Aguirre M, Venema K. Challenges in simulating the human gut for understanding the role of the microbiota in obesity

Submitted
Social relevance

According to the World Health Organization (WHO), over 600 million adults around the world were obese in 2014 (Fig. 1) [1]. A 39% of adults were overweight and around 13% were obese. Facts also show that obesity is killing more than underweight (!). If this is not disturbingly enough, an increase in obesity is not only seen in adults, but this health problem also extends to children: 42 million children under the age of 5 were overweight or obese in 2013. However, the important message here is that obesity is preventable.

A lot of research has been performed in obesity and associated diseases. Looking at genetics it appears to be the case that the most common gene-environment interactions do not seem to explain the major part of the obesity problem. In the last 10 years the gut microbiota has been increasingly recognized as a contributing factor for obesity. This was the main focus of this project: looking at the diet-microbe relationship including microbial products like short chain fatty acids (SCFA: acetate, propionate, butyrate) which are produced by the microbiota when they ferment indigestible dietary compounds. These microbial products may have an important impact in our metabolism. On the one hand they may affect signaling of satiety-coupled receptors and on the other hand SCFA may enter the circulation and have more systemic effects (fat accumulation, substrate metabolism in the skeletal muscle, adipose tissue and liver, processes of inflammation, etc.) [2].

Fig. 1. Key facts about obesity and overweight.
It is clear that there is an urgent need to tackle obesity, and the possibility of manipulating the gut microbiota as a therapeutic way for either prevention or treatment has taken force in recent years. The scientific outcome of this thesis describing how the fermentation of different dietary compounds occurs in the microbiota from different subjects is one concrete step to elucidate the impact of these communities on host energy balance. This project sets a precedent for future 
\textit{in vivo} studies aiming on personalized nutrition research.

\textbf{Target groups}

Besides the large portion of the population suffering from overweight or obesity specified in the above section, the results from the present study benefit the sector of the food industry focused on prebiotic and probiotic products. Scientific evidence from this project is provided for the use of some prebiotics and how they could modulate the intestinal community as well as for identifying microorganisms which could be key players in a lean profile.

\textbf{Activities/Products}

The results from the present study could be translated and shaped into:

\textit{Bacterial cocktails to be used for treating obesity}

As explained in the introduction chapter, the modification of the gut bacteria could be achieved by administrating different bacteria isolated from the human gut. The concept of having a cocktail of a defined community containing specific bacteria could be used to replace dysfunctional ecosystems with a healthy customized ecosystem [3]. As explained by Petrof \textit{et al.} [4], the use of a defined community to manipulate the gut microbiota offers a wide range of advantages which include: i) the freedom of selecting specific bacteria for making a defined composition, ii) the capacity for individual growth of bacterial species within such defined composition, and iii) better safety profile when compared to complex communities derived from feces. The optimal design and growth of defined communities can be a biological relevant model not only as a new approach to study gut microbial dysbiosis but also for its treatment.

\textit{Optimizing in vitro models when studying obesity}

The development of physiological relevant \textit{in vitro} models for studying the role of the human gut microbiota in obesity could accelerate the development of strategies...
to tackle the elevated incidence of cases of obesity in the worldwide population as well as the potential reduction of animal testing. It is of high importance to optimize in vitro systems to model human colonic fermentation in obesity. Special attention needs to be focused on: culturing media, the use of a representative control, study of the effect of fats, shear stress, redox potential, anaerobiosis and the simulation of transit time.

**Culturing media, when sometimes the proof of the pudding is in the eating**

The main substrates available for microbial fermentation in the large intestine are a mixture of components which, for different reasons, were not (efficiently) digested or absorbed in the small intestine [5,6]. These include dietary oligosaccharides, polysaccharides, peptides and proteins as well as host epithelial cells, mucins and pancreatic secretions [7,8]. Members of the gut microbiota preferably ferment carbohydrates [9]. For this reason, the metabolic activity of the residing bacteria in the proximal colon is mostly dedicated to the production of SCFA (with some intermediate metabolites, such as lactate, formate, succinate, acrylate, ethanol) and gases including H$_2$, CH$_4$, CO$_2$, which consequently causes a low pH (~5.4-5.9) in this part of the colon [10]. Due to the depletion of available carbohydrates and the relative increased protein content, the metabolic activity of the bacteria reaching the distal colon switches to proteolytic fermentation with the production of branched-chain fatty acids (BCFA) and toxic compounds including phenols, indoles and ammonia, increasing the pH (~6.6-6.9) [10].

A culture medium mimicking the terminal ileal chyme from an average Western diet has been widely used in fermentation studies. The composition of this medium has been developed by testing a wide range of different nutritional ratios of polysaccharides (mainly arabinogalactan, pectin, xylan, dextrins and starch), proteins (peptone, caseine), glycoproteins (mucin) and minerals and vitamins against in vivo values of composition and activity (metabolite and gas production, and enzymatic essays) of the microbiota found in samples of the intestinal content of sudden death individuals [11-13]. In the case of batch fermentations, the trophic status can be classified as oligotrophic or eutrophic as described by Long et al. [14]. Oligotrophic systems are typically inoculated with a high dense fecal slurry (5 to 20% w/v) which is meant to also provide nutrients, and the media (usually consisting on a phosphate-buffered saline system –PBS-) is not always supplemented with vitamins or minerals [14]. In contrast, the concentration of the inoculum of eutrophic systems is lower (~1% w/v) but a basal medium (usually basal culture medium –BCM-) fortified with bile salts, yeast extract and peptone is used [14].
It has been observed that differences in the nutritional availability in fermentation systems account for discrepancies in the development of the microbiota even in the presence or absence of a prebiotic [14-16]. Part of the media’s modulatory effect is hypothesized to be derived from differences in the availability of bile salts and peptides [14].

When studying obesity, the use of a proper medium has to be evaluated in order to properly reproduce the nutritional components to which the microbiota from obese subjects are exposed. Despite the efforts of closely simulating the Western diet, fats are not incorporated in the media for *in vitro* studies yet. In fact, to our knowledge, there is no *in vitro* research performed so far on the effect of fats on the composition and activity of the human gut microbiota. Many of these studies focused on testing primarily carbohydrates, also excluding research on the effect of proteins, a drawback that limits the evaluation of the impact of microbe and diet interaction. In an average Western diet, about 15.4% and 32.8% of the energy intake comes from protein and fat, respectively [17]. Another important aspect to take into consideration is the concentration of the test compound to be evaluated. Short-term studies preferably use a high concentration of the test compound while long-term studies use relatively lower concentrations with the aim to detect more gradual changes such as the case of experiments in TIM-2 or SHIME [18].

**The use of a representative control**

A representative control for the *in vitro* investigation of the role of the gut microbiota in obesity is needed. Basically, two important points need to be addressed: one is reaching a consensus about what could be considered as a control in the experimental set up and the other one about the complexity of the media which is provided to the microbiota to grow. First, part of the inconsistency in the results of testing the effect of prebiotics in batch fermentations may come from the differences in the control used [14]. As explained by Long *et al.* [14], some studies regard samples taken at the initial point (t₀) as the baseline control to measure the response of the microbiota to the different substrates. Others, use a negative control which is basically a parallel fermentation in which the tested substrate is not added [14]. However, the use of the last case is not recommended due to the fact that these fermentations present a minimal production of SCFA accompanied by minimal changes in pH which has been interpreted as an indicator of lack of fermentation [19]. Second, as previously mentioned, the preparation of a complex media which primarily contains polymerized carbon and nitrogen sources has been considered representative to stimulate the optimal growth of communities simulating those residing in the intestine in *in vitro* fermentation studies [20]. However, this does not reflect entirely the environment to which the gut microbiota
from obese subjects is exposed. The microbiota from obese subjects has been exposed to a repeated high energy diet [21]. To our knowledge only one study has attempted to replicate the high energy content in the media composition simulating a Western diet. In this study, the authors also compared the administration of normal energy and low energy in the simulation of a child gut. In order to accurately simulate the different nutrient loads and dietary conditions, the composition of the media used in this study was based on an extensive investigation of dietary records from anorexic, normal weight and obese children [21].

The importance of testing the effect of fats on gut microbiota
Dietary fat supplies around 9 kcal/g of energy, more than double of what is contributed by carbohydrate or protein (estimated to be around 4 kcal/g) [22]. The nutritional dietary lipid recommendations for adults is around 20-35% of daily calories ingested [23].

Nowadays it is widely acknowledged from animal studies that fat overconsumption leads to obesity [24]. However, it is unclear how much fat reaches the colon. Some initial studies have indicated that from a load of 70g/day, 3.5g enters the large intestine [25]. Still, more research is needed in this area.

There is evidence that indicates that dietary fat has a larger effect on the modulation of the composition of the gut microbiota than on the development of obesity itself. Such evidence mainly comes from studies in mice and to our knowledge there are no in vitro studies focusing on this matter. For instance, de Wit et al. [26] observed that the overflow of fat to the distal intestine triggered changes in the composition of the microbiota in mice. Specifically, it may reduce diversity and increase the Firmicutes-to-Bacteroidetes ratio. In addition, the microbiota from mice fed with a high fat diet for 12 weeks showed a strong response in terms of composition but returned indistinguishably to baseline after 10 weeks of switching back to the normal diet when compared to the control [27]. Therefore, it has been suggested that the response of the microbiota to fat exposure can be reversible indicating diet as a treatment for obesity [28]. Still, some studies indicate that the response may also depend on the specific diet composition as observed in conventional and germ-free mice [29].

Mechanics of a dynamic environment
The simulation of the mechanically dynamic environment characteristic for the human intestine, which includes intraluminal fluid flow and peristaltic motions, is poorly simulated in the current available in vitro continuous models of the human
gut [30]. However, though limited, there are a number of models where at least shear forces are applied from peristaltic to stirring motions. Shear forces are a key factor regulating bacterial gene expression and physiology, stress resistance, and adhesion of bacteria to the gut wall [31-34]. In general, shear stress (stirring) is applied in a bioreactor with the aim of increasing oxygen transfer in the media. In such case agitation speed has been found to be crucial for achieving high yields of enzyme production [35]. However, current models of the human gut solely aim to simulate the luminal colon. In an anaerobic system as such, the stirring of the culture media is performed with the purpose of homogenizing the suspension to guarantee that heat and food distribution is even in the system. The application of shear forces in an in vitro system simulating the human gut becomes crucial when it comes to the integration of cell lines in the experimental design. The cells in the human intestinal epithelial layer are exposed to rhythmic mechanical deformations originating from the peristaltic motion of the intestine [30]. Such conditions promote the differentiation of the cells and shape the height and polarization of these cells [30], which are factors that are important in microbe-host interaction.

Redox potential
The redox potential has been defined as the predisposition for a compound to gain electrons [36]. The interpretation of values measuring the redox potential in the media where cells are found can be used as an indicator of intracellular metabolism in which redox balance and electron transfer play an important role [36]. Therefore, redox potential has been considered as a sensitive tool to provide information about subtle changes in intracellular metabolism [36]. Environmental factors that affect the redox potential in a fermentation process include anaerobic conditions, dissolved oxygen, temperature and ratio of oxidizing to reducing compounds (represented most of the time by nutritional substrates provided in the medium, such as cysteine) [36-38]. These factors form part of the typical stress conditions to which the gut microbiota is exposed after the transfer from human gut to in vitro model.

Biologically speaking, many functions of the cells are affected by fluctuating redox potential values. These include altered gene expression, enzyme synthesis (directly affecting signal sensing and transduction) and metabolic profiles [39-41]. Therefore, it is important to reach homeostasis in terms of redox potential in the gut model to optimally simulate in vivo conditions. Such homeostasis is crucial for an optimal intracellular metabolism in the cells stimulating growth of these bacteria and their survival.
Anaerobiosis

Linked to redox-potential is maintenance of physiological oxygen gradient concentration values, which is also important for proper simulation of the human gut. This implies a high level of complexity because oxygen is supplied by host tissue which consequently explains the higher oxygen content found close to the mucosal surface when compared to the anaerobic lumen [42], and the existence of an oxygen gradient along the radial axis of the gut. At present it is unclear whether there also is a longitudinal gradient along the proximal to distal gut.

Many in vitro models are restricted to the simulation of the colonic lumen where $N_2$ is continuously flushed in order to maintain an $O_2$-free atmosphere [11-13,43,44]. However, an anaerobic atmosphere can also be established by initially flushing with $N_2$ and then be kept by the usual gases produced by the fermentative activity of the microbiota as suggested by Feria-Gervasio et al. [45]. According to the authors, this method is believed to represent in vivo conditions better since it facilitates the action of hydrogenotrophs and decreases the $H_2$ pressure. This could offer the advantage of studying reductive acetogens, sulphate reducing prokaryotes and methanogens at a higher resolution [45]. Furthermore, bacteria utilizing hydrogen are needed to prevent that fermentation reactions are blocked [46], this may be important in obesity research. For instance, it has been observed that propionate production increases by the inhibition of methanogens which are the main hydrogen utilizers [47].

pH

In in vitro systems, pH has been found to influence the total amount of SCFA produced by the microbiota and their ratio. Furthermore, previous studies indicate that gut bacteria differ in their growth performance in response to changes in the pH of the media [48]. Such response, in terms of susceptibility or tolerance, could be directly associated with specific groups, including Bacteroides [48]. Examples of the effect of pH on the development of in vitro cultures include the study by Rajilic-Stojanovic et al. [49] who observed that the total amount of SCFA were lower for fermentations at pH 5.8 when compared to pH 6.4 and 7.0. Additionally, not only the final amounts were found to be affected but also the different molar ratios and diversity of the microbiota [49]. In the same manner, Walker et al.[48] observed substantial butyrate production in a static in vitro system using human microbiota fed with a polysaccharide composite and maintained at pH 5.5. However, when all conditions were maintained with the exception of pH, which was increased to 6.5, the authors observed a drastic drop in the concentration of butyrate accompanied with a substantial decreased of some bacteria (mainly Roseburia spp.). This emphasizes the fact that even a one-unit
shift in pH shows a significant effect on metabolic profiles produced by the microbiota. As mentioned earlier colonic pH varies as a function of host dietary intake [50]. Therefore, in view of the importance of reproducing a dense and active microbiota which will be physiologically relevant to understand its role in health and disease, it is crucial to set a proper pH value in the \textit{in vitro} fermentation system when simulating obese subjects whose consumption of fermentable carbohydrates may vary when compared to lean subjects.

Transit time
Transit time is a physiological parameter that defines the time frame for the fermentation of a substrate in the colon [46]. Small intestinal transit time has been considered to play an important role in weight balance since it determines how much of the nutrients from the diet can be absorbed [51]. Therefore, a prolonged transit time has been suggested to promote a higher absorption and consequently the promotion of weight gain [51]. In contrast, a short transit time may be interpreted as a shorter time for absorption and it probably leads to weight loss or weight maintenance [51]. Obese subjects are part of the population where transit time is believed to be different when compared to other groups [52-55]. Furthermore, differences in transit time have shown to be dependent on the energy content of meals. For instance, low and high energy meals clearly differed in the small intestinal transit time in obese subjects (64 and 123 min, respectively) [56]. Despite that a large part of nutrients are absorbed in the small intestine, differences in transit time in this part of the tract influence the substrates which are finally available in the colon for both fermentation and absorption. This can also have an impact on host energy balance. Average transit time in the colon of a healthy subject has been reported to range from 12 to 24 h [57]. Unfortunately, studies addressing colonic motility in obesity are limited [51]. However, evidence indicates a delayed total colonic transit time in obese children when compared to lean [54]. With respect to \textit{in vitro} fermentation systems, the modification of the retention time has previously been shown to have an effect on the establishment of certain types of bacteria together with changes in their metabolic activities [58]. This is explained by the fact that substrate availability highly depends on retention time, and the growth rate of bacteria is based on how fast microorganisms are able to metabolize the available substrate and compete with others [58]. For instance, changes in retention time have been shown to significantly affect quantities of
lactobacilli and bifidobacteria [45], carbohydrate degradation patterns [12,59] and production of specific metabolites such as butyrate [60,61]. Special emphasis in *in vitro* models simulating the human gut in obesity has therefore also to be done with respect to the correlation between transit time and the reciprocal dilution rate as suggested by Cinquin *et al.*[43].

**Future perspectives**

The next step to proceed in the area of research described in this thesis is to determine the number of donors which could be used in the preparation. This makes it feasible in finding a representative inoculum to perform a wide range of *in vitro* studies for food and pharmaceutical studies. Further experiments are recommended to i) test the optimal time and alternatives for thawing as e.g., discussed by Hamilton *et al.*[62] who tested thawing using an ice-bath, ii) study the extent of the effects of preparing a human fecal inoculum on *in vitro* experiments fermenting specific substrates and iii) determine the effect of different treatments on microbial enzyme activities and gas production.

Future research should also be focused on improving the standard operating procedures of the TIM-2 system. This includes the inoculation of the model, the stabilization during the propagation of the inoculum during the first hours in the system, the pH chosen for the fermentation protocol, to name a few. Furthermore, studies including *in vivo* tests are suggested to explore the similarities in the response of diet in different individuals.

Another interesting area to explore in the (*in vitro*) study of human obesity is the investigation of the role of methanogens in energy balance. Although the prevalence of methanogens in the human gut has not been directly related with gender, it is believed to be affected by age, diet, geography and host genetic background [63]. In obesity, the study of archaeal species might be of interest due to the hypothesis that hydrogen transfer between bacteria and archaea may optimize the uptake of energy in individuals by increasing the production of SCFA [64].

The potential influence of methanogens in energy balance has been corroborated after finding an elevated presence of archaea in anorexic patients, speculated to be due to an adaptation of the microbiota with the aim to optimally use the low calorie diet ingested by the anorexic host. In obese and type 2 diabetic patients an enrichment in archaea has also been observed [65,66]. However, findings about the abundance of archaea after a diet intervention in obese subjects are contradictory. Methanogenic archaea did not show a significant change during a controlled diet in
overweight men [67] while it was found to be more prevalent (together with *Akkermansia*) after a weight reduction intervention [68].

*In vitro* models of the human gut are of great potential to study the impact of methanogens in the dynamics of a gut bacterial community since the establishment of *M. smithii* in conventionalized rodents with human gut microbiota has failed [69,70] while it has shown to be successful in an *in vitro* model [71].
References


