HYFFI (444270) Final report

The overall aim of the project is to realize a commercial opportunity to produce low molecular weight polysaccharides (LMWP) from alginate- and agar-bearing seaweeds for applications in food & health, and wellness products. In order to exploit the use of agars and alginates in this area a number of technological and scientific problems need to be solved and these are addressed within the project.

1. Development of novel LMWP derivatives of agar and alginates (WP2)

1.1. Laboratory scale production

The aim of this research was to develop reproducible laboratory and pilot scale processes for the depolymerisation of agar and alginate substrates in order to produce low molecular weight food grade powders for assessment in vitro and in vivo for prebiotic potential.

Development of laboratory scale processes was based on two key considerations: (i) all processes had to be transferable to pilot scale production if necessary and (ii) the resulting products had to be cold soluble and of sufficient organoleptic quality to be incorporated into a delivery product for the human intervention study.

Starting materials used

Agar and alginate starting materials are detailed in Table 1 and consisted of (i) commercially available high molecular weight (HWM) alginate and agar powders and (ii) source seaweeds containing either alginate (*Ascophyllum nodosum*) or agar (*Gelidium sequipidale & Gracillaria spp*). Two different alginate powders were used. Alginate powder 1 (Manugel DMB) was used for all preliminary work and laboratory development. Alginate powder 2 (Manucol DM) was identified later in the project and was used for optimisation of protocols and scale up activities and for the final production of the LMW product.

Manucol DM alginate has a high content of alginate that is derived from Irish and Icelandic sourced *Ascophyllum nodosum*. To the best of our knowledge there are no commercially available alginates containing higher quantities of *Ascophyllum nodosum* alginate and none containing Scottish material.

The initial concentration of starting material was pre-defined by the necessity to spray dry or roller dry the end products. As both drying processes operate more efficiently and cost effectively when the solids content of the material being dried is high (approx 10-30%), initial concentrations of 10 – 30% were assessed here.

Table 1. Starting materials used for laboratory scale production.

Substrate	Supplier	CC Number
Alginate Powder 1 - Manugel DMB	FMC Biopolymer	1721
Alginate powder 2 - Manucol DM	FMC Biopolymer	2015
Agar powder from Gracilaria spp	Industrias Roko (RGM 08040285)	1617
Agar powder from *Gelidium sesquipidale*	Industrias Roko (RG 07110351)	1618
Ascophyllum nodosum seaweed	Hebridean Seaweed Company	1616
Gracilaria spp. seaweed	Industrias Roko	1619
Gelidium sesquipidale seaweed	Industrias Roko	1620

Depolymerisation methods used

Three different depolymerisation techniques were used:

- mild acid hydrolysis using 1M acetic acid
- free radical hydrolysis using Fenton's reagent – iron II sulphate and hydrogen peroxide (H2O2)
- free radical hydrolysis using ascorbic acid and hydrogen peroxide (H2O2)

A reduction in viscosity was taken as indicative of depolymerisation in those reactions involving alginate. Viscosity was measured in centipoise (cps) using a Brookfield digital viscometer at a speed of 30rpm. Whereas, a reduction in gel break strength was taken as indicative of depolymerisation in those reactions involving agar. Gel break strength was measured using a Stable Micro Systems TA-XT2i texture analyser in g/cm2 .

Molecular weight analysis of starting materials (agar and alginate powders only) and all LMW end products was conducted using SEC MALLS - Size Exclusion Chromatography Multi-angle Laser Light Scattering at the Owen Glyndwr University, UK. It was not possible to determine the MW of the seaweed raw materials as the substrates were far to complex.

Laboratory scale processes

All reaction conditions were experimentally derived and then optimised in the laboratory. The baseline process for each depolymerisation method is as follows:

Acid hydrolysis – 10% - 20% (w/v) mixture of powder or seaweed made up in 1M acetic acid; reacted under optimal conditions in heated water baths, intermittent stirring; cooled; neutralised with potassium hydroxide; blended and roller dried.

Free radical degradation with Fentons reagent - 10% - 20% (w/v) mixture of powder or seaweed made up in DI water; 0.04% (w/v) $FeSO_4$ added; 0.75% - 3.5% (w/v) hydrogen peroxide added (30% puriss grade); reacted until hydrogen peroxide exhausted under optimal conditions in heated water baths, intermittent stirring; residual hydrogen peroxide checked using Quantofix TM peroxide indicator strips; cooled; blended and roller dried.

Free radical degradation with Ascorbic acid - 10% - 20% (w/v) mixture of powder or seaweed made up in DI water; 0.05% (w/v) ascorbic acid added; 0.3% - 1% (w/v) hydrogen peroxide added (30% puriss grade); reacted until hydrogen peroxide exhausted under optimal conditions in heated water baths, intermittent stirring; residual hydrogen peroxide checked using Quantofix TM peroxide indicator strips; cooled; blended and roller dried.

The molecular weight of all LMW products was determined using size exclusion chromatography multi angle laser light scattering (SEC-MALLS) . 19 LMW products were sent for *in vitro* screening at the University of Reading (Table. 2).

The use of a sodium alginate extraction step (as routinely used in the alginate industry) was also assessed as part of the degradation process for *Ascophyllum.* Samples of depolymerised *Ascophyllum* were forwarded for *in vitro* screening but the process was not optimised on account of high salts being introduced to the depolymerised samples. This severely affected the organoleptic qualities of the products.

1.2. Pilot scale production

The aim of this phase of the research was to produce approximately 40kgs each of two candidate LMWPs for *in vivo* evaluation through (i) transfer of processes developed in the laboratory to pilot scale, (ii) scale up production of LMWPs and (iii) incorporation of candidate LMWPs into a product suitable for the in vivo study.

The depolymerisation method using ascorbic acid was trialled at pilot scale for the following: Alginate powder (Manucol DM, FMC Biopolymer), average molecular weight given by manufacturer as approximately 130 000Da; *Ascophyllum* seaweed, dried seaweed meal (Hebridean Seaweed Company); *Gelidium* derived agar (Roko), average molecular weight 176 000Da; *Gelidium* seaweed, dried whole seaweed (Roko).

Pilot scale validation

All processes were transferred directly from those previously optimised in the laboratory and outlined above. A number of difficulties were encountered on account of scaling issues and use of different equipment but most were resolvable. All end products were spray dried and not roller dried.

Table 2. Low molecular weight polysaccharides (LMWPs) used in batch culture experiments.

Low molecular weight polysaccharide extracts	Molecular weight (kDa)	Depolymerisation method
Gracilaria seaweed CC2247	320.75	Acid hydrolysis
Alginate powder CC2238	212.05	Fentons degraded
Gelidium seaweedCC2246	201.15	Acid hydrolysis
Gracilaria seaweed CC2251	143.80	Fentons degraded
Alginate powder CC2235	97.09	Acid hydrolysis
Gracilaria derived agar powder CC2245	81.03	Acid hydrolysis
Gelidium seaweed CC2250	71.34	Fentons degraded
Gracilaria derived agar powder CC2249	67.03	Fentons degraded
Gelidium seaweed extract CC2253	64.64	Free radical degradation with L-ascorbic acid
Ascophyllum seaweed CC2236	55.97	Acid hydrolysis
Ascophyllum seaweed CC2237	49.43	Acid hydrolysis with extraction step
Alginate powder (Manugel DMB) CC2241	38.43	Free radical degradation with L-ascorbic acid
Ascophyllum seaweed CC2239	31.04	Fentons degraded
Gelidium derived agar powder CC2248	28.24	Fentons degraded
Ascophyllum seaweed CC2240	27.83	Fentons degraded + extraction step
Ascophyllum seaweed CC2243	21.33	Free radical degradation with L-ascorbic acid
Gelidium derived agar powder CC2244	16.30	Acid hydrolysis
Gelidium derived agar powder CC2252	11.34	Free radical degradation with L-ascorbic acid
Alginate powder (Protonal) CC2242	-	Free radical degradation with L-ascorbic acid

Alginate powder – in general, the process transferred well, there were some issues with separation of insoluble matter but these were resolved by introducing a second separation step. Due to the low volume of material being processed, a number of fixed and variable losses *e.g.* fine particles being lost in the drier chimney were expected. 60kg of LMW product were produced from 100kg of starting material.

The MW of the end product was 40380 Da which compares very well with the MW achieved using the laboratory process (38403 Da). The end product was tasteless, odourless, cold soluble with slight golden colour. The product was deemed suitable from an organoleptic perspective to be incorporated into a delivery product for the *in vivo* studies.

Ascophyllum seaweed - in general, the process transferred well, there were some issues with separation of insoluble matter but these were resolved by introducing a clarification step. Product yield was expected to be relatively low on account of the high amounts of insoluble material contained in the seaweed matrix and also due to fixed and variable processing losses. 31kg of LMW product was produced from 100kg of dried seaweed meal.

The end product was cold soluble and flavourless, had a slight "seaweed" odour but very dark in colour. The product was deemed not suitable to be incorporated into a delivery product for the *in vivo* studies on account of the colour and odour.

Gelidium derived agar - the procedure optimized in the laboratory was transferred to this scale up stage successfully with a few resolvable issues. Fixed and variable losses were expected but a total of 61kg of LMW product was produced from 100kg of starting material.

The MW of this product was 11739 Da which compares very well with the MW of the laboratory produced sample (11335 Da). The end product was slightly golden in colour, cold

soluble and odourless with a slight off taste. The product was deemed suitable to be incorporated into a delivery product for the *in vivo* studies.

Gelidium seaweed - two separate scale up trials were carried out for this product and a number of difficulties were encountered in transferring the process. Neither trial was successful and although a LMW product was produced, losses were substantial and yields were very low. From 200kg of starting material, a total of 31.4kg of LMW product was produced.

The end product was cold soluble, slightly yellow in colour, odourless but with a definite seaweed flavour. The product was deemed not suitable to be incorporated into a delivery product for the *in vivo* studies on account of the taste. Also, without further optimisation, this process is not considered commercially viable.

Incorporation into a delivery product for in vivo studies

The focus of this phase of the research was to produce a drink product to act as a carrier for four test substances in the *in vivo* study (i) LMW agar (ii) LMW alginate (iii) Inulin - positive control (iv) Maltodextrin - negative control (Task 4.2). The primary aim was to produce four drinks that were not readily differentiated on colour, odour, taste or mouthfeel. The drink product also had to fulfil the following criteria (i) be water based and not greater than 250mls (ii) contain 8g of one of the test substances (iii) minimal added flavour or colour (iv) nothing added to affect prebiotic potential (v) nothing added to affect growth & activity of gut microflora (vi) be microbiologically stable for about 10 months.

Formulation of the drink recipe was essentially self defining, using the in house sensory capability at CyberColloids to optimise the recipe. The following formulation was chosen to best mask the various organoleptic characteristics of the four test ingredients. These characters ranged from very sweet and sickly (Inulin and maltodextrin) to slightly salty (agar); colourless (inulin and maltodextrin) to slightly coloured (agar and alginate); all had slightly different viscosities and mouthfeel.

An investigation of the potential effects of UHT treatment was also approximated in the laboratory as UHT treatment is known to affect the colour and flavour of treated products. Laboratory based assessments indicated that sensory changes as a consequence of heat treatment were negligible.

Table 3. Drink formulation for *in vivo* study

Ingredient	% content	Ingredient	% content
Test substance (agar, alginate, inulin or maltodextrin)	3.20%	Sodium citrate (buffer)	0.05%
water	90.52%	Sodium benzoate (preservative)	0.01%
Sucrose (for flavour)	6.00%	Raspberry flavour (Calaf Nuances)	0.02%
Citric acid (for flavour and acidity)	0.20%	Artificial colour (CyberColors Ltd.)	0.01%

All dried ingredients were shipped to DrinkPac UK, where they were blended, packed into 250ml TetraPak cartons and UHT treated with a holding temperature of 93^{0}C and time of 15 seconds, as recommended by the company. Opaque packaging with an opaque drinking straw attached was selected so as to minimise the visibility of the drink being taken.

Microbiological testing of each drink product was carried out by ENVA Ireland Ltd, Co. Cork, prior to the commencement of the *in vivo* study and was repeated during each wash out period to ensure that all drinks were microbiologically fit for human consumption. The following organisms were screened - *Enterobacteriaceae, E.Coli, Staphlococcus aureus, Listeria monocytogenes, Salmonella,* Yeast & Moulds, Total Viable Count. An accelerated shelf life study was also conducted to assess microbial growth through time under ambient

(18^0C) and elevated (35^0C) temperature.

2. In vitro testing (WP3): Investigation of potential of LMWPs to increase calcium absorption in the gut (Task 3.3)

Having isolated a variety of the LMWP derivatives, their ability to be fermented to short chain fatty acids and support the growth of potentially beneficial bacteria in the gut was assessed by University of Reading using batch cultures. (WP3)

2.1 Batch culture screening of LMWP's for potential beneficial effects on gut microflora composition (bifidogenic activity) and SCFA production (Tasks 3.1 & 3.2)

Methods:
Nineteen batch culture fermentation vessels (300ml) containing 135 ml basal medium were set up for each of the LMWPs (Table 2). Two additional cultures were included, one containing a well characterized prebiotic- inulin served as positive control and another containing cellulose served as negative control.. All substrates were added at a final concentration of 1%w/v. The vessels were continuously stirred, maintained at a temperature of 37°C, pH 6.8 and inoculated with 10%v/v faecal slurry. Anaerobic conditions were maintained by sparging the vessels with oxygen free nitrogen gas. All batches were run in triplicate with faecal samples obtained from three different donors. Samples were removed at intervals of 0, 5, 10 and 24 h respectively after inoculation and addition of substrates. In order to monitor changes in bacterial flora, samples were fixed in 4%w/v paraformaldehyde and stored at -20˚C. Molecular characterisation of these samples was performed using fluorescent *in situ* hybridization using synthetic oligonucleotide probes targeting specific regions of the 16S rRNA gene. Oligonucleotide probes targeting specific regions of the 16SrRNA gene labelled with the fluorescent dye Cy 3 were used (Sigma Aldrich Ltd., UK). The bacterial groups were selected based on their predominance and contribution to the colonic microbiota. The probes used were Eub mix (Eub, EubII, EubIII) (Daims et al. 1999), Bif164 (Langendjik et al. 1995), Lab158 (Harmsen et al. 1999), His150 (Franks et al. 1998), Bac303 (Manz et al. 1996); Prop (Walker et al. 2005); Fprau (Hold et al. 2003) and Erec482 (Franks et al. 1998) specific for total bacteria, bifidobacteria, *Lactobacillus/ Enterococcus* spp., *Clostridium perfringens/ histolyticum* subgroup, *Bacteroides/ Prevotella group* and *Clostridium coccoides/ Eubacterium rectale* respectively.
For analysis of short chain fatty acids, 1ml samples from the batches were removed at each time point and centrifuged to remove cells and particulate matter. The samples were acidified with HCl and run through a 5890 series II GC system (HP, Crawley, West Sussex, UK) fitted with a FFAP column. Acetic, Propionic, i-butyric, butyric, i-valeric, valeric and caproic acid concentrations were obtained using calibration curves for each of these acids.

Results:
Changes in bacterial populations after 0, 5, 10 and 24h of incubation in batch culture fermenters following supplementation with LMWPs, inulin or cellulose were determined using fluorescent in-situ hybridization are shown in Table 4. Bifidobacterial numbers increased over 24h fermentation for most of the polysaccharides tested. Of these, only *Gelidium* seaweed extract CC2253 produced a significant increase in bifidobacterial population after 24h fermentation from $\log_{10}$ 8.06±0.20 at 0h to $\log_{10}$ 8.55±0.11 at 24h (p=0.018). The positive control inulin, however induced a higher increase in bifidobacterial numbers from 8.00±0.23 at 0h to 8.88±0.30 at 24h (p=0.003). A comparison between bacterial populations at 0 and 24h for *Gelidium* seaweed extract CC2253 and inulin is shown in Figure 1. For total bacterial populations, alginate powder CC2238 produced a significant increase from $\log_{10}$ 9.01±0.27 at 0h to $\log_{10}$ 9.58±0.13 at 24h (p=0.032). Inulin produced an increase from $\log_{10}$ 8.98±0.14 at 0h to $\log_{10}$ 9.44±0.10 at 24h (p=0.007). The seaweed extracts had little or no effect on the other bacterial populations *viz.* Lactobacilli, Bacteroides, *Eubacterium rectale/ Clostridium coccoides* and *Clostridium histolyticum*.

Table 4 Bacterial populations (log cells/ml ± SD) in pH controlled batch cultures at 0, 5, 10 and 24h in presence of low molecular weight polysaccharides (LMWPs) and controls

Substrates (Controls and LMWP extracts)	Time (h)	Total bacteria	*Bifidobacterium* genus	*Bacteroides Prevotella* group	*Lactobacillus Enterococcus* group	*Eubacterium rectale, C. coccoides* group	*C. histolyticum*
Inulin	0	8.98 ± 0.14	8.00 ± 0.23	8.15 ± 0.28	6.52 ± 0.44	8.59 ± 0.18	6.32 ± 0.26
	5	9.04 ± 0.12	8.37 ± 0.30	8.25 ± 0.36	6.50 ± 0.54	8.64 ± 0.28	6.49 ± 0.37
	10	9.35 ± 0.23	8.57 ± 0.32*	8.23 ± 030	6.51 ± 0.51	8.89 ± 027	6.45 ± 0.30
	24	9.44 ± 0.10**	8.88 ± 0.30**	8.30 ± 0.29	6.58 ± 0.52	8.90 ± 0.27	6.44 ± 0.35
Cellulose	0	8.99 ± 0.19	7.99 ± 0.37	8.28 ± 0.30	6.36 ± 0.55	8.58 ± 0.19	6.29 ± 0.41
	5	9.03 ± 0.16	7.96 ± 0.22	8.25 ± 0.32	6.43 ± 0.41	8.48 ± 0.07	6.43 ± 0.33
	10	9.07 ± 0.09	8.04 ± 0.26	8.24 ± 0.33	6.49 ± 0.47	8.45 ± 0.13	6.25 ± 0.24
	24	9.03 ± 0.11	8.05 ± 0.29	8.26 ± 0.42	6.45 ± 0.44	8.43 ± 0.15	6.26 ± 0.37
Gracilaria seaweed CC2247	0	9.02 ± 0.06	7.80 ± 0.31	8.05 ± 0.05	6.43 ± 0.57	8.37 ± 0.20	6.18 ± 0.57
	5	8.89 ± 0.07	8.07 ± 0.49	8.05 ± 0.06	6.45 ± 0.39	8.63 ± 0.17	6.31 ± 0.32
	10	9.05 ± 0.26	8.04 ± 0.40	8.03 ± 0.04	6.39 ± 0.52	8.57 ± 0.04	6.68 ± 0.07
	24	9.27 ± 0.34	7.97 ± 0.46	8.09 ± 0.07	6.77 ± 0.86	8.73 ± 0.29	6.37 ± 0.09
Alginate powder CC2238	0	9.01 ± 0.27	8.21 ± 0.15	8.08 ± 0.00	6.18 ± 0.58	8.55 ± 0.36	6.31 ± 0.31
	5	9.01 ± 0.34	8.28 ± 0.20	8.08 ± 0.03	6.46 ± 0.15	8.60 ± 0.32	6.17 ± 0.46
	10	9.27 ± 0.33	7.97 ± 0.12	8.04 ± 0.05	6.36 ± 0.48	8.50 ± 0.25	6.39 ± 0.59
	24	9.58 ± 0.13*	8.09 ± 0.01	8.07 ± 0.03	6.46 ± 0.33	8.56 ± 0.17	6.21 ± 0.48
Gelidium seaweed CC2246	0	8.97 ± 0.28	7.87 ± 0.23	8.03 ± 0.10	6.38 ± 0.58	8.61 ± 0.16	6.26 ± 0.36
	5	8.72 ± 0.21	7.88 ± 0.22	8.02 ± 0.04	6.52 ± 0.61	8.55 ± 0.19	6.55 ± 0.51
	10	8.99 ± 0.54	7.88 ± 0.35	8.05 ± 0.05	6.21 ± 0.64	8.35 ± 0.16	6.73 ± 0.38
	24	9.11 ± 0.33	8.19 ± 0.57	8.12 ± 0.13	6.21 ± 0.64	8.63± 0.18	6.59 ± 0.24
Gracilaria seaweed CC2251	0	8.99 ± 0.23	8.14 ± 0.11	8.05 ± 0.05	6.73 ± 0.51	8.61 ± 0.16	6.15 ± 0.29
	5	9.09 ± 0.14	8.46 ± 0.22	8.06 ± 0.12	6.99 ± 0.68	8.70 ± 0.20	6.38 ± 0.91
	10	9.27 ± 0.42	8.44 ± 0.23	8.10 ± 0.02	6.70 ± 0.56	8.68 ± 0.27	6.14 ± 1.10
	24	9.27 ± 0.50	8.41 ± 0.15	8.06 ± 0.12	6.69 ± 0.42	8.63± 0.12†	6.18 ± 0.83
Alginate powder CC2235	0	8.91 ± 0.40	7.82 ± 0.08	8.06 ± 0.03	6.55 ± 0.64	8.45 ± 0.16	5.77 ± 0.17
	5	8.76 ± 0.30	8.05 ± 0.19	8.05 ± 0.07	6.51 ± 0.63	8.29 ± 0.21	6.30 ± 0.81
	10	9.08 ± 0.39	7.89 ± 0.10	8.06 ± 0.02	6.39 ± 0.59	8.19 ± 0.30	6.26 ± 0.32
	24	9.14 ± 0.26	7.92 ± 0.27	8.04 ± 0.05	6.49 ± 0.30	8.34± 0.15	6.44 ± 0.44
Gracilaria derived agar powder CC2245	0	8.94 ± 0.21	8.01 ± 0.23	8.04 ± 0.07	6.86 ± 0.48	8.82 ± 0.59	6.33 ± 0.12
	5	9.12 ± 0.21	8.26 ± 0.25	8.06 ± 0.06	7.06 ± 0.54	8.84 ± 0.35	6.60 ± 0.60
	10	9.26 ± 0.47	8.13 ± 0.42	8.13 ± 0.09	6.81 ± 0.33	8.90 ± 0.50	6.58 ± 0.71
	24	9.36 ± 0.21	8.10 ± 0.05	8.07 ± 0.13	6.80 ± 0.33	8.80 ± 0.35	6.54 ± 0.71
Gelidium seaweed CC2250	0	9.10 ± 0.10	7.79 ± 0.14	8.12 ± 0.09	6.71 ± 0.79	8.56 ± 0.28	6.32 ± 0.31
	5	9.06 ± 0.33	8.03 ± 0.24	8.05 ± 0.06	6.81 ± 0.48	8.66 ± 0.28	6.72 ± 0.10
	10	9.08 ± 0.19	8.13 ± 0.19	8.04 ± 0.01	6.38 ± 0.68	8.52 ± 0.30	6.55 ± 0.07
	24	8.83 ± 0.09	8.09 ± 0.54	8.08 ± 0.06	6.75 ± 0.40	8.49 ± 0.42	6.74 ± 0.14
Gracilaria derived agar powder CC2249	0	8.88 ± 0.23	8.08 ± 0.18	8.01 ± 0.02	6.78 ± 0.73	8.44 ± 0.46	6.43 ± 0.23
	5	8.90 ± 0.20	8.14 ± 0.02	8.07 ± 0.05	6.83 ± 0.38	8.49 ± 0.48	6.48 ± 0.42
	10	9.02 ± 0.29	8.08 ± 0.30	8.05 ± 0.02	6.65 ± 0.44	8.39 ± 0.28	6.40 ± 0.35
	24	9.32 ± 0.31	8.36 ± 0.19	8.07 ± 0.02	6.88 ± 0.50	8.54± 0.28	6.49 ± 0.39
Gelidium seaweed extract CC2253	0	8.87 ± 0.24	8.06 ± 0.20	8.03 ± 0.10	6.74 ± 0.28	8.65 ± 0.37	6.35 ± 0.50
	5	8.95 ± 0.07	8.33 ± 0.16	8.03 ± 0.06	6.80 ± 0.35	8.73 ± 0.36	6.60 ± 0.29
	10	9.13 ± 0.24	8.36 ± 0.18	8.01 ± 0.03	7.21 ± 0.75	8.80 ± 0.34	6.43 ± 0.36
	24	9.36 ± 0.24	8.55 ± 0.11*	8.11 ± 0.08	7.14 ± 0.57	8.68± 0.36	6.27 ± 0.38
Ascophyllum seaweed CC2236	0	9.32 ± 0.44	7.86 ± 0.27	8.02 ± 0.16	6.68 ± 0.45	8.55 ± 0.22	6.24 ± 0.25
	5	8.90 ± 0.32	7.85 ± 0.22	8.04 ± 0.05	6.72 ± 0.51	8.63 ± 0.28	6.29 ± 0.27
	10	9.22 ± 0.15	7.87 ± 0.18	8.04 ± 0.06	6.82 ± 0.34	8.93 ± 0.42	6.28 ± 0.44
	24	9.30 ± 0.71	7.94 ± 0.37	8.03 ± 0.12	6.82 ± 0.33	8.86± 0.33	6.48 ± 0.32
Ascophyllum seaweed CC2237	0	8.97 ± 0.28	8.00 ± 0.33	8.08 ± 0.10	6.82 ± 0.61	8.61± 0.16	6.67 ± 0.42
	5	9.01 ± 0.29	8.01 ± 0.12	8.08 ± 0.13	6.61 ± 0.43	8.67 ± 0.16	6.66 ± 0.17
	10	9.09 ± 0.48	8.20 ± 0.22	8.04 ± 0.01	6.81 ± 0.44	8.48 ± 0.12	6.53 ± 0.23
	24	9.20 ± 0.37	8.29 ± 0.41	8.06 ± 0.04	6.99 ± 0.47	8.46± 0.32	6.14 ± 0.34
Alginate powder (Manugel DMB) CC2241	0	8.95 ± 0.19	7.95 ± 0.39	8.06 ± 0.03	6.86 ± 0.21	8.54 ± 0.11	6.36 ± 0.37
	5	8.95 ± 0.34	8.03 ± 0.38	8.05 ± 0.01	6.48 ± 0.60	8.37 ± 0.23	6.09 ± 0.62
	10	8.90 ± 0.36	8.05 ± 0.15	8.00 ± 0.03	6.75 ± 0.39	8.06 ± 0.42	6.30 ± 0.44
	24	9.10 ± 0.34	7.94 ± 0.29	8.14 ± 0.10	6.66 ± 0.31	7.92 ± 0.11	6.10 ± 0.24

Ascophyllum seaweed CC2239	0	9.10 ± 0.27	8.00 ± 0.17	8.06 ± 0.02	6.73 ± 0.55	8.57 ± 0.25	6.45 ± 0.26
	5	9.09 ± 0.25	7.95 ± 0.11	8.03 ± 0.06	6.84 ± 0.35	8.63 ± 0.32	6.78 ± 0.15
	10	9.21 ± 0.10	7.92 ± 0.24	8.05 ± 0.09	6.59 ± 0.37	8.70 ± 0.17	6.60 ± 0.22
	24	9.30 ± 0.23	8.13 ± 0.26	8.06 ± 0.05	6.82 ± 0.44	8.62± 0.23	6.35 ± 0.43
Gelidium derived agar powder CC2248	0	8.98 ± 0.10	8.00 ± 0.25	8.27 ± 0.36	6.45 ± 0.67	8.58 ± 0.14	6.26 ± 0.49
	5	9.09 ± 0.07	8.08 ± 0.14	8.19 ± 0.45	6.62 ± 0.59	8.65 ± 0.21	6.55 ± 0.48
	10	9.08 ± 0.15	8.06 ± 0.13	8.15 ± 0.47	6.69 ± 0.82	8.50 ± 0.14	6.55 ± 0.28
	24	9.16 ± 0.27	8.27 ± 0.23	8.27 ± 0.38	6.56 ± 0.69	8.50 ± 0.14	6.60 ± 0.17
Ascophyllum seaweed CC2240	0	8.94 ± 0.36	7.84 ± 0.20	8.07 ± 0.04	6.68 ± 0.57	8.47 ± 0.05	6.30 ± 0.29
	5	8.84 ± 0.33	7.82 ± 0.13	8.02 ± 0.03	6.34 ± 0.85	8.50 ± 0.04	6.45 ± 0.55
	10	9.15 ± 0.38	8.04 ± 0.30	8.06 ± 0.04	6.56 ± 0.58	8.63 ± 0.10†	6.52 ± 0.54
	24	9.06 ± 0.30	8.13 ± 0.59	8.11 ± 0.13	6.44 ± 0.66	8.43 ± 0.22	6.49 ± 0.31
Ascophyllum seaweed CC2243	0	9.15 ± 0.27	7.96 ± 0.31	8.07 ± 0.09	6.63 ± 0.71	8.56 ± 0.10	6.12 ± 0.64
	5	9.19 ± 0.22	8.20 ± 0.19	8.06 ± 0.02	6.72 ± 0.46	8.67 ± 0.30	6.54 ± 0.31
	10	9.27 ± 0.29	8.19 ± 0.17	8.07 ± 0.04	7.09 ± 0.66	8.58 ± 0.27	6.05 ± 0.37
	24	9.24 ± 0.29	8.24 ± 0.26	8.19 ± 0.22	7.26 ± 0.65	8.54± 0.17	6.38 ± 0.20
Gelidium derived agar powder CC2244	0	9.03 ± 0.23	8.10 ± 0.34	8.03 ± 0.07	6.50 ± 0.62	8.78 ± 0.31	6.21 ± 0.47
	5	9.01 ± 0.08	7.97 ± 0.06	8.05 ± 0.04	6.76 ± 0.27	8.74 ± 0.27	6.58 ± 0.07
	10	9.09 ± 0.29	7.90 ± 0.11	7.99 ± 0.05	6.80 ± 0.59	8.72 ± 0.16†	6.25 ± 0.06
	24	9.09 ± 0.32	8.06 ± 0.31	8.02 ± 0.05	6.82 ± 0.51	8.61± 0.20	6.34 ± 0.21
Gelidium derived agar powder CC2252	0	8.99 ± 0.36	7.97 ± 0.30	8.01 ± 0.06	7.02 ± 0.70	8.55 ± 0.35	6.30 ± 0.21
	5	9.10 ± 0.28	7.76 ± 0.06	8.05 ± 0.04	6.74 ± 0.42	8.51 ± 0.20	6.44 ± 0.07
	10	9.17 ± 0.32	7.89 ± 0.20	7.99 ± 0.06	6.52 ± 0.41	8.66 ± 0.16	6.32 ± 0.24
	24	9.09 ± 0.52	7.94 ± 0.35	8.04 ± 0.02	6.54 ± 0.70	8.61± 0.24	6.17 ± 0.17
Alginate powder (Protonal) CC2242	0	8.98 ± 0.13	8.13 ± 0.32	8.00 ± 0.03	6.71 ± 0.34	8.56 ± 0.26	6.36 ± 0.18
	5	9.08 ± 0.31	8.19 ± 0.13	8.07 ± 0.09	6.60 ± 0.41	8.47 ± 0.10	6.25 ± 0.51
	10	9.02 ± 0.40	8.02 ± 0.09	8.05 ± 0.12	6.66 ± 0.41	8.27 ± 0.18	6.34 ± 0.36
	24	9.37 ± 0.40	8.30 ± 0.25	7.98 ± 0.11	6.79 ± 0.62	8.01 ± 0.40†	5.99 ± 0.59

In order to obtain a general quantitative measure of the prebiotic effect of the LMWP extracts, a prebiotic index was calculated as described in the Materials and Methods section above. The PI values at 5 and 10h were considerably lower than 24h (Table 5). At 10h, a highly positive PI score of 7.16 was obtained for alginate powder (Protonal) CC2242, however, the standard deviation value of 12.16 suggested that there was a substantial difference in response of the microflora from the three donors for this extract. At 24h, alginate powder CC2238, *Gracilaria* derived agar powder CC2249, *Gelidium* seaweed extract CC2253 and *Ascophyllum* seaweed CC2239 indicated a positive PI score of 0.39, 0.55, 0.44 and 2.16 respectively with their error bars consistently over zero and thus may be considered as potential prebiotics. The PI score for inulin was 3.65.

Table 5 PI scores of LMWP extracts, inulin and cellulose after 5, 10 and 24h of anaerobic fermentation at pH 6.8 and 37°C.

Substrates (Controls and LMWP extracts)	**PI at**		
	5h	**10h**	**24h**
Inulin	0.42 ± 2.02	0.32 ± 0.26	3.65 ± 0.44
Cellulose	-6.62 ± 14.03	-1.03 ± 2.23	0.00 ± 3.52
Gracilaria seaweed CC2247	-0.29 ± 0.49	-0.08 ± 0.08	0.87 ± 7.60
Alginate powder CC2238	0.01 ± 0.06	0.07 ± 0.16	0.39 ± 0.48
Gelidium seaweedCC2246	-0.05 ± 0.16	-0.12 ± 0.10	-1.02 ± 1.67
Gracilaria seaweed CC2251	-0.69 ± 1.90	-0.42 ± 0.88	0.57 ± 8.13
Alginate powder CC2235	0.0 ± 0.17	0.00 ± 0.01	0.15 ± 0.47
Gracilaria derived agar powder CC2245	0.09 ± 0.14	0.02 ± 0.04	1.60 ± 27.30
Gelidium seaweed CC2250	0.03 ± 0.16	-0.53 ± 0.87	-1.43 ± 1.28
Gracilaria derived agar powder CC2249	-0.66 ± 0.87	-0.23 ± 0.90	0.55 ± 0.68
Gelidium seaweed extract CC2253	0.10 ± 0.36	0.36 ± 0.30	0.44 ± 0.58

Ascophyllum seaweed CC2236	0.00 ± 0.10	0.15 ± 0.23	0.00 ± 0.00
Ascophyllum seaweed CC2237	0.65 ± 0.79	0.00 ± 0.00	0.62 ± 8.54
Alginate powder (Manugel DMB) CC2241	0.24 ± 0.19	-0.09 ± 0.23	0.15 ± 1.65
Ascophyllum seaweed CC2239	1.68 ± 1.89	0.30 ± 0.21	2.16 ± 2.11
Gelidium derived agar powder CC2248	-0.04 ± 0.19	-0.26 ± 0.36	1.35 ± 2.22
Ascophyllum seaweed CC2240	0.15 ± 0.19	0.00 ± 0.00	1.57 ± 2.12
Ascophyllum seaweed CC2243	0.29 ± 0.30	0.16 ± 0.12	1.59 ± 2.33
Gelidium derived agar powder CC2244	-0.41 ± 0.46	0.30 ± 0.61	-1.83 ± 3.71
Gelidium derived agar powder CC2252	-0.78 ± 1.21	0.68 ± 0.32	1.81 ± 6.07
Alginate powder (Protonal) CC2242	1.74 ± 2.60	7.16 ± 12.16	1.92 ± 2.92

There did not seem to be an increase in bacterial populations with respect to decreasing molecular weights as expected *Gelidium* seaweed extract CC2253 of molecular weight 64.64Kda seemed to be the best prebiotic among the seaweed extracts tested with the ability to increase bifidobacterial numbers significantly after 24h incubation.

Contrasting to the lack of modulation of gut microbiota by most seaweeds, the SCFA profiles of all extracts except Alginate powder (Manugel DMB) CC2241, *Gelidium* derived agar powder CC2248 and alginate powder (Protonal) CC2242 indicated significantly high levels of total SCFA particularly acetate and propionate (Table 6). Highest levels of total SCFA (87.61% acetate and 11.28% propionate) were produced by *Gelidium* seaweed extract CC2253. The chemical composition and linkages between seaweed polysaccharides may influence susceptibility of the extracts to faecal flora, thus affecting fermentability and proportion of SCFA produced. In addition, variations in faecal microflora between individuals may also affect their degradation (Bourquin et al. 1996; Salazar et al 2009).

Table 6. SCFA concentration (mM) in pH controlled batch cultures

Substrates (Controls and LMWP extracts)	Time (h)	Total SCFA	Acetic acid	Propionic acid	Butyric acid
Inulin	0	7.79 ± 5.36	6.80 ± 6.15	0.75 ± 0.65	0.24 ± 0.25
	5	24.69 ± 4.48*	21.47 ± 4.02*	2.48 ± 0.25*	0.74 ± 0.32
	10	39.21 ± 10.63*	32.06 ± 11.75*	5.82 ± 1.89*	1.33 ± 0.88
	24	61.50 ± 4.06**	46.62 ± 4.53**	12.85 ± 3.90**	2.04 ± 0.98*
Cellulose	0	2.23 ± 0.18	2.23 ± 0.18	0.00 ± 0.00	0.00 ± 0.00
	5	2.66 ± 0.37	2.66 ± 0.37	0.00 ± 0.00	0.00 ± 0.00
	10	2.48 ± 0.21	2.48 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
	24	2.04 ± 0.06	2.04 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
Gracilaria seaweed CC2247	0	2.74 ± 0.56	2.74 ± 0.56	0.00 ± 0.00	0.00 ± 0.00
	5	12.90 ± 1.26**	11.77 ± 0.88**	1.14 ± 1.29	0.04 ± 0.00
	10	16.02 ± 1.97**	13.63 ± 1.17**	2.36 ± 1.12*	0.58 ± 0.07
	24	24.99 ± 3.27**	17.78 ± 1.77**	6.63 ± 0.99**	0.00 ± 0.51
Alginate powder CC2238	0	2.73 ± 1.02	2.66 ± 1.07	0.08 ± 0.07	0.00 ± 0.00
	5	6.31 ± 1.64**	4.70 ± 2.11	1.61 ± 0.53**	0.00 ± 0.00
	10	14.60 ± 2.21**	12.79 ± 1.69**	1.81 ± 0.59**	0.00 ± 0.00
	24	47.23 ± 4.45**	39.80 ± 4.87**	7.43 ± 0.67**	0.00 ± 0.00
Gelidium seaweed CC2246	0	5.36 ± 2.69	5.36 ± 2.69	0.00 ± 0.00	0.00 ± 0.00
	5	19.33 ± 3.48**	14.84 ± 3.48*	4.49 ± 1.19**	0.00 ± 0.00
	10	31.53 ± 5.77**	29.17 ± 5.74**	2.35 ± 0.66**	0.00 ± 0.00
	24	15.98 ± 5.88**	14.31 ± 6.09**	1.66 ± 0.61**	0.00 ± 0.00
Gracilaria seaweed CC2251	0	6.00 ± 6.07	6.00 ± 6.07	0.00 ± 0.00	0.00 ± 0.00
	5	17.40 ± 1.62*	14.08 ± 1.25	3.32 ± 0.88**	0.00 ± 0.00
	10	30.57 ± 1.82*	23.86 ± 2.67**	6.72 ± 0.91**	0.00 ± 0.00
	24	38.10 ± 1.92**	31.33 ± 1.41**	6.77 ± 0.58**	0.00 ± 0.00

Alginate powder CC2235	0	27.66 ± 7.45	27.66 ± 7.45	0.00 ± 0.00	0.00 ± 0.00
	5	36.36 ± 2.50	36.36 ± 2.50	0.00 ± 0.00	0.00 ± 0.00
	10	56.84± 2.39**	55.61± 1.37**	1.23 ± 1.29	0.00 ± 0.00
	24	22.89 ± 1.58	21.19 ± 1.10	1.70 ± 2.08	0.00 ± 0.00
Gracilaria derived agar powder CC2245	0	22.57 ± 0.49	22.57 ± 0.49	0.00 ± 0.00	0.00 ± 0.00
	5	39.50 ± 2.73	38.05 ± 1.85	1.45 ± 0.94	0.00 ± 0.00
	10	37.12 ± 1.66	34.62 ± 0.95	2.5 ± 0.87*	0.00 ± 0.07
	24	48.77 ± 3.51*	43.45 ± 2.50*	5.33 ± 0.81	0.00 ± 0.23
Gelidium seaweed CC2250	0	4.00 ± 0.00	4.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	5	7.08 ± 2.73	5.34 ± 1.85	1.74 ± 0.94*	0.00 ± 0.00
	10	11.95 ± 1.66**	7.82 ± 0.95**	4.09 ± 0.87**	0.04 ± 0.07
	24	22.76 ± 3.51**	15.10 ± 2.50**	7.40 ± 0.81**	0.26 ± 0.23
Gracilaria derived agar powder CC2249	0	3.32 ± 1.27	3.32 ± 1.27	0.00 ± 0.00	0.00 ± 0.00
	5	8.64 ± 1.82*	7.51 ± 0.85**	1.14 ± 1.09	0.00 ± 0.00
	10	15.29 ± 2.75**	12.96 ± 2.00**	2.27 ± 0.73**	0.07 ± 0.12
	24	27.13 ± 3.79**	21.91 ± 2.95**	4.47 ± 0.74**	0.76 ± 0.11**
Gelidium seaweed extract CC2253	0	1.47 ± 0.46	1.47 ± 0.46	0.00 ± 0.00	0.00 ± 0.00
	5	4.05 ± 1.29*	3.64 ± 1.55	0.41 ± 0.27	0.00 ± 0.00
	10	47.81 ± 7.47**	40.80 ± 7.85**	6.43 ± 0.76**	0.59 ± 0.41
	24	73.46 ± 6.44**	64.36 ± 6.53**	8.29 ± 0.84**	0.81 ± 0.31*
Ascophyllum seaweed CC2236	0	13.02 ± 12.61	11.18 ± 7.97	1.84 ± 0.54	0.00 ± 0.00
	5	50.70 ± 12.99*	46.59 ± 12.41*	4.10 ± 0.21**	0.00 ± 0.00
	10	45.02 ± 12.83*	41.21 ± 13.18*	3.81 ± 0.58*	0.00 ± 0.00
	24	48.80 ± 2.11**	45.82 ± 12.70**	2.94 ± 0.71*	0.04 ± 0.06
Ascophyllum seaweed CC2237	0	20.78 ± 5.17	20.78 ± 5.17	0.00 ± 0.00	0.00 ± 0.00
	5	18.00 ± 2.96	17.79 ± 3.02	0.22 ± 0.38	0.00 ± 0.00
	10	39.02 ± 19.22	38.69 ± 18.87	0.33 ± 0.38	0.00 ± 0.00
	24	68.37 ± 7.13**	66.46 ± 6.92**	1.91 ± 0.40**	0.00 ± 0.00
Alginate powder (Manugel DMB) CC2241	0	1.94 ± 0.86	1.78 ± 0.84	0.00 ± 0.00	0.16 ± 0.28
	5	1.72 ± 0.55	1.53 ± 0.58	0.19 ± 0.32	0.00 ± 0.00
	10	2.37 ± 0.44	2.37 ± 0.44	0.00 ± 0.00	0.00 ± 0.00
	24	4.79 ± 0.68	4.28 ± 0.90	0.51 ± 0.45	0.00 ± 0.00
Ascophyllum seaweed CC2239	0	7.94 ± 1.57	7.12 ± 1.00	0.81 ± 0.60	0.00 ± 0.00
	5	15.11 ± 2.35*	12.93 ± 2.98*	2.18 ± 1.98	0.00 ± 0.00
	10	15.24 ± 3.97*	12.11 ± 1.91*	3.00 ± 1.89	0.13 ± 0.23
	24	26.44 ± 3.92**	22.16 ± 1.10**	4.02 ± 2.97	0.26 ± 0.45
Gelidium derived agar powder CC2248	0	0.82 ± 0.86	0.51 ± 0.45	0.31 ± 0.54	0.00 ± 0.00
	5	1.36 ± 0.95	1.33 ± 0.99	0.04 ± 0.06	0.00 ± 0.00
	10	1.27 ± 1.12	0.82 ± 0.84	0.45 ± 0.45	0.00 ± 0.00
	24	4.72 ± 2.90	2.90 ± 2.19	1.82 ± 0.73*	0.00 ± 0.00
Ascophyllum seaweed CC2240	0	10.47 ± 6.09	10.08 ± 5.64	0.39 ± 0.67	0.00 ± 0.00
	5	20.02 ± 14.43	20.43 ± 13.59	1.59 ± 0.98	0.00 ± 0.00
	10	30.58 ± 11.14	27.74 ± 10.37	2.28 ± 0.49*	0.56 ± 0.63
	24	50.31 ± 11.36**	44.06 ± 9.45**	5.80 ± 1.51**	0.46 ± 0.41
Ascophyllum seaweed CC2243	0	1.20 ± 0.69	1.06 ± 0.80	0.15 ± 0.25	0.00 ± 0.00
	5	1.65 ± 1.22	1.48 ± 1.11	0.17 ± 0.29	0.00 ± 0.00
	10	13.97 ± 2.53**	12.55 ± 2.22**	1.42 ± 1.43	0.00 ± 0.00
	24	18.93 ± 0.42**	16.49 ± 1.48**	2.44 ± 1.16*	0.00 ± 0.00
Gelidium derived agar powder CC2244	0	4.72 ± 4.49	4.72 ± 4.49	0.00 ± 0.00	0.00 ± 0.00
	5	1.82 ± 1.10	1.82 ± 1.10	0.00 ± 0.00	0.00 ± 0.00
	10	6.73 ± 2.99	5.70 ± 2.78	1.03 ± 0.21**	0.00 ± 0.00
	24	14.44 ± 2.83*	12.85 ± 3.06**	1.59 ± 0.46**	0.00 ± 0.00
Gelidium derived agar powder CC2252	0	1.87 ± 1.25	1.57 ± 0.99	0.30 ± 0.27	0.00 ± 0.00
	5	7.14 ± 3.43	5.68 ± 2.65	1.46 ± 0.80	0.00 ± 0.00
	10	18.19 ± 3.27**	15.78 ±1.89**	2.41 ± 1.38*	0.00 ± 0.00
	24	27.70 ± 2.47**	23.04 ± 1.91**	4.66 ± 1.63**	0.00 ± 0.00
Alginate powder (Protonal) CC2242	0	1.11 ± 0.68	0.98 ± 0.48	0.14 ± 0.24	0.00 ± 0.00
	5	0.45 ± 0.40	0.44 ± 0.40	0.00 ± 0.00	0.00 ± 0.00
	10	4.73 ± 3.41	4.10 ± 2.81	0.63 ± 0.62	0.00 ± 0.00
	24	2.10 ± 1.28	1.30 ± 1.39	0.80 ± 0.81	0.00 ± 0.00

In conclusion, we have shown that low molecular weight extracts derived from agar and alginate bearing seaweeds were fermentable by gut microbiota as indicated by noticeable increases in SCFA. *Gelidium* seaweed extract CC2253 exhibited potential to be used as a prebiotic with significant increases in bifidobacterial populations, positive prebiotic index and concomitant increase in acetate and propionate. There did not seem to be a correlation between molecular weight and prebiotic properties of seaweed extracts.

2.2 Detailed assessment of 2 candidate LMWPs for prebiotics effects in an in vitro model of the human colon (Tasks 3.4, 3.5).

Two LMWP samples (AgarH1CC2013 and Alginate H1CC2012) used in the human intervention study, were also investigated in 3 stage continuous culture fermenter which models many of the physiological and biochemical conditions in the human colon The 3-stages represent the proximal, transverse, and distal colon and allow diet-induced changes in gut microflora and SCFA production to be assessed.

<u>*Methods:*</u>
Simulated human digestion of LMWP (from mouth to small intestine)
To remove any material from the LMWP samples that might be metabolized in the stomach or small intestine and hence not reach the colon in vivo, we pre-digested the extracts as described by Connolly *et al.*2010. Four 60g of LMWP extract were weighed into stomacher bags (Seward Classic 400), 150ml of sterile distilled water was added and the mix blended for 5 min at normal speed using a stomacher (Seward Norfolk, UK). 20mg α-amylase (Sigma A4551) was dissolved in 6.25 ml filter sterilized $CaCl_2$ (1mM, pH 7). The solution was added to 500ml of a glass Duran bottle and transferred to the seaweed mix which was then incubated at 37°C for 30 min on a shaker (IKA labortechnik KS501) set at 150rpm. The pH was acidified to 2.0 with HCl (6M). Pepsin solution was prepared by adding 2.7g of pepsin (Sigma P7000) in 125 ml of 0.1M HCl. This was then added to the seaweed mixture and incubated under similar shaking conditions as above for 2 h at 37°C. 560mg Pancreatin (Sigma P8096) and 3.5g Bile (Sigma B8631) was dissolved in 125ml of 0.5M $NaHCO_3$ and dispensed into the mix. The pH was adjusted to 7.0 with either 6M HCl or 6M NaOH and incubated at 37°C for 3h under similar shaking conditions as above. The sample was then transferred to a 100-500Da cut off membrane (VWR, UK) and incubated in cold, sterile NaCl (10mM) solution for 15h. The dialysis was replaced with fresh NaCl solution and incubated for a further 2h. The dialysis tubes were then cut open and contents dispensed into a clean sterile plastic container. 150ml of the dialysed seaweed mix was then dispensed into several 250 ml bottles and covered with a filter paper and sealed using rubber bands. The samples were stored at -80°C overnight. The frozen samples were then freeze dried (IEC lyoprep 3000) for 5 days to remove all fluid content.

In vitro three stage continuous system
The gut model consisted of a cascade of three glass fermenters, V1, V2 and V3 connected in series and ultimately to a waste vessel. The three vessels, V1, V2 and V3 simulate the proximal, transverse and distal colon conditions respectively. The operating volumes for the three vessels were 280, 300 and 320ml and pH 5.5, 6.2 and 6.8 respectively. Each vessel was magnetically stirred and continuously sparged with O_2 free N_2 gas. Temperature was maintained at 37°C by a water bath and culture pH automatically controlled by addition of 1N NaOH and HCl. After overnight gassing of the basal medium (135ml) in the three vessels, each vessel was inoculated with 100ml of 20% (w/v) faecal slurry from a healthy human donor. The system was allowed to initially operate in the batch culture mode for 24h by clamping each of the vessels. After this, 5L medium reservoir was connected to vessel 1 and flow rate adjusted to 36ml/h using a peristaltic pump. Steady state 1 (SS1) conditions were determined by stabilization of total bacterial numbers and SCFA profiles as assessed by 4',6-

diamidino-2-phenylindole (DAPI) and gas chromatography (GC) respectively. SS1 was established after approximately 10 days where the fermentation of new biomass was balanced by the loss of cells in the vessels. This state was achieved after approximately seven turnovers of the medium. 6ml sample was removed from each vessel- V1, V2 and V3 to assess bacteriology and SCFA concentrations. Samples for SS1 were taken after 10 days for three consecutive days *viz.* day 10, 11 and 12. After SS1, 8g of the dialysed seaweed extract was dissolved in 20 ml sterile gut model basal medium and subsequently added daily to the system via feeding inlet of V1 until the second steady state (SS2) was achieved. 6ml samples were taken after 10 days of continuous feeding at three consecutive days - 20, 21 and 22 day for bacteriology and SCFA profiles. Thus, samples were collected at days 10, 11, 12- SS1 and 20, 21 and 22-SS2 for bacteriology and SCFA concentrations.
Changes in the faecal bacterial populations before (SS1) and after feeding (SS2) with seaweed extracts were assessed using fluorescent in-situ hybridization (FISH) as described above. The short chain fatty acids (SCFA) acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and caproate were analyzed as their salyl derivatives by GC.

Statistical analysis: All statistical analyses were performed using paired t tests to compare the changes in the bacterial populations and SCFAs before (SS1) and after feeding (SS2) the model with the respective substrates. The significance level was set at $p<0.05$.

<u>Results</u>

Microbial changes : Figure 1 and 2 show a comparison of bacterial counts for the different bacterial populations tested in the three vessels of the gut model at steady state 1 (SS1), without addition of the substrate and at steady state 2 (SS2) after feeding the model with agar and alginate respectively. Results from feeding the gut model with Agar (Figure 1) show that there was a significant reduction in *Atopobium* (Figure 1c) and *Clostridium histolyticum* (Figure 1h) numbers in the transverse colon (V2) from $\log_{10}$ 8.94 to 8.73 ($p<0.001$) and $\log_{10}$ 6.87 to 6.77 ($p=0.002$) respectively. There were no significant changes observed for other bacterial populations analysed *viz. Bifidobacterium,* Total bacteria, *Bacteroides*, Lactobacilli, *Faecalibacterium prausnitzii*, Propionibacteria and *Eubacterium rectale/ Clostridium coccoides* group.

Results from feeding the gut model with alginate (Figure 2) show that there was a significant decrease in total bacterial numbers from $\log_{10}$ 9.58 to 9.49 ($p=0.02$) in the transverse colon (V2). On the contrary, significant increases in *Faecalibacterium prausnitzii* bacterial numbers were observed for transverse colon (V2) from $\log_{10}$ 9.10 to 9.19 ($p=0.03$) and distal colon (V3) from $\log_{10}$ 9.07 to 9.24 ($p=0.01$). There were no significant changes observed for other bacterial populations analysed.

Increases in *Faecalibacterium prausnitzii* are considered beneficial since this organism has been associated with anti-inflammatory effects. However overall the specific changes observed in *F. prausnitzii* and other bacterial populations were of relatively minor magnitude and are probably of limited biological significance. In particular there was no change in bifidobacteria numbers with either LMWP , which was the prebiotic effect sought.

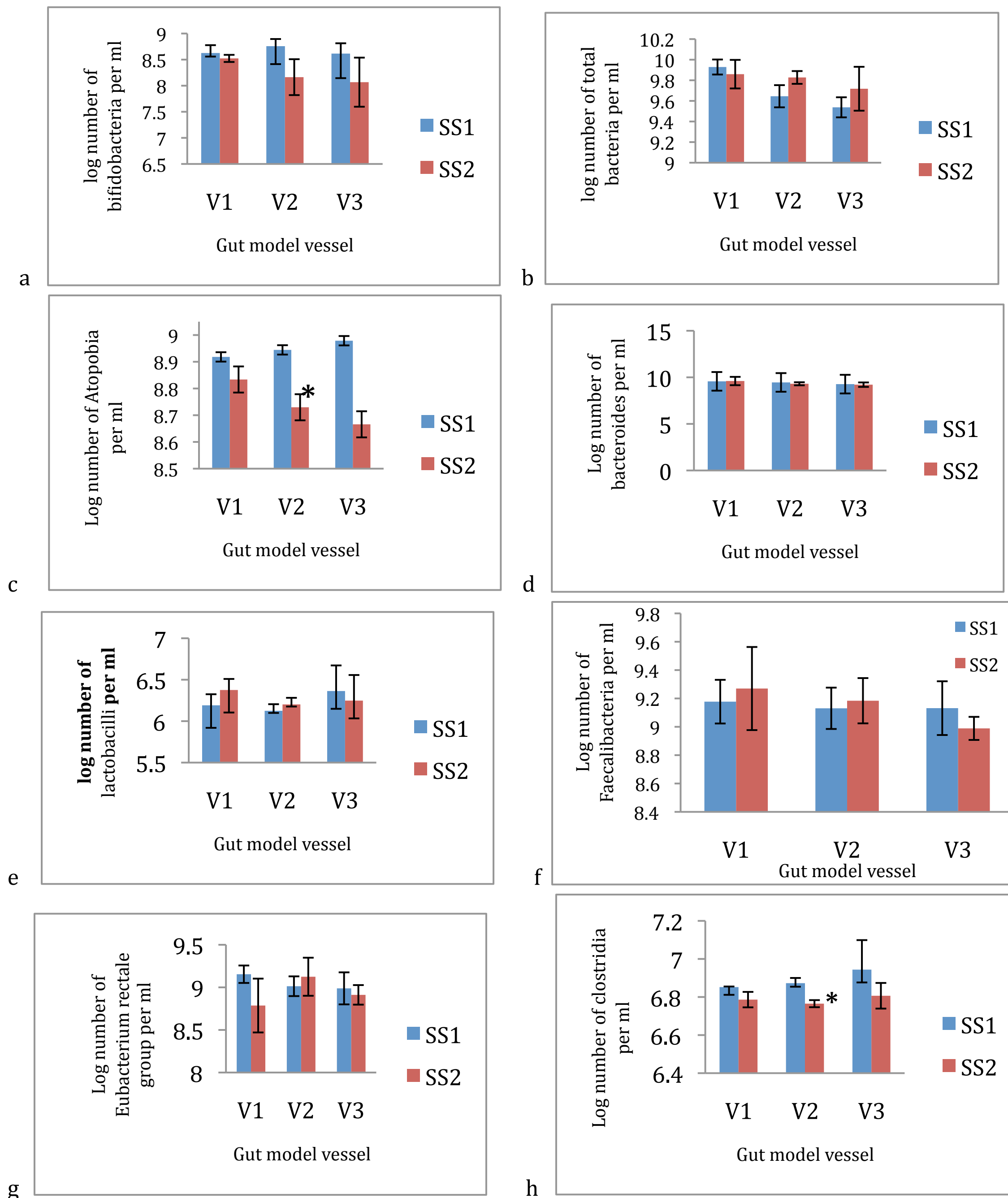

Figure1. Changes in numbers of a) Bifidobacteria, b) Total bacteria, c) Atopobia, d) *Bacteroides*, e) Lactobacilli, f) *Faecalibacterium prausnitzii*, g) *Eubacterium rectale/ Clostridium coccoides* group, h) *Clostridium histolyticum* group in the three gut model vessels- V1, V2 and V3 fed with AgarH1CC2013 at SS1 (day 10,11 and 12 without addition of substrate) and at SS2 (day 20,21 and 22 after addition of agar 1). The data represent a mean of triplicate values derived from three consecutive days for each model and a mean of three models with faecal samples from three different donors.

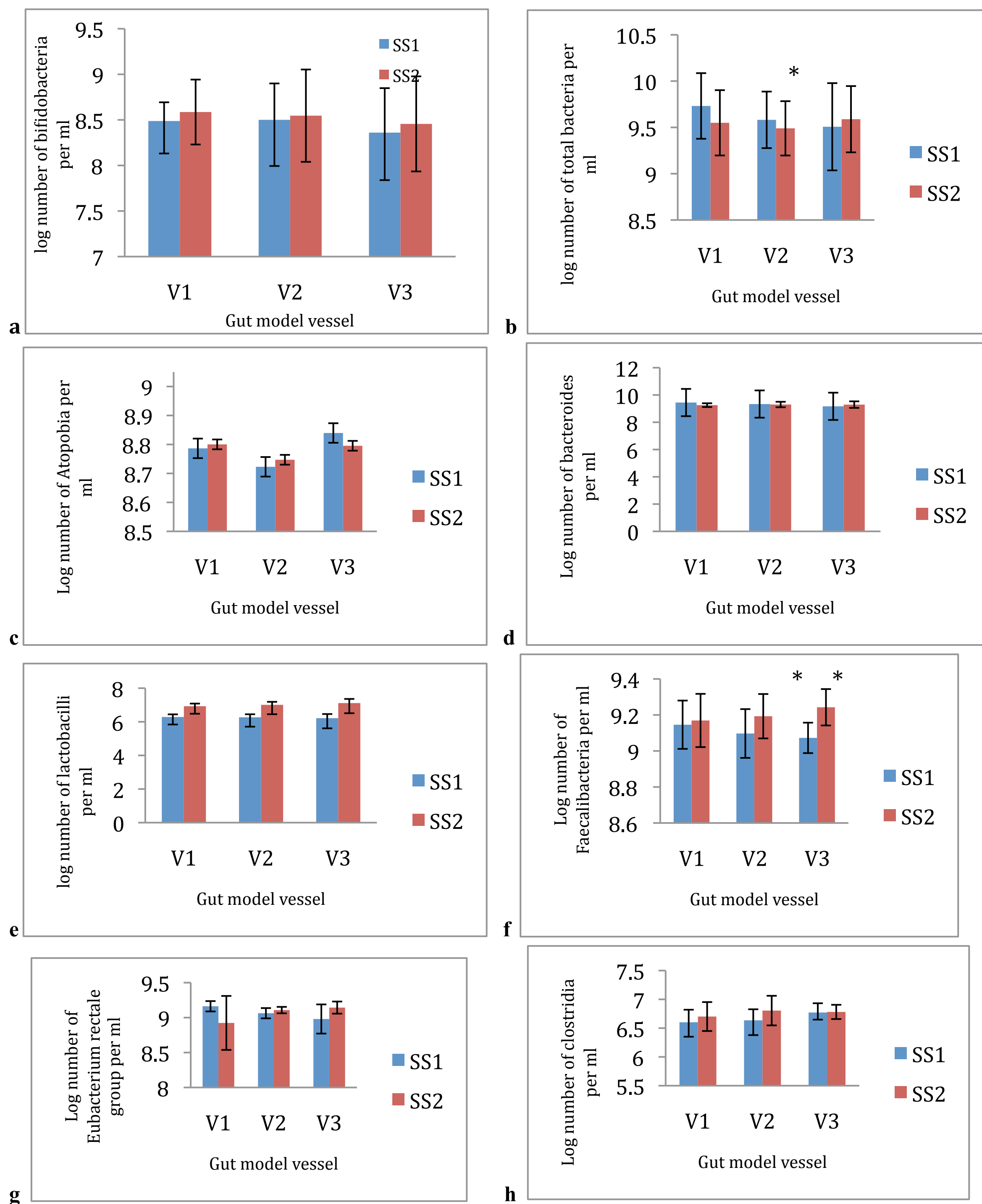

Figure 2. Changes in numbers of a) Bifidobacteria, b) Total bacteria, c) Atopobia, d) *Bacteroides*, e) Lactobacilli, f) *Faecalibacterium prausnitzii*, g) *Eubacterium rectale/ Clostridium coccoides* group h) *Clostridium histolyticum* group, in the three gut model vessels- V1, V2 and V3 fed with AlginateH1CC2012 at SS1 (day 10,11 and 12 without addition of substrate) and at SS2 (day 20, 21 and 22 after addition of Alginate). The data represent a mean of triplicate values derived from three consecutive days for each model and a mean of three models with faecal samples from three different donors.

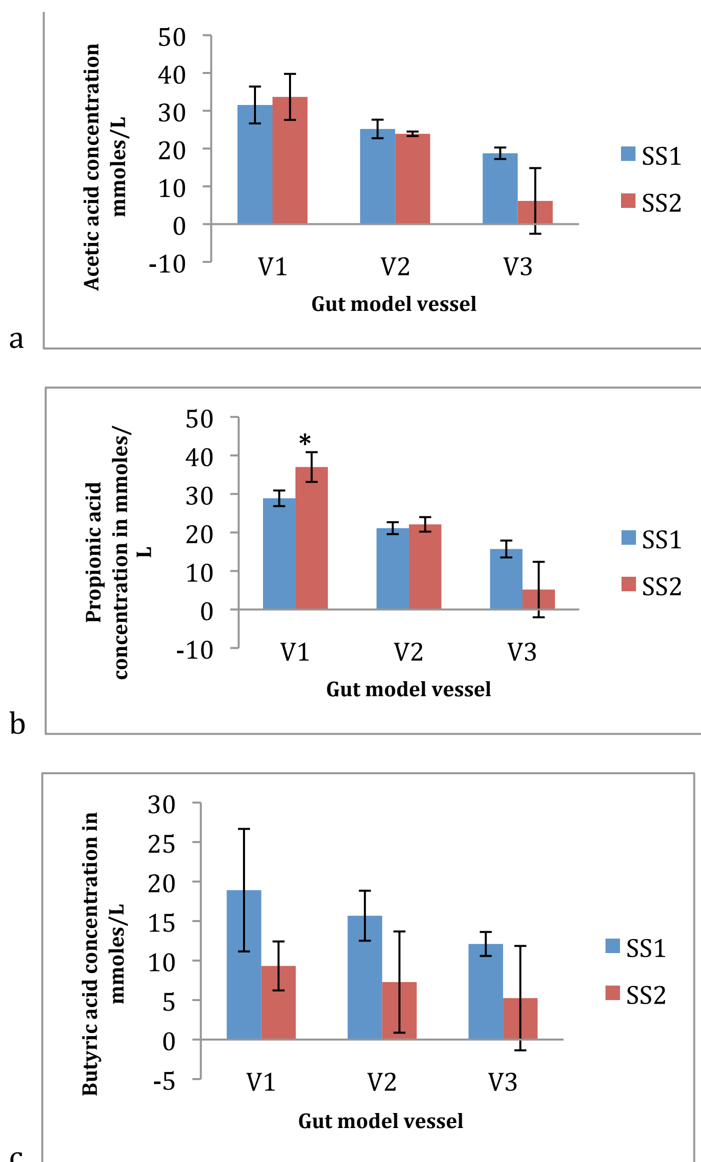

Figure 3. SCFA changes a) Acetic acid, b) Propionic acid and c) Butyric acid in the three gut model vessels- V1, V2 and V3 fed with AgarH1CC2013 at SS1 (day 10, 11 and 12 without addition of substrate) and at SS2 (day 20, 21 and 22 after addition of Agar). The data represent a mean of triplicate values derived from three consecutive days for each model and a mean of three models with faecal samples from three different donors.

Short chain fatty acid production : The concentrations of SCFA in the three vessels- V1, V2 and V3 fed with agar are shown in Figure 3. Feeding with LMWP Agar resulted in elevated levels of propionate from 28.87mM to 36.99mM in proximal colon (vessel 1) ($p=0.03$) (Figure 3b). However, there were no other differences tested in any of the other vessels for steady state 2 (SS2) compared to steady state 1 (SS1). Gut models with Alginate showed no significant changes in SCFA concentrations (data not shown)

2.3 Effects on calcium transport (Task 3.3)

Objective: To screen agar and alginate LMWPS provided by Cybercolloids for effect on calcium transport across a cell monolayer using a Caco-2 cell based assay.

Methods: AgarH1CC2013 and Alginate H1CC2012 LMWP were tested on the Caco2 cell model for epithelial function in the presence and absence of Aquamin (Calcium source derived from a calcified seaweed). Neither LMWP significantly improved barrier function in the CaCo-2 epithelial layer as measured by TER (electrical resistance measure) nor did addition of Aquamin in conjunction with either improve barrier function in the cell model.

Addition of Aquamin in conjunction with LMWPS (Agar and Alginate) did not improve calcium absorption across the epithelial layer (Fig 4)

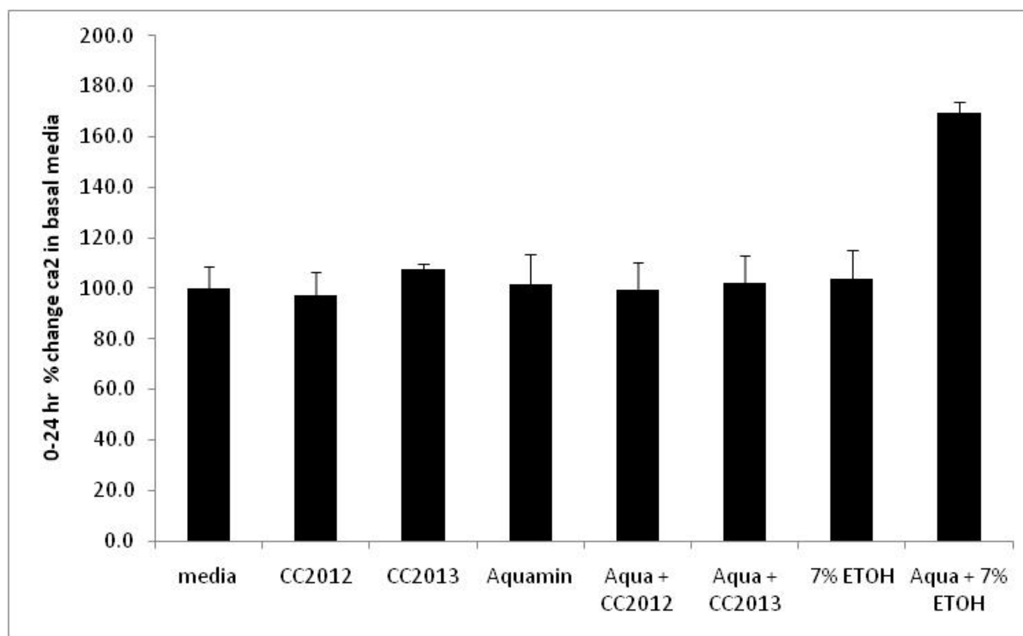

Fig 4 Effect of Alginate and Agar LMWPs on calcium transport in vitro. No statistical significance N=3 independent experiments.

3. Efficacy of selected LMWPs in human volunteers in terms of bifidogenic activity, gut health, blood glucose and plasma lipids (WP4; WP5)

3.1 Methods

Subjects.

Sixty volunteers, mean age 35.9 +/- 8.73 years, mean BMI 25.8 +/- 3.6, 30 males, 30 females completed the study. All volunteers were healthy non vegan, non-users of dietary supplements or medications, as determined using a pre-screening health and lifestyle questionnaire. The study was conducted with the prior approval of the ethics committee of the University of Ulster and with the informed consent of volunteers and fully complied with the requirements of the Ethics review.

Study design

The study design was a 3-way randomized double blind placebo controlled study. The volunteers were randomly assigned to either of the two LMWP candidates (AgarH1CC2013 and Alginate H1CC2012) or a placebo control (Maltodextrin) during the first phase of the study. It was ensured that each of these groups contained equal numbers of males/females. During the 28 day treatment phase, subjects consumed daily one 250 ml drink containing 8g of test agent (agar, alginate or maltodextrin) in addition to their normal diet. During the washout phase (28 days), between treatment phases subjects were asked to maintain their habitual diet. Faecal samples and 4-day food diaries were provided before and after each treatment phase (weeks 1, 4, 8, 12, 16, 20) for each of six sampling visits. Fasting blood samples were collected, only before and after the alginate and the placebo treatment phases, by venepuncture into EDTA or non additive tubes as required. All blood samples were processed on ice. Plasma and serum were immediately stored at –20 ^{0}C. All biological measurements were carried out at the end of the intervention, in batches containing equal number of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses.

Food diaries and bowel habit questionnaires

All subjects were requested to provide information on there habitual diet at each time point of the study using a 4-day "food diary" assessment. The information on the dietary composition of the volunteers' intake was generated using the nutritional software package NETWISP. Subjects were also requested to keep a diary during each treatment period to monitor stool frequency and consistency (constipation, hard, soft or diarrhoea), abdominal pain (none, mild, moderate or severe), intestinal bloating (none, mild, moderate or severe), flatulence (none, mild, moderate or severe).

Faecal samples and processing

Faecal samples provided pre and post each 28 day treatment period were processed in accordance with the method described in Gill *et al* 2007. In brief faecal samples were collected and stored for no longer than two hours prior to processing. Individual samples were scored for stool type (Bristol stool chart, colour, consistency), weighed and the pH measured, a portion of the sample was removed and frozen directly for bacterial composition analysis (WP6, task 6.1). The remaining sample was used to produce faecal water by mixing 1:1 wt/vol with ice cold PBS then homogenised. The faecal slurry was then ultra centrifuged (Beckman XL 80 Ultracentrifuge, 50,000g, 2hrs, 4^0C), the supernatant removed, filter sterilised (0.22 μm) and stored at –80 °C. Faecal water samples will be used to measure short chain fatty acid levels and faecal water barrier function bioactivity (WP5, task 5.2, 5.3).

Faecal water activity TER

Tissue culture.: The CACO2 human adenocarcinoma cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). Dulbecco's Minimal Essential Medium (MEM) was obtained from Gibco Life Technologies Ltd., Paisley, Scotland. CACO2 cells cultured in Roux flasks as monolayers in MEM (containing 10% foetal bovine serum, 2mM glutamine and 100 units per litre penicillin/streptomycin). Cells were cultured for 7 days (<75% confluence) at 37^oC with 5% CO_2 and 95% filtered air. The medium was changed every 2 days. Thereafter cells were washed with phosphate buffered saline (PBS) for 2 minutes and re-suspended by the addition of trypsin (0.25% trypsin-EDTA) at 37°C for 5 minutes. Cells were centrifuged at 258xg for 3 minutes and re-suspended in the appropriate medium.

Trans-epithelial resistance assay (TER): CACO2 cells are capable of enterocytic differentiation forming atypical brush border membranes and tight junctions and provide an appropriate and frequently used model for permeability, barrier function and transport studies. The complexity and the number of tight-junction strands correlate with the electrical resistance of the barrier. Briefly, the CACO2 cell suspension was seeded in 6 well plates with Transwell inserts (0.1% rat tail collagen coated polyethyleneterapthalate membranes, BD Biosciences, Bedford, UK) at a density of 2.5 x10^5 cells per insert. The culture medium was replaced (1.5ml for apical, 2.5ml for basal side) every other day for 14 days. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. From day 11-14 the integrity of the monolayer was evaluated by measuring the trans-epithelial resistance (TER) (expressed as Ωcm^2) using an EVOM™ epithelial voltohmmeter (World Precision instruments Ltd, Aston, UK). Once the TER values had stabilised the inserts were ready for experimentation. The TER of the CACO2 cell monolayers was measured at 0h, then 24, 48 h. after the addition of neat faecal water to the apical compartment to reach a final dilution of faecal water 1/10. The values were in the range of 600-700 Ωcm^2 at baseline. The activity of the samples was assessed in duplicate and the mean taken. Values were calculated as a % difference from the baseline (0 hr) TER value.

Plasma Lipid profile

Plasma total cholesterol, HDL cholesterol and triglycerides were measured on the Hitachi 912 autoanalyser using commercial kits (Roche diagnostics, Lewis, UK) according to kit manufacturer's protocols and plasma LDL cholesterol was calculated from the other three lipid profile parameters using the Friedewald formula.

<u>Statistical analysis</u>
All biological measurements were carried out at the end of the intervention, in batches containing equal number of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses. All values are expressed as mean ± SD, unless otherwise specified. The mean values are shown for all subjects (n=60) during their treatment phase (alginate and agar) and during their placebo control (maltodextrin) phase. All blood and faecal water analysis were conducted in duplicates and the average of the two values taken as the final result. For the blood lipid measurements and faecal water activity, the results are presented as treatment affects. This was undertaken by calculating individual differences between pre- and post- values for both control and treatment phases for each subject. The statistical tests were then carried out on the difference (post treatment- minus pre treatment-) in values between treatment (agar and alginate) and placebo control phase (maltodextrin). For analysis on microbial composition and SCFA levels, paired t tests were used to compare changes in the bacterial population proportions from baseline to the end of each treatment for each of the bacterial groups monitored and SCFA in the in vivo study. Significance level was set at $p<0.05$. All statistical analyses were performed using the SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA).

3.2 Results

<u>Diet</u>
No significant changes in BMI were observed in the volunteers over the duration of the study. Further no significant alterations to dietary habits occurred as a result of any of the interventions (Table 7)

Table 7 Group Mean dietary intake (MDI), all time points (tp), N=60.

Group MDI (n=60)	**Protein (g)**	sd	**Total Fat (g)**	sd	**CHO (g)**	sd	**Energy (kcal)**	sd	**Fibre Englyst (g)**	sd
tp1	77.06	27.67	71.98	29.59	222.38	64.86	1920.02	565.67	13.09	8.62
tp2	77.39	26.07	69.09	30.05	217.56	64.99	1915.47	616.55	11.79	5.14
tp3	75.59	28.92	69.98	25.66	210.89	71.26	1904.15	563.18	10.92	3.82
tp4	77.74	20.50	72.89	26.27	219.91	78.91	1897.73	551.35	11.87	4.83
tp5	73.48	23.05	65.59	26.47	201.87	63.47	1789.67	515.92	10.74	4.18
tp6	73.58	22.27	66.86	20.80	201.78	63.09	1807.61	482.21	11.69	5.61

<u>Stool characteristics</u>
Table 8 describes the stool characteristics in response to intervention. The average group stool weight (N=60) at time point 1 was 85.97 g +/- 73.66, with a range of 282.96 g to 10.86 g; average group stool pH was 6.86 =/- 0.64 with a 7 day average group stool frequency was 11.36 +/- 8.14 and the median stool type was 4 on the Bristol chart. Stool weight for volunteers on the agar treatment was observed to increase, with the average stool weight increasing by 9.67 g, in comparison to stool weight on placebo which decreased by 18.16, while alginate treatment increased stool weight by 1.18g. Thus, a significant treatment effect was observed for both agar ($p=0.002$) and alginate ($p=0.02$) in comparison to placebo treatment, indicating a pronounced stool bulking capacity for the alginate and agar based

treatments of approximately 30% and 20% respectively relative to placebo. Treatment with agar also caused a minor but significant reduction in stool pH. Neither treatment resulted in a significant alteration in 7 day stool frequency.

Table 8 Changes in stool characteristics in response to treatment

Stool weight (g)	Pre-treatment	sd	Post-treatment	sd	Treatment effect Post treat- Pre treat
Placebo	82.32	71.05	64.16	47.97	-18.16[a]
Agar	75.12	58.68	84.79	52.06	9.67[b]
Alginate (N=59)	67.56	58.20	68.75	52.04	1.18[b]
Stool pH					
Placebo (N=59)	6.96	0.66	7.15	0.50	0.19[a]
Agar	7.06	0.56	6.89	0.46	-0.17[b]
Alginate	6.94	0.62	7.05	0.60	0.11[a]
Stool type					
Placebo	4	2	3	2	-0.41
Agar	4	2	4	1	0.68
Alginate	4	2	3	1	-0.18
Stool Frequency					
Placebo (N=51)	10.16	5.34	10.20	6.43	0.04
Agar (N=50)	11.52	8.14	10.86	5.70	-0.66
Alginate (N=46)	10.04	4.23	10.33	4.13	0.28

[a,b] matching letters in superscript denote no significant difference between values. Wilcoxon signed ranked test P<0.05.

Faecal water bioactivity, barrier function.

The group average faecal water activity for time point 1 at 48hrs was 106.6% +/- 18.09. No significant effects (outliers removed) were observed for either alginate or agar treatment on FW barrier function as measured by the TER (Table 9). However, when the population was adjusted to remove subjects already meeting the approximate RDA for fibre (in this case an intake greater than17g p.d.) a significant reduction in TER of 6.5% (p=0.037) was observed for the individuals with moderate fibre consumption >9-<17g p.d. consuming the alginate LMWP (outliers removed) . A greater reduction in FW activity compared to placebo was observed when individuals with a low fibre intake were considered (9.2%) however this failed to reach significance P=0.057 (1-tailed), most likely as a result of the diminished population size N=16.

Table 9 Effect of intervention on faecal water activity (TER, barrier function).

Treatment	All subjects (n=60)	Moderate & low fibre intake (n=55)	Moderate fibre intake (n=39)	Low fibre intake (n=16)
Placebo	1.0%	1.7% [a]	-0.2%	5.4%
Agar	-0.6%	-0.4%	-1.8%	4.3%
Alginate	-4. %	-5.2% [b]	-6.3%	-3.8%

Treatment effect (post treatment – pre treatment values) of intervention on FW activity Total study population, stratifies in relation to habitual levels of fibre intake , UK Fibre RDA 18 g/p.d., stratified groups as follows High fibre intake > 17g p.d. Moderate fibre intake <17- >9 g p.d., Low fibre <9g p.d. [a,b] matching letters in superscript denote no significant difference between values. Wilcoxon signed ranked test P<0.05.

Microbial changes

The predominant bacterial groups within the faecal microbiota were enumerated using fluorescent in situ hybridization (Table 10). A comparison of baseline levels with treatment after consumption of Agar 1 for 28 days, led to a significant increase in *Faecalibacterium prausnitzii* group from $\log_{10}$ 8.81 to 8.91 (p=0.04). *Clostridium histolyticum* group showed a decrease in bacterial populations after comparison of baseline vs. treatment from $\log_{10}$ 6.79 to 6.72 with near borderline significance (p=0.08). None of the other bacterial groups showed any significant changes after feeding with Agar LMWP. Comparing baseline vs. treatment after feeding with Alginate LMWP, a significant decrease in lactobacilli numbers was observed from $\log_{10}$ 6.85 to 6.72 (p=0.05). For *Atopobium* group, a decrease in bacterial populations from $\log_{10}$ 8.30 to 8.21 was observed with near borderline significance (p=0.08). None of the other bacterial groups showed any significant changes after feeding with Alginate. For control, Maltodextrin, a comparison of baseline vs. treatment values showed a decrease in bacterial numbers for *Clostridium histolyticum* group from $\log_{10}$ 6.78 to 6.71 (p=0.03). Bifidobacterial numbers showed an increase from $\log_{10}$ 8.16 to 8.25 with near borderline significance (p=0.10). Also, none of the other bacterial groups showed any significant changes after feeding with control maltodextrin.

Table 10 Faecal bacterial numbers for sixty volunteers over the trial period. Bacterial counts in stool samples as determined by fluorescence in situ hybridization as mean $\log_{10}$ cells/g faeces.

Bacterial group	Maltodextrin		Agar		Alginate	
	Baseline	Treatment	Baseline	Treatment	Baseline	Treatment
Total bacteria	9.73±0.21	9.75±0.26	9.74±0.22	9.76±0.23	9.76±0.27	9.74±0.20
***Bacteroides* spp.**	9.07±0.26	9.07±0.33	9.03±0.26	9.06±0.36	9.02±0.30	9.04±0.32
***Eubacterium rectale* subgroup**	8.81±0.24	8.88±0.33	8.81±0.28	8.90±0.30[b]	8.86±0.32	8.89±0.31
***Bifidobacterium* spp.**	8.16±0.38	8.25±0.37[b]	8.16±0.34	8.20±0.32	8.17±0.36	8.20±0.35
***Atopobium* spp.**	8.27±0.37	8.28±0.35	8.31±0.31	8.27±0.37	8.30±0.33	8.21±0.35[b]
***C. histolyticum* subgroup**	6.78±0.19	6.71±0.21[a]	6.79±0.21	6.72±0.23[b]	6.78±0.21	6.73±0.18
Lactobacilli/Enterococci	6.83±0.37	6.74±0.46	6.82±0.39	6.84±0.48	6.85±0.36	6.72±0.40[a]
Propionibacteria	8.80±0.31	8.81±0.34	8.82±0.26	8.79±0.28	8.89±0.26	8.83±0.30
Faecalibacterium prausnitzii	8.84±0.29	8.88±0.21	8.81±0.24	8.91±0.28[a]	8.90±0.22	8.98±0.25

a: Treatment significantly different from baseline values (p<0.05)
b: Treatment borderline different from baseline values (p=0.05-0.10)

SCFA analysis

Table 11 shows concentrations of faecal SCFA. The levels of SCFA detected in all the treatments were very low. Significant increase in levels of propionic acid and butyric acid were observed before and after feeding with agar, alginate and control (maltodextrin). For maltodextrin, agar and alginate the propionic acid levels increased from 0.84 to 1.3 mmoles/L ($p=0.006$), 0.86 to 1.18 mmoles/L ($p=0.001$) and 0.96 to 1.29 mmoles/L ($p=0.003$) respectively. Butyric acid levels increased from 0.85 to 1.42 mmoles/L ($p<0.001$), 0.82 to 1.44 mmoles/L ($p<0.001$) and 0.97 to 1.57 mmoles/L ($p<0.001$) for agar, alginate and control (maltodextrin) respectively. No significant changes were observed for acetic acid concentrations for agar or alginate. However, maltodextrin showed a significant increase in acetic acid concentrations from 1.67 to 2.03mmoles/L ($p=0.05$).

Table 11. SCFA concentrations in faecal samples collected from sixty volunteers over the course of the trial measured by gas chromatography

SCFA	Maltodextrin		Agar		Alginate	
	Baseline	Treatment	Baseline	Treatment	Baseline	Treatment
Acetic acid	1.67±0.93	2.03±1.29[a]	1.66±1.11	1.91±1.15	1.87±1.12	2.09±1.41
Propionic acid	0.84±0.59	1.30±0.57[a]	0.86±0.60	1.18±0.45[a]	0.96±0.63	1.29±0.53[a]
Butyric acid	0.97±0.62	1.57±0.57[a]	0.82±0.54	1.44±0.50[a]	0.97±0.62	1.57±0.57[a]

a: Treatment significantly different from baseline values ($p<0.05$)

Blood lipid profile.

The mean group (n=60) blood cholesterol level at time point 1 was 4.82 +/- 1.01 mmol/l; HDL 1.57 +/-.39 mmol/l; LDL 2.7 +/-0.69 mmol/l and triglycerides 1.19 +/- 0.68 mmol/l. No significant effect was observed on fasting blood lipid profiles in response to consumption of alginate treatment in comparison to placebo treatment as is reported table 12.

Table 12 Effect of alginate LMWP on blood lipid profile.

	Pre-treatment	sd	Post treatment	sd	Treatment effect Post treat-Pre treat
Tryglyceride mmol/l					
Placebo (n=59)	1.21	0.76	1.26	0.69	0.04
Alginate (n=60)	1.20	0.72	1.22	0.58	0.02
LDL mmol/l					
Placebo (n=59)	2.79	0.76	2.82	0.78	0.01
Alginate (n=60)	2.75	0.73	2.79	0.82	0.03
HDL mmol/l					
Placebo (n=59)	1.72	0.45	1.68	0.47	-0.03
Alginate(n=60)	1.64	0.45	1.66	0.44	0.01
Cholesterol mmol/l					
Placebo (n=59)	5.06	0.93	5.02	1.05	-0.04
Alginate(n=60)	4.94	0.98	5.01	0.97	0.05

3.3 Effect of alginate LMWP on blood glucose level.

Methods

Twenty volunteers undertook an acute glycaemic response study , mean age, 33.0 +/- 8.51 years, mean BMI 26.6 +/- 4.01) 11 males, 9 females completed the study. All subjects were healthy non vegan, non-users of dietary supplements or medications, as determined using a pre-screening health and lifestyle questionnaire. The study was conducted with the prior approval of the ethics committee of the University of Ulster and with the informed consent of volunteers.

Study design : The acute study design was a 2-way randomized double blind placebo controlled study. The volunteers were randomly assigned to either an alginate LMWP or a placebo control (Maltodextrin) during the first phase of the study. Fasting volunteers were cannulated 30 minutes prior to consumption of the test drink either a 250 ml drink (66% water 30% glucose) containing either 8g alginate LMWP or placebo. Blood glucose response was determined for 180min post-prandially (0, 15, 30, 60, 90, 120,150, 180 min) by cannulated venepuncture into sodium heparin/sodium fluoride tubes as required. All blood samples were processed on ice. Plasma and serum were immediately stored at –20 ^{0}C. All biological measurements were carried out at the end of the intervention, in batches containing equal number of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses. 1 subject did not complete the crossover treatment, therefore analysis presented for n=19 subjects using paired T test $P<0.05$.

Blood glucose profile: Serum glucose was measured on the Hitachi 912 autoanalyser using commercial kits (Randox) according to kit manufacturer's protocols. Glucose levels were also measured in whole blood at time of sampling using the haemocue analysis system. Analysis of blood glucose was conducted using area under the curve (AUC) performed using the SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Consumption of alginate significantly reduced the glycaemic response to a glucose challenge measured using AUC analysis ($P<0.026$) when measured in whole blood using the haemocue analysis system (Table 13). A similar effect was seen when the effect was measured using the serum glucose assay, although the decrease just failed to reach significance ($P=0.062$, paired T test, 1 tailed). The results suggest that alginate LMWP could be useful as a means of modulating beneficially post-prandial glucose levels although further work is needed to confirm the result and to determine the optimum dose to be used. .

Table 13 Effect of alginate on blood glucose.

Treatment	Whole blood Glucose level AUC (Haemocue analysis)	sd	Serum Glucose level AUC (ilab analysis)	sd
Placebo (n=19)	15.03^{a}	2.77	15.9^{a}	3.3
Alginate (n=19)	14.03^{b}	2.36	15.22^{a}	2.6

^{a,b} matching letters in superscript denote no significant difference between values. Paired T Test.

4. Conclusions

Batch culture screening of 19 novel LMWP derivatives of seaweed derived polysaccharides revealed that several of such compounds had potential prebiotic properties. Two LMWPs (one agar- and one alginate-derived) were selected on the basis of technological and organoleptic suitability for more detailed assessment. In an *in vitro* gut model and in a human intervention study, small changes in gut microflora profile were seen with both LMWP products. In some cases changes were observed in groups with potentially beneficial effects, eg the increase in *Faecalibacterium prausnitzii* associated with agar intake in the human volunteer study. Although statistically significant, the results however are unlikely to be of biological importance due to the small magnitude of the changes. Similarly, only small changes in SCFA profile were seen which probably reflect the minor changes in gut bacterial numbers. We conclude therefore that the agar and alginate LMWPs have no prebiotic effects in human volunteers. There were however potentially beneficial changes in gut function characteristic of dietary fibre in the volunteers consuming the agar or alginate LMWP derivatives. During consumption of the LMWPs there were significant increases in stool weight by comparison with the placebo group and the agar LMWP reduced faecal pH. Furthermore, post prandial glucose absorption was decreased in subjects consuming modest amounts (8g/day) of alginate LMWP. Such effects indicate that despite the considerable reduction in molecular weight of the polysaccharides and the associated decrease in viscosity, fibre-related properties in the gut were maintained.