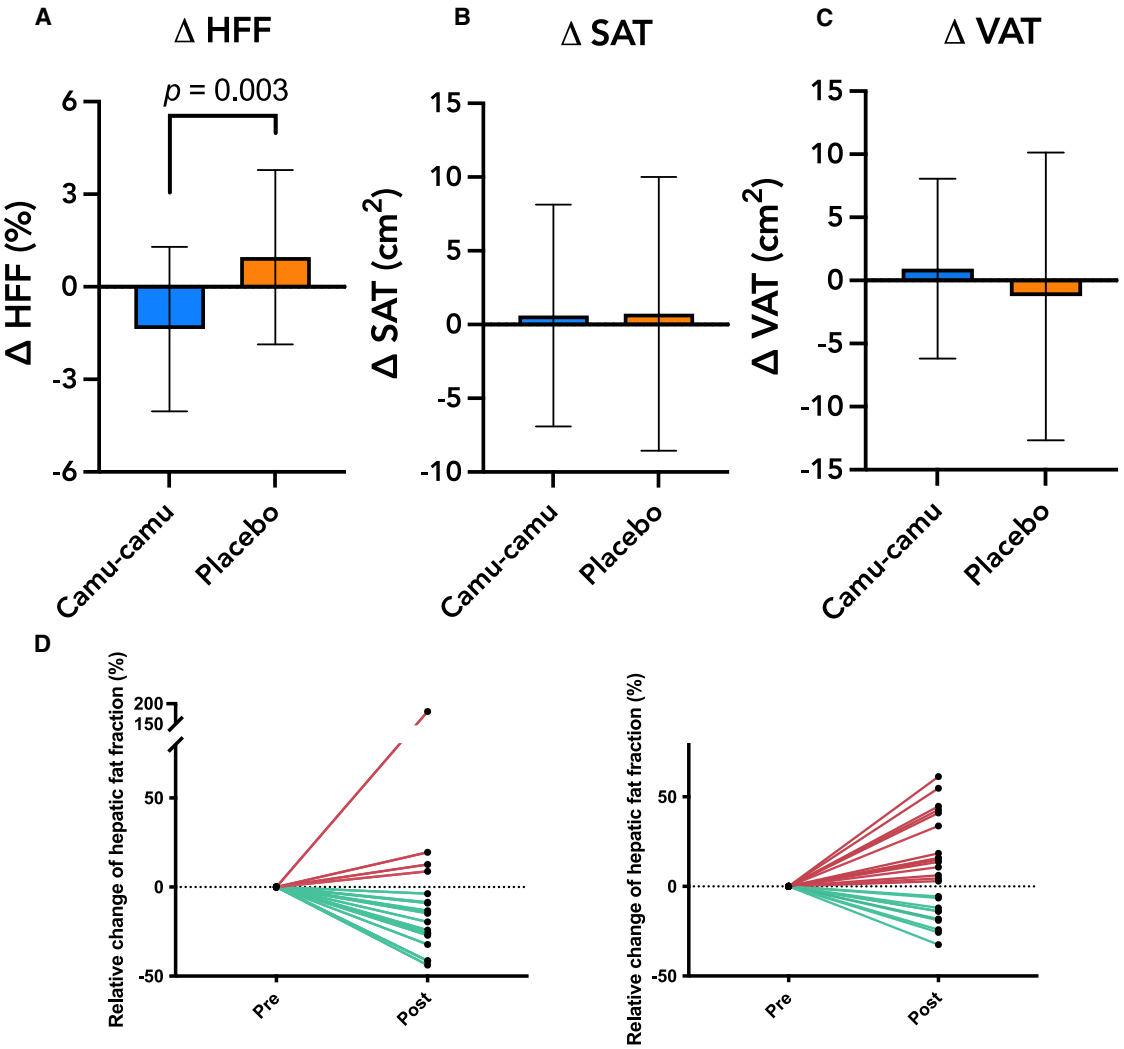


Figure 2. Effects of treatment on MRI-measured hepatic and abdominal fat distribution

(A) Δ hepatic fat fraction (HFF) for CC: $n = 20$ and placebo: $n = 25$; $p = 0.003$.

(B) Δ volume of subcutaneous adipose tissue (SAT) for CC: $n = 20$ and placebo: $n = 24$.

(C) Δ volume of visceral adipose tissue (VAT) for CC: $n = 20$ and placebo: $n = 25$.

(D) Individual relative change of percentage of HFF from baseline after CC (left) or placebo (right) phases. Results are presented as raw means ± SD. Analyses were conducted with SAS Studio and comparisons were made using the MIXED procedure adjusted for age, gender, BMI, baseline, sequence, interaction between treatment and sequence. See also Tables S1, S2, S3, and S4.

shown to prevent deterioration of obesity-induced glucose and insulin tolerance in preclinical studies.[9,20] Moreover, an *in vitro* study previously demonstrated that freeze-dried CC powder effectively inhibited α-glucosidase, an enzyme involved in carbohydrate digestion and absorption.[28] The impact of CC administration on post-prandial glycemia was also reported in humans, where the consumption of a single serving (300 mL) of clarified CC juice decreased glycemia compared to water after a meal test consisting of 50 g of white bread.[29] Nonetheless, in this trial, we did not observe any significant changes in glucose homeostasis indices following the 12-week CC administration. As participants were instructed to fast overnight prior to the oral glucose tolerance test, and no CC was ingested before the test, this may have prevented us from observing an effect on postprandial glucose metabolism. Moreover, participants included in this trial were not selected based on abnormal glucose homeostasis, which could have prevented us from observing an improvement in the latter parameter.

As an exploratory outcome, we have also tested the hypothesis that CC supplementation for 12 weeks induces changes in gut microbiota composition and function in the study participants. This was based on our previous preclinical work showing that CC exerted strong microbiome-dependent activity against obesity and hepatic steatosis in obese mice.[9] When compared to placebo, CC increased the relative abundance of *Enterococcus*, *Lactobacillus*, and *Lactococcus*, and metagenomic analyses further

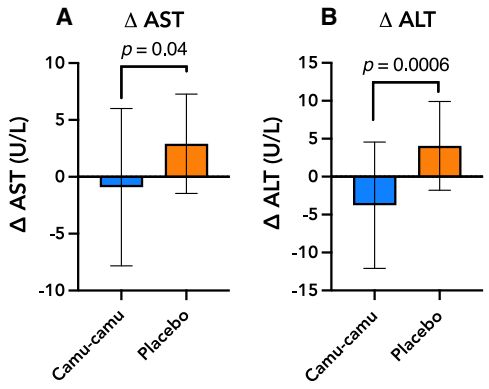


Figure 3. Effects of treatment on liver steatosis and hepatic health markers

(A) Δ aspartate aminotransferase (AST) for CC: $n = 22$ and placebo: $n = 23$; $p = 0.04$. (B) D alanine aminotransferase (ALT) for CC: $n = 29$ and placebo: $n = 30$; $p = 0.0006$. Results are presented as raw means ± SD. Analyses were conducted with SAS Studio and comparisons were made using the MIXED procedure adjusted for age, gender, BMI, baseline, sequence, and interaction between treatment and sequence. See also Table S3.

revealed an increase in bacterial species such as *Pediococcus pentosaceus*, *Latilactobacillus sakei*, and *Lactobacillus acidophilus*. CC treatment also decreased the abundance of *Intestinibacter*, *Shuttleworthia, Adlercreutzia*, and *Erysipelatoclostridium.* It has been previously reported that both *Erysipelatoclostridium* and *Adlercreutzia* were more abundant in the feces of mice with NAFLD.[30,31] *Intestinibacter* was also identified as a major contributor to NAFLD progression and correlated with severe intestinal disorders in humans.[32,33] Therefore, the decreased abundance of these NAFLD-associated bacteria by CC could be beneficial.

Metagenomic analysis further revealed that CC consumption altered pathways previously associated with metabolites implicated in NAFLD pathogenesis, such as a decrease in pathways related to tyrosine biosynthesis[34–36] and an increase in pathways related to the biosynthesis of 3-phenylpropanoate[37] and mycolate.[38] CC treatment was further associated with a decrease in a pathway implicated in the degradation of myo-inositol. This is of potent interest since it was previously reported that myo-inositol supplementation exhibits anti-inflammatory effects and improves body composition and liver-related markers in obese patients with NAFLD.[39,40] Future studies will be needed to investigate whether these changes in gut microbiota composition and function upon CC treatment are causally related to changes in liver fat accretion in our participants.

Limitations of the study

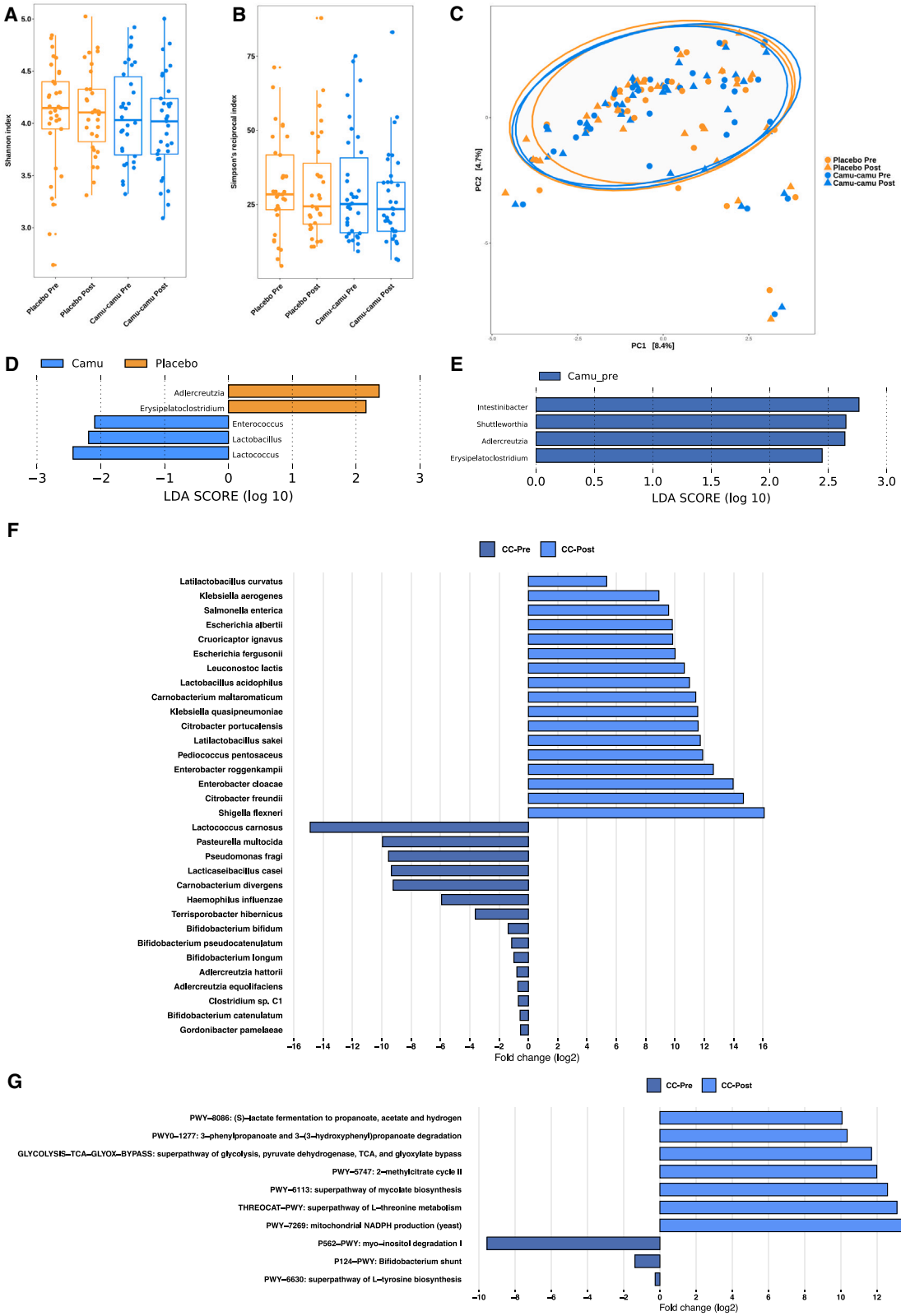
There are some limitations to this randomized, double-blind, placebo-controlled crossover trial. The number of participants

Table 3. Indices of cardiometabolic health over time by intervention group

	CC		Placebo		Delta change ($n = 30$)		Adjusted
Variable	Pre ($n = 30$)	Post ($n = 30$)	Pre ($n = 30$)	Post ($n = 30$)	CC	Placebo	p value
Weight (kg)	85.8 ± 16.5	86.1 ± 16.7	85.7 ± 16.9	85.0 ± 17.2	0.28 ± 1.28	0.32 ± 1.48	0.95
BMI (kg/m^2)	30.6 ± 4.8	30.9 ± 4.7	30.6 ± 4.9	30.7 ± 4.9	0.21 ± 0.81	0.11 ± 0.51	0.92
Waist circ. (cm)	104.7 ± 10.2	104.8 ± 10.6	104.2 ± 10.8	104.3 ± 11.2	0.4 ± 1.17	0.07 ± 0.93	0.11
SBP (mmHg)	122.6 ± 14.3	123.2 ± 14.4	122.3 ± 11.9	122.2 ± 14.8	0.6 ± 11.9	−0.3 ± 9.8	0.73
DBP (mmHg)	76.0 ± 9.0	75.3 ± 8.8	75.6 ± 9.4	77.1 ± 11.0	−0.7 ± 6.1	1.5 ± 5.5	0.11
Heart rate (bpm)	70.6 ± 10.8	69.8 ± 10.2	70.2 ± 8.8	70.6 ± 10.6	−0.8 ± 7.1	0.2 ± 6.8	0.60
TG (mmol/L)	1.79 ± 0.70	1.78 ± 0.72	1.68 ± 0.71	1.67 ± 0.6	0.02 ± 0.56	−0.01 ± 0.41	0.82
Total-C (mmol/L)	5.8 ± 1.1	5.59 ± 1.04	5.75 ± 1.11	5.64 ± 1.06	−0.2 ± 0.5	−0.1 ± 0.7	0.67
HDL-C (mmol/L)	1.4 ± 0.4	1.44 ± 0.41	1.44 ± 0.39	1.4 ± 0.4	0 ± 0.14	−0.03 ± 0.12	0.21
LDL-C (mmol/L)	3.6 ± 0.9	3.32 ± 0.85	3.54 ± 0.97	3.47 ± 0.94	−0.21 ± 0.45	−0.08 ± 0.57	0.35
Total-C/HDL-C	4.4 ± 1.4	4.14 ± 1.4	4.26 ± 1.36	4.29 ± 1.32	−0.17 ± 0.61	0.02 ± 0.41	0.13
Free fatty acids (μM/L)	0.55 ± 0.19	0.57 ± 0.18	0.54 ± 0.16	0.56 ± 0.16	0.02 ± 0.29	0.02 ± 0.18	0.58
HbA1c (%)	0.055 ± 0.003	0.056 ± 0.003	0.055 ± 0.003	0.056 ± 0.004	0.001 ± 0.002	0.001 ± 0.001	0.77
C-reactive protein (mg/L)	2.9 ± 3.0	2.9 ± 2.5	3.0 ± 2.6	3.9 ± 6.0	0.4 ± 1.1	1.0 ± 5.5	0.51
Apo-B (g/L)	1.1 ± 0.3	1.11 ± 0.25	1.1 ± 0.24	1.1 ± 0.23	−0.001 ± 0.104	−0.001 ± 0.142	0.86
AST (U/L)	21.58 ± 6.64	20.54 ± 7.79	18.11 ± 6.65	22.44 ± 6.83	−0.91 ± 6.91	2.91 ± 4.37	0.04*
ALT (U/L)	33.93 ± 13.58	30.41 ± 10.34	30.3 ± 11.02	34.37 ± 12.68	−3.76 ± 8.33	4.07 ± 5.85	0.0006*
BARD score	1.00 ± 0.59	1.00 ± 0.66	0.89 ± 0.83	1.04 ± 0.89	−0.09 ± 0.81	0.04 ± 0.93	0.55

BMI, body mass index; Waist circ., waist circumference; Hip circ., hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; Apo-B, apolipoprotein B; Total-C, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; total-C/HDL-C, total cholesterol/high-density lipoprotein cholesterol; HbA1C, glycated hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Results are presented as raw means ± SD. Analyses were conducted with SAS Studio and comparisons were made using the MIXED procedure adjusted for age, gender, BMI, baseline, sequence, and interaction between treatment and sequence. For C-reactive protein, normal distribution was not obtained, and analyses were made using NPAR1WAY procedure with SAS studio.

*p value < 0.05.



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that completed MRI for liver fat determination was lower than originally planned due to COVID-19. The imaging platform at the Québec Heart and Lung Institute (IUCPQ) research center was at times restricted access by sanitary regulations and at other times commandeered for urgent clinical care as IUCPQ was transformed into a primary COVID-19 care center. Nonetheless, the sample size is comparable to other clinical trials investigating the impact of polyphenols on gut microbiota,[41–43] but this may have prevented us from finding potential correlation between gut microbiota features and HFF in the present study, as we previously found in preclinical studies. In addition, liver biopsies were not performed in the present study, which may limit the comprehensive assessment of the full etiology of NAFLD, including fibrosis. While recognizing the valuable information that liver biopsy provides on inflammation and fibrosis, it is important to note that this type of intervention was not performed due to its invasiveness. Still, we acknowledge the importance of exploring alternative, noninvasive tests for fibrosis in future studies. Promising options such as FibroScan, Fibrosis-4 (FIB-4), or Enhanced Liver Fibrosis (ELF) tests may offer valuable insights into liver health without the constraints associated with repeated invasive procedure. However, a major strength of our study was the crossover design with the subject being its own control, which certainly contributed to limit the effect of the inter-individual variability observed in such studies.

In conclusion, supplementation for 12 weeks with 1.5 g/day of CC in participants with metabolic alterations decreased hepatic steatosis by 15.85% and plasma levels of AST and ALT as compared to placebo. These improvements were associated with changes in the gut microbiota composition, suggesting that CC exhibits a prebiotic-like action. These results support the hepato-protective potential of CC against NAFLD. Considering the increasing prevalence of liver disease worldwide and the lack of pharmacological treatments, these findings are of clinical relevance. Further long-term studies on larger sample size are needed to confirm the beneficial effects of CC on NAFLD and cardiometabolic health in humans.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101682.

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AUTHOR CONTRIBUTIONS

A. Morissette prepared the first draft of the manuscript, with the help of A.-L.A., L.D., G.P., and A. Marette. A.-L.A. conducted the statistical analysis. A. Morissette and A.-L.A. performed the laboratory analysis. J.M. was the clinical coordinator for this study. T.V.V. performed the gut microbiota analysis. E.L. supervised the MRI analysis. T.G. and A.-M.C. calculated the glucose homeostasis indices. A.-M.C. and C.G. medically supervised this trial. A. Marette, M.-C.V., G.P., and J.M. designed this study. All authors reviewed/revised and approved the final manuscript.

DECLARATION OF INTERESTS

A. Morissette, L.D., and T.G. received studentship from *Fonds de recherche du Québec – Santé* (FRQS). A.-L.A. is supported by the *Fondation du Centre Hospitalier Universitaire de Québec*. A. Marette holds a Pfizer/CIHR Partnered Research Chair on the pathogenesis of insulin resistance and cardiovascular diseases. Y.D. holds an NSERC-Diana Food Industrial Partnership Chair on prebiotic effects of polyphenols. M.-C.V. is the recipient of a Canada Research Chair in Genomics Applied to Nutrition and Metabolic Health. A.-M.C. and C.G. received a career award from the FRQS.

Figure 4. Effects of treatment of gut microbiota composition

(A) Shannon index.

(B) Simpson's reciprocal index.

(C) Principal component analysis of fecal microbial diversity.

(D) Linear discriminant analysis (LDA) effect size (LEfSe) was calculated to explore the taxa that most strongly discriminated between all participants after the intervention (post-placebo vs. post-CC).

(E) LEfSe was also calculated to explore the changes induced by CC following the 12 weeks intervention (pre-CC vs. post-CC).

(F) DESeq2 analysis on shotgun sequencing data was performed to explore the taxa or pathways (G) that were significantly different between the gut microbiota before and after CC consumption (pre-CC vs. post-CC). For plots A–C: CC: $n = 30$; placebo: $n = 30$. See also Figure S2.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human plasma samples	This study	N/A
Human stool samples	This study	N/A
Critical commercial assays		
Human Insulin ELISA Kit	Alpco	80-INSHU-E01.1
QIAamp Fast DNA Stool Mini Kit	Qiagen	Cat No./ID: 51604
Quant-iT™ PicoGreen™ dsDNA Assay Kit – Invitrogen™	ThermoFisher Scientific	Cat No: P7589
NEBNext® Ultra™ II DNA Library Prep Kit	Illumina	NEB #E7645L
Deposited data		
16S rRNA sequencing data	European Nucleotide Archive	[ENA]: PRJEB76302
Shotgun metagenomics data	European Nucleotide Archive	[ENA]: PRJEB76307
Software and algorithms		
R v.4.3.3	R Project	https://www.r-project.org; RRID: SCF_001905
DADA2 v1.16	Callahan et al.[28]	https://benjjneb.github.io/dada2/index.html; RRID: SCR_023519
Rdp classifier v2.2	Wang et al.[29]	https://sourceforge.net/projects/rdp-classifier/; RRID: SCR_022773
LEfSe	Segata et al.[31]	https://huttenhower.sph.harvard.edu/lefse/; RRID: SCR_014609
Trimmomatic v0.39	Bolger et al.[32]	http://www.usadellab.org/cms/index.php?page=trimmomatic; RRID: SCF_011848
bowtie2 v2.4.2	Langmead and Salzberg,[33]	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml; RRIC: SCR_005476
Kraken2 v2.1.21	Wood et al.[34]	https://ccb.jhu.edu/software/kraken2/
Bracken	Lu et al.[35]	https://ccb.jhu.edu/software/bracken/
HUMAnN 3.0	Beghini et al.[36]	https://huttenhower.sph.harvard.edu/humann/; RRID: SCR_014620
MetaCyc	Caspi et al.[37]	https://metacyc.org/; RRID: SCR_007778
SILVA database version 138.1	German Network for Bioinformatics Infrastructure	https://www.arb-silva.de/documentation/release-1381/; RRID: SCF_006423
phyloseq R package version 1.48.0	McMurdie and Holmes,[38]	https://joey711.github.io/phyloseq/; RRID: SCR_013080
DESeq2 R package version 1.44.0	Love et al.[39]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html; RRID: SCR_015687
SAS® Studio v3.8	SAS	https://welcome.oda.sas.com/
GraphPad Prism Version 9.5.0	GraphPad Software, Inc	https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, André Marette (andre.marette@criucpq.ulaval.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The 16S rRNA and shotgun sequencing datasets generated during this study are available at the European Nucleotide Archive: PRJEB76302 and PRJEB76307, respectively.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study design and participants

This study is a randomized, double-blind, crossover trial (two 12-week intervention periods, separated by a 4-week washout period) (Figure 1). After a pre-selection visit, a 2-week run-in period was carried out to stabilize dietary and health habits. Participants were asked not to change their dietary patterns, to maintain a constant level of physical activity, to keep their body weight stable, and to avoid participating in other concurrent research projects. With regard to dietary habits, participants were asked to limit their alcohol consumption to a maximum of 4 drinks per week, with a maximum of 2 red wine drinks per week. In addition, they were advised to limit their consumption of berries to a maximum of 4 servings (1 serving = ½ cup of fresh or frozen fruits) per week.

During each of the 12-week treatment periods, participants were asked to consume daily either 1.5 g (3 capsules of 500mg) of whole CC berry powder (purchased in a single-batch bulk from Ecoideas, CA) or placebo (maltodextrin) before breakfast. Treatment sequence was randomly assigned. The sequence codes were revealed after the completion of the statistical analyses for hepatic fat. Compliance was assessed by measuring the number of capsules returned. Volunteers were also asked to complete a daily checklist to complete the compliance assessment.

Sample size calculation was based on the mean and standard deviation of the post-intervention change in liver fat fraction (HFF), in line with a previous study showing a mean change in HFF from baseline at week 12 of $-1.7\% \pm 3.1\%$ in 18 participants treated with 400 mg of magnolia officinalis extract.[44] This study demonstrated the potential impact of this plant extract on HFF, serving as a benchmark for our CC expectations. Based on this study, we calculated, with a statistical power of 80% and an alpha error of 0.05, that a sample size of 27 participants was necessary to observe significant changes in HFF. We therefore sought to recruit 32 participants, assuming an attrition rate of 15%.

To meet the inclusion criteria, males and females had to be between 18 and 75 years old, with a body mass index (BMI) from 25 to 40 kg/m^2 and have fasting plasma triglycerides $\geq$1.35 mmol/L. Exclusion criteria included smoking, medications affecting glucose metabolism or plasma lipids levels; having concentrations of HbA1c >6.5% or fasting glycemia >7 mmol/L; gastrointestinal malabsorption, cirrhosis, chronic kidney disease; medications that can cause fatty liver; consumption of fruit or polyphenol supplements in the last 3 months; allergy or intolerance for CC or for an ingredient of the placebo; regular alcohol drinkers (>2 drinks/d); loss of 5% of body weight over the past 3 months; major surgery in the last 3 months; pregnancy or breastfeeding; take of antibiotics or probiotics in the last 3 months. Recruited and randomized subjects' profile are shown in Table 1.

During the study, subjects were asked to avoid the use of natural health products and to limit the consumption of berries, red wine, polyphenol supplements and all products containing berries or wine to quantities similar to those specified during the initial run-in period. Using group electronic messages sent to university and Institute of Nutrition and Functional Foods (INAF) members as well as social media advertisements, a total of 257 individuals contacted the researchers.

Ethical approval

This study was conducted according to the guidelines described in the Declaration of Helsinki and was approved by the Research Ethics Committee of IUCPQ-Université Laval (ethics committee approbation number 2020–3350, 21854). All participants signed a written informed consent prior their participation in the study.

METHOD DETAILS

Dietary assessment and questionnaires

Web-based validated self-administered Food frequency questionnaires (FFQ) were used to evaluate the participant's usual diet before and after each 12-week intervention period.[45] This FFQ is based on typical food items available in Québec and contains 136 questions divided in 8 categories: dairy products, fruits, vegetables, meat and alternatives, cereals and grain products, beverages, "other foods" and supplements. The frequency at which each item was consumed per day, per week, per month or none at all in the last month was asked, and examples of portion item was provided within the online FFQ for a better estimation of portions consumed.

Anthropometric, blood pressure and heart rate measurements

Waist circumference was measured according to the procedures recommended at the Airlie conference on the standardization of anthropometric measurements.[46] A beam scale with measuring rod graduated in centimeters was used to obtain a measurement of fasting weight, height, and BMI was calculated in kilograms per square meter. Fat mass and lean mass were measured by Dual Energy X-ray Absorptiometry (DEXA). Blood pressure and heart rate were measured three times following a 10-min rest using an automatic tensiometer (Digital Blood Pressure Monitor, model HEM-907XL; OMRON, Kyoto, Japan).

Hepatic enzymes measurements

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by Dimension Vista AST and ALT methods, adapted from the recommended methods of International Federation of Clinical Chemistry with the use of the coenzyme pyridoxal-5-phosphate (P5P). Laboratory analyzes were performed at CHU of Québec (QC, Canada).

Evaluation of insulin sensitivity, glucose homeostasis

A 3-h 75g oral glucose tolerance test was performed for calculations of post-prandial and fasting glycemia, insulin sensitivity, insulin secretion and pancreatic beta cell function indices. Blood samples were collected at timepoints 0, 15, 30, 60, 90, 120 and 180 min. Plasma glucose, C-peptide and HbA1c were measured as described elsewhere.[47] Plasma insulin concentration was measured using an ELISA assay (Alcpo human insulin kit) according to the manufacturer's instructions. Whole-body insulin sensitivity (SI), whole-body dynamic insulin sensitivity taking into account timing of insulin action (SI^D) and total insulin secretion (Phi_{tot}) indices were assessed using the Oral Minimal Model method[48] implemented in the SAAM II software v2.2 (The Epsilon group, Charlottesville, VA, USA). The insulin secretion oral minimal model was adjusted for 0–180 min sampling with a Bayesian prior applied on parameter alpha (mean = 0.09 and SD = 20%) and forcing function constraint denominator set to 2*(G-gss+0.00001). Disposition indices, accounting for pancreatic beta cell function, were calculated by multiplying Phi_{tot} with SI and SI^D indices to respectively generate the disposition index (DI_{tot}) and the dynamic disposition index (DId_{tot}). Fasting hepatic insulin sensitivity (HOMA2%S) and fasting pancreatic β cell function (C-Peptide HOMA2%B) indices were calculated using the Homeostatic Model Assessment 2.[49] Fasting adipose tissue insulin resistance was derived from the ADIPO-IR index.[50] Insulin clearance was estimated using C-Peptide/Insulin incremental area under the curve ratios between 0-30 min and 0–120 min.[51]

Magnetic resonance imaging

Abdominal adiposity and liver fat accumulation was measured by magnetic resonance imaging (MRI) as previously detailed.[52] Images were obtained using a 3T clinical system (Ingenia, Philips Healthcare, software version R5.1.9). They were analyzed according to standardized protocols and software (cvi42, v.5.3.4, Circle Cardiovascular Imaging Inc.; IntelliSpace Portal, v.7.0, Philips). Subcutaneous and visceral adipose tissue: Abdominal water/fat imaging was performed using mDixon 3D echo-gradient sequence (in-phase, out-of-phase, water and fat) with TR = 3.4 ms and incremental echo timesTE1 = 1.19ms; TE2 = 2.1 ms) spanning the abdomen from L2 to S2 lumbar vertebrae (5 mm thickness, 0 mm space, 1.75 mm resolution on location). Liver fat fraction was measured using a 6-point mDixon 3D gradient-echo (mDixon Quant) sequence with complex fat modeling (TR = 6.2 ms, TE1 = 0.95 ms, delta TE = 0.8 ms, 2.5 mm in-plane resolution, mm slice thickness). A representative liver sample of 5 regions of interest ($100mm^2$ each) from segments IV (left lobe) and V (right lobe) was collected on at least 3 different slices (IntelliSpace Portal, Philips), excluding vessels, ducts and ligaments, as previously reported. Each participant was imaged at baseline and follow-up, and change was reported in matched regions of interest. Participant were not required to fast before undergoing MRI. Image analysis was performed offline in a standardized core laboratory (*Laboratoire d'Imagerie Cardiovasculaire Avancée*, CRIUCPQ-UL) by a single trained technician supervised by a certified Level 3 MRI reader. Both were blinded to the study hypothesis and patient data.

Bacterial DNA extraction and 16S rRNA gene sequencing

Stool samples were collected before the beginning of each phase and after CC or placebo treatments using the EasySampler Stool Collection Kit (ALPCO, Salem, NH, USA). Samples were stored at −20°C until they were delivered to the research center, at which point they were stored at −80°C until analysis. Bacterial genomic DNA was extracted from 1 g of fecal material from each sample. Samples were homogenized with 10 mL of Inhibitex (Qiagen, Valencia, CA, USA). Part of lysate was transferred to tubes containing 0.1 mm zirconium beads, 50 mg of lysozyme and 500 U of mutanolysine. Tubes were incubated at 37°C for 1 h. Then, tubes were vortexed twice for 1 min using a bead beater. The suspension was heated for 5 min at 95°C. Bacterial DNA was then extracted (Qiagen QIamp DNA Stool Mini Kit) following manufacturer's instructions. DNA concentrations were evaluated with Quant-IT PicoGreen dsDNA reagent (Invitrogen, Waltham, MA, USA). Extracted DNA was stored at −20°C until it was sent to *Center d'expertise et de services Génome Québec*.

The fecal microbiota composition was determined by 16S rRNA amplicon sequencing and was performed by the sequencing platform at *Center d'expertise et de services Génome Québec* for PCR amplification of the V3-V4 region using the primers 341F and 805R, and followed by sequencing on an Illumina MiSeq sequencing platform (Illumina, CA, USA), as detailed previously.[9,53]

Metagenomics shotgun profiling

Shotgun metagenomics sequencing was also performed to further compare the gut microbiota composition and function before and after the CC intervention. Metagenomics libraries were prepared with NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) as per the manufacturer's recommendations. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). The average size fragment was determined using a LabChip GX (PerkinElmer) instrument. Libraries were sequenced on the Illumina NovaSeq X platform using a 2 × 150 bp paired end run.

Fecal short-chain fatty acids

Fecal SCFA were quantified by gas chromatography. Feces were collected and kept frozen at – 80°C until extraction. After addition of 1mL H_2O per 100 mg of material, fecal suspensions were homogenized 2 min then centrifuged at 18000 g for 10 min at 4°C. Supernatant were spiked with 4-methylvaleric acid and acidified with phosphoric acid 10%. To extract SCFAs, samples were mixed 2 min with an equal volume of diethyl ether then centrifuged at 18000 g for 10 min at 4°C. Organic phase analysis was performed on a GC-FID system (Shimadzu), constituted of a GC 2010 Plus gas chromatograph equipped with an AOC-20s auto-sampler, an AOC-20i auto-injector and a flame ionization detector. The system was controlled by GC solution software. SCFA were separated on a Nukol capillary GC column (30 m × 0.25 mm id, 0.25 μM film thickness, Supelco analytical). The column flow was constant at 1.3 mL/min of hydrogen. The injector was set on 230°C and the detector on 250°C. The oven temperature was initially programmed at 60°C, then increased to 200 °C at 12 °C/min, hold 2 min. SCFA were quantified using a 5-points calibration curve prepared with a mix of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and internal standard 4-methyl valeric acid. Phosphoric acid was purchased from VWR. Diethyl ether (99.5%) and all the 99% grade standards (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and internal standard 4-methyl valeric acid) were purchased from Sigma-Aldrich.

QUANTIFICATION AND STATISTICAL ANALYSIS

16S rRNA gene statistical analyses

Sequences were processed using the DADA2 package (v1.16)[54] in the R environment (http://www.R-project.org) and taxonomic annotation of reads was obtained using the RDP classifier algorithm (v2.2)[55] trained against the Silva database 138.1.[56] To quantify bacterial alpha diversity, Shannon and Simpson's reciprocal indexes were calculated. Principal component analysis (PCA) was performed on Aitchison distance matrix in order to measure beta diversity. Identification of differentially abundant bacteria between two distinct biological conditions was measured with LEfSe using a linear discriminant analysis (LDA) score threshold of $\geq$2.5.[57] A p-value <0.05 was considered statistically significant.

Metagenomics shotgun statistical analyses

Metagenomic reads were quality-filtered using Trimmomatic with a cutoff $\geq$ Q20[58] and further filtered to remove the host-origin reads using Bowtie2.[59] Host-decontaminated reads were taxonomically profiled using Kraken2[60] against the RefSeq bacterial genomes. The abundance of species in annotated reads from a metagenomic sample was computed using Bracken.[61] Species that were not present in at least 10% of all samples were discarded. Functional analyses were performed using HUMAnN3,[62] and MetaCyc[63] pathways were generated from HUMAnN3 output. The R Phyloseq package[64] was used to perform all diversity analyses. Shannon and Simpson diversity indices were used to reflect the richness and evenness of microbial representation in a sample. Additionally, the similarity of microbial communities between samples was calculated using principal component analysis (PCA) based on the Aitchison distance. Detection of differentially abundant taxa or pathways between groups was performed with DESeq2[65] with significance set at 0.05.

Clinical characteristics statistical analyses

Statistical analyses were performed using SAS Studio (SAS Institute, Inc., Cary, NC, USA) version 3.8. Normality of the data was verified using skewness and kurtosis. Non-normally distributed variables were transformed (log10, natural log or squared roots). If the normality of the transformed variable was not reached, a nonparametric analysis using Mann Whitney U tests was performed. Outcome deltas (**D)** were calculated as the difference between post-intervention and pre-intervention for each intervention. The PROC mixed procedure was used to test for differences in outcomes between CC and placebo groups. The model included fixed effects for treatment and a random effect for the subject. Potential confounders of the outcome such as age, gender, BMI, values before intervention (baseline), treatment sequence, and sequence × time interaction were included in the models. Differences were considered significant when $p < 0.05$. Least square means were used to perform post hoc comparisons between groups. Data from participants who dropped out of the study were not considered in the analyses.

ADDITIONAL RESOURCES

The study is registered at clinicaltrials.gov as NCT04130321.