***In vitro* colonization of the distal colon by *Akkermansia muciniphila* is largely mucin and pH dependent.**

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Running title: *A. muciniphila* colonization in the distal colon

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ABSTRACT

Host mucin is the main constituent of the mucus layer that covers the gut epithelium of the host, and an important source of glycans for the bacteria colonizing the intestine. *Akkermansia muciniphila* is a mucin-degrading bacterium, abundant in the human gut, that is able to produce acetate and propionate during this degradation process. *A. muciniphila* has been correlated with human health in previous studies, but a mechanistic explanation is lacking. In this study, the main site of colonization was characterized alongside additional conditions, such as differences in colon pH, prebiotic supplementation and variable mucin supply. A dynamic *in vitro* gut model, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) was used to perform this in-depth exploration of the ecological behavior of *A. muciniphila* in one biological environment and to overcome the limitations of *in vivo* studies. *A. muciniphila* was found to colonize the distal colon (± 8 log copies/mL) more abundantly than the proximal colon (± 4 log copies/mL) and this colonization pattern was pH-dependent. The addition of mucin caused a specific increase of *A. muciniphila* (± 4.5 log increase over two days), far exceeding the response of any other bacteria present, together with an increase in propionate. This indicates that *A. muciniphila* is a specialized mucin degrader highly sensitive to low pH and mucin concentration. Our results indicate the preference of *A. muciniphila* for the distal colon environment due to its higher pH and uncovered the quick and stable response of *A. muciniphila* to mucin supplementation.

Keywords: Simulator of the Human Intestinal Ecosystem (SHIME), mucin degradation, propionate, qPCR, Illumina sequencing

INTRODUCTION

Over recent decades, multiple correlations have been established between human health and the composition of the gut microbiota (Round and Mazmanian, 2009). *Akkermansia muciniphila* is a commensal gut bacterium that represents 1-3% of the total microbiota (Derrien *et al.*, 2008) and has been associated with beneficial health effects in several studies. It was shown to be absent in patients suffering from inflammatory bowel disease (IBD), while being abundantly present in healthy individuals (Png *et al.*, 2010). Lower *A. muciniphila* numbers have been encountered in patients with obesity, autism and type 2 diabetes (Wang *et al.*, 2011; Zhang *et al.*, 2009; Zhang *et al.*, 2013), and the initial correlation with type 2 diabetes (Qin *et al.*, 2012) could be attributed to the confounding effect of metformin, which stimulates the growth of *A. muciniphila* (Forslund *et al.*, 2015; Lee and Ko, 2014; Shin *et al.*, 2014). *A. muciniphila* supplementation reversed fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance in obese mice that received a high-fat diet (Everard *et al.*, 2013). Not only probiotic treatment with *A. muciniphila* but also treatment of mice with dietary compounds such as cranberry extract and fish oil, rich in polyphenols and omega-3 fatty acids respectively, resulted in higher abundances of *A. muciniphila* and metabolically healthier mice/phenotypes (Anhe *et al.*, 2015; Caesar *et al.*, 2015). The direct impact of *A. muciniphila* on the host, its interactions with other beneficial intestinal microbes and its susceptibility to prebiotics remain to be elucidated.

Mucins are the preferred substrate of *A. muciniphila*, yielding acetate and propionate as by-products of their fermentation (Derrien *et al.*, 2004). Mucin glycans constitute 80% of the dry weight of the mucus layer that covers the intestinal epithelium (Johansson *et al.*, 2008). They are composed of O-glycosylated protein backbones, with chains of 2 to 12 monosaccharides, mostly galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and sialic acid. They may also be N-glycosylated with the same compounds to a lesser extent (Lai *et al.*, 2009). Mucins are not only confined to the mucus layer but are also present in the luminal content, due to the continuous mucin production, roughly between 6 and 15 g per day, and the constant mucus desquamation (Atuma *et al.*, 2001; Faure *et al.*, 2002; Johansson, 2012; Wilson, 2005). The degradation of host- and diet-derived glycans results in production of short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate. Butyrate serves as an energy source for colonocytes, while it may also protect from inflammatory disorders and suppress the growth of colonic tumours (Guilloteau *et al.*, 2010). Acetate and propionate enter the portal vein more efficiently than butyrate. Acetate can be used as a substrate for fatty acid synthesis and cholesterol in the liver, increases colonic blood flow and oxygen uptake, and it is important to protect from enteric infections (Fukuda *et al.*, 2011). In contrast, propionate inhibits the incorporation of acetate into fatty acids and cholesterol and has been related with specific health benefits. Propionate induces satiety and it may therefore play an important role in obesity (Hosseini *et al.*, 2011; Nishina and Freedland, 1990; Ruijschop *et al.*, 2008). In monoculture, *A. muciniphila* produces acetate and propionate (60:40 molar ratio) from mucins, which impacts the host genes involved in lipid metabolism (Hnf4 α) and proliferation (Tp53 and Tp73) (Derrien *et al.*, 2004; Lukovac *et al.*, 2014). Although multiple studies associate *A. muciniphila* with human health indicators (Anhe *et al.*, 2015; Belzer and de Vos, 2012; Everard *et al.*, 2013; Png *et al.*, 2010; Santacruz *et al.*, 2010; Schneeberger *et al.*, 2015; Swidsinski *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2009), the specific factors that govern *A. muciniphila* colonization within a mixed community and its contribution to the overall acetate, propionate and/or butyrate production have not been addressed.

88 Production of mucin by the host epithelium is dependent on specific food ingredients (Van den
89 Abbeele *et al.*, 2011), drugs (Wlodarska *et al.*, 2011) or disease states (Fyderek *et al.*, 2009), thus
90 interfering with the abundance of mucin degraders. For example, consumption of prebiotics was
91 shown to shift mucin degradation to the distal colon (Van den Abbeele *et al.*, 2011). Therefore,
92 sampling along different sites of the intestine becomes essential for understanding the ecological
93 behaviour and colonization potential of mucin degraders in the gut. Also, in patients with Crohn's
94 disease and ulcerative colitis the mucus layer is three times thinner than in healthy people
95 (Fyderek *et al.*, 2009) and antibiotic treatment with metronidazole resulted in a thinner inner
96 mucus layer (Wlodarska *et al.*, 2011). These are confounding factors that can be overcome with
97 dynamic *in vitro* gut models, like the SHIME[®], that exclude the host environment and allow
98 simulating different sites of the intestine. Such *in vitro* models provide a great opportunity for
99 mechanistic research that aims at unraveling the ecology of mucin degraders, such as *Bacteroides*
100 sp. and *A. muciniphila*, which have been shown to thrive in these models (Van den Abbeele *et al.*,
101 2010).

102 The aims of this study were to evaluate the colonization behaviour of *A. muciniphila* in a mucin
103 rich environment in presence of a complex microbial community. Since this research serves as a
104 start to characterize *A. muciniphila*, a donor was selected to ensure efficient and abundant
105 colonization of *A. muciniphila* a donor was selected with high amounts of *A. muciniphila*
106 previously shown to successfully colonize the SHIME (Van den Abbeele *et al.*, 2013; Van den
107 Abbeele *et al.*, 2011; Van den Abbeele *et al.*, 2010). Hence this inoculum guaranteed that we
108 could see the effects of the variable conditions, such as the stabilization period, differences in
109 colon pH, prebiotic supplementation and variable mucin supply, on *A. muciniphila* and
110 dynamically monitor its ecological behaviour. However, this only provides us with results from
111 one microbial background and further research considering multiple donors, will need to be
112 performed to investigate the effect of the microbial parameter on *A. muciniphila*. We assessed
113 the overall microbial and metabolic changes associated with evolving numbers of *A.*
114 *muciniphila*..

MATERIALS AND METHODS

Chemicals, growth media and bacterial strains

Chemicals were obtained from Sigma (Bornem, Belgium), unless stated otherwise. The nutritional medium for the SHIME consisted of (in g/L) arabinogalactan (1.0), arabinoxylan (2.0) (BioActor, Maastricht, The Netherlands), starch (2) (Anco, Roeselare, Belgium), xylan (1.0), pectin (2.0), D-(+)-glucose (0.4), yeast extract (3.0), peptone (1.0), cysteine (0.5) and commercial pig gastric mucin (4.0). The monosaccharide composition of mucin was (in g/ 100g DM): L-arabinose (0.05), D-xylose (0.04), D-mannose (0.28), D-galactose (7.47) and D-glucose (1.95). This medium was autoclaved and acidified to pH 2.0. The pancreatic juice contained (in g/L) NaHCO₃ (12.5), bile salts (6.0) (Difco, Bierbeek, Belgium) and pancreatin (0.9).

Our previous experience with a faecal microbial inoculum from a 28-year old male individual (Van den Abbeele *et al.*, 2013; Van den Abbeele *et al.*, 2011; Van den Abbeele *et al.*, 2010) showed that *A. muciniphila* colonization was consistently abundant and efficient. Hence, this was an ideal inoculum to subject *A. muciniphila* to variable conditions and dynamically monitor its ecological behaviour. Faecal samples were collected and prepared within 1h according to standard procedures (Molly *et al.*, 1993). In short, aliquots (20 g) of freshly voided faecal samples were diluted and homogenized with 100 mL 0.1 M phosphate buffer (8.8 g K₂HPO₄.L-1 and 6.8 g KH₂PO₄.L-1, pH 6.8) containing 1 g.L-1 sodium thioglycolate as reducing agent. After removal of the particulate material by centrifugation (2 min, 500 g) the faecal suspension was used as inoculum.

Long-term dynamic in vitro gut model for the luminal colon microbiota (SHIME)

The long-term colonization of *A. muciniphila* within a mixed human gut microbiota was assessed in the dynamic in vitro gut model, SHIME® (ProDigest-Ghent University, Ghent, Belgium). The model consists of five compartments that simulate the stomach, the small intestine and three or two colon regions (namely ascending, transverse and descending colon or proximal and distal colon, respectively) (Van den Abbeele *et al.*, 2010). Each anaerobic compartment was continuously stirred at 37°C and flushed with N₂ (15 min/day) to ensure anaerobic conditions after sampling. On day 0, the colon compartments were filled with nutritional medium and inoculated with 40 mL of 20% (w/v) faecal slurry. Following an overnight static incubation of the colon compartments (16 h), the stomach and small intestine compartments operate on the fill and draw principle, with peristaltic pumps adding 140 mL nutritional medium and 60 mL pancreatic juice three times a day and gradually emptying the small intestine compartment into the colon compartments after gastro-intestinal digestion. The volume in the colon compartments is kept constant by the simultaneous fluid flow in and out of compartments (Possemiers *et al.*, 2004). Samples were taken from the vessels, daily (10h00) before new feed entered the colon compartments.

By applying relevant environmental conditions (retention time, nutrition and pH) to each colon region, the faecal microbiota evolves to establish region-specific microbial communities. This stabilization process requires two weeks and it is reproducible for SHIME-units that are run simultaneously (Van den Abbeele *et al.*, 2010).

Samples from a previous experiment were analysed to provide the preliminary data for the experiments performed in this study (Van den Abbeele *et al.*, 2010). *A. muciniphila* colonization was studied in the three colon regions of the SHIME, using *A. muciniphila*-specific primers and qPCR on samples of a published SHIME-study (Van den Abbeele *et al.*, 2010). Samples were collected on day 19 and 26 after inoculation from two stable SHIME-units run simultaneously. The net SCFA production was determined to link the SCFA production to the numbers of *A. muciniphila* in specific colon regions. As steady conditions prevailed upon stabilization, the net SCFA production equals to the difference between the concentrations in subsequent colon regions.

Three SHIME experiments were performed in this study. For the purpose of the first one, the “**stabilization experiment**”, investigating the stabilization of *A. muciniphila*, the SHIME set-up consisted of two colon compartments, proximal and distal (Figure S1A). The second one, the “**pH and inulin experiment**”, focusing on pH and prebiotics, consisted of three parallel distal colon vessels at different pH intervals: 6.6 to 6.9 (Distal-high pH, Dh), 6.15 to 6.4 (Distal-medium pH, Dm) and 5.6 to 5.9 (Distal-low pH, Dl) (Figure 1A). The same proximal colon vessel (PC) was used to feed all three to ensure identical nutritional conditions in the different distal colon vessels. After 11 days of normal nutritional feeding, 5 g/L of inulin were supplemented to the feed. This allowed the investigation of the prebiotic effect of inulin in the proximal colon and in the distal colon at different pH intervals. For the third one, the “**Mucin experiment**” evaluating the effect of mucin supplementation, the set-up of the experiment consisted of one proximal (PC) and three parallel distal colon vessels (Distal 1-3) (Figure 4A). After supplementing a mucin-free nutritional medium during the first 10 days, 8g/L of mucin were delivered to the proximal colon vessel on day 10, and from day 12 to 15, 4g/L mucin were supplied. *A. muciniphila* numbers were quantified with qPCR and mucin and SCFA concentrations were determined. Based on qPCR results of the pH and mucin experiment, respectively 32 and 20 samples of the most important time points, were characterised using next-generation sequencing.

Microbial community analysis

The DNA extraction procedure was adapted from Boon *et al.* (Boon *et al.*, 2003) with modifications to increase the release of DNA from microbial cells. 1% SDS was added during the first extraction step and mechanic lysis was performed. Copy number of the 16S rRNA gene of *A. muciniphila* was estimated by quantitative PCR on 10- or 100-fold diluted DNA, using specific primers for *A. muciniphila* (AM1 and AM2) (Collado *et al.*, 2007). Primer concentration was 300 nM. Standard curves were constructed with serial dilutions (10^2 to 10^8 copies. μL^{-1}) of plasmid DNA from clones of *A. muciniphila*. PCR was performed to amplify the plasmid containing the sequence insert using described protocols (Collado *et al.*, 2007). The specificity of all primers was verified by the amplification of amplicons of the correct size from the target products in all samples. Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems, Ghent, Belgium) using *Power SYBR[®] Green PCR 2X Master Mix* (Applied Biosystems, Ghent, Belgium) according to the manufacturer’s instructions. The results were expressed as log copies/mL of initial sample (Collado *et al.*, 2007).

Biodiversity was analysed using Illumina high throughput sequencing (MiSeq, Illumina, Hayward, CA, USA). The V5-V6 hypervariable region of the 16S rRNA gene was amplified

using primers 807F and 1050R (Bohorquez *et al.*, 2012). Libraries were prepared by pooling equimolar ratios of amplicons (200 ng of each sample), and tagged with a unique barcode (Camarinha-Silva *et al.*, 2014). Resulting libraries were sequenced on a Miseq (Illumina, Hayward, CA, USA) using 250 bp single-end sequencing chemistry. Single reads were trimmed to 120 nucleotides and quality filters were performed as previously described (Camarinha-Silva *et al.*, 2014). Samples from both experiments were analysed separately. From the pH experiment (32 samples) and the mucin experiment (20 samples), 905284 and 544294 operational taxonomic units (OTUs), respectively, were retrieved and were clustered into 132 unique taxa. Sequence composition was compared using the RDP Classifier tool (Wang *et al.*, 2007) and SILVA database (Pruesse *et al.*, 2007). Data were randomly subsampled to the sequence count of the sample with the lowest sequence count using the function `rarefy_even_depth` from the `phyloseq` package from R (McMurdie and Holmes, 2013); relative abundances of the top twelve taxa, with their deepest possible RDP classification up to the family level were determined (Kerckhof *et al.*, 2014). Rarefaction curves, richness and biodiversity indices were obtained with the `vegan` package in R (Oksanen *et al.*, 2011). Sequences are deposited and publically available in the European Nucleotides Archives under the accession numbers LN832064-LN832188.

Metabolic activity analysis: SCFA production and mucin concentration

Acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate and isocaproate were measured as described previously (De Weirdt *et al.*, 2010). Data were analysed using the SPSS 19 software (SPSS Inc., Chicago, USA). Normality was determined with a Kolmogorov-Smirnov test before investigating probability of intergroup differences. Significant differences between treatments were detected using one-way ANOVA or the non-parametric Mann-Whitney U test in case of non-normality. Post hoc analysis was performed using the Bonferroni correction for equal variances or Dunnett test when variances were assumed to be different. Pearson correlation coefficients were calculated to investigate the relation between the SCFA and *A. muciniphila*. Significance was set at 0.05.

Multivariate statistical analysis

Multiple Factor Analysis (MFA) was employed to assess the variations on the relative bacterial abundances in the pH experiment on each SHIME vessel at different pH, following inulin supplementation. MFA was employed to simplify the data by reducing the dimensionality of the dataset encompassing the bacterial families and the qualitative descriptors (pH and sampling time). In this way, MFA allowed for balancing the influence of each group of bacterial families, for investigating the associations between time and pH, and to produce a representation of the individual samples grouped according to their similarities regarding the relative bacterial abundances. Bacterial abundances were weighted on a global PCA and results were explained in a factor map (de Tayrac *et al.*, 2009), where the value of the abundance of each bacterial family (vector) for the corresponding pH (factor) was plotted (Grunert *et al.*, 2016). The function MFA from the `FactoMineR` package (Le *et al.*, 2008) was performed in R. Parametric bootstrapping was applied to construct confidence ellipses around the barycentre of the abundances on each SHIME vessel to visualise whether the bacterial abundances were significantly different among colon vessels. Confidence intervals of the average coordinates of the bacterial abundances were represented by the ellipses, indicating 95% of similarity among bacterial abundances. If the ellipses were not overlapping, the bacterial abundances were significantly different; incomplete

overlap indicated that bacterial abundances were significantly different in the samples outside the ellipse (Dehlholm *et al.*, 2012). Hence, non-overlapping confidence “ellipses” denoted that two vessels differed at significant level ($P > 0.05$).

Correspondence analysis (CA) using the Single Value Decomposition (SVD) highlighted the variations in relative bacterial abundance in the mucin experiment of the 3 distal colon vessel on the SHIME pre- and post-mucin treatment. Computations were performed in the R language, using the ca package (Nenadic and Greenacre, 2007). The percentages of explained variance (inertias) for the bacterial abundances (rows) and time point (columns) on each dimension of the CA were determined. Total variance was explained in 4 factors or dimensions. The graphical display of the CA showed relationships among the relative abundances of specific bacterial groups (Hernandez-Sanabria *et al.*, 2013). The area of the point symbols plotted in the CA indicates the mass. Masses are the marginal proportions of the relative bacterial abundances (row variable). These proportions are used to weight all the bacterial abundances when computing the distance between the abundance of an “x” bacterial family to the centroid of the abundance of all bacterial families. This weighting has the effect of compensating for unequal numbers of cases (Nenadic and Greenacre, 2007). Therefore, the area of a point in the graph will indicate the relative contribution of a particular bacterial family to the total variance. The colour intensity of the point symbol and that of the arrow are proportional to the absolute contribution of the points in terms of a percentage of explained variance. Darker colours of the arrows indicate higher percentages of variance attributed to a particular time point. Point symbols with darker colour represent the bacterial abundances that contributed more to the variance.

RESULTS

Stabilization of *A. muciniphila* in the colon environment

We previously demonstrated an 8-fold increase of *A. muciniphila* in the complex microbial community of the distal colon regions (transverse and descending) compared to the proximal colon by applying a phylogenetic microarray (HITChip) on consecutive colon regions of a stabilized SHIME-model (19-26 days after inoculation with a faecal sample) (Table S1) (Van den Abbeele *et al.*, 2010). QPCR analysis of *A. muciniphila* confirmed that *A. muciniphila* increased in the distal colon regions ($> 10^5$ -fold increase), with no difference between transverse and descending colon compartments ($P=0.102$) (Table S1). During the stabilization experiment, we monitored *A. muciniphila* copy number and mucin degradation upon inoculation of the *in vitro* gut model with a human faecal sample to gain insight in the temporal patterns of *A. muciniphila* colonization in the distal colon (Figure S1B-C). Initially (day 0-3), *A. muciniphila* increased in both the proximal and the distal colon region, coinciding with efficient mucin degradation in both compartments. Starting from day 3 after inoculation, *A. muciniphila* washed out from the proximal colon, as measured with qPCR and mucin started to accumulate in this colon region. At the same time, *A. muciniphila* abundantly colonized the distal colon, which was characterized by almost complete mucin degradation. *A. muciniphila* numbers stabilized 6 days after inoculation.

Effect of pH and inulin on *A. muciniphila* in the distal colon

As previous experiments showed that *A. muciniphila* proliferates in the distal but not the proximal colon compartment of the SHIME (Van den Abbeele *et al.*, 2010), we focused on the

distal colon during the pH experiment to evaluate the effect of pH on *A. muciniphila* colonization (Figure 1A). Quantitative PCR analysis confirmed that *A. muciniphila* was present the proximal colon compartment, yet at abundances close to the detection limit (Figure 1B). Moreover, a strong pH-dependent colonization of the distal colon was observed; the average abundance over time was measured at 7.98 ± 0.07 log copies/ml with pH between 6.15-6.4 (Dm) and 8.35 ± 0.05 log copies/ml at pH 6.6-6.9 (Dh). Despite the identical nutritional conditions in the distal colon region with low pH (5.6-5.9), *A. muciniphila* numbers decreased dramatically to around the detection limit (= 4 log copies/mL), similar to the abundances in the proximal colon. *A. muciniphila* thrived at neutral pH values. Further, prebiotic treatment with inulin at day 11 did not significantly affect *A. muciniphila* numbers (Table S2).

Figure 1: pH and inulin experiment

SCFA analysis revealed that the distal colon regions at higher pH (Dh/Dm) contained high acetate, propionate and initially also valerate/branched SCFA levels and low butyrate levels (Figure 2). Administration of inulin resulted in increased acetate and butyrate in comparison with levels of branched SCFA in the proximal colon and a slight increase in butyrate and propionate levels in the distal colon vessels.

Figure 2: Short chain fatty acid concentration of the pH and inulin experiment

Microbial composition analyses of the colon environment

Illumina sequencing showed high relative abundance of *A. muciniphila* in the distal colon with medium and high pH, compared to the distal colon with low pH and the proximal colon, confirming the qPCR results (Figure 3A). This increase of *A. muciniphila* is at the expense of several other species like *Clostridium* sp. and *Bacteroides* sp. and coincides with higher relative abundances of *Alistipes*, *Parabacteroides* and *Bilophila* species (Figure 3). It is remarkable that the differences in the pH lead to such a strong response in one species, *A. muciniphila*, compared to the other species in this complex microbial community.

Multiple factor analysis was employed to interpret how the differences in pH impacted the bacterial abundances in each region of the colon and whether the relative abundances remained stable before and after the supplementation with inulin (Figure 3B). The goal of this analysis was to discriminate whether the relative abundances of each colon fraction were similar among them. In the figure the coloured symbols, representing the bacterial abundances in the different environments at different time points, were plotted in a two dimensional space. The variables described on each dimension are included in Table S3. The first dimension comprised of the relative bacterial abundances associated with day 6 in particular, and in the distal colon with high pH, while the second dimension explained the relative abundances in the distal colon on day 10 (Table S3). These two dimensions accounted for 54% of the differences among relative abundances on the different locations and time points. The third dimension included the relative abundances that are correlated with days 14 and 15 and it accounts for 14% of the total variance. It may be suggested that the first dimension describes the initial colonizers, because it is significantly correlated with day 6, whereas the second dimension explains the genus correlated with high pH (distal colon) and the third dimension describes the genera correlated with the inulin treatment, because day 14 and 15 are comprised in this dimension. Genera that are positively correlated with the second dimension are *Alistipes*, *Bilophila* and *Akkermansia* and

with the third dimension are *Lactobacillus*, *Succiniclasticum*, *Propionispora* and *Lachnoclostridium* whereas *Bacteroides* is negatively correlated with this dimension (Table S3). The fourth dimension included the genera correlated with location with low pH (distal colon).

At day 6, the bacterial abundances are different between locations, which can be due to the initial adaptation to pH and other environmental conditions (Figure 3B). Proximal and distal colon (with low pH) partially overlap, which indicates that the relative bacterial abundances are similar in the time points inside the overlapping area. The partial overlap may be explained by the pH, which is low in both locations. The area of the confidence ellipse is smaller in the high pH, indicating that the relative abundances in high pH tended to be not significantly different (Figure 3B). MFA showed that in the distal colon with higher pH, the relative abundances on day 8 and 10 (pre-inulin) are significantly different from the relative abundances on day 14 (post-inulin). However, the effect was not consistent when pH was low.

Figure 3: Illumina sequencing results of the pH and inulin experiment

The effect of variable mucin concentration in the colon environment on *A. muciniphila* abundance

To further unravel the behaviour of *A. muciniphila* in a complex microbial community, the effect of mucin presence was investigated. In this mucin experiment, the aim was to wash out *A. muciniphila* from the distal colon compartment by feeding mucin-free nutritional medium to the SHIME (Figure 4A). Based on qPCR, *A. muciniphila* decreased during administration of mucin-free feed but did not wash out entirely from the distal colon at day 10 (Figure 4B). The initial high numbers are probably caused by growth on mucins present in the inoculum. Upon the supplementation of mucin, *A. muciniphila* numbers increased (3.03 ± 0.13 log copies/mL from day 10 to 11) in all distal colon regions ($P=0.0006$) but not in the proximal colon ($P=0.3$) (Figure 4B).

Figure 4: Mucin experiment

There was a distal increase of acetate, propionate, butyrate, valerate and branched SCFA upon addition of mucins (Figure 5). Only acetate, valerate and especially propionate increased in the distal colon compartment when accounting for the proximal SCFA production, while butyrate and branched SCFA were already produced in the proximal colon compartment. The *A. muciniphila* increase in the distal colon on day 11 thus correlates with increased propionate levels. As can be seen from the qPCR and SCFA results, the three distal colon vessels were stable throughout the experimental period.

Figure 5: Short chain fatty acid concentration of the mucin experiment

Microbial composition analyses of a colon environment with variable mucin supply

Illumina sequencing data showed a strong increase in relative abundance of *A. muciniphila* after mucin addition, confirming the qPCR results (Figure 6A). This increase coincides with a decrease of almost all the other genera, except for *Parabacteroides*, which increase together with *A. muciniphila*. *Lactobacillus* and some *Clostridium* species are significantly more abundant after mucin supplementation (Table S5). Correspondence analysis was used to further explain the variations in relative abundances (Figure 6B). The area of the points in the figure indicates the relative frequency of a particular genus and darker colours of the arrows indicate higher percentages of variance attributed to a particular time point. Point symbols with darker colour

represents the bacterial abundances that contributed more to the variance among time points (Table S5). The presence of a distinctive microbial community before and after the supplementation with mucin was validated based on the relative abundances of the bacteria associated with each time point. It is clear that dimension 1, which accounted for 87% of the total variance among samples, shows the distinction between the community before and after mucin supplementation. Day 8 and 10 are characterized by *Lachnoclostridium*, *Bacteroides* and *Parasutterella* whereas day 12 and 15 are characterized by *Akkermansia*, *Parabacteroides* and *Lactobacillus* (Table S5). Changes in the abundance of *Akkermansia* alone explain 53.76% of total variance. Dimension 2 accounts for 9% of the total variance and is mostly characterized by the community in transition.

Figure 6: Illumina sequencing results of the mucin experiment

DISCUSSION

We used a dynamic *in vitro* gut model (SHIME) to gain insight in the colonization and metabolic performance of the mucin-degrading gut symbiont *A. muciniphila* within the three regions of the human colon and in the presence of a complex gut microbiota. This overcame limitations that are inherently associated with human *in vivo* studies, such as the difficult access to the different regions of the colon and confounding effects of processes that may indirectly impact host mucin production and fitness of mucin degraders. Due to the successful and abundant colonization of *A. muciniphila*, we created an ideal biological environment to study the ecological behaviour of *A. muciniphila* and the impact of pH and mucin.

Using the *in vitro* SHIME model and a specific inoculum with high abundances of *A. muciniphila*, the combination of qPCR analysis, HITChip and Illumina sequencing demonstrated the preferential colonization of the distal colon ecosystem by *A. muciniphila* (Table S1, Figure S1, Figure 1, Figure 3, Figure 4, Figure S2) (22, 23). The preference of *A. muciniphila* for distal colon colonization was shown, yet partial mucin degradation also took place in the proximal colon. This process was probably performed by *Bacteroides* species, which are colonizers of the proximal colon compartment (Figure 3A, Figure S2) and which are known as versatile glycan-degrading microbes (Martens *et al.*, 2009; Salyers *et al.*, 1977). In contrast, our results suggested that *A. muciniphila* was the specialist mucin degrader in the distal colon compartment, because its abundant colonization significantly correlates with mucin concentrations (Figure S1, Figure 4, Figure 6) and mucin degradation (Figure S1). Propionate is a major end product of *A. muciniphila* metabolism, hence the increase in propionate after mucin supplementation indicated that *A. muciniphila* was degrading mucin in the distal colon (Derrien *et al.*, 2004) (Figure 5). When the food bolus arrives in the distal colon, the carbohydrates are already fermented and the proteolytic fermentation starts. This leads to the formation of phenols, branched short chain fatty acids, amines and other end products, many of which have toxic effects (Macfarlane and Macfarlane, 2012; Nyangale *et al.*, 2012). The presence of *A. muciniphila* in the distal colon and its production of beneficial short chain fatty acids from mucins can thus be a protective strategy by prolonging the saccharolytic fermentation and maybe counteracting some of the deleterious effects of the proteolytic fermentation.. This location preference of *A. muciniphila* for the distal colon compartment of the SHIME can derive from parameters, such as pH, nutrient availability, residence time, and the microbial community, which differ between colon regions.

Further research into these different parameters, shows that the preferential colonization of *A. muciniphila* seems to be determined by the pH (Figuren!), because *A. muciniphila* numbers were different between distal colon vessels that only differ in pH (getallen!). This confirms results from *A. muciniphila* grown in monoculture, where the optimum pH for growth was 6.5 and no growth was observed below pH 5.5 or above pH 8 (Derrien *et al.*, 2004). So *A. muciniphila* thrives at the more neutral pH (6.6 to 6.9) of the distal colon compartment and is less competitive in the proximal colon with lower pH (5.6 to 5.9) (Cummings, 1997). *A. muciniphila* might be locally outcompeted by *Bacteroides* species, which have the ability to grow and compete across a broad pH range (Macfarlane *et al.*, 1995). *A. muciniphila* prefers colon environment with high pH and is correlated with higher acetate and propionate production but lower butyrate concentration. In the vessels with pH 6.6 to 6.9, more acetate and propionate were produced but less butyrate, which has been previously reported (Walker *et al.*, 2005). The decrease in butyrate may be a direct effect of pH as butyrate producers proliferate at lower pH (Duncan *et al.*, 2009). The increase in propionate may be attributed to higher *A. muciniphila* abundances, as other genera correlated with higher pH in this experiment are not known for their high propionate production. *Bilophila* and *Alistipes* have been previously associated with undesirable gut health traits, like most species that thrive at more neutral pH in the distal colon (Cummings, 1997; da Silva *et al.*, 2008; Nyangale *et al.*, 2012; Rautio *et al.*, 2003). However, it cannot be ruled out that other factors play a role. For instance, the pH may have an effect on the mucin structure or its enzymatic accessibility and also the microbial background may play a role since we only tested it in one microbial environment.

Not only was the effect of pH and inulin investigated, but also the effect of variable mucin availability. The most obvious result of the administration of mucin after mucin depletion was the increase in *A. muciniphila* numbers in the distal colon compartment. Mucin supplementation also produced an increase in butyrate production in the proximal colon (Figure 5). This increased butyrate production was potentially established via cross-feeding with *Bacteroides* and other species that can degrade mucin (Martens *et al.*, 2008; Martens *et al.*, 2009; Salyers *et al.*, 1977). An increase in the relative abundance of *Bifidobacterium* species was observed in the proximal colon at day 15. This may indicate that those species are capable of partial mucin degradation and they could also contribute to the increase in butyrate (Hoskins *et al.*, 1985; Killer and Marounnek, 2011; Png *et al.*, 2010) (Figure S2). In the distal colon compartment, the mucin administration caused a significant increase in propionate production that can be attributed to *A. muciniphila* and *Parabacteroides* species, which both characterize the new mucin-associated community (Figure 6). *Parabacteroides* sp. produce acetate and succinate, which can be used by other species to produce propionate, for example *Phascolarctobacterium*, which relies for its carbon-source on succinate (Deldot *et al.*, 1993; Sakamoto and Benno, 2006; Watanabe *et al.*, 2012). Increase in propionate production in a mucin degrading environment might thus be a biological marker for *A. muciniphila*.

We observed that colonization of *A. muciniphila* using this specific inoculum in our *in vitro* system is dependent on pH and mucin, but not on inulin.. In a rat study (Van den Abbeele *et al.*, 2011) it was shown that inulin shifted *A. muciniphila* from the caecum to more distal regions and increased the faecal numbers of *A. muciniphila*. As mentioned above, the colonization of *A. muciniphila* is very pH dependent. Therefore, as inulin supplementation is known to lower pH (Van den Abbeele *et al.*, 2011; Welters *et al.*, 2002), it might force *A. muciniphila* to colonize more distal regions in the rats. Inulin does not only affect pH but it also stimulates mucin

secretion by the host ((Barcelo *et al.*, 2000; Schmidt-Wittig *et al.*, 1996; Shimotoyodome *et al.*, 2000)). Since *A. muciniphila* is a known mucin degrader and susceptible to changes in mucin availability, this is another way by which inulin can exert an indirect effect on *A. muciniphila* numbers. In our *in vitro* study however, the pH and the mucin concentration were maintained constant and no effect of inulin on *A. muciniphila* could be observed. Inulin treatment did stimulate *Lactobacillus* species, which has been described in many other studies (Kleessen *et al.*, 2001; Macfarlane *et al.*, 2006; Makras *et al.*, 2005; Sghir *et al.*, 1998). Also propionate producing bacteria *Succiniclacticum* and *Propionispora* increased after inulin treatment, which can explain the higher propionate concentration (Abou-Zeid *et al.*, 2004; van Gylswyk, 1995). The increased butyrate concentration however could not be linked to higher abundances of butyrate producing bacteria (Table S2).

Our experiments showed the mechanism behind the effect of inulin, pH and mucin on the colonization of *A. muciniphila* and confirmed our hypothesis. In this way, prebiotics like inulin may exert a beneficial effect along the entire length of the colon, by stimulating mucin secretion and shifting the mucin degradation to distal regions. This process may yield higher propionate and acetate production. Other dietary compounds that can favour *A. muciniphila* are for example cranberries, which are rich in polyphenols and have been shown to increase the *A. muciniphila* population in diet-induced obese mice (Anhe *et al.*, 2015). Also in mice fed with fish-oil, rich in omega-3 fatty acids, *A. muciniphila* was enriched (Caesar *et al.*, 2015). The manner in which these products stimulate *A. muciniphila* is not yet clear and, like with inulin, the effect might not be the same in our *in vitro* system. Human milk oligosaccharides (HMO) resemble mucins in their structure and *A. muciniphila* has the capacity to grow on these HMOs. So treatment with human milk oligosaccharides might directly affect *A. muciniphila*, just like mucin treatment, and thus be useful in our *in vitro* SHIME model.

For the experiments performed in this paper a microbial environment was created with pronounced *A. muciniphila* colonisation to ensure the study of its ecological behaviour. The experiments were carried out in the *in vitro* SHIME model that is perfect for these mechanistic studies. Although there were hundreds of other species present, only *A. muciniphila* reacted that strongly to the changes in the SHIME environment, i.e. different pH and mucin concentration. Further research considering multiple donors, providing different microbial environments with different *A. muciniphila* abundances, should be performed to elucidate whether the sensitivity of *A. muciniphila* is dependent on the microbial background or not. The results shown in this paper are indicative of a high sensitivity of *A. muciniphila* to environmental changes. This high sensitivity could explain why its abundance changes drastically *in vivo* when the conditions in the colon change, for example due to certain disease states (Everard *et al.*, 2011; Png *et al.*, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2009). This could clarify why *A. muciniphila* is so often correlated with diseases and might be an indicator species for gut health.

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743

FIGURE LEGENDS

FIGURE 1: pH and inulin experiment. (A) Experimental set-up of the SHIME run to investigate the effect of pH on the colonization of *A. muciniphila*. After receiving normal nutritional medium during the first 11 days, 5g/L inulin was supplemented to the feed from day 11 until day 15. (B) *A. muciniphila* abundance (log (copies/mL)) measured with qPCR as a function of time after inoculation (days) for the proximal colon (PC) and the three distal colon regions on different pH values: Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9). Technical variation was never higher than 3%.

FIGURE 2: Short chain fatty acid (SCFA) concentration (mM) as a function of time after inoculation (days) in the different colon regions, proximal and distal with high, medium and low pH. Inulin (5g/L) was supplemented to the feed from day 11 until day 15. Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9).

FIGURE 3: Illumina sequencing results of the pH and inulin experiment. Inulin (5g/L) was supplemented to the feed from day 11 until day 15. Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9). (A) An overview of the relative abundance (% of the total community) of the 11 most abundant genera in the different colon vessels for the different time points (days). (B) Multiple Factor Analysis (MFA) was employed to assess the relative bacterial abundances detected in each SHIME vessel and for the different time points based on the Illumina data from the different samples.($p < 0.05$)

FIGURE 4: Mucin experiment. (A) Experimental set-up of the SHIME run to investigate the effect of mucin depletion and administration on the colonization of *A. muciniphila*. After receiving mucin depleted nutritional medium during the first 10 days, 8g/L was dosed to the proximal colon on day 10, while 4g/L was dosed from day 12-15. (B) *A. muciniphila* abundance (log (copies/mL)) measured with qPCR as a function of time (days) after inoculation for the proximal colon and the three replicate distal colon regions (Distal 1-3). Technical variation was never higher than 3%.

FIGURE 5: SCFA levels (mM) as a function of time (days) after inoculation for the proximal colon and the three distal colon regions that were fed a mucin-free feed until day 10 after which 8g/L (day 10-12) and 4g/L mucin (day 12-15) was administered.

FIGURE 6: Illumina sequencing results of the mucin experiment where a mucin-free feed was fed until day 10 after which 8g/L (day 10-12) and 4g/L mucin (day 12-15) was administered. (A) An overview of the relative abundance (% of the total community) of the 11 most abundant genera in the distal colon vessels (averaged) for the different time points (days). (B) Correspondence analysis (CA) using the Single Value Decomposition (SVD) was employed to highlight the variations in relative bacterial abundance of the distal colon vessel on the SHIME pre- and post-mucin treatment ($p < 0.05$)

A

- 0 → Day 0: Inoculation of SHIME with human fecal inoculum
- Day 0-11: Study effect of pH on *A. muciniphila* colonization
- 11 → Day 11: Administration of inulin
- Day 11-15: Study effect of inulin on *A. muciniphila* levels

B

---◆--- Proximal
—■— Distal-high pH
---▲--- Distal-medium pH
.....×..... Distal-low pH

A **B**