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Article

Impact of Blueberry Consumption on the Human Fecal Bileacidome: A Pilot Study

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Abstract: Cholesterol-derived bile acids (BAs) affect numerous physiological functions such as glucose homeostasis, lipid metabolism and absorption, intestinal inflammation and immunity, as well as intestinal microbiota diversity. Diet influences the composition of the BA pool. The present study analyzes the impact of a dietary supplementation with a freeze-dried blueberry powder (BBP) on the fecal BA pool composition. The diet of 11 men and 13 women at risk for metabolic syndrome was supplemented with 50g/day of BBP for 8 weeks, and feces were harvested before (pre) and after (post) BBP consumption. BAs were profiled using liquid chromatography coupled to tandem mass spectrometry. No significant changes in total BAs were detected when comparing pre- vs post-BBP consumption samples. However, post-BBP consumption samples exhibited significant accumulations of glycine-conjugated BAs (p=0.04), glycochenodeoxycholic (p=0.01) and glycoursodeoxycholic (p=0.01) acids, as well as a significant reduction (p=0.03) of the secondary BA levels, when compared to pre-BBP feces (p=0.03). In conclusion, the fecal bileacidome is significantly altered after the consumption of BBP for 8 weeks. While additional studies are needed to fully understand the underlying mechanisms and physiological implications of these changes, our data suggest that the consumption of blueberries can modulate toxic BAs elimination.

Keywords: blueberries; bile acids; dietary supplements; polyphenols; LC-MS/MS profiling

1. Introduction

Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary bile acids (BAs) formed from cholesterol in the liver, and stored in the gallbladder under the forms of conjugates to the amino acids glycine or taurine also known as bile salts [1]. Glyco- and tauro-conjugated BAs can then be secreted into the biliary tract to reach the intestinal lumen where they favour the absorption of fat-soluble nutrients [1]. Due to their amphipathic nature, BAs have the ability to form micelles which promote intestinal absorption of dietary lipids and other lipophilic compounds such as vitamins A, D, K and E [2]. From the intestinal lumen, BAs are recycled through an enterohepatic recirculation. The majority of BAs are actively reabsorbed in the ileum *via* captation by the apical sodium-dependent bile salt transporter from enterocytes [3]. Then, BAs can be secreted from the ileocytes to the portal circulation to return to the liver [3]. This mechanism, together with low passive absorption throughout the intestinal tract, is responsible for the effective recycling of 95% of the BAs secreted in the intestine [1]. The remaining 5% (roughly 500 mg per day) are excreted in feces [4]. BAs that are not reabsorbed will undergo many transformations by the microbiota most of which occur in the ileum and colon. Gut bacteria can deconjugate conjugated-BAs *via* bile salt hydrolase enzymes to form unconjugated BAs, which can

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then be dehydroxylated into the secondary lithocholic acid (LCA) and deoxycholic acid (DCA) *via* the 7α-dehydroxylase enzyme [5].

BAs and their derivatives play major roles in the regulation of cholesterol, and act as endogenous ligands for several nuclear and membrane receptors (namely the Farnesoid X receptor (FXR), Pregnane X receptor (PXR), vitamin D receptor (VDR), Constitutive androstane receptor (CAR) and Takeda G protein–coupled receptor 5 (TGR5)), to regulate functions such as glucose and lipid metabolism, inflammation and immunity [1,4,6,7]. BAs display different capabilities for binding to and activating these receptors. For example, as a FXR agonist, the primary acid CDCA and its tauro-conjugates play a determinant role in the intracrine negative feedback loop in which BAs control their own hepatic synthesis [4]. By contrast, the secondary acid, glycoursodeoxycholic acid (GUDCA) can act as an FXR antagonist [8]. Intestinal secondary acids such as DCA and LCA also act as natural antibiotics and thus influence microbiota diversity [9]. In such a context, minimal changes in the BA pool composition can have a major impact on human health.

To ensure an optimal level for these biological functions, the formation and metabolism of BAs are highly and specifically regulated by endo- and exogenous factors such as negative feedback loops, feeding state, circadian rhythm and enteric reabsorption [1]. In addition, the composition of the BAs pool can be influenced by dietary factors, microbiota composition, metabolic disorders, and several pathologies of the gut-liver axis [10]. Such a complex regulation process results in different BA profiles, particularly following specific dietary intervention [11].

Dietary polyphenols are among the food components currently investigated in depth regarding human health are polyphenols [12,13]. Recent observations revealed that consumption of polyphenol-rich fruits can affect the bileacidome [13,14]. Because blueberries are highly concentrated in anthocyanins, a type of polyphenol that give the characteristic colors of the berries [15] and that are known to influence the microbiome [16,17], we sought to test the possibility that an 8-week consumption of 50 g/day of freeze-dried blueberry powder (BBP) may impact the fecal bile acid profile in humans.

2. Methods

2.1. Ethics Statement

This study was approved by the Ethics Committees of Université Laval and the CHU de Québec Research Centre. The study is registered at https://clinicaltrials.gov/ (NCT03266055). All subjects signed a written informed consent prior to their participation in the study.

2.2. Participants and Original Design

Samples analysed in this study were originally collected from volunteers of a randomized, double-blind, placebo-controlled trial measuring the impact of BBP supplementation on immune related pathways (see reference [18]). Briefly, the original study was conducted between 2017 and 2019 at the Institute of Nutrition and Functional Foods (INAF) of Université Laval in the Quebec City area (Canada). Caucasian men and women (premenopausal), in good health and aged 18-55 years were recruited. The original trial involved 49 participants randomized in either the placebo (n=24) or BBP intervention group (n=25).

From the 49 participants involved in the original trial [18] only those from the intervention group were selected for the present proof of concept study on bile acids. A sample from one participant in the BBP group could not be used as it was in insufficient quantity to be profiled for BAs. In sum, the final cohort involved in the present investigations comprised 24 Caucasian individuals (11 men and 13 premenopausal, non-pregnant and non-lactating women) of the BBP intervention group that are at risk of metabolic syndrome (Table 1).

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Table 1. General baseline characteristics of participants from whom fecal samples were used in the present study. Adapted from Rousseau M and colleagues [18].

		Mean	[Range]
Weight (kg)		89.5	[62.5 - 126.5]
Height (m)		1,71	[1.53 - 1.88]
BMI (kg/m²)		30.8	[23.4 - 47.1]
Waist circumference (cm)		101.5	[81.0 - 131.3]
Hip circumference (cm)		110.2	[92.2 - 141.8]
SBP (mmHg)		115.2	[99.0 - 131.7]
DBP (mmHg)		72.5	[57.0 - 82.33]
Apo B (g/L)		0.91	[0.6 - 1.41]
Total-C (mmol/L)		4.56	[2.99 - 6.41]
TG (mmol/L)		1.54	[0.53 - 3.98]
HDL-C (mmol/L)		1.17	[0.74 - 1.99]
LDL-C (mmol/L)		2.68	[1.58 - 4.05]
Total-C/HDL-C		4.06	[2.42 - 6.97]
HbA1c		0.051	[0.045 - 0.056]
Age (year)		35.0	[23 - 53]
Sex	**[n]**		
Men	*11*		
Women	*13*		
Annual household income $CDN	**[n]**		
0–39,999	*7*		
40,000–79,000	*8*		
80,000–99,000	*4*		
≥100,000	*4*		
Non disclosed	*1*		
Highest education level completed	**[n]**		
High school	*2*		
College	*6*		
University	*16*		

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, triglycerides; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; HbA1c, glycated hemoglobin.

As extensively described in Rousseau M. and colleagues [18], inclusion criteria were a BMI of 25.0–40.0 kg/m² or abdominal obesity (waist circumference ≥ 94 cm for men and ≥ 80 cm for women); triglycerides ≥ 1.35. mmol/L or fasting insulin concentration ≥ 42 pmol/L [19]. Participants were excluded if, prior to the study, they were diagnosed with diabetes, hypercholesterolemia, or hypertension, and if they were taking medications for these conditions. Participants were also excluded if they took antibiotics during of up to months before the study [18].

2.3. Intervention

Participants were asked to consume 50g of a freeze-dried BBP per day (2 doses of 25g separated by at least 8 hours) for 8 weeks. Fifty grams BBP is roughly equivalent to 350g of fresh blueberries [18]. The BBP was provided by the US Highbush Blueberry Council

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(Folsom, CA). It consisted of a blend of milled freeze-dried highbush blueberries from two cultivars (*Vaccinium virgatum (ashei)* and *Vaccinium corymbosum*), in a 1:1 ratio [18]. Participants were asked to add the BBP to 300ml of water or to mix it in food that would not compromise the phytochemicals [18]. They were instructed not to make major changes in their lifestyle and dietary pattern for the duration of the study. Restrictions were imposed on whole berry consumption as well as on certain products with high content in phytochemical like red wine, coca products, tea, and coffee [18].

At week 0 (pre-) and 8 (post-treatment), participants were asked to collect stool samples at home at a bowel movement as close as possible to the visit planned to the research center [18]. They had to keep the samples in their freezer (-20 °C) until they brought it to the research center. An icepack was provided to keep the samples frozen during transportation. Thereafter, stool samples were stored at -80 °C at the research center until BA profiling.

2.4. Measurement of fecal bile acids

A total of 19 BA species were extracted, separated and quantified using a previously reported liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method [20,21]**.** Briefly, fecal samples were lyophilized under nitrogen and resolubilized in a water: methanol (50:50) solution containing 0.1% formic acid. Stainless-steel beads were added to the resulting suspension and blended at 4°C to obtain a homogenate (Blender, Next Advance, NY). Organic fraction was evaporated under nitrogen before resuspension in water containing 0.1% formic acid. Deuterated BAs were added as an internal standard. After centrifugation at 5,000 g for 5 min, the supernatant was collected and the organic fraction was evaporated under nitrogen before resuspension in water containing 0.1% formic acid. The solution containing BAs then underwent a solid-phase extraction using Strata-X 60 mg columns (Phenomenex, Torrance, USA). As extensively reported [20,21], the LC-MS/MS system consisted in a Nexera ultra-high-pressure liquid chromatography (UHPLC) instrument (Shimadzu Scientific Instruments, Columbia, USA) coupled with an API6500 tandem mass spectrometer (Applied Biosystems, Concord, Canada).

HPLC-grade solvents were purchased from VWR Canlab (Montréal, Canada). Ammonium formate was bought from Laboratoire Mat (Québec, Canada). Deutered BAs standards (d_4-CA, d_4-CDCA, d_4-LCA d_4-DCA, d_4-GCA and d_4-TCA) were purchased from C/D/N Isotopes (Montréal, Canada) and Toronto Research Chemicals (Toronto, Canada). The separation column for chromatographic separation used was a Poroshell 120 EC-C18 2.7 µm; 2.1x150mm (Agilent).

2.5. Bile acid analysis

As previously reported [21,22], total BA concentrations correspond to the sum of all BA measured. Total sums of glyco- and tauro-conjugates were calculated by the summation of concentrations of conjugated BA: CDCA, CA, DCA, LCA. UDCA, and hyodeoxycholic acid (HDCA). The sum of unconjugated BA also included HCA concentration. The total of primary, secondary, and hydroxylated BA species was determined by adding all unconjugated and/or conjugated species of CDCA + CA, LCA + DCA, or HDCA + hyocholic acid (HCA), respectively. Total of CA, CDCA, LCA, DCA, HDCA or HCA were obtained by summing the concentrations of all forms (unconjugated and conjugated) for each species.

2.6. Statistical Analysis

All data are presented as mean±SEM. Bile acid levels showed neither normal or lognormal distribution using normality and lognormality hypothesis tests (Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test) and failed visual test for normality with a Quantile-Quantile plot. Thus, non-parametric tests were chosen for all the analyses. To assess differences in BA levels after the intervention

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compared to baseline, the Wilcoxon matched-pairs signed rank tests also corrected for multiple comparisons using the Bonferroni-Dunn method. For differences between sex in BA profiles, the Mann-Whitney test corrected for multiple comparisons with the Bonferroni-Dunn method was used. Descriptive statistics (Mean, Standard error of the mean (SEM), Coefficient of variation (CV), Range, etc.) were used to present the data and to characterize interindividual variation. All statistical analyses were made using *Prism 9* from GraphPad Software™ (San Diego, CA). Significance threshold was set for p values <0.05 (two sided).

3. Results

3.1. Fecal bile acid profiles sustain large inter-individual variability.

As illustrated in Table 2, unconjugated BAs were the main component of the fecal pool representing 95.4% ± 0.7% at the beginning of the study. Most of the unconjugated species corresponded to secondary acids, which represented 90.2 % ± 2.0% of total BAs pool (Table 2). With respective levels of 2.91±0.31 and 2.53±0.23 nmol/mg of feces, the unconjugated forms of the secondary acids, DCA and LCA were the most abundant species (Table 2). On the other hand, glycine- and taurine-conjugated BAs together represented only 1.75% ± 0.52% of fecal BAs.

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Table 2. Bile acids concentrations before and after an 8-week period of consumption of freeze-dried blueberry powder.

Bile acids	Before (Baseline)		After (Week 8)		Mean Diff.	Ajusted p value
	Mean	SEM	Mean	SEM		
CA	0.1887	± 0.0734	0.2732	± 0.1672	0.0844	>0.999
CDCA	0.0967	± 0.0278	0.1296	± 0.0673	0.0328	>0.999
DCA	2.9097	± 0.3159	2.1280	± 0.2336	-0.7817	0.103
LCA	2.5275	± 0.2297	1.9844	± 0.1574	-0.5431	0.103
HDCA	0.0087	± 0.0011	0.0064	± 0.0008	-0.0023	0.375
HCA	0.0023	± 0.0007	0.0019	± 0.0006	-0.0005	>0.999
UDCA	0.0323	± 0.0093	0.0518	± 0.0192	0.0195	>0.999
GCA	0.0159	± 0.0076	0.0279	± 0.0090	0.0120	0.115
GCDCA	0.0102	± 0.0032	0.0170	± 0.0043	0.0068	**0.010**
GDCA	0.0132	± 0.0031	0.0145	± 0.0018	0.0013	>0.999
GLCA	0.0003	± 0.0001	0.0003	± 0.0000	-0.0001	>0.999
GUDCA	0.0010	± 0.0002	0.0017	± 0.0004	0.0008	**0.011**
TCA	0.0153	± 0.0081	0.0212	± 0.0098	0.0059	>0.999
TCDCA	0.0073	± 0.0030	0.0188	± 0.0095	0.0115	>0.999
TDCA	0.0306	± 0.0117	0.0203	± 0.0065	-0.0103	>0.999
TLCA	0.0015	± 0.0007	0.0011	± 0.0004	-0.0004	>0.999
TUDCA	0.0007	± 0.0004	0.0014	± 0.0008	0.0007	>0.999
TOTAL BA	6.0068	± 0.5721	4.8358	± 0.4598	-1.1710	0.517
Unconjugated	5.7660	± 0.5556	4.5752	± 0.4564	-1.1908	0.393
Taurine-conjugated	0.0554	± 0.0228	0.0628	± 0.0231	0.0074	>0.999
Glycine-conjugated	0.0406	± 0.0139	0.0614	± 0.0141	0.0208	**0.045**
Primary	0.3343	± 0.1075	0.4877	± 0.2482	0.1534	>0.999
Secondary	5.4828	± 0.5251	4.1486	± 0.3707	-1.3343	**0.030**
6α-hydroxylated	0.0110	± 0.0015	0.0083	± 0.0008	-0.0027	0.940
Total CA	0.2200	± 0.0820	0.3223	± 0.1735	0.1023	>0.999
Total CDCA	0.1144	± 0.0299	0.1659	± 0.0760	0.0515	>0.999
Total DCA	2.9536	± 0.3213	2.1632	± 0.2368	-0.7905	0.115
Total LCA	2.5294	± 0.2299	1.9859	± 0.1574	-0.5435	0.103
Total HDCA	0.0087	± 0.0011	0.0065	± 0.0008	-0.0023	0.414
Total HCA	0.0023	± 0.0007	0.0019	± 0.0006	-0.0004	>0.999

Values are presented as mean concentration (nmol/mg of feces) of the 24 pre- and post-diet samples ± SEM (standard error of the mean).

Mean Diff., namely the difference between pre- vs post-treatment were calculated for each of the participants (11 men and 13 women), and values represent the mean±SEM.

P values were calculated using Wilcoxon matched-pairs signed rank test adjusted for multiple comparisons using the Bonferroni-Dunn method. Bile acids composition analyses were performed as detailed in the materials and method section.

CA: cholic acid; CDCA: chenodeoxycholic acid; LCA: lithocholic acid; DCA: deoxycholic acid; HDCA: hyodeoxycholic acid; HCA: hyocholic acid; UDCA: Ursodeoxycholic acid. G: glyco; T: tauro.

At week 0 (Supplemental Table 1), the initial profile of BAs sustained a large interindividual variability in both male and female volunteers. Indeed, the total BA concentration ranged from 0.47 to 12.29 nmol/mg of feces (CV: 62.1%) in men, and from 3.71 to 10.20 nmol/mg of feces in women (CV: 34.6%) (Supplemental table 1). The largest variations were observed with glycine conjugated species in men (CV: 177.0%), and with taurine conjugated species in women (CV: 182.3%) (Supplemental table 1). An important variation was also observed among CA species for both sexes with total CA values ranging from 0.01 to 0.69 nmol/mg of feces in men (CV: 160.0%) and from 0.01 to 1.64 nmol/mg of feces

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in women (CV: 175.5%). The less variable parameters were the 6α-hydroxylated BAs in men and total LCA species in women with values ranging from 0.0007 to 0.0199 nmol/mg of feces (CV: 56.6%) and from 1.67 to 4.24 nmol/mg of feces (CV: 32.9%), respectively.

3.2. No sexual dimorphism is detected in the fecal bileacidome.

No significant changes were detected between men and women concerning the fecal BA profiles in samples harvested before (Supplemental Table 1) and after (Supplemental Table 2) the BBP consumption period.

3.3. The fecal bileacidome exhibit significant alteration after the 8-week consumption period of freeze-dried blueberry powder

As summarized in Table 1, the 8-week consumption period of BBP led to an altered the fecal BA profile. While the changes in specific parameters (namely GCDCA, GUDCA, the sum of glycine-conjugated and secondary acids) reached the statistical significance (Fig. 1), most of the parameters remained either unaffected or only tended to be modified in a non-significant manner (Table 2). For example, the reduction of total BA levels from 6.01±0.57 to 4.84±0.46 of feces failed to reach statistical significance (Table 2 and Fig. 1A). The same also applies to unconjugated bile acids (Fig. 1B). By contrast, the level of glycine-conjugated BAs was significantly 1.5-times increased in feces harvested after the diet, when compared to initial levels (Fig. 1C). This increase reflected a significant accumulation of GCDCA (p=0.010) and GUDCA (p=0.011) (Fig. 1D&E), while the 1.75-fold increase in GCA fecal levels remained non-significant (p=0.115) (Table 2 and Fig. 1F).

Beyond conjugated and unconjugated forms, BA species can also be divided into primary and secondary species. While the increase from 0.33±0.11 to 0.49±0.25 nmol/mg of feces in primary BAs remained statistically nonsignificant (Fig. 1G), the reduction in levels of secondary BAs (from 5.48±0.52 to 4.15±0.37 nmol/mg of feces) observed in post-BBP samples reached the statistical significance (p=0.030; Fig. 1H). Interestingly, fecal concentrations of secondary DCA and LCA acids, also tended to be reduced (from 2.91±0.32 to 2.13±0.23 nmol/mg of feces for DCA, and from 2.53±0.23 to 1.98±0.16 nmol/mg of feces for LCA) after BBP consumption (Fig. 1I&J; p=0.103 for both metabolites).

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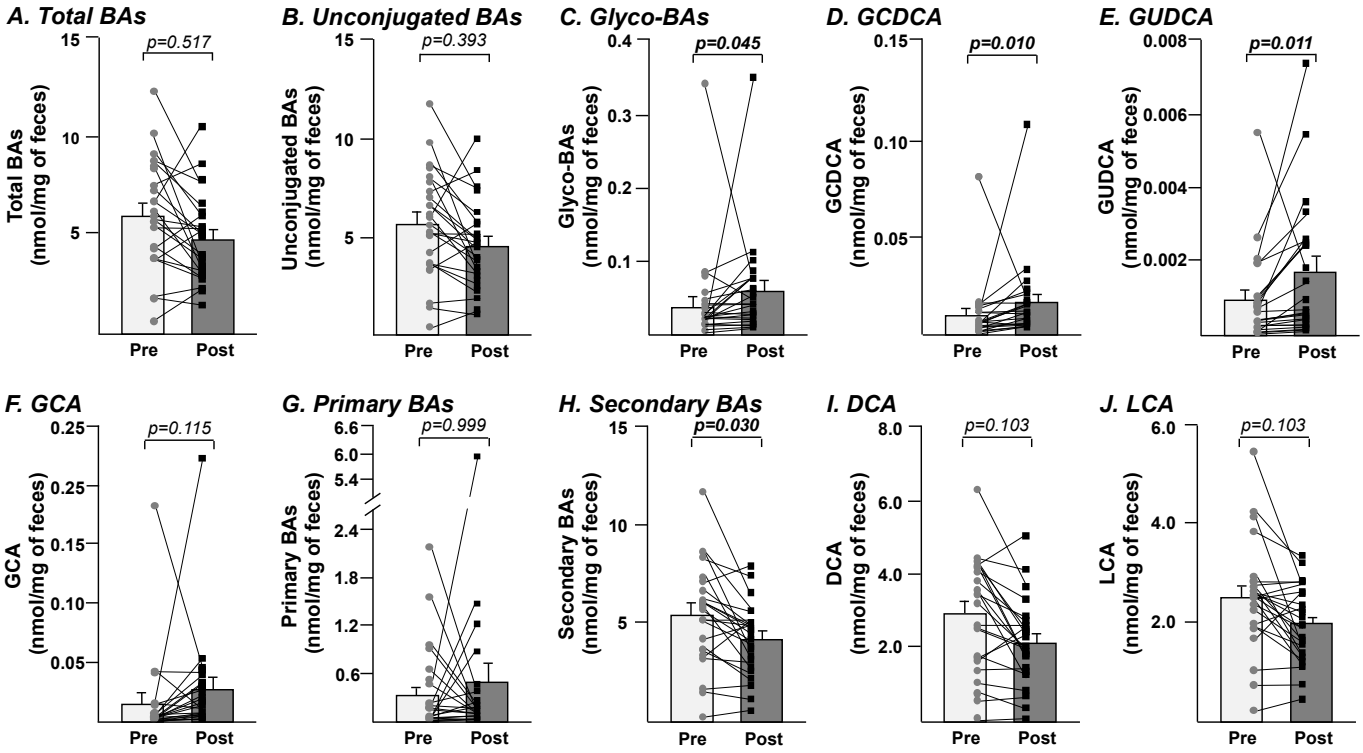

Figure 1. Fecal samples from the post-freeze-dried blueberry powder consumption period display significant accumulations of glycine-conjugated bile acids, GCDCA and GUDCA, while secondary bile acids levels are reduced. Bile acids from pre- and post-study samples were profiled using LC-MS/MS measurements, and bile acids composition analyses were performed as detailed in the materials and method section. Bars represent the mean±SEM of the pre- (light grey) and post-BBP (dark grey) concentrations of total bile acids (A), unconjugated bile acids (B), glycine-conjugated bile acids (C), GCDCA (D), GUDCA (E), GCA (F), primary (G) and secondary (H) bile acids, as well as DCA (I) and LCA (J). Individual values are plotted and linked for baseline (circle) and after 8-weeks treatment (square). Lines represent the specific trend for each individual. Statistical significance of the differences was assessed using Wilcoxon matched-pairs signed rank test and then adjusted for multiple comparisons using the Bonferroni-Dunn method and are expressed as adjusted p values. BAs; bile acids; glyco-BAs: glycine-conjugated bile acids; GCDCA: glycochenodeoxycholic acid; GUDCA: glycoursodeoxycholic acid; GCA: glycocholic acid; DCA: deoxycholic acid; LCA: lithocholic acid.

Overall, these observations indicate that BBP treatment significantly alters the fecal BA profile.

4. Discussion

In this pilot study, significant alterations of the human fecal bileacidome are detected after an 8-week consumption of BBP. The most significant changes corresponded to increased concentrations of glycine-conjugated BAs, and a decrease concentration of secondary BAs.

Profiling the pre-diet samples revealed that secondary acids such as the LCA and DCA dominate the fecal BA profile, which is in accordance with previous reports [23]. An important interindividual variability is observed both at baseline and at the end of the study for all BAs. This important inter-individual variability in fecal BA profiles is also consistent with previous studies in overweight patients at high risk for metabolic syndrome [24]. No significant sex-dependent differences were detected either in the pre- and post-BBP samples. While such differences were previously reported for the circulating BA profile [25], the present observations are consistent with the lack of study reporting that sex affects the fecal excretion of BAs [26]. On the other hand, parameters such as the changes in DCA and LCA levels tended to be reduced but failed to reach statistical significance, which is likely due to insufficient statistical power. It can be envisioned that a

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study with a larger number of volunteers might find significant differences in these parameters. Because BAs secreted from the liver sustain major changes by the intestinal microbiota [27], it can be envisioned that the above-mentioned changes in the fecal BA profile could reflect a modulation of the intestinal microbiota composition. Indeed, blueberries are particularly rich in anthocyanins, a subclass of flavonoid polyphenolic compounds with a characteristic 3-ring structure [28,29]. A recent meta-analysis showed that anthocyanins alter the ratio of two major Bacteria phyla present in the human microbiota (i.e Firmicutes and Bacterioidetes) [30]. Bacteria from these phyla are highly involved in the microbiota-dependent modifications of BAs in the intestine [31,32]. It can be hypothesized that the accumulation of glyco-BAs and the reduction of secondary acids can reflect an altered relative abundance of bacteria belonging to Firmicutes and Bacterioidetes species. Beyond the modification of microbiota composition, polyphenols can also stimulate BA excretion through the formation of complexes with amidated BAs (i.e. glycine- and taurine-conjugated acids) [33]. Thus, additional investigations are warranted to not only further ascertain the role of polyphenols in the changes detected in post-BBP samples, but also to decipher the mechanisms governing such changes.

A second plausible explanation for these changes relates to the high levels of dietary fibers present in blueberries [34]. Indeed, previous results from the same study revealed that consumption of BBP significantly increase daily dietary fibers consumed by the participants [18]. Dietary fibers enhance fecal BAs excretion (reviewed in [35]). While the underlying mechanisms beyond such enhancement remain to be fully elucidated, but a recent review article by Naumann & al. [34] proposed two potential mechanisms: 1) fibers may cause an increased viscosity of the intestinal content that interferes with the micellar properties of BAs; and 2) fibers may form hydrophobic interactions that complex BAs to plant compounds. Both mechanisms may increase the fecal BA content *via* the hydrophobic properties of specific BA species [34]. Glycine-conjugated BAs, such as GUDCA and GCDCA, which are significantly altered after the post-BBP diet, are among the most hydrophobic BAs [36].

While we cannot exclude the possibility that the reported changes in the BA profile could be also due to other secondary effects of the intervention, we are confident that alterations of the fecal bileacidome are actually reflecting the impact of the BBP consumption, since each individual was compared to its own baseline. In addition, all participants were carefully instructed not to change their usual lifestyle habits.

Beyond their role in cholesterol elimination and intestinal absorption, BAs also play numerous roles as endocrine regulators, but their amphipathic nature can have toxic impacts when they accumulate at high concentrations [4]. Thus, one can speculate that alteration of the bileacidome detected in this pilot study may translate into important effects for human health. For example, GCDCA promotes liver fibrosis in experimental models of hepatocellular cholestasis [37], and a blueberry-enriched diet could be viewed as a mean to reduce liver fibrosis in cholestatic patients. On the opposite, GUDCA has been proposed as being part of the mechanism by which metformin improves obesity-induced glucose intolerance and insulin resistance [8]. The post-BBP diet reduction of this acid may be considered as a potential drug-diet interference for metformin-treated patients. In the same vein, post-BBP samples tended to present lower levels of the secondary DCA and LCA acids. While LCA is a pro-inflammatory molecule in the intestine, some of its derivatives produced by bacteria exert important immunomodulating effects in the intestinal epithelium, and act as promoters of the intestinal barrier integrity *via* interactions with TGR5 [38]. A similar ambivalence also applies to DCA, the levels of which are associated to colon cancer, inflammation and cell proliferation, but which is a potent antibiotic protecting against the growth of opportunistic bacteria known to cause health problems such as *Clostridioides difficile* [39]. In sum, how the changes detected in the BA profiles after BBP consumption will have beneficial or deleterious consequences for human health is likely to be the subject of important inter-individual variability depending on the subject's health status.

In conclusion, results of this pilot study indicate that the fecal bileacidome of participants at high risk of metabolic syndrome was significantly influenced following an 8-week intake of freeze-dried BBP consumption. Both the mechanisms leading to such modifications and their consequences for human health require additional investigations with larger populations and specific study designs to be fully understood. Nevertheless, our work suggests that the consumption of blueberries could be considered as a potential mean to increase toxic BAs elimination.

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