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RESPONSE OF THE HUMAN COLONIC MICROBIOTA TO DIETARY CHANGE

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SUMMARY

Molecular methodologies allow increasingly detailed profiling of the microbial communities in terms of species composition and gene complement. This information needs to be linked to functionality, and the availability of representative cultured isolates can make an extremely important contribution by allowing functionally significant microbial markers to be monitored. A number of recent human dietary studies have shown that the species composition of the human faecal microbiota is significantly influenced by the type and quantity of non-digestible carbohydrates in the diet. Supplementation with prebiotics such as inulin not only increases bifidobacterial populations, but also those of important groups of anaerobes such as *Faecalibacterium prausnitzii* that may promote gut health. Reductions in total carbohydrate intake in weight loss diets for obese volunteers result in greatly reduced populations of the *Roseburia/E. rectale* group of butyrate producers that parallel the decrease in faecal butyrate. Most studies have so far relied on identifying phylogenetic groups that share a common function (e.g. butyrate formation) and that can be tracked using 16S rRNA-based methods. An alternative approach involves amplification of functionally relevant genes, and this has now been explored for butyrate-producing bacteria using the recently identified butyryl CoA:acetate CoA transferase gene. This type of targeted metagenomic approach allows the monitoring of previously uncultured, as well as cultured, groups of butyrate-producing bacteria. In general, there appear to be very good prospects for identifying new microbial biomarkers that are relevant to gut health.

INTRODUCTION

An unprecedented array of molecular tools is now available for analyzing the human gut microbiota. Over the past 15 years these have been based mainly on 16S rRNA sequences, allowing the diversity and abundance of different phylogenetic groupings of gut bacteria to be described in increasing detail (*Wilson* and *Blitchington*, 1996; *Suau* et al., 1999; *Hold* et al., 2002, *Eckburg* et al., 2005). Metagenomics can now provide information on the majority gene complement found in gut samples (*Gill* et al., 2006; *Kurokawa* et al.,

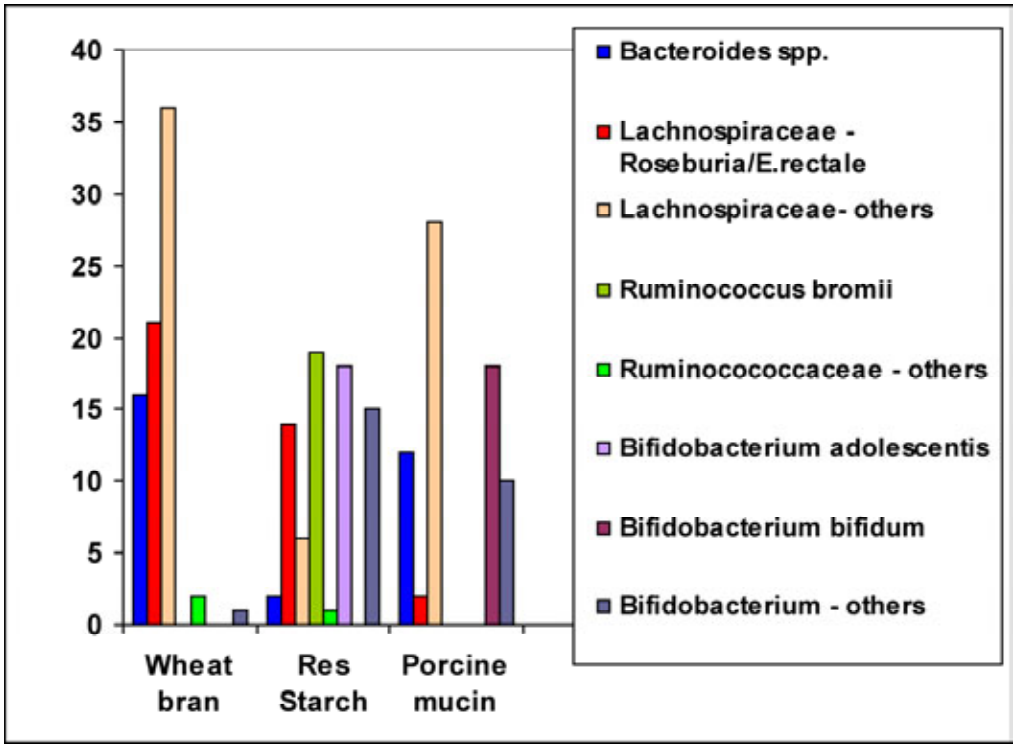

Figure 1: Selective colonization of insoluble substrates by human faecal bacteria in an *in vitro* fermentor system (*Leitch* et al., 2007). Bacterial colonization was assessed by sequencing of 16S rRNA amplicons recovered from washed residual substrate after 24 h incubation.

2007). Further technical advances make it feasible to use this information for the development of diagnostic methods, and various array-based detection approaches are being developed (*Rajilic-Stojanovic* et al., 2009).

In the rush to exploit new technologies, however, we should not overlook the importance of gaining functional information on key strains and species of gut microorganism. While acknowledging the importance of horizontal gene transfer and metabolic cross-feeding, the microbial cell remains the fundamental unit of propagation of chromosomal DNA, and of metabolism. There is great potential from combining molecular approaches with work on isolated cultures of gut bacteria through organism-based approaches (*Flint* et al., 2007).

There is increasing evidence that diet can modify the species composition of the gut microbiota. This article will consider some recent analyses of diet-induced responses as defined by molecular ecological approaches, and will then consider briefly the consequences that such shifts in the community may have upon the host.

KEY FUNCTIONAL GROUPS OF COLONIC BACTERIA

Intestinal microorganisms can be divided into functional groups based on metabolic, immunological or other criteria. As examples we will consider

here selected groups that are defined by substrate utilization and metabolic product formation.

Utilization of carbohydrate substrates: Fibre- and starch-degraders

Although many gut bacteria possess polysaccharidases, the ability to degrade plant polysaccharides, particularly when present in insoluble food particles, is less widespread (*Flint* et al., 2008). The classical approach for defining functional groups depends on the ability of isolated colonies to utilise particular substrates for growth (*Chassard* et al., 2008). A different approach recently used an *in vitro* fermentor system together with 16S rRNA sequencing to detect bacteria from human faecal samples that colonised insoluble particles of starch, wheat bran and mucin (*Leitch* et al., 2007). This revealed that colonisation was highly selective (Figure 1); in the case of starch most of the colonisers were known cultured species, but for the other two substrates many of the dominant colonisers were unknown. Such specificity is less easily detected in *in vivo* studies, since particles consist of a mixture of undigested material. Nevertheless, fractionation of faecal samples revealed that particular groups of ruminococci were significantly more abundant in the particulate than in the liquid phase (*Walker* et al., 2008). Some representatives of this group were previously isolated by *Robert* and *Bernalier-Donadille* (2003) as cellulolytic bacteria. Conversely, *Bacteroides* relatives were relatively less abundant in the particle-attached than liquid phase communities. These ecological differences are likely to reflect the different organization of polysaccharide degrading enzymes ad transport systems in different intestinal bacteria (*Flint* et al., 2008). Another novel approach is provided by stable isotope probing, which was recently used to reveal the dominant bacteria that utilise starch (*Kovacheva-Datchary* et al., 2009). Interestingly the same species were detected that attached to starch in fermentor studies (*Leitch* et al., 2007).

Product formation: Butyrate-producers

Microbially-produced butyrate is considered to be particularly important for colonic health because of its role as an energy source for the colonic epithelium and in the prevention of colorectal cancer (*Hamer* et al., 2008; *Pryde* et al., 2002). The diversity of butyrate producing bacteria in the human gastrointestinal tract has been explored by cultivation under anaerobic conditions (*Barcenilla* et al., 2000). The abundance of these cultured species has been confirmed by molecular approaches such as 16S rRNA-based fluorescent *in situ* hybridization and real time PCR, and more recently by analysis of the butyryl CoA:acetate CoA transferase gene (Figure 2; *Louis* and *Flint*, 2007; *Louis* et al., 2010). The latter technique also allows the identification of uncultured butyrate-producers, and approximately one third of the sequence groups (OTUs) detected did not correspond to cultured strains and species. These investigations have revealed three major phylogenetic groups that also display potentially significant functional differences.

Eubacterium rectale, together with four species of *Roseburia*, form one coherent group of human colonic butyrate-producers belonging to the *Lachnospiraceae*, based on 16S rRNA sequencing. They are estimated to account for 5-15% of total faecal bacteria (*Aminov* et al., 2006). Despite their extreme oxygen sensitivity, the culturability of this group appears to be high, as only one branch on the phylogenetic tree of available 16S rRNA clone li-

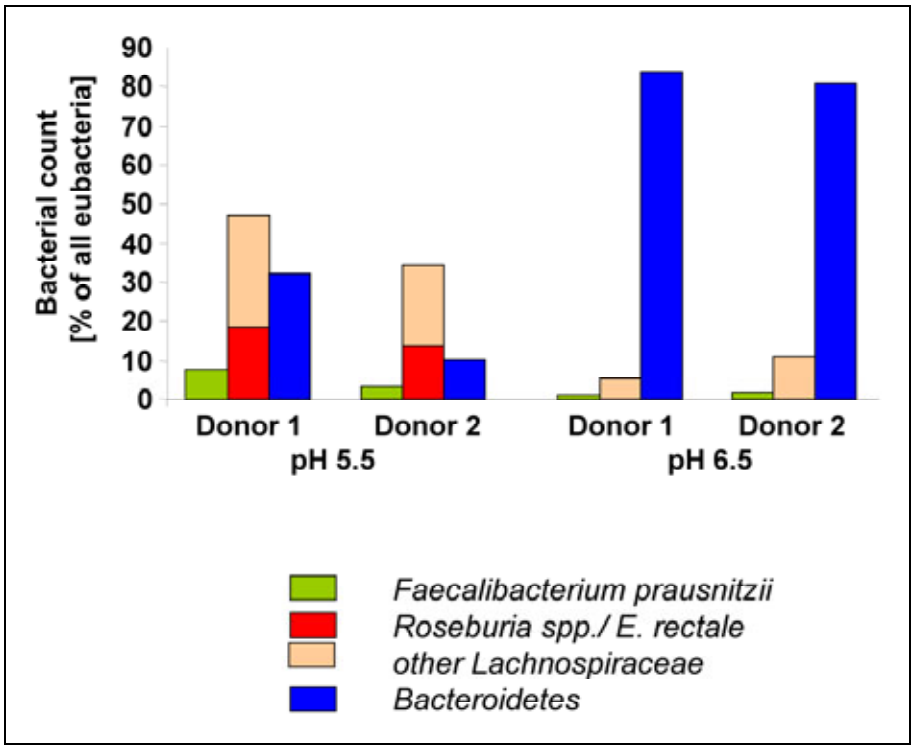

Figure 2: Abundance of presumed butyrate-producing bacteria estimated by 16S rRNA-based FISH detection (left hand columns: black = *Roseburia* + *E. rectale*, grey = *F. prausnitzii*) and by butyryl CoA: acetate CoA-transferase based quantitative PCR (right hand columns, stippled) (*Louis* and *Flint*, 2007). The samples analyzed were from an *in vitro* fermentor study on the effects of pH on human intestinal microbial communities (*Walker* et al., 2005).

brary sequences was found to lack a cultured representative (*Aminov* et al., 2006, *Flint* et al., 2007).Species of this group share several potentially significant features. They utilise acetate while forming butyrate during growth in the presence of short chain fatty acids (SCFA) and they share a unique arrangement of central butyrate pathway genes (*Louis* and *Flint*, 2009). Most are able to utilise polysaccharides, especially starch, for growth (*Ramsay* et al., 2006; *Chassard* et al., 2006). These bacteria are motile, possessing multiple flagella, and show tolerance of mildly acidic pH (*Duncan* et al., 2009).

Faecalibacterium prausnitzii represents one of the most abundant species found in human faeces, belonging to the *Ruminococcaceae*, and is also estimated to account for 5-15% of colonic bacteria. Relatively few isolates are available, but 16S rRNA sequences of the available cultured strains suggest that there is considerable diversity within the species (*Duncan* et al., 2002). Recent evidence suggests that *F. prausnitzii* may have an important role in suppressing inflammation in the gut lining (*Sokol* et al., 2008). Neither of these two prominent groups of butyrate producers shows the ability to grow with lactate as energy source. On the other hand, a third group includes species found to utilise D- and L-lactate (*E. hallii, A. caccae*) or D-lactate alone (an as yet un-named species represented by strain SS2/1) (*Duncan* et al.,

2004; *Flint* et al., 2007). This activity may be significant in stabilizing the colonic microbial community by preventing lactate accumulation and dramatic decreases in pH (*Belenguer* et al., 2007).

A recent analysis of almost 6000 16S rRNA sequences from six obese male volunteers revealed 16 dominant bacterial phylotypes that were present in all six subjects (*Walker* et al., in preparation). Eight of these were butyrate-producers: Three *F. prausnitzii,* three *Roseburia spp./E. rectale,* plus *E. hallii* and SS2/1, showing that these butyrate-producers are dominant core species within the human colonic microbiota.

IMPACT OF DIETARY CARBOHYDRATES UPON THE COLONIC MICROBIAL COMMUNITY

Profiling of the human faecal microbiota by DGGE has suggested that the bacterial species composition is relatively stable within an adult individual over time periods of a few weeks (*Zoetendal* et al., 1998). This approach provides qualitative information, however, and tends to emphasise a few very abundant bacterial ribotypes. When examined by more quantitative approaches such as FISH microscopy it is apparent that the proportions of different bacterial groups fluctuate considerably over time (*Franks* et al., 1998; *Duncan* et al., unpublished results). Such fluctuations are not surprising; different foods are consumed throughout the day, and dietary patterns typically vary through the week. Gut transit times also vary, largely in response to dietary intake. Since the gut environment and substrate availability in the large intestine must therefore change with time, the conditions for proliferation of different groups of gut bacteria will necessarily be affected.

Impact of low carbohydrate weight-loss diets on the colonic microbiota

Temporal variation clearly poses a problem for human studies aimed at understanding the impact of diet on the colonic microbiota. This problem can be minimised however if dietary intake is carefully controlled, as in some recent studies on weight loss diets in obese human subjects. *Duncan* et al. (2007) looked at the impact of two high protein weight loss diets containing reduced carbohydrates, supplied for four weeks each in a cross-over design. The low carbohydrate diet resulted in a two-fold reduction of total faecal SCFA that can be ascribed to the reduction in fibre and the virtual elimination of starch in this diet. Faecal butyrate was reduced disproportionately, around four-fold (Figure 3). Similarly *Brinkworth* et al. (2009) showed significant reductions in faecal output and in faecal butyrate and total SCFA over 8 weeks on high fat, low carbohydrate weight-loss diet. In the study by *Duncan* et al. (2007) FISH analysis of major groups within the faecal microbiota showed no significant change in the proportions of *Bacteroides* or in the overall proportion of *Clostridium*-related gut anaerobes. Two major groups of butyrate-producing *Clostridium*-related bacteria, however, behaved very differently. *F. prausnitzii* decreased only slightly as a proportion of total bacteria, but relatives of *Roseburia* and *E. rectale* decreased dramatically (Figure 4). The simplest explanation for this is that

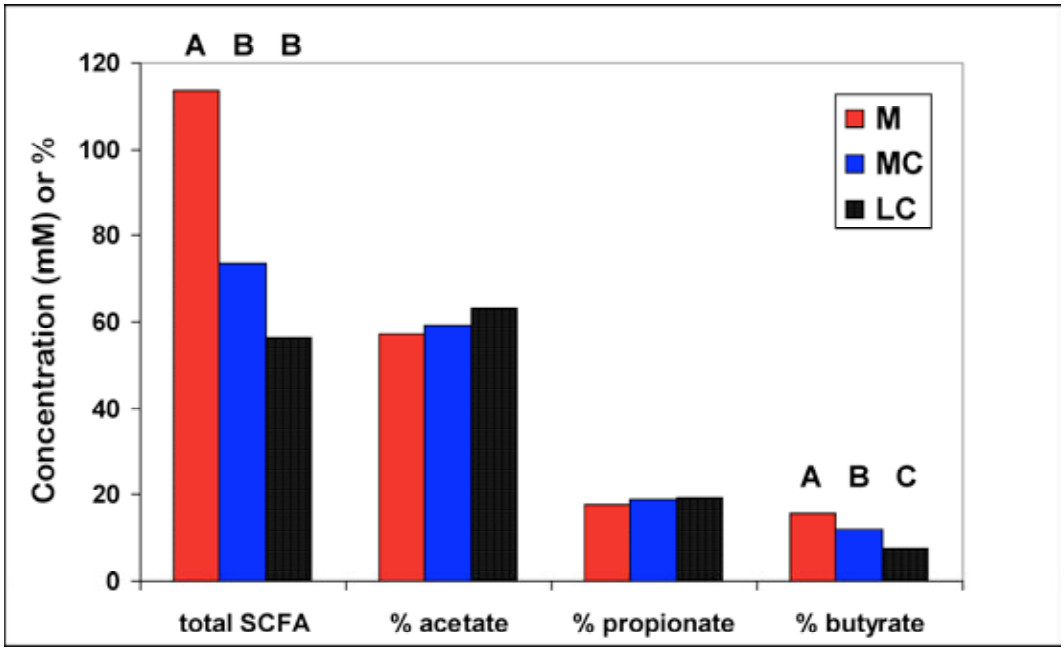

Figure 3: Impact of reduced carbohydrate weight loss diets upon faecal short chain fatty acids in a group of 17 obese male volunteers (from *Duncan* et al., 2007). Volunteers were given weight maintenance (M - mean of 399 g CHO/day for 3 days), medium carbohydrate (MC -127 g CHO/day, 4 weeks) or low carbohydrate (LC - 24 g CHO/day, 4 weeks) diets. For each metabolite shown, columns carrying different letters differed significantly ($p<0.05$).

members of the *Roseburia* group are particularly dependent on dietary resistant starch as an energy source for growth. An important compounding factor may be the effect of pH changes in response to active fermentation of dietary carbohydrates in the proximal colon, as will be discussed further below. The cross-over design of thisstudy allowed us to conclude that these changes in colonic bacteria and their metabolites were driven by diet, and not by a change in host physiology accompanying weight loss (*Duncan* et al., 2008). Both *Duncan* et al. (2007) and *Brinkworth* et al. (2009) reported reduced bifidobacterial populations on low carbohydrate, high fat diets. *Ley* et al. (2006) followed twelve obese subjects who were on either reduced fat or reduced carbohydrate weight-loss diets over 52 weeks. Their data, based on 16S rRNA clone library analysis, indicate a progressive increase on the percentage *Bacteroidetes* in the faecal microbiota with increasing weight loss. The starting percentage of *Bacteroidetes* was however far lower than has been reported in other studies on obese subjects (*Duncan* et al., 2007; *Zhang* et al., 2008; *Turnbaugh* et al., 2008).

Impact of dietary supplementation with specific carbohydrates (including prebiotics)

There is now quite extensive evidence for the modification of faecal microbiota as a result of prebiotic supplementation (e.g. *Bouhnik* et al., 2004; *Kruse* et al., 1999). Most studies have focussed on target groups such as *Bifidobacterium* and *Lactobacillus*, but some have surveyed the whole gut community. *Ramirez-Farias* et al. (2009) for example detected a significant increase in *F. prausnitzii* as well

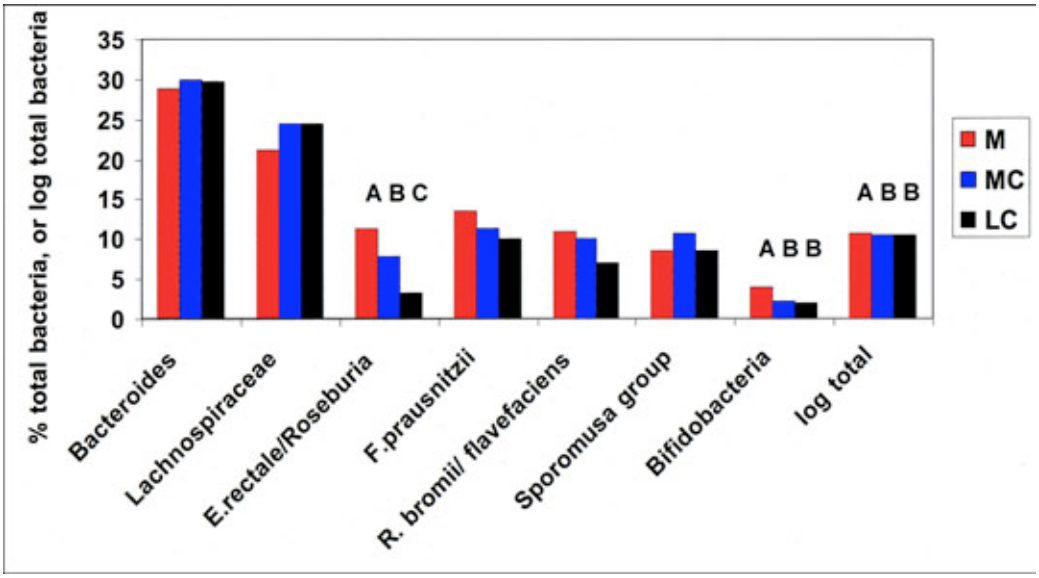

Figure 4: Impact of reduced carbohydrate weight loss diets upon bacterial populations detected in faecal samples for a group of 17 obese male volunteers (from *Duncan* et al., 2007). Diets are as described for Figure 3, and data refer to the same samples. Bacterial numbers were determined by fluorescent *in situ* hybridization (FISH) (for details of probes and conditions please see *Duncan* et al., 2007).

as in *Bifidobacterium* spp. in volunteers receiving inulin. Dietary supplementation with resistant starch (RS) also leads to significant alterations in the microbial community. Based on DGGE analysis, *Abell* et al. (2009) showed that *Ruminococcus bromii* was stimulated by RS, and this finding fits in well with recent *in vitro* work on starch utilization by faecal bacteria discussed earlier (*Leitch* et al., 2007; *Kovacheva-Datchary* et al., 2009).

Mechanisms underlying shifts in the microbial community

The most obvious explanation for a change in microbiota species composition following increased carbohydrate intake is selective stimulation of bacteria able to utilise the substrate. The overall effects are however likely to be far more complex than this. Metabolic cross-feeding, for example, will produce changes in other groups (*Belenguer* et al., 2006; *Falony* et al., 2006; *Flint* et al., 2007). In addition increased intake of fibre, resistant starch or prebiotics is likely to increase gut transit, thus influencing the gut environment and potentially altering the balance of the whole community (*Stephen* et al., 1987; *Lewis* and *Heaton*, 1997). In particular increased fermentation leads to decreased luminal pH, and this is likely to be a key factor modulating the competition and metabolism within the colonic microbiota with mildly acidic conditions shown to promote butyrate-producing bacteria and curtail *Bacteroides* spp. (*Walker* et al., 2005; *Duncan* et al., 2009; Figure 2). Very low colonic pH, reported in severe ulcerative colitis, is associated with the accumulation of lactic acid largely due to an inhibition of lactate utilization (*Belenguer* et al., 2007). In healthy subjects the pH of the proximal colon is mildly acidic whilst that of the distal region in usually closer to neutrality.

POTENTIAL CONSEQUENCES OF SHIFTS IN MICROBIAL COMMUNITY COMPOSITION

Pathogenesis

The presence of pathogenic microorganisms within the gut community presents an obvious threat of infection. This threat depends to varying degrees on the gut environment, health status, immune status and the presence of other microorganisms in the gut community. Thus certain pathogens are opportunistic, infecting only when there is an alteration in normal gut barrier function or in the resident community. For food-borne pathogens, the infectious dose is influenced by whether conditions in the gut permit survival and replication. Therefore the carriage of potentially pathogenic microorganisms and the antagonistic influence of the dominant commensal species against pathogens, are both determined by community composition. Interestingly there is evidence from a mouse model that colonisation with *Salmonella enterica* can modify the remainder of the gut community (*Stecher* et al., 2007).

Gut metabolism

Even with a gut community of constant composition it is expected that the metabolic outputs will respond to changes in the quantity and type of substrate arriving in the large intestine. The species *R. inulinivorans* for example metabolises glucose largely to butyrate, but fucose largely to propionate (*Scott* et al., 2006). Because sustained changes in substrate supply also alter community composition, as discussed above, these compositional changes will generally amplify the impact on metabolism. Thus the reduction in SCFA formation in response to decreased carbohydrate intake in obese subjects on weight loss diets (*Duncan* et al., 2007) can be attributed partly to mass action effects, but the disproportionate decrease in faecal butyrate is explained by the decreased proportion of butyrate-producing bacteria. The proportions of the major SCFA fermentation products may have an important influence upon health. Acetate may promote lipogenesis, whereas propionate is thought to help suppress cholesterol. There is increasing evidence to suggest that the proportions of the major SCFA influence gut health and metabolic health through their roles as energy sources and through their interactions with gut receptors that influence gut motility and immune responses (*Brown* et al., 2003; *Hamer* et al., 2008).

A vast array of metabolites detected in serum and urine can be ascribed to the activities of intestinal microorganisms and many of these have important health implications (*Nicolson* et al., 2005; *Holmes* et al., 2008). Some of these metabolic transformations can be attributed to minor components of the intestinal community that may show significant inter-individual variation. To give one example, *Oxalobacter formigenes* is one of the few oxalate-utilizing species in the gut community, but is found to be absent in many adults (possibly eliminated as a result of antibiotic treatment) (*Stewart* et al., 2004). These individuals show higher circulating concentrations of oxalate that may contribute to their risk of kidney stones. Interestingly, inoculation with *O. formigenes* was successful in restoring its population and reversing oxalate accumulation (*Duncan* et al., 2002). We have little idea how many significant metabolic transformations may be determined in this way by minor components of the microbiota, but they may well include metabolism of a variety of drugs and toxins.

Impact on energy supply to the host

Microbial fermentation in the large intestine is estimated to supply around 10% of the daily energy intake in humans, depending of course on the diet (*McNeil,* 1984). This energy is obtained through the uptake and metabolism of SCFA. Per mole of sugar, non-digestible carbohydrates supply less energy to the host than carbohydrates that are digested in the upper GI tract, since a significant fraction is diverted into microbial metabolism and growth (*Roberfroid,* 1999). Furthermore, many non-digestible carbohydrates such as cellulose are only partially degraded in the large intestine. This raises the interesting possibility that the extent of degradation of certain food components will be influenced by the species present in an individual's gut microbiota. *Robert* and *Bernalier-Donadille* (2003) suggested that individuals who produce methane possess different dominant species of cellulose-degrading bacteria than non-methanogenic individuals. This might be explained by the energetic advantages from the association of hydrogen-producing bacteria with methanogens and also by the likely relationship between slow transit time and methanogenesis (*El Oufir* et al., 1996). It has yet to be established however whether the extent of cellulose breakdown differs between these two groups of individuals. It has been suggested that genetically obese mice gain more energy from the diet than do lean mice, and that this reflects the higher proportion of Firmicutes than Bacteroidetes in the intestinal microbiota of the obese animals (*Turnbaugh* et al., 2006)

Impacts on the gut mucosa and the immune system

Microbial metabolites such as SCFA are known to interact with gut receptors that influence immune responses and exert anti-inflammatory effects (*Brown* et al., 2003). Furthermore there is a host of other potentially bioactive metabolites that can be formed or released from dietary components by microbial activity (*Nicolson* et al., 2005). In addition, Toll-like receptors on host cells respond to different molecular signals of microbial origin (PAMPs) such as flagellin and lipopolysaccharide (*Vijay-Kumar* et al., 2007). Changes in the distribution of such metabolic and molecular signals within the colonic community therefore have the potential to influence a wide variety of host responses. Some bacterial groups also degrade mucin or metabolise sugar residues associated with gut receptors that coat the surface of the intestinal tract (*Sonnenburg* et al., 2005). Furthermore colonisation of the gut by bacteria can influence the state of glycosylation of gut receptors (*Bry* et al., 1996).

CONCLUSIONS

Recent advances in microbial profiling and detection suggest that the human faecal microbiota can provide biomarkers for intestinal health, metabolism or dietary intake. Recent work indicated for example that the population of the *Roseburia*/*E. rectale* group was particularly dependent upon dietary non-digestible carbohydrate in the diet in obese subject on weight loss diets (*Duncan* et al., 2007). It remains to be seen however whether this relationship will hold for other subject groups and dietary regimes. One complicating factor is the extent of inter-individual variation in the gut microbiota. An-

other is the fact that most phylogenetic groups show considerable diversity and flexibility with respect to substrate utilization and this is likely to result in varied responses to different diet combinations. More promising perhaps than markers for dietary intake is the potential to correlate metabolic products or signals impacting on the host (e.g. PAMPs) with specific microbial signatures. Returning to the example above, it can be argued that populations of *Roseburia* spp. detected in faeces may provide a better measure of butyrate production in the proximal colon than can be obtained from faecal butyrate concentration. Considerably more evidence is required to test these possibilities, but such functionally-linked investigations of the gut microbiota certainly have the potential to provide valuable new biomarkers for gut health.

ACKNOWLEDGEMENTS

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