

## A double-blind, placebo-controlled, cross-over study to establish the bifidogenic effect of a very-long-chain inulin extracted from globe artichoke (*Cynara scolymus*) in healthy human subjects

Adele Costabile<sup>1\*</sup>, Sofia Kolida<sup>1</sup>, Annett Klinder<sup>1</sup>, Eva Gietl<sup>2</sup>, Michael Bäuerlein<sup>3</sup>, Claus Froberg<sup>2</sup>, Volker Landschütze<sup>2</sup> and Glenn R. Gibson<sup>1</sup>

<sup>1</sup>Food Microbial Sciences, School of Food and Nutritional Sciences, University of Reading, Reading RG6 6AP, UK

<sup>2</sup>Bayer BioScience GmbH, Hermannswerder 20a, 14473 Potsdam, Germany

<sup>3</sup>Bayer Cropscience AG, Alfred-Nobel-Strasse 50, 40789 Monheim, Germany

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There is growing interest in the use of inulins as substrates for the selective growth of beneficial gut bacteria such as bifidobacteria and lactobacilli because recent studies have established that their prebiotic effect is linked to several health benefits. In the present study, the impact of a very-long-chain inulin (VLCI), derived from globe artichoke (*Cynara scolymus*), on the human intestinal microbiota compared with maltodextrin was determined. A double-blind, cross-over study was carried out in thirty-two healthy adults who were randomised into two groups and consumed 10 g/d of either VLCI or maltodextrin, for two 3-week study periods, separated by a 3-week washout period. Numbers of faecal bifidobacteria and lactobacilli were significantly higher upon VLCI ingestion compared with the placebo. Additionally, levels of *Atopobium* group significantly increased, while *Bacteroides-Prevotella* numbers were significantly reduced. No significant changes in faecal SCFA concentrations were observed. There were no adverse gastrointestinal symptoms apart from a significant increase in mild and moderate bloating upon VLCI ingestion. These observations were also confirmed by *in vitro* gas production measurements. In conclusion, daily consumption of VLCI extracted from globe artichoke exerted a pronounced prebiotic effect on the human faecal microbiota composition and was well tolerated by all volunteers.

### Prebiotics: Bifidogenic effect: Gas production: Intestinal microflora

In the last decade functional foods in human and animal nutrition have gained in importance. Within this field of research, scientific concepts underpinning directed modulation of the human gut microbiota towards a more beneficial composition have been developed<sup>(1,2)</sup>. The prebiotic approach advocates targeting selected indigenous beneficial bacteria through non-viable food ingredients<sup>(3)</sup>. The concept was recently updated by Gibson *et al.*<sup>(4)</sup>, and the weight of evidence for established and emerging prebiotics reviewed<sup>(5)</sup>. Much of the interest in the development of prebiotics aims at non-digestible oligosaccharides. These are short-chain carbohydrates that consist of two to twenty saccharide units. Examples include inulin-type fructans, galacto-oligosaccharides, isomalto-oligosaccharides, xylo-oligosaccharides, soya-oligosaccharides, gluco-oligosaccharides and lacto-sucrose<sup>(6,7)</sup>, although each of these varies in their prebiotic potential.

Inulin and fructo-oligosaccharides are plant  $\beta$  (2-1) fructans with a degree of polymerisation (DP) ranging from 2 to 60 or more for inulin, or from 2 to 10 for oligofructose<sup>(8)</sup>.

There is a high degree of variability in DP distribution among products derived from different plant origins. Inulin derived from globe artichoke (*Cynara scolymus*) generally

has the highest DP<sup>(9)</sup> whereas onion, garlic, Jerusalem artichoke (*Helianthus tuberosus*), leeks, asparagus and banana have lower DP<sup>(8)</sup>. The inulin content of Jerusalem artichoke ranges from 17 to 20.5% on a fresh weight basis. Praznik *et al.*<sup>(10)</sup> found that 74% of Jerusalem artichoke inulin had a DP < 19 while 20% was between DP 19 and 40 and 6% had a DP > 40. The inulin content of chicory (*Chichorium intybus*), harvested from roots, ranged from 15.2 to 20.5%, of which 55% had a DP of 2 to 19, 28% with DP between 19 and 40, and 17% with DP > 40<sup>(8)</sup>. The globe artichoke root contains inulin in a range from 30.6 to 36.7% on a DM basis<sup>(11)</sup> with a maximum DP of about 200<sup>(12)</sup>.

DP distribution and molecular weight are influenced by the harvesting conditions and additionally the extraction process could influence the molecular weight distribution or composition of the inulin and fructo-oligosaccharides<sup>(13)</sup>. DP distribution and molecular weight in turn influence the physico-chemical properties of inulins such as solubility, thickening power and resistance to crystal formation.

Research, to date, on prebiotic potential has been limited to inulin-type fructans derived from chicory roots, whereas inulin or fructo-oligosaccharides derived from other plant sources,

**Abbreviations:** DP, degree of polymerisation; FISH, fluorescence *in situ* hybridisation; VLCI, very-long-chain inulin.

\* **Corresponding author:** Dr A. Costabile, fax +44 118 931 0080, email a.costabile@reading.ac.uk

such as globe artichoke, have received little attention. Only recently the interest in their tubers for food purposes has increased mainly due to their content of several potentially bio-active ingredients such as inulin and fructo-oligosaccharides, naturally occurring fructose, minerals, essential amino acids, vitamins and flavonoids<sup>(14)</sup>.

Very-long-chain inulin (VLCI) which is extracted from globe artichoke has an average DP of between 50 and 103 and is used for inhibiting the growth of ice crystals in frozen foodstuffs, preferably frozen confections. Its DP is considerably higher than that of the long-chain inulin Beneo<sup>®</sup>HP investigated so far. Beneo<sup>®</sup>HP is extracted from chicory roots by applying physical separation techniques to eliminate all oligomers resulting in an average DP higher than 23 but mainly lower than 60.

Inulin has thus far been reported to have a positive effect on the gut microbiota at doses ranging from 4 to 40 g/d<sup>(15–21)</sup>. Most physiological changes that inulin induces in the host are probably effected by bacterial metabolites. The most important metabolites are the SCFA acetate, propionate and butyrate<sup>(22)</sup>. SCFA acidify the colonic environment, which is beneficial for certain bacteria such as bifidobacteria and lactobacilli, and detrimental to the growth of potential pathogenic species<sup>(23,24)</sup>. SCFA are rapidly absorbed from the colon and are then metabolised in various tissues: butyrate by the colonic epithelium, propionate and acetate partly by the liver and acetate partly by muscle and other peripheral tissues<sup>(3,24)</sup>.

In the present study, the potential of VLCI to beneficially modulate the gastrointestinal microbiota and their activities was determined against maltodextrin (placebo). The objective was to assess the capacity of VLCI compared with the placebo to selectively increase numbers of bifidobacteria and lactobacilli in faeces.

Here, we present the findings of a double-blind, placebo-controlled cross-over study with thirty-two free-living healthy subjects randomised into two groups and fed either VLCI (10 g/d) or maltodextrin (10 g/d) for 3 weeks. Changes within the gut microbiota and fermentation profiles were determined in stool samples before and after treatment and gastrointestinal symptoms and stool characteristics were recorded. In order to corroborate the *in vivo* results on gas production without the bias of volunteer perception, the *in vitro* gas production kinetics in five randomly selected volunteers at baseline and after the 3 weeks of inulin or maltodextrin intake was also examined.

## Materials and methods

### *Molecular-weight analysis of very-long-chain inulin by gel permeation chromatography with light scattering and refractive index detection (GPC-RI-MALLS system)*

VLCI (no. WO/2009/000535 – Use of very long chain inulin for inhibiting the growth of ice crystals in a frozen foodstuff, patent of Bayer Cropscience AG, Monheim, Germany) and Beneo<sup>®</sup>HP (Orafti, Tienen, Belgium) were dissolved in extra-pure water at a concentration of 0.5% (w/v). The solutions were heated at 95°C for 30 min. Then, the polymers were analysed using the following devices: Alliance chromatography system (Waters Corp., Milford, MA, USA), DAWN-EOS light scattering detector (Wyatt Technology,

Santa Barbara, CA, USA) with  $\lambda_0 = 658$  nm and sixteen detectors in the range of angles from 14.4 to 163.3°C, K5 flow cell. The polymers were fractionated on a pre-column and three columns having the separation ranges of 300–10<sup>4</sup>, 5 × 10<sup>4</sup>–2 × 10<sup>6</sup> and 10<sup>6</sup>–10<sup>8</sup> (SUPREMA-Gel; PSS Polymer Standards Service GmbH, Mainz, Germany). For fractionation of the samples 100 µl of this solution were injected at a temperature of 30°C and a flow rate of 0.8 ml/min with 0.05 mM-NaNO<sub>3</sub> as the eluent. The Astra V 5.1.8.0 program (Wyatt Technology) was used to analyse the molecular-weight distribution of the samples.

### *Polymer chain distribution analysis of inulin by high-performance anion exchange chromatography coupled with pulsed amperometric detection (known as HPAEC-PAD)*

VLCI and Beneo<sup>®</sup>HP (Orafti) inulins (5%, w/v) were solubilised completely in boiling water and filtered through a 0.22 µm filter. Filtrates with a concentration of 0.02% inulin were used for analysis. To map VLCI profiles and to determine the DP, a Dionex series 500 ion chromatograph (DX 500; Dionex, Idstein, Germany) coupled with a pulsed amperometric detector (Carbo-Pac PA-100 column) was used. During the analysis, the carbohydrates were eluted with 150 mM-NaOH and 1 M-sodium acetate in 150 mM-NaOH solution being used as eluents A and B, according to the following time and composition program (eluents A and B were expressed as a percentage (v/v)): 0 to 5 min, 100; 5 to 40 min, 72 and 28; 40 to 70 min, 50 and 45; 70 to 75 min, 0 and 100; 75 to 80 min, 100; 80 to 85 min, 100 and 0; 85 to 90 min, 100. The flow rate was set at 1 ml/min. All mobile phases were kept under pressure by sparging with He in order to prevent the subsequent production of carbonate, which would act as a displacing ion and reduce retention times. Chromatographic data were collected and plotted using the Dionex 500 chromatography workstation.

### *Subjects*

Thirty-two healthy volunteers (aged 20–42 years, mean age 25 years; eighteen females, fourteen males) were recruited from the Reading area. Written consent was obtained from all participants and the study protocol was approved by the Ethics and Research Committee of the University of Reading.

Inclusion criteria for participation in the study were: aged 18–50 years; BMI of 20–30 kg/m<sup>2</sup>; good general health. Volunteers with evidence of physical or mental disease, major surgery, history of drug abuse, severe allergy or a history of severe abnormal drug reaction and smokers were excluded. Volunteers were also excluded if pregnant, lactating or planning pregnancy. Intake of an experimental drug within 4 weeks before study, former participation in a prebiotic, probiotic or laxative trial within 3 months, or use of antibiotics within 6 months before the study, chronic constipation, diarrhoea or other chronic gastrointestinal complaint (for example, irritable bowel syndrome) were all exclusion criteria. Any intake of drugs active on gastrointestinal motility, antibiotic treatment or any class of laxative was not permitted. Any medication taken throughout the duration of the study was recorded in diaries. Volunteers were instructed not to alter their usual diet or fluid intake during the trial periods;

however, they were asked to refrain from consuming prebiotics and probiotics including live yoghurts and fermented milk drinks.

#### *Dietary intervention study*

The dietary intervention study was performed in a double-blind, randomised, placebo-controlled cross-over manner. Thirty-two healthy volunteers were recruited onto the study, but one volunteer dropped out due to personal reasons. For a period of 14 d before the dietary intervention study, volunteers followed a restricted diet as described earlier. Thirty-one subjects were randomly allocated into one of two groups.

The first group ( $n$  18) consumed VLCI (10 g/d; Bayer BioScience GmbH, Hermannswerder, Potsdam, Germany) for 3 weeks, and then after a 3-week washout period, they consumed the placebo (maltodextrin; Roquette, Lestrem, France) (10 g/d) for 3 weeks. The second group ( $n$  13) received first the placebo (10 g/d) for 3 weeks, and then after a 3-week washout, they consumed VLCI (10 g/d) for another 3-week treatment period. Each product was given for 3 weeks, followed by 3-week washout periods during which no prebiotic was consumed. The composition of globe artichoke root (cv. Madrigal; Nunhems, Haelen, The Netherlands) derived VLCI was as follows: inulin, 97.5 %, moisture, 3.1 %, sugars (glucose, fructose, sucrose), 0.2 %; ash, 0.4 %.

All test products were packaged, labelled and randomised by Bayer BioScience GmbH (Hermannswerder, Potsdam, Germany) before the study, and the study was blinded to both the investigators in the University of Reading and the volunteers. Volunteers were asked to keep diaries throughout the study to record stool frequency, consistency (constipation, hard, formed, soft or diarrhoea), abdominal pain (none, mild, moderate or severe), intestinal bloating (none, mild, moderate or severe) and flatulence (none, mild, moderate or severe) on a daily basis. Any concomitant medication and adverse events were also recorded.

Faecal samples were collected from each volunteer at five different points, before and after each treatment: at baseline, before inulin intake (pre-VLCI), during VLCI intake, at washout 1 (post-VLCI), before maltodextrin (pre-maltodextrin), during maltodextrin intake, at washout 2 (post-maltodextrin).

#### *Collection and stool sample preparation*

Freshly voided faecal samples were stored in an anaerobic cabinet (10 % H<sub>2</sub>; 10 % CO<sub>2</sub>; 80 % N<sub>2</sub>) for no longer than 2 h before processing. Samples were diluted 1:10 (w/w) with PBS (0.1 M; pH 7.0) and mixed in a Stomacher 400 (Seward, Thetford, Norfolk, UK) for 2 min at normal speed.

#### *Enumeration of faecal microbial populations by fluorescence in situ hybridisation*

Fluorescence *in situ* hybridisation (FISH) was performed as described by Martín-Peláez *et al.*<sup>(25)</sup>. The composition of hybridisation and wash buffers depends on the rRNA-targeted oligonucleotide probe as reported in probeBase (<http://www.microbial-ecology.net/probebase>) and was used accordingly. Briefly, samples (375 µl) of 1:10 (w/w) stool sample suspension in PBS were fixed overnight at 4°C with 4 % (w/v)

filtered paraformaldehyde (pH 7.2) in a ratio of 1:4 (v/v), washed twice with filtered PBS (0.2 µm pore size), re-suspended in 300 ml of a PBS–ethanol mixture (1:1, v/v) and then stored at –20°C for up to 3 months. For the hybridisations, 20 µl of each sample were pipetted onto Teflon- and poly-L-lysine-coated, six-well (10 mm diameter each) slides (Tekdon Inc., Myakka City, FL, USA). To permeabilise the cells for use with probe Lab158, samples were treated with 50 µl of lysozyme (1 mg/ml in 100 mM-2-amino-2-hydroxy-methyl-propane-1,3-diol-HCl, pH 8.0) at 37°C for 15 min before being washed (2–3 s) in water and were finally dehydrated in the ethanol series (50 %, 80 % and 96 % (v/v) ethanol, 3 min each). The probe (5 ng/µl probe in hybridisation buffer) was applied onto the surface of each well. Hybridisations were performed for 4 h in an ISO20 oven (Grant Boekel, Cambridge, Cambs, UK). For the washing step, slides were placed in 50 ml of wash buffer containing 20 µl of 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/µl; Sigma, St Louis, MO, USA) for 15 min. They were then washed (2–3 s) in ice-cold water and dried under a stream of compressed air. Then 5 µl of antifade reagent (polyvinyl alcohol mounting medium with DABCO™ antifading; Sigma) were added to each well and a coverslip was applied. Slides were stored in the dark at 4°C (for a maximum of 3 d) until cells were counted under a Nikon E400 Eclipse microscope (Nikon, Kingston upon Thames Surrey, UK). DAPI slides were visualised with the aid of a DM 400 filter and probe slides with the aid of a DM 575 filter. All probes were Cy3-labelled and synthesised by Sigma Aldrich. Table 1 gives the details of probes used in the present study<sup>(26–33)</sup>.

#### *Faecal SCFA analysis*

Samples (1 ml) of 1:10 (w/w) stool sample suspension in PBS were dispensed into 1.5 ml tubes and centrifuged at 13 000 g for 5 min to pellet bacteria and other solids. Supernatant fractions were filtered using 0.2 µm polycarbonate syringe filters (Whatman International Ltd, Maidstone, Kent, UK) and injected (200 µl) into an HPLC system (Merck, Whitehouse Station, NJ, USA) equipped with refractive index (RI) detection. The column used was an ion-exclusion REZEX ROA organic acid column (Phenomenex, Inc., Macclesfield, Cheshire, UK) maintained at 85°C. Sulfuric acid in HPLC-grade water (0.0025 M) was used as the eluent and the flow rate was maintained at 0.5 ml/min<sup>(25)</sup>. The carbonic acids in the samples were quantified through calibration curves of lactic, acetic, propionic and butyric in concentrations ranging between 2.5 and 100 mM. Peaks were integrated using Atlas Lab managing software (Thermo Lab Systems, Mainz, Germany).

#### *In vitro rate of gas production*

A pressure technique<sup>(34)</sup> was used to monitor the cumulative and rate of gas production in five randomly selected volunteers participating in the feeding study at baseline, and upon completion of treatments (VLCI and maltodextrin).

Pre-reduced gut model culture medium (80 ml)<sup>(35)</sup> was dispensed into clean serum vials (125 ml working volume; Belco Glass Inc., Vineland, NJ, USA) in an anaerobic cabinet (10 % H<sub>2</sub>, 10 % CO<sub>2</sub>, 80 % N<sub>2</sub>) and sealed with a gas-impermeable butyl rubber septum and aluminium cap. Vials were

**Table 1.** Probes used for fluorescence *in situ* hybridisation analysis of bacterial populations in human faeces

Short name	Accession no.	Full name†	Sequence (5' to 3')	Target species	Reference
Bif164	pB-00037	S-G-Bif-0164-a-A-18	CATCCGGCATTACCACCC	Most <i>Bifidobacterium</i> spp. and <i>Parascardovia denticolens</i>	Langendijk <i>et al.</i> (1995) <sup>(26)</sup>
Lab158	ND	S-G-Lab-0158-a-A-20	GGTATTAGCAYCTGTTTCCA	Most <i>Lactobacillus</i> , <i>Leuconostoc</i> and <i>Weissella</i> spp.; <i>Lactococcus lactis</i> ; all <i>Vagococcus</i> , <i>Enterococcus</i> , <i>Melisococcus</i> , <i>Tetragenococcus</i> , <i>Catelliococcus</i> , <i>Pediococcus</i> and <i>Paralactobacillus</i> spp.	Harmsen <i>et al.</i> (1999) <sup>(27)</sup>
Bac303	pB-00031	S*-Bacto-0303-a-A-17	CCAATGTGGGGGACCTT	Most <i>Bacteroides sensu stricto</i> and <i>Prevotella</i> spp.; all <i>Parabacteroides</i> ; <i>Barnesiella viscericola</i> and <i>Odoribacter splanchnicus</i>	Manz <i>et al.</i> (1996) <sup>(28)</sup>
Chis150	pB-00962	S*-Chis-0150-a-A-23	TTATGCGGTATTAATCTYCCTTT	Most members of <i>Clostridium</i> cluster I; all members of <i>Clostridium</i> cluster II; <i>Clostridium tyrobutyricum</i> ; <i>Adhaeribacter aquaticus</i> and <i>Flexibacter canadensis</i> (family Flexibacteriaceae); ( <i>Eubacterium</i> ) <i>combesii</i> (family Propionibacteriaceae)	Franks <i>et al.</i> (1998) <sup>(29)</sup>
Rbro730‡	pB-00558	S*-Rbro-730-a-A-18	TAAAGCCCAGYAGGCCGC	<i>Ruminococcus bromii</i> -like; <i>Clostridium sporosphaeroides</i> and <i>Clostridium leptum</i>	Harmsen <i>et al.</i> (2002) <sup>(30)</sup>
Rfla729‡	pB-00557	S*-Rfla-729-a-A-18	AAAGCCCAGTAAGCCGCC	<i>Ruminococcus albus</i> and <i>Ruminococcus flavefaciens</i>	Harmsen <i>et al.</i> (2002) <sup>(30)</sup>
Ato291	pB-00943	S*-Ato-0291-a-A-17	GGTCGGTCTCTCAACCC	<i>Atopobium</i> , <i>Colinsella</i> , <i>Olsenella</i> and <i>Eggerthella</i> spp.; <i>Cryptobacterium curtum</i> ; <i>Mycoplasma equigenitalium</i> and <i>Mycoplasma elephantis</i>	Harmsen <i>et al.</i> (2000) <sup>(31)</sup>
Erec482	pB-00963	S*-Erec-0482-a-A-19	GCTTCTTAGTCARGTACCG	Most members of <i>Clostridium</i> cluster XIVa; <i>Syntrophococcus sucromutans</i> , ( <i>Bacteroides</i> ) <i>galacturonicus</i> and ( <i>Bacteroides</i> ) <i>xylanolyticus</i> , <i>Lachnospira pectinschiza</i> and <i>Clostridium saccharolyticum</i>	Franks <i>et al.</i> (1998) <sup>(29)</sup>
Ehal1469	pB-00554	ND	CCA GTT ACC GGC TCC ACC	<i>Eubacterium hallii</i> , <i>Clostridium herbivorans</i> , <i>Clostridium polysaccharolyticum</i>	Harmsen <i>et al.</i> (2002) <sup>(30)</sup>
Ecy1387	pB-00556	ND	CGC GGC ATT GCT CGT TCA	<i>Eubacterium cylindroides</i> , <i>Clostridium innocuum</i> , <i>Eubacterium bifforme</i> , <i>Eubacterium tortuosum</i> , <i>Eubacterium dolichum</i> , <i>Streptococcus pleomorphus</i>	Harmsen <i>et al.</i> (2002) <sup>(30)</sup>
Eub I-II-III	pB-00159-160-161	S-D-Bact-0338-a-A-18	GCT GCC TCC CGT AGG AGT	Total bacteria	Amann <i>et al.</i> (1990) <sup>(32)</sup>
Clit135	pB-00961	*-Clit-0135-a-A-19	GTTATCCGTGTGTACAGG	Some of the <i>Clostridium lituseburense</i> group ( <i>Clostridium</i> cluster XI)	Franks <i>et al.</i> (1998) <sup>(29)</sup>
Rrec584	ND	ND	TCAGACTTGCCG(C/T)ACCGC	<i>Roseburia</i> – <i>Eubacterium rectale</i> (a component of cluster XIVa)	Walker <i>et al.</i> (2005) <sup>(33)</sup>
EC1531	ND	L-S-Eco-1531-a-A-21	CACCGTAGTGCCTCGTCATC	<i>Escherichia coli</i>	Walker <i>et al.</i> (2005) <sup>(33)</sup>
Prop853	ND	ND	ATTGCGTAACTCCGGCAC	<i>Clostridium</i> cluster IX	Walker <i>et al.</i> (2005) <sup>(33)</sup>

ND, no information relating to these probes has been deposited in probeBase (<http://www.microbial-ecology.net/probebase>).

† Probe designation according to probeBase.

‡ These probes were used together in equimolar concentrations (both at 50 ng/μl). Formamide (20%) was included in the hybridisation buffer.



autoclaved and placed overnight in a 37°C incubator. The culture medium consisted of the following (g/l) dissolved in distilled water: starch (potato), 5.0; pectin (citrus), 2.0; guar gum, 1.0; mucin (porcine gastric type III), 4.0; xylan (oatspelt), 2.0; arabinogalactan (larch wood), 2.0; inulin (Beneo®ST; Orafiti), 1.0; casein (BDH Ltd, Poole, Dorset, UK), 3.0; peptone water (Oxoid, Basingstoke, Hants, UK), 5.0; tryptone (Oxoid), 5.0; bile salts no. 3 (Oxoid), 0.4; yeast extract (Oxoid), 4.5; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.005; NaCl (Fisher Scientific, Springfield, NJ, USA), 4.5; KCl (VWR International, Lutterworth, Leics, UK), 4.5; KH<sub>2</sub>PO<sub>4</sub>, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.25; CaCl<sub>2</sub>·6H<sub>2</sub>O, 0.15; NaHCO<sub>3</sub>, 1.5; cysteine HCl, 0.8; hemin, 0.05; Tween 80, 1.0 ml; vitamin K<sub>1</sub>, 10 µl. Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK).

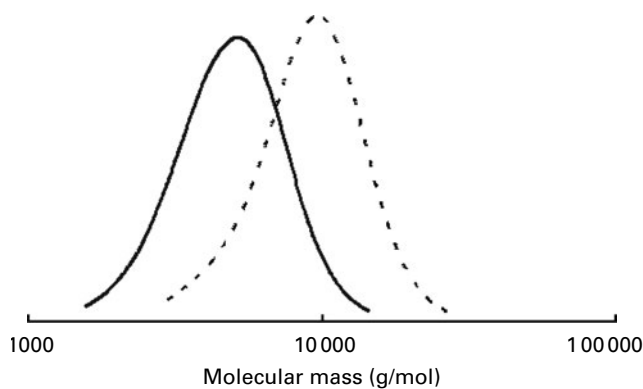
Each vial containing a volume of 90 ml was inoculated with 10 ml of 1:10 diluted faecal slurry, prepared as above, via a sterile syringe, giving a final volume of 100 ml.

The gas production potential of the faecal flora after treatment was evaluated by recording the head space pressure (pounds per square inch; psi) of each vial. Seven replicate vials were prepared for each donor. Vials were incubated at 37°C and were continuously shaken. Pressure readings were obtained every 3 h for a 27 h fermentation period by piercing the rubber caps with a U200/66 needle adaptor connected to a pressure transducer (type 2200BGF150WD3DA; Keller Ltd, Dorchester, Dorset, UK) with a T443A digital panel meter (Bailey and Makey Ltd, Birmingham, UK). Pressure readings (psi) were converted into gas volume (ml) using an established linear regression of pressure recorded in the same vials with known air volumes at the incubation temperature. Gas production data were fitted to the model proposed by France *et al.*<sup>(36)</sup> using the program CurveExpert (version 1.37; Daniel G Hyams, Hixson, TN, USA) to provide parameters describing cumulative gas release in terms of potential gas production (A; ml), lag time (T; h), time to half-asymptote (T/2; h) and fractional rate of gas production at T/2 (µ; per h).

#### Statistical analysis

A general linear model was used to test for time-dependent effects (Genstat® for Windows® 8th edition; VSN International, Hemel Hempstead, Herts, UK). When there was no significant effect for 'sequence' all time points were included and subsequent statistical analyses were performed using one-way ANOVA, including *post hoc* tests appropriate for the individual datasets (Tukey's post test with significance set at  $P < 0.05$ ). We used GraphPad Prism (version 5.0; Graph-Pad Software, Inc., San Diego, CA, USA) for FISH, SCFA and gas production analysis.

The occurrence and intensity of gastrointestinal symptoms were scored in the run-in period during the intervention period (3 weeks). Volunteers were requested to rank their wellbeing with respect to gastrointestinal complaints, such as abdominal pain, intestinal bloating, flatulence, nausea, borborygmi, diarrhoea and constipation. The respective ranks given were: 0 = none; 1 = mild; 2 = moderate; 3 = severe. Moreover, stool frequency and consistency were also monitored and ranked as follows: 0 = watery/diarrhoeal; 1 = soft; 2 = normal; 3 = hard. Statistical analyses were also conducted using GraphPad Prism (version 5; Graph-Pad Software, Inc.).



**Fig. 1.** Gel permeation chromatography with light scattering and refractive index detection (GPC-RI-MALLS system; Mainz, Germany). (—), Beneo®HP (Orafiti, Tienen, Belgium); (---), globe artichoke (*Cynara scolymus*) inulin (very-long-chain inulin).

The average cumulative gas production *in vitro* was fitted iteratively to the model<sup>(36)</sup>, using the program CurveExpert (version 1.37; Daniel G Hyams).

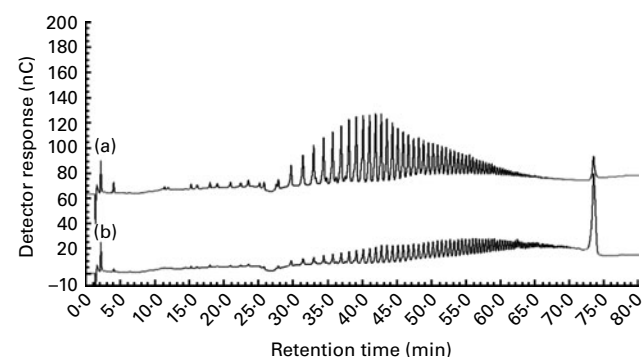
#### Results

##### *Molecular-weight analysis of very-long-chain inulin by gel permeation chromatography with light scattering and refractive index detection (GPC-RI-MALLS system)*

GPC-RI-MALLS chromatograms of VLICI and commercially available chicory root inulin (Beneo®HP) are shown in Fig. 1. It is obvious that VLICI has a markedly higher molecular-weight distribution compared with Beneo®HP. The average DP of VLICI was >55 as compared with >23 in the case of Beneo®HP.

##### *Polymer chain distribution analysis of inulin by high-performance anion exchange chromatography coupled with pulsed amperometric detection (known as HPAEC-PAD)*

The inulin profiles of chicory (Beneo®HP) and globe artichoke tubers (VLICI) were separated into defined DP ranges. As shown in Fig. 2, chicory inulin (Fig. 2(a)) contains a much higher amount of shorter molecules as compared with globe artichoke inulin (Fig. 2(b)) and the range of high-DP species detected in the VLICI profile clearly exceeds that of chicory.



**Fig. 2.** High-performance anion-exchange chromatography with pulsed amperometric detection (DX 500 Dionex, Idstein, Germany) of (a) Beneo®HP (Orafiti, Tienen, Belgium) and (b) globe artichoke (*Cynara scolymus*) inulin (very-long-chain inulin).

*Faecal microflora*

The main objective of the study was to assess the impact of consumption of inulin extracted from globe artichoke on the intestinal microbiota. Fourteen 16S rRNA-based FISH probes were used to target predominant groups or species of human faecal bacteria (Table 1) covering 70–80.6 % of total bacteria detected in stool samples. Bacterial numbers at the end of each treatment period – either 10 g VLICI/d or 10 g maltodextrin/d – were compared with samples obtained before treatment (pre-) and after washout (post-) of the respective treatment. Statistical significance of the results was evaluated using one-way ANOVA with Tukey’s post test (95 % CI). Average faecal bacterial numbers for thirty-one healthy volunteers over the trial period as determined by FISH and expressed as log<sub>10</sub> cells/g faeces (*n* 31) are shown in Table 2.

Total bacterial levels (detected by Eub I-II-III) remained unaffected by intervention. A significant increase in *Bifidobacterium* numbers was observed during inulin intake compared with samples taken immediately before inulin intervention (2.82-fold increase; *P*<0.05). There was also a significant difference in bacterial population levels in faeces when comparing inulin and maltodextrin treatment samples. Numbers of bifidobacteria were significantly higher following ingestion of inulin compared with the maltodextrin treatment period (2.75-fold increase; *P*<0.001).

Numbers of faecal lactobacilli–enterococci increased significantly with ingestion of VLICI compared with pre-inulin levels (2.42-fold increase; *P*<0.01) and the increase was even more pronounced when compared with maltodextrin (5.88-fold increase; *P*<0.05) in thirty-one volunteers. During the ingestion of maltodextrin, the *Lactobacillus*–*Enterococcus* group significantly decreased with respect to pre-maltodextrin (2.64-fold decrease; *P*<0.001).

No significant changes were observed between pre-treatment and post-treatment samples for bifidobacteria as well as for lactobacilli counts.

A significant increase in the *Atopobium* group was observed (2.83-fold increase; *P*<0.05) whereas numbers of the *Bacteroides*–*Prevotella* group were reduced (1.77-fold decrease; *P*<0.05) after consumption of VLICI compared with maltodextrin (Table 2).

No differences were noted in the levels of *Escherichia coli* and the *Eubacterium rectale*–*Clostridium coccooides* group enumerated by the Erec482 probe. The Ehal1469 probe specific for subgroups within the Erec482 (*Clostridium* cluster XIVa) group did not show significant differences between treatment and placebo. Similarly, the butyrate-producing *E. rectale*–*Roseburia* group, also a component of cluster XIVa, was present at no more than 10 % of the total *E. rectale*–*C. coccooides* group at all time points analysed, when enumerated by Rrec584.

Cluster IX representatives (detected by Prop853) and *Ruminococcus* numbers (*Clostridium* cluster IV, detected by the Rfla729/Rbro730 probes) did not exhibit any statistically significant changes upon ingestion either of the placebo or VLICI (*P*>0.05). The Clit135 probe, specific for some members of the *C. lituseburensense* group (*C. lituseburensense* group – cluster XI) and *C. histolyticum* accounted for no more than 1 % of the total faecal bacteria population.

Specific members of *Clostridium* cluster XVI (detected by the Ecy1387 probe) did not exhibit changes throughout the duration of the intervention study (*P*>0.05).

*SCFA concentrations*

Table 3 shows the concentrations of faecal SCFA. No significant changes in faecal concentrations of acetic, propionic

**Table 2.** Average faecal bacterial numbers for thirty-one healthy volunteers over the trial period as determined by fluorescence *in situ* hybridisation (FISH), expressed as log<sub>10</sub> cells/g faeces (Mean values and standard deviations)

FISH probe name	Pre-VLICI		VLICI		Post-VLICI		Pre-maltodextrin		Maltodextrin		Post-maltodextrin	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Eub I-II-III	10.84	0.1356	10.94	0.1310	10.83	0.1768	10.81	0.1806	10.93	0.1574	10.86	0.1554
Bac303	9.696	0.2489	9.608	0.2817	9.534	0.2274	9.609	0.2027	9.856†	0.3680	9.539	0.1864
Ecy1387	9.132	0.2386	9.132	0.2386	9.182	0.2284	9.155	0.2426	9.250	0.2740	9.210	0.2584
Erec482	9.749	1.643	9.812	1.649	9.857	1.651	10.06	0.2217	10.12	0.2670	9.813	1.648
Bif164	9.231	0.3441	9.681*	0.2191	9.354†	0.2823	9.276	0.3206	9.241†	0.2799	9.323	0.2175
Ato291	9.225	0.4536	9.677*	0.3246	9.413	0.5510	9.547	0.4569	9.419†	0.2705	9.727	0.2275
Clit135	7.714	0.2786	7.767	0.3538	7.866	0.3307	7.861	0.3894	7.779	0.3336	7.588	0.3583
Lab158	8.536	0.2044	8.919*	0.2301	8.373	0.2160	8.570	0.2877	8.149†§	0.3234	8.233	0.2747
EC1531	7.188	0.3784	7.204	0.4624	7.183	0.5049	7.193	0.4155	7.142	0.5030	7.236	0.5377
Prop853	9.363	0.3213	9.455	0.2216	9.294	0.2885	9.302	0.2728	9.417	0.2489	9.225	0.3079
Chis150	7.727	0.2669	7.739	0.4365	7.830	0.3587	7.442	0.3689	7.755	0.2988	7.962	0.3117
Rbro730-Rfla729‡	9.894	0.2174	9.803	0.3043	9.684	0.3496	9.747	0.3119	9.672	0.3304	9.644	0.2315
Ehal1469	8.859	0.2889	8.840	0.2664	8.807	0.3305	8.842	0.3012	8.851	0.3075	8.846	0.2815
Rrec584	9.583	0.2470	9.612	0.2702	9.575	0.2749	9.566	0.3059	9.549	0.3070	9.552	0.1890

VLICI, very-long-chain inulin.

\* Mean value was significantly different from that for pre-VLICI (*P*<0.05; Tukey’s post test).

† Mean value was significantly different from that for VLICI (*P*<0.05; Tukey’s post test).

‡ Mean value was significantly different from that for post-VLICI (*P*<0.05; Tukey’s post test).

§ Mean value was significantly different from that for pre-maltodextrin (*P*<0.05; Tukey’s post test).

|| Mean value was significantly different from that for maltodextrin (*P*<0.05; Tukey’s post test).

**Table 3.** SCFA concentrations measured by HPLC present in faecal samples collected from thirty-one volunteers over the course of the trial (Mean values and standard deviations)

SCFA (mM)	Pre-VLCI		VLCI		Pre-maltodextrin		Maltodextrin	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acetic acid	29.08	16.45	29.68	16.71	28.57	14.73	35.4	23.04
Propionic acid	8.518	6.239	10.19	6.505	9.252	6.88	11.21	5.711
Butyric acid	5.299	6.061	7.779	7.825	7.442	5.651	10.95	14.04

VLCI, very-long-chain inulin.

and butyric acids were observed over the course of the trial, neither between pre-inulin and inulin and maltodextrin and pre-maltodextrin nor when inulin or maltodextrin were compared.

#### Analysis of bowel habit and gastrointestinal symptoms

Table 4 summarises data on bowel habit and gastrointestinal symptoms, as recorded by the volunteers during intake of VLCI or maltodextrin. Stool frequency did not differ during inulin ingestion compared with placebo. Stool consistency, qualitatively graded by volunteers as hard, formed, or soft, varied greatly between individuals. No diarrhoea was reported by any subjects, while there was a significant increase in bloating upon ingestion of VLCI ( $P < 0.05$ ). However, on average, even the increased bloating was still rated as mild. There were no significant differences between treatments with regard to flatulence and abdominal pain. None of the gastrointestinal symptoms was severe enough for volunteers to discontinue the study.

#### Gas production kinetics

Fig. 3 shows the cumulative gas production at 3 h intervals for a 27 h fermentation period, in five randomly selected volunteers participating in the feeding study upon completion of treatments with VLCI and maltodextrin. In general, VLCI showed higher gas volumes than maltodextrin from 5 h onwards. There was a significant increase in the rate of gas production pattern during the first 5 h of *in vitro* fermentation, in particular with faecal samples after the intake of VLCI compared with the maltodextrin (Fig. 3).

Both treatment groups appeared to generate gas at 3 h after inoculation of faecal slurry and up to 27 h (Fig. 3).

The cumulative gas production after maltodextrin treatment was significantly lower up to 27 h fermentation as compared with VLCI. Apart from volunteer 4, samples of the other volunteers generated similar levels of cumulative gas production during the 24 h fermentation period. Volunteer 4 had markedly higher rates of gas production compared with the other donors.

The gas production pattern over the 27 h of incubation and the results of the cumulative gas production are presented in Table 5. Samples after VLCI intervention generated higher gas volume production (A) than after maltodextrin intervention with lower lag time and fractional degradation rate at 12 and 24 h. These data are in accordance with bloating ratings recorded in the volunteers' diaries during the course of the trial.

#### Discussion

The bifidogenic nature of chicory inulin is very well established<sup>(3,18,37–44)</sup>. Positive shifts in the composition of microflora have been shown *in vivo* in human studies at doses ranging from 4 to 40 g/d<sup>(15–21)</sup>. While the majority of studies to date have investigated the effect of chicory inulin on the human gut microbiota using culture-based methodologies<sup>(3,18,19,45,46)</sup>, more recent studies have applied molecular methods such as FISH and real-time PCR for the bacterial analyses<sup>(14,47–55)</sup>.

However, the *in vivo* effect of globe artichoke-derived inulin in human subjects has not been studied to date. It is clear that globe artichoke inulin differs in DP distribution as well as in average DP<sup>(8)</sup>. Recent *in vitro* studies reported that DP may be a factor affecting not only the prebiotic efficacy of a substrate, but also its persistence in the colon and the manifestation of undesirable gastrointestinal side effects<sup>(56,57)</sup>. The inulin with the highest average DP to

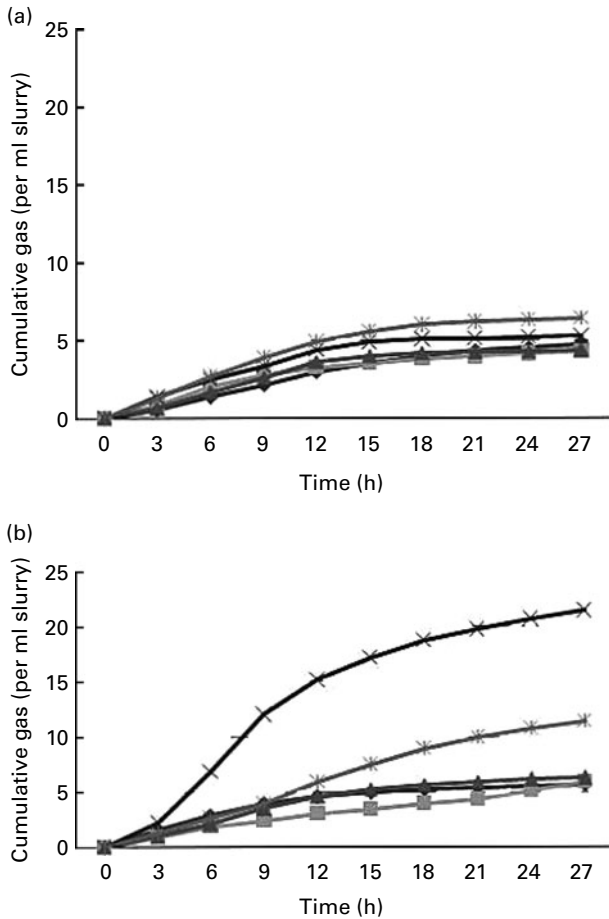
**Table 4.** Summary of bowel habit and gastrointestinal symptom data for thirty volunteers over the course of the trial† (Mean values and standard deviations)

	Bowel habit and gastrointestinal symptoms									
	Stool number (per d)		Stool consistency		Abdominal pain		Intestinal bloating		Flatulence	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
VLCI	1.34	0.64	1.30	0.47	1.13	0.86	1.33*	0.76	1.24	0.63
Maltodextrin	1.45	0.57	1.27	0.45	0.83	0.59	0.87	0.57	1.20	0.55

VLCI, very-long-chain inulin.

\* Mean value was significantly different from that for maltodextrin ( $P < 0.05$ ; paired *t* test, two-tailed).

† Analysis was performed by allocating numbers to defined conditions. Stool consistency: hard = 0; formed = 1; soft = 2. Abdominal pain, bloating and flatulence: none = 0; mild = 1; moderate = 2; severe = 3.



**Fig. 3.** Cumulative gas production over 27h with maltodextrin (a) and very-long-chain inulin (b). Values are means of five volunteers (seven replicates for each volunteer on each respective treatment). (—◆—), Volunteer 1; (—■—), volunteer 2; (—▲—), volunteer 3; (—×—), volunteer 4; (—\*—), volunteer 5.

be studied *in vivo* in human subjects so far has been Beneo® HP<sup>(18,54)</sup>.

Using direct molecular-based enumeration of faecal bacteria, we have shown that VLCI derived from globe artichoke selectively stimulates the levels of faecal bifidobacteria. This is concordant with other *in vivo* intervention studies with fructans which often reported increases between 0.5–1.0 log<sub>10</sub> bifidobacteria counts<sup>(58)</sup>. However, the magnitude of increase seems to depend on the baseline values, and higher increases

are often observed in volunteers with low initial bifidobacteria numbers<sup>(55)</sup>. While the bifidogenic potential is well proven, the effect of consumption of inulin on other bacterial groups is less well established. A decrease in Clostridia was mainly reported by culture-based bacteria analysis<sup>(3,47)</sup>; however, a recent study which used FISH also found decreased bacteria numbers for the *C. histolyticum* group and *C. lituseburensis* group after inulin ingestion<sup>(14)</sup>. In the same study<sup>(14)</sup> there was also a decrease in *Bacteroides* numbers after inulin consumption. We also found that *Bacteroides* numbers were significantly lower after VLCI than after maltodextrin intervention. Additionally, we observed a significant increase in lactobacilli–enterococci after VLCI treatment. While there was a small increase after inulin consumption in one human intervention trial<sup>(59)</sup> and some indication from *in vitro* fermentation studies with inulins and fructo-oligosaccharides<sup>(60,61)</sup> that the lactobacilli–enterococci group could be influenced by inulin, this is the first time that such a high increase in this bacterial group has been reported for inulin in a human intervention study. The only other bacterial group in the present study being stimulated after VLCI ingestion was *Atopobium*. The role of *Atopobium* in the human colon is not clear. However, they are commonly isolated from healthy human faeces and are present at 1.5–3% of the total faecal populations<sup>(62)</sup>. It is not clear from the results of the present study whether *Atopobium* can ferment VLCI or if the increase is due to cross-feeding between different bacterial groups. It is noteworthy that the increase in bifidobacteria and lactobacilli–enterococci in the present study were paralleled by a decrease in the levels of potentially harmful bacteria such as *Bacteroides–Prevotella*. As total bacterial levels remained unchanged throughout the study, changes upon VLCI ingestion appear to be inter-population only.

No differences were observed in faecal SCFA throughout the study. This is not surprising, as over 95% of SCFA produced in the human large intestine are thought to be rapidly absorbed within the colon, and only a small proportion of all SCFA produced in the large bowel is excreted in the faeces<sup>(63,64)</sup>.

Several studies have shown that fermentation of inulin-type fructans may stimulate bowel movements and may normalise stool frequency<sup>(18–19,21)</sup>. Increased bloating and mild flatulence upon inulin-type fructan ingestion is a well-known and accepted side effect<sup>(19,20,64–67)</sup>.

Analysis of the gastrointestinal symptom volunteer diaries indicates a significant increase in intestinal bloating during VLCI intake in healthy volunteers. The formation of H<sub>2</sub>,

**Table 5.** Gas production kinetics obtained by the *in vitro* fermentation of very-long-chain inulin (VLCI) and maltodextrin\*

Parameters...	A		T		μ <sub>12</sub>		μ <sub>24</sub>	
	VLCI	Maltodextrin	VLCI	Maltodextrin	VLCI	Maltodextrin	VLCI	Maltodextrin
1	6.06 <sup>c</sup>	4.62 <sup>c</sup>	1.28 <sup>d</sup>	1.22 <sup>b</sup>	0.046 <sup>a</sup>	–0.002 <sup>d</sup>	0.032 <sup>a</sup>	–0.001 <sup>b</sup>
2	6.53 <sup>c</sup>	5.40 <sup>b</sup>	0.03	1.75 <sup>a</sup>	0.008 <sup>b</sup>	0.065 <sup>a</sup>	0.005 <sup>b</sup>	0.046 <sup>a</sup>
3	6.51 <sup>c</sup>	4.76 <sup>c</sup>	1.61 <sup>c</sup>	1.84 <sup>a</sup>	–0.006 <sup>c</sup>	–0.010 <sup>c</sup>	–0.005 <sup>c</sup>	–0.007 <sup>d</sup>
4	23.09 <sup>a</sup>	5.61 <sup>b</sup>	1.88 <sup>b</sup>	0.58 <sup>c</sup>	–0.008 <sup>c</sup>	0.005 <sup>b</sup>	–0.005	0.003 <sup>c</sup>
5	14.08 <sup>b</sup>	6.65 <sup>a</sup>	2.33 <sup>a</sup>	1.12 <sup>b</sup>	–0.006 <sup>c</sup>	–0.002 <sup>d</sup>	–0.004 <sup>c</sup>	–0.00 <sup>b</sup>

a,b,c,d Values within a column with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Parameters obtained from the adjustment of gas production by France *et al.*<sup>(36)</sup>. Potential gas production (A; ml); lag time (T; h), time to half-asymptote (T/2; h) and fractional rate of gas production at T/2 (μ; per h).



which is a metabolic endproduct of bacterial fermentation in the colon, is probably the major cause of this symptom<sup>(14)</sup>.

However, it has to be emphasised that bifidobacteria, the numbers of which increased significantly with inulin, are not gas producers. Several studies have reported increased gas production upon the ingestion of inulin, which is not accompanied by a concomitant increase in gas-producing bacterial groups and the authors have commonly attributed this effect to consumer perception<sup>(54,55)</sup>. Therefore the gas-generating potential of samples obtained from a group of randomly selected volunteers was also followed *in vitro*, at baseline and at the end of each treatment.

The *in vitro* observations confirmed the *in vivo* reports, as after ingestion of VLICI the rate of gas production in faeces increased and led to higher cumulative gas levels compared with the maltodextrin faeces. VLICI selectively increased the faecal levels of bifidobacteria and lactobacilli–enterococci *in vivo*, whilst Clostridia, which are prolific gas producers in faeces, remained stable. However, increased gas production may be due to increased or altered metabolic activity of these bacteria facilitated probably by a higher availability of potential substrates such as acetate. Bacterial enumeration cannot account for possible increases in metabolic activity. Furthermore, there may be shifts at species levels promoting the growth of strains that possess higher gas-producing capacities. Further clarification of the relationship between specific intestinal bacteria and gas production as a result of colonic fermentation is required. In general, both maltodextrin and VLICI treatments were well accepted by the human volunteers and all subjects completed the study without severe side effects being noted.

The prebiotic ability of a VLICI may have further implications *in vivo*. While short-chain fructans are more rapidly fermented<sup>(61)</sup>, higher molecular weight and low solubility may improve persistence in the gut<sup>(56,68)</sup>. Thus, due to its physico-chemical characteristics – high molecular weight and low solubility – VLICI is expected to prolong saccharolytic fermentation into distal colonic areas and *in vivo* animal studies are under way to investigate the effect of VLICI on the site of fermentation and the selectivity compared with Beneo<sup>®</sup>HP.

In conclusion, the present study has demonstrated the prebiotic efficacy of VLICI from globe artichoke, which combined significant increases in the levels of both bifidobacteria and lactobacilli in a selective manner with little adverse gastrointestinal effects at 10 g/d.

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A. C. was the principal investigator for conducting the experiment and writing the manuscript. A. K. contributed to data interpretation, manuscript preparation and the literature search. S. K. contributed to the analysis of *in vitro* rate of gas production. G. R. G. supervised the study design along with drafting the manuscript. E. G. contributed to the analysis of VLICI by the GPC-RI MALLS and HPAEC-PAD methods. V. L. was the overall coordinator of the VLICI project. M. B. was responsible for the preparation of the VLICI material

(harvest, isolation, purification, quality check). C. F. was involved in the functionality testings of VLICI. All other authors provided suggestions, alterations to the manuscript and comments for the analyses.

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