Effects on weight gain and gut microbiota in rats given bacterial supplement and a high-energy dense diet from foetal life through six months of age

DNA extraction

DNA from caecal content was isolated and purified by QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in combination with glass bead beating and usage of BioRobot EZ1 (tissue kit and card; Qiagen). Caecal samples (180-220 mg) were placed in tubes with glass beads (2 mm in diameter), homogenized in 1·4 ml of ASL-buffer and lysed at 95°C for 5 min. Further preparation was performed as previously described[1].

PCR amplification, purification, and measurement of DNA concentration

The 16S rRNA genes were amplified with the universal forward primer FAM-ENV1 (5´-AGA GTT TGA TII TGG CTC AG-3´), fluorescently labelled with carboxyfluorescein (6-FAM) at the 5´ end, and the reverse primer ENV2 (5´-CGG ITA CCT TGT TAC GAC TT-3´) (I=inosine), which anneal by 8-27 bp and 1511-1492 bp respectively. The PCR reaction mixture contained 0·5 µM FAM-ENV1 primer, 0·2 µM ENV2 primer, 0·2 mM of each deoxyribonucleotide triphosphate (Roche Diagnostics, Indianapolis, IN), 5 µl of 10 x PCR reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 8·3), 2·5 U/µl Taq polymerase (Roche Diagnostics, Mannheim, Germany) and 0·2-5 µl of template, in a final volume of 50 µl. Amplification was made for 30 cycles as described by Karlsson *et al.* and PCR products were verified and purified as described elsewhere[2]. DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) using 1 µl purified PCR product.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

Aliquots of 200 ng purified PCR products were digested at 37ºC in a Mastercycler® 5333 (Eppendorf, Hamburg, Germany) with 10 U of the restriction enzymes *Msp*I and *Alu*I (Fermentas GmbH, St. Leon-Rot, Germany), separately, for 5 and 2 hours respectively, in a total volume of 10 µl. Inactivation was made by heating at 65ºC for 20 minutes. Digestion products were stored at -20˚C until analysis by capillary electrophoresis at the core facility DNAlab at Skåne University Hospital, Malmö, Sweden. Prior to analysis, samples were diluted 1:5 in sterile distilled water. One microlitre of this dilution was mixed with 9 µl GeneScan™-600 LIZ® internal size standard (Applied Biosystems) that was diluted 1:75 with formamide (Applied Biosystems) before being denatured at 94˚C for 5 minutes and immediately chilled to 4˚C before loading to 3130xl Genetic Analyzer (Applied Biosystems), using POP-7 polymer (Applied Biosystems), 46 seconds injection time, 1·2 kV injection voltage, 15 kV run voltage and 60˚C run temperature. Electropherograms were analysed with GeneMapper® version 4.0 (Applied Biosystems, Foster city, CA, USA) and the fragment sizes and peak areas were estimated using the Local Southern method. Threshold for internal standard and T-RFs was set to 50 fluorescence units.

Cloning and sequencing

For one animal in group C and one in group Lp, and for two animals in group Ec, that demonstrated the highest diversity according to T-RFLP profiles, four PCR reactions were made with ENV1 and ENV2 primers as described above. PCR products from the same animal were pooled and run on 1·5% agarose gel in TAE buffer. Bands were excised and DNA was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA).

Purified PCR products were ligated into the pGEM®-T vector system and transformed into *E. coli* JM 109 high efficiency competent cells according to the manufacturer's instructions (Promega). Colonies were blue/white screened on Luria-Bertani agar with Ampicillin (100 µg/ml, Sigma, St. Louis, USA), IPTG (0·5 mM, Promega) and X-Gal (80 µg/ml, Promega). White colonies were randomly picked from each animal and stored in freezing medium at -80°C. For each animal 41-44 clones were sequenced by Eurofins MWG Operon (Martinsried, Germany) using primer Univ0519d (5´-GWA TTA CCG CGG CKG CTG-3´) (W= A or T, K= G or T). Sequences were checked and edited in BioEdit version 7.0.9.0. prior to submission to Ribosomal Database Project for comparison with closest clone, isolate and type strain. Clone libraries were compared using LibCompare with Naïve Bayesian rRNA Classifier[3] with confidence threshold of 80%.

1. Wang M, Karlsson C, Olsson C*, et al.* (2008) Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol* **121**, 129-134.
2. Karlsson C, Ahrne S, Molin G*, et al.* (2010) Probiotic therapy to men with incipient arteriosclerosis initiates increased bacterial diversity in colon: A randomized controlled trial. *Atherosclerosis* **208**, 228-233.
3. Wang Q, Garrity GM, Tiedje JM*, et al.* (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261-5267.

Table S1. Composition of the high-energy dense diet (HEDD)[1]

	g/kg
Casein, 80 Mesh	226·43
L-Cysteine	3·40
Corn Starch	82·08
Maltodextrin 10	113·21
Sucrose	230·96
Dextrose	8·38
Fructose	10·19
Cellulose, BW200	56·61
Soybean Oil	28·30
Lard	175·48
Mineral Mix S10026	11·32
DiCalcium Phosphate	14·72
Calcium Carbonate	6·23
Potassium Citrate, 1 H_2O	18·68
Vitamin Mix V10001	11·32
L-Ascorbic Acid Phosphate (35% active)	0·43
Choline Bitartrate	2·26
Protein	20
Carbohydrate	45·6
Fat	20
	E%
Protein	18
Carbohydrate	41
Fat	41
Energy	18·70 kJ/g

[1] Produced by Research Diets (New Brunswick, NJ, USA)

Table S2. Incidence of T-RFs that differed significantly between groups Lp and C, and between groups Ec and C.

T-RF size	*P*-value[†] Lp[*] vs C[*]	group Lp[*]	group C[*]	group Ec[*]	*P*-value[†] Ec[*] vs C[*]
305bp, *Msp*I			8/9	1/8	0·003
488bp, *Msp*I			6/9	0/8	0·007
90bp, *Alu*I			7/8	0/8	0·001
180bp, *Alu*I			6/8	1/8	0·021
203bp, *Alu*I			9/9	1/8	<0·001
258bp, *Alu*I			7/8	0/8	0·001
80bp, *Msp*I	0·033	7/9	2/9		
294bp, *Msp*I	0·005	0/9	6/9		
488bp, *Msp*I	0·005	0/9	6/9		
515bp, *Msp*I	0·026	6/9	1/9		

[*]C, control; Lp, *L. plantarum* DSM 15313; Ec, *E. coli* CCUG 29300[T].

[†]Significance according to Fisher's Exact test with two-tailed *P*-values and T-RF sizes according to GeneMapper v. 4·0.