British Journal of Nutrition (2020), 124, 521–530
doi:10.1017/S0007114520001142

Diet and the gut microbiome: from hype to hypothesis

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(Submitted 21 November 2019 – Final revision received 17 March 2020 – Accepted 20 March 2020 – First published online 2 April 2020)

Abstract

Microbiome research in the last two decades has delivered as a key finding that the human intestine hosts a unique and complex ecosystem with many variables affecting the composition of the microbiota and in turn its function in metabolism and immune defence. Hundreds of external (environmental) factors have meanwhile been identified as significantly associated with bacterial biomass and diversity and, amongst these, diet is considered as a key determinant of microbial populations. However, dietary intervention studies, including those with fermentable substrates that have bulk effects on bowel functions, have revealed only very minor effects on overall microbiome composition and usually show only a very few species changing in population size. What that means in the context of hundreds of different species coexisting in competition or mutualism in the human colon is far from understood. This review addresses some of the current limits in research on diet effects by taking anatomical and physiological features of the intestine into consideration. It also provides some recommendations on future human studies needed to assess how the diet influences the microbiome and associated effects on metabolic health.

Key words: **Microbiota mass: Diversity: Colon: Energy balance: Gut physiology: Transit time**

Bioscience has seldom seen such acceleration in the proliferation of scientific publications in relation to human health as that for microbiome research in recent years. For the gut microbiome alone, about twenty to thirty new papers per d appear in PubMed and there seems to be no day where the public is not informed about 'news from microbiome research'. The field is driven mainly by the ability to undertake large-scale and high-throughput sequencing since the costs per sample have declined rapidly allowing large sample sizes and huge cohorts to be analysed. The most recent metagenomic analysis of 11 850 human gut microbiomes from different countries and ethnicities predicted 1952 different uncultured candidate bacterial species[1]. However, in almost all individuals, the microbiota is dominated by the phyla Bacteriodetes and Firmicutes, while Actinobacteria, Proteobacteria and Verrucomicrobia constitute minor phyla[2]. And, a single strain per species usually dominates the microbiome and that 'individual-specific strain' also seems to preserve the stability of the human gut microbiome over time[3].

Despite ten-of-thousands of papers and huge efforts in characterising the gut microbiota of humans across the planet, science is still not able to define a 'healthy microbiome' or to give specific recommendation on how to alter the microbiome to affect health outcomes. Moreover, only about 20 % of the microbial diversity found in faecal samples has been associated with intrinsic and extrinsic factors[4], leaving more than 80 % of bacterial diversity in faecal samples unexplained. Very little is known about the effects of diet on the gut microbiome in humans and, particularly, on its contribution to whole-body energy balance. However, diet is considered as a key environmental factor in the microbiome–health relationship, and the evidence for that shall be critically discussed here. In addition, I shall address the role of gut physiology that is usually not taken into account, when interpreting findings of faecal microbiome analysis.

How much bacterial biomass constitutes the gut microbiome?

It seems to be generally accepted that the gut microbiome makes up 1·5 to 2 kg in mass. This estimate is communicated again and again, even in scientific literature. Similarly, the number of bacteria residing in and on the human was claimed to exceed ten times the number of human body cells. It is amazing to realise that these numbers trace back to a rough estimate provided in a paper published in 1972[5], which has never been questioned until recently. In 2016, Sender *et al.* analysed the evidence carefully and recalculated the ratio of bacteria:human cells and provided as the best estimate a total of 4×10^{13} of bacteria *v.* 3×10^{13} of human cells resulting in a close to 1:1 ratio[6]. The difference

Abbreviations: BSS, Bristol Stool Scale; FMT, faecal microbiome transfer; GOS, galacto-oligosaccharides; TMAO, trimethylamine-oxide; TT, transit time.

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between the old and new estimate results mainly from different intestinal volumes (colonic volume) used to calculate total biomass based on bacterial density per g or volume unit. Sender *et al.* finally estimated a volume of 150–200 ml of colonic content by using information on the daily stool output and a mean colonic transit time and by using published estimates of bacterial numbers from stool samples. They concluded that about 200 g of total bacterial mass was residing in and on humans of which the large intestine hosts the majority[7]. This new estimate of gut microbiome mass matches quite well with data reported by John Cummings in 1990[8] based on the weight of the total contents taken out of the large intestine at autopsy of forty-six sudden-death victims from Europe and Africa. In this study, the mean value of the intestinal contents of colon (wet weight) was 222 g (58–904 g) of which 95 g represented bacteria (mean value by wet weight) which, after freeze-drying, equated to 36 g DM (mean value) in the total colonic content. Taken together, the best evidence currently available argues for about 200–250 g of colonic content with about 100 g of bacteria representing the gut microbiome. The amount of this biomass excreted in human stool per d accounts for about 30–80 g of which half represents non-viable bacteria[2,7]. It is striking to realise that the quantity of stool is seldomly reported in microbiome publications, but knowing that the daily mass excreted can vary several fold, it can be anticipated that differences in the abundance of individual bacteria species and genera may very much depend on the 'dilution' from volume variation. Most recently, the first studies have started using quantitative microbiome analysis to determine not only bacterial diversity (richness) derived from sequences but also number of (viable) bacterial cells in the stool sample and, consequently, it is not surprising that cell counts ranged up to ten-fold, giving a quite different picture than relative abundance only[9]. Taking these findings together, one may conclude that the quantitative aspects of gut microbiome research need much more attention. Life science without considering masses is like analysing the universe and not taking the physics into account.

A stool sample and what it can tell us

The huge differences in colonic contents in sudden death victims from Africa when compared with those from Europe mirrors the huge differences in daily stool output between individuals[9,10]. That seems to be due to the much higher intakes of non-digestible carbohydrates/fibres in Africa compared with intake via typical European diets. But even within Europe, daily stool output varies over >3-fold[10]. In addition to the wide distribution in mean stool weight across the population, there is huge day-to-day variation in stool weight for the same individual, ranging between 50 and more than 400 g[11]. Stool also varies in appearance, by colour and consistency. That fact has been recognised recently as a critical determinant in analysis of gut microbiome composition. Clinical diagnosis of gastrointestinal disorders associated with obstipation or diarrhoea uses the so-called Bristol Stool Scale (BSS) or stool chart with pictures on colouring and consistency of stool sample to classify stool on a range from one to seven. In various studies, the richness of the faecal microbiome as assessed by relative species abundance showed a clear association with the BSS score and, in turn, stool consistency is related to stool water content[12,13]. Moreover, in metagenomics analyses of stool samples from cohorts, the BSS was amongst the most relevant determinants of microbiota richness and variance[4]. Consequently, the identified faecal enterotypes also show a strong clustering with moisture content of stool samples[12]. Water content of stool associates with the gastrointestinal transit time and in particular with the residence time of contents in the colon and rectum[14], and with stool frequency[15] and all of this affects microbial diversity. Moreover, colonic and faecal water content in mammals can oscillate and that depends on circadian alterations in aldosterone concentrations that act via the colonic receptors for this mineral corticoid to modulate electrolyte and water transport processes[13,14].

The mouth to anus transit time in Europeans is about 60 h of which 7–24 h is accounted for by transit through the caecum and right colon and 9–30 h for the left colon. The recto-sigmoid accounts for additional 9–15 h[15]. There are also marked sex-dependent differences in transit time with about 15 h faster transit in men than in women[10]. Transit time[16] and stool frequency[17] are strongly associated with stool consistency, and this is strongly associated with microbiota richness[18]. Care should thus be taken when comparing and interpreting findings on relative abundance of bacterial species or taxa variance across ethnic groups consuming their habitual diets. In addition, care should also be taken when interpreting data obtained from a single stool sample of an individual and how its composition relates to metabolic differences and responses to diet or even disease risks. Direct-to-customer businesses based on the microbiome analysis of a single stool sample are flourishing and, for the reasons outlined above, these should be viewed very critically. The huge variability in composition of the stool – day by day, or even within a day – and by variation of bacterial density in a faecal sample[11] makes analysis of a single sample and its interpretation questionable. Moreover, the diagnostic value of microbiome analysis for predicting metabolic health effects (see below) has been questioned recently by a study from China that revealed a major influence of the geographical region (fourteen districts) in which the volunteer lives on composition of the stool microbiota. This 'regional effect' overruled by far all other determinants of microbiome diversity such as age, sex, BMI, BSS or diet variables[19]. In addition, models developed to assess metabolic disease risks based on the microbiome data obtained in one district failed to work in another district. The authors therefore recommended the models to be built for given geographical areas[19].

Currently, there are no standardised and generally accepted protocols for stool sampling and processing, or for downstream analysis, nor are there any reference microbiomes that could be used as a quality assurance measure. Therefore, it is not surprising that a single stool sample analysed in different laboratories shows an impressive variance in composition[20]. However, this problem of insufficient reproducibility has been recognised, and some activities have been launched to standardise better current procedures[21,22].

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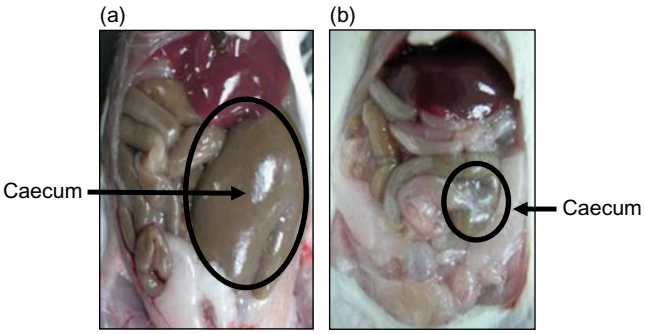

Fig. 1. Photograph of the arrangement of the gastrointestinal tract of C57BL6 mice kept under germ-free conditions or in a conventional animal facility but fed diets with identical composition (photograph: H. Daniel). (a) Germ-free mouse; (b) conventional mouse.

The intestinal tract has longitudinal and radial compartmentation. In addition, the mucosa shows huge morphological differences from the small to the large intestine in its surface-enhancing substructures. Further, the anatomy of the gut differs between species. That becomes relevant when, for example, findings in rodents are translated to the human condition. This applies particularly to mice which as germ-free animals are widely used in the microbiome research. Gnotobiotic mice serve here as a host to explore the phenotypic consequences after inoculation of a microbiome from a donor, including human samples. The anatomy of the mouse large intestine is dominated by a huge caecum that serves as a fermentation chamber and that, in germ-free animals, fills the abdomen almost completely (see Fig. 1). That suggests that the microbiota is part of a feedback loop that controls the growth of the tissue. Germ-free mice also have a markedly increased transit time[23] and that of course has a major effect on the availability of nutrients and energy for absorption. The entire surface of the intestine is lined by mucus. In the colon, the mucus covering the epithelium is comprised of an inner firmly adherent layer of about 100 μm in thickness and a looser outer layer that ranges in thickness from 500 to 800 μm[24]. When the mucus layer is stained for bacteria, the inner layer appears sterile, whereas the outer layer with a fluffy appearance shows the presence of bacteria in the meshwork[25,26]. This anatomical compartmentation means that, except for tiny regions and transiently, bacteria are not directly in contact with the apical membranes of the different cells lining the epithelium. The inner mucus layer also has a distinct microenvironment that does not mix easily with the outer mucus layer and the lumen. This is seen, for example, in marked differences not only in ionic composition and pH but also in O_2 tension[27]. These parameters all affect the growth of different bacteria in different ways. The presence of a higher pO_2 in the mucus layer that by diffusion from the tissue into the lumen forms a steep gradient may well explain that analysis of samples from the mucus layer show a different pattern in microbiota at the phylum and genus level than samples taken from the lumen. Although only very few studies have explored these differences in humans, studies in mice (with all restrictions for translation) suggest that the differences between luminal and mucosal (mucus-associated) microbiomes become larger in more distal segments of the intestine[28]. When faecal samples and those taken out of the intestine by biopsy in the same individual were compared, the bacterial signatures were quite different[29]. This means that care should be taken when extrapolating from stool data corresponding bacterial functions and the effects on the mucosa and host because (i) metabolic processes that are relevant in the vicinity of the epithelium (where there is a distinct microenvironment) may be quite different from those in the lumen and (ii) bacteria are mostly not in contact with the epithelial surface.

Diet effects on bacterial composition

As noted above, only about 15–20 % of the variance in composition of the microbiome can so far be explained despite huge research efforts. What is most striking is the small effect of the host genome on the gut microbiota in humans. Both, population studies in cohorts of different ethnicities[30] and twin studies with over 1000 twin pairs[31] revealed that only between 2 and 9 % of the taxa identified in stool samples appear to be inherited. And, except for the gene encoding in a variant lactase persistence, no significant SNP have been found to associate with the gut microbiota[30]. That leaves the environment as the key effector of the gut microbiome. Numerous studies have addressed individual environmental variables and determinants with significant effects on gut microbiome composition. In a comprehensive analysis of environmental and endogenous effectors in two cohorts from Flanders (Belgium) and the Netherlands, a total of 126 factors (at a false discovery rate of <0·1) were identified as significant covariates of the gut microbiome[4]. Out of 503 variables investigated, the BSS (self-reported) ranked as number one with the most pronounced effects on microbiome composition which confirmed previous findings that moisture content of stool samples is strongly associated with bacterial diversity[9]. The 126 significant covariates of microbiome composition included sex and age, a variety of diseases, various drugs and numerous food items. However, individual dietary factors generally ranked much lower with similar effect sizes observed, for example, energy intake, total carbohydrate content of the diet, and amounts of beer, coffee or red wine consumed[4]. Despite the comprehensive coverage of a large number of variables in these cohorts, 92·3 % of microbiome composition remained unexplained.

Systematic studies addressing effects of diets or individual dietary factors on the gut microbiome in humans are sparse. David *et al.* investigated the impact of a plant-based diet when compared with an animal food-based diet (provided *ad libitum*) over a 5-d period in a cross-over design in healthy volunteers (six male and four female) with a baseline and a wash-out period of a couple of days each[32]. Despite major differences in intakes of fibre, fat and protein, the α-diversity (Shannon diversity index) in stool samples did not change when microbiome composition was analysed each day. The β-diversity (Jenssen-Shannon-distance; used as a surrogate measure of the induced changes) showed significant differences when volunteers were consuming a diet based on animal products but only when compared with the respective baseline values. Marked differences were obtained for the concentration of acetate, butyrate and isovalerate and isobutyrate (based on wet weight) in stool samples.

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Whereas acetate and butyrate levels declined on the animal-based diet, those of the SCFA derived from degradation of branched-chain amino acids increased more than two-fold, likely as a consequence of a three-fold higher protein intake. In contrast, the plant-based diet provided about 25 g of fibre/d with almost zero intake on the animal-based diet[32]. However, since stool output (volume) and faecal water content were not reported, the stool microbiota may also be affected by these parameters. Although feeding the two extreme diets overruled inter-individual variation in microbiome composition, the overall effects of diets in changing bacterial diversity were small. A similar finding was reported by Wu *et al.* in ninety-eight individuals with recorded short-term (24-h recall) and long-term (FFQ) food intake. In this study, volunteers could be clustered into two distinct microbiota enterotypes with characteristic reversed compositions in *Bacteroides* and *Prevotella* taxa which were associated with long-term dietary patterns. However, a short-term dietary intervention trial in the same volunteers comparing high-fat/low-fibre with low-fat/high-fibre diets did not cause a change in the enterotype, albeit alterations in microbiome composition were found already at 24 h after switching diets[33]. In a recently published study with thirty-four healthy human subjects who collected stool samples each day for seventeen consecutive days, faecal shotgun metagenome analysis revealed huge variability across the volunteer group but rather stable individual microbiomes despite considerable variability in the food groups consumed[34]. Most strikingly, analysis of stool from two volunteers who consumed a meal replacement in the form of a liquid diet over the observation period did not reveal any obvious differences in microbiome composition (at the genus level) when compared with volunteers consuming ordinary diets. Since differences in macronutrient intake did not associate with diversity of bacteria at the genus or functional module level, the authors[34] concluded that 'food-based interventions seeking to modulate the gut microbiota may need to be tailored to the individual microbiome'. When taken together, studies examining effects of diet on faecal microbiomes have not delivered strong evidence that diet is a major changer of composition and even diets high in fibre content provoked rather small effects on overall bacterial diversity.

Two recent human studies have assessed the effects of two types of fermentable fibres on stool microbiome composition. In a randomised double-blind placebo-controlled 12-week study with forty-four healthy volunteers consuming 12 g/d of inulin (or placebo) for 4 weeks, microbiome analysis revealed that the variation induced by inulin consumption accounted for 0·8 % of global microbiome composition, whereas inter-individual variation ranged from 63·9 % to 77·2 %[35]. However, significantly increased *Bifidobacterium* and decreased *Bilophila* numbers were observed during inulin intake and those changes associated weakly with softer stools. Contrary to expectations, BSS and stool frequency did not show significant alteration although inulin has been shown in numerous studies to have beneficial effects on constipation and even carries a health claim provided by the European Food Standard Agency for these effects. Because of their 'bifidogenic' effects, galacto-oligosaccharides (GOS) were substituted for human milk oligosaccharides in cows' milk-based formula diets some decades ago to provide infants with a 'fermentable fibre'. In a double-blinded placebo-controlled parallel intervention study with forty-four obese adults given 15 g GOS per d over 12 weeks, this 'bifidogenic' effect was confirmed with a five-fold increase in the abundance of *Bifidobacterium* species[36]. Despite these specific changes, neither microbial richness in faecal samples nor overall microbiome diversity was affected by GOS intake. Comprehensive metabolic phenotyping of volunteers receiving GOS, compared with placebo, did not reveal any differences in SCFA concentrations in stool or in plasma nor any changes in gut-derived hormones or markers of inflammation in systemic circulation. In addition, there were no significant alterations in peripheral and adipose tissue insulin sensitivity, body composition or energy/substrate metabolism[36]. In summary, these carefully conducted fibre supplementation studies revealed that overall bacterial composition does not change significantly even with rather high daily intakes of either inulin or GOS, whereas selective effects on *Bifidobacteria* – considered to be beneficial – were found in both studies. These findings match quite well with those of various other studies on effects of fibre and resistant starch in humans employing other analytical techniques such as microarray, pyrosequencing or FISH[37] that all revealed selective effects but rather small, or no, changes in overall bacterial composition. Factors that limit the interpretation of these studies on effects of fibre on the gut microbiome were identified in a workshop organised by the National Institutes of Health and the United States Department of Agriculture with some recommendations developed on better study designs when assessing diet/fibre effects[38].

Does the microbiome make you slim or obese?

One of the mysteries in microbiome research has been the contribution of the gut microbiome to overall energy homoeostasis. Some studies suggest that the microbiome is an energy harvester providing net energy to the host in the form of SCFA and other metabolic products and thus contributing to an obesity phenotype, whereas other studies suggest that the microbiome is an energy sink. The latter are mainly studies from the animal nutrition sphere in which the use of antibiotics or other compounds that reduce the bacterial load in the large intestine of pigs or poultry has proven that less bacteria in the large intestine lead to higher weight gain[39–41]. In contrast, studies that suggest that the microbiome contributes to obesity are mainly from use of gnotobiotic (germ-free) mice, with or without transfer of microbiomes from lean or obese animals or even human feacal samples, and from studies with high-fat diets[42,43]. Although some of the findings in germ-free animals are remarkable, these studies may be a bit misleading when translated to the human condition because of the experimental conditions used. Rodents kept in SPF-facilities or kept sterile are fed diets that are sterilised by dry heat in autoclaves or that have been irradiated in the dry state. In either case, digestibility of the dietary constituents such as carbohydrates (starch) or protein by pancreatic enzymes is limited resulting in higher quantities of non-digested products reaching the large intestine. In mice that is predominantly the caecum with the character of a large fermentation chamber.

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Even in conventional animals, rather large particles from the dry, very hard food pellets can be recovered from the caecum, demonstrating that these diets *per se* have a very low digestibility in the upper small intestine. Therefore, in rodents, the gut bacteria assist in extracting energy from such a diet. A lack of proper weight gain in germ-free mice may be the best evidence for that. However, whereas the contents of caecum and colon constitute about 1 % of total body mass in a C57BL6 mouse, in humans the colonic contents equate to only about 0·2 % of total body mass. The capacity to ferment is thus considerably higher in mice than in humans, and moreover, most human diets are a mixture of raw and heat-treated foods with a much higher availability of energy by digestion and absorption in the upper small intestine. When diets for mice are heat-treated and compared with the same diets fed raw, body weight changes are more pronounced and support the notion of a loss of energy via the fermentation route[44,45]. In humans, this issue can be exemplified with non-digestible starches and other polysaccharides which, when fermented in colon, provide an estimated 8·3 kJ mainly via SCFA[46] from each gram of carbohydrate (gross energy = 16·7 kJ) reaching the colon. That means that only 50 % of the energy contained in the carbohydrate is delivered to the host and the other 50 % is likely consumed by bacteria for their needs in growth and maintenance of biomass. In studies combining collection of ileostomy efflux followed by fermentation *ex vivo*, it has been estimated that in humans, SCFA could deliver 3–11 % of total energy needs per d[47]. In addition, studies comparing digestibility of raw and cooked protein suggest that fermentation of dietary protein may also contribute to energy extraction in colon. Cooked egg white has a digestibility of 91 % in humans, but the digestibility is reduced to 50 % when the protein is given raw[48] with much more protein, and thus amino acids, reaching the colon. In summary, different quantities of fermentable/utilisable nutrients reach the large intestine from raw or heat-treated food to satisfy the energetic needs of bacteria with the delivery of some extra energy to the host that otherwise would not be available.

Studies in volunteers or patients with a stoma in the terminal ileum provide rough estimates of how much energy passes from the lower small intestine across the ileocaecal valve to serve as 'bacterial feed'. Data compiled from various studies with analysis of ileal output reveal that about 1–5 g of carbohydrates, 2–10 g of protein, 2–5 g of fat and some 20 g of fibre per d pass from the ileum into colon[49–52]. The quantity of non-digestible starch and NSP that reach the colon seems particularly relevant since their contents in the diet vary considerably and correlate closely with the bacterial biomass[49]. In addition, 2·3 g mucus per d was recovered from ileostomy effluent[50]. Further, constituents of gastrointestinal secretions and epithelial cells lost by apoptosis are sources of energy for bacteria. To maintain a microbiome during fasting and starvation, these sources become relevant. However, only a few bacterial species are known for their ability to utilise the glycoproteins as the major constituent of mucus. Taken together, a rough estimate is that fermentation of these substrates could yield about 1050 kJ/d. This calculation comes close to the estimation of energy available for absorption in colon from a study in ileostomy patients fed diets containing different types of carbohydrates combined with an *in vitro* digestion of the ileal effluent to quantify the SCFA produced[50]. Assuming an energy intake of 10 500 kJ/d, findings from this study would translate into 300–1200 kJ of that absorbed in colon. The daily energy output in faeces is about 500 kJ, but ranges from 300 to 700 kJ/d[11] and is thus almost as large as the amount that crosses the ileocaecal valve to be absorbed in colon. Based on this 'back of the envelope' calculation of the 'energetic balance' across the human colon, one may conclude that the microbiota provides only very small energy quantities to the host – if any – compared with its own energy demands. Taken together, the energy balance in and across the human (and mouse) large intestine remains as the 'dark side' and does currently not allow a final conclusion to be drawn on whether the microbiome 'makes us obese or slim'. Yet, the extent to which experts in human and animal nutrition interrogate the same biology with quite opposing perceptions (as either an energy-delivering or an energy-consuming system) is striking. Some of the confusing views may relate to findings in rodents that need more care when translated to the human condition because of different anatomy, physiology, diets and other environmental factors. Fig. 2 summarises some of the critical physiological determinants of intestinal functions in humans that contribute significantly to microbiome quantity and diversity including the estimated nutrient/energy balance.

Evidence of different microbiome compositions in obese and lean humans has been used to push the concept that net energy delivery to the host contributes to overweight and obesity[53]. Predominantly changes in the ratio of Firmicutes:Bacteriodetes were found as associated with higher BMI and body fat content[54]. However, more recent analysis has questioned these findings since, usually, the between-study variability in the relative abundance of Bacteriodetes and Firmicutes is far greater than the within-study differences found in lean and obese individuals[53]. Whether the reported differences between lean and obese individuals hold up needs further studies with standardised analytical procedures and reference materials; it could well be that the gut microbiome is just another read-out of an obese phenotype rather than a cause.

The limits of models and how to better assess microbiome biology

There can be no doubt that the current methods and techniques used in microbiome research have limits and are prone to deliver misleading information. Besides the analytical constraints when it comes to characterisation of the microbiota, the importance of the microbial ecosystem as an interface between diet and host metabolism remains elusive and is quite often exaggerated. One of the most important tools to address the role of the microbiota for host and host health is gnotobiotic animals, inoculated with either faecal samples or discrete bacterial species or microbial populations. A recent critical review assessing the value of these approaches concludes that the findings are limited by conceptual flaws, limited in biological interpretation and limited in translation to the human condition[55]. As addressed above, the gastrointestinal tract of a rodent is quite different on grounds of anatomy, morphology and biochemistry. And, even the very limited number of strains used is a critical constraint. Mouse strains

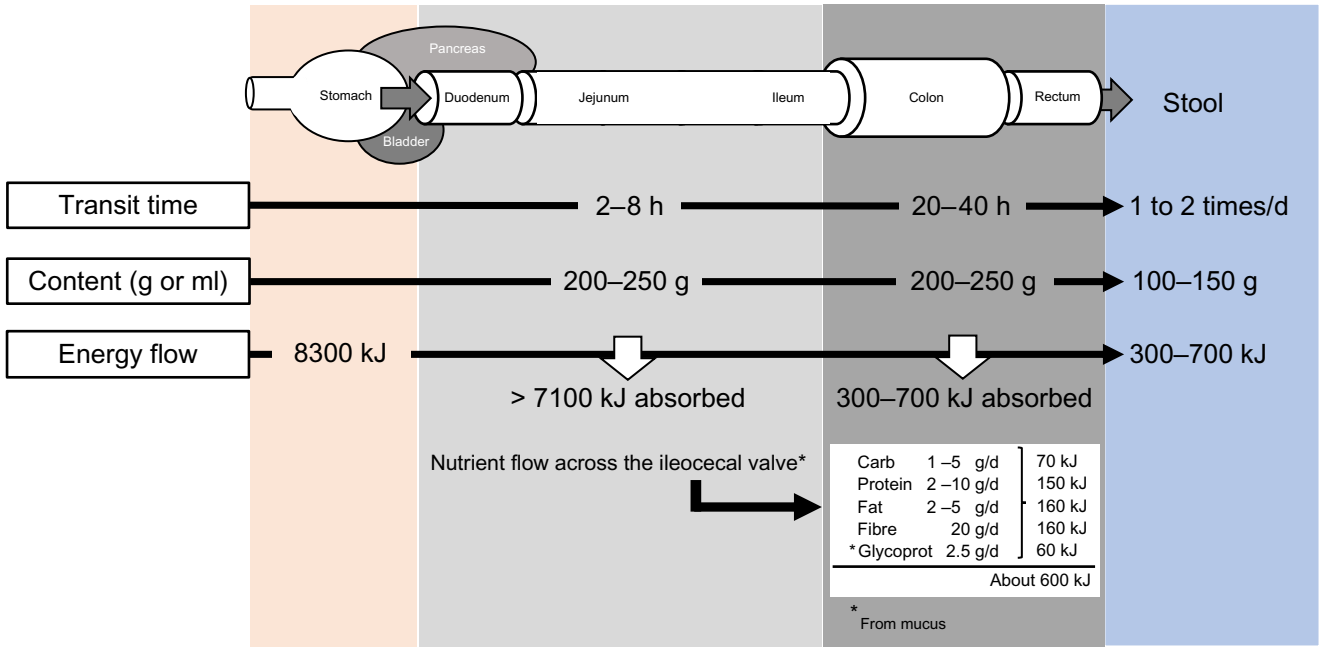

Fig. 2. Selected gastrointestinal functions and parameters known to affect stool sample mass and composition as well as substrate flow across the ileocaecal valve for metabolic use by the microbiota (for details see text). Carb, carbohydrate; Glycoprot, glycoprotein.

display impressive differences in metabolic phenotype. Microbiome research should therefore preferentially use humanised mouse models in which immune and metabolic functions are made more human-like. More appropriate animals such as pigs may as well be employed. They can be fed like a human with all kinds of processed food in contrast to rodents and that would also allow matrix effects of food items to be studied which is not done or more difficult to do in mice. There is also the option to use humanised pigs[56] for those type of studies. Non-human primates appear attractive as well, but their use has limits by both, the resources and infrastructures needed and by the much higher financial requirements.

Over the last decade, various *in vitro* methods and technology platforms have been developed to simulate the human (animal) gastrointestinal tract for studying digestion and bioavailability of nutrients or assessing the effects of dietary constituents on the microbiota. Although some intestinal processes can be mimicked quiet well *in vitro*[57] and data gained may be translated, others could not be confirmed *in vivo*. This was recently demonstrated by comparing the effects of inulin on the microbiome and the production of SCFA *in vitro* and in human studies[58]. What currently *in vitro* models cannot simulate appropriately is the autoregulatory loops that control motility and fluid handling via the enteric nervous system and the numerous gastrointestinal hormones produced in the gut that continuously adapt the physiology to the recorded status of digestion and absorption. What rarely is considered in microbiome research – and thus also not included in most *in vitro* studies – is the secretory component. About 5 litres of endogenous fluid enter the intestine per d and are almost completely reabsorbed together with the fluids drunken and bound in food leaving about 100 ml excreted with stool. The endogenous secretions provide a continuous flow of solutes into the intestinal system comprised of not only electrolytes but also low molecular metabolites such as sugars, amino acids and urea. Depending on protein intake, humans produce 25–50 g of urea per d of which about 25 % cycle through the gastrointestinal system[59] with rapid hydrolysis to ammonia (mainly in colon) by bacteria possessing urease. This is associated with marked effects on pH which in turn affects the microbiota. It is thus a very interesting approach to use the effluents of volunteers with an ileostoma for *ex vivo* studies of microbiota[47]. The effluent represents the 'physiological substrate' that would pass into the colon for further fermentation and when collected from volunteers consuming different diets, new insights into how diet can alter the microbiota may be gained.

Microbiome and metabolic health

Since faecal transplantation of 'healthy stool' to patients with recurrent *Clostridium difficile* infections has been established as a therapy[60], the principle of transferring microbiomes has also been applied to study metabolic health effects.

As an example, faecal microbiome transfer (FMT) was used very recently to study the effects of trimethylamine-oxide (TMAO) on cardiometabolic health in individuals with the metabolic syndrome[61]. TMAO is a suggested mediator of microbiome effects on the cardiovascular system since elevated plasma concentrations of TMAO associate with increased cardiovascular risk and mortality[62]. In the gut microbiome, trimethylamine is produced from dietary carnitine, choline and phosphatidylcholine and is then oxidised in liver to TMAO. Since a vegetarian diet delivers far less of the dietary precursors for microbial trimethylamine production, the corresponding microbiome thus could have a much lower capacity to produce trimethylamine resulting also in a lower cardiovascular burden.

This hypothesis was tested in a FMT study using stool samples from a vegetarian donor and autologous (own) faecal samples as control provided to individuals with the metabolic syndrome combined with analysis of markers for cardiovascular health[55]. Although the intervention resulted in some changes in microbiome composition in the recipients, TMAO concentrations in blood and measures of vascular health remained unchanged. In addition, the TMAO hypothesis has recently been challenged by a study that used a Mendelian randomisation approach to reassess studies on TMAO in the context of CVD in cohort studies[63]. It was concluded that the observed TMAO levels in plasma likely result from slight impairments in renal clearance of TMAO in the risk cohorts and that TMAO thus may not be causative but rather serve as a biomarker in the disease trajectory.

Obesity and insulin resistance appear to be associated with alterations in faecal microbiome signatures and moreover, inoculation of faecal samples from obese or lean individuals into gnotobiotic mice affected differently animal weight gain. That provided support for the hypothesis that the gut microbiome contributes to an obese phenotype and the development of the metabolic syndrome. A recent study in twenty-two obese individuals tested whether an FMT approach with a stool sample of a single donor of BMI 17·5 kg/m^2 provided via capsules to eleven test volunteers can cause changes in body weight. The study period was 12 weeks with eleven other volunteers receiving placebo capsules as control. Treatment started with a dose of thirty capsules at week 4 and a second dose of twelve capsules at week 8. Each capsule contained 0·75 g stool of the donor. Careful analysis of weight changes, microbiome composition and numerous metabolic parameters revealed that the procedure *per se* was safe but failed to cause any significant alterations in the volunteers receiving stool from the lean donor[64]. For the metabolic syndrome, a trial with eighteen patients of which nine received FMT with samples from lean human donors (provided as lavage via duodenal tube) and nine obese individuals serving as control and receiving autologous FMT reported improved insulin sensitivity after 6 weeks in the group undergoing allogenic FMT[65]. However, the same research group failed to show any metabolic improvements or microbiome changes after 18 weeks in a subsequent study with twenty-six patients undergoing allogenic FMT with twelve controls receiving autologous FMT[66]. Another approach to assess the contribution of the microbiome to an insulin resistance phenotype is the treatment with antibiotics. However, a human study with type 2 diabetic volunteers who received either amoxicillin or vancomycin for 7 d failed to provide evidence for any metabolic improvements despite significant alterations in SCFA and bile acid concentrations in stool and major changes in the microbiome[67].

A close link between microbiome composition and plasma glucose responses has been derived from a study conducted with originally 800 volunteers carrying an interstitial glucose monitor to obtain blood glucose responses to given meals and individual food items. The study revealed huge differences in glucose profiles between individuals when challenged with food items of seemingly the same carbohydrate load (but not identical in composition with respect to starch or sucrose, non-digestible starch or NSP content or accompanying fat and protein content). However, the observed different responses in glucose levels were associated with microbiome signatures and the authors developed an algorithm that included some phenotypic measures and microbiome information that was able to predict blood glucose profiles in an independent cohort with an accuracy of 60–70 %[68]. This algorithm has been applied in other cohorts and proven to predict glycaemic responses with similar precision[69,70]. These findings, of course, suggest a causal link between microbiome composition, its metabolic activity and the increase in plasma glucose concentration and/or glucose clearance in response to a given food. Such a relationship may be mediated by the gastrointestinal hormone system, including the incretins that act as central regulators of insulin secretion and thus plasma glucose concentration. However, it is more difficult to provide a mechanistic explanation for the possible role of the gut bacteria in these acute glycaemic responses to foods because the bacteria reside mainly in colon. This part of the gastrointestinal tract is well equipped with a high density of enteroendocrine cells and is known to secrete glucagon-like peptide 1 and peptide YY that contribute to upper small intestinal physiology, for example, by delaying gastric emptying and providing some satiety signals. However, the peak in blood glucose concentration after food intake occurs usually within 30–60 min and that is long before any food residues or digested products have reached the colon to provide via the microbiome a feedback that could modulate glucose absorption. However, inter-individual differences in gastrointestinal motility and transit time (TT) are an underlying phenomenon that would affect blood glucose responses and microbiome signatures in a coupled manner. TT can be altered by drugs such as loperamide or erythromycin and agents such as senna alkaloids or magnesium citrate. These manoeuvres that increase or decrease TT have been shown to cause changes in bacterial density in stool samples[71] and microbiome composition[16]. Indeed, the bacterial mass in stool (g/d) can be altered almost 3-fold by these transit time modifying agents and there is a close relationship between mass and the log of transit time[71]. In humans, colonic transit time measured by radio-opaque markers via x-ray correlated with Shannon index or the operational taxonomic units richness as diversity markers of the faecal microbiome[16]. In addition, stool water content and BSS are key determinants of the four enterotypes that are frequently defined as core microbiome groups with distinct compositions and these are intrinsically linked to TT[12]. Further, TT is a critical determinant of the rate of glucose absorption in the upper small intestine. For postprandial glucose profiles, compounds such as loperamide that delay gastric emptying reduce plasma glucose responses, whereas prokinetic agents such as metoclopramide that increase gastric emptying lead to faster, and higher, rises in postprandial glucose concentration[72,73]. As a consequence, gastrointestinal motility and transit through stomach and intestine that affect both upper and lower intestinal physiology link microbiome effects with individual glucose responses. They appear as two read-outs of a common intestinal phenotype with marked intraindividual differences. Interpretation of studies of post-prandial glycaemia and the microbiome should also take into account the evidence that high postprandial glucose concentrations (as observed in individuals with insulin resistance or type 2 diabetes) can alter

gastric, pancreatic and intestinal responses to diet and change the TT and that these may well be factors contributing to microbiome changes reported in these disease states[72,73]. A direct proof of the hypothesis that the individual's TT is a critical determinant of both, microbiome mass and diversity and postprandial glucose responses requires studies with comprehensive analysis of all related parameters.

A new quality of gut microbiome research

William Hanage in 2014 asked for a 'healthy dose of scepticism' in microbiome science and proposed some key questions including whether experiments can detect differences that matter, whether studies can reveal evidence of causation and not just correlation or whether anything else could explain the findings[74]. Asking these questions seems as valid today as 6 years ago despite the thousands of new papers published since then. A very recent commentary asks as well for more rigor and critique in microbiome science to avoid and prevent unrealistic expectations that may undermine the credibility of science *per se*[69]. It all starts with better standardisation of all procedures for sampling and processing of samples and for analysis of microbiomes of which some are being addressed through activities such as the microbiome quality control project that was launched a couple of years ago[22,23] and that delivered some 'best practise' recommendations[37]. The research area also needs reference materials (stool samples or defined mixtures of bacteria) to be included for quality control purposes and such quality assurance measures should be made mandatory, and enforced, by funders and research journals.

Microbiome science should no longer ignore mass issues and basic intestinal (and whole body) physiology. Stool output, BSS, water content and bacterial biomass in the sample should be measured routinely. Moreover, microbiology beyond sequencing efforts needs to become quantitative by measuring the true abundance of bacteria (by numbers for at least the dominating species) and not only relative species abundance.

We need hypothesis-driven research on the effects of diet and on whether the microbiome is a net energy delivery or energy-consuming biosystem. Such studies in humans, combined with studies in volunteers with ileostoma, should include careful analysis of energy going in (energetic intake) and energy excreted per d (by bomb calorimetry) of total stool and urine. Iliostomised human volunteers receiving the same diet as the healthy control would allow by collection and analysis of stoma effluent how much energy and which nutrients pass into the colon allowing to assess the energy balance across the colon in humans. Of course, some alterations in the physiology and microbiology in ileostomy patients (due to the previous disease such as colorectal cancer, Crohn's disease or colitis) may hamper the direct translation to the healthy individual, at present, there appears no better system to quantify total substrate inflow into the gut microbiome.

Although most studies with fermentable substrates showed only modest effects on overall microbiome diversity with persistent changes in a few species only[36], all non-digestible starches and other NSP are known to cause changes in stool volume. An important question is how these changes in stool output *per se* affect the microbiome in mass and composition (diversity). For such studies, the effects of different types and quantities of carbohydrates/fibres on the microbiome should be studied in the same volunteers combined with standardised microbiome profiling. That could well be done in pan-European/international projects with different microbiome backgrounds and with standardised diets and standardised analytical procedures. The recent study from China[19] demonstrating the great importance of the geographical/regional influence on microbiome composition suggests strongly that diet effects may need to be studied at such regional levels to enable the development of generalisable conclusions and recommendations for public health.

In addition, when investigating the effects of diet, it is important to consider the technology used for food processing and whether the food is consumed raw or heat-treated. For example, in volunteers with ileostoma between 4 and 19 g of non-digestible starch and NSP (about 2 g) were recovered from the effluent when two bananas were consumed – depending on their state of ripeness. In raw banana, the starch in the granules is in a highly crystallised form and seems to resist hydrolysis by α-amylase[75]. Starch structure in general is an important factor in digestibility, a certain fraction is always resistant to hydrolysis and that fraction is altered when food rich in starch undergo heating and cooling (or reheating). Whereas cooling can reduce digestibility of potato starch, for example, by about 10 %, reheating does not lead to full recovery of digestibility. For many food and in particular for convenience products (frozen or microwave prepared) that may well be relevant. To date, there are no systematic studies on how food processing affects the gut microbiome and that also applies to the fundamental question on how raw *v.* heat-treated food in a diet affect overall energy balance and microbiome composition. Heat treatment not only changes digestibility of starch but also that of protein and fat. Using the same batch of food products and a cross-over design (and if possible with volunteers carrying an ileostoma as control for determining small intestinal digestibility), such a study could yield interesting findings on the shift of nutrient and energy harvest from large to small intestine when early hominids employed fire and started cooking; estimated to have happened about a million years ago and considered to be a milestone in the evolution of our species and its intestinal microbiome.

Acknowledgements

This paper was written in honour of John H. Cummings, Professor Emeritus, who provided over decades seminal work on the physiology, biochemistry and diet effects of the human large intestine.

There are no conflicts of interest.

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