

Anthocyanin-enriched bilberry extract attenuates glycaemic response in overweight volunteers without changes in insulin



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ABSTRACT

This study investigated the effect of a three week supplementation of bilberry extract on glucose metabolism. Overweight volunteers (n = 16; BMI 31) were given three capsules of either 0.47 g bilberry extract (36% (w/w) anthocyanins) or placebo per day for three weeks in a double blinded cross over intervention.

The ingestion of the bilberry extract decreased the OGTT AUCi for glucose (20%; p = 0.008) but not for insulin compared with the placebo. There was no change in anti-inflammatory markers (hsCRP, MCP-1, leptin), vascular health markers (Apo A1, sVCAM-1, sICAM-1), reducing potential/ radical scavenging capacity or faecal bacterial microbiota. In vitro studies showed that the bilberry extract decreased α -amylase activity (p < 0.0001), α -glucosidase activity (p < 0.0001) and glucose uptake (p < 0.0001).

This study demonstrates that the ingestion of a bilberry extract over a three week intervention period reduces postprandial glycaemia without changes in insulin. This is probably due to reduced rates of carbohydrate digestion and/or absorption.

1. Introduction

Dietary strategies for alleviating health complications, such as premature vascular disease, associated with type 2 diabetes (T2D) and obesity are actively being pursued as alternatives to pharmaceutical interventions (Ceriello & Testa, 2009). Such a strategy could complement other key lifestyle interventions such as avoiding being overweight and the need to take regular exercise. The genus *Vaccinium* (e.g. blueberry, bilberry, cranberry) has been used traditionally as a source of folk remedies for established diabetic symptoms.

Berries from this genus are enriched in anthocyanins, a group of polyphenols recognized for their ability to provide and activate cellular

antioxidant protection and inhibit inflammatory gene expression (Neto, 2007; Tsuda, 2008; Zafra-Stone et al., 2007), activities that may contribute to the efficacy of the *Vaccinium* genus as ameliorators for type 2 diabetes. For example consumption of a freeze-dried blueberry beverage for an 8 week period decreased plasma concentrations of the cardiovascular risk factors oxidized LDL cholesterol, malondialdehyde and hydroxynonenal (Basu et al., 2010). In another trial, blueberries improved insulin sensitivity in obese insulin-resistant men and women (Stull, Cash, Johnson, Champagne, & Cefalu, 2010). In both these studies the investigators reported no change in inflammatory markers following supplementation. However another study with bilberry juice was shown to decrease plasma markers of inflammation C-reactive

Abbreviations: T2D, type 2 diabetes; BMI, body mass index; OGTT, oral glucose tolerance test; w/w, weight for weight; AUCi, incremental area under the curve; MCP-1, monocyte chemoattractant protein-1; TEAC, trolox equivalent antioxidant capacity; FRAP, fluorescence recovery after photobleaching; LDL, low-density lipoprotein; IL-6, interleukin 6; COPD, chronic obstructive pulmonary disease; NOSRES, north of Scotland research ethics; HOMA, homeostasis model assessment; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; NEFAs, non-esterified fatty acids; SD, standard deviation; GI, gastrointestinal; SGLT1, sodium glucose co-transporter 1; hsCRP, high sensitivity C-Reactive Protein; ApoA1, Apolipoprotein A1; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intracellular adhesion molecule-1; HOMA-IR, Homeostatic model assessment of insulin resistance; HOMA-beta, Homeostatic model assessment of beta cell function; ApoB, Apolipoprotein B-100; HbA1c, Haemoglobin A1c; TNF α , Tumour necrosis factor alpha; BODIPYL, boron-dipyrromethene; pNPG, 4-Nitrophenyl-beta-D-glucopyranoside; EMEM, Eagle's Minimum Essential Medium; CTB, cell titre blue; AUC, area under curve; 2DG, 2-Deoxy-D-glucose; GLUT2, glucose transporter 2; RESAS, Rural and Environment Science and Analytical Services

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protein and IL-6 in subjects with increased risk of cardiovascular disease (Karlsen et al., 2010) and a second study reported a decrease in inflammation with a diet rich in bilberries (Kolehmainen et al., 2012). These beneficial responses from human studies (Crespo & Visioli, 2017) are supported by data that demonstrate long-term beneficial effects of anthocyanins from mouse models of obesity and diabetes (DeFuria et al., 2009; Guo, Li, Ling, Feng, & Xia, 2011; Jayaprakasam, Olson, Schutzki, Tai, & Nair, 2006; Prior et al., 2008, 2010).

There are also a number of studies in vitro and in vivo that suggest that polyphenols influence carbohydrate digestion and absorption, resulting in improved postprandial glycaemia. Polyphenols from acerola (Hanamura, Hagiwara, & Kawagishi, 2005; Hanamura, 2006) and viburnum dilatatum (Iwai, Kim, Onodera, & Matsue, 2006) along with catechins and theaflavins (Matsui et al., 2007) inhibit intestinal α -glucosidase activity. Quercetin glucosides (Cermak, Landgraf, & Wolfram, 2004), polyphenols from acerola (Hanamura, 2006), green tea (Kobayashi et al., 2000) as well as dietary polyphenols (Johnston, Sharp, Clifford, & Morgan, 2005; Welsch, Lachance, & Wasserman, 1989) and flavonoids (Song et al., 2002) have all been shown to inhibit glucose transport in vitro. In humans, several studies have examined the effect of polyphenols on the post-prandial glycaemic response (Törrönen et al., 2010). In our previous human intervention study the ingestion of a bilberry extract given as a single capsule (0.47 g standardised bilberry extract (36% w/w anthocyanins) followed by an OGTT in an acute crossover design in volunteers with T2D resulted in a significant decrease in the OGTT AUCi for both glucose and insulin compared with the placebo (Hoggard et al., 2013).

Overall evidence suggests that consuming edible berries, particularly from the genus Vaccinium, that have high concentrations of anthocyanins could provide a supplementary intervention to improve glycaemia in subjects with T2D or impaired glucose tolerance (Castro-Acosta, Lenihan-Geels, Corpe, & Hall, 2016; Coe & Ryan, 2016; Crespo & Visioli, 2017). The object of this study was to determine the early mechanisms of action which may be responsible for the reported changes in glucose metabolism. This study investigated the effect of a three week supplementation of standardised bilberry extract (36% w/w anthocyanins) on glucose metabolism in overweight volunteers.

2. Materials and methods

2.1. Subjects

Overweight male and postmenopausal female volunteer subjects ($n = 16$, BMI 31 SD 4; aged 62 SD 7 years) with ($n = 9$) or without ($n = 7$) type 2 diabetes controlled by diet and lifestyle alone (Table 1), were recruited from the Aberdeen area of the UK. Subjects were only included if they were not on any special religious or prescribed diet and had a stable weight. Medical exclusion criteria included chronic illnesses, such as thromboembolic or coagulation problems, thyroid disease, renal or hepatic disease, severe gastrointestinal disorders, pulmonary disease (e.g. chronic bronchitis, COPD), alcohol or any other substance abuse, eating disorders or psychiatric disorders. Volunteers were also excluded if they were taking oral steroids, tricyclic antidepressants, neuroleptics anticoagulants, digoxin and anti-arrhythmics, or chronically using anti-inflammatories (e.g. high doses of aspirin, ibuprofen). These criteria were checked with each participant's primary care physician. All subjects provided informed written consent before inclusion in the study which was approved by the North of Scotland Research Ethics Committee (NOSRES). The study was registered at clinicaltrials.gov number NCT01180712. On all visits, all anthropometric measurements were made following an overnight fast.

2.2. Study design

In a cross-over design, volunteers ($n = 16$) were randomised and double blinded into two groups matched for BMI as well as age and

Table 1

Baseline characteristics of the overweight non diabetic/diabetic study volunteers. Data are mean (SD) for all parameters.

	Mean	SD
n (male/post-menopausal female)	16 (10/6)	
Age	62	7.3
Diet controlled T2D	9	
Body mass index (Kg/m ²)	31.3	4
Body weight (kg)	93.1	15.1
% Body fat	34.9	9.3
Blood pressure (mmHg)		
systolic	137.7	12.8
diastolic	79.4	7
Plasma Cholesterol (mmol/l)	4.6	1.1
Plasma Glucose (mmol/l)	6.2	0.8
Plasma HDL cholesterol (mmol/l)	1.2	0.3
Plasma LDL cholesterol (mmol/l)	2.8	1
Triglycerides (pmol/l)	1.5	0.8
NEFAs (mmol/l)	0.7	0.1
Leptin (ng/ml)	22.3	17.8
Fasting plasma insulin (pg/ml)	N/A end point only	
HbA1c	6.1	0.5
HOMA-IR	N/A	
HOMA-beta	N/A	

given three capsules of either 0.47 g of Mirtoselect® [a standardized bilberry extract (36% (w/w) anthocyanins)] per day, which equates to ~150 g of fresh bilberries per day, formulated in gelatin capsules or a control capsule consisting of microcrystalline cellulose in an opaque gelatine capsule, over a three week period. The opposite procedure was conducted following a two week washout period. Each volunteer therefore visited the Human nutrition unit on four occasions when an overnight fasted plasma blood sample and anthropometric data were collected. The placebo capsules were not matched for the small amount of carbohydrate in the bilberry extract. The volunteers were asked to record what they ate in a food diary over a three day period prior to each study visit, Food diaries were analysed using NETWISP V3.0 (Tinuviel software) by a trained dietician. Subjects were reimbursed travelling expenses on completion of the study. Based on previous published studies we expected between volunteer SD of glucose AUC to be about 20–22%. Within volunteer variation was expected to be less, and if it is about half this value then 16 volunteers in a cross-over study would give us 80% power to detect treatment effects of about 10–12% (Rebello, Burton, Heiman, & Greenway, 2015).

Although the volunteers consumed their habitual diet while taking the supplements, syringic acid (35OMe4OHBA), used as a potential marker of compliance showed a significant difference in the plasma between placebo and extract (ANCOVA $p < 0.001$; Appendix Fig. 1). This hydroxybenzoic acid is ubiquitous in soft fruits and unlike several of the hydroxycinnamic acids is detected in the plasma and urine following consumption (Russell, Scobbie, Labat, & Duthie, 2009). In addition to monitor compliance volunteers were asked to record when they took a capsule and to return any unused capsules to the Human nutrition unit which were then counted. The capsules were supplied in excess so that some should have been left over each week.

2.3. Oral glucose tolerance testing (OGTT)

In the evening before the test on each visit the volunteers were asked to consume the same meal. Volunteers were fasted (10–12hrs) overnight prior to the OGTT. The last capsule (bilberry extract or placebo) was ingested in the morning approximately two hours prior to the venous blood sample being taken at the Rowett Institute.

Venous blood samples were taken through an indwelling cannula inserted into a forearm vein, at -15 , -10 , -5 (fasted) and 15, 30, 45, 60, 90, 120, 150, 180 and 240 min after consuming 75 g of Polycal liquid (<http://www.nutricia.co.uk>). Polycal was selected as the main

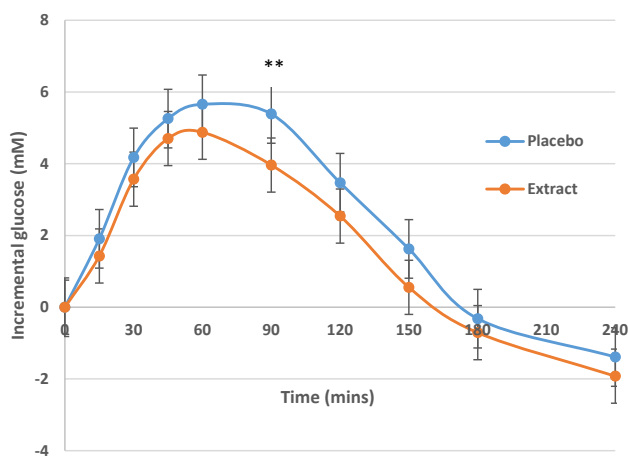


Fig. 1. Plasma incremental glucose concentrations following consumption of a glucose load following three weeks of either the bilberry extract capsule or placebo control (3×0.47 g capsules/day) ($n = 16$ mean \pm SEM) $**P < 0.01$, (end point comparison) Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

carbohydrate as it is in the form of polysaccharides and this is closer to normal dietary consumption than glucose only. Movement during the 240 min OGTT was kept to a minimum. Plasma glucose concentrations were measured in triplicate using an automated clinical analyser (Kone Oyj, Espoo, Finland). Plasma insulin was measured in duplicate using an ELISA assay (MercoDia, Uppsala, Sweden). The inter- and intra-assay coefficients of variation were 2.6%–3.6% and 2.8%–3.4%, respectively. Homeostasis models of assessment of insulin resistance (HOMA, HOMA-IR and HOMA-beta) was measured using the fasting glucose and insulin values.

2.4. Metabolic profile

An automated clinical analyser (Kone Oyj) was used for the analysis of plasma hsCRP, glucose, triacylglycerol, LDL cholesterol, HDL cholesterol, NEFAs, Apo A1, Apo B and cholesterol using commercial kits (Microgenics GmbH; Hemel Hempstead, Herts., UK). HbA1c was measured using an Afinion AS100 Analyser (Abbott, Stirling, UK)

2.5. Mirtoselect®

Mirtoselect® is a standardized extract of bilberries (manufactured by Indena S.p.A., Milan, Italy), prepared by an industrial process to ensure a reproducible anthocyanin composition (36%, w/w). Predominant anthocyanin constituents are delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, cyanidin-3-galactoside and cyanidin-3-glucoside (Indena datasheet). Other anthocyanins include cyanidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside, and malvidin-3-arabinoside. Mirtoselect® also contains other polyphenols (phenolic acids, flavonols, proanthocyanidins; ~18%), carbohydrates and aliphatic organic alcohols (~29%), fats (~0.04%), nitrogen compounds (~1%), ash (~0.7%), with the remaining 15% undefined. The main sugar composition of the Mirtoselect® is fructose 13%, glucose 14%; and sucrose 4% (w/w). No side effects of the Mirtoselect® supplementation were noted.

Local wild bilberries were picked from one season's growth in one area from Aberdeenshire, UK. Bilberries were vacuum-freeze dried (Freezone vacuum Freeze dryer, Labconco, USA) followed by freeze-milling (Freezer mill Spex CertiPrep 6800, UK) into powder, vacuum-sealed and stored in -70°C until further use.

2.6. Plasma ELISAs

Plasma was collected in heparinised EDTA tubes (BD) containing aprotinin with DPP-IV Inhibitor (Millipore Ltd, Watford Hertfordshire, UK) added at $10 \mu\text{l/ml}$ and stored at -70°C until analysis. Plasma leptin, MCP-1 and TNF α were measured using the commercial Milliplex multiplex ELISA kit (HADK2MAG-61K) while plasma sICAM-1, sVCAM-1 were measured using the commercial Milliplex multiplex ELISA kit (HSP1MAG-63K). All according to the manufacturer's instructions (Millipore Ltd, Watford Hertfordshire, UK). All samples were assayed in triplicate.

The minimum level of detection of leptin was 38 pg/ml and the intra- and inter-assay coefficients of variation were 10% and 15%, respectively consistent with the manufacturer's specification. Similar values for all the other analytes can be found on the Millipore website (www.millipore.com/).

2.7. Reducing and radical scavenging activity in plasma

The ferric reducing activity of plasma assay (FRAP; Benzie & Strain, 1996) and the Trolox equivalent antioxidant capacity assay (TEAC; Dragsted et al., 2004) were used to define changes to plasma anti-oxidant capacity. FRAP was assayed in a microplate format, using $6 \mu\text{l}$ of plasma to which $18 \mu\text{l}$ of sterile distilled water and $180 \mu\text{l}$ of freshly prepared FRAP reagent (250 mM sodium acetate (pH3.6), 1.6 mM iron (III) chloride and 0.83 mM 2,4,6-Tri(2-pyridyl)-s-triazine) was added. Samples were incubated at 37°C for 4 min and absorbance ($A_{593\text{nm}}$; Dynex Technologies, USA) recorded after 4 min and after a further 30 min at 37°C . The difference in A_{593} between samples and blanks was calculated and compared to a standard curve of Fe(II) standard solutions (50–1000 μM). For TEAC analysis a proprietary assay (TEAC Assay, Sigma CS0970) was used and conducted according to the manufacturer's instructions. Briefly, $10 \mu\text{l}$ of plasma was added to $20 \mu\text{l}$ of myoglobin solution and $150 \mu\text{l}$ of ABTS substrate working solution. Samples were incubated for 5 min at 21°C (as optimised), the reaction stopped and the $A_{405\text{nm}}$ recorded (Dynex Technologies, USA). The antioxidant capacity was reported as trolox equivalents by referral to the equation obtained from the linear regression of a trolox standard curve.

2.8. Microbial analysis

Faecal samples were collected at the beginning and end of each of the three-week intervention periods. They were kept at 4°C and processed within 5 h of collection. Each sample was mixed and diluted in sterile phosphate-saline buffer as described before (Walker et al., 2011). An aliquot of $450 \mu\text{l}$ faecal slurry was transferred to Lysing Matrix E tubes of the FastDNA® spin kit for soil (MB Biomedicals) and stored at -70°C until DNA extraction with the same kit according to the manufacturer's instructions (MB Biomedicals).

Microbiota analysis was carried out for twelve volunteers who had not been on antibiotics for the three month period prior to providing a sample and who were willing to provide a sample. DNA was quantified using a Qubit dsDNA BR Assay kit on a Qubit™ 3.0 Fluorimeter (Thermo Fisher Scientific, Renfrew, UK) as per the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed with iTaq™ Universal SYBR® Green Supermix with a CFX384™ Real-time System (Bio-Rad, Watford, UK) as described before (Vollmer et al., 2017). Samples were amplified with universal primers against total bacteria and with specific primers against 21 different microbial groups. Primers and annealing temperatures are given in Table 2. The data are expressed as 16S rRNA gene copies per g faeces.

2.9. In vitro analysis

2.9.1. α -amylase assay

Human salivary α -amylase was purchased from Sigma-Aldrich, UK

Table 2
Quantitative PCR primers and annealing temperatures used in this study.

Target group	Primer	Primer sequence 5'-3'	Annealing temperature	Reference
total bacteria	UniF	GTGSTGCAYGGYYGTCGTCA	60	Fuller et al. (2007)
	UniR	ACGTCRTCCMCNCCTTCCCTC		
<i>Bacteroides</i> spp.	G-Bfra-R-Fmod	GCTCAACCKTAAAAATTGCAGTTG	63	Reichardt et al. (2018)
	Bac708Rmod	GCAATCGGRGTTCTTCGTG		
<i>Prevotella</i> spp.	g-Prevo-Fmod	CRCRCRGTAAACGATGGATG	65	Reichardt et al. (2018)
	BacPreRmod	TTGAGTTTCACCGTTGCCGG		
Ruminococcaceae ¹	sg-Clept-F	GCACAAGCAGTGGAGT	60	Vollmer et al. (2017)
	sg-Clept-Fm2	GCACAAGCGGTGGATT		
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA		
<i>Faecalibacterium prausnitzii</i> ²	FPR-2F	GGAGGAAGAAGGTCCTTCGG	60	Ramirez-Farias et al. (2009)
	Fprau645mR	AATTCGGCTACCTCTGCACT		
<i>Ruminococcus bromii</i>	Rflbr730F	GGCGGCTRYGGGCTTT	60	Salonen et al. (2014)
	RbromR	CAACTTTCGCCGAAGGGACCTA		
<i>Ruminococcus albus/bicirculans</i>	400Fmod1	GACTGCCGTTGACAAAAC	60	Salonen et al. (2014)
	Ralbr	CCTACAATCCGAACCTAAGACTGC		
<i>Ruminococcus flavefaciens/callidus/champenellensis</i>	400Fmod1	GACTGCCGTTGACAAAAC	60	Salonen et al. (2014)
	Rfla + R	CCTRCAATCTGAACCTGGGACAA		
<i>Oscillibacter/Oscillospira</i> -related ¹	Osc370F1	GGCAATGGACGCAAGTCTGACC	60	Walker et al. (2011)
	Osc370F2	GGCAATGGGCGCAAGCCTGACC		
	Osc455R1	ATTCGTACAGTACCGTCTTCTRCTC		
	Osc455R2	ATTCGCCAGGTACCGTCTTCTACTC		
	Osc455R3	ATTCAAGDGGTACCGTCTTCTRCTC		
	Osc455R4	ATTCTAGAGGTACMGTTCTTCTRCTC		
	Osc455R5	GTTTTCAGGGTACCGTCTTCTGCTC		
<i>Negativicutes</i> (Cluster IX) ¹	IX552F	GTTTTCGGGAATYATTGGGC	63	Reichardt et al. (2014)
	IX854R2A	ATTGGGTTAACTCCGGCACA		
	IX854R2G	ATTGGGTTAACTCCGGCACG		
<i>Roseburia/Eubacterium rectale</i> group	RrecF	GCGGTRCGGCAAGTCTGA	63	Ramirez-Farias et al. (2009)
	Rrec630mR	CCTCCGACACTCTAGTMCAGC		
<i>Eubacterium hallii</i>	EhalF	GCGTAGGTGGCAGTGCAA	60	Ramirez-Farias et al. (2009)
	EhalR	GCACCGRAGCCTATACGG		
<i>Anaerostipes hadrus</i>	SSC2F2	CTTTAGTAGCCAGCATATAAGG	60	Louis, Young, Holtrop, and Flint (2010)
	SSC2R	TTGCTCAGTCTCAGGAGGCT		
<i>Coprococcus eutactus</i>	CeutFext/L2-50Fext	TTCCAGTAGCCAGCAGTMAGAT	63	Reichardt et al. (2018)
	CeutR	CAATCCGAAGTGGACAGCC		
<i>Dorea</i> spp.	DoreaF	GCAGCTAACGCAATAAGCAG	60	Reichardt et al. (2018)
	DoreaR	CATTACGAAGCGGTATCG		
<i>Eubacterium eligens</i>	EelF	CGACAATGTTGCATGACAT	63	Chung et al. (2016)
	EelR	CTTAGTCAGGTACCGTCACTA		
<i>Blautia</i> spp.	BlautiaF2	CGCGTGAAGGAAGAAGTATC	63	Reichardt et al. (2018)
	BlautiaR2	GAGCCTCAACGTCAGTTACC		
<i>Lactobacillus</i> spp.	LAC-1	AGCAGTAGGGAATCTTCCA	60	Walter et al. (2001)
	Lab 0677	CACCGCTACACATGGAG		Heilig et al. (2002)
<i>Bifidobacterium</i> spp.	BifF	TCCGCTCYGGTGTGAAAAG	60	Fuller et al. (2007)
	g-Bifid-R	GGTGTCTTCCCAGATATCTACA		
<i>Bifidobacterium adolescentis</i>	Bif164F	GGGTGGTAATGCCGATG	60	Ramirez-Farias et al. (2009)
	BiADO-2	CGAAGGGCTTGCTCCAGT		
<i>Desulfovibrio</i> spp.	Dsv691F	CCGTAGATATCTGGAGGAACATCAG	63	Fite et al. (2004)
	Dsv826R	ACATCTAGCATCCATCGTTTACAGC		
Enterobacteria	EnterobactDmod2F	GACCTCGCGAGAGCA	60	Hansen et al. (2012)
	Enter1432mod	CCTACTTCTTTTGAACCCA		
Methanogenic Archaea	Met630F	GGATTAGATACCCSGGTAGT	60	Walker et al. (2011)
	Met803R	GTTGARTCCAATTAACCGCA		

Primer references: Chung et al. (2016), Fite et al. (2004), Fuller et al. (2007), Hansen et al. (2012), Heilig et al. (2002), Louis et al. (2010), Ramirez-Farias et al. (2009), Reichardt et al. (2014), Reichardt et al. (2018), Salonen et al. (2014), Vollmer et al. (2017), Walter et al. (2001), Walker et al. (2011).

¹ Where more than one forward or reverse primer is given, all primers are used together at equimolar concentrations.

² The primers exhibit some cross-reactivity with *Subdoligranulum variabile*.

(A0521) and the assay was performed using an EnzChek® Ultra Amylase Assay Kit (E33651), Invitrogen, UK according to the manufacturer's instructions.

Briefly all the berry extracts and reagents were prepared in 1X reaction buffer, provided in the assay kit as instructed. The assay contained a total reaction volume of 100 µl per well in a 96-well plate. 50 µl of samples were added to 50 µl of 200 µg/ml of DQ substrate solution (DQ starch from corn, BODIPYL conjugate) while mixing. Each assay contained the α-amylase enzyme standard curve (50 µl at 0–10 mU/ml) and blank (reaction buffer only, without any enzyme or test samples). Acarbose at 1 mg/ml was taken as the positive inhibitor. The samples were then incubated at room temperature protected from light for 15 min and then read using a 96-well fluorescence microplate reader

(Spectramax Gemini XS- Molecular Devices, USA) at a wavelength of 502/512 nm. For each data point, the background fluorescence was corrected by subtracting the value derived from the no-enzyme blank. Samples were read against the standard curve to determine the concentration of α-amylase in the test samples.

2.9.2. α-Glucosidase assay

Berry extracts were analysed in triplicate along with no substrate and no enzyme controls. The enzyme, inhibitor and substrate solutions were prepared in phosphate buffer (0.1 M; pH 6.9). Yeast α-glucosidase inhibition was analysed using an end-point assay in a 96-well plate format. A reaction mixture containing 20 µl of phosphate buffer, 20 µl of α-glucosidase (0.5 U/ml) and 20 µl of berry extracts of varying

Table 3

Averages for dietary intake and nutrient composition readings obtained from repeated four day diet records (for the three days prior to and on the day of each study visit) compiled by study subjects taking either three weeks of bilberry extract treatment or three weeks of placebo. Data are presented as average \pm SEM. n = 16 (ns = no significant difference). Comparisons were based on t-tests carried out in Graphpad Prism.

	Study week	Placebo	Extract	t-test
Energy (KJ)	0	9480 \pm 466	8580 \pm 476	ns
	3	9890 \pm 500	9680 \pm 464	ns
Energy (Kcal)	0	2260 \pm 111	2040 \pm 113	ns
	3	2352 \pm 118	2304 \pm 110	ns
Protein (g)	0	96 \pm 5	87 \pm 9	ns
	3	97 \pm 6	92 \pm 5	ns
Fat (g)	0	90 \pm 7	83 \pm 4	ns
	3	92 \pm 5	96 \pm 5	ns
Carbohydrates (g)	0	266 \pm 16	230 \pm 18	ns
	3	276 \pm 17	260 \pm 16	ns
Sucrose (g)	0	99 \pm 6	89 \pm 9	ns
	3	103 \pm 9	103 \pm 8	ns
Starch (g)	0	150 \pm 13	136 \pm 11	ns
	3	167 \pm 12	151 \pm 10	ns
Fibre (g)	0	24 \pm 2	24 \pm 2	ns
	3	26 \pm 2	24 \pm 2	ns
Alcohol (g)	0	13 \pm 4	10 \pm 4	ns

concentrations within the physiological range was prepared and pre-incubated for 15 min at 37 °C, 3 mM pNPG (40 μ l) was then added to the mixture as the substrate. The reaction was stopped by adding 150 μ l of sodium carbonate (0.2 M) to the reaction mixture. Absorbance was read at 405 nm by a microplate reader (μ Quant™ Microplate Spectrophotometer, BioTek Instruments Inc. USA) and compared to a control which had 200 μ l of buffer solution only. Acarbose was used as an inhibitor at a concentration of 1 mg/ml for the assay. The α -glucosidase inhibitory activity was expressed as inhibition percentage (%) and was calculated as follows;

$$\% \text{ inhibition} = \left\{ \frac{\Delta A \text{ Control}(405 \text{ nm}) - \Delta A \text{ Sample}(405 \text{ nm})}{\Delta A \text{ Control}(405 \text{ nm})} \right\} \times 100$$

where; ΔA = Individual absorbance recorded at 405 nm after subtracting from the blank.

2.9.3. Glucose uptake

Cell culture

CaCo-2 cells obtained from ATCC were cultured in Eagle's minimum Essential medium, 12-662 (EMEM; Lonza), supplemented with 10% fetal bovine serum (Gibco), 100 IU/ml penicillin G, 100 μ g/ml streptomycin sulfate (Sigma-Aldrich) and 0.2 mM L-glutamine G7513 (Sigma-Aldrich). Cells were cultured in a humidified 37 °C incubator with 5% CO₂.

Glucose uptake

The amount of glucose uptake in CaCo-2 cells was measured using the Glucose Uptake-Glo Assay Kit (Promega) according to the manufacturer's instructions. Cells were seeded in a 12 well plates and cultured for 19–20 days post confluence. Cells were incubated in serum free media overnight (16 hrs), the following day culture medium was removed and cells were washed twice with PBS. The cells were incubated in glucose free media containing the treatments for 2 hrs prior to the addition of glucose. Freshly prepared 10 mM 2-deoxyglucose was added and incubated for 20 min. The uptake process was stopped, 75 μ l transferred to a 96 well white walled plate (Gibco) then neutralized and detection reagent added, protocol carried out as per manufacturer's instructions. Luciferase activities were measured on Tecan plate reader. The rate of glucose uptake was analyzed according to the manufacturer's instructions.

Both berry extracts used in this study were not cytotoxic to the CaCo-2 cells at all the concentrations used. This was determined using

by the CellTiter-Blue (CTB) cell viability assay kit (Promega-G8081, UK) (Data not shown).

2.10. Statistical analysis

The area under the curve (AUC) was calculated using the trapezoid approximation. For the incremental version (AUC_i), only the extent of interpolated values above baseline contributed.

Treatment effects were assessed by Analyses of variance of the endpoint of each intervention period, with terms for volunteer (random effect), period (1st or 2nd) and treatment (placebo or extract) and baseline values as a covariate.

For the in vitro analysis all the samples were run in triplicates for each experiment, and the results were presented as a mean \pm standard error. Statistical analysis was performed using GraphPad Prism 7.0 for Windows. The data was analysed using One-Way-Analysis of Variance (ANOVA) to compare the groups and Tukey's test was performed to account for multiple comparisons.

Microbiota analysis: The endpoint data was analysed by Analysis of Variance with volunteer as random effect and supplement (prebiotic or placebo) as fixed effect, and with the corresponding baseline measurements as covariate. Order (order in which the two supplements were given) and gender or diabetic status were included as fixed effects to investigate whether these modified the response to treatment.

3. Results

3.1. Volunteers

The mean body weight of the volunteers remained stable during the study: 93.2 (SD 14.8) kg on visit 1 compared with 93 (SD 15.4) kg, 93.6 (SD 14.8) kg and 94.0 (SD 14.8) kg on visits 2, 3 and 4. Similarly the percentage body fat of the volunteers also remained relatively stable during the study: 34.9% (SD 9.3%) on visit 1 compared with 36.1% (SD 8.6%), 35.8% (SD 8.9) and 36.7% (SD 8.7%) on visits 2, 3 and 4. The order in which the capsules were taken (control vs bilberry extract) did not significantly influence any variable. There was no significant difference between any of the variables measured, the diabetic status or sex of the volunteers. Therefore all subsequent data is presented as one group ingesting the bilberry extract or the placebo control over the three week intervention period.

The averages for dietary intake and nutrient composition readings obtained from repeated four day diet records (for three days prior to and on the day of each study visit) compiled by the study subjects taking either three weeks of bilberry extract treatment or three weeks of placebo are shown in (Table 3). No significant changes were recorded in energy intake or any of the other macronutrients measured when the volunteers were on the bilberry extract compared to when the volunteers were on the placebo (Table 3). There was no significant change in fasting plasma glucose levels in volunteers taking the bilberry extract compared with the control (Table 4).

3.2. Oral glucose tolerance test (OGTT)

3.2.1. Plasma glucose

The ingestion of the bilberry extract over the three week intervention period resulted in lower OGTT venous plasma glucose AUC_i compared with placebo with a 20% decrease in the glucose (656 vs 523; p = 0.008; Fig. 1). Eleven of the sixteen volunteers showed a decrease in plasma glucose AUC_i when taking the bilberry extract compared with the placebo (data not shown). The incremental plasma glucose concentrations were significantly lower at 90 min after taking the bilberry extract compared with the placebo control (p < 0.01, Fig. 1). Although the differences in glucose following the OGTT was only significant at 90mins the differences in the curves suggest that the bilberry extract is having a greater effect on glucose in the second half of the curve

Table 4

Markers of glucose, lipid metabolism, vascular health and inflammatory adipokines /markers taken fasted at the beginning of each intervention and at the end of each three week intervention period following the ingestion of three capsules a day of either bilberry (*Vaccinium myrtillus* L.) extract or the placebo. Values are means of triplicate replicates of all sixteen subjects, with standard errors. Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

		Placebo				Extract				p
		Baseline 1	SD	Change over 3 weeks	SD	Baseline 2	SD	Change over 3 weeks	SD	
Glucose	Glucose (mmol/L)	6.34	0.76	0.03	0.44	6.42	0.96	-0.18	0.48	ns
Lipid metabolism	Cholesterol (mmol/L)	4.65	1.21	0.02	0.20	4.49	1.11	-0.12	0.38	ns
	HDL Chol (mmol/L)	1.21	0.35	0.02	0.09	1.18	0.35	-0.07	0.18	ns
	LDL-Chol (mmol/L)	2.79	1.00	-0.10	0.29	2.71	1.01	-0.15	0.24	ns
	NEFA (mmol/L)	0.67	0.12	-0.08	0.20	0.69	0.20	0.03	0.21	ns
	TRIGLY (mmol/L)	1.54	0.74	-0.19	0.42	1.55	0.72	0.03	0.41	ns
Vascular health	Apo A1 (g/L)	1.31	0.17	0.00	0.18	1.30	0.20	-0.01	0.13	ns
	Apo B (g/L)	0.84	0.29	-0.04	0.08	0.82	0.26	-0.02	0.15	ns
	sVCAM-1 (pg/ml)	442,000	170,000	684	152,000	434,000	129,000	6468	65,294	ns
Inflammatory adipokines/markers	sICAM-1 (pg/ml)	118,000	87,000	9730	50,500	108,000	40,300	3670	13,593	ns
	Leptin (pg/ml)	23,500	20,800	1760	6250	21,400	15,000	-1600	7720	ns
	MCP-1 (pg/ml)	178	65.3	14.2	30.5	177	56.8	12.0	41.6	ns
	TNF α (pg/ml)	5.89	2.24	0.14	1.39	6.68	4.28	0.64	3.23	ns
	hsCRP (mg/L)	4.04	5.29	0.60	4.67	3.16	4.74	-1.56	6.43	ns

compared with the placebo.

3.2.2. Plasma insulin

When comparing the OGTTs at the end of the three week interventions (bilberry extract intervention vs placebo intervention) the ingestion of the bilberry extract had no significant effect on the venous plasma insulin compared with placebo ($p = 0.72$; Fig. 2).

3.3. Lipid metabolism

The bilberry extract had no effect on fasting plasma concentrations of cholesterol, HDL-cholesterol, LDL cholesterol, NEFA or triglyceride compared with placebo control (Table 4).

3.4. Inflammatory adipokines/markers

The bilberry extract had no effect on fasting plasma concentrations of the inflammatory adipokine MCP-1, TNF- α , leptin, hsCRP (Table 4) compared with placebo control.

3.5. Markers of cardiovascular health

The bilberry extract had no effect on fasting plasma concentrations of Apo A1, Apo B, sVCAM-1 or sICAM-1 (Table 4) compared with

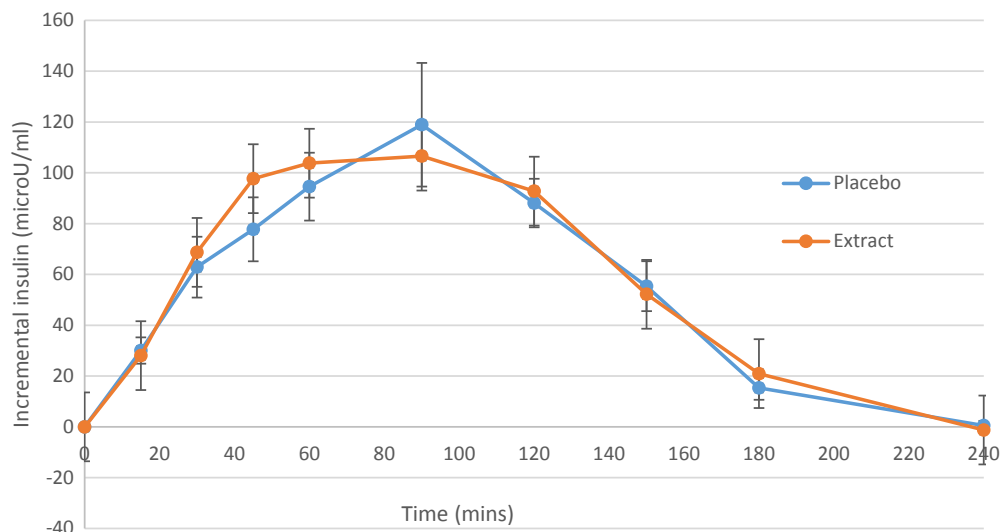


Fig. 2. Plasma incremental insulin concentrations, following consumption of a glucose load following three weeks of bilberry extract or placebo control capsule (3×0.47 g capsules/day) ($n = 16$ mean \pm SEM). $**P < 0.01$, (end point comparison) Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

placebo control.

3.6. Oxidative state reducing activity and radical scavenging potential

Similarly there was no effect of the bilberry extract on the reducing activity or radical scavenging features of plasma as measured by FRAP and TEAC assays compared with placebo (Table 5).

3.7. Microbial analysis

Microbiota composition at the start and end of each intervention period was determined by qPCR against total bacteria and 21 microbial families, genera and species belonging to Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Archaea. No effect of treatment was found for either total bacterial abundance or the microbial groups examined (Fig. 3).

3.8. In vitro analysis

The concentration of extracts used for the in-vitro analysis were based on Cai et al. (2011) who measured the anthocyanins and metabolites in the urine by HPLC-MS/MS following a single dose of the same bilberry extract used our human intervention.

Table 5

Plasma concentrations for ferric-reducing ability of plasma (FRAP) Trolox equivalent antioxidant capacity (TEAC) taken at the beginning of each intervention and at the end of each three week intervention period following the ingestion of three capsules a day of either bilberry (*Vaccinium myrtillus* L.) extract or the placebo. Values are means of triplicate replicates of all sixteen subjects, with standard errors. Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

	Placebo				Extract				p
	Baseline 1	SD	Change over 3 weeks	SD	Baseline 1	SD	Change over 3 weeks	SD	
FRAP (Fe II mmol/L)	1430	177	20	187	1510	178	15	168	ns
Teac mmol Trolox equivalents/L	469	126	16	149	470	143	18	94	ns

3.8.1. Bilberry extract and freeze dried wild bilberries inhibit salivary α -amylase

We determined if bilberry extract and freeze dried wild bilberries can inhibit the activity of human salivary α -amylase in vitro. Both bilberry extract and wild freeze dried bilberries extracts showed a dose-dependent inhibition of human salivary α -amylase (ANOVA $P < 0.0001$; Fig. 4). The bilberry extract showed 61% ($P < 0.0001$), 57% ($P < 0.0001$) and 55% ($P = 0.0001$) inhibition of human salivary α -amylase at 66, 6.6 and 0.66 $\mu\text{g/ml}$ respectively, while freeze-dried wild freeze dried bilberries showed 78% ($P < 0.0001$), 73% ($P < 0.0001$) and 68% ($P = 0.0001$) inhibition of human salivary α -amylase at the same respective concentrations. Both the extracts showed greater inhibition of salivary α -amylase than the commercial inhibitor acarbose at 1 mg/ml (31%, $P = 0.003$). No activity was observed with either no enzyme or no substrate controls.

3.8.2. Bilberry extract inhibits yeast α -glucosidase but not the freeze dried wild bilberries

We determined the effects of bilberry extract and freeze dried wild bilberries on the activity of yeast α -glucosidase derived from *S. cerevisiae*. The bilberry extract significantly inhibited yeast α -glucosidase activity (ANOVA $P < 0.0001$; Fig. 5). The inhibition of yeast α -glucosidase activity by the bilberry extract was dose-dependent (77% inhibition at 66 $\mu\text{g/ml}$; ($P < 0.0001$); 45% inhibition at 13.2 $\mu\text{g/ml}$; ($P < 0.0001$) and 17% inhibition at 6.6 $\mu\text{g/ml}$; ($P = 0.003$). At the same concentrations, freeze dried wild bilberries showed no effect on yeast α -glucosidase activity (Fig. 2B). Acarbose, the known inhibitor of yeast α -glucosidase activity inhibited the enzyme activity by 47% ($P \leq 0.001$) at 1 mg/ml. No activity was observed with no enzyme and no substrate controls.

3.8.3. Bilberry extract and freeze dried wild bilberries inhibit 2DG uptake

We then determined the effect of Bilberry extract and freeze dried wild bilberries extract on 2DG uptake in vitro. Bilberry extract and wild freeze dried bilberry extract extracts showed an inhibition of 2DG uptake (ANOVA $P < 0.0001$; Fig. 6) in CaCo-2 cells. Bilberry extract showed 61% ($P < 0.0001$) inhibition of 2DG uptake in CaCo-2 cells at 6.6 $\mu\text{g/ml}$, but not at 0.66 or 0.066 $\mu\text{g/ml}$ respectively, while freeze-dried wild freeze bilberry extract showed 73% ($P < 0.0001$), 50% ($P < 0.0001$) and 32% ($P = 0.0001$) of inhibition of 2DG uptake in CaCo-2 cells at the respective concentrations tested. As a control, the glucose transporter inhibitor, Phloretin (1 mM) showed a 63% inhibition of 2DG uptake in the CaCo-2 cells ($P < 0.0001$).

4. Discussion

The present study shows that the ingestion of concentrated bilberry extract as three capsules per day containing concentrated bilberry extract over a three week period reduces the post-prandial glycaemic response in overweight volunteers compared to an inert placebo capsule. The bilberry extract over the same period had no effect on post prandial plasma insulin compared with the control. We should say that we cannot totally rule out an acute effect of the capsule on the glucose metabolism (Hoggard et al., 2013) as the last capsule was taken approximately two hours before the OGTT. Huang, Park, Edirisinghe, & Burton-Freeman (2016) have shown significantly reduced glucose concentrations over the 10-h day when strawberries were given 2 h before a meal compared to consuming strawberries with the meal suggesting it may be important for strawberry constituents, such as anthocyanins, to be absorbed and be available prior to maximal nutrient absorption, possibly priming cellular processes for managing the impending metabolic stress resulting in improved nutrient management

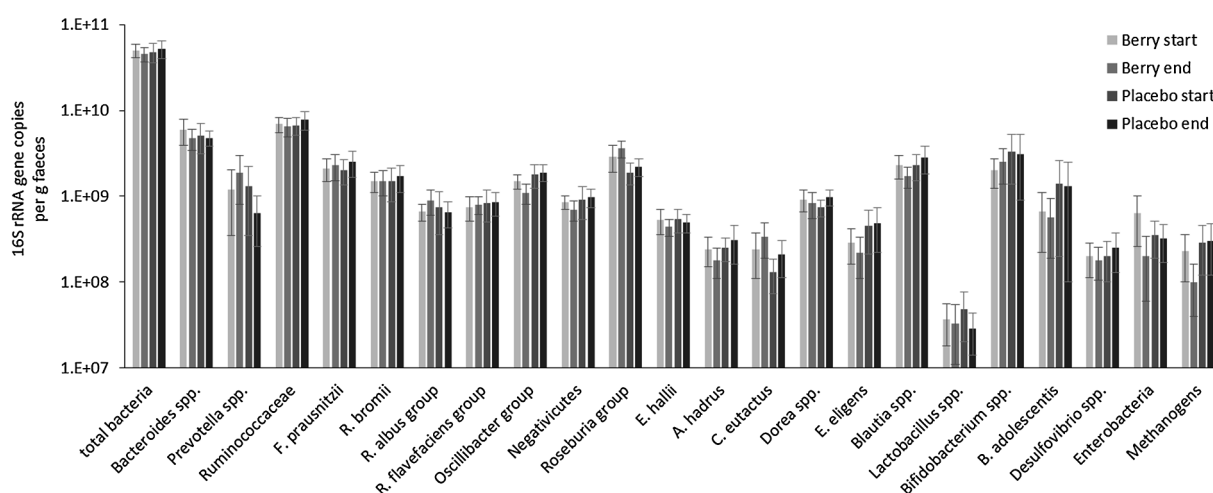


Fig. 3. Faecal microbiota composition before and after three weeks of either the bilberry extract or placebo control (3×0.47 g capsules/day) determined by quantitative PCR targeting the 16S rRNA gene of different microbial groups. Average and standard error of 12 volunteers. Treatment order was found not to modify the response and no significant differences were found between bilberry extract or placebo for any of the microbial groups determined. Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

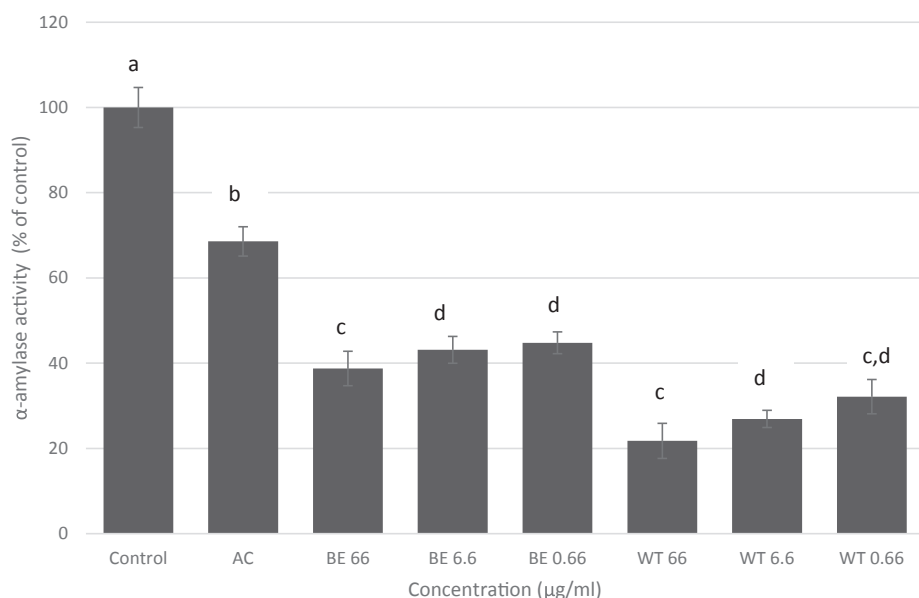


Fig. 4. Effect of bilberry and wild freeze dried bilberries extract on salivary α -amylase activity. Effect of bilberry and wild freeze dried bilberries extract (66, 6.6 and 0.66 $\mu\text{g/ml}$) and the commercial inhibitor acarbose (1 mg/ml) on salivary α -amylase activity. Enzyme activity was determined as a percentage of the control. All the samples were run in triplicate and are expressed as percentage activity \pm SD. Experiments were repeated independently at least three times on separate days with the similar results. Statistical analysis was done by one-way analysis of variance of Tukey's multiple comparison tests. Values without a common letter are significantly different ($P < 0.05$). AC: Acarbose, BE: Bilberry extract, WT: Wild freeze dried bilberries. Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

and reduced inflammatory burden.

One study has shown that consuming a smoothie supplemented with blueberries (from the same genus as bilberry) for six weeks increased insulin sensitivity as determined by hyperinsulinemic –euglycemic clamp in obese and pre-diabetics when compared to the control group consuming a placebo smoothie (Stull et al., 2010). Another study showed that subjects with T2D that consumed capsules containing 80 mg of anthocyanins (purified from bilberry and blackcurrant) taken twice a day for 24 weeks had a significant improvement in HOMA_{IR} (i.e. increased insulin sensitivity) (Li, Zhang, Liu, Sun, & Xia, 2015). However some studies have reported no changes in insulin sensitivity between the berry and placebo groups (Basu et al., 2010; Stull et al., 2015).

We looked at a shorter three week intervention period to determine the early effects of the bilberry extracts.

Bilberries are enriched in anthocyanins recognized for their ability to provide and activate cellular antioxidant protection, inhibit inflammatory gene expression, and consequently protect against oxidant-induced and inflammatory cell damage and cytotoxicity (Neto, 2007;

Tsuda, 2008; Zafra-Stone et al., 2007). In light of this we investigated in the effects of the bilberry extract on the inflammatory markers hsCRP, MCP-1, TNF α , and leptin that play a role in the recruitment of monocytes due to the low grade inflammation associated with obesity (Nakamura, Fuster, & Walsh, 2014). However in this study we did not see any changes in plasma levels of hsCRP, MCP-1, TNF α or leptin due to the ingestion of the bilberry extract compared with the control. Similarly we could not detect any alterations in plasma TEAC or FRAP values, both indicative of changes with activities/capacities frequently linked to changes in antioxidant status within environments such as plasma (Apak et al., 2016). Antioxidant status assay in themselves not de facto link to nor should they infer an effect on oxidative stress. Nevertheless, the absence of change reported here indicates that the extract does not dramatically affect antioxidant capacity.

T2D is closely linked with cardiovascular health and in light of this we looked for changes in plasma markers of cardiovascular health (Harding, Pavkov, Magliano, Jonathan, & Edward, 2019). We observed no changes in Apo A1, Apo B1, sVCAM-1 or ICAM-1 due to the ingestion of the bilberry extract compared with the control.

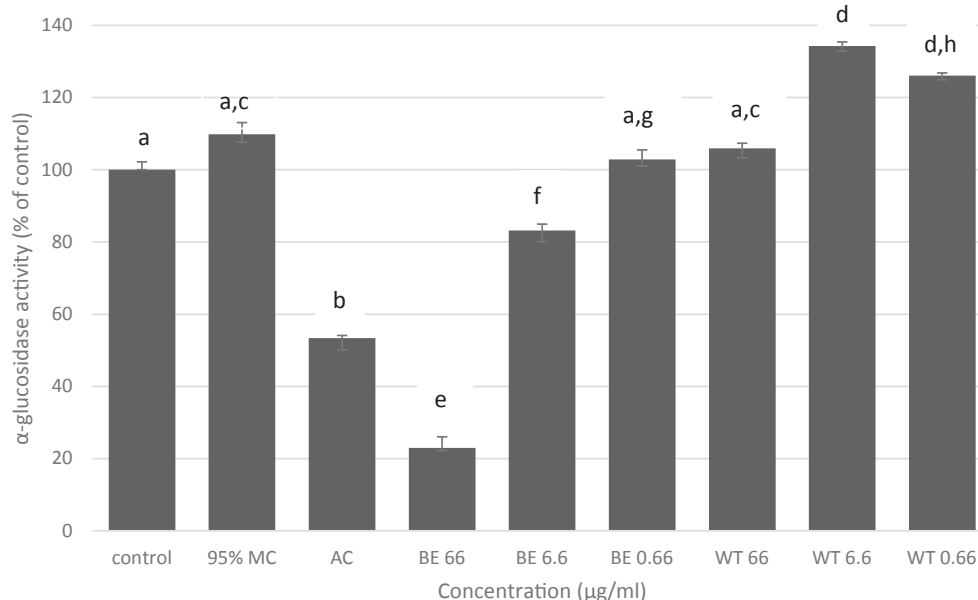


Fig. 5. Effect of bilberry and wild freeze dried bilberries extract on α -glucosidase activity. Effect of bilberry and wild freeze dried bilberries extract (66, 6.6 and 0.66 $\mu\text{g/ml}$) and the commercial inhibitor agarose (1 mg/ml) on α -glucosidase activity. Enzyme activity was determined as a percentage of the control. The 95% methanol control was shown not significantly different from the control. All the samples were run in triplicate and are expressed as percentage activity \pm SD. Experiments were repeated independently at least three times on separate days with the similar results. Statistical analysis was done by one-way analysis of variance of Tukey's multiple comparison tests. Values without a common letter are significantly different ($P < 0.05$). AC: Acarbose, MC: 95% methanol control, BE: Bilberry extract, WT: Wild freeze dried bilberries. Change from baseline in the two treatments was compared by analysis of variance in Graphpad Prism.

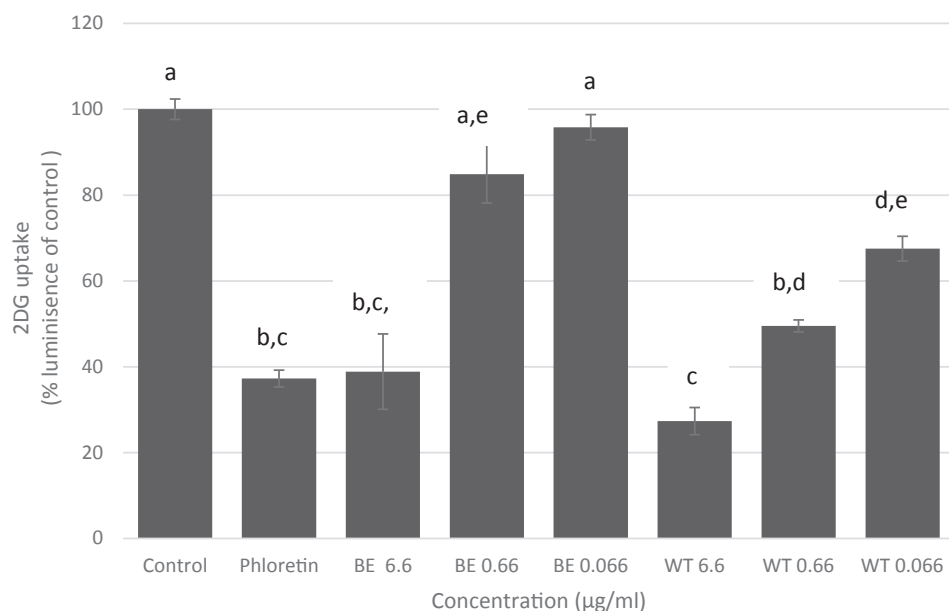


Fig. 6. Effect of bilberry and wild freeze dried bilberries extract on 2DG uptake. Effect of bilberry and wild freeze dried bilberries extract (6.6, 0.66 and 0.066 µg/ml) and the commercial inhibitor phloretin (1 nM) on 2DG uptake in 21 day post-confluent caco-2 cells. 2DG uptake was determined as a percentage of the control. All the samples were run in triplicate and are expressed as percentage activity \pm SD. Experiments were repeated independently at least three times on separate days with the similar results. Statistical analysis was done by one-way analysis of variance of Tukey's multiple comparison tests. Values without a common letter are significantly different ($P < 0.05$).

Disruption of the ecological equilibrium in the gut has been associated with several pathological processes, including obesity and its related comorbidities, with diet being a strong determinant of gut microbial balance. In vitro studies suggested that polyphenols exert antimicrobial effect on detrimental bacterial species while favouring the growth of beneficial bacteria. Species potentially beneficial, as *Lactobacillus spp.* and *Bifidobacterium spp.*, have been reported to increase in the presence of polyphenols especially anthocyanin whereas the growth of potentially detrimental species, such as *Enterobacteria*, is decreased (Anhê et al., 2015; Duda-Chodak, Tarko, Satora, & Sroka, 2015; Faria, Fernandes, Norberto, Mateus, & Calhau, 2014; Jamar, Estadella, & Pisani, 2017). We have seen no change in the faecal bacterial microbiota in a sub population of twelve volunteers with the addition of the bilberry extract compared with the placebo control. It is possible that the three week intervention period in our study is simply too short a period to see changes in the faecal bacterial microbiota.

Although dietary fibre has been shown to affect post-prandial glucose (Wood, 2007), the bilberry extract used in each capsule only contains 18 mg of dietary fibre (15 mg in soluble form). This small quantity is unlikely to explain the reduced glycaemic response.

It has been suggested that berry polyphenols inhibit α -glucosidase, the enzyme responsible for the digestion of sucrose to glucose in the intestinal epithelium. Two anthocyanins cyanidin-3-rutinoside (Adisakwattana et al., 2004) and cyanidin-3-galactoside (Adisakwattana, Charoenlertkul, & Yibchok-Anun, 2009) have been shown in vitro as inhibitors of α -glucosidase. Cyanidin-3-galactoside is present in bilberries (Buchert et al., 2005) and cranberries (Wilson et al., 2008), and has shown a synergistic effect with acarbose (Adisakwattana et al., 2009). Acarbose is used as an inhibitor of α -glucosidase in the treatment of diabetes. Also proanthocyanidins have shown potent α -glucosidase inhibitory activity (Kumar, Narwal, Kumar, & Prakash, 2011; Schäfer & Högger, 2007). The anthocyanins in the bilberry extract are well documented (Cai et al., 2011; Cooke et al., 2006) and include both cyanidin-3-galactoside and proanthocyanidins. Similarly some studies have suggested that berry polyphenols inhibit salivary α -amylase the enzyme responsible for the digestion of starch into maltose and dextrin (Grussu, Stewart, & McDougall, 2011).

We used polycal, which is composed of complex carbohydrates (glucose syrup, maltodextrin), as the glucose load in this study for the OGTT. Therefore the reduced postprandial glycaemia in response to the three week bilberry extract intervention may be due in part to a reduction in the breakdown of carbohydrates. To investigate this further

we looked at the effect in vitro of the bilberry extract as well as a freeze dried wild bilberry extract on both α -amylase activity and α -glucosidase enzyme activity. The bilberry extract at physiological concentrations (Cooke et al., 2006) inhibits the human salivary α -amylase and as well as the yeast α -glucosidase enzyme activity which supports the theory that the breakdown of carbohydrates in digestion is a key mechanism of action in the decrease of postprandial glucose. The freeze dried wild bilberry extract at the concentrations tested only decreased the human salivary α -amylase enzyme activity. The bilberry extract is higher in anthocyanins compared to the freeze dried wild bilberry extract which may account for the difference but this is something that needs further investigation. We are currently investigating the effects of in vitro digested berries and the individual polyphenols which are present in the bilberries to try and identify the active polyphenols.

There is also evidence that polyphenols and bilberry anthocyanins can affect the absorption of glucose across the intestine (Baron et al., 2017; Zou et al., 2014). This is thought to be mediated by active Na-dependent transport via sodium glucose co-transporter 1 (SGLT1) and facilitated Na-independent transport via GLUT2 (Levin, 1994). The Na⁺-dependent SGLT1-mediated glucose uptake appears to be inhibited by several phenolic acids (e.g. chlorogenic, ferulic and caffeic acids) (Welsch et al., 1989) as well as by glycosides of quercetin (Cermak et al., 2004). The glucose transport by GLUT2 was inhibited by the flavonols quercetin and myricetin (Johnston et al., 2005; Song et al., 2002). These polyphenols and flavonols with inhibitory activity against intestinal glucose uptake are common polyphenolic constituents of berries (Määttä-Riihinen, Kamal-Eldin, Mattila, González-Paramás, & Törrönen, 2004). To investigate this further we looked at the effect in vitro of the bilberry extract as well as the freeze dried wild bilberry extract on the uptake of fluorescently labelled 2-deoxyglucose uptake. Both the bilberry extract as well as the freeze dried wild bilberry inhibited glucose uptake in human CaCo-2 cells compared to the untreated control cells.

Thus both reduced breakdown of carbohydrates and lowered intestinal absorption of carbohydrates appear to be key events leading to the improved glycaemic excursion we observe in this study.

5. Conclusion

In conclusion, the ingestion of a concentrated bilberry extract over a three week period at amounts that can be easily tolerated produces a reduced AUCi postprandial glycaemia in overweight volunteers in

response to an OGTT. This suggests that this is the initial effect of the ingestion of the bilberry extract over the three week intervention period which may lead to changes in insulin response (Stull, 2016). We show that the reduced rates of carbohydrate digestion and/or absorption are the most likely cause of the observed decreases in glycaemic response.

In our intervention study no changes in faecal gut microbiota, plasma inflammatory/oxidative markers or markers of cardiovascular health were observed in volunteers taking bilberry extract compared with the control which suggests that these are potentially later events to the observed changes in glucose metabolism.

The use of berry polyphenols as phytochemicals capable of lowering the glycaemia response to carbohydrates not only in subjects with diabetes but also in those with impaired glucose tolerance control may prove to be useful in helping control blood sugar. Such a strategy could complement other lifestyle interventions such as avoiding being overweight and the need to take regular exercise.

Declaration of Competing Interest

The authors declare that they have no known competing financial

Appendix A

See Fig. A1.

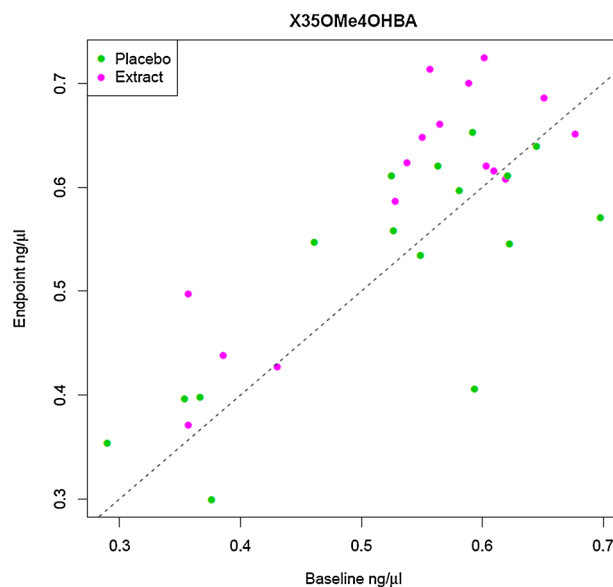


Fig. A1. Overweight volunteers ($n = 16$; BMI 31, SD 4), nine of whom have T2D controlled by diet and lifestyle alone, were given three oral capsules of either 0.47 g standardized bilberry extract (36% (w/w) anthocyanins) or placebo per day for three weeks in a double blinded cross over intervention. Samples were taken before the start of the three week intervention (baseline) and at the end (endpoint). Syringic acid (35OMe4OHBA) as measured by mass spectrometry showed a significant differences between placebo and extract (ANCOVA $p < 0.001$).

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interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement

I declare that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) in accordance with experiments involving humans

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