Report from the Intestinal Microflora and Dietary Modulation Working Group

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Introduction

The human gastrointestinal tract contains a diverse and active microbiota. For example in the colon, numbers approach 10^{12} organisms for every gram of contents. The concerted activities of the microbial flora make the hindgut the most metabolically active organ in the body, although there is also much activity in more proximal areas of the alimentary tract (Tannock, 1999). Carbohydrates and proteins, provided by the diet or through indigenous sources, are fermented anaerobically to produce organic acids and gases. Through the formation of such end products gut bacteria can impact upon host health and welfare. The gut flora also contains components which may excrete toxins or other deleterious compounds. There is interest therefore in attempting to alleviate gut disorder by influencing the composition and activities of the resident microbiota. Both probiotics and prebiotics do so by increasing populations seen as 'beneficial.'

Justification for the use of Probiotics and Prebiotics to Modulate the Gut Flora Composition

Diseases of the gastrointestinal tract are of major economic and medical concern. For example, reported infections from agents of foodborne disease such as *Campylobacter* spp., *E. coli* and *Salmonella* spp. continue to increase. This is further exacerbated by the continuous emergence of novel variants of established pathogens. Such acute infections are extremely ubiquitous and are said to affect almost everyone at some point in their lives. On a chronic basis, inflammatory bowel disease, colon cancer and irritable bowel syndrome have all been linked to intestinal microorganisms and their activities (Marteau *et al.*, 1993; Chadwick and Anderson, 1995; Burns and Rowland, 2000). The gut flora may also be linked with certain systemic states. The site of action, namely the human gut, is a relatively under explored ecosystem and yet affords the best opportunity for reducing the impact of food related disease. This is amplified by the fact that few working therapies exist for most gut disorders, often the approach is to attempt to manage conditions through non specific approaches involving anti-inflammatory drugs or antibiotics. In the severest of cases, surgery may be required and some states like colorectal cancer can be fatal (Yancik *et al.*1998). As such, clinicians, patients and medical authorities are becoming increasingly interested in defining alternative approaches that may be either prophylactic or curative. Probiotics and prebiotics have a track record of being safe and a long history of use in humans (Adams and Marteau, 1995) and are popular 'dietary intervention' tools for modulating the gut microbiota composition and activities. It is suspected that pathogenic bacteria are the aetiological agents of many acute and chronic gut disorders and probiotics/prebiotics may exert suppressant effects on such components of the flora.

Current State of the Science
Carbohydrates said to be prebiotics have been variably tested for modulating the gut flora activities. For example, fructooligosaccharides, galactooligosaccharides and lactulose are recognised for their bifidogenic effects in laboratory, animal and human trials carried out in multiple centres (Gibson *et al.,* 2000). Some data are conflicting but these materials appear to be the current market leaders. In Japan a much wider list of prebiotics exists which includes soyoligosaccharides, xylooligosaccharides, isomaltooligosaccharides, gentiooligosaccharides, lactosucrose and glucooligosaccharides. These are currently being tested in Europe and elsewhere (Cummings *et al*. 2001). Resistant starches and some sugar alcohols have also been touted as prebiotics. New prebiotics with multiple functionality are also under development. With new advances in molecular based diagnostic procedures for characterising the gut flora responses to dietary change, a more reliable database of effects should ensue (Gibson *et al.,* 2000).

Similarly, improved studies on probiotic efficacy should result from genotypic approaches that allow fed strains to be discriminated from those indigenous to the gut (Tannock, 1999). Most commercial probiotics have been tested in vitro for their resistance to gastric acidity, bile salts, etc. but there are few data on in situ survivability. Maintenance of product viability and integrity during processing and after feeding is a major issue for probiotic approaches. It is largely agreed that probiotics may have effects in the small and large intestine but it is unclear how robust the strains are therein.

Many studies on probiotic recovery and prebiotic functionality have been carried out in healthy persons and there is now a requirement to assess the clinical impact of this. Similarly, their feeding to companion animals and farm livestock may improve nutritional status but the health values are much less known. One major question for probiotics and prebiotics is therefore 'what are the consequences of gut microflora modulation and how do they occur?' This accepts that the best products will modify the gut microbiota composition but addresses the applied consequences of this. Moreover, given the lack of mechanistic data on their use it is imperative to generate hypothesis driven research that determines functionality. A harnessing of multiple disciplines that exploit the best technologies available should now address these issues.

Gaps in the Knowledge
A number of issues need to be addressed for a more full clarification of the functional and mechanistic effects of gut flora modulation. These may consequence as health relevant values at the local and/or systemic levels. Some impact on probiotics alone, others prebiotics only and some questions are relevant to both approaches.

To help define how prebiotics operate, there is a need for more structure to function studies. A selective fermentation is one requirement for an efficient prebiotic, with certain oligosaccharides seemingly preferring the bifidobacteria. However, it is not clear why this is the case or why certain linkages induce selective changes in a mixed microbial ecosystem. As more information on the biochemical, physiological and ecological capabilities of the target organisms is generated, such information will become more apparent.

Survival of probiotics under various physicochemical conditions that are imposed during both processing and after intake (in the gastrointestinal tract) varies between strains. This needs further explanation and inter-species differences determined. This will lead towards a focusing upon the most reliable strains.

Many human gut disorders are of left colonic origin. For example, both colon cancer and ulcerative colitis are prevalent in the distal large intestine. This may be related to increased levels of proteolysis, which leads to the formation of toxic metabolites. A challenge here is to enhance survival of probiotics through the hindgut and also generate a more persistent metabolism of prebiotics (the danger being that these small molecular weight carbohydrates are fully utilised in the proximal colon).

Trials carried out in multiple centres are desirable but not mainstream as yet. For determining changes in the microbiota composition, genetic tools and the technology involved can be transferred between laboratories but the wealth of data produced needs good bioinformatics back up, especially if large intervention studies are planned.

For probiotics, nomenclature (taxonomical changes), poor stability and inaccurate labelling make sound conclusions difficult. Similarly there is a lack of quantification on what constitutes a transient, persistent or colonising effect in the gut flora.

For prebiotics, there are problems if the gut flora does not contain their target microorganisms and there are a more limited range of available products than there are probiotics. Effects of prebiotics would be easier to define if their influences on the gut microbiota could be standardised i.e. are any changes that ensue consistent?

The usefulness of alterations in faecal flora from human trials is debateable and gives a low amount of information on interactions at the mucosal surface and in sites other than the distal bowel. However, faeces is routinely accessible material.

The long term physiological effects of dietary intervention also need clarification.

Suggested Strategies to Fill the Gaps
Research strategies that harness best technologies and complementary disciplines may help to answer the above questions and propel the science of probiotics and prebiotics forward. Areas relevant to the intestinal microflora and its modulation are as follows.

A variety of model systems exist for determining the effects of probiotics and prebiotics on the gut flora. These help to better inform and plan well conducted human/animal trials. There are various limitations and advantages. For example, multiple stage chemostat systems allow a prediction on the site of interaction in the gastrointestinal tract and are useful for 'challenge' tests not possible in humans e.g. with pathogens or genetically engineered strains. Laboratory animals can be used to determine immunological effects. In vitro cell lines are useful for attachment studies and cytokine expression work. Biopsy collections give information on microbiology at the mucosal interface. Useful biomarkers of functionality (organic acids, bioactive molecules) should be used in concert with reliable indices of flora change. Various complementary systems have been developed and should be applied with the research hypothesis in mind.

In recent years advances in molecular technologies based on rRNA have shed new light on the diversity of the gut microbiota (Vaughan *et al*., 2000). In particular rRNA gene sequencing studies have revealed the presence of a myriad of previously undiscovered species (Suau *et al*. 1999). It is now clear that a major gap in our knowledge exists concerning the diversity of organisms resident within the human gut. Some of the new diversity studies are likely to uncover hitherto unrecognised probiotics, these may have advantages over existing strains that are in use. rRNA

sequencing provides an excellent means of characterising organisms in terms of resolving power but is time consuming. New research has now been commissioned to increase the throughput of such procedures. The ultimate aim is to characterise the 'microflora at a glance.' Technologies available include genetic probing strategies by microscopy, image analysis or flow cytometry; microarray developments; genetic fingerprinting studies; direct community analysis; RT-PCR, etc. These genotypic methods should be used in conjunction with conventional cultural techniques to improve our knowledge of the gut flora and its interactions. Some techniques are fully qualitative and give an overall picture of the diversity present, others are quantitative but require a prior knowledge of the target organisms. Again, a multiplicity of approaches and recognition of limitations is desirable.

Such genomic approaches have allowed improved probe design (in some cases at the species or strain levels) and are being increasingly applied to both probiotic and prebiotic research. One fundamental observation is that there are age related changes in the gut microbiota composition. Moreover, there may be geographical variation and little commonality between individuals. Nevertheless, the database on flora diversity studies has expanded markedly in recent years and provided much needed information on groups who are more likely to benefit from pro/prebiotic intake.

A further advance would be the use of MALDI-ToF-MS, which provides high resolution proteomic-based comparisons of whole bacterial cells. It is planned to use MALDI-ToF-MS to assemble a database of species-characteristic profiles to facilitate the rapid identification of gut anaerobes. Intact cells, single colonies, can be analysed (using a MALDI-Linear time of flight mass spectrometer) and the automated acquisition of mass spectra from 96 well target plates (single run), will be ideally suited to the high throughput required for examining population dynamics of large numbers of samples. Profiles of all species known to reside in the human gut would be generated. Use of a proteomic approach in conjunction with ongoing genomic data (extensive 16S rRNA gene sequence) will greatly facilitate the recognition of gut microflora composition. Strains giving rise to unidentified proteomic profiles may be subjected to gene sequence analysis to facilitate their detailed phylogenetic characterisation. This will permit a parallel updating of proteomic and genomic databases which will provide an invaluable resource for future gut ecological studies.

In terms of functionality, transcriptomics could look at activity through mRNA expression studies, whilst metabolomics, in concert with NMR spectroscopy could be used to assay, in an unambiguous manner, diagnostic sets of biomarkers including microbial metabolites. Transcriptomics and metabolomics are still in their infancy from the microbial perspective but progression is rapid and promises to allow activity measurements to also be encompassed in new studies. It is proposed that current and emerging molecular based information be collated into a functional approach and directed these towards disorders for which treatment is ill defined or even lacking, but has the potential to be managed by pro, pre and synbiotics. The trials should be done in multiple countries and would be a good progression for current work which is developing the technology, generating new test materials, exploring mechanisms, determining safety, identifying best products, etc. The application of post-genomic principles in gut microbial studies will help to fully explore human gut microflora diversity, develop reliable model systems, test a new generation of purpose designed pro/prebiotic molecules with enhanced functionality and determine the effectiveness of dietary intervention in the clinical situation.

A synbiotic is a probiotic combined with a prebiotic. This may be a rational way in which to progress dietary intervention studies. Use of an appropriate carbohydrate

should help to fortify the live addition in the gut, whilst the dual advantages of both approaches may also be realised. To help deliver probiotics to the lower bowel, encapsulation is possible. No products exist that use prebiotics as the encapsulation material but an appropriate choice of molecular weight may help persistence throughout the colon.

A reference database of known probiotic traits will help to standardise procedures and increase reproducibility. This could incorporate both in vitro and in vivo effects. The database would clarify useful products from others and develop a framework against which new strains could be compared. The same approach could be taken for prebiotics. One aspect is dose. For probiotics that are proven to be safe there is probably no upper limit. However, excessive use of prebiotics may compromise selectivity of the fermentation leading to undesirable side effects like excessive gas formation.

Substantiation

Comparative studies in multiple centres have clear advantages, as long as the technology transfer is reliable. Hypothesis based research can help product development. Such developments ought to produce more targeted pro, pre and synbiotics that help specific disease states. The targets should be planned around situations where a defined aetiology is suspected or confirmed. The main intention is to address the health consequences of flora modulation through exploiting current technological developments. Ultimately, both the effects and the mechanisms behind them should be unravelled i.e. provide consumers with the definitive health aspects but also give accurate information on why they occur.

Future Directions

Main areas of interest are as follows:

- Environmental genomics and large scale trials
- Use of complementary in vitro and in vivo approaches
- Determining host-cell cross talk (are the 'messages' a probiotic transmits different to those of a pathogen)
- Population dynamics and modulation
- Design of a database of effects to improve current status (including multiple functionality)
- In situ activity of pro, pre and synbiotics (applied genomics) and the clinical consequences of intake
- Linking of functional diversity to phylogenetic diversity

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