CoMiniGut - a small volume in vitro colon model for the screening of gut microbial fermentation processes

Maria Wiese [Corresp., 1] , Bekzod Khakimov [1, 2] , Sebastian Nielsen [3] , Helena Sørensen [1] , Frans van den Berg [1] , Dennis Sandris Nielsen [1]

[1] Department of Food Science, University of Copenhagen, Frederiksberg, Denmark
[2] Department of Plant and Environmental Sciences, Copenhagen Plant Science Center, University of Copenhagen, Frederiksberg, Denmark
[3] Plant Facilities and Workshops, University of Copenhagen, Frederiksberg, Denmark

Corresponding Author: Maria Wiese
Email address: mwiese@food.ku.dk

Driven by the growing recognition of the influence of the gut microbiota (GM) on human health and disease, there is a rapidly increasing interest in understanding how dietary components, pharmaceuticals and pre- and probiotics influence GM. *In vitro* colon models represent an attractive tool for this purpose. With the dual objective of facilitating the investigation of rare and expensive compounds, as well as an increased throughput, we have developed a prototype *in vitro* parallel gut microbial fermentation screening tool with a working volume of only 5 ml consisting of 5 parallel reactor units, that can be expanded with multiples of five to increase throughput. This allows e.g. the investigation of interpersonal variations in gut microbial dynamics and the acquisition of larger data sets with enhanced statistical inference.The functionality of the *in vitro* colon model, Copenhagen MiniGut (CoMiniGut) was first demonstrated in experiments with two common prebiotics using the oligosaccharide inulin and the disaccharide lactulose at 1% (w/v). We then investigated fermentation of the scarce and expensive human milk oligosaccharides (HMOs) 3-Fucosyllactose, 3-Sialyllactose, 6-Sialyllactose and the more common Fructooligosaccharide in fermentations with infant gut microbial communities. Investigations of microbial community composition dynamics in the CoMiniGut reactors by MiSeq-based 16S rRNA gene amplicon high throughput sequencing showed excellent experimental reproducibility and allowed us to extract significant differences in gut microbial composition after 24h of fermentation for all investigated substrates and fecal donors. Furthermore, short chain fatty acids (SCFAs) were quantified for all treatments and donors. Fermentations with inulin and lactulose showed that inulin leads to a microbiota dominated by obligate anaerobes, with high relative abundance of Bacteroidetes, while the more easily fermented lactulose leads to higher relative abundance of Proteobacteria. The subsequent study on the influence of HMOs on two infant GM communities, revealed the strongest bifidogenic effect for 3´SL for both infants. Inter-individual differences of infant

GM, especially with regards to the occurrence of Bacteroidetes and differences in Bifidobacterial species composition, correlated with varying degrees of HMO utilization foremost of 6´SL and 3´FL, indicating species and strain related differences in HMO utilization which was also reflected in SCFAs concentrations, with 3´SL and 6´SL resulting in significantly higher butyrate production compared to 3´FL. In conclusion, the increased throughput of CoMiniGut strengthens experimental conclusions through elimination of statistical interferences originating from low number of repetitions. Its small working volume moreover allows the investigation of rare and expensive bioactives.

1 **CoMiniGut - a small volume *in vitro* colon model for the screening of gut microbial**

2 **fermentation processes**

4 Maria Wiese[1], Bekzod Khakimov[1,2], Sebastian Nielsen[3], Helena Sørensen[1], Frans van den Berg[1],

5 Dennis Sandris Nielsen[1]

7 [1] Department of Food Science, University of Copenhagen, Frederiksberg C, Denmark

8 [2] Department of Plant and Environmental Sciences, Copenhagen Plant Science Center,

9 University of Copenhagen, Frederiksberg C, Denmark

10 [3] Plant Facilities and Workshops, University of Copenhagen Frederiksberg C, Denmark

12 Corresponding Author:

13 Maria Wiese[1]

14 Rolighedsvej 26, 1958 Frederiksberg, Denmark

15 E-mail address: mwiese@food.ku.dk

Abstract

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and disease, there is a rapidly increasing interest in understanding how dietary components,
pharmaceuticals and pre- and probiotics influence GM. *In vitro* colon models represent an
attractive tool for this purpose. With the dual objective of facilitating the investigation of rare
and expensive compounds, as well as an increased throughput, we have developed a prototype *in
vitro* parallel gut microbial fermentation screening tool with a working volume of only 5 ml
consisting of 5 parallel reactor units, that can be expanded with multiples of five to increase
throughput. This allows e.g. the investigation of interpersonal variations in gut microbial
dynamics and the acquisition of larger data sets with enhanced statistical inference.

The functionality of the *in vitro* colon model, Copenhagen MiniGut (CoMiniGut) was first
demonstrated in experiments with two common prebiotics using the oligosaccharide inulin and
the disaccharide lactulose at 1% (w/v). We then investigated fermentation of the scarce and
expensive human milk oligosaccharides (HMOs) 3-Fucosyllactose, 3-Sialyllactose, 6-
Sialyllactose and the more common Fructooligosaccharide in fermentations with infant gut
microbial communities.

Investigations of microbial community composition dynamics in the CoMiniGut reactors by
MiSeq-based 16S rRNA gene amplicon high throughput sequencing showed excellent
experimental reproducibility and allowed us to extract significant differences in gut microbial
composition after 24h of fermentation for all investigated substrates and fecal donors.
Furthermore, short chain fatty acids (SCFAs) were quantified for all treatments and donors.
Fermentations with inulin and lactulose showed that inulin leads to a microbiota dominated by
obligate anaerobes, with high relative abundance of Bacteroidetes, while the more easily

40   fermented lactulose leads to higher relative abundance of Proteobacteria. The subsequent study

41   on the influence of HMOs on two infant GM communities, revealed the strongest bifidogenic

42   effect for 3′SL for both infants. Inter-individual differences of infant GM, especially with

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44   composition, correlated with varying degrees of HMO utilization foremost of 6′SL and 3′FL,

45   indicating species and strain related differences in HMO utilization which was also reflected in

46   SCFAs concentrations, with 3′SL and 6′SL resulting in significantly higher butyrate production

47   compared to 3′FL.

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50   small working volume moreover allows the investigation of rare and expensive bioactives.

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52

53 **Introduction**

54 Growing evidence shows that human health and disease are linked to gut microbiota (GM)

55 composition and functionality (Marchesi et al. 2016). This has led to an increasing interest in

56 investigations on the effect of dietary components, pharmaceuticals, and pre- and probiotics on

57 GM composition and function (Payne et al. 2012). Human intervention studies are the "gold

58 standard" in the study of GM and GM-host interactions, but these are also expensive and

59 hampered by ethical constrains. Similarly, animal models are essential for the study of e.g. the

60 impact of GM on many diseases, but animal GM composition differs from the human GM and

61 for ethical reasons the use of animal experiments should be minimised if alternatives are

62 available (Fenwick et al. 2009; Krych et al. 2013).

63 *In vitro* colon models facilitate investigations circumventing ethical constrains and lowering

64 study costs. These models provide unlimited screening possibilities of new products, facilitating

65 continuous monitoring and sampling possibilities under standardized conditions. A variety of *in*

66 *vitro* colon models exists as reviewed previously (Macfarlane & Macfarlane 2007; Payne et al.

67 2012; Venema & Van den Abbeele 2013). The models range from batch to continuous cultures,

68 consisting of single or multistage setups. The latter design facilitates the simulation of spatial,

69 temporal, nutritional and physicochemical properties of the colon through a series of three

70 connected chemostats, simulating the proximal, transverse and distal colon regions (Payne et al.

71 2012).

72 Along the colon a continuous increment in pH and lowered redox potential is seen due to a

73 varying degree of SCFAs production, cross-feeding of SCFAs by GM members, absorption by

74 the host and increased proteolysis (Fallingborg et al. 1993).

75 *In vitro* simulations of the colon which are operated with larger volumes do generally not

76 facilitate investigations on the GM modulating effects of expensive compounds or novel

77 synthesized materials only available in small amounts e.g. due to high costs. Furthermore, the

78 usually low throughput-number of larger *in vitro* models with only a few or no technical

79 replicates is reducing the statistical strength of experimental data and scientific conclusions.

80 HMOs are a family of structurally diverse unconjugated glycans, which constitute the third most

81 abundant human milk component (Newburg 1996). HMOs display resistance to the low pH in

82 the infant`s stomach as well as hydrolysis by host enzymes and gastrointestinal absorption

83 (Engfer et al. 2000) and are selectively fermented by the infant GM. Variations in infant GM

84 composition due to feeding and delivery mode have been described, and inter-individual

85 differences in GM prevail even when grouped according to delivery and feeding mode (Azad et

86 al. 2013). Characteristic for breast-fed infants is the selective nourishment and support of a

87 protective, co-evolved gut microbiota dominated by bifidobacteria (Bäckhed et al. 2005;

88 Haarman & Knol 2005).

89 HMOs are composed of lactose reducing ends elongated with up to 25 *N*-acetyllactosamine units,

90 which can be extensively fucosylated and/or sialylated through R-glycosidic linkages

91 (Ninonuevo et al. 2006). This translates to over 200 different known human milk oligosaccharide

92 (HMO) structures (Ninonuevo et al. 2006). Emerging data indicates that HMO profiles are

93 dynamic, revealing fluctuations between and within lactation in individual mothers (Ninonuevo

94 et al. 2006; Thurl et al. 2010; Totten et al. 2012). Knowledge on the relationship between the

95 levels of specific milk oligosaccharides and their role in maternal and infant health and

96 development is still scarce (Zivkovic et al. 2011). However, in general HMOs are associated

97 with an array of health benefits, of which many are thought to be directly or indirectly associated

98 with the infants gut microbiota (Bode 2012). Further, HMO shaped microbial communities can

99   aid to prevent the colonization by pathogens through competitive exclusion (Morrow et al.

100   2004). HMOs have been also reported to act as decoy pathogen binding sites directly inhibiting

101   the adhesion and invasion of pathogenic microbes (Marcobal et al. 2010) modulating intestinal

102   epithelial cell growth and infant immune system as reviewed previously (Bode 2006; Bode 2012;

103   German et al. 2008).

104   The utilization of specific HMOs by the infant GM is still being explored. HMO consumption is

105   conserved in some infant-associated types of bifidobacteria, such as the *B. longum* subsp. *infantis*

106   lineage, whereas other display strain-specific phenotypic variation (LoCascio et al. 2007; Ward

107   et al. 2006). Several studies have investigated the utilization of HMOs in anaerobic cultures with

108   single species (Marcobal et al. 2010; Sela et al. 2012; Sela et al. 2011; Ward et al. 2007; Ward et

109   al. 2006; Yu et al. 2013), where e.g. Marcobal et al. (2010) reported the metabolization of HMOs

110   by *Bacteroides fragilis* and *Bacteroides vulgatus* strains with high efficiency (Marcobal et al.

111   2010).

112   Functional HMO alternatives such as galacto-oligosaccharides (GOS) are produced by glycoside

113   hydrolases (GH) using lactose as substrate (Torres et al. 2010) and fructo-oligosaccharides

114   (FOS), can be produced through transfructosylation of sucrose or hydrolysis of inulin by

115   endoinulinases (Singh & Singh 2010), are the main prebiotics currently added to infant formula

116   (Rinne et al. 2005).

117   With the assumption that the human colon can be simulated *in vitro* in small volumes, and with

118   the dual objective of facilitating the investigation of rare and expensive compounds, as well as an

119   increase of throughput, we have developed a prototype *in vitro* colon model with a working

120   volume of only 5 ml mimicking an average colon transit time of 24h. Multiples of 5-plex

121   CoMiniGuts can be aligned and constitute, in combination with high throughput molecular

122   biology techniques and advanced analytics, an efficient screening platform to investigate GM

123   dynamics and fecal metabolomics. Here we demonstrate as proof of principle the use of

124   CoMiniGut for the investigations of the two common, well studied prebiotics inulin and

125   lactulose. We then investigate the fermentation of the rare and expensive compounds, and most

126   original prebiotics, namely human milk oligosaccharides and compare the effect with that of

127   fructo-oligosaccharides (Bode 2012; Marcobal et al. 2010).

128

129   **Material and Methods**

130   **The CoMiniGut**

131   The CoMiniGut prototype consists of a climate box with five parallel single-vessel, stirred,

132   anaerobic reactor units, which are pH monitored and controlled (Fig. 1). Each anaerobic reactor

133   unit consists of a fused quartz glass vial (FQ-2010, Fused quartz crucible, cylindrical, 10 ml, OD

134   22 mm x H 33 mm AdValue Technology, Tucson, AZ85713 USA, 5 ml working volume)

135   positioned in a 150 ml polymethylmethacrylat (PMMA) compartment. Anaerobic conditions are

136   achieved using either Anaerogen compact sachets (AN0020D, ThermoScientific) positioned

137   inside the PMMA compartments or via integrated gas in- and outlets for flushing the

138   compartments with nitrogen (99.8%) to maintain anaerobiosis (this also facilitates gas or

139   headspace sampling). Resazurin soaked indicators are used to signal anaerobiosis (Anaerobe

140   Indicator Test, Sigma Aldrich). The lid of the PMMA compartments is composed of a PMMA

141   ring and an exchangeable vacuum greased silicon rubber septa (20420-U SUPELCO GR-2

142   Rubber Sheet Stockmaterial, Sigma) containing a pH probe inlet. The rubber septa are penetrable

143   by needles for pH control, sampling, as well as feeding of substrate if desired. The parallel

144   alignment of the five reactor vessels in one unit is based on a magnetic stirrer with five stirring

145   positions. The climate box is kept at a temperature of 37°C by a circulating water bath connected

146   to a heat-exchange plate inside the climate box and an external temperature probe positioned in

147   the box for feedback control. A ventilation system secures even temperature distribution, and

148   temperature logging (Temp 101A MadgeTech Temperature data logger) is performed throughout

149   experiments. The pH is monitored via a 6 channel pH meter and data logger (Consort multi-

150   parameter analyser C3040). The pH meter is connected to a laptop running in-house Matlab

151   scripts for pH control (ver. R2015a, The MathWorks, Inc., Massachusetts, USA), which

152   regulates a multichannel syringe pump charged with syringes containing 1 M NaOH. The

153   syringes (10 ml, BD Biosciences) are connected via tubing (VWR) and injection needles

154   (Frisenette) into the fermentation compartments.

155   **Fecal Inoculum**

156   For inulin and lactulose fermentations fecal samples from two healthy adults (F1, F2) were

157   individually homogenized in a 1:1 ratio with 1M PBS/20% glycerol (v/v) in a stomacher bag for

158   2 x 60 sec using the Stomacher (Stomacher 400, Seward) at normal speed. For HMO

159   fermentations fecal samples were prepared as stated above for two infant fecal donors.

160   Participants and parents of infants provided consent, sampling and use of fecal material for

161   inoculation of the CoMiniGut fermentations has been approved by the Ethical Committee (E) for

162   the Capital Region of Denmark (H-15001754).

163   Both infant donors were healthy males, approximately 6 months of age, born vaginally, have

164   been exclusively breast-fed and did not receive antibiotic treatment nor probiotics. Fecal slurries,

165   with a final glycerol concentration of 10% (a standard glycerol concentration used for fecal

166   transplants (Hamilton et al. 2012) were then aliquoted into cryo vials and stored at -60°C until

167   use.

168   Fecal glycerol stocks were thawed and further diluted with 0.1M PBS pH 5.6, ratio 1:4, on the

169   day of the experiment. CoMiniGut reaction vessels containing 4.5 ml of media were then

170   inoculated with 0.5 ml of fecal slurry to achieve an inoculation at 10% of the fermentation

171   volume (hence 1% original fecal matter), diluting the glycerol out to 0.2% (v/v).The viability of

172   feces derived microbiota of frozen and fresh fecal slurries was evaluated through the counting of

173   colony forming units (CFUs) on GAM and BHI agar plates after anaerobic incubation for 72h at

174   37°C.

175

176   ***In vitro* fermentation media and conditions**

177   Stirred batch-culture fermentations (5 ml working volume) were set up and aseptically filled with

178   4.5 ml basal medium (0.5 g/l bile salts, peptone water 2 g/l, 2 g/l yeast extract (all purchased

179   from Oxoid), 0.1 g/l NaCl, 0.04g/l K_2HPO_4, 0.04 g/l KH_2PO_4 (all purchased from Merck), 0.01

180   g/l $MgSO_4 \times 7H_2O$, 0.01 g/l $CaCl_2 \times 6H_2O$, 2g/l $NaHCO_3^-$, Hemin 0.002 g/l, Vitamin K_1 10 µl,

181   Tween-80 2 ml (all purchased from Sigma-Aldrich), 0.5 g/l L-Cysteine HCl (Calbiochem). The

182   basal colon medium was chosen for screening purposes as also done previously (Fooks & Gibson

183   2002; Sanz et al. 2005; Sarbini et al. 2013; Saulnier et al. 2008).

184   For the fermentations of inulin and lactulose the media was supplemented with 50 mM MES 2-

185   (*N*-morpholino) ethanesulfonic acid (SIGMA-Aldrich) buffer, divided into two batches and one

186   batch was supplemented with 1% inulin (w/v) from chicory (SIGMA-Aldrich) and the other

187   batch with 1% lactulose (w/v) (SIGMA-Aldrich). For the HMO fermentations basal colon media

188   supplemented with 50 mM MES (SIGMA-Aldrich) buffer, was divided into 5 batches and 4

189   batches were supplemented with each one of the 4 HMOs 1% (w/v). HMO used were (3-

190   Fucosyllactose (3FL) (Oligotech, France), 3-Sialyllactose (3SL) and 6-Sialyllactose (6SL)

191 (Carbosynth, United Kingdom), Oligofructose (FOS) (Orafti P95, Belgium) and a carbohydrate

192 negative control. The fermentations were performed for each substrate and fecal sample in

193 quadruplicates. Fermentations were performed with pH control. The pH was set to increase from

194 5.7 to 6.0 during the first 8h of fermentation simulating pH conditions prevalent in the proximal

195 colon, followed by an 8h pH increment from pH 6.0 to 6.5 representing the pH conditions in the

196 transverse colon and finally a pH increment from 6.5 to 6.9 for distal colon. Samples were taken

197 for endpoint analysis after 24h of fermentation as previously described (Glei et al. 2006; Kneifel

198 2000; Stein et al. 2011). The HMOs were fermented in quadruplicates, the negative control

199 without HMOs are represented in triplicates. GraphPad Prism software (Version 6.0) was used

200 for statistical analyses of the pH controlled fermentations.

201 **DNA extraction**

202 One ml of each fermentation endpoint (at 24h) was pelleted via centrifugation at 13.000 g for 10

203 min and gDNA was extracted from the pellet using the Power Soil Kit protocol (MoBio

204 Laboratories). The FastPrep bead-beating step was performed in 3 cycles of 15 s each at a speed

205 of 6.5 M/s in a FastPrep-24™ Homogenizer (MP). DNA quantity and quality were measured

206 using a NanoDrop 1000 (Thermo Scientific).

207 **16S rRNA gene library preparation**

208 The fecal microbiota composition of *in vitro* fermentation samples were determined using tag-

209 encoded 16S rRNA gene MiSeq-based (Illumina, CA, USA) high throughput sequencing. The

210 V3 region of the 16S rRNA gene was amplified using primers compatible with the Nextera Index

211 Kit (Illumina) NXt_338_F:5'-

212 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG -3'

213 and NXt_518_R: 5'-

214   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG -3''

215   (Ovreås et al. 1997) the PCR reactions and library preparation was conducted as described in

216   (Kristensen et al. 2016).

217   **High throughput sequencing and data treatment**

218   The raw dataset containing pair-ended reads with corresponding quality scores were merged

219   and trimmed using *fastq_mergepairs* and *fastq_filter* scripts implemented in the UPARSE

220   pipeline. The minimum overlap length was set to 10 base pairs (bp). The minimum length of

221   merged reads was 150 bp, the maximum expected error E was 2.0, and the first truncating

222   position with quality score was N≤4. Purging the dataset from chimeric reads and constructing

223   de novo Operational Taxonomic Units (OTU) were conducted using the UPARSE pipeline

224   (Edgar 2013). The Green Genes (13.8) 16S rRNA gene collection was used as a reference

225   database (McDonald et al. 2012). Quantitative Insight Into Microbial Ecology (QIIME) open

226   source software (Caporaso et al. 2010) (1.7.0 and 1.8.0) was used for the subsequent analysis

227   steps. Principal coordinate analysis (PCoA) plots were generated with the Jackknifed Beta

228   Diversity workflow based on 10 UniFrac distance metrics calculated using 10 subsampled OTU

229   tables. The number of sequences taken for each jackknife subset was set to 90% of the sequence

230   number within the most indigent sample, hence 77000 reads per sample for the inulin and

231   lactulose 16S rRNA gene amplicon sequencing based analysis and 87000 reads/sample for the

232   HMO 16S rRNA gene amplicon sequencing based analysis. Analysis of similarities (ANOSIM)

233   was used to evaluate group differences using weighted and unweighted (Lozupone & Knight

234   2005) UniFrac distance metrics that were generated based on rarefied (77000 reads/sample and

235   87000 reads/sample, respectively) OTU tables. The relative distribution of the genera registered

236   was calculated for unified and summarized in genus level OTU tables. Alpha diversity measures

237   expressed as observed species values (sequence similarity 97%) were computed for rarefied

238   OTU tables (77000 reads/sample and 87000 reads/sample respectively) using the alpha

239   rarefaction workflow. Differences in alpha diversity were determined using a t-test-based

240   approach employing the non-parametric (Monte Carlo) method (999 permutations) implemented

241   in the compare alpha diversity workflow. The ANOVA determined significance of quantitative

242   (relative abundance) association of OTUs with given categories, p values were False Discovery

243   Rate (FDR) corrected. These were calculated based on 1000 subsampled OTU-tables rarefied to

244   an equal number of reads (77000 reads/sample and 87000 reads/sample, respectively).

245   **SCFA extraction and analysis**

246   SCFAs profiles were extracted from 1 ml of pH controlled fermentations. In brief: 2 ml of 0.3M

247   oxalic acid was added to the sample and vortexed for 1 min, followed by centrifugation at 2800 g

248   for 15 min. Subsequently 800 µl was filtered through 0.45 µm pore size Ultrafree-MC-HV filters

249   (Millipore, Cork, Ireland). 600 µl of the filtrate was transferred into HPLC vials containing 30 µl

250   of the internal standard 50 mM 2 ethyl butyrate (Sigma-Aldrich), in water. The samples were

251   then analyzed using GC-MS (Agilent 7890A GC and an Agilent 5973 series MSD). GC

252   separation was performed on a Phenomenex Zebron ZB-WAXplus column (30 m × 250 µm ×

253   0.25 µm). A sample volume of 1 µl was injected into a split/split-less inlet at 285°C using a 2:1

254   split ratio. Septum purge flow and split flow were set to 13 ml/min and 2 ml/min, respectively.

255   Hydrogen was used as carrier gas, at a constant flow rate of 1.0 ml/min. The GC oven program

256   was as follows: initial temperature 100°C, equilibration time 1.0 min, heat up to 120°C at the rate

257   of 10°C/min, hold for 5 min, then heat at the rate of 40°C/min until 230°C and hold for 2 min.

258   Mass spectra were recorded in Selected Ion Monitoring (SIM) mode and the following *m/z* ion

259   were detected at a dwell time of 50 msec: 41, 43, 45, 57, 60, 73, 74, 84 and the MS detector was

260 switched off during the 1 min of solvent delay time. The transfer line, ion source and quadrupole

261 MS temperatures were set to 230, 230 and 150°C, respectively. The mass spectrometer was

262 tuned according to manufacturer's recommendation using perfluorotributylamine (PFTBA).

263 Dilution series of SCFAs standards of acetic, propionic, butyric, isobutyric, 2-methyl isobutyric,

264 valeric and isovaleric acid (Sigma-Aldrich) were prepared at the concentrations of 1.000, 0.500,

265 0.250, 0.125, 0.060 and 0.030 mM for the construction of standard curves for quantification. For

266 the analysis of SCFAs in the HMO fermentations, the amounts were subtracted by the SCFAs

267 produced in the control fermentations, in order to extract the HMO effect.

268 **SCFAs Data Analysis**

269 Initial analysis and visualization of the GC-MS data was performed using MSD ChemStation

270 software (ver. E.02.02.1431, Agilent Technologies, Inc., Germany). Mass spectra of SCFAs were

271 compared against the NIST11 library (NIST, Maryland, USA). SCFAs peak areas were

272 integrated from SIM chromatograms using in-house scripts written in Matlab (ver. R2015a, The

273 MathWorks, Inc., Massachusetts, USA). Two SCFAs, 2-methyl isobutyric acid and isovaleric

274 acid, co-eluted at the retention time range of 4.22-4.45 min; peak areas were determined by de-

275 convoluting these peaks using base peaks at m/z ion 74 for 2-methyl isobutyric acid and m/z ion

276 60 for isovaleric acid. T-test and one-way ANOVA with post-hoc tests (significance level 0.05)

277 were performed using GraphPad Prism.

278

279 **Results**

280 **Fermentations of Inulin and Lactulose**

281 The pH-profiles of the inulin and lactulose fermentations with and without pH control are

282 displayed in Fig. 2. The pH controlled fermentations were conducted with an average standard

283   deviation of 0.07 to 0.10 pH units between experimental replicates over the course of 24 h, with

284   no significant difference between F1 and F2 fermentations for each treatment (ANOVA, p=0.4).

285   Uncontrolled fermentations displayed an average standard deviation ranging from 0.23 to 0.36

286   pH units and differed significantly (ANOVA $p < 0.001$). The uncontrolled pH profiles displayed

287   inter- individual differences as well as differences between fermentation substrates.

288   16S rRNA gene amplicon sequencing analysis yielded 7.796.212 reads with on average of

289   194.905.3 ± 80.366.8 reads per sample fulfilling the quality control requirements (minimum

290   sequence length ≥ 180 bp, minimum average quality score ≥ 25). The alpha diversity of the fecal

291   donor GM used as fermentation inoculum in this study did not differ significantly between the

292   samples, with the number of observed species for F1 being 393 and for F2 318. The mean phyla

293   composition was dominated by Firmicutes (F1: 59.7%, F2: 46.7%) and Bacteroidetes (F1:

294   37.2%, F2: 51.1%) for both donors with Cyanobacteria, Tenericutes, Verrumicrobia,

295   Proteobacteria, Actinobacteria, Lentisphaerae, and Fusobacteria all constituting minor parts of

296   the inoculum (0.01-1.5%). Principal Coordinates Analysis (PCoA) revealed a clear separation

297   between the GM of the fecal inoculum prior and after *in vitro* fermentation (Fig. 3).

298   Experimental replicates clustered together and statistical analysis on unweighted and weighted

299   UniFrac matrices revealed significant separation between the treatments (ANOSIM unweighted

300   inulin and lactulose F1 and F2 R- stat 0.1948, p-value 0.039; ANOSIM weighted inulin and

301   lactulose F1and F2 R-stat 0.931, p-value 0.001) and displayed a clear donor effect, (Fig. 3). The

302   relative abundance of Bacteroidetes differed significantly with 65% of the reads constituting

303   Bacteroidetes for inulin versus 12% for lactulose ($p < 0.001$). The abundance of Proteobacteria

304   also differed significantly with a mean relative abundance of 30% for inulin vs 79% for lactulose

305   fermentations ($p < 0.001$) (Fig. 4A). The bifidogenic effect of the two tested prebiotics differed

306   between the two tested GMs, with bifidobacterial abundance being significantly higher in inulin

307   fermentations for the GM of F2 (inulin 0.5% vs. lactulose 0.07%, p=0.005), while for F1 the

308   opposite picture was seen with a stronger (though non-significant) bifidogenic effect for

309   lactulose (inulin 0.6% and lactulose 7.2%).

310   Seven SCFAs were successfully identified in the samples at a concentration range of 0.06 - 34

311   mM. In addition, one unknown peak at component retention time 5.85 min, was consistently

312   detected in all samples (Supp. Fig. 1). It was not possible to identify this compound using the

313   metabolite library in NIST11 (details found in Supp. Fig. 2). Individual GMs within the lactulose

314   treatment, differed significantly in production of propionic (p<0.0001) and acetic acid

315   (p<0.0001) (Fig. 4B). When fermenting inulin, the donor GM showed no significant difference

316   in propionic and butyric acid production. The individual average SCFAs profiles (%) of the

317   fermentations are shown in Fig. 4B.

318   SCFA production with inulin and lactulose as substrates differed for both donors with regard to

319   butyric acid levels, which were higher in inulin fermentations (1.28 ± 0.59 vs 0.06 ± 0.05 mM,

320   p=0.003). Similarly, more isobutyric acid (0.12 ± 0.00 vs 0.09 ± 0.01 mM, p=0.05) and propionic

321   acid (6.02 ± 1.90 vs 3.32 ±1.24 mM, p=0.006) was detected in inulin fermentates compared to

322   the lactulose fermentates.

323   **Fermentations of HMOs**

324   Alpha diversity of the infant fecal samples did not differ significantly (t-stat p=0.08). The GM

325   composition of both infant donors at the phyla level was dominated by Actinobacteria p<0.01

326   (B1 73.12%, B2 96.70%), but differed significantly in other phyla abundance levels. The GM of

327   baby 2 feces had relatively low abundance of Firmicutes (B1 14.40%, B2 2.41%, p<0.01) and

328   Proteobacteria B1 9.00%, B2 0.88%, p<0.01) Bacteroidetes represented 0.009% of the relative

329 abundance in baby 2 whereas they represented 3.25% of the reads in baby 1.

330 Of the investigated HMOs 3´SL clearly had the strongest bifidogenic effect and induced the

331 highest relative abundance (21.5%) of Actinobacteria after 24h of fermentation followed by 3´FL

332 (9.9%) and 6´SL (8.4%), 2.4 % in FOS and 5.7 % in the control, the abundances differed

333 significantly between treatments, p=0.03 (Fig. 5).

334 Looking at the babies separately, the highest abundances of Actinobacteria were identified in

335 fermentations supplemented with sialyated HMOs for baby 1 with 3´SL 17.3% and 16.2% for

336 6´SL supplemented fermentations (Fig.5). In the fermentations of HMOs inoculated with GM

337 from baby 1 higher abundances of Actinobacteria were observed in all supplemented media

338 compared to the negative control with the exception of 3´FL. The 3´FL fermentation also

339 displayed the highest abundance of Bacteroidetes (42.8%), while Firmicutes were most abundant

340 in the 3´SL fermentations (35.9%). Proteobacteria were lower in HMO supplemented treatments

341 compared with FOS and non-supplemented control (Fig.5). In fermentations inoculated with

342 baby 2 GM, which barely contained representatives of the phyla Bacteroidetes, the three

343 sialyated supplemented fermentations displayed the highest relative abundance of 25.7%

344 Actinobacteria. Significant differences in Actinobacteria were detected between treatments

345 p=0.015. In contrast to baby 1, 6´SL did not induce growth of Actinobacteria in baby 2, to any

346 larger extent.

347 For fermentations with GM of baby 1 (Table 1) significant differences in the relative abundance

348 of an unassigned Veillonellaceae (p=0.0001) and the OTU *Veillonella* (p=0.0001), were

349 identified between treatments. Both were low abundant OTUs (< 1 %) in all fermentations, but

350 growing in 3´FL fermentations with 2.8% and 5.5 % relative abundance respectively.

351 In baby 1 phyla Bacteroidetes was represented by nine OTUs on the species level, with the

352 species *Parabacteroides distasonis* displaying the highest abundances in all HMO fermentations

353 (14-31%) compared to the control and FOS fermentations (1-2%) (Table 1, included all OTUs

354 with 0.03% relative abundance in at least one of the treatments). *Bifidobacterium breve* was

355 highly abundant in the 3'SL and 6'SL fermentations (15-16%) in baby 1, but only constituting 2-

356 4% of the GM in the 6'FL, FOS and control fermentations (Table 1).

357 Baby 2 harbored four species of Bifidobacteria, *B. bifidum*, *B. adolescentis, B. longum and B.*

358 *faecale. B. bifidum* were the most abundant bifidobacteria, reaching the highest relative

359 abundance in the 3'FL and 3'SL fermentations (at 5-13%) while constituting less than 1% in the

360 6'SL, FOS and control fermentations (Table 2, included all OTUs with 0.03% relative abundance

361 in at least one of the treatments). The bifidobacterial species of baby 2 did not seem to ferment

362 6´SL. This is in contrast to baby 1, where especially *Bifidobacterium breve* increased in relative

363 abundance, when growing with 6´SL supplemented media. 3´FL induced the least bifidogenic

364 effect in baby 1 with small increase in growth of *Bifidobacterium breve*, but had the second

365 largest bifidogenic effect for baby 2 promoting growth of *Bifidobacterium bifidum* (7.6%) and *B.*

366 *adolescentis* (5.6 %).

367

368 **SCFAs results**

369 When looking at the SCFAs excluding unknowns the HMO treatments did not differ

370 significantly from the control (p=0.35), but display trends of increased amounts of propionic acid

371 in 3´FL and highest butyric acid in FOS. When subtracting the SCFAs found in the control from

372 all other treatments Fig. 6, it becomes clear that Isovaleric and Valeric acid as well as 2- Methyl-

373 Butyric acid mainly originate from substrates derived from media and or feces. For both babies

374 marked substrate effects on SCFAs-production were observed. For baby 1, with significantly

375   higher propionic acid production (p<0.006) and lower amount of butyric acid (p= 0.01) in the

376   3´FL fermentations compared to 3´SL, 6´SL and FOS (Fig. 6). For baby 2, with the same pattern

377   of significantly higher propionic acid production in the 3`FL treatment (p<0.0001) and lower

378   butyric acid production compared to the other treatments. A significant difference was detected

379   for FOS fermentations, with higher butyric acid production in fermentations with baby 1 GM

380   (p=0.01), Fig. 6.

381

382   **Discussion**

383   Here we describe a novel, low-volume *in vitro* colon model with increased throughput.

384   CoMiniGut's automated pH control set-up allows a flexible experimental design, with optional

385   settings for e.g. the simulation of only one colon region within the vessel or - as presented in this

386   study – the passage through the colon simulated by a pH increment over time. Sampling of

387   fermentate is facilitated at all times through a needle inlet. Similarly, standard media can be

388   altered and different feeds can be added gradually or at specific time points in a programmed

389   manner via a syringe pump.

390   Due to the small amount of fecal sample needed for the inoculations of each experiment in

391   CoMiniGut (250 μg of the fecal matter for the inoculation of 5 reactors), fecal cryo-stock

392   libraries can be constructed, that way facilitating multiple experimental repetitions, something

393   that is not possible, when fresh inoculum is used. Frozen stocks furthermore facilitate an

394   increased comparability between the donors, as samples can be processed consistently from

395   delivery to inoculation. A synchronized delivery of fresh fecal material especially from e.g.

396   infants is difficult to achieve. No significant differences in culturable cell counts were observed

397   between fresh fecal samples and the frozen samples, using the same conditions as for CoMiniGut

398   fecal inoculum implemented in this study (Suppl. Fig.3)

399   Due to its small working volume CoMiniGut will also allow for the investigation of expensive

400   compounds or novel synthesized materials only available in small amounts, which otherwise

401   would be very costly or simply impossible. For the conduction of a fermentation at 1% (w/v)

402   only 50 mg of substrate is needed per fermentation reaction. The requirement of only small

403   amounts of substrate and fecal material facilitates the screening of a plethora of different

404   bioactives, in different doses and combinations, for one or multiple fecal donors.

405   Using inulin and lactulose as substrate for fermentations inoculated with fecal slurry from two

406   adult donors significant substrate as well as inter-individual differences in microbiota

407   composition and SCFAs-profiles were demonstrated. Rycroft et al. (2001) investigated *in vitro*

408   simulated colon fermentations of several prebiotics including inulin and lactulose using

409   fluorescence *in situ* hybridization (FISH) to investigate the effect on microbiota composition.

410   Similar to Rycroft et al we observed higher acetic acid levels after 24h of fermentation of

411   lactulose *in vitro* compared to inulin fermentations, though in the present study the differences in

412   acetic acid production were not significant (Rycroft et al. 2001). Venema *et al*. also investigated

413   the effects of lactulose on the composition of GM and SCFAs in human volunteers and using the

414   TIM-2 *in vitro* model. They, similar to the present study, found low levels of butyrate production

415   when fermenting lactulose *in vitro* (German et al. 2008). Further, when comparing the ratios of

416   the SCFAs (butyrate, propionate and acetate) it is seen that the ratios of SCFAs found in our

417   study are comparable to those observed by Venema *et al.* (2003) in the adapted microbial

418   communities. This shows that SCFAs-results obtained using the CoMiniGut are comparable to

419   existing *in vitro* models with a higher working volumes and lower throughput. The model further

420   facilitated the fermentations of the rare and expensive HMOs only available in small amounts

421 with two infant GMs. We have extracted inter-individual difference of HMO utilization capacity

422 especially with regards to 6-sialyated HMOs.

423 The site of sialyation 3 vs 6` SL impacts the degree of gut microbial growth and its utilization

424 depends on the metabolic capacity and the ability to utilize it within a given microbial

425 consortium. In this study we have found the strongest average bifidogenic effect to be induced by

426 3`SL overall when looking at both infants. The lack of a bifidogenic effect in the 6`SL-

427 supplemented fermentations of baby 2 compared to the significant population (≈16%) of

428 Actinobacteria in 6`SL fermentations of GM from baby 1 (Fig. 5) is striking.

429 The GM of both infants harbored different bifidobacteria species. While both babies harboured

430 members of *Bifidobacterium bifidum* and *B. longum*, the GM of baby 1 also harboured

431 *Bifidobacterium breve*, whereas baby 2 harboured *B. adolescentis* and *Bifidobacterium faecale*.

432 None of the bifidobacterial species present in the baby 1 GM did increase in relative abundance

433 during 6´SL fermentations (Table 1), whereas *Bifidobacterium breve* (baby 2) grew in 6´SL

434 fermentations. Different Bifidobacterial species have varying physiologies leading to varying

435 capacities of HMO utilization, and even strain dependent utilization of HMOs has for instance

436 been described for *Bifidobacterium breve* (LoCascio et al. 2007; LoCascio et al. 2009; Ruiz-

437 Moyano et al. 2013). It has been suggested that *B. longum* subsp. *infantis* has an inherent and

438 constitutive ability to process sialyated compounds (Yu et al. 2013). Nevertheless the *B. longum*

439 strain of baby 2 did not seem to utilize 6´SL, which might be due to the specific site of

440 sialyation. Another explanation for the observed discrepancies is a syntrophic interaction

441 between members of the phyla Bacteroidetes, especially *Parabacteroides distasonis,* which are

442 present in baby 1 but barely present in baby 2, and bifidobacteria.

443 Also the utilization of 3´FL varies between baby 1 and baby 2. It is possible that this is due to

444 different strains of the species *B. bifidum* and *B. longum* in baby 2 vs. baby1. Nevertheless, it is

445 also possible that the discrepancies between baby 1 and baby 2 relate to the difference in overall

446 microbial community and the presence of Bacteroidetes, which are capable of HMO utilization.

447 Yu et al reported that when supplemented with 3′FL, all *Bifidobacterium* spp. and *Bacteroides*

448 spp. displayed appreciable induction of Alfa-L-Fucosidase, AFU activity and consumed 40% or

449 greater of the 3′FL in their study (Yu et al. 2013). In the present study it is hence likely that

450 Bacteroidetes thrive on 3′FL and compete with the bifidobacterial strains explaining comparably

451 lower bifidobacterial abundance in baby 1, when growing together with 3′FL utilizing

452 Bacteroidetes.

453 **Conclusions**

454 The construction of fecal libraries of specific target groups such as babies, healthy adults,

455 elderly, diabetics, or obese donors allows for the assessment of single or multiple dietary

456 components, and their impact on individual gut microbial populations and subsequent changes to

457 their metabolism. In combination with advanced high throughput sequencing protocols

458 CoMiniGut constitutes an excellent tool for the investigation of not only bacterial species but

459 also interactions between bacteriophages and eukaryotic microorganisms and various microbe-

460 microbe and microbe-bioactive interactions *in vitro*. In this study we show superior advantages

461 of the newly developed *in vitro* colon model with regards to its throughput, reproducibility and

462 its potential application for the investigation of rare and expensive compounds. To conclude, the

463 CoMiniGut facilitates next generation *in vitro* colon simulations with high statistical inference

464 and simultaneous reduction of resources.

465

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469

470 **References**

471 Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, Sears MR, Becker AB, Scott

472   JA, and Kozyrskyj AL. 2013. Gut microbiota of healthy Canadian infants: profiles by

473   mode of delivery and infant diet at 4 months. *Canadian Medical Association Journal*

474   185:385-394.

475 Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, and Gordon JI. 2005. Host-bacterial

476   mutualism in the human intestine. *science* 307:1915-1920.

477 Bode L. 2006. Recent advances on structure, metabolism, and function of human milk

478   oligosaccharides. *The Journal of Nutrition* 136:2127-2130.

479 Bode L. 2012. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*

480   22:1147-1162.

481 Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena

482   AG, Goodrich JK, and Gordon JI. 2010. QIIME allows analysis of high-throughput

483   community sequencing data. *Nature methods* 7:335-336.

484 Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads.

485   *Nature methods* 10:996-998.

486 Engfer MB, Stahl B, Finke B, Sawatzki G, and Daniel H. 2000. Human milk oligosaccharides

487   are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *The American*

488   *journal of clinical nutrition* 71:1589-1596.

489 Fallingborg J, Christensen LA, Jacobsen BA, and Rasmussen SN. 1993. Very low intraluminal

490   colonic pH in patients with active ulcerative colitis. *Digestive diseases and sciences*

491   38:1989-1993.

492 Fenwick N, Griffin G, and Gauthier C. 2009. The welfare of animals used in science: How the

493   "Three Rs" ethic guides improvements. *The Canadian Veterinary Journal* 50:523.

494 Fooks LJ, and Gibson GR. 2002. In vitro investigations of the effect of probiotics and prebiotics

495   on selected human intestinal pathogens. *FEMS Microbiology Ecology* 39:67-75.

496 German J, Freeman S, Lebrilla C, and Mills D. 2008. Human milk oligosaccharides: evolution,
497   structures and bioselectivity as substrates for intestinal bacteria. *Personalized Nutrition*
498   *for the Diverse Needs of Infants and Children*: Karger Publishers, 205-222.
499 Glei M, Hofmann T, Küster K, Hollmann J, Lindhauer MG, and Pool-Zobel BL. 2006. Both
500   wheat (Triticum aestivum) bran arabinoxylans and gut flora-mediated fermentation
501   products protect human colon cells from genotoxic activities of 4-hydroxynonenal and
502   hydrogen peroxide. *Journal of agricultural and food chemistry* 54:2088-2095.
503 Haarman M, and Knol J. 2005. Quantitative real-time PCR assays to identify and quantify fecal
504   Bifidobacterium species in infants receiving a prebiotic infant formula. *Applied and*
505   *environmental microbiology* 71:2318-2324.
506 Hamilton MJ, Weingarden AR, Sadowsky MJ, and Khoruts A. 2012. Standardized frozen
507   preparation for transplantation of fecal microbiota for recurrent Clostridium difficile
508   infection. *The American journal of gastroenterology* 107:761-767.
509 Kneifel W. 2000. In vitro growth behaviour of probiotic bacteria in culture media with
510   carbohydrates of prebiotic importance. *Microbial Ecology in Health and Disease* 12:27-
511   34.
512 Kristensen KHS, Wiese M, Rytter MJH, Özçam M, Hansen LH, Namusoke H, Friis H, and
513   Nielsen DS. 2016. Gut microbiota in children hospitalized with oedematous and non-
514   oedematous severe acute malnutrition in Uganda. *PLoS Negl Trop Dis* 10:e0004369.
515 Krych L, Hansen CH, Hansen AK, van den Berg FW, and Nielsen DS. 2013. Quantitatively
516   different, yet qualitatively alike: a meta-analysis of the mouse core gut microbiome with
517   a view towards the human gut microbiome. *PLoS One* 8:e62578.
518 LoCascio RG, Ninonuevo MR, Freeman SL, Sela DA, Grimm R, Lebrilla CB, Mills DA, and
519   German JB. 2007. Glycoprofiling of bifidobacterial consumption of human milk
520   oligosaccharides demonstrates strain specific, preferential consumption of small chain
521   glycans secreted in early human lactation. *Journal of agricultural and food chemistry*
522   55:8914-8919.
523 LoCascio RG, Niñonuevo MR, Kronewitter SR, Freeman SL, German JB, Lebrilla CB, and
524   Mills DA. 2009. A versatile and scalable strategy for glycoprofiling bifidobacterial
525   consumption of human milk oligosaccharides. *Microbial biotechnology* 2:333-342.

526 Lozupone C, and Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial
527   communities. *Applied and environmental microbiology* 71:8228-8235.

528 Macfarlane GT, and Macfarlane S. 2007. Models for intestinal fermentation: association between
529   food components, delivery systems, bioavailability and functional interactions in the gut.
530   *Current opinion in biotechnology* 18:156-162.

531 Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross
532   J, Smidt H, and Tuohy KM. 2016. The gut microbiota and host health: a new clinical
533   frontier. *Gut* 65:330-339.

534 Marcobal A, Barboza M, Froehlich JW, Block DE, German JB, Lebrilla CB, and Mills DA.
535   2010. Consumption of human milk oligosaccharides by gut-related microbes. *Journal of*
536   *agricultural and food chemistry* 58:5334.

537 McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL,
538   Knight R, and Hugenholtz P. 2012. An improved Greengenes taxonomy with explicit
539   ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*
540   6:610-618.

541 Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, Farkas T,
542   Chaturvedi P, Pickering LK, and Newburg DS. 2004. Human milk oligosaccharides are
543   associated with protection against diarrhea in breast-fed infants. *The Journal of pediatrics*
544   145:297-303.

545 Newburg DS. 1996. Oligosaccharides and glycoconjugates in human milk: their role in host
546   defense. *Journal of mammary gland biology and neoplasia* 1:271-283.

547 Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL,
548   Killeen K, and Grimm R. 2006. A strategy for annotating the human milk glycome.
549   *Journal of agricultural and food chemistry* 54:7471-7480.

550 Ovreås L, Forney L, Daae FL, and Torsvik V. 1997. Distribution of bacterioplankton in
551   meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis
552   of PCR-amplified gene fragments coding for 16S rRNA. *Applied and environmental*
553   *microbiology* 63:3367-3373.

554 Payne AN, Zihler A, Chassard C, and Lacroix C. 2012. Advances and perspectives in in vitro
555   human gut fermentation modeling. *Trends in biotechnology* 30:17-25.

556 Rinne MM, Gueimonde M, Kalliomäki M, Hoppu U, Salminen SJ, and Isolauri E. 2005. Similar
557   bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and
558   breastfeeding on infant gut microbiota. *FEMS Immunology & Medical Microbiology*
559   43:59-65.
560 Ruiz-Moyano S, Totten SM, Garrido DA, Smilowitz JT, German JB, Lebrilla CB, and Mills DA.
561   2013. Variation in consumption of human milk oligosaccharides by infant gut-associated
562   strains of Bifidobacterium breve. *Applied and environmental microbiology* 79:6040-
563   6049.
564 Rycroft C, Jones M, Gibson GR, and Rastall R. 2001. A comparative in vitro evaluation of the
565   fermentation properties of prebiotic oligosaccharides. *Journal of applied microbiology*
566   91:878-887.
567 Sanz ML, Gibson GR, and Rastall RA. 2005. Influence of disaccharide structure on prebiotic
568   selectivity in vitro. *Journal of agricultural and food chemistry* 53:5192-5199.
569 Sarbini SR, Kolida S, Naeye T, Einerhand AW, Gibson GR, and Rastall RA. 2013. The prebiotic
570   effect of α-1, 2 branched, low molecular weight dextran in the batch and continuous
571   faecal fermentation system. *Journal of Functional Foods* 5:1938-1946.
572 Saulnier DM, Gibson GR, and Kolida S. 2008. In vitro effects of selected synbiotics on the
573   human faecal microbiota composition. *FEMS Microbiology Ecology* 66:516-527.
574 Sela DA, Garrido D, Lerno L, Wu S, Tan K, Eom H-J, Joachimiak A, Lebrilla CB, and Mills
575   DA. 2012. Bifidobacterium longum subsp. infantis ATCC 15697 α-fucosidases are active
576   on fucosylated human milk oligosaccharides. *Applied and environmental microbiology*
577   78:795-803.
578 Sela DA, Li Y, Lerno L, Wu S, Marcobal AM, German JB, Chen X, Lebrilla CB, and Mills DA.
579   2011. An infant-associated bacterial commensal utilizes breast milk
580   sialyloligosaccharides. *Journal of Biological Chemistry* 286:11909-11918.
581 Singh RS, and Singh RP. 2010. Production of fructooligosaccharides from inulin by
582   endoinulinases and their prebiotic potential. *Food Technology and Biotechnology* 48:435.
583 Stein K, Borowicki A, Scharlau D, Scheu K, Brenner-Weiß G, Obst U, Hollmann J, Lindhauer
584   M, Wachter N, and Glei M. 2011. Modification of an in vitro model simulating the whole
585   digestive process to investigate cellular endpoints of chemoprevention. *British Journal of*
586   *Nutrition* 105:678-687.

587   Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, and Stahl B. 2010.

588   Variation of human milk oligosaccharides in relation to milk groups and lactational

589   periods. *British Journal of Nutrition* 104:1261-1271.

590   Torres DP, Gonçalves MdPF, Teixeira JA, and Rodrigues LR. 2010. Galacto-oligosaccharides:

591   Production, properties, applications, and significance as prebiotics. *Comprehensive*

592   *Reviews in Food Science and Food Safety* 9:438-454.

593   Totten SM, Zivkovic AM, Wu S, Ngyuen U, Freeman SL, Ruhaak LR, Darboe MK, German JB,

594   Prentice AM, and Lebrilla CB. 2012. Comprehensive profiles of human milk

595   oligosaccharides yield highly sensitive and specific markers for determining secretor

596   status in lactating mothers. *Journal of proteome research* 11:6124-6133.

597   Venema K, and Van den Abbeele P. 2013. Experimental models of the gut microbiome. *Best*

598   *Practice & Research Clinical Gastroenterology* 27:115-126.

599   Ward RE, Niæonuevo M, Mills DA, Lebrilla CB, and German JB. 2007. Research Article In

600   vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria.

601   *Mol Nutr Food Res* 51:1398-1405.

602   Ward RE, Ninonuevo M, Mills DA, Lebrilla CB, and German JB. 2006. In vitro fermentation of

603   breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri.

604   *Applied and environmental microbiology* 72:4497-4499.

605   Yu Z-T, Chen C, and Newburg DS. 2013. Utilization of major fucosylated and sialylated human

606   milk oligosaccharides by isolated human gut microbes. *Glycobiology*:cwt065.

607   Zivkovic AM, German JB, Lebrilla CB, and Mills DA. 2011. Human milk glycobiome and its

608   impact on the infant gastrointestinal microbiota. *Proceedings of the National Academy of*

609   *Sciences* 108:4653-4658.

610   **Table legends**

611   **Table1.** Microbiota composition of all fermentations (3'SL, 6'FL, 6'SL, FOS and control) for

612   baby 1, as determined by 16S rRNA gene amplicon sequencing.

613   **Table 2.** Microbiota composition of all fermentations (3'SL, 6'FL, 6'SL, FOS and control) for

614   baby 2 as determined by 16S rRNA gene amplicon sequencing.

615

616   **Figure legends**

617   **Fig. 1:** CoMiniGut 5-Plex Overview: 1. Climate Box, 2. Water bath, 3. Heating plate, 4.

618   Temperature probe, 5. Temperature data logger, 6. Ventilators, 7. Lab-jack table, 8. Magnetic

619   stirrer, 9. PMMA compartment, 10. Reaction vial, 11. Gas inlet, 12. Gas outlet, 13. Flow meters,

620   14. Nitrogen /gas tap, 15. pH probe and needle inlets, 16. Lid with septa, 17. Multichannel

621   syringe pump, 18. pH meter, 19. Computer.

622   **Fig. 2:** Fermentation pH profiles of controlled and uncontrolled inulin (A) and lactulose (B) (1%

623   w/v) fermentations using fecal inocula from adult donors F1 and F2 (n = 4, line is mean value,

624   shadow area is convex hull).

625   **Fig. 3:** PCoA score plot of 16S rRNA gene tag-encoded sequence reads based on weighted

626   UniFrac distance metrics (n = 4, for all six experimental conditions).

627   **Fig. 4A:** Phyla composition (as determined by 16S rRNA gene amplicon sequencing) of pH

628   controlled fermentations (24 h) of inulin and lactulose (1% w/v) using fecal inocula from adult

629   donors F1 and F2 in CoMiniGut. ANOVA analysis of F1 and F2 inulin fermentations revealed

630   significant differences (phylum level) in the relative abundances of Bacteroidetes, Proteobacteria

631   and Firmicutes ($p<0.01$), while no significant differences were detected between donors for

632   Fusobacteriap ($p=0.1$) and Actinobacteria ($p=0.5$). Variation between technical replicates was

633   not significant (n=4, F1 $p=0.4$, F2 $p=0.4$). For lactulose fermentations all phyla differed

634   significantly between the two donors ($p\leq0.05$), while variation between technical replicates was

635   not significant for both donors, (n=4, F1 $p= 0.6$; F2 $p= 0.8$).

636   **Fig. 4B:** Relative concentrations of short chain fatty acid profiles of pH controlled CoMiniGut

637   fermentations with inulin and or lactulose (1% w/v) using fecal inocula from adults donors F1

638   and F2 (n = 4).

639   **Fig. 5:** Average Phyla composition, (relative % fraction of total) all fermentations for baby 1 and

640   baby 2 with 3'SL, 6'FL, 6'SL and FOS as substrates as determined by 16S rRNA gene amplicon

641   sequencing.3`FL fermentations differed significantly in the relative abundance of assigned phyla

642   between donors $p\leq0.02$, but no significant differences were found between technical replicates

643   (n=4, B1 $p=0.7$, B2 $p=0.6$). For 3`SL fermentations significant differences between donors were

644   detected for Proteobacteria and Firmicutes $p< 0.01$, whereas no significant variation was detected

645   between technical replicates (n=4, B1 $p=0.8$, B2 $p=0.6$). For 6`SL fermentations, relative

646   abundance were significantly different for all phyla $p\leq0.05$, whereas no significant variation was

647   detected for technical replicates (n=4, B1 $p=0.8$, B2 $p=0.5$). For FOS significant differences were

648   detected for Bacteroidetes, Firmicutes and Proteobacteria $p\leq0.05$, whereas no significant

649 differences were detected between technical replicates (n=4, B1 p=0.8, B2 p=0.5). For the

650 negative control no significant differences between phyla composition were detected between

651 donors ≥0.3 and technical replicates (n=4, B1 p=0.6, B2 p=0.3).

652

653

654 **Fig. 6:** Quantified SCFAs (µM) produced after 24h of fermentation of 3'SL, 6'FL, 6'SL and

655 FOS by GM of baby 1 and 2. Amounts displayed represent values after subtraction of SCFAs

656 produced in the control fermentations.

657 **Supplementary Fig. 1:** GC-MS chromatogram displaying the identified peaks (retention times

658 in minutes): acetic acid (2.6), formic acid (3.0; product of oxalic acid), propionic acid (3.3),

659 isobutyric acid (3.6), butyric acid (4.3), isovaleric acid (4.9) and 2-methyl-butyric (4.9; co-elute),

660 valeric acid (6.2), 2 ethyl butyrate (6.7); overlaid with a sample chromatogram also showing the

661 unknown peak at 5.85 min.

662 **Supplementary Fig. 2:** RI and EI-MS profiles of the unknown peak and 2-methyl butanoic acid.

663

664 **Supplementary Fig.3:** CFU/g counts of fresh and frozen fecal slurries on GAM and BHI media

665 after anaerobic incubation for 72h. No significant difference in the viability of bacteria from

666 fresh and frozen fecal slurries was detected for individual weighted plate mean CFU/g (Students

667 Paired T-test (p = 0.080).

668

CoMiniGut model overview

1. Climate Box, 2. Water bath, 3. Heating plate, 4. Temperature probe, 5. Temperature data logger, 6. Ventilators, 7. Lab-jack table, 8. Magnetic stirrer, 9. PMMA compartment, 10. Reaction vial, 11. Gas inlet, 12. Gas outlet, 13. Flow meters, 14. Nitrogen /gas tap, 15. pH probe and needle inlets, 16. Lid with septa, 17. Multichannel syringe pump, 18. pH meter, 19. Computer.

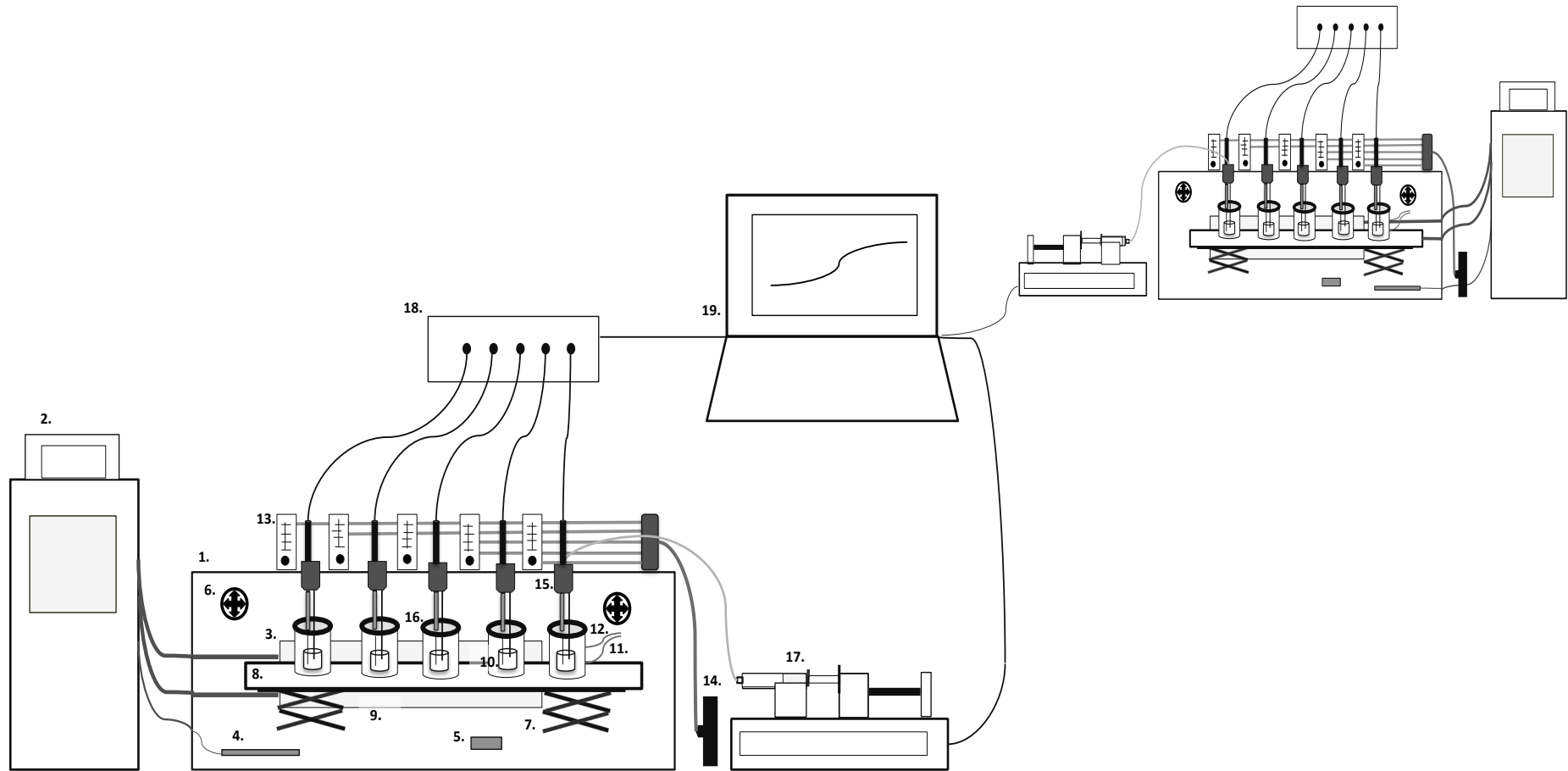



Figure 2

pH profiles of inulin and lactulose fermentations

Fermentation pH profiles of controlled and uncontrolled inulin (A) and lactulose (B) (1% w/v) fermentations using fecal inocula from adult donors F1 and F2 (n = 4, line is mean value, shadow area is convex hull).

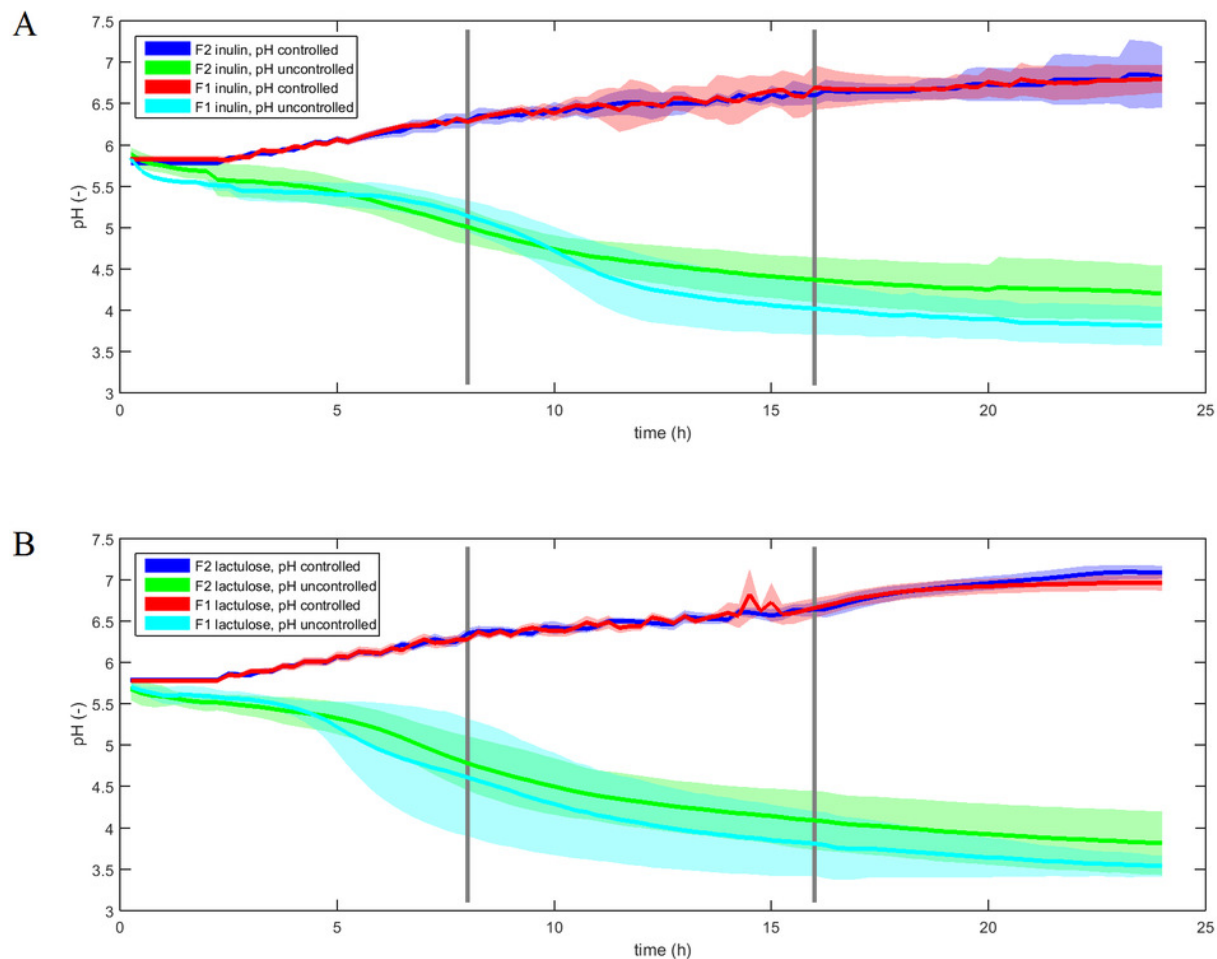



Figure 3

PCoA plot of 16S rRNA gene sequences

PCoA score plot of 16S rRNA gene tag-encoded sequence reads based on weighted UniFrac distance metrics (n = 4, for all six experimental conditions).

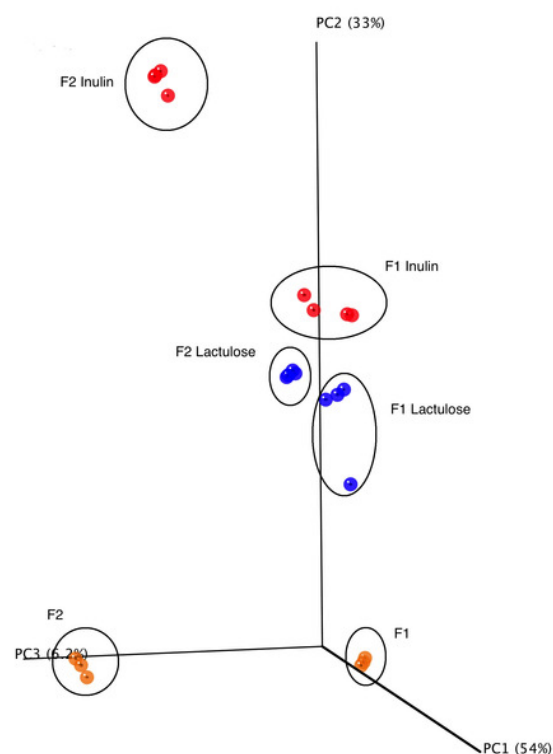



Figure 4

Phyla (A) and SCFA (B) composition of the inulin and lactulose fermentations

A: Phyla composition (as determined by 16S rRNA gene amplicon sequencing) of pH controlled fermentations (24 h) of inulin and lactulose (1% w/v) using fecal inocula from adult donors F1 and F2 in CoMiniGut. ANOVA analysis of F1 and F2 inulin fermentations revealed significant differences (phylum level) in the relative abundances of Bacteroidetes, Proteobacteria and Firmicutes ($p<0.01$), while no significant differences were detected between donors for Fusobacteriap ($p=0.1$) and Actinobacteria ($p=0.5$). Variation between technical replicates was not significant (n=4, F1 $p=0.4$, F2 $p=0.4$). For lactulose fermentations all phyla differed significantly between the two donors ($p≤0.05$), while variation between technical replicates was not significant for both donors, (n=4, F1 $p=0.6$; F2 $p=0.8$). B: Relative concentrations of short chain fatty acid profiles of pH controlled CoMiniGut fermentations with inulin and or lactulose (1% w/v) using fecal inocula from adults donors F1 and F2 (n = 4).

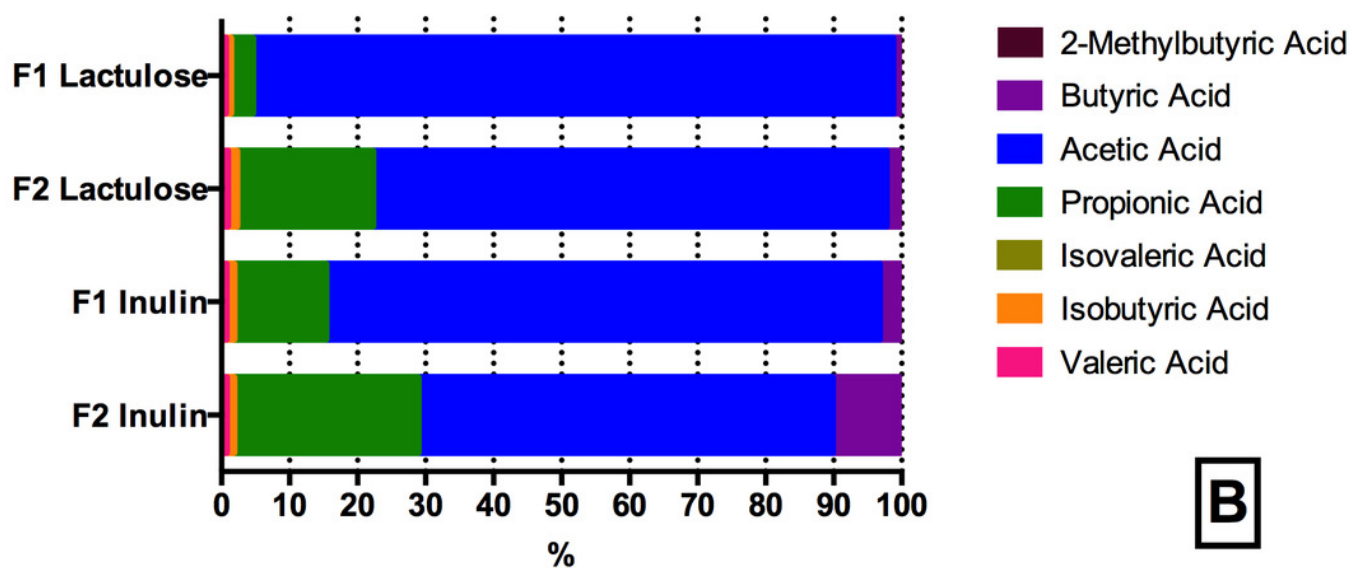
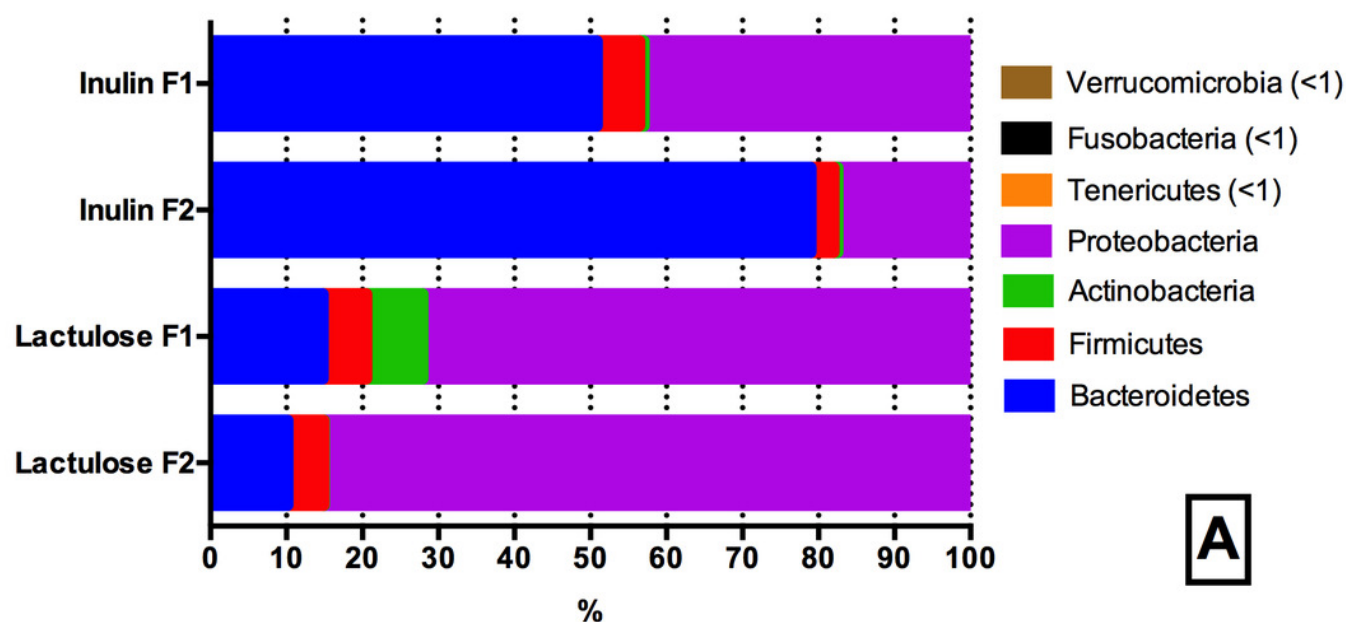



Figure 5

Average Phyla composition of HMO fermentations

Average Phyla composition, (relative % fraction of total) all fermentations for baby 1 and baby 2 with 3'SL, 6'FL, 6'SL and FOS as substrates as determined by 16S rRNA gene amplicon sequencing.3`FL fermentations differed significantly in the relative abundance of assigned phyla between donors p≤0.02, but no significant differences were found between technical replicates (n=4, B1 p=0.7, B2 p=0.6). For 3`SL fermentations significant differences between donors were detected for Proteobacteria and Firmicutes p< 0.01, whereas no significant variation was detected between technical replicates (n=4, B1 p=0.8, B2 p=0.6). For 6`SL fermentations, relative abundance were significantly different for all phyla p≤0.05, whereas no significant variation was detected for technical replicates (n=4, B1 p=0.8, B2 p=0.5). For FOS significant differences were detected for Bacteroidetes, Firmicutes and Proteobacteria p≤0.05, whereas no significant differences were detected between technical replicates (n=4, B1 p=0.8, B2 p=0.5). For the negative control no significant differences between phyla composition were detected between donors ≥0.3 and technical replicates (n=4, B1 p=0.6, B2 p=0.3).

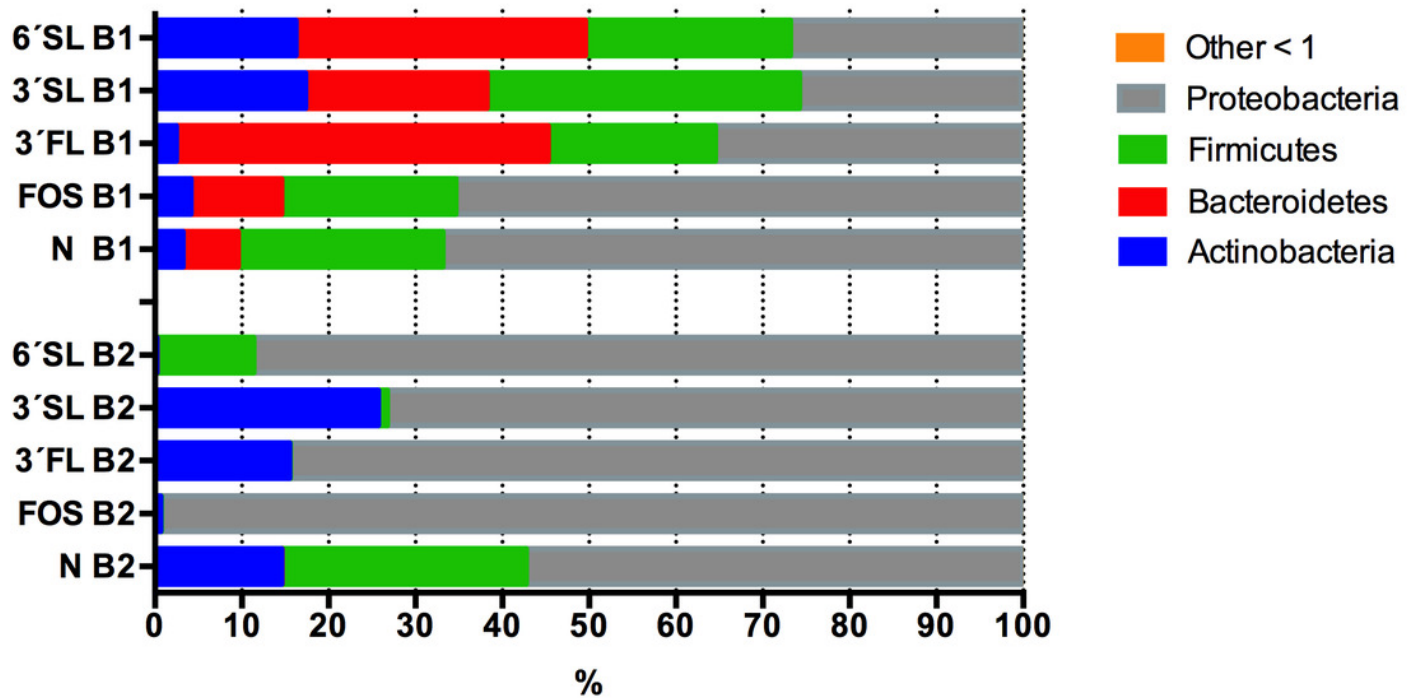
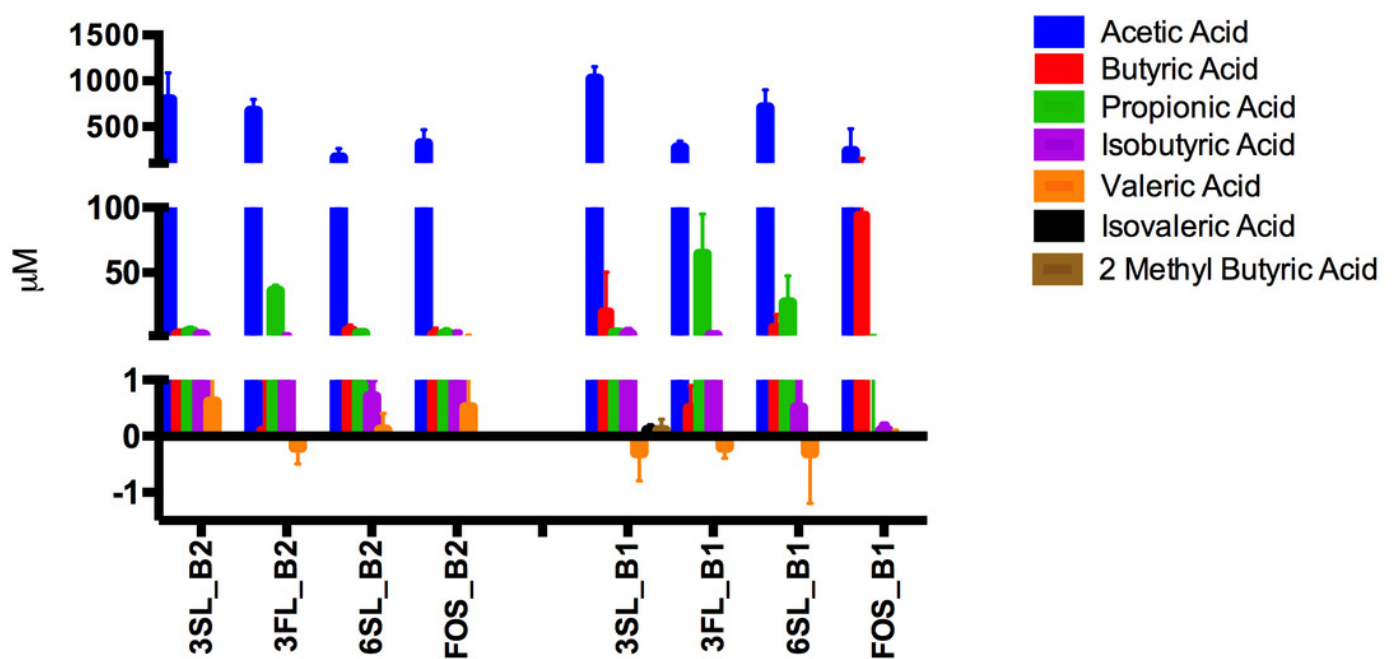



Figure 6

SCFA content of HMO fermentates

Quantified SCFAs (µM) produced after 24h of fermentation of 3'SL, 6'SL, 3'FL and FOS by GM of baby 1 and 2. Amounts displayed represent values after subtraction of SCFAs produced in the control fermentations.

Species compositions of HMO fermentations with GM of baby 1

Microbiota composition of all fermentations (3'SL, 3'FL, 6'SL, FOS and control) for baby 1, as determined by 16S rRNA gene amplicon sequencing.

BABY1				Control	FOS	6SL	3SL	3FL	p-value
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	bifidum (*99.4)	0.03	0.05	0.21	0.21	0.05	0.28
	Bifidobacteriaceae	*Bifidobacterium*	longum	0.6	0.37	0.24	1.38	0.38	0.5
	Bifidobacteriaceae	*Bifidobacterium*	breve (*97.8)	2.53	3.68	15.76	15.68	1.99	0.35
Bacteroidetes	Porphyromonadaceae	*Parabacteroides*	distasonis	1.76	1.44	24.07	14.23	31.39	0.07
	Porphyromonadaceae	*Parabacteroides*	gordonii (*96.9)	0.01	0	0.12	0.06	0.17	0.06
	Other	*Other*	Other	0.02	0.03	0.19	0.09	0.19	0.05
	Bacteroidaceae	*Bacteroides*	ovatus	1.31	0.32	0.84	0.78	1.08	0.65
	Bacteroidaceae	*Bacteroides*	caccae	0.46	3.61	0.39	0.37	0.58	0.19
	Bacteroidaceae	*Bacteroides*		2.14	4.68	6.67	4.62	8.63	0.64
	Bacteroidaceae	*Bacteroides*	uniformis	0.19	0.19	0.59	0.39	0.25	0.64
	Bacteroidaceae	*Bacteroides*	acidifaciens	0.37	0.14	0.21	0.22	0.26	0.76
	Bacteroidaceae	*Bacteroides*	Other	0.11	0.04	0.23	0.14	0.28	0.25
Firmicutes	Lactobacillaceae	*Lactobacillus*	zeae	0	0.2	0	0.11	0.01	0.75
	Clostridiaceae	*Clostridium*	Other	11.15	9.6	9.81	13.51	5.54	0.85
	Clostridiaceae	*Clostridium*	perfringens	7.35	6.38	6.54	9.12	3.67	0.85
	Veillonellaceae			0.02	0.01	0.03	0.01	2.83	<0.001
	Veillonellaceae	*Veillonella*		0.04	0.02	0.07	0.01	5.55	<0.001
	Clostridiaceae	*Clostridium*		3.08	2.65	5.31	9.4	0.99	0.63
	Peptostreptococcaceae	*Clostridium*	Other	0.05	0	0	0.43	0	0.67
	Other	*Other*	Other	0.03	0.01	0.02	0.14	0.01	0.65
	Enterococcaceae	*Enterococcus*		0.53	0.09	0.16	0.24	0.15	0.58
	Clostridiaceae			0.84	0.77	1.26	2.19	0.28	0.64
	Clostridiaceae	*Other*	Other	0.33	0.27	0.31	0.62	0.15	0.34
	Enterococcaceae	*Other*	Other	0.07	0.03	0.05	0.09	0.02	0.5
Proteobacteria	Enterobacteriaceae	*Dickeya*		0.07	0.34	0.12	0.01	0.01	0.03
	Enterobacteriaceae	*Enterobacter*	Other	0.32	0.58	0.16	0.03	0.07	0.15
	Enterobacteriaceae	*Klebsiella*		19.74	23.69	6.18	0.6	1	0.18
	Enterobacteriaceae	*Other*	Other	3.97	3.35	1.05	0.85	0.57	0.34
	Enterobacteriaceae	*Erwinia*	Other	0.09	0.12	0.07	0.02	0.02	0.27
	Enterobacteriaceae			42.52	37.17	19	23.97	33.64	0.4
	Other	*Other*	Other	0.12	0.04	0.06	0.11	0.1	0.54

Species composition of HMO fermentations with GM of baby 2

Microbiota composition of all fermentations (3'SL, 3'FL, 6'SL, FOS and control) for baby 2 as determined by 16S rRNA gene amplicon sequencing.

Baby 2				Control	FOS	6SL	3SL	3FL	p value
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*bifidum*	0.09	0.26	0.08	13.25	7.58	<0.001
	Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	0.04	0.17	0.03	10.37	5.64	<0.001
	Bifidobacteriaceae	*Bifidobacterium*	*longum*	0.07	0.19	0.06	1.94	2.14	0.02
	Bifidobacteriaceae	*Bifidobacterium*	*fecale (*97.24)*	0	0	0	0.15	0.12	0.04
Firmicutes	Ruminococcaceae			0.21	0	0	0	0	0.28
	Lachnospiraceae	*Blautia*		0.91	0	0	0	0	0.26
	Staphylococcaceae	*Staphylococcus*	*aureus*	3.93	0	0	0	0	0.24
	Lachnospiraceae			0.44	0	0	0	0	0.23
	Clostridiaceae	*Clostridium*		1.57	0	7.56	0	0	0.23
	Clostridiaceae			0.4	0	1.75	0	0	0.22
	Enterococcaceae	*Other*	*Other*	0	0	0.09	0.04	0	0.21
	Clostridiaceae	*Clostridium*	*Other*	0.18	0.02	0.77	0.02	0.01	0.2
	Clostridiaceae	*Other*	*Other*	0.05	0	0.15	0	0	0.2
	Enterococcaceae	*Enterococcus*		0.01	0.02	0.72	0.3	0.02	0.29
	Streptococcaceae	*Streptococcus*		0	0	0.01	0	0	0.42
	Lactobacillaceae	*Lactobacillus*		0	0.01	0.05	0.65	0.01	0.62
	Clostridiaceae	*Clostridium*	*perfringens*	0	0.01	0.01	0.01	0.01	0.91
Proteobacteria	Enterobacteriaceae	*Erwinia*	*Other*	0.15	0.11	0.17	0.04	0.19	0.39
	Other	*Other*	*Other*	0.23	0.24	0.32	0.28	0.43	0.44
	Enterobacteriaceae	*Klebsiella*		11.2	7.21	19.89	1.41	11.38	0.44
	Enterobacteriaceae	*Proteus*		0.01	0	0	0	0.01	0.21
	Enterobacteriaceae	*Enterobacter*	*cloacae*	0.01	0.02	0	0.07	0.13	0.07
	Enterobacteriaceae	*Enterobacter*	*Other*	0.33	0.48	0.36	0.09	0.38	0.13
	Enterobacteriaceae			37.28	77.23	63.89	69.03	66.64	0.16
	Enterobacteriaceae	*Dickeya*		0.05	0	0.02	0	0	0.29
	Enterobacteriaceae	*Other*	*Other*	3.86	13.88	3.98	2.29	5.19	0.03
	Enterobacteriaceae	*Enterobacter*	*ludwigii*	0.02	0.05	0.01	0.01	0.01	0.04
	Enterobacteriaceae	*Trabulsiella*		0.01	0.02	0.01	0.01	0.01	0.27