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MIMICKING THE HUMAN GUT MICROBIOME COMPOSITION AND FUNCTIONALITY

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SUMMARY

This paper highlights the current *in vitro* technological advances in simulating the gut microbiome composition and functionality with enabling technologies related to the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). While *in vitro* models offer the standard advantages over *in vivo* studies (low costs, no ethical constraints, multi-parametric testing), their core value lies in their ability to simulate regional and micro-environmental differences that occur along the longitudinal and radial axis of the gut. We will provide evidence that these models can be used:

1. to simulate microbiome differences between proximal and distal colon regions,
2. to establish distinct luminal and mucus-associated microbial communities, and
3. to enable intimate host-microbe interactions near the gut epithelial surface.

More specifically, we will present published and unpublished data obtained with the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) and the Host-Microbe Interaction (HMI-) module. Finally, our insights into the specific micro-environmental behaviour of gut microbes will be used to propose some future perspectives for (*in vitro*) gut microbiome research.

INTRODUCTION

Human health is influenced by a multitude of determinants: genetics, way of delivery, diet, lifestyle, medical practices, hygiene, the exposome… Also the human microbiome is considered a major factor in the health equation, either directly or through interaction with any of the aforementioned factors. In the last decade of scientific research, different -omics approaches have revolutionized the field by shedding light on correlations between health status and microbiome composition, specific expression of genes, translation into proteins or production of specific metabolites. The number of these studies has exponentially grown in recent years, yet scientists struggle to find preventive or therapeutic measures that tackle any of the microbial processes (*de Vrieze*, 2015). This is mainly caused by the lack of mechanistic insight in host-microbe interaction processes and the lack of appropriate model systems that allow dynamic sampling of specific environments.

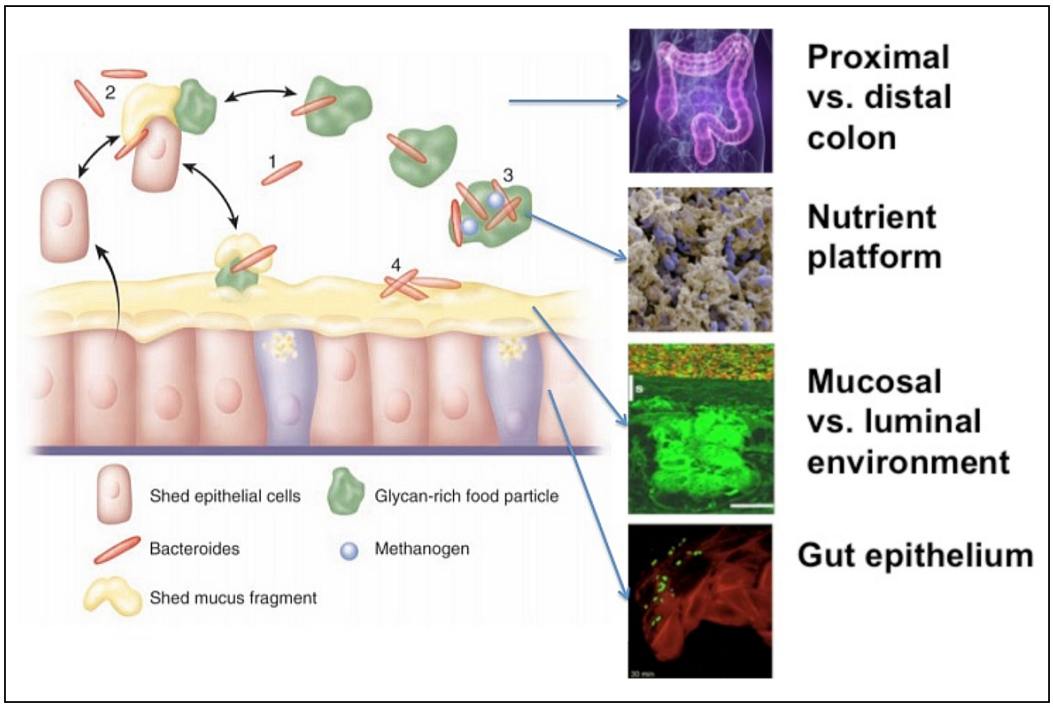

Figure 1: Depiction of different gut micro-environments and altered microbial colonization profiles (picture adapted from *Bäckhed* et al., 2005).

GUT MICROENVIRONMENTS

"The" gut microbiome is composed of different microbial consortia that are composition-wise and functionally highly diverse because of their association with different gut micro-environments (Figure 1) (*Bäckhed* et al., 2005). Because of differences in residence time, physicochemical or enzymatic stressors in the upper digestive tract, diverse presence of M-cells or variable thickness of the mucus layer along the gastrointestinal tract, the microbial colonization is distinct and highly specific. Already in the colon alone, there are significant differences between the proximal and distal region when it comes down to microbiome composition and the fermentative metabolism of dietary substances or the metabolic potency towards secondary plant metabolites, pharmaceuticals or pollutants. The suspension in the gut lumen cannot just be considered as a homogeneous mixture of non-digestible dietary components, microbiota and host secretions. It is rather a heterogeneous suspension where nutrient platforms offer a scaffold for microorganisms to adhere to and where they can interact with one another, for example to cross-feed on dietary fibre resulting in the production of a diverse short chain fatty acid profile. When focusing on the mucus layer overlying the epithelium, one must appreciate the existence of specific physicochemical and immunological gradients that dictate what microorganisms have a higher preference (or success rate) to become part of the mucosal microbiome. The existence of an oxygen gradient over the epithelium and mucus

layer can result in altered colonization and even modulated gene expression, for example by *Shigella flexnerii* pathotypes (*Marteyn* et al., 2010). The mucosal surface and intervillus region is also known for their altered fluid shear forces, with low fluid shear prevailing near the mucosa and for example directing virulence gene expression by pathogenic *Salmonella* strains (*Höner zu Bentrup* et al., 2006). These mucosal microbes are interesting from a health point of view, because of their close proximity to the gut epithelium and, hence, higher potency to interact with the human host. Specific microorganisms can even further migrate through the mucus layer and actually reach epithelial cells where they can more profoundly interact with the host, sometimes leading to actual pathogenesis. Finally, because of the gut being an open ecosystem, changes in the aforementioned determinants (diet, medical practices, lifestyle…) add an additional layer of complexity to the microbiome composition. This necessitates a dynamic monitoring of the gut microbiota and a correlation with changing environmental parameters.

NEED FOR MODEL SYSTEMS

For obvious reasons, human microbiome research is physiologically most representative when conducted on biological samples from human subjects. However, only relying on the analysis of faecal microbiota does not give an accurate view on the colonization ability and dynamics of the microbial consortia in the different gut microenvironments. While biopsy samples may give a closer view, the sampling procedure is far from straightforward and it does not allow to dynamically monitoring the microbiome upon dietary shifts or during disease progress. While animal models - even the gnotobiotic models that are humanized with human microbiota - give some more flexibility in the analysis of different gut regions, also these samples are restricted to endpoint measurements. Moreover, interpretation of the scientific data must always take into account to what extent these models are representative for human biological processes.

To address the issues of gut microenvironment differences and microbiome dynamics, specific lab-scale model systems have been developed over recent years. While the biggest limitation of these model systems is obviously the lack of a physiological environment, they do provide certain advantages over *in vivo* observations. Firstly, specific microenvironments can be simulated, both in a longitudinal direction (proximal vs. distal gut regions) as in a cross-sectional direction (luminal vs. mucosal regions). Secondly, these environments can be sampled in a dynamic way, allowing the study of microbial adaptations to a changing environment. Finally, and this is probably the biggest *in vitro* asset, due to the control over several digestive, enzymatic and physicochemical parameters, scientists are able to conduct mechanistic research. These benefits can however not be overestimated. *In vitro* models always need a proper validation against human *in vivo* data and the generated scientific insights need to be used to support *in vivo* observations or to direct research prior to entering clinical research.

One of the models that has tried to encompass most of the digestive processes going from the upper digestive

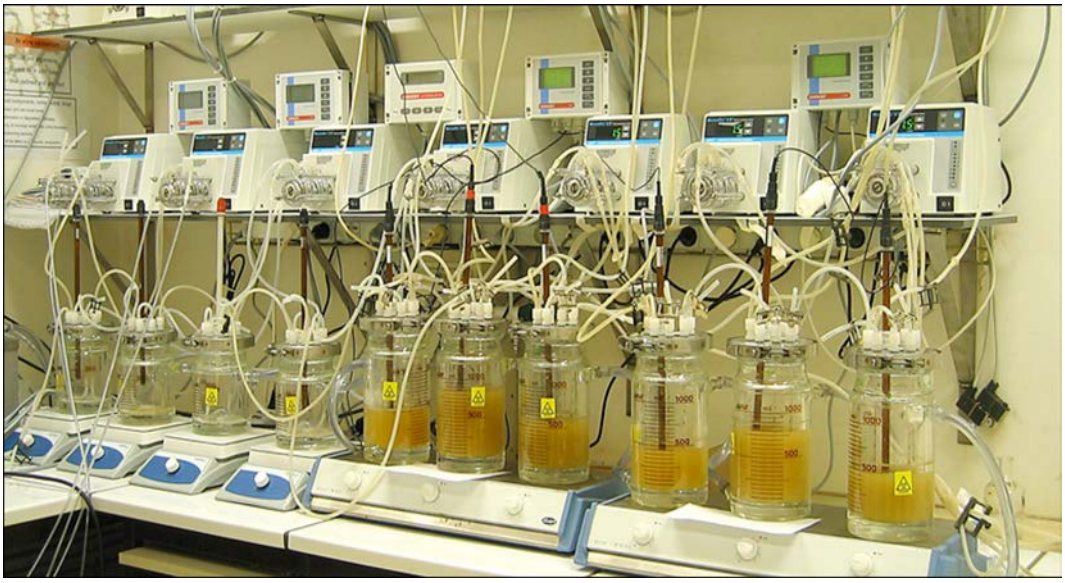

Figure 2: Experimental setup of a TWIN-SHIME system, composed of 2 parallel units of stomach, small intestine, ascending colon, transverse colon and descending colon compartments.

tract (stomach, small intestine) to the lower gut (proximal and distal colon) is the simulator of the human microbial ecosystem, or SHIME® (registered name by Ghent University and ProDigest) (Figure 2) (*Molly* et al., 1993). The model system has been validated against human *in vivo* data both for microbiome composition and fermentative activity (*Molly* et al. 1994) as for very specific metabolic conversions that are distinguishing individuals from one another (*Possemiers* et al., 2006). While at first, SHIME research primarily focused on metabolic interactions in the lumen, the last 5 years have seen a significant improvement of the model by including a mucosal environment by incorporation of mucus coated microcosms (*Van den Abbeele* et al., 2013). This so-called M-SHIME (mucosal SHIME) (Figure 3) allows not only the luminal microbes to settle in the system, but also the colonization of mucosal microbes on surfaces that are representative of the *in vivo* situation, at least from the glycoprotein perspective.

Another improvement of the SHIME model is the host-microbe interaction (HMI) module (Figure 4) (*Marzorati* et al., 2014). This bicompartmental module separates gut microbes from epithelial cells through a mucin-covered semi-permeable polyamide membrane. Unlike Transwell® systems, the HMI module has in- and outlets at both the microbial and host side of the membrane. This enables connecting the microbial compartment to the M-SHIME and seeding the host compartment with 3D organotypic colon epithelium. In a proof-of-concept, this module was successfully used to study host responses upon prebiotic modulation of the intestinal microbiota (*Possemiers* et al., 2013).

Interestingly, two specific physicochemical conditions prevailing near the mucosa can also be simulated with the HMI device. Firstly, by providing oxygen from the host compartment and maintaining anaerobiosis in the microbial compartment it is possible to

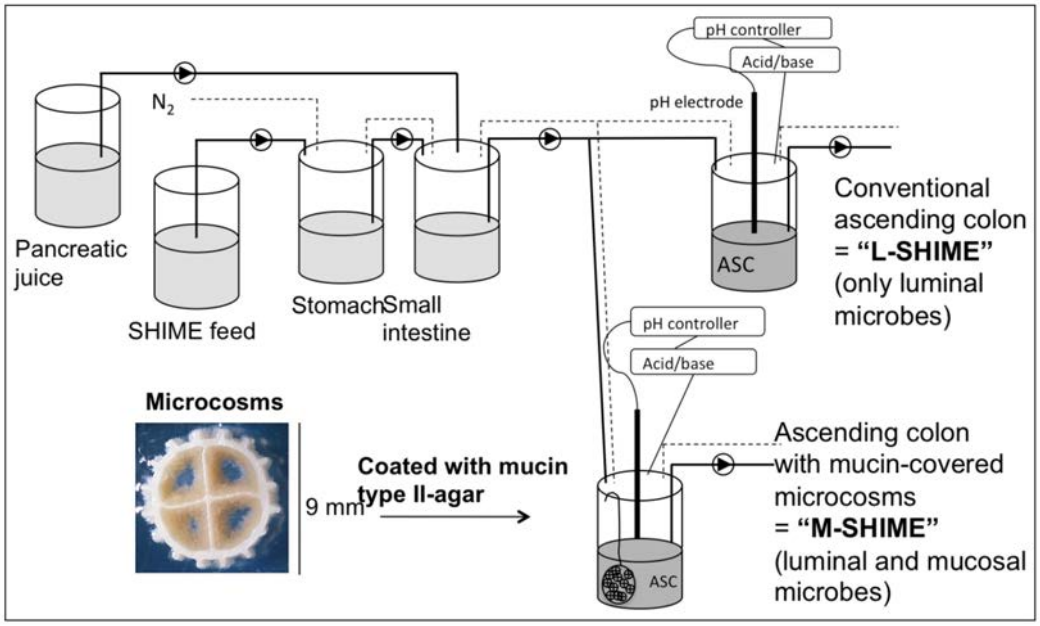

Figure 3: Experimental setup of the M-SHIME system. Mucin coated microcosms or mucus beads are brought into the respective colon compartments thereby creating a mucosal contact surface next to the luminal suspension (*Van den Abbeele* et al., 2013).

establish an oxygen gradient over the mucus layer and thereby mimic the mucosal environment more closely. This is for example interesting to study what impact hypoxic stress may have towards microbiome colonization in a scenario of insufficient mucosal oxygenation with IBD patients. Secondly, the design of the HMI module is such that it allows control over fluid shear conditions over the mucus layer, another important parameter that not only affects morphology and gene expression of epithelial cells, but also the gene expression of microorganisms in that environment.

MIMICKING THE MUCOSAL MICROBIOME AND MICROBIAL BEHAVIOUR IN THE MUCOSA

In this paper, we primarily focus on the mucosal microbiome and the role that specific microorganisms may play in health and disease. *In vivo* data have demonstrated the microbiome from biopsy samples to have a distinct composition as opposed to faecal microbiota (*Zoetendal* et al., 2002; *Swidsinski* et al., 2002). Moreover, there seems to be specific correlations between specific mucosal microorganisms and health status: for example, typical mucosal colonizers such as the butyrate producing microorganisms such as *Faecalibacterium prausnitzii,* downregulated in patients with Crohn's disease (*Willing* et al., 2009) and metabolic syndrome. More detailed *in vivo* analysis on murine samples also indicates the higher preference of butyrate producing Clostridia to colonize the mucosal environment (*Nava* et al., 2011). In that respect, it is interesting to note that the M-SHIME system

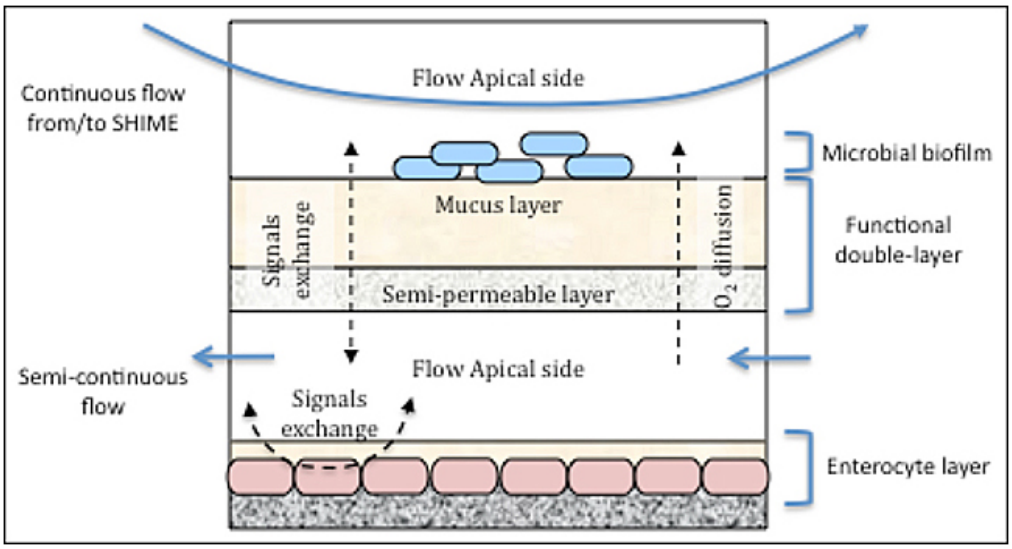

Figure 4: Host microbe interaction module comprising flow cell with low fluid shear conditions representing the mucosal microbial compartment and a host compartment (*Marzorati* et al., 2014).

displays similar colonization profiles with much higher abundances of *Clostridium* clusters IV and XIVa in the mucosal environment as opposed to the luminal suspension (*Van den Abbeele* et al., 2013). In addition, micro-array analysis for 1100 microbiome phylotypes also demonstrates M-SHIME to preserve unique microbiome features that distinguish individuals from one another.

The finding of a more pronounced colonization of the mucosal environment by butyrate producers requires some further attention. Whole genome shotgun data from the human microbiome project demonstrate these butyrate producing *Clostridium* cluster IV and XIVa to reach abundances up to 30% of the entire microbiome (*Karlsson* et al., 2012). It thus plays a dominant role in the gut ecosystem. Moreover, monoassociation of gnotobiotic mice with several of these butyrate producing Clostridia was found to result in an upregulation of Foxp3+ in CD4 cells, indicating the role of these microorganisms in regulatory T cell function (*Atarashi* et al., 2011). This can either occur through bacterial cell-associated antigens or through secreted compounds. To exemplify, *Faecalibacterium prausnitzii* was previously found to be an anti-inflammatory commensal following microbiome analysis of Crohn's disease patients (*Sokol* et al., 2009). Only recently, the compound thought to elicit the anti-inflammatory effects in epithelial cell culture models and an animal model of chemical-induced colitis, was revealed to be a 15 kDa protein, termed MAM (microbial anti-inflammatory molecule) (*Quévrain* et al., 2015).

The example of *F. prausnitzii*'s anti-inflammatory properties is particularly interesting. As mentioned above, Clostridium cluster IV butyrate producers, from which *F. prausnitzii* is the most abundant, are strong colonizers of the mucosal environment (*Willing* et al., 2009). Butyrate producing clostridia are typically anaerobic and given the fact that oxygen gradients may exist over the mucus layer, it is intriguing to find out what mechanism lies behind

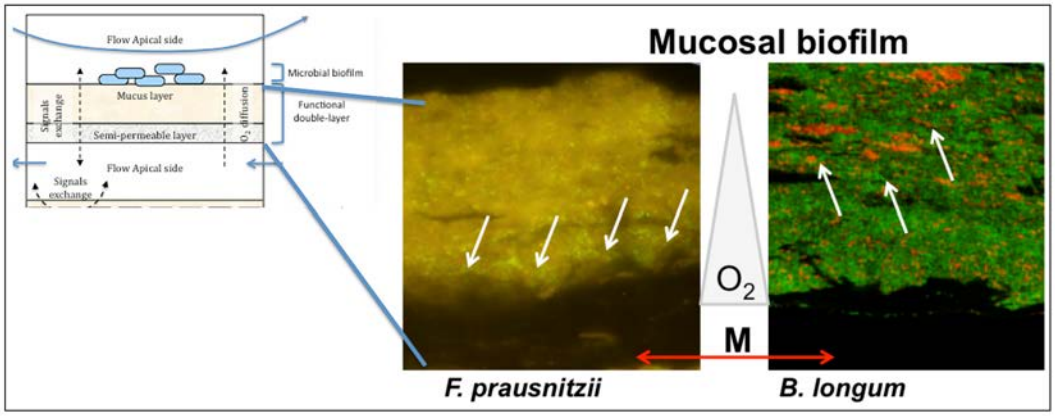

Figure 5. FISH analyses a) positioning of F. prausnitzii (left panel - fluorescent microscopy) and bifidobacteria (right panel - Confocal Laser Scanning Microscopy) in the microbial biofilm with respect to the membrane and mucus layer (M), as indicated by the white arrows. Oxygen concentration (O_2) is assumed to decrease from the bottom to the top of the biofilm. The green background is auto-fluorescence of the matrix: EPS, and non-responding bacteria in the left panel, while in the right panel it corresponds to bacteria stained with the EUB338 probe FITC labeled, and also some auto-fluorescent EPS (adapted from *Marzorati* et al., 2014).

their successful colonization. To monitor this mucosal colonization process more in-depth, the HMI module is particularly useful. Coupling the HMI to SHIME enables *in vitro* cultured gut microbiota to colonize the mucus layer on top of the semi-permeable membrane in the HMI module. This results in the establishment of an early stage biofilm where the positioning of different microorganisms of interest can be determined. Using FISH probes, *Marzorati* et al., (2014) monitored the mucosal colonization of Bifidobacteria and *Faecalibacterium prausnitzii* with the HMI module and came to interesting findings. While the strictly anaerobic bifidobacteria tended to colonize the upper side of the mucus layer, the anaerobic *F. prausnitzii was* mainly found back in the lower part of the mucus, i.e. at the anoxic/oxic interphase (Figure 5). *Khan* et al., (2012) demonstrated that *F. prausnitzi*i can grow in the oxic-anoxic interphase due to the fact that this microorganism, despite being oxygen sensitive, copes with O2 because of a special extracellular electron shuttle of flavins and thiols. Similar to the *in vivo* situation - where small amounts of oxygen permeate from blood vessels towards the gut lumen - in the HMI module, oxygen diffusion from the aerobic lower chamber to the anaerobic upper chamber (Figure 4) probably results in more oxidative conditions at the base of the biofilm. Availability of the flavin/thiol electron shuttle gives *F. prausnitzii* a selective advantage over other gut microbes enabling it specific mucosal colonization. These findings were supported by the dynamic monitoring of *F. prausnitzii* with qPCR. Over a 48h experiment, a decreasing concentration of *F. prausnitzii* was noted in the luminal compartment and an increasing one in the mucus layer, as opposed to an unchanging bifidobacteria concentration in the lumen and decreasing bifidobacteria concentrations in the mucus layer. This demonstrates the potency of the HMI module to maintain the preferential mucosal colonization of specific gut microorganisms within the mucus layer and the possibility to complement

in vivo observations with mechanistic explanatory data.

This mucosa-specific functional behaviour is not solely confined to *F. prausnitzii*. Using hydrodynamic chronoamperometry with a rotating disk electrode *Prévoteau* et al., (2015) recently demonstrated that also *Butyricicoccus pullicaecorum* - another butyrate producing Clostridia cluster IV member with anti-inflammatory potential (*Eeckhaut* et al., 2012) - has the ability to use riboflavins as electron shuttle for coping with oxidative stress. In a scenario of inflammation, a status of epithelial hypoxia is often encountered due to impaired perfusion or the metabolic demands of localized inflammatory cells (*Marteyn* et al., 2011). This may be one of the reasons where flavin-using electron shuttling butyrate producing Clostridia may loose their selective colonization advantage over other strict anaerobes that cannot make use of electron shuttles to cope with oxidative stress. It is clear that such specific micro-environment functions of gut microorganisms cannot be studied *in vivo,* and that *in vitro* model systems are required to elucidate these mechanisms.

The abundance of butyrate producing Clostridia is not merely correlative with health status. Mucosal butyrate producers may also confer stability to the gut microbiome when challenged by stress factors. We provide one example from dietary related stress and one example from chronic gut inflammation.

One of the most recent insights has come from Western diets that have become increasingly rich in linoleic acid (LA). Just like other poly-unsaturated fatty acids linoleic acid has a strong antimicrobial activity, thereby potentially compromising the gut microbiome. In addition, rumen microbiology has learned us that certain bacteria can convert linoleic acid to more saturated products. This process is called biohydrogenation, in which hydrogen gas is combined with linoleic acid to gradually saturate the double bounds to subsequently vaccenic acid and the completely saturated end-product stearic acid. With each biohydrogenation step the antimicrobial activity is decreased. Interestingly, *Devillard* et al. (2007) have previously described that among a wide range of human gut bacteria, the most important biohydrogenating species are *Roseburia* and *Butyrivibrio* species, which are specialists in converting linoleic acid towards vaccenic acid. Both genera belong to the butyrate producing Clostrida cluster XIVa. It was thus hypothesized that these biohydrogenating and butyrate producing species may confer stability upon challenging an *in vitro* cultured gut microbiome with linoleic acid.

Using M-SHIME (containing both a mucosal as luminal environment) as opposed to L-SHIME (which only has a luminal environment), De Weirdt et al. (unpublished) investigated the importance of the presence of a mucosal environment. Exposing either M-SHIME or L-SHIME to 1 g/L of linoleic acid (which corresponds to the theoretical concentration colon bacteria would experience upon consumption of a Western diet) revealed that the biohydrogenating potential in the presence of a mucus layer (M-SHIME) was 6 times higher than in the absence of a mucus layer (L-SHIME). This indicates a higher biohydrogenating potential of the gut microbiome when mucosal bacteria are able to thrive.

De Weirdt et al. (unpublished data) then investigated to what extent this difference in biohydrogenating functionality was also reflected in the microbiome composition. Co-occurrence analysis on Illumina based NGS of the microbiome's 16S rRNA genes

revealed two distinct microbial populations under control conditions: one large population affiliates with the lumen and has many positive correlations between mainly fermentative bacteria, while a smaller mucosal population contains several of the aforementioned butyrate producing Clostridia clusters including the biohydrogenating Roseburia and Pseudobutyrivibrio. Construction of co-occurrence networks for samples that had been exposed to LA, reveals an interesting shift. It is no longer possible to distinguish the 2 populations with region-specific affinity. Instead, the two specialist biohydrogenating genera move to the centre of a new co-occurrence network where they interact with a lot more genera than was the case for the control situation. These data show that members of the mucosal microbiome with specific functional behaviour (i.e. biohydrogenation) may bring stability to the entire gut microbiome upon dietary stressors such as linoleic acid.

The possibility of using butyrate-producing Clostridia to protect the gut microbiome may also extend towards disease scenarios. Particularly with respect to chronic gut inflammation, clear correlations with microbial dysbiosis and abundance of butyrate producing Clostridia have been observed before. *Willing* et al. (2009) showed that butyrate producing clostridia were lower in abundance in patients with IBD. In addition, *Faecalibacterium prausnitzii* and *Butyricoccus pullicaecorum*, both members from the Clostridium cluster IV, have been shown to elicit a protective effect towards gut barrier function both *in vitro* as *in vivo* (*Sokol* et al., 2009; *Eeckhaut* et al., 2012). Again, model systems of the gut mucosa can reveal more in depth what role any of these microorganisms may fulfil with respect to protection of gut barrier function.

Geirnaert et al. (unpublished) recently inoculated colon compartments from M-SHIME model systems with microbiota from either Crohn's patients during active disease or in remission. While microbiome analysis showed significantly different colonization in the lumen and mucus, metabolic analysis revealed that the cultured colon microbiota originating from active disease had a significantly reduced (30%) butyrate production. M-SHIME colon compartments were then treated with a cocktail of 6 butyrate producing Clostridia from both cluster IV as XIVa. Intestinal water was prepared from the different colon compartments and subjected to Caco-2 epithelial cell cultures during their differentiation process. Trans-epithelial electrical resistance measurements and trans-epithelial lucifer yellow transport experiments were then conducted to find out to the impact on epithelial barrier function.

Intestinal water generated from untreated SHIME colon samples resulted in a 50% drop of the TEER value compared to the control situation. This indicates that secreted metabolites from gut bacterial origin pose a certain stress towards tight junction proteins. These findings were supported by a drop in mitochondrial activity and a sharp increase in paracellular transport of lucifer yellow. As a positive control, addition of 2 mM butyrate (a physiological concentration expected *in vivo)* restored TEER values indicating the protective effect from butyrate towards epithelial barrier functioning. Interestingly, intestinal water derived from colon vessels treated with the cocktail of butyrate producing Clostridia also resulted in normalized TEER values and lucifer yellow transport, independent of butyrate levels. These recent findings with M-SHIME systems connected to Caco-2 epithelial cell cultures can help to support previous findings on the

ability of butyrate producing bacteria to improve gut barrier functioning with bioactive peptides (*Quévrain* et al., 2015) or to stimulate regulatory T-cells (*Atarashi* et al., 2011).

CONCLUSION

To conclude, the importance of micro-environment differences for the preferential colonization of gut microbiota and their functionality cannot be underestimated. While *in vivo* samples cannot always grasp these differences, several *in vitro* model systems have been developed that can mimic these different micro-environments. While very useful in providing mechanistic information to support *in vivo* observations, *in vitro* data can neither be overestimated. Each model system comes with its boundaries, and needs a proper validation with human *in vivo* data.

Another advantage of model systems of the gut micro-environment is the ability to study the micro-environment behaviour of specific microorganisms. This not only contributes to our understanding of their role in host-microbe interactions, but it also provides opportunities for exploring these environments with the purpose of isolating microorganisms with novel metabolic traits etc… (Van den Abbeele et al., 2013).

The increasing insight in the putative health-promoting role of functionally important groups such as butyrate producing bacteria indicate that next generation probiotics will no longer belong solely to the classic lactic acid producing strain, but will also include strains from other phyla. Such efforts obviously need to come with the necessary risk assessment steps and depending on the domain of application (pharmaceutical vs. nutraceutical) and the type of application (preventive vs. therapeutic), selection criteria will be more stringent. In addition, current legislation is not yet adapted for the inclusion of these novel strains and a debate between scientists, regulators and industry is highly warranted.

Finally, the last aspect where *in vitro* model systems can become very useful is the exploration of and preclinical testing of novel biotherapeutics. The last couple of years have brought a revolution in this field, especially with respect to ecosystem restoration. The best-known example is the highly successful application of faecal microbial transplants to cure *Clostridium difficile* associated diarrhoea. While the success of FMT for CDAD primarily lies in the sudden diversification of the microbiome, thereby tackling microbial dysbiosis (the primary reason why *Clostridium difficile* is so successful), this success cannot be simply extrapolated to other pathologies. Especially when considering FMT for disease where inherent gut barrier function may be compromised (Crohn's disease, ulcerative colitis, metabolic syndrome…), extreme caution is necessary given the badly characterized nature of faecal transplants and the risk of disease or allergen transmission from the donor to the receiving patient. Already now, research groups have started to tackle these FMT disadvantages by making cocktails of microorganisms of diverse phylogeny and functionality. This has already resulted in the successful treatment of 2 CDAD patients with a defined consortium of 33 microorganisms, isolated from a healthy individual's faecal microbiome (Petrof et al., 2013).

Knowledge about microbial composition and functionality in specific gut

micro-environments may result in new candidate strains that can be taken up in such defined microbial cocktails. *In vitro* model systems will become useful tools to help in the identification and isolation of such microorganisms and in the preclinical testing of the resulting biotherapeutic products.

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