Arctic berry extracts target the gut–liver axis to alleviate metabolic endotoxaemia, insulin resistance and hepatic steatosis in diet-induced obese mice

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Abstract

Aims/hypothesis There is growing evidence that fruit polyphenols exert beneficial effects on the metabolic syndrome, but the underlying mechanisms remain poorly understood. In the present study, we aimed to analyse the effects of polyphenolic extracts from five types of Arctic berries in a model of diet-induced obesity.

Methods Male C57BL/6 J mice were fed a high-fat/high-sucrose (HFHS) diet and orally treated with extracts of bog blueberry (BBE), cloudberry (CLE), crowberry (CRE), alpine bearberry (ABE), lingonberry (LGE) or vehicle (HFHS) for 8 weeks. An additional group of standard-chow-fed, vehicle-treated mice was included as a reference control for diet-induced obesity. OGTTs and insulin tolerance tests were conducted, and both plasma insulin and C-peptide were assessed throughout the OGTT. Quantitative PCR, western blot analysis and ELISAs were used to assess enterohepatic immunometabolic features. Faecal DNA was extracted and 16S rRNA gene-based analysis was used to profile the gut microbiota.

Results Treatment with CLE, ABE and LGE, but not with BBE or CRE, prevented both fasting hyperinsulinaemia (mean $\pm$ SEM [pmol/l]: chow 67.2 ± 12.3 , HFHS 153.9 ± 19.3 , BBE 114.4 ± 14.3 , CLE 82.5 ± 13.0 , CRE 152.3 ± 24.4 , ABE 90.6 ± 18.0 , LGE 95.4 ± 10.5) and postprandial hyperinsulinaemia (mean $\pm$ SEM AUC [pmol/l $\times$ min]: chow 14.3 ± 1.4 , HFHS 31.4 ± 3.1 , BBE 27.2 ± 4.0 , CLE 17.7 ± 2.2 , CRE 32.6 ± 6.3 , ABE 22.7 ± 18.0 , LGE 23.9 ± 2.5). None of the berry extracts affected C-peptide levels or body weight gain. Levels of hepatic serine phosphorylated Akt were 1.6-, 1.5- and 1.2-fold higher with CLE, ABE and LGE treatment, respectively, and hepatic carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 tyrosine phosphorylation was 0.6-, 0.7- and 0.9-fold increased in these mice vs vehicle-treated, HFHS-fed mice. These changes were associated with reduced liver triacylglycerol deposition, lower circulating endotoxins, alleviated hepatic and intestinal inflammation, and major gut microbial alterations (e.g. bloom of *Akkermansia muciniphila*, *Turicibacter* and *Oscillibacter*) in CLE-, ABE- and LGE-treated mice.

Conclusions/interpretation Our findings reveal novel mechanisms by which polyphenolic extracts from ABE, LGE and especially CLE target the gut–liver axis to protect diet-induced obese mice against metabolic endotoxaemia, insulin resistance and hepatic steatosis, which importantly improves hepatic insulin clearance. These results support the potential benefits of these

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Research in context

What is already known about this subject?

- Diets rich in fruits and vegetables are strongly associated with better health and reduced all-cause mortality
- Both animal and human studies support a role for the gut microbiota in the health benefits of dietary polyphenols

What is the key question?

- What mechanisms underlie the ability of dietary polyphenols to alleviate the metabolic syndrome?

What are the new findings?

- Treatment with extracts of cloudberry (CLE), alpine bearberry (ABE) and lingonberry (LGE), at nutritionally relevant doses, reduced fasting and postprandial hyperinsulinaemia without affecting fat-mass accretion in diet-induced obese mice
- CLE, ABE and LGE improved insulin sensitivity, prevented liver triacylglycerol deposition and enhanced hepatic insulin clearance in diet-induced obese mice
- CLE, ABE and LGE alleviated metabolic endotoxaemia and intestinal inflammation; these findings were linked to expansion of *Akkermansia muciniphila*, *Turicibacter* and *Oscillibacter* populations in the gut microbiota

How might this impact on clinical practice in the foreseeable future?

- Our findings support the benefits of CLE, ABE and LGE consumption and their integration into health programmes to help attenuate metabolic disorders, and point to the use of specific classes of polyphenols as promising strategies against the enterohepatic complications of the metabolic syndrome

Arctic berries and their integration into health programmes to help attenuate obesity-related chronic inflammation and metabolic disorders.

Data availability All raw sequences have been deposited in the public European Nucleotide Archive server under accession number PRJEB19783 (<https://www.ebi.ac.uk/ena/data/view/PRJEB19783>).

Keywords *Akkermansia muciniphila* · Arctic berries · CEACAM-1 · Gut microbiota · Insulin clearance

Abbreviations

ABE	Alpine bearberry extract
BBE	Bog blueberry extract
BCAA	Branched-chain amino acids
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule 1
CLE	Cloudberry extract
CRE	Crowberry extract
DIO	Diet-induced obesity
HFHS	High-fat/high-sucrose
IPITT	i.p. insulin tolerance test
KEGG	Kyoto Encyclopedia of Genes and Genomes
LGE	Lingonberry extract
PAC	Proanthocyanidin
SCFA	Short-chain fatty acids

Introduction

Obesity has reached pandemic proportions, increasing the rates of cardiovascular disease and type 2 diabetes [1].

Excessive accumulation of visceral fat promotes metabolic alterations that are triggered and sustained by a low-grade chronic inflammatory state [2]. While features of the metabolic syndrome have been associated with major taxonomic and functional changes in the gut microbiota [3, 4], diet-induced obesity (DIO) has been linked to gut-barrier disruption and the leakage of microbial-derived endotoxins into the circulation (i.e. metabolic endotoxaemia) [5], which contributes to the onset and progression of insulin resistance. Metabolic endotoxaemia also plays a role in the development of non-alcoholic fatty liver disease, which stems from the functional connection between the gut and the liver through the enterohepatic circulation [6].

There is compelling epidemiological evidence that diets rich in fruits and vegetables are strongly associated with better health and reduced all-cause mortality [7, 8]. While several studies using animal models have confirmed the positive impact of polyphenols on metabolic health [9], pharmacological doses with little nutritional relevance are often tested. Here, we analyse the effects of polyphenol-rich extracts of five types of Arctic berries (i.e. bog blueberry [BBE], cloudberry [CLE],

crowberry [CRE], alpine bearberry [ABE] and lingonberry [LGE]), at nutritionally relevant doses, on intestinal inflammation, metabolic endotoxaemia and features of metabolic syndrome in a murine model of DIO.

Metagenome-wide association studies have revealed that the consumption of fruits and other polyphenol-rich foods (e.g. dark chocolate, red wine, coffee, tea) are among the strongest factors explaining alterations in gut microbial communities in humans [10, 11]. Accordingly, we and others have previously reported that berry polyphenols have a marked impact on the gut microbiota [12–15]. In this study, we therefore applied 16S rRNA-based analysis in order to investigate the potential role of the gut microbiota in the effects of Arctic berry extracts.

Methods

Animals Eight-week-old C57BL/6 J male mice (Jackson, Bar Harbor, ME, USA) were bred in the animal facility of the Institute of Nutrition and Functional Foods (Québec, QC, Canada). Mice were housed two per cage in a controlled environment (12 h day/night cycle, lights off at 18:00) with food and water ad libitum. Mice were randomly divided into six groups ($n = 12$) and fed a high-fat/high-sucrose (HFHS) diet (see ESM Table 1). Treatment started concomitantly with the introduction of the HFHS diet and consisted of daily oral doses (200 mg powdered extract/kg body weight) of resuspended BBE (*Vaccinium uliginosum* L.), CLE (*Rubus chamaemorus* L.), CRE (*Empetrum nigrum* L.), ABE (*Arctostaphylos alpina* L. Spreng.) or LGE (*Vaccinium vitis-idaea* L.) or the vehicle used to resuspend these extracts (i.e. the drinking water of the animal facility) throughout 8 weeks. An additional group ($n = 12$) of standard-chow-fed (Teklad 2018, Envigo, Huntingdon, UK), vehicle-treated mice was included as a reference control for HFHS-induced obesity. Body weight gain and food intake were assessed twice a week. At week 8, animals were anaesthetised in chambers saturated with isoflurane and then euthanised by cardiac puncture. Tissues were harvested and blood was drawn in tubes containing 2 IU heparin and immediately centrifuged in order to

separate plasma from cells. All group assignments and outcome assessments carried out in this study were blinded. Absence of dermatitis, dehydration and weight gain were absolute inclusion criteria for animals. Non-immediate storage at -80°C and repetitive freeze–thaw cycles were exclusion criteria for samples. Values lower than $Q1 - 1.5 \times \text{IQR}$ or greater than $Q3 + 1.5 \times \text{IQR}$ were treated as potential outliers ($Q1$, first quartile; $Q3$, third quartile, IQR, interquartile range [$Q3 - Q1$]). None of these criteria led to the exclusion of animals, samples or data from this work. This study followed the *Guide for the care and use of laboratory animals* and all procedures had been previously approved by the Laval University Animal Ethics Committee.

Extracts of Arctic berries Bog blueberries, cloudberry, crowberries, alpine bearberries and lingonberries (refer to Table 1 for a complete list of names) were harvested in Nunavik (QC, Canada) and stored frozen (-20°C). After grinding, fresh-frozen berries underwent two extractions using 100% ethanol; the obtained solution was then filtered, rotoevaporated and freeze dried. The phenolic profiles of the extracts studied herein are described in Table 2. Harvest sites, along with more detailed phenolic profiles of the berries used in this study, have been published elsewhere [16].

Glucose homeostasis i.p. insulin tolerance tests (IPITTs) and OGTTs were performed as previously described [12]. Briefly, at week 6 mice were fasted for 6 h and insulin tolerance tests were carried out followed by i.p. insulin injections; at week 7, after overnight fasting (12 h), animals were given a glucose load (1 g/kg) and blood glucose was measured before (0 min) and after (15, 30, 60, 90 and 120 min) the glucose challenge. Blood samples were collected at each time point during OGTTs for determination of insulin and C-peptide levels.

Analytical methods Plasma insulin and C-peptide concentrations were measured using an ultra-sensitive ELISA kit (Alpco, Salem, NH, USA). Liver triacylglycerol was assessed after chloroform–methanol extraction and enzymatic reactions using a commercial kit (Randox Laboratories, Crumlin, UK). Plasma endotoxin (lipopolysaccharide) concentrations were

Table 1 The Arctic berries under investigation in this study

Scientific name	Inuktitut name	Common names
<i>Vaccinium uliginosum</i> L.	Kigutanginaq	Bog blueberry (BBE) ^a , bog bilberry, blueberry, alpine blueberry, northern blueberry
<i>Rubus chamaemorus</i> L.	Arpik	Cloudberry (CLE) ^a , bakeapple, knotberry, lowbush salmonberry, averin
<i>Empetrum nigrum</i> L.	Paurngaq	Crowberry (CRE) ^a , blackberry, black crowberry
<i>Arctostaphylos alpina</i> L. Spreng.	Kallaq	Alpine bearberry (ABE) ^a , bearberry, mountain bearberry, black bearberry
<i>Vaccinium vitis-idaea</i> L.	Kimminaq	Lingonberry (LGE) ^a , redberry, cowberry, mountain cranberry, partridgeberry, red whortleberry

^a Common names and abbreviations used in the manuscript

Table 2 Phenolic profiles of Arctic berry extracts

Daily dose ($\mu\text{g/kg}$ body weight) ^a	BBE	CLE	CRE	ABE	LGE
Total polyphenols	4061.7	3035.2	13,937.8	14,172.8	8478.6
Phenolic acids	118.0	239.7	372.9	321.8	448.4
Anthocyanins	1767.0	94.0	15,487.9	3589.4	3738.6
Flavonols	1712.4	528.8	837.8	797.8	10.5
PACs (condensed tannins)	708.8	170.0	2162.0	2994.2	4242.5
Monomers	147.7	57.5	442.6	1860.8	690.1
Dimers	266.2	103.9	906.2	721.2	1494.8
Trimers	125.8	8.5	220.6	173.2	723.7
Tetramers	73.9	–	182.7	89.4	479.7
Pentamers	41.5	–	69.2	51.4	311.5
Hexamers	30.6	–	40.6	38.3	236.6
Heptamers	11.4	–	18.9	15.0	96.5
Octamers	5.5	–	12.2	8.8	61.7
Nonamers	3.0	–	11.4	7.4	45.3
Decamers	–	–	2.9	1.3	10.1
Polymers	3.1	–	254.7	27.6	92.4
Ellagitannins (hydrolysable tannins)	–	538.7	–	523.1	–
Free ellagic acid	–	21.6	–	2.1	22.8

^a Calculated based on the amount in 200 mg extract/kg body weight

determined using a kit based on a reaction with *Limulus* amoebocyte lysate (QCL1000, Lonza, Allendale, NJ, USA), as previously described [12]. Plasma branched-chain amino acid (BCAA; leucine, isoleucine and valine) concentrations were determined using a colorimetric assay kit (BioVision, Milpitas, CA, USA). Faecal short-chain fatty acids (SCFA) and branched-chain SCFAs were assessed by gas chromatography as previously described [17]. Faecal pellets collected at week 6 were dried and gross energy density was determined using adiabatic bomb calorimetry (Parr Instruments, Moline, IL, USA). Plasma bile acids were assessed as previously described [18].

Western blot Analysis of total carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), total Akt, tyrosine-phosphorylated (p)-CEACAM-1 and phospho (p)-Akt (serine 473) was performed as previously described [19, 20]. Briefly, mice were injected (i.v.) with saline (154 mmol/l NaCl) or insulin (3.8 IU/kg) 5 min before euthanasia by cardiac puncture; aliquots of freeze-powdered livers were processed to yield total protein lysates and immunoblotted against anti-CEACAM-1 (1:1000; no. 3759, Beauchemin Lab, McGill University, Montréal, QC, Canada), anti-Akt (rabbit no. 9272S, 1:1000; Cell Signaling, Danvers, MA, USA) and anti-p-Akt serine 473 (rabbit no. 9271 L, 1:1000; Cell Signaling). CEACAM-1 was immunoprecipitated from total liver lysates and a cocktail of monoclonal anti-phosphotyrosine antibodies (rabbit no. 9411, 1:1000; Cell Signaling; and clone 4G10, 1:1000; Millipore, St. Louis,

MO, USA) was used to assess p-CEACAM-1. Anti-actin was used as the loading control (mouse sc-8432, 1:5000; Santa Cruz Biotechnology, Paso Robles, CA, USA).

mRNA quantification by real-time PCR Sections of jejunum and colon (approximately 0.5 cm) and freeze-powdered livers were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific, Haverhill, MA, USA) and total RNA was purified using an RNeasy Mini Kit (Qiagen, Frederick, MD, USA). After RNA reverse transcription, real-time PCR was performed using a SYBR Green Jump-Start Gene Expression Kit (Sigma-Aldrich, Oakville, ON, Canada) or with commercially available TaqMan primers and probe sets and TaqMan Master Mix (Life Technologies, San Diego, CA, USA). *Acaca*, *Adgre1*, *Cd11c* (also known as *Itgax*), *Cox2*, *Gcg*, *Il1b*, *Klf4*, *Marco*, *Nos2*, *Ocln*, *Ppara*, *Reg3g*, *Tjp1*, *Tlr4* and *Tnfa* gene expression was assessed using the $\Delta\Delta C_t$ method and *Actb* or *Hprt* were used as reference genes. Primer sequences are shown in ESM Table 2.

Bacterial genomic DNA extraction and 16S rRNA gene-based gut microbial analysis Faecal samples were freshly collected at week 8 and immediately stored at -80°C . Bacterial genomic DNA was extracted as previously described [12]. 16S rRNA gene-based profiling of the faecal microbiota was carried out as previously described [21]. All raw sequences were deposited in the public European Nucleotide Archive server under accession number PRJEB19783 (<https://www.ebi.ac.uk/ena/data/view/PRJEB19783>).

Functional prediction of gut bacterial communities

Prediction of functional genes was performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, available at <http://picrust.github.io/picrust>). Briefly, from 16S rRNA sequencing data and from the Kyoto Encyclopedia of Genes and Genomes (KEGG) reference genomes (www.genome.jp/kegg), PICRUSt was used to estimate gene-family abundance based on an extended ancestral-state reconstruction algorithm and then to produce a predicted metagenome functional content for each sample. This software was also used to determine the contribution of each Operational Taxonomic Unit (OTU) to a given KEGG pathway.

Statistical analysis Data are expressed as means $\pm$ SEM. One-way ANOVA with a post hoc Student–Newman–Keuls test and unpaired Student's *t* test were used to assign significance to the comparisons between HFHS-fed controls vs Arctic berry-treated groups and chow- vs HFHS-fed controls, respectively (GraphPad, La Jolla, CA, USA). Time points within different groups were compared using two-way repeated measures ANOVA with a Student–Newman–Keuls post-test (SigmaPlot, San Jose, CA, USA). All results were considered statistically significant at $p < 0.05$.

A weighted UniFrac distance matrix was calculated at the genus level based on taxa having at least 1% of total relative abundance. Principal coordinates analysis was performed on the resulting distance matrix using the ‘phyloseq’ R package (version 1.16.2, available at <https://www.r-project.org>). The statistical significance of differentially abundant and biologically relevant taxonomical and functional biomarkers among groups was measured using a linear discriminant analysis effect size with a threshold of 2 or 2.5. A *p* value of < 0.05 was considered to indicate statistical significance for the factorial Kruskal–Wallis rank-sum test.

Results

Administration of the Arctic berry extracts had no impact on HFHS-induced weight gain, fat-mass accretion (Table 3) or elevated fasting blood glucose (Fig. 1a). However, CLE-, ABE- and LGE-treated mice had lower plasma insulin levels than vehicle-treated, HFHS-fed controls (Fig. 1b; mean $\pm$ SEM [pmol/l]: chow 67.2 ± 12.3 , HFHS 153.9 ± 19.3 , BBE 114.4 ± 14.3 , CLE 82.5 ± 13.0 , CRE 152.3 ± 24.4 , ABE 90.6 ± 18.0 , LGE 95.4 ± 10.5). The glucose-lowering effect of insulin measured during IPITTs was improved at the 10 and 90 min time points in CLE- and LGE-treated mice as compared with vehicle-treated animals, indicating an insulin sensitising effect (Fig. 1). C-peptide levels were increased by HFHS feeding but not affected by any of the berry extracts (Fig. 1c), suggesting that changes in insulin clearance, and not in insulin secretion, may underlie the reduced fasting hyperinsulinaemia in CLE-, ABE- and LGE-treated mice.

We next sought to investigate the dynamic glycaemic/insulinaemic responses. None of the extracts improved HFHS-induced glucose intolerance, and blood glucose was slightly higher in mice treated with ABE (90 min time point) and LGE (90 and 120 time points) post-glucose challenge (Fig. 2a), although this was not enough to promote glucose intolerance based on glucose AUCs (Fig. 2a insert). Plasma insulin during OGTT in CLE-treated mice was lower than in vehicle-treated, HFHS-fed control mice, reaching the level of significance at the 15 and 30 min time points and also when calculating the total insulin AUC. ABE (30 and 90 min) and LGE (30 and 120 min) treatments also lowered insulin levels at specific time points, although this was not enough to significantly impact the overall insulin AUCs (Fig. 2b; mean $\pm$ SEM AUC [pmol/l $\times$ min]: chow 14.3 ± 1.4 , HFHS 31.4 ± 3.1 , BBE 27.2 ± 4.0 , CLE 17.7 ± 2.2 , CRE 32.6 ± 6.3 , ABE 22.7 ± 18.0 , LGE 23.9 ± 2.5). Similarly to in the fasting state, there were no differences in glucose-induced insulin secretion as revealed by C-peptide responses during OGTT (Fig. 2c), again suggesting that these three berry extracts (ABE, CLE

Table 3 Body characteristics after administration of Arctic berry extracts for 8 weeks

Characteristic	Chow	HFHS	BBE	CLE	CRE	ABE	LGE
Total weight gain (g)	5.08 $\pm$ 0.46	9.45 $\pm$ 0.43 ^{†††}	9.21 $\pm$ 0.64	9.1 $\pm$ 1.05	8.25 $\pm$ 1.04	7.98 $\pm$ 0.74	8.15 $\pm$ 0.75
Total energy intake (kJ)	1671.0 $\pm$ 234.09	1687.4 $\pm$ 138.2	1710.8 $\pm$ 258.1	1703.7 $\pm$ 301.1	1524.2 $\pm$ 352.4	1667.3 $\pm$ 321.5	1336.7 $\pm$ 78.5
Visceral fat pad (g)	0.96 $\pm$ 0.08	2.08 $\pm$ 0.22 ^{†††}	2.07 $\pm$ 0.2	1.73 $\pm$ 0.14	1.97 $\pm$ 0.14	1.67 $\pm$ 0.20	1.83 $\pm$ 0.11
Subcutaneous fat pad (g)	0.40 $\pm$ 0.03	0.75 $\pm$ 0.07 ^{†††}	0.82 $\pm$ 0.08	0.84 $\pm$ 0.13	0.91 $\pm$ 0.11	0.72 $\pm$ 0.08	0.80 $\pm$ 0.10
Interscapular brown fat pad (g)	0.18 $\pm$ 0.07	0.17 $\pm$ 0.05	0.11 $\pm$ 0.08	0.105 $\pm$ 0.05	0.110 $\pm$ 0.07	0.105 $\pm$ 0.07	0.106 $\pm$ 0.08
Gastrocnemius (g)	0.32 $\pm$ 0.01	0.32 $\pm$ 0.08	0.32 $\pm$ 0.07	0.31 $\pm$ 0.08	0.30 $\pm$ 0.07	0.310 $\pm$ 0.07	0.31 $\pm$ 0.01
Liver (g)	1.11 $\pm$ 0.04	1.03 $\pm$ 0.04	1.04 $\pm$ 0.04	0.99 $\pm$ 0.04	0.97 $\pm$ 0.04	0.97 $\pm$ 0.04	0.95 $\pm$ 0.04

Data are expressed as means $\pm$ SEM

^{†††} $p < 0.001$ vs chow-fed mice

Fig. 1 Impact of Arctic berry extracts on fasting blood glucose, plasma insulin and plasma C-peptide and on insulin sensitivity in DIO mice. **(a)** Fasting blood glucose ($n = 12$), **(b)** plasma insulin ($n = 12$) and **(c)** C-peptide ($n = 9$ for chow, BBE, CRE and ABE; $n = 10$ for HFHS, CLE and LGE). Chow-fed animals are represented by a reference line and the significance between chow and HFHS was calculated using an unpaired two-tailed Student's t test ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$). One-way ANOVA with a Student–Newman–Keuls post hoc test was used to calculate the significance between HFHS and the groups treated with Arctic berry extracts ($^*p < 0.05$, $^{**}p < 0.01$). **(d)** IPITT (0.65 IU/kg, $n = 12$). Two-way repeated measures ANOVA with a Student–Newman–Keuls post hoc test was used to calculate the significance of the differences between time points ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.001$ for chow vs HFHS; $^{**}p < 0.01$ for CLE-treated groups vs HFHS; $^{**}p < 0.01$ for LGE-treated groups vs HFHS). Data are expressed as means $\pm$ SEM

and LGE), especially CLE, reduce hyperinsulinaemia through an effect on insulin clearance rather than on insulin secretion. Consistent with improved hepatic insulin sensitivity, we found a 1.6-, 1.5- and 1.2-fold increase in Akt phosphorylation at serine 473 in the livers of CLE-, ABE- and LGE-treated mice, respectively, compared with vehicle-treated HFHS-fed mice (Fig. 2d).

Tyrosine phosphorylation of CEACAM-1 is a key event involved in receptor-mediated insulin endocytosis and degradation in the hepatocyte [22] and a major pathway for hepatic insulin clearance in vivo [19, 20]. Despite no changes in total CEACAM-1 content (Fig. 2e), mice treated with CLE, ABE and LGE displayed, respectively, 0.6-, 0.7- and 0.9-fold more tyrosine-phosphorylated CEACAM-1 in comparison with

HFHS-fed controls (Fig. 2f). Taken together, our findings suggest that these three berry extracts prevented DIO-induced hyperinsulinaemia by protecting the liver from the detrimental consequences of HFHS feeding, therefore improving the hepatic capacity to clear insulin from the circulation.

Treatment with CLE, ABE and LGE significantly reduced HFHS-induced hepatic triacylglycerol accumulation and triacylglycerolaemia in comparison with vehicle-treated, HFHS-fed controls (Fig. 2g,h). These findings suggest that increased lipid oxidation in the liver, rather than decreased lipid uptake, is an important driver of the benefits of CLE, ABE and LGE on liver homeostasis. Consistent with increased lipid catabolism, the mRNA expression of *Ppara* and *Acaca* (acetyl-CoA carboxylase) was higher and lower, respectively, in the livers of CLE-, ABE- and LGE-treated mice versus HFHS-fed controls (Fig. 2i).

To investigate the inflammatory profile in the livers of CLE-, ABE- and LGE-treated mice, we measured the mRNA expression of several genes involved in immune regulation. While the mRNA expression of *Adgre1* (which encodes F4/80) and *Cd11c* (both markers of macrophages) was unchanged, CLE, ABE and LGE treatment markedly down-regulated *Marco* and upregulated *Klf4* mRNA expression (Fig. 2i). Since *Marco* and *Klf4* are markers of polarisation towards M1 and M2 populations [23, 24], respectively, these findings suggest that these three extracts did not alter the number of macrophages but triggered their polarisation towards a less proinflammatory activity. Accordingly, CLE-, ABE- and LGE-treated mice expressed lower levels of *Il1b* transcripts and non-significantly expressed less *Tnfa* than vehicle-treated, HFHS-fed mice (Fig. 2i). *Cox2* mRNA expression was not altered in CLE-, ABE- and LGE-treated mice, but since HFHS-fed mice did not show higher *Cox2* mRNA expression than chow-fed mice, this suggests that this enzyme is not particularly regulated at the transcriptional level in our model. The mRNA expression of *Tlr4* was lower in CLE-, ABE- and LGE-treated mice when compared with vehicle-treated, HFHS-fed mice (Fig. 2i), suggesting that a reduction in the activation of the hepatic innate immune response by microbial-associated molecular patterns contributes to alleviating inflammation in the liver of CLE-, ABE- and LGE-treated mice.

Consistent with our previous observations, CLE, ABE and LGE administration significantly blunted metabolic endotoxaemia (Fig. 3a), suggesting improved intestinal barrier integrity. The mRNA expression of *Nos2* (encoding inducible nitric oxide synthase), was decreased in the jejunum of CLE- and LGE-treated mice and was non-significantly lower in ABE-treated mice as compared with HFHS-fed controls (Fig. 3b). In the colon, the HFHS-induced increase in *Nos2* expression was significantly prevented by CLE, ABE and LGE treatment (Fig. 3j). The mRNA expression of *Tnfa* was decreased in the jejunum of LGE-treated mice (Fig. 3c) and in

Fig. 2 Impact of Arctic berry extracts on postprandial blood glucose, plasma insulin and C-peptide, and liver homeostasis in DIO mice. **(a)** OGTTs ($n = 12$). **(b, c)** Blood collected during the OGTT was used to assess insulin levels ($n = 12$) and C-peptide ($n = 9$ for chow, BBE, CRE and ABE; $n = 10$ for HFHS, CLE and LGE) before and after glucose challenge. **(a–c)** Two-way repeated measures ANOVA with a Student–Newman–Keuls post hoc test was used to calculate the significance of the differences between time points ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for chow vs HFHS; $^{*}p < 0.05$, $^{**}p < 0.01$ for LGE-treated groups vs HFHS; $^{*}p < 0.05$, $^{**}p < 0.01$ for ABE-treated groups vs HFHS; $^{§}p < 0.05$, $^{§§}p < 0.01$ for CLE-treated groups vs HFHS). In the inset bar charts, chow-fed animals are represented by a reference line and the significance between chow- and HFHS-fed mice was calculated using an unpaired two-tailed Student’s t test ($^{\dagger\dagger\dagger}p < 0.001$). **(d)** Liver Akt ($n =$

12), p-Akt^{ser473} ($n = 12$), **(e)** CEACAM-1 (IB, immunoblotting, $n = 12$) and **(f)** p-CEACAM-1 tyrosine (IP, immunoprecipitation, $n = 9$ for chow; $n = 11$ for HFHS and LGE; $n = 12$ for CLE and ABE). **(g)** Liver triacylglycerol, **(h)** Plasma triacylglycerol and **(i)** Liver mRNA expression of *Adgre1*, *Cd11c*, *Marco*, *Klf4*, *Tnfr*, *Il1b*, *Cox2*, *Tlr4*, *Ppara* and *Acaca* at week 8. White bars, HFHS-fed control mice; black bars, CLE; grey bars, ABE; light grey bars, LGE. **(d–i)** Chow-fed animals are represented by a reference line and the significance between chow- and HFHS-fed mice was calculated using an unpaired two-tailed Student’s t test ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$). One-way ANOVA with a Student–Newman–Keuls post hoc test was used to calculate the significance between HFHS-fed mice and the groups treated with Arctic berry extracts ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

the colon of CLE-, ABE- and LGE-treated mice (Fig. 3k) vs vehicle-treated, HFHS-fed controls. We found lower mRNA expression of *Adgre1* in the jejunum and a non-significant

reduction in the colon of CLE-, ABE- and LGE-treated mice (Fig. 3d,l). Similarly, the mRNA expression of *Cd11c* (also present in macrophages) was non-significantly reduced in the

jejunum and significantly reduced in the colon of CLE-, ABE- and LGE-treated mice (Fig. 3e,m). No significant changes in the mRNA expression of *Tjp1* and *Ocln* were seen in the jejunum or colon (Fig. 3f,g,n,o), although a non-significant increase in *Tjp1* gene expression was seen in the colon of CLE-, ABE- and LGE-treated mice (Fig. 3n). Furthermore, while CLE, but not ABE or LGE, treatment prevented the

Fig. 3 CLE, ABE and LGE improve intestinal inflammation, gut permeability and metabolic endotoxaemia in DIO mice. (a) *Limulus* amoebocyte lysate-based assay was used to assess circulating lipopolysaccharides/endotoxins. Jejunal mRNA expression of (b) *Nos2*, (c) *Tnfa*, (d) *Adgre1*, (e) *Cd11c*, (f) *Tjp1*, (g) *Ocln*, (h) *Reg3g* and (i) *Gcg*. Colonic mRNA expression of (j) *Nos2*, (k) *Tnfa*, (l) *Adgre1*, (m) *Cd11c*, (n) *Tjp1*, (o) *Ocln*, (p) *Reg3g* and (q) *Gcg*. Data are expressed as means $\pm$ SEM. Chow-fed animals are represented by a reference line and the significance between chow and HFHS was calculated using unpaired two-tailed Student's *t* test ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$). One-way ANOVA with a Student–Newman–Keuls post hoc test was used to calculate the significance between HFHS and the groups treated with Arctic berry extracts ($^*p < 0.05$)

HFHS-induced decrease in the mRNA expression of the anti-microbial peptide *Reg3g* at both sites (Fig. 3h,p), the mRNA expression of the proglucagon gene *Gcg* was not altered by any of the extracts (Fig. 3i,q).

We next explored the impact of CLE, ABE and LGE administration on the gut–liver axis by applying 16S rRNA gene-based analysis of faecal DNA samples. Principal coordinates analysis on weighted UniFrac distances showed a separation between the gut microbiota of chow- and HFHS-fed mice (Fig. 4a). The microbial communities of CLE-, ABE- and, to a lesser extent, LGE-treated mice clustered apart from those of HFHS-fed controls (Fig. 4a), indicating altered beta-diversity in CLE-, ABE- and LGE-treated mice vs vehicle-treated, HFHS-fed mice. The Firmicutes to Bacteroidetes ratio was drastically increased in HFHS- vs chow-fed mice, whereas CLE, ABE and LGE administration attenuated this increase (ESM Table 3). The gut microbial communities of CLE-treated mice were discriminated from those of HFHS-fed control mice by an increased presence of Peptostreptococcaceae, *Akkermansia muciniphila* and *Turicibacter*, and by a lower representation of taxa assigned to the genera *Lactobacillus* and *Bifidobacterium* in the faecal microbiota (Fig. 4c). Overrepresentation of *Oscillibacter* and *A. muciniphila* were identified as the main features discriminating ABE from vehicle-treated, HFHS-fed mice metagenomes (Fig. 4d), whereas an increased presence of *Oscillibacter* and *Turicibacter* were characteristic gut microbial features of LGE-treated mice vs vehicle-treated, HFHS-fed mice (Fig. 4e). Relative abundances are described in ESM Tables 3–7.

We applied the PICRUSt method to predict functional alterations in the gut microbiome of HFHS-fed mice treated with CLE, ABE and LGE. As previously reported [14, 25], functions related to cell motility (i.e. bacterial motility, flagellar assembly, bacterial chemotaxis) were more represented in the gut microbiota of HFHS-fed mice than in chow-fed mice (Fig. 5a). Our analysis revealed an overall increase in functional pathways related to cofactor and vitamin metabolism (e.g. folate, riboflavin and biotin metabolism) and metabolism of terpenoids (e.g. limonene, pinene and geraniol degradation) in CLE-, ABE- and LGE-treated mice vs HFHS-fed controls

Fig. 4 CLE, ABE and LGE administration is associated with changes in the gut microbial profile of DIO mice. Faecal samples of chow-fed ($n = 9$) and HFHS-fed ($n = 11$) mice, as well as of HFHS-fed mice orally treated with ABE ($n = 4$), CLE ($n = 6$) and LGE ($n = 7$), were freshly harvested at week 8. Genomic DNA was extracted from faeces and 16S rRNA-based analysis profiling was performed. **(a)** Beta-diversity between groups was initially observed by means of principal coordinates analysis (PCoA) on weighted UniFrac distance. The linear discriminant analysis (LDA) effect size was calculated in order to explore the taxa within genus levels that more strongly discriminated between the gut microbiota of mice fed with **(b)** chow and HFHS, **(c)** HFHS and CLE, **(d)** HFHS and ABE and **(e)** HFHS and LGE. Families followed by the label ‘_g’ indicate unidentified genera. Dark grey bars, chow; white bars, HFHS-fed control mice bars; black bars, CLE; medium grey bars, ABE; light grey bars, LGE

(Fig. 5b–d). Increased amino acid, fatty acid, carbohydrate (e.g. tricarboxylic acid cycle) and energy metabolism functions were overrepresented in the gut microbiota of CLE-, ABE- and LGE-treated mice (Fig. 5b–d). Microbial pathways assigned to valine, isoleucine and leucine (BCAA) degradation were increased in CLE- and ABE-treated mice vs vehicle-treated, HFHS-fed animals (Fig. 5b–d). Altogether, the PICRUST analysis highlights important adaptations in the intestinal microbiome in response to phytochemicals and points

to a general impact of CLE, ABE and LGE administration on bacterial substrate utilisation.

We next assessed key metabolome components involved in the gut microbiota–host interaction. None of the extracts significantly affected the profile or the total amount of faecal SCFA (ESM Fig. 1a) or plasma bile acids (ESM Fig. 2). Interestingly, faecal energy density was found to be lower in CLE-, ABE- and LGE-treated mice vs vehicle-treated, HFHS-fed controls (ESM Fig. 1c). Moreover, the concentration of

Fig. 5 CLE, ABE and LGE alter metabolic pathways in the gut microbiota of DIO mice. Prediction of the functional genes in the sampled bacterial community was performed using PICRUSt. 16S rRNA sequencing data and KEGG reference genomes were used to estimate gene-family abundance based on an extended ancestral-state reconstruction algorithm and then to produce a metagenome prediction for each sample. The linear

discriminant analysis (LDA) effect size was calculated in order to explore the microbial functions that more strongly discriminate between the gut microbiota of mice fed with (a) chow and HFHS, (b) HFHS and CLE, (c) HFHS and ABE, (d) HFHS and LGE. Horizontal stripes, chow; white, HFHS-fed control mice; black, CLE; dark grey, ABE; light grey, LGE

branched-chain SCFA, which are by-products of microbial BCAA degradation, were non-significantly higher in the faeces of CLE-treated mice (ESM Fig. 1b), indicating lower BCAA availability to the host. We therefore assessed the circulating levels of BCAA and found a non-significant ($p = 0.06$) decrease in circulating BCAA in CLE-treated vs vehicle-treated, HFHS-fed mice (ESM Fig. 1d).

Discussion

Our work shows that the daily administration of CLE, ABE and LGE alleviates HFHS-induced intestinal inflammation and metabolic endotoxaemia in DIO mice. This was found to be independent of an anti-obesity effect and was associated with improved liver function, ameliorated hepatic insulin

sensitivity and attenuated hyperinsulinaemia. Our data also provide evidence of an adaptive response of intestinal bacterial communities to the presence of CLE, ABE and LGE, leading to altered taxonomic and functional profiles.

In line with our findings, the administration of LGE to DIO mice has been previously associated with attenuated hyperinsulinaemia, reduced liver steatosis [26, 27], alleviated circulating lipopolysaccharide-binding protein and major taxonomic and functional alterations in the gut microbiota, such as bloom of *A. muciniphila* and increased microbial pathways linked to substrate utilisation [14]. While in this latter study treatment with LGE has been shown to prevent high-fat DIO [14], in our model LGE did not affect fat-mass accretion. This is possibly explained by three major factors: (1) differences in the composition of the extracts; (2) differences in the diet used; and (3) the mode of administration of the extracts (gavage vs mixing the extract into the diet). Interestingly, the same group has shown that LGE extracts from different batches exert distinct effects on body-fat accumulation [14]; in agreement with our results, the batch that did not affect obesity still improved hepatic steatosis, liver inflammation and plasma lipopolysaccharide-binding protein levels [14]. Importantly, our study further demonstrates that the obesity-independent benefits of LGE involve better hepatic insulin sensitivity, improved insulin clearance and alleviated metabolic endotoxaemia.

By assessing C-peptide secretion and hepatic CEACAM-1 activation, our study provides novel mechanistic insights into the metabolic benefits of LGE, CLE and ABE, with improved hepatic insulin clearance likely to be the key mechanism underlying lower hyperinsulinaemia in this study. As liver-specific CEACAM-1 overexpression in HFHS-fed mice was shown to prevent hyperinsulinaemia, insulin resistance and hepatic lipid accumulation by increasing hepatic β -oxidation [28], it is possible that CLE, ABE and LGE target CEACAM-1 to improve both insulin clearance and hepatic steatosis. It is also conceivable that alleviation of HFHS-induced intestinal inflammation and lipopolysaccharide leakage in CLE-, ABE- and LGE-treated mice contributes to reduce the proinflammatory load reaching the liver, and to therefore improve hepatic metabolism. While CLE, ABE and LGE administration were not associated with improved glucose tolerance, as determined from an OGTT, the glucose-induced insulinaemic responses were reduced and accompanied by increased hepatic insulin clearance, whereas pancreatic insulin secretion remained unchanged. It is possible that an 8-week long treatment with CLE, ABE and LGE exerts beneficial effects that are more restricted to the gut–liver axis, resulting in a milder impact on the ability of muscles to take up glucose while predominantly improving hepatic insulin resistance and limiting chronic hyperinsulinaemia. This is in line with the concept that hyperinsulinaemia is an early event leading to type 2 diabetes [29].

Our results clearly indicate that the effectiveness of polyphenolic extracts against features of the metabolic syndrome varies considerably. Ellagitannins were present only in CLE (538.7 $\mu\text{g/kg}$) and ABE (523.1 $\mu\text{g/kg}$), while low amounts of free ellagic acid were present in CLE (21.6 $\mu\text{g/kg}$), ABE (2.1 $\mu\text{g/kg}$) and LGE (22.8 $\mu\text{g/kg}$). The bioactivity of ellagic acid and its hydrolysable polymeric form (i.e. ellagitannins) is dependent on gut microbial hydrolysis of ellagitannins (yielding ellagic acid) and processing of ellagic acid into urolithins [30]. While urolithin A has been implicated in mitophagy and a prolonged lifespan in *Caenorhabditis elegans* [31], ellagic acid administration has been shown to improve metabolic health in HFHS-fed rats [32]. LGE is particularly rich in proanthocyanidins (PACs), with a degree of polymerisation ranging from 2 to 10, and in polymeric PACs (degree of polymerisation >10). PACs are poorly bioavailable and have been linked to major gut microbial changes, blunted gut inflammation and enhanced metabolism in obese mice [12, 33]. However, other subclasses of polyphenols, non-flavonoid secondary metabolites, vitamins, minerals and soluble fibres were also found to be present in the Arctic berry extracts and may potentially account for the effects seen.

A. muciniphila is a Gram-negative, mucin-degrading bacterium that has been highly associated with a better health status [11], causally implicated in the improvement of diet-induced insulin resistance [34, 35] and closely associated with the intake of polyphenol-rich fruit extracts [36]. *A. muciniphila* uses ellagic acid as a substrate [37], which may partially explain the higher presence of *A. muciniphila* in the gut microbiota of CLE- and ABE-treated mice. The effect of PACs on *A. muciniphila* still warrants further investigation, but it has been hypothesised to be a response to a PAC-stimulated increase in mucus production, which creates a propitious environment for *A. muciniphila* to thrive [36]. CLE-, but not ABE and LGE-treated mice, showed a marked upregulation of *Reg3g* mRNA expression in both the jejunum and colon. REG3 γ (regenerating islet-derived 3 γ) is an antimicrobial peptide and an important component of the intestinal barrier. In agreement with previous works showing a tight relationship between *A. muciniphila* and the expression of *Reg3g* [34], CLE-treated mice in the current study displayed the highest abundance of this bacterium (ESM Table 7). However, since *A. muciniphila* is not a major coloniser in the jejunum, CLE may enhance REG3 γ secretion in the proximal bowel probably by mechanisms independent of *A. muciniphila*. It is noteworthy that as ABE- and LGE-treated mice showed improved gut barrier, as suggested by lower endotoxaemia in these mice, it is plausible that factors beyond REG3 γ play a part in the benefits of ABE and LGE on intestinal homeostasis.

None of the extracts affected the profile or the total amount of faecal SCFA and plasma bile acids. While our results suggest that the effects of CLE, ABE and

LGE on the gut microbiota and on host physiology are independent of major alterations in the synthesis of SCFA and in the bile acid profile, we cannot rule out a relevance of these molecules to the phenotype of CLE-, ABE- and LGE-treated mice. Further analysis of SCFA in plasma and tissues (e.g. liver) and a broader profile of bile acids (faeces, gallbladder and plasma) are warranted.

Lower energy density in the faeces of CLE-, ABE- and LGE-treated mice coupled with PICRUST analysis suggest increased bacterial amino acid, fatty acid and carbohydrate utilisation. However, gut transit was not taken into account in our study and is a key variable in the energy excretion equation. Importantly, since none of the treatments significantly affected weight gain and there were no differences in energy intake among the groups, it is reasonable to conclude that bacterial use of dietary substrates was not modified to an extent that impacted energy availability to the host.

Microbial BCAA degradation was predicted to be over-represented in CLE- and ABE-treated mice and similar results have been reported in rats supplemented with lowbush blueberries (*Vaccinium angustifolium*) [38]. Further analysis in the current study revealed a non-significant reduction in BCAA availability to the host in CLE-treated mice. This is of particular interest because increased circulating BCAA levels have been established as an early biomarker of insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease [39–41]. While our study does not provide definitive proof that reduced BCAA availability to the host contributes to improved insulin sensitivity and hepatic homeostasis in CLE-treated mice, the results call attention to this promising line of investigation. For instance, our analysis revealed that *Akkermansia*, *Peptostreptococcaceae* and *Oscillospira* together accounted for more than 50% of the predicted increase in microbial BCAA degradation in CLE-treated mice (ESM Fig. 3), meaning that these constitute promising targets for future studies linking dietary polyphenols, BCAA bioavailability and insulin resistance.

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Data availability All raw sequences have been deposited in the public European Nucleotide Archive server under accession number PRJEB19783 (available at <https://www.ebi.ac.uk/ena/data/view/PRJEB19783>). The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Contribution statement This study was conceived by AM, MLe, MLu and CSH, and designed by FFA, AM and GP. FFA, TV, MLe and SD performed the experiments. All authors were involved in the analysis and discussion of the data. FFA wrote the manuscript. All authors reviewed and approved the final manuscript. AM and FFA are responsible for the integrity of the work as a whole.

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