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# Intestinal Microbiota in Functional Bowel Disorders: A Rome Foundation Report

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## 3. INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are defined by symptom-based diagnostic criteria, including various combinations of chronic or recurrent symptoms attributable to the gastrointestinal tract, not explained by other pathologically based disorders [1]. The FGIDs are classified into 6 major categories for adults: oesophageal, gastroduodenal, bowel, functional abdominal pain syndrome, biliary and anorectal. Of these, the functional bowel disorders constitute one of the most common reasons to seek health care [2], and they are associated with poor health-related quality of life [3-5] and substantial costs to society [6-9]. The functional bowel disorders are characterized by combinations of bowel related symptoms (figure 3.1), and include irritable bowel syndrome (IBS), functional bloating, functional diarrhoea, functional constipation and unspecified functional bowel disorder. Moreover, IBS can be further divided into subgroups, namely, IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U) [10]. The pathophysiological mechanisms underlying these disorders are incompletely known, but abnormal gastrointestinal motility, visceral hypersensitivity, altered brain-gut function, low-grade inflammation, psychosocial disturbance, and gastrointestinal microbiota may contribute[11-13].
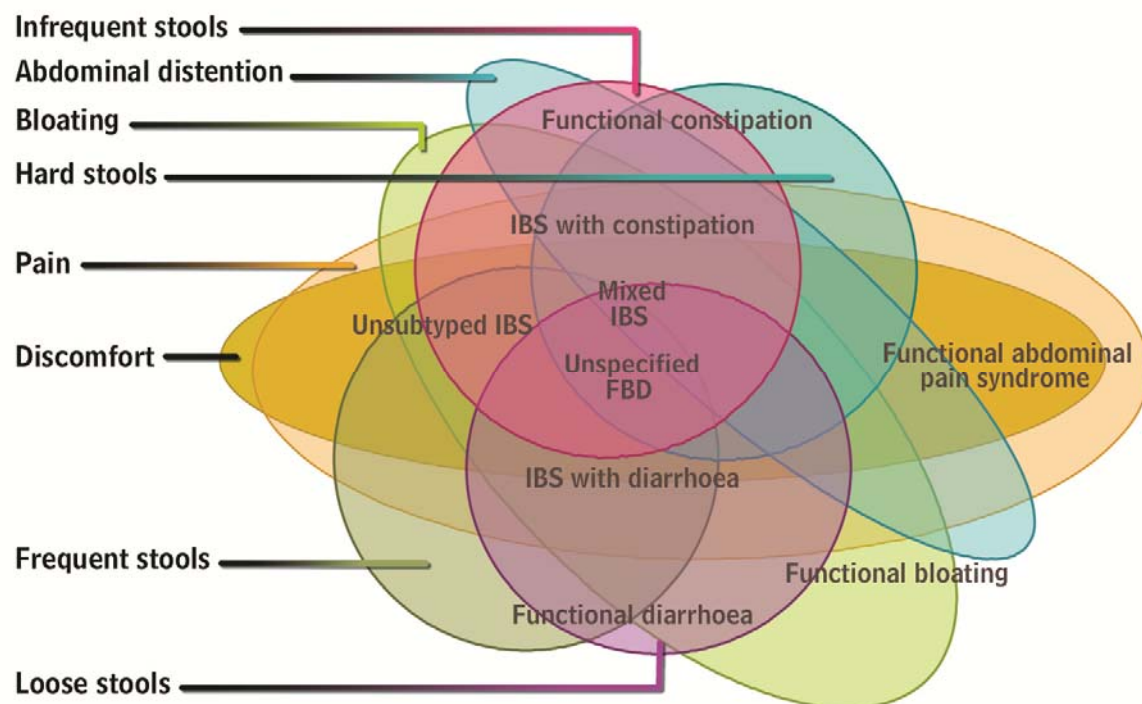


**Fig. 3.1.** Overlap of symptoms defining functional bowel disorders and functional abdominal pain [10, 14].

The human body is inhabited by a complex community of microbes, collectively referred to as microbiota [15]. It is estimated that the human microbiota contains $10^{14}$ cells, which outnumber the human cells in our bodies by a factor of ten[16]. A vast majority of these are found in the gastrointestinal tract, with a continuum from $10^1$ to $10^3$ bacteria per gram of content in the stomach and duodenum, to $10^{11}$-$10^{12}$ cells per gram in the colon[17]. Moreover, the microbial composition differs between these sites[18], and there are also significant differences between the microbiota present in the gut lumen and the microbiota attached to and embedded in the mucus layer of the gastrointestinal tract[19] (figure 3.2).  The microbiota is taxonomically classified via the classical biological nomenclature (figure 3.3), and today more than 50 bacterial phyla have been described, but the colon is totally dominated by three of these: the Firmicutes, Bacteroidetes and the Actinobacteria; other sites display a different microbial composition[20, 21]. It is now apparent that the gut microbiota has co-evolved with us and that it can manipulate and complement our biology in ways that are mutually beneficial, but ecological or genetic changes may also result in diseases[20-22]. A problem for research and clinical work is that most of the microbial diversity in the human GI tract is not currently represented by available cultured species [23], but during recent years, the use of culture-independent techniques to study the gut microbiota has increased the understanding of the role of gut microbiota in health and disease[16].
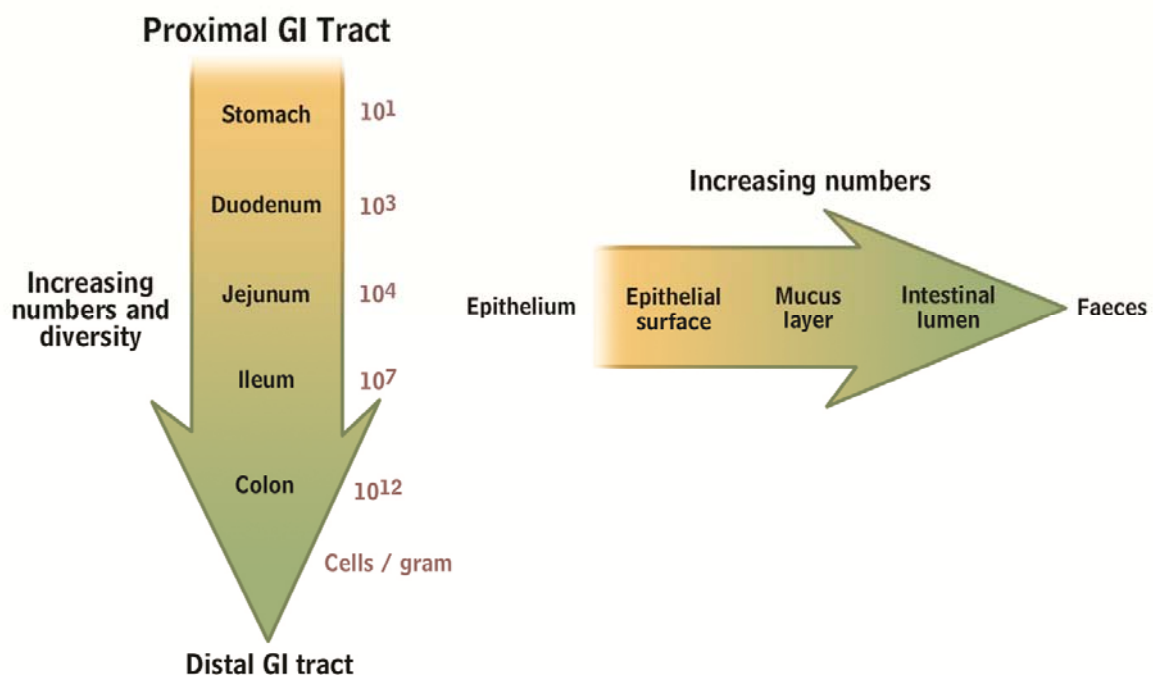


**Fig. 3.2.** Variation in the microbiota composition in the GI tract both with respect to distance from the mouth and form the lumen.

| Nomenclature | Examples |
|---|---|
| Phylum | Firmicutes |
| Class | Bacilli |
| Order | Lactobacillales |
| Family | Lactobacillaceae |
| Genus | *Lactobacillus* |
| Species | *Lactobacillus acidophilus* |

**Fig. 3.3.** Example of bacterial taxonomic classification.

Several lines of evidence now suggest that bacteria may be involved in the pathogenesis and pathophysiology of functional bowel disorders, through the metabolic capacity of the luminal microbiota, and the potential of the mucosa-associated microbiota to influence the host via immune-microbial interactions [24]. For instance, many subjects with IBS report onset of their GI symptoms following an enteric infection[25]. There are also studies reporting positive effects of treatments aiming at manipulating the gut microbiota in patients with functional bowel disorders[26, 27]. Moreover, small intestinal bacterial overgrowth[28], and altered intestinal microbiota[29] have been found in at least subgroups of patients with functional bowel disorders. However, the clinical relevance of these findings is at this stage unclear, and therefore, the aim of this Rome Foundation Working Team Committee was to critically review the existing literature on the role of intestinal microbiota in functional bowel disorders, mainly IBS. Based on this literature search we aim to provide recommendations for how to implement the current knowledge into clinical practice and to guide future research to improve the current knowledge of the role of intestinal microbiota in functional bowel disorders.

## 4. CURRENT KNOWLEDGE OF THE MICROBIOME

**Key points**

- Humans are born with almost sterile intestines but are rapidly colonised by maternal and later close family members' microbiota
- Initially idiosyncratic, gut microbiota show convergence to a norm with age but remain unique to each individual
- Diet has a powerful influence with substantial differences seen between rural, high fibre, low fat diets versus more refined diets seen in industrialised societies
- Host genetics and in particular the host immune response to microbiota strongly influence gut morphology and function

### a. Introduction

Years of co-evolution have allowed the host and the intestinal microbiota to peacefully coexist under steady state conditions. Evidence, mostly from animal, but also from some human studies, supports the concept of a bidirectional interaction between the host and its intestinal microbiome. A conceptual framework is emerging where host genetic and immune, as well as environmental factors, influence intestinal microbiota composition; while, in turn, the intestinal microbiota contributes to shape host immunity and physiology, within and beyond the gut.

### b. Complexity of the human gut microbiota

Humans acquire bacteria randomly from their environment (initially mainly from the mother in a conventional birth) and whether these bacteria are transients or become permanent colonizers depends on genetic makeup, diet, age, infection (reviewed in chapter 6) and lifestyle, including drug intake such as antibiotics. There is a dominant intestinal microbiota community in mammals consisting of thousands of bacterial species that belong to a small number of phyla[20, 30, 31]. These include Firmicutes, Bacteroidetes, and Actinobacteria; with a substantially greater number of Firmicutes compared to the other two in both humans and mice[32]. However, despite conservation at the highest taxonomic ranks, most studies find considerable inter-individual variability as assessed by similarity indices. It is not surprising that several human studies using sequence analysis of cloned small- subunit ribosomal RNA genes [16S ribosomal DNA] have demonstrated a hitherto unimagined complexity of the human gut microbiota of which most identified bacteria have not previously been cultured [23].

It needs to be emphasized that most studies investigating microbiota composition have been performed using fecal samples. A study in healthy volunteers detected differences in the total

number of cultivatable aerobic bacteria in faeces and at the mucosal surface. However, in IBS-D patients the difference found between faecal and mucosal sites was less pronounced[33]. Thus, comparisons between luminal and mucosal-associated intestinal microbiota may yield important pathophysiological insights.

**c. Effect of age on the intestinal microbiota**

The inter-individual differences in intestinal microbiota composition are maximum in the neonatal period and decrease with ageing. Babies are in general considered to be born with sterile intestines but are rapidly colonised. Those born vaginally acquire maternal vaginal and fecal bacteria while those born by caesarean section have reduced *Bifidobacterium spp., Lactobacillus spp.*[34] and *Bacteroides fragilis* with increased risk of *Clostridium difficile* at 1 month of age[35]. Early studies using conventional culture techniques showed breast-fed babies had a simpler microbiota during the first few months dominated by *Bifidobacterium spp* and few enterobacteria while formula-fed babies had more strict anaerobes and a higher diversity of bacteria [36]. Introduction of solid food caused a successive appearance of first enterococci, followed by the strict anaerobes such as *Bacteroides spp.*, *Clostridium spp*. and anaerobic streptococci[36]. A more detailed analysis of healthy babies using a microarray followed by sequencing showed a rapid increase in total bacterial counts from $10^4$ to $10^8$ 16S ribosomal RNA (rRNA) gene copies per gram faeces[137], rapidly reduced by courses of antibiotics. The dominant phyla were in all cases Firmicutes, Bacteroidetes and Proteobacteria , but the precise species were unique to each individual and relatively stable. Early colonisers were mainly aerobes (staphylococci, streptococci and enterobacteria while late colonisers were strict anaerobes (*Eubacterium* spp. and clostridia). As babies grew, "uneven" profiles where one species dominated became less common and their profiles approached the more complex adult pattern. Early sudden changes in individual bacterial numbers were not uncommon even in those not receiving antibiotics, possibly reflecting phage attacks. The same study showed that the efficiency of PCR for bifidobacteria was 8 fold less than for clostridia, leading to systematic underestimation of numbers, a problem that is frequently seen in 16S rRNA gene PCR-based approaches, but often neglected in the biological interpretation.

Denaturing Gradient Gel Electrophoresis (DGGE) of PCR amplified 16S rRNA genes demonstrated that in the first 4-weeks of life preterm infants had rather simple band patterns that increased in similarity (11-57%) with other preterm babies with time. By contrast breast-fed remained more individual with only 11% interindividual similarity[38]. The KOALA project in the Netherlands showed exclusively formula fed babies had more *E. coli*, *C. difficile* and *B. fragilis*, a difference which is lost if the formula contains oligosaccharides as prebiotics[35].

There are few studies of the evolution of faecal microbiota from 1 year to adulthood. One study from India, where most were lactovegetarians, showed relatively stable *Bifidobacterium spp.* up to age 17 followed by a steep decline in adults, while from 2-17 there was a gradual increase in *Bacteroides spp*. to adult levels[39]. *Lactobacillus spp.* declined markedly after age 5 years while *Faecalibacterium prausnitzii* increased and then declined. These changes may well reflect changes in diet though this has yet to be proven.

Extreme old age is associated with a decrease in *Bacteroides spp.* and increases in *Enterococcus spp.* and *E. coli*[40].

**d. Effect of diet on the intestinal microbiota**

Diet is a modifiable factor significantly affecting human health. The interaction between diet and intestinal bacteria begins soon after birth and is a complex process. Human milk contains up to $10^9$ microbes/L including staphylococci, bifidobacteria[41] and lactic acid bacteria (LAB)[42]. It also contains oligosaccharides which act as prebiotics, increasing the number of bifidobacteria in the gut[43]. Therefore, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are now commonly added to baby formula because of their perceived benefits. On weaning, young babies are traditionally fed fermented dairy products containing LAB such as yogurt and cheese. A microbiological study of a randomized controlled trial (RCT) of the effect of a probiotic *L. paracaseii A* in 12-24 month old infants showed minimal changes overall in the faecal microbiota with a small decline in *Clostridium spp*. over 4 weeks and increase in *Lactobacillus spp.*[44]. African children living in rural areas with a polysaccharide-rich diet, when compared with Italian city children, showed a significant enrichment in Bacteroidetes and depletion in Firmicutes together with an increase in *Prevotella spp.* and *Xylanibacter spp.*, bacteria known to contain genes for cellulose and xylan hydrolysis[45]. See Figure 4.1.

## Different Microbiota in Italian vs African Children
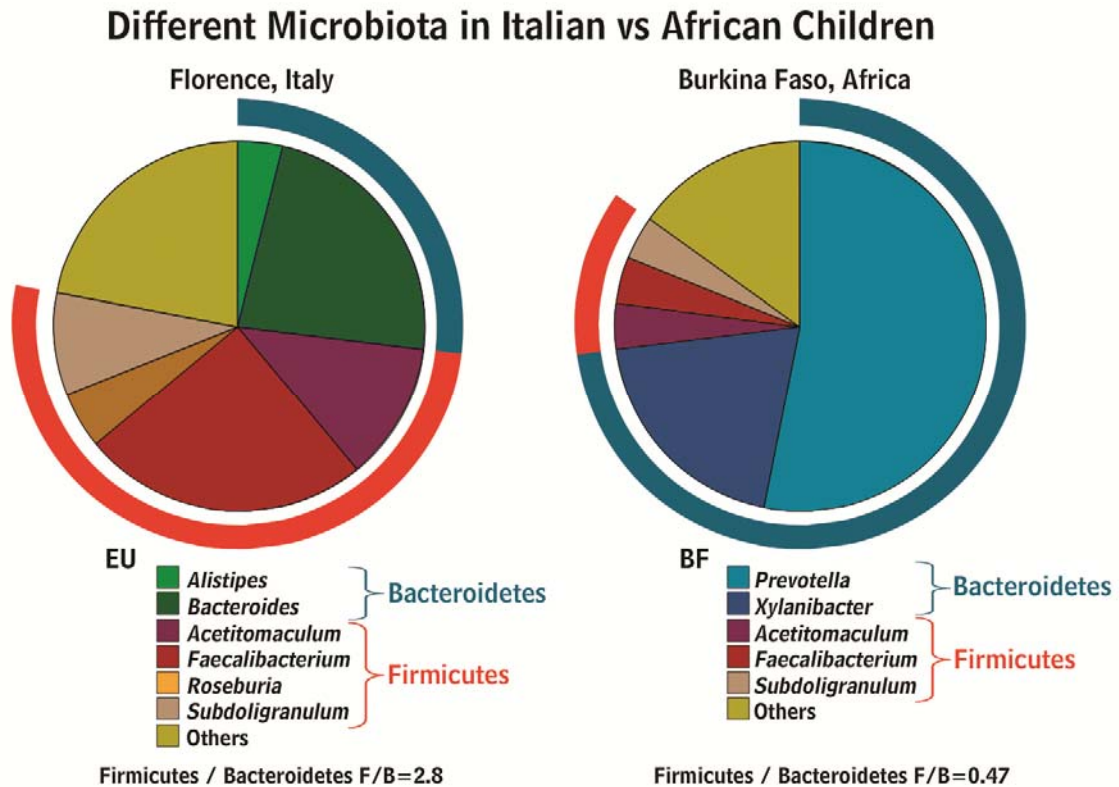


**Fig 4.1.** Gut microbiota composition in African children living in rural areas with a polysaccharide-rich diet when compared with Italian city children [45]. (Redrawn from DeFilippo et al [45])

Adding 48 g of whole grain cereals daily for 3 weeks to the diet in a RCT increased numbers of faecal *Bifidobacterium spp.* and *Lactobacillus spp.* compared with 48gm wheat bran[46]. Conversely removing dietary fibre by using enteral low residue diets in paediatric Crohn's disease showed marked reduction of numbers of *Eubacterium spp.*, *Bifidobacterium spp.*, *Bacteroides-Prevotella* and *Clostridium leptum*[47]. Obesity was in one study associated with a decrease in Bacteroidetes and decrease in diversity suggesting the abnormally high energy input may allow the selective increase of particular species, though in humans few species accounts for more than 0.5% of the total[48].

Clear demonstration of the effect of dietary manipulation on microbiota in adults has been limited by ability to make substantial changes in diet. However, carefully-controlled diets high in resistant starch (RS) have been compared with diets high in wheat bran recently in 14 obese human subjects. This showed a reversible stimulation of two main groups of Firmicutes bacteria among faecal bacteria with additional RS intake [49]. Interestingly, however, despite significant mean changes, responses differed markedly between individuals. Meanwhile, weight loss diets with carbohydrate contents have been shown to decrease one group of butyrate-producing bacteria (*Roseburia* spp. and relatives) as well as faecal butyrate concentrations [50]. Humanised mice have also been used to show

striking changes in the microbiota induced by switch from low fat high plant polysaccharide diet to Western diet[51]. Rats with human faecal microbiota show that brussel sprouts and inulin increase *Bifidobacterium spp*. numbers with an increase in butyrate and beta-glucoronidase activity[52].

There have been several RCTS looking at the prebiotic, oligofructose which increases faecal *Bifidobacterium spp.* and reduces time to resolution of *C. difficile*-associated diarrhoea during antibiotic treatment from 6 to 3 days[53].

Lactulose is a synthetic disaccharide, not absorbed by the small intestine but metabolized by colonic bacteria and widely used as a laxative. The ingestion of lactulose acidifies the proximal colon[54], stimulating propulsive motility[54, 55] and leading to an increase in *Bifidobacterium spp*. counts in healthy humans[56, 57]. A prospective, randomized parallel-group trial to evaluate the effects of lactulose and polyethylene glycol-4000 on colonic microbiota in chronic idiopathic constipation showed that lactulose induced a significant increase in faecal *Bifidobacterium spp*. counts and b-galactosidase activity. The metabolic activity of the faecal microbiota remained stable in the lactulose group, but a significant decrease in total short-chain fatty acids, acetate, butyrate and faecal bacterial mass was observed in the PEG-treated group[58]. It is unclear whether this matter since PEG is at least as effective as lactulose and generally better tolerated[59].

Other prebiotics such as lupin kernel can increase *Bifidobacterium spp.* in humans[60] as can inulin-containing juices[61] and 10 gm of arabinoxylan-oligosaccharides[62].


**e. Effect of transit on the intestinal microbiota**

Modulating intestinal transit by accelerating it with senna or slowing it with loperamide has been used to explore how this alters the microbiota. Faster transit, which may impair absorption in the small bowel and increase available nutrients in the colon, increased faecal sulphate, sulphide, bile acids, methionine and the production rates of acetic acid but reduced fecal methanogens and methane production. The reverse effects were seen with loperamide[63].

Cisapride-reduced transit time was associated with a significant rise in the concentrations of total SCFAs ($P<0.05$), propionic and butyric acids ($P<0.05$) and appeared to alter the microbiota as seen by the increase in the rate of metabolism of beet substrate when incubated with faecal innocula for 24 h[64].

Endogenous bacterially produced SCFAs modulate gut motility to maintain homeostasis. Thus SCFAs activate propulsive ileal motor patterns in both dog[65] and human[66], a pattern which ensures that bacteria and their products are scarce in the ileum and largely confined to the colon. Recent data indicate that there are specific G protein coupled receptors, GRP41 and 43, located in the distal ileal and colonic enteroendocrine and mast cells which detect SCFAs and whose activation leads to changes in motility[67]. Acetate, which predominates in the colonic contents, is largely inhibitory while

propionate and butyrate stimulate motility. The GRP43 positive enteroendocrine cells contain PYY, a hormone which slows gastric emptying and inhibits intestinal motility and secretion. In addition to these acute effects there are also longer term trophic effects on the enteric nerves. Chronic exposure to resistant starch or intracaecal infusion of butyrate increases the proportion of nerves expressing choline acetyl transferase (ChAT) and enhances cholinergic muscle contractility[68]. Thus the circuitry exists whereby colonic bacteria, through their metabolic products can modulate motility and hence their own environment.

### f. Effect of host genes on the intestinal microbiota

Similarities in microbiota composition in homozygotic twins raise the possibility that host genotype can affect the gut microbiome[69]. Twin studies comparing those concordant or discordant for obesity show strong interfamily similarities in microbiota unrelated to BMI[48] and using genetically modified animals, it is possible to show clearly how host mutations in bacterial sensing molecules can alter the composition of the microbiome[70, 71]. Genus-specific 16S rRNA analysis demonstrated that Bacteroides were barely detectable in wild-type and heterozygous control mice but, in mice deficient of the bacterial sensing receptor nucleotide oligomerization domain (NOD)-2, significant amounts of Bacteroides as well as Firmicutes were detected. This raises the hypothesis that genetic profiles associated with certain inflammatory conditions of the gut may contribute to selection of specific microbiome components with pro-inflammatory or altered metabolic capacity. This may be particularly relevant to the patients with Post infective IBS which has been associated with certain single nucleotide polymorphisms (SNPs) in the bacterial recognition receptor Toll-like receptor (TLR)9 [72].

### g. Effect of the immune system on the intestinal microbiome

Innate and adaptive mechanisms can aid in the control and establishment of a balanced intestinal microbiota. α-defensins or cryptidins are antimicrobial peptides produced in Paneth cells in the small intestine against enteric pathogens. Recent reports have indicated increased levels of defensins in patients with IBS though at present its significance is uncertain[73]. Significant changes in microbiota composition were detected mice expressing a human α-defensin gene (DEFA5) and in mice lacking matrix metalloproteinase 7 (MMP7) an enzyme required for the processing of mouse α-defensins[74]. Activation of TLRs by commensals is important for the maintenance of gut homeostasis via the stimulation of cytokines and epithelial reparative factors[75], and adaptive immunity cooperates with innate immunity to maintain host-commensal homeostasis[76]. The absence of normal secretory IgA (IgAs) has been shown to lead to a significant shift in anaerobe populations in the small intestine in

mice[77]. In addition, IgAs limit translocation of commensal bacteria to mesenteric lymph nodes[78], and mice lacking sIgA develop low-grade inflammation in the gut[79].

**h. Host-microbiota bidirectional interaction:**

***h.i. The intestinal microbiome is a driving force for gut immunity and physiology***

Discrimination between commensals and pathogens is in part achieved by a family of receptors that recognize bacterial components. Two major classes of innate receptors have been identified: 1) TLRs involved in the detection of extracellular bacterial components[80-82] and 2) cytosolic NOD-like receptors (NLRs) that detect intracellular bacterial components[83]. Bacterial pattern recognition receptors are crucial for maintenance of host-microbial homeostasis and play a key role in the innate immune response that is responsible for retaining the intestinal microbiota in the mucosal compartment[75]. Commensal bacteria regulate innate defense and extrinsic components of the intestinal barrier such as mucus production and secretion of antimicrobial peptides by intestinal Paneth cells[84-86]. The intestinal microbiome induces the development of both the mucosal and systemic immune systems. In germ-free mice, there is a paucity of lamina propria T cells, IgA producing B cells, and intraepithelial T cells. The absence of microbiota also affects systemic immunity since germ-free mice have decreased serum immunoglobulin levels and CD4 T-cells in the spleen[78, 87]. It has been determined that commensals induce a local, mucosal immune response without activating systemic immune responses. This "systemic ignorance" allows the preservation of systemic immune responses to commensals in the event that small breeches in mucosal barrier occur[78].

Evidence that intestinal bacteria are important for normal gastrointestinal function has been demonstrated in animal models under germ-free conditions that are generated and bred within a sterile environment. The immune system of germ-free animals is underdeveloped and introduction of commensals induces significant histological, metabolic and functional changes in the host[78, 88-90]. Intestinal motility is markedly abnormal in germ-free animals. The intervals between phase III fronts of the migrating motor complex (MMC) are prolonged, and upon colonization with a specific pathogen-free (SPF) microbiota, motor patterns are normalized[91, 92]. Normalization of motor patterns does not occur with all species of the intestinal microbiota, and animals that are monocolonized with *E. coli* exhibit opposite effects than those colonized with lactobacilli, bifidobacteria, or clostridia[93] Colonization with a common commensal, *Bacteroides thetaiotaomicron*, modifies, in addition to a multitude of genes involved in immunity and barrier function, the expression of genes involved in motility and neurotransmission[94]. This selective modulation of gut motility by components of the intestinal microbiome raises therapeutic possibilities to modulate altered bowel habits using probiotic bacteria. Studies in animal models of IBS have demonstrated that the probiotic strain

*Lactobacillus paracasei* NCC2461, its secreted products, or metabolites, modulate contractility of intestinal smooth muscle[95]. The probiotic strains belonging to *L. rhamnosus* R0011 and *L. helveticus* R0052 improved gastric emptying in a model of post-infectious gastric dysmotility[96]. Conditioned media from *E. coli* Nissle 1917 was shown to modulate contractility of muscle strips isolated from humans[97]. Other studies have shown that co-administration of conditioned media from *L. paracasei* NCC2461 with antibiotics reduced visceral hypersensitivity associated with antibiotic treatment and normalized sensory neurotransmitter expression in the myenteric and submucosal plexuses[98]. *L. acidophilus* NCFM and *L. paracasei* NCC2461 have also shown capacity to modulate visceral and pain perception in other models of visceral hypersensitivity[99, 100]. It is likely that the pathways affected by these specific probiotics differ according to the strains and model used, and their effectiveness in attenuating visceral pain in humans remains to be determined.

Direct proof that the intestinal microbiome determines gut function in humans is lacking however there is good evidence that bacterial metabolites particularly SCFAs do as already discussed (see section e), and indirect evidence from studies in patients receiving antibiotics (see Chapter 8).

### h.ii. The intestinal microbiome and the gut-brain axis

Gastrointestinal function is controlled by the central nervous system (CNS). The gut brain axis is a bidirectional communication system that integrates brain and gastrointestinal (GI) functions. Evidence for a microbiota–gut–brain axis that influences brain biochemistry and modulates behaviour in adult mice comes from a recent study showing that transient perturbation of the microbiota with antimicrobials increased hippocampal BDNF and exploratory behavior. These changes were reversible upon normalization of the microbiota after antimicrobial treatment. Interestingly, antimicrobials did not affect behaviour in germ-free mice, but colonization of germ-free mice with a specific pathogen free (SPF) microbiota altered behaviour. Moreover, using two germ free mouse strains as recipients, and a cross-over design, SPF microbiota from mouse strains with opposite behavioural phenotype, determined behaviour and brain BDNF level in the recipient mice[101]. This is an exciting new area that raises the hypothesis of a critical contribution of the intestinal microbiota in behaviour and central neurotrophin expression. These results may have important clinical implications for the understanding of IBS and its psycho-social co morbidity (reviewed in Chapter 7).
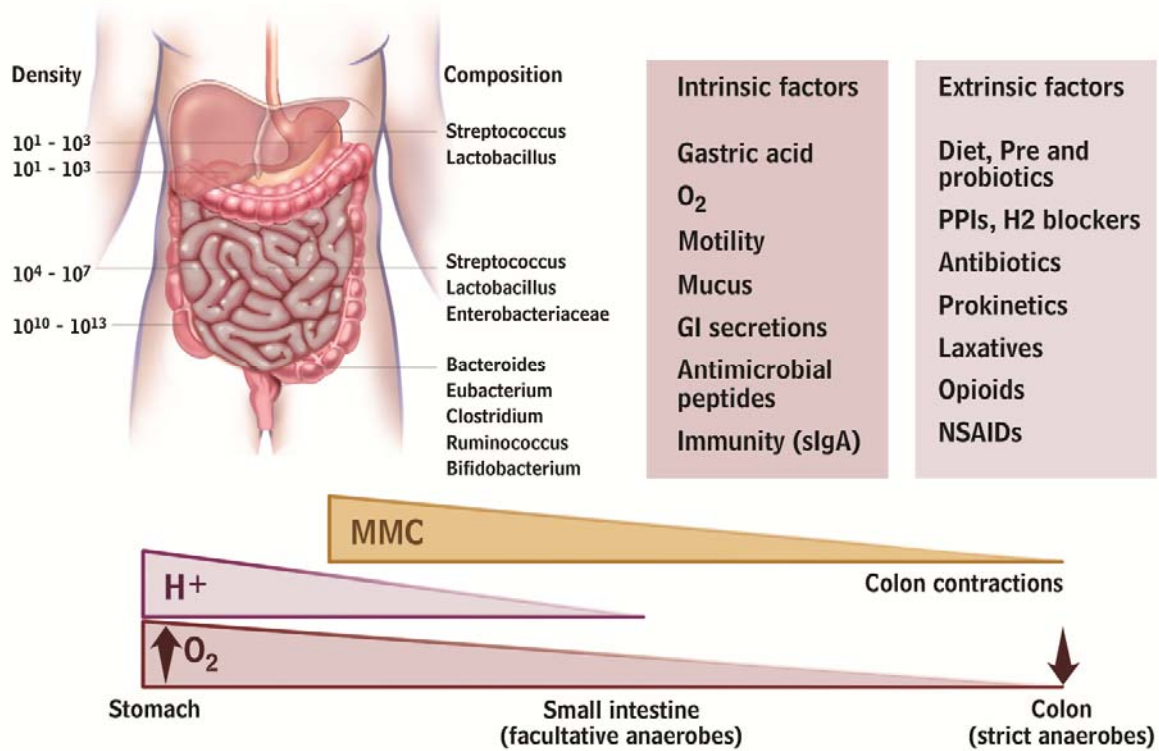
**Fig 4.2.** Gut microbiota and the intrinsic and extrinsic factors that can affect its distribution and composition. A number of host mechanisms participate in gut microbiota modulation, including gastric acid secretion, fluid, anti-commensal sIgA and antimicrobial peptide production, and GI motility. Drugs that block acid secretion and affect GI motility, can indirectly alter the microbiota. Antibiotics, depending on spectrum and dosage, will directly affect microbiota composition inducing dysbiosis. Dietary modifications, including probiotic and fibre supplements will also affect microbiota composition. (MMC: migrating motor complex; PPI: proton pump inhibitor; NSAID: non steroidal anti-inflammatory drugs).

# 5. APPROACHES TO THE STUDY OF MICROBIOTA

**Key points**

- Breath tests are not validated to accurately detect SIBO.

- Rapid molecular approaches have largely replaced cultural approaches for enumeration of the dominant GI tract microbiota; nevertheless cultural microbiology remains crucial for investigating microbial diversity and for the selective isolation of representatives of key functional groups, including pathogens

- Culture-independent approaches to study the GI tract microbiota have revolutionized our insight into this complex microbial ecosystem.

- The possibilities of using high throughput approaches and their depth of analysis are increasing explosively, but it is important they are applied with careful reference to well-defined scientific questions.

Interest in the GI tract microbiota in relation to health and disease is increasing as it is evident that the microbiota plays a crucial role. For more than a decade insights into the GI tract microbiota have increased which is in part due to the emerging developments in culture-independent technologies to study different aspects of the microbiota (Table 5.1). This chapter will provide an overview of the different approaches with focus on its application in GI tract microbiota research.

## a. Breath testing

*a.i. Overview.* The lactulose (LBT) or glucose (GBT) breath tests have become the most widely used breath tests to determine small intestinal bacterial overgrowth (SIBO) in for example patients with irritable bowel syndrome (IBS) [28, 102-104]. These tests involve serial measurements of breath hydrogen ($H_2$) or methane gas ($CH_4$) levels following the ingestion of a standard dose of the sugar substrate. A complete technical review of these tests has recently been published[105]. Other sugar substrates and radio-labelled sugars, including fructose, sucrose, and xylose, as well as bile acids have also been used in a small number of studies. They do not offer any apparent advantages [106] and will not be discussed further. The results of the LBT in IBS studies, and to a lesser degree the GBT, were pivotal in developing the concept that SIBO is associated with the irritable bowel syndrome (IBS) [102, 107-110]. This has led to much greater use of these diagnostic tests in North America and Europe for the assessment of patients with IBS. While the results continue to be promoted as a rationale for using antibiotics to treat these patients [110-112], there is considerable controversy

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concerning their accuracy for diagnosing SIBO in IBS patients and hence their contribution to understanding the pathogenesis of IBS [113-116].

***a.ii. Rationale underlying the use of the GBT and LBT in IBS patients.*** The proposed rationale for using the LBT and GBT tests is based on the fact that $H_2$ and $CH_4$ gas are produced almost exclusively in the gastrointestinal tract [117] and that bacteria (colonic microbiota) can produce measureable changes in breath $H_2$ gas levels within a few minutes of contact with small quantities of these sugar substrates [118, 119]. For example, in healthy control subjects where lactulose was infused directly into the cecum or just a few cm proximally within the distal terminal ileum, and simultaneous measurements of breath $H_2$ were obtained, infusion of 0.5 g lactulose (i.e. 5% of 10 g test dose) was sufficient to increase $H_2$ gas levels 5-10 parts per million (PPM) in breath samples within 2 min [118]. Thus, if abnormal numbers and/or possibly microbial species were present in the small intestine this would theoretically lead to an early rise in $H_2$ and/or $CH_4$ production. The use of the LBT versus the GBT also has implications for detecting SIBO in IBS because of the differences in the transit of glucose and lactulose in the small bowel [102, 105, 109]. Glucose has been reported to provide a better measure of fermentation in the proximal small intestine because it can be absorbed before reaching the distal small bowel whereas lactulose is a non-absorbable sugar and therefore also provides a measure of fermentation in the distal small bowel or colon. Proponents of the SIBO hypothesis in IBS have suggested the pivotal event is the migration of colonic microbiota into the distal small intestine in these patients and therefore the LBT would be a much more accurate means of detection [109, 110, 117]. This reasoning however has not been consistently applied in the literature. For example, the same authors have argued that using glucose instead of lactulose can be problematic [106, 110] and may explain why others have not found evidence for SIBO in IBS [120], yet in their recent meta-analysis of breathing testing in IBS, 6 of the 11 studies examined used glucose or a related sugar other than lactulose. The results of this analysis were then used to suggest, despite their conclusion in yet another study that currently there are no tests that can accurately diagnose SIBO [106], that breath testing is merited in IBS and that the results support the idea that SIBO is important in IBS [104, 110].

The separate measurement of $CH_4$ gas in LBTs and GBTs may also be important because changes in this gas were strongly correlated with constipation-predominant IBS [121], methanogens may falsely lower $H_2$ gas levels, $CH_4$ gas may play a pathogenic role in constipation, possibly by inhibiting motility[122], and may provide a useful measurement in patients where changes in $H_2$ gas is not detectable [105]. However, this association is also found in non-IBS constipated subjects and in high numbers of controls and thus the significance of this finding in IBS remains to be clarified [102].

***a.iii. Summary of Test Criteria and Study Results.*** In IBS studies, a number of criteria have been used to define an abnormal breath test.  In studies employing the LBT, the most commonly used criteria for $H_2$ gas were:  1) a double peak rise in $H_2$ (variably defined), 2) a rise in $H_2 > 20$ PPM by 180 min, or 3) a rise in $H_2 > 20$ PPM by 90 min (which currently appears to be most widely recommended) [102, 123]. Criteria for changes in $CH_4$ gas have also been variable between studies with some studies using a rise of 1 PPM or more by 90 min after lactulose ingestion [108] or a rise in $CH_4 > 20$ PPM [121] in the same time period.  Similarly, various criteria for $H_2$ gas have been used to define an abnormal GBT but most commonly it has been defined as a rise in $H_2$ of 12 PPM above baseline [104 105].  When these criteria were applied in LBTs in the early clinical trials, a very strong association between SIBO and IBS was reported, with prevalence rate as high as 84% [123].   Two meta-analyses of studies using breath testing to diagnose SIBO in IBS have been performed, one evaluated 12 studies (1921 patients) [104] and the other 11 studies [28].   Both studies reported that abnormal breath testing was more common among IBS patients than healthy controls (OR = 4.46, 95% CI = 1.69 -11.80 and OR = 3.45, 95% CI, 0.9-12.7, respectively).   However, there was tremendous heterogeneity in the studies, including study design, the type of sugar substrate ingested, and criteria used to define a positive test, as well as evidence of a possible publication bias, and as a result, the implications of the findings are unclear.

***a.iv. Limitations of the Breath Tests.*** Although there are technical issues [105] and other possible confounding variables, the critical limitation of breath testing for diagnosing  SIBO in IBS patients is the lack  of validated criteria to accurately define an abnormal test [102, 105, 106].  As highlighted above, these criteria have varied widely between investigators and even in different studies conducted by the same groups [102, 124].  The negative implications of using criteria which had not been validated were underscored by several studies which compared the prevalence of an abnormal LBT test in IBS patients and healthy controls.  These studies found no difference in the prevalence of positive tests between IBS patients and controls, regardless of the criteria applied [115, 116, 124, 125].

There have been very few attempts to validate specific criteria, in large part because there is no readily accepted gold standard.  Traditionally, cultures obtained from small bowel aspirates have been considered the gold standard (> $10^5$ CFUs/ml colonic microbiota) [117].  This test has been criticized because of technical difficulties in ensuring accurate cultures and that these cultures would not be representative in IBS patients because the overgrowth occurs in the more distal small intestine where aspirates cannot be readily obtained [106].   However when this technique has been applied in IBS patients [115], no increase in overgrowth was detected, although a post-hoc analysis using a cut-off of  $\geq 5 \times 10^3$ CFU/ml detected in increase IBS patients (43% ) vs. controls (12%). Another approach has been to measure combined LBT with oral $^{99m}$ Tc scintigraphy to determine whether the rise in $H_2$ occurs before or after the test meal reaches the cecum and more distal large

bowel [114].  This study found that 63% of the 40 Rome II positive IBS patients had an abnormal LHBT and that, in 88% of cases (22/25), the $^{99m}$Tc had > 5% accumulation in the cecum before the rise in $H_2$ occurred.  This suggested that the "abnormal" LHT reflected variations in small intestinal transit in most cases rather than the presence of SIBO.

*a.v. Summary.* In summary, breath tests do not provide an accurate means of diagnosing SIBO due to the absence of proper validation studies of the diagnostic criteria; they should not be used in routine clinical practice to screen for or confirm presence of SIBO.  The use of a test - treat - test/outcome paradigm [112]  may also problematic because antibiotics will also suppress microbiota in the colon, which could account for changes in subsequent  testing and or symptoms, and thus obscure the true meaning of an altered post-antibiotic result.  It may be advisable not to use breath tests in clinical care settings for IBS until they are validated. If SIBO is suspected, the GBT may be the most specific test, particularly if high thresholds are applied, because it is not as vulnerable to changes in small bowel transit compared to the LBT.

**b. Culture-based approaches to microbial diversity and enumeration**

Culturing of GI tract microbes has for many decades been the gold standard for detection and classification of microbes from the human GI tract. A major advantage of cultivation of microbes is that pure cultures of microbes are obtained and hence, their function can be studied in detail with respect to their physiology, genetics and interactions with hosts and other microbes in controlled experiments. However, numerous molecular surveys of the human faecal and colonic microbiota have reported that 70-80% of the phylotypes, i.e. species defined by 16S rRNA sequencing (see paragraph 5e for details) do not correspond to well characterized, cultured species of bacteria [23, 49, 126].  On the other hand, it appears that the most abundant phylotypes are also the most likely to have been cultured. Walker *et al* [127]found that 66% of phylotypes that individually accounted for >0.5% of sequences corresponded to cultured species, while only 30% of those accounting for <0.5% of total sequences corresponded to cultured species. This suggests that the majority of bacterial cells in normal faecal samples will be related to cultured species, whilst it is the rarer species that are under-represented by cultures. This also suggests that a lack of well-characterized isolates, rather than any intrinsic resistance to cultivation, may be an additional reason for the low coverage of gut microbial diversity by cultured strains.

With the advent of sequence-based molecular methodologies there is a tendency to dismiss culture-based approaches as 'old technology'. In the past enormous effort went into describing the human faecal microbiota using careful anaerobic cultivation methods (eg.[128-130]). Although it has been widely assumed that cultivation methods must severely bias the bacteria that can be recovered, recent work

suggests that the dominant species described in such studies correspond quite closely to the most dominant species detected by molecular surveys[127]. Conversely clone libraries of amplified 16S rRNA sequences have tended to underestimate certain groups such as bifidobacteria that are readily recovered by cultivation [131].

The sheer laboriousness of cultural approaches makes it unlikely that large scale anaerobic culture work will be undertaken as an approach to describing microbial diversity in the future. On the other hand, viable counts offer a straightforward estimate of total bacterial numbers in gut samples. Total anaerobe counts estimated in faecal samples from healthy adults using 'non-selective' media typically yield >$10^{11}$ cfu/g compared with around $10^8$/g for facultative anaerobes[132] although facultative numbers are higher in the elderly [133]. Total bacterial numbers can also be estimated by DAPI (4',6-diamidino-2-phenylindole) staining and by FISH (fluorescence in situ hybridization) microscopy using total bacterial probes, giving numbers that are of the same order [132] or somewhat higher [134] than those obtained by viable counting. Most other molecular methods such as qPCR and direct sequencing either do not give reliable estimates of total numbers, or give only relative proportions. Selective plating methodologies using anaerobic media include Beerens medium, considered selective for bifidobacteria, and MRS medium considered selective for lactobacilli and lactococci [133, 135, 136]. Verifying that counts obtained on such media correspond to a given phylogenetic group, however, requires further molecular analysis of the colonies obtained. Another problem is in verifying that all representatives of a given groups are equally likely to be recovered on the selective medium. For these reasons anaerobic selective plating has been largely discontinued as an approach to the enumeration of particular phylogenetic groups, being replaced by molecular methodologies, although its value in describing functional groups of gut bacteria remains crucial as discussed further below. In principle, improved selective approaches could be developed in the future for enumerating key groups of obligately anaerobic bacteria. This would however require a detailed knowledge of growth characteristics that is currently lacking. It has proved possible to detect single strains of butyrate-producing strict anaerobes when introduced into a background of total human gut bacteria in fermentor simulations, but this relied on introducing antibiotic resistance mutations [137].

In contrast, selective culture-based approaches remain of great value for the enumeration of specific groups, especially the many important pathogens that are facultative anaerobes. The predominant obligate anaerobes are eliminated under aerobic conditions, and media can be made highly selective for the relevant target organisms. Furthermore they allow detailed strain typing and characterization, which is particularly important for pathogens. Magnetic beads carrying antibodies that selectively bind a given serotype have also been used to enhance recovery of bacteria present in low numbers eg. for *E. coli* O157 [138].

### c. Isolation and function-based molecular detection

An important benefit from the ability to culture micro-organisms is that functions can be identified and putatively associated with phylogroups defined by 16S rRNA (or other) sequences [139]. Relevant functions that appear to show limited phylogenetic distribution among organisms that colonise the human large intestine include methanogenesis (confined to certain *Archaea*) and sulphate reduction (certain genera of Proteobacteria). Functions such as butyrate production or lactate utilization occur within several different phylogenetic groups, but particular subgroups that are responsible for these traits can be identified and independently targeted [140]. 16S rRNA –based probes and PCR detection methods that target relevant functional/phylogenetic groups have now provided important information on responses to dietary change and disease states in a number of studies [141] [142, 143].

The use of selective culture media provides an important approach for estimating populations of organisms concerned with particular functions, especially the utilization of specific substrates[132, 144] or cross-feeding of fermentation products [145]. This has led to the isolation and description of new species with defined functions, e.g. the utilization of lactate [146], mucin [147] or cellulose [148]. Isolations from non-selective media have also been used to identify organisms that produce specific short chain fatty acids [149]. Isolated strains are providing fundamental information on substrate utilization, and on microbe-microbe and microbe- host interactions for key members of the microbial community [139, 150].

Valuable functional information can also be obtained from studies that combine cultivation of the mixed community with molecular analyses. One such approach is stable isotope probing, in which the substrate of interest is labelled, usually with $^{13}$C, and incubated with mixed faecal microorganisms. Organisms that utilize the substrate will incorporate $^{13}$C label into their RNA and DNA, and the labelled nucleic acid can be recovered after density gradient centrifugation. Amplification and sequencing of 16S rRNA genes can then be used to identify the organisms involved. This approach has been applied successfully to utilization of starch [151]. Another approach allows identification of organisms that selectively adhere to insoluble substrates [152]. Mixed faecal bacteria are incubated with substrate in a fermentor system, and attached bacteria again identified by 16S rRNA sequencing.

### d. Functionally relevant gene targets

One application of functional information from cultured strains has been to identify functional gene targets that can be used for culture-independent molecular enumeration. In this approach, the molecular characterization of the community is not based on species or phylotype identity, but on functional properties. This is extremely useful for functions that can be performed by micro-organisms from different phylogenetic groups or functions that are only represented in specific

strains from a species, since these functions cannot be directly extrapolated from species or phylotype data. For example, it has been shown that most isolated human colonic bacteria that produce butyrate rely on the butyryl CoA: CoA transferase gene for the final step in butyrate formation [153, 154]. Degenerate PCR primers have now been developed that allow the amplification of this gene from phylogenetically diverse Gram-positive bacteria [155]. Other examples of this approach are provided by the sulfatase [156] and *Archaeal mcrA* genes [157]. While the same information could in principle be obtained by metagenomics, such targeted approaches can be quicker and cheaper and remove the need for extensive bioinformatics.

**e. From culturing to 16S rRNA gene characterization of microbial communities.**

Although large numbers of bacterial strains are continuously isolated with help of recent innovations in high throughput microbial cultivation [158] and targeted cultivation of microbial groups with specific functions such as butyrate production [142] or mucin degradation [147], it is evident that laborious cultivation approaches are not suitable for studying the diversity and population dynamics of the gastrointestinal tract microbiota. Therefore, approaches that do not rely on selective cultivation are indispensable to obtain insight into the true diversity. About two decades ago, Carl Woese [159-161] discovered ribosomal RNA (rRNA) to be an extremely useful phylogenetic marker for microbial identification and systematics and this has led to the discovery of the *Archaea* as the third domain of life and the construction of the famous tree of life [159, 162].  rRNA is part of the ribosomes, which are also called the "factories" of protein synthesis. Since ribosomes are universal in structure and function in all life forms, it has very low mutation and transformation rates. Therefore, differences in rRNA sequences are inversely correlated to the relatedness between the organisms they derive from and hence, rRNA is ideal for phylogenetic identification of organisms. From the three rRNA molecules the small subunit (SSU) RNA or (16S rRNA in *Bacteria* and *Archaea*, 18S rRNA in  *Eukarya*) is the most widely used marker due to its manageable size (approximately 1.5 kb) and its discriminative power. It contains nine hypervariable regions that enable identification and differentiation of specific species. Since 16S rRNA and its corresponding gene can be directly obtained from any environmental sample without cultivation procedures, it has been recognized as a marker to detect and identify basically all members of the respective ecosystem, including the microbes that have never been obtained in culture. This has led to the new research expertise called molecular microbial ecology and as a result the number of nearly complete 16S rRNA sequences in the databases is approximately 1.5 million, which is far more than what can be found for any other gene [163]. A variety of 16S rRNA based approaches has been developed and include classical cloning and sequencing, fingerprinting, FISH, and qPCR, which have been employed to determine the bacterial diversity of ecosytems or to detect

and quantify specific bacterial groups in the human GI tract [164]. It has to be realized that none of the approaches gives an unbiased view of the diversity of the microbiota and abundance of its different members. Therefore, it should be noted that these nowadays called classical approaches are not competitors of each other, but can best be seen as complementing approaches as they differ in terms of sensitivity, selectivity and phylogenetic resolution (Figure 5.1).

The most commonly used 16S rRNA approach is the PCR amplification of 16S rRNA genes followed by subsequent cloning and sequencing to directly access the phylogenetic diversity of bacteria within the GI tract using universal primers that cover most currently known microbes [164]. This classical approach still provides the highest phylogenetic information as nearly complete 16S rRNA genes are sequenced. Exploring diversity based on sequencing 16S rRNA genes requires a novel different classification system because cultivation is not involved. Therefore, classical taxonomic approaches, which include physiological characterization, cannot be applied to classify uncultured microbes into species. As the 16S rRNA gene is the only marker to detect uncultured microbes, classification is based on comparative sequence analysis that results in species-level phylogenetic types or phylotypes. Phylotypes are defined as groups of 16S rRNA gene sequences with a certain level of similarity. Phylotype definition is based on a cut-off value of 16S rRNA sequence similarity and currently that is not consistently used with variations between 97 and 99%. As a consequence different cut-off values will result in different estimates of diversity. Moreover, it has to be emphasized that 16S rRNA gene sequences and their copy numbers may vary within the same microbial genome and hence it is difficult to extrapolate 16S rRNA gene numbers to actual cell numbers. Cloning and sequencing of 16S rRNA genes has been used to characterize a wide variety of ecosystems, including the GI tract.

Sequence specific fingerprinting of 16S rRNA genes has also been frequently applied in studying the GI tract microbiota composition and population dynamics[164]. These approaches do not provide direct insight into the microbiota composition, but provide a so-called fingerprint of the 16S rRNA gene diversity within a sample. Denaturing- and Temperature Gradient Gel Electrophoresis (D/TGGE) as well as terminal-restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes are the most commonly applied approaches. Sequence specific separation of 16S rRNA amplicons by D/TGGE relies on the melting behaviour of melting domains in the amplicons within a temperature or chemical gradient of a gel while T-RFLP based separation relies on the size of amplicons after being cut by sequence specific restriction enzymes [164]. This results in a banding pattern that represents the diversity of the different rRNA gene sequences present in the sample. The intensity of a band is a semi-quantitative measure for the relative abundance of this sequence in the population. The banding profiles do not provide any phylogenetic information unless amplicons are excised from the gel and sequenced. The benefit of fingerprinting is that multiple samples can be easily compared and

provide information about the overall community and its dynamics. Moreover, fingerprinting can monitor population dynamics of the total community as well as that of specific groups of microbes, which can be determined by the choice of primers [165-168].

Fingerpinting of 16S rRNA genes or other approaches based on classical PCR do not provide quantitative information about the microbiota. Real-time or quantitative PCR (qPCR) on the other hand is a method that is used to quantify the amount of DNA or a gene-of-interest present in biological samples. During qPCR the amount of DNA produced at the end of each amplification cycle is quantified using fluorescent technology. In contrast to regular PCR, qPCR focuses on the onset of the logarithmic phase (threshold) of PCR product accumulation rather than the end-point abundance of PCR product since this is a more accurate estimate of the amount of PCR product obtained because it is less affected by the amplification efficiency of the reaction or depletion of reagents. The power of 16S rRNA gene-specific qPCR is that it can be used to quantify any population of interest and can target even low abundance microbes [169, 170]. However, it remains difficult to extrapolate 16S rRNA gene copies to actual numbers due to various numbers of gene copies per species and cell lysis efficiency that can be different in environmental samples compared to pure cultures. Another 16S rRNA-specific quantification approach is Fluorescent *in situ* hybridization (FISH). This is a technique using a specific fluorescent oligonucleotide probe binding to rRNA in whole cells to detect, identify and enumerate bacteria in complex ecosystems. FISH using an array of 16S rRNA oligonucleotide probes can be combined with automated microscopy-based enumeration or flow cytometric enumeration[171-173]. FISH is the currently the most accurate quantification method as it targets whole cells rather than extracted DNA. However, cell permeability and ribosome accessibility are crucial for FISH and the efficiency differs between species. Moreover, FISH quantification of bacteria in tissue is difficult.

These classical 16S rRNA (gene)-based approaches have already been frequently applied to characterize the human GI tract microbiota and due to their relatively low throughput the approach of choice depended largely on the research question to be addressed. The combination of these approaches in the different studies resulted in a phylogenetic framework of the GI tract microbiota. The pioneering study by Suau and colleagues demonstrated the high diversity of the GI tract microbiota of which the fast majority has not been identified by culturing [49]. These findings have been confirmed repeatedly, including a recent extensive study that looked at faecal and colonic mucosa-associated communities [23]. A striking observation with most of the studies of cloning and sequencing approaches have underestimated the number of bifidobacteria and other bacteria belonging to the phylum Actinobacteria, which is likely due to the mismatches of the most frequently used "universal primers" to target them in combination with the high G+C content in the genomes of

Actinobacteria. This became evident when FISH is used to quantify different groups of microbes, as has been demonstrated by FISH using SSU rRNA-targeted oligonucleotide probes [171, 174]. The first sequence-specific fingerprinting of GI tract communities demonstrated that the dominant microbes are host-specific and relatively stable in time in healthy adults [175], which has been confirmed frequently. In contrast, GI tract community structure and that the microbiota composition is unstable when individuals are suffering from intestinal disorders, such as inflammatory bowel diseases (IBD) [176-180].

Faecal samples have the benefit that they contain a dense microbiota and that they can be easily collected without invasive procedures. However, a major disadvantage is that feces only represents the end of the colon as has been demonstrated by comparative analysis with mucosal biopsies from the intestine [23, 181-185]. Moreover, microbiotas of the small intestine are different in composition compared to those from the colon, which is most likely explained by the different conditions microbes encounter in the respective parts of the GI tract [183, 186, 187]. This indicates that care must be taken when conclusions about the microbiota in relation to health, disease and diet are solely based on fecal sample analysis.


**f. High throughput 16S rRNA characterization of the microbiota**

Since the past years classical 16S rRNA gene based approaches have been complemented by novel high throughput approaches. These novel approaches provide deeper insight into the microbiota composition and enable characterization of multiple samples in a high throughput manner, allowing more and better powered observations. These high throughput approaches include phylogenetic microarraying and barcoded pyrosequencing. Phylogenetic microarray analysis is based on arrays spotted with 16S rRNA targeting oligonucleotides that are developed on 16S rRNA gene sequences that exist in DNA databases. The barcoded pyrosequencing approach is different because it does not rely on current 16S rRNA gene sequences in databases, but is based on the novel sequencing tools, 454 pyrosequencing, and hence can be considered as de novo community profiling.

The principle of phylogenetic microarray analysis is similar to those of comparative genomics and transcriptomics. The principle of phylogenetic microarraying is based on a DNA microarray, a glass or membrane surface of a microscopic slide that is spotted with thousands of covalently linked 16S rRNA-gene specific probes, which are subsequently hybridized with DNA or RNA for diversity analysis. In this way thousands of 16S rRNAs or their respective genes can be specifically identified and quantified in a single experiment. After publication of the first phylogenetic microarray, specific for nitrifying bacteria, these microarrays have been implemented in a variety of studies focusing the microbial diversity different ecosystems, varying from specific microarrays focusing on sulphate reducers as well as general microarrays covering thousands of microbes [188-190]. Besides general or

group specific microarrays, ecosystem-specific microarrays have also been developed including those focusing on the human GI tract. The first microarray was developed to monitor 40 bacterial species in the GI Tract [191]. Afterwards, several phylogenetic microarrays have been developed and applied to study the human GI tract microarray in a high throuput matter [192-195]. These studies have demonstrated the power of phylogenetic microarraying since it combines the power of fingerprinting and phylogenetic analysis with the quantitative power of FISH in a single analysis per sample. Another major benefit of phylogenetic microarray analysis is that the array data can be stored in a database and this allows multiple sample comparisons as has been demonstrated in a recent meta-analytic study on phylogenetic profiles originating from 1,000 samples [196].

Another high throughput 16S rRNA gene approach that is increasing in popularity is 454 barcoded pyrosequencing.  454 pyrosequencing in its current status enables the generation of 1 million reads of approximately 400bp in a single analysis and hence provides far more sequence information than what is obtained by classical Sanger sequencing. Another major benefit of 454 pyrosequencing and other novel sequencing technologies is that sequence targets do not have to be cloned prior to sequence analysis, as the separation of the different sequencing targets is included in these technologies. By using primers that contain a specific barcode consisting of four or more nucleotides, amplicons from multiple samples can be analysed concurrently in a single picotiterplate sequence run can be binned according to their original sample afterwards [197]. This allows the analysis of multiple samples in a single run.  Pyrosequencing of 16S rRNA genes has already been applied frequently to compare and contrast microbiotas inhabiting various body sites, including the human GI tract [48, 197-199].

Despite all the advantages of pyrosequencing, it has been established that pyrosequencing overestimates diversity as has been recently demonstrated [200]. Although some strategies for correcting for sequencing errors have been proposed, it is almost impossible to differentiate between sequence difference and sequence errors, and thus estimating the diversity when characterizing microbial ecosystems. However, recently solexa sequencing based microbiota profiling has been developed [201, 202]. Solexa sequencing or Illumina sequencing is like 454 pyrosequencing a high throughput sequencing technique for which mixed templates do not have to be separated by cloning. Solexa sequencing has a drastically lower error rate and provides far more reads in a single run compared to 454 pyrosequencing. Another advantage of solexa sequencing is that reads can be generated in a paired end or mate pair mode. This allows sequencing of different variable regions of the 16S rRNA gene independent on their location in the gene. However, read sizes are still relatively short (100 bp) which limits its phylogenetic depth. Nevertheless, the enormous speed in developments with respect to read length and quantity of read generation, it will be a very promising approach for future profiling of the microbiota.

Despite their totally different principle, both high throughput sequencing of 16S rRNA genes and phylogenetic microarraying give similar microbiota profiles for faecal samples as was demonstrated for the Human Intestinal Tract Chip (HITChip) [203, 204]. Nevertheless it has to be mentioned that the dynamic range of the HITChip compares to approximately 200,000 pyrosequences per sample and that both approaches provide more distinct profiles for the small intestinal microbiota[203, 204]. Nevertheless, it is evident that both, phylogenetic microarraying and high throughput sequencing of 16S rRNA genes are promising approaches which will help researchers to link specific members of the microbiota to host characteristics in health and disease.
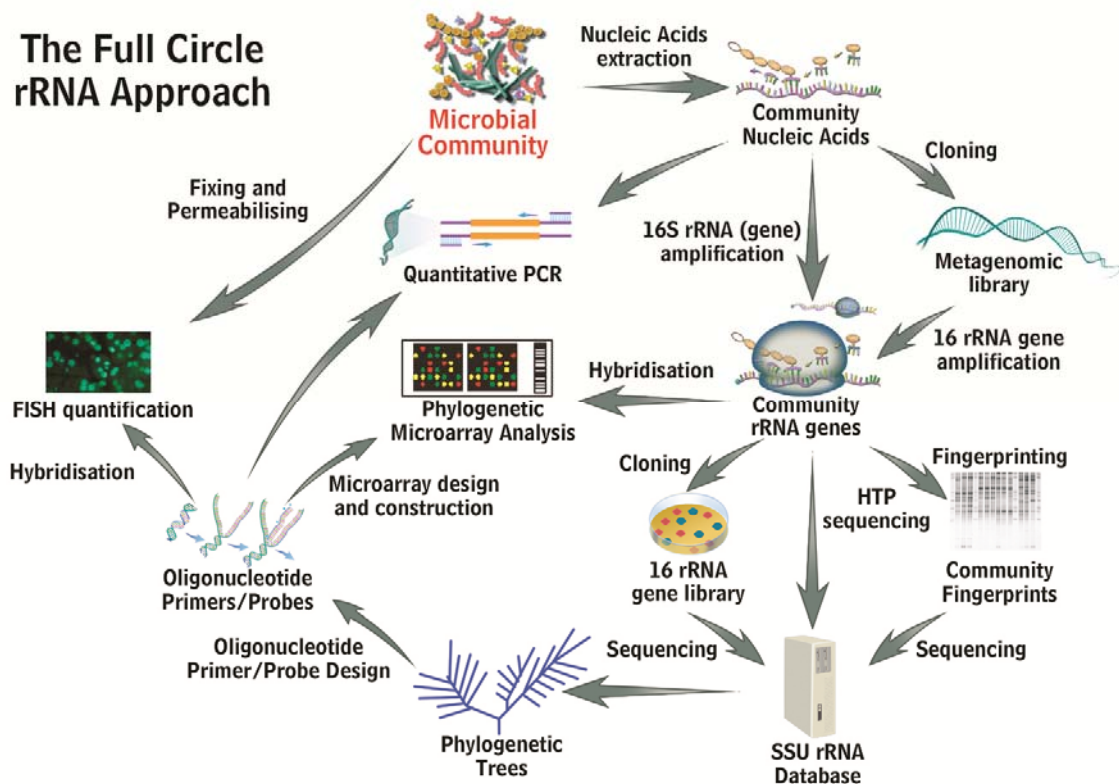
**Fig 5.1.** The full circle rRNA approach.

**g. Metagenomics**

As indicated above 16S rRNA gene-based approaches provide valuable information about the identity, number and diversity of microbial communities. However, further insights into the functional properties of microbes cannot be extrapolated from 16S rRNA and its respective genes. To get insight into the functional potential encoded in the community, metagenomics approaches are needed [205]. Metagenomics is defined as the study of genomes recovered from environmental samples which will provide information about the functional capabilities and the phylogenetic distribution of an ecosystem. Thus, instead of looking at one genome with classical genomics approaches, with metagenomics you study the genomes from all microorganisms in an ecosystem simultaneously. Depending on the research question, metagenomic information can be gathered from short insert clone libraries (1-10 Kb), large insert clone libraries (20-100 kb) or direct randomly generated sequences using the novel high throughput sequencing approaches, such as 454 pyrosequencing and Solexa sequencing. Short insert libraries can be used for individual gene-function screening such as identification of specific enzymatic activities [206], while large insert libraries favors the discovery of multiple genes or operons, potentially linked to phylogenetic markers that supplement specific functional pathways to the cloning host, which is in most cases *Escherichia coli*

[207]. The benefit of direct random sequencing is that DNA can be directly assessed without any cloning procedure as indicated previously. In general, metagenomics-based studies can be divided into two main strategies: sequence-based screening and function-based screening [205, 208]. Sequence-based screening employs large scale sequence determination and mining to unravel gene functions in order to describe and understand the microbial community [209], while function-based screening mainly focuses on the identification of gene sequences of clones that were screened for a particular function or activity in a heterologous expression host.

### g.i. Sequence-based metagenomics

Sequence-based metagenomics, or random sequencing of DNA fragments from environmental samples, can be performed in several ways. One way is to construct a metagomic library and screen for sequences of interest via PCR or microarray strategies [205]. When 16S rRNA gene is used as a screening target, such an approach will unravel some of the genetic potential of specific (uncultured) microbes as genes flanking the 16S rRNA genes will be sequenced. However, this way of screening can also be used to screen for the diversity of specific genes, such as  those encoding butyryl-CoA:acetate-CoA transferase in the fecal microbiota [140].

Most sequence-based sequencing strategies are based on random shotgun sequencing or random pyrosequencing or solexa sequencing. The latter ones have been primed by the explosive developments in sequencing technologies as has been discussed earlier. Random sequencing strategies generate vast amounts of sequence information which require advanced computational approaches for assembly and gene-function assignment [209] and a large variety of reference genome sequences from GI tract bacterial species [210]. The first shotgun sequencing of the human GI tract microbiota revealed the discovery of genes that complement our human genome with respect to GI tract functionality [211, 212]. The latter study also demonstrated that microbiotas of different individuals are rather diverse in functional categories in infants, while those in adults are more similar [212], especially between family relatives [48]. Recently, deep paired end solexa sequencing of the fecal microbiota  of more than 100 individuals enabled the definition of an intestinal microbiota core-metagenome, and allowed the detection of gene sets specifically enriched within this microbial consortium [210].

Besides random sequencing of DNA, more specified sequencing of ecosystem members has also been performed. Transposon-aided capture (TRACA) of plasmids from the human GI tract microbiota has been demonstrated to get insight into the mobile metagenome of the human GI tract microbiota [213] and recently the enormous diversity of our virome has also been revealed by metagenomic sequencing of virus-like particles isolated from human faeces [214].

It is evident that sequence-based metagenomics of the human GI tract microbiota will expand drastically the coming years. There are worldwide many initiatives in sequencing this microbiota all

around the world, including the EU FP7 project MetaHIT (http://www.metahit.eu) and the Human Microbiome Initiative from the USA (http://nihroadmap.nih.gov/hmp/). All these efforts will result in a large catalogue of microbial genes that are associated with our GI tract.

### g.ii. Function-based metagenomics

Function-based metagenomics is based on construction of small or large-insert libraries in a cloning host. In contrast to the random sequencing approach in sequence-based metagenomics, this approach focuses on screening metagenomes for functional properties that are encoded on cloned DNA fragments. In order to enable detection of functions, heterologous expression of genes located in the insert as well as suitable screening assays are needed [205]. This might be complicated for functions that involve multi-gene pathways or that need to be transported across the membrane(s). Despite these restrictions, function-based metagomics has been successfully applied to screen for microbial functions in the human GI tract. Screening the human GI tract metagenome library for bile salt hydrolase (BSH) activity has revealed that it is a conserved activity of the microbiota of individual human hosts that can be found in *Bacteria* and *Archaea* [215]. In another study, two cloned inserts encoding mucin degradation capacity were identified in a fosmid library derived from a human ileum biopsy specimen of which one of the inserts encoded a putative novel pathway for mucin degradation with highest similarity to genes from *Entercoccus* [216].

One of the major problems with functional screening is that large numbers of clones have to be screened in order to identify one or a few positive clones and therefore, high throughput screening technologies are crucial. Substrate-Induced Gene Expression (SIGEX), which employs a cloning vector for operon-trapping and fusion to green fluorescent protein (GFP), is one way of high throughput screening as it can be coupled to Fluorescence Activated Cell Sorting (FACS) of positive cells [217]. In a similar way, a high throughput "intracellular" screening called "metabolite-regulated expression" METREX was developed, in which metagenomic DNA is cloned into a host cell containing a biosensor for compounds that induce bacterial quorum sensing [218]. Neither of these screening systems has been applied to the GI tract ecosystem yet, but could be attractive to set-up a screening assay for induction of microbial gene expression by host-derived substrates such as mucus or specific bacterial metabolites such as short chain fatty acids.

A novel way to unravel microbial functions in the GI tract is by cell-line based host-response phenotype screening [219]. In this approach fosmid clones were screened for their capacity to induce or inhibit epithelial-cell proliferation. Specific genes in the fosmid clones were identified by transposon-mutagenesis and included ABC systems, a glutamate synthase subunit, a RecD homologue, and a V-type ATPase subunit. In a similar way, several fosmid clones were detected that has an impact on the NF-κB signaling pathway [220]. This revealed the identification of genes encoding an ABC transport system and lipoprotein with highest similarity to *Bacteroides*. These examples indicate the power of

these screening approaches to couple microbial genes to specific functions in the GI tract. Since metagenomics uses DNA as a target, it only provides a catalogue of the genetic potential within an ecosystem. It has to be realized that the presence of a gene or function does not indicate that it will be expressed in situ. It is even possible that the genetic information originates from dead cells. To gain insight into the activity of microbes, markers of activity, such as messenger RNA (mRNA), proteins or metabolites need to serve as targets.

### h. Metatranscriptomics

Metatranscriptomics covers the overall or gene-subset-specific transcriptome analysis of microbes within an ecosystem [221]. With metatranscriptomics the overall gene-expression within a community is determined which reflects its overall activity. Since bacterial RNA is rather instable, proper storage of samples, and a direct and efficient RNA isolation protocol are crucial to detect genes that are expressed in an ecosystem, such as the GI tract, accurately [222]. Moreover, enrichment of mRNA, which reflects the actual transcript activity, is required since more than 95% of total bacterial RNA consists of rRNA. This can be done by selective capturing of rRNA or selective exonuclease reaction to digest specifically rRNA [221, 223].

Classical transcriptomics is basically done with DNA microarrays or gene-specific Reverse transcription qPCR (RT-qPCR) which are both well established. However, designing microrrays or gene-specific qPCR assays for metatranscriptomics of diverse communities is very challenging, because these require prior knowledge of the genes that are present in the community.

One way to reduce this complexity is designing microarrays that target a specific subgroup of the community and determine its gene expression in situ. Such an approach has been recently applied by determining the gene-expression pattern of bifidobacteria in the infant gut by using a mixed species array of bifidobacteria [224]. This study demonstrated that genes involved in metabolism of host-specific substrates, such as mucin or milk oligosaccharides are expressed by the infant bifidobacterial population in the GI tract.

Instead of microarrays, other approaches, which do not require prior sequence knowledge, have also shown to be successful for metatransciptomics of GI tract communities. cDNA-Amplified Fragment Length polymorphism (cDNA-AFLP) combined with sequencing of excised amplicons from the gel has demonstrated to be a powerful fingerprinting approach for microbial gene in the human GI tract [223]. This study indicated that expression profiles differ between subjects and that carbohydrate metabolism is among the dominantly expressed function by the GI tract microbiota. Another powerful metatranscriptomics strategy that does not require prior sequence knowledge is sequencing of mRNA-derived cDNA since it has been demonstrated that it is able to elucidate transcriptome responses of single organisms under different growth conditions [225, 226] and with

quantitative accuracy that is comparable to microarrays and RT-qPCR [227]. Metatranscriptome analysis of the fecal microbiota in twin pairs via direct cDNA sequencing showed that Clusters of Orthologous Groups categories for translation, energy metabolism, chaperones and hypothetical genes with unknown functions were among the highly expressed genes [228]. In addition, Gosalbes and colleagues demonstrated in feces expression of microbial genes involved in carbohydrate metabolism, energy production and cellular components[229]. Such metatranscriptomics studies are expected to be used extensively in the near future since technological improvements of sequencing are developing fast. It should be noted however that bacterial mRNAs have very short half-lives. This means that gene expression monitored by this approach in faecal samples is likely to reflect the environment of the faecal sample more than that of the gut itself. A recent study has demonstrated that RNA can also be directly sequenced without the conversion of RNA into cDNA and by eliminating this step, direct RNA sequencing will result in more accurate and high-throughput transcript analyses using only femtomoles of RNA [230]. Although direct RNA sequencing has to date only been demonstrated for single organisms, such as *Saccharomyces cerevisiae*, it is evident that this technology opens avenues for metatransciptomics analysis of complex ecosystems, such as the GI tract.

**i. Metaproteomics**

Metaproteomics encompasses the study of the proteome produced by the community in an ecosystem and is a very powerful strategy for understanding overall microbial-ecosystem functioning [231]. Similar to metatranscriptomics, metaproteomics provides insights into the microbiota overall activity as reflected by its protein profiles. Classical proteome consists of two-dimensional (2D) gel electrophoresis, often combined matrix assisted laser desorption ionization-time of flight mass spectometry (MALDI-TOF MS) and the first gut metaproteomics study of the infant microbiota revealed its metaproteome dynamics and the identification of a bifidobacterial transaldolase [232]. However, a major impetus in metaproteomics of the human GI tract microbiota came from a novel high-throughput, non-targeted mass spectrometry (MS) based on the shotgun metaproteomic approach for detection and identification of all proteins without gel-based separation [233]. A high abundance of proteins involved in translation, energy production and carbohydrate metabolism were among the dominantly produced proteins by the GI tract microbiota. Recently, it has been demonstrated that metaproteomics data analysis can even be enhanced by using an approach based on a synthetic metagenome that combines the power of sequence diversity contained in metagenomic diversity and the high reliability of predicted proteins[234]. Although metaproteomics is still in its infancy, the technological developments are increasing fast and hence, it is expected that its application in studying the human GI tract microbiota will increase in the near future.

**j. Metabonomics/metabolomics**

Metabolite profiling can be divided into metabolomics and metabonomics. With metabolomics small metabolite molecules in complex biological samples, are characterized and quantified, while with metabonomics the global and dynamic metabolic response to environmental factors or genetic manipulations of ecosystems or other multicellular biological systems are measured[235]. In contrast to the other meta-omics approaches, metabonomics and metabolomics focus on the produced metabolites and therefore, cannot be linked to any genetic information. Hence, they do not provide details about the microbes that are responsible for the produced metabolites. Although the field of metabolite profiling in ecology is recent, it has already been applied to associate variation in human metabolic phenotypes to several factors, such as host genotype, age, sex, lifestyle, nutrition, health status and commensal microbial [236]. Metabolic profiling of faecal samples from IBD patients and healthy controls indicated marked metabolic differences between these groups [237]. A recent correlation analysis of the gut microbiota composition and the variation in metabolic phenotypes measured in human faecal and urinary samples of seven Chinese individuals suggested profound host-microbiota symbiotic associations that have an important influence on the global metabolism, regardless of the genetic background across a range of pathways or environmental conditions of the host[70]. In addition, another recent study demonstrated that the gut microbiota plays a crucial role in the production of metabolites in plasma are predictors of cardiovascular disease risk, while major shifts have been detected in faecal metabolites in obese subjects on high protein, low carbohydrate weight loss diets [238, 239]. It is evident that these metabonomics studies, combined with other meta-omics' approaches will improve our understanding of host-microbe interactions and how that affects our global metabolism as a superorganism of human and microbial cells.

**Table 5.1.** Overview of approaches to study the microbiome.

| Question | Target | Approach | Data generated | Can microbes be identified directly? | Main benefit | Main Limitation |
|---|---|---|---|---|---|---|
| Which microbes are present in the GI tract? | isolates | Cultivation | Phenotypic characterization | Yes | Accurate species identification | Not representative |
| | 16S rRNA gene | Cloning and Sanger Sequencing | Phylogenetic identification | Yes | Complete 16S rRNA gene sequence data | Cloning bias |
| | 16S rRNA gene | High throughput sequencing | Phylogenetic identification | Yes | High throughput data generation | Short reads |
| | 16S rRNA gene | Fingerpinting | Community profile | No | Fast comparison between communities | No direct link with phylogeny |
| | 16S rRNA | FISH | Cell numbers | Yes | Accurate enumeration | Dependent on 16S rRNA databases |
| | 16S rRNA gene | qPCR | 16S rRNA gene abundances | Yes | Wide dynamic range | Dependent on 16S rRNA databases |
| | 16S rRNA gene | Phylogenetic microarray | Phylogenetic identification | Yes | High throughput phylogenetic profiling | Dependent on 16S rRNA databases |
| What microbial genes are present in the GI tract? | community DNA | Sequence-based metagenomics | Gene sequences | Not always | High throughput data generation | Function mainly based on predictions |
| | community DNA | Function-based metagenomics | Functional properties encoded on DNA fragment | Not always | Functional properties linked to DNA sequences | Suitable cloning host/system and screening assays needed |
| What are GI tract microbes doing? | mRNA | Metatranscriptomics | Community gene expression | Not always | Direct information about microbial activity | Community RNA extraction challenging |
| | proteins | Metaproteomics | Community protein production | Not always | Direct information about microbial activity | No uniform protocol for all cell fractions |
| | metabonomics | Metabonomics/ metabolomics | Community metabolite profiles | No | Microbiota activity representation | No link with microbes or its function |
| | Lactulose Hydrogen Breath Test | Measuring GI tract gas production | Hydrogen and methane breath content | No | Unclear, simple test but not validated for diagnosing SIBO | May simply measure small intestinal transit time to cecum |
| | Glucose Hydrogen Breath Test | Measuring GI tract gas production | Hydrogen breath content | No | Same as above | Poor sensitivity; Misses distal SIBO. |

6. DIFFERENCES IN THE MICROBIOME IN FUNCTIONAL BOWEL DISORDERS

**Key points**

- Very little is known about small bowel commensals.

- Using a conventional cut-off of $>10^5$ cfu/ml in duodenal aspirate the incidence of small intestinal bacterial overgrowth is <5% in IBS, no different from healthy controls

- The lactulose breath test is not validated for diagnosing SIBO and many of the abnormalities may reflect rapid oro-caecal transit.

- A number of confounding factors, including acid inhibition by concomitant proton pump inhibitors, may also account for abnormal lactulose breath tests and/or differences in numbers of bacteria in the small intestine.

- Colonic commensals form complex interrelated communities.

- Heterogeneity of IBS and variation in methods used to study the fecal microbiota has resulted in conflicting reports of differences from healthy controls

- There are no data on changes in postinfectious IBS but studies of acute gastroenteritis show a "dysbiosis" with loss of diversity, depletion of anaerobes often associated with overgrowth of enterobacteria.

- The microbiome may contribute to IBS symptoms by altering gut neuromotor-sensory function, barrier function, and/or the brain-gut axis.

**a. Small Intestine Microbiome in Healthy controls and IBS patients**

The hypothesis that overgrowth of bacteria in the small intestine is an important pathogenic mechanism underlying IBS[123] has generated considerable interest. While breath testing has been the main diagnostic technique employed to substantiate this hypothesis, the lack of validated criteria for diagnosing SIBO[102] undermines the ability to draw accurate conclusions (see Chapter 5). The following discussion highlights what is known about the small intestine microbiome using classical culture techniques as well as the recent application of molecular techniques and how these techniques have influenced the debate concerning SIBO in IBS. The relative advantages and limitations of these techniques have been outlined in detail above (see Chapter 5).

***a.i. Small intestinal microbiome in healthy controls***

*Culture*. Despite the interest in the small intestine microbiota there is relatively little known, largely because of the limited access to this region of the GI tract[223]. Most of what we know has been

obtained using culture techniques on effluent and mucosal samples obtained using endoscopic and radiologic tube/capsule placement in healthy volunteers, samples obtained from trauma patients and at autopsy, and those from ileostomies (Table 6.1). Despite the well-recognized limitations of culture techniques[106, 208], important differences compared to the colon have been described. There are considerably fewer bacteria in the small bowel compared to the colon with a gradient of viable counts and diversity of organisms from the duodenum to the ileum. Estimates based on cultures are in the following ranges - duodenum and jejunum $10^{0-4}$ cfu/ml, $10^{0-5}$ cfu/ml in the proximal ileum, $10^{5-8}$ cfu/ml in the terminal ileum, and $10^{10-12}$ cfu/ml in the caecum [240-242]. These bacteria are typically gram positive aerobes proximally, gram negative and positive anaerobes and facultative anaerobes in the terminal ileum.

*Culture-independent analysis.* The application of molecular techniques to the study of human small intestine microbiome is in its relative infancy but emerging studies highlight the potential complexity of these microbial communities and suggest that previous consideration of this microbiota may be overly simplistic[208]. Studies using 16S rRNA gene libraries and terminal restriction fragment length polymorphism (T-RFLP) identified jejunal and ileal microbiota consisting of streptococci, lactobacilli, 'Gammaproteobacteria", the *Enterococcus* group and the *Bacteroides* group. Most species were facultative anaerobes or aerobes [183]. In ileostomy effluent, streptococci, *Veillonella*, and different clostridia groups predominate[187]. However, these and other studies also highlighted marked individual differences in the composition of the microbiota [183, 187], large fluctuations over time even during a single day suggesting relative instability, possible age related differences, and several phylotypes not previously identified[186, 187, 243].

### a.ii. Small intestinal microbiome in IBS

*Culture.* In addition to the general limitations of this technique, two specific issues related to IBS have presented challenges for diagnosing SIBO in this disorder. Firstly, while many experts suggest that SIBO should be defined as ≥$10^5$ cfu/ml of colonic bacteria, this definition has not been uniformly accepted [106, 244]. Proponents of the SIBO-IBS hypothesis, suggest that these criteria are derived from studies of major diseases predisposing to SIBO e.g. stagnant loop in Bilroth II gastrectomy with associated steatorrhea and may not be relevant to IBS[106, 112]. Secondly, if the SIBO-IBS hypothesis depends upon migration of colonic bacteria into the ileum and distal jejunum as initially proposed, sampling the more proximal bowel would not detect these changes [245]. There is only one large prospective study of well characterized IBS patients[115] and a few retrospective or small studies of patients with probable or possible IBS[244, 246-249] (where results from small bowel cultures were obtained (Table 1). Together, these studies show the duodenum and proximal jejunum of most IBS

patients contains relatively small numbers of bacteria and no obvious differences in content compared to controls. The largest study did show a significantly greater proportion of patients had ≥ 5 X $10^3$ cfu/ml compared to healthy controls (42% vs. 12%) in a post-hoc analysis but no difference between groups using a ≥ 5 X $10^5$ cfu/ml cut-off. A recent preliminary report found similar findings using the lower cut-off, but identified the IBS patients using Rome III criteria retrospectively from a group of patients admitted with upper gastrointestinal bleeding[250]. Further studies are needed to determine the significance of these finding, particularly as increased numbers have been documented in other diseases [106] and in healthy controls. There are currently no studies of patients which have cultured samples from the distal jejunum and ileum nor systematically applied molecular techniques in any region of the small intestine to determine whether important qualitative changes in the microbiota exist.

*Culture-independent analysis.* To date there are very few studies which have applied these techniques to the study of the microbiome in IBS patients but these studies should be important in resolving the debate concerning the role of SIBO in IBS. One study examined jejunal aspirates in small numbers of IBS patients (n=10) and healthy controls using qPCR combined with culture techniques and breath testing [106]. The molecular analysis did not reveal any differences in total bacterial counts or the composition of the microbiota including the numbers of colonic-type bacteria (see however limitations of qPCR above).

**Table 6.1.** Summary of Studies Culturing Small Bowel Microbiome [115, 244, 246-253]

| Study | Number of patients | Sample type | Microbiology results | Comments |
|---|---|---|---|---|
| Drasar et al. Gut 1969[247] | 13 diarrhoea, all investigations negative | jejunal capsule | no difference from controls; no increased numbers pathogens or non-pathogens | possible IBS but not defined as IBS |
| Lewis et al. Dig Dis Sci 1997[248] | 23 with functional bowel disorders | duodenal endoscopic aspirate | mean control count 3.2 x $10^2$ cfu/ml, no aneraobes, no sterile samples | no specific IBS, defined as Functional Bowel Disorders |
| Corazza et al. Gastroenterology 1990[246] | 31 chronic diarrhoea, no predisposing cause | proximal jejunal aspirate | 10 had SIBO ($\geq 10^6$ cfu/ml or colonic bacteria), 2 IBS, 8 others multiple other diagnoses | IBS not defined, and total IBS not clear |
| Bardhan et al. Scand J Gastroenterol 1992[251] | 10 controls 4 irritable colon; 22 others | endoscopic aspirates from proximal jejunum | no positive cultures in irritable colons | positive cultures in 11 cases, many post-surgical |
| Rumessen et al Scand J Gastroenterol 1985[253] | 60 patients suspected of SIBO | proximal jejunal aspirate | 15 with no predisposing cause had no evidence SIBO; of 23 with SIBO, 4 had no predisposing cause | groups poorly defined, 8 IBS identified and all negative for SIBO; 22 cases considered inconclusive |
| Posserud et al. Gut 2007[115] | 162 IBS 42 controls | proximal jejunal aspirate | 4%$\geq 10^5$ cfu, same as controls. Sub-analysis using $\geq 5$ x $10^3$, 43% IBS vs 12% controls | no correlation with motor pattern in IBS group |
| Sullivan et al. Anaerobe 2003[249] | 7 IBS 20 controls | proximal jejunal biopsy from using Watson capsule | No differences, flora similar to normal oropharyngeal flora | Colonic pathogen in 2 healthy subjects |
| Kerckhoffs et al. J Clin Gastroenterol 2008[244] | 8 IBS 9 controls | proximal jejunal aspirate | no differences number diagnosed with SIBO using multiple definitions | no differences also using molecular-based counts |
| Choung et al. AlimentPharmacolTher 2011[252] | 148 IBS 542 "other indications to test for SIBO" | Duodenal endoscopic aspirate | 2% IBS > $10^5$ cfu/ml 10% in 'other' indications | retrospective study 18% IBS >0 <$10^5$ cfu/ml |
| Pyleris et al Gastroenterology (Abstract) 2011[250] | 85 IBS 150 non-IBS | Duodenal endoscopic aspirate | 37% IBS > $10^3$ cfu/ml 15.11% non-IBS | All investigated because of UGI bleed |

**b. SIBO and IBS: Confounding Factors**

*b.i. Confounding Factors between SIBO and IBS*

The lack of consistency in the data linking SIBO to IBS[28] raises the possibility of confounding by known factors such as proton pump inhibitors (PPIs), impaired clearance of fasting secretions by disordered motility or other unknown factors.

*b.ii. Could IBS be Linked to SIBO through PPIs?*

The use of proton pump inhibitors (PPIs) which is commoner in IBS than controls could be a major confounder[254] since PPI therapy may promote both gastric bacterial overgrowth[255] and SIBO by eliminating gastric acid[256-261]. However most studies linking SIBO to IBS have not adjusted for or excluded the use of PPI therapy.

The commonest side effects of PPIs include abdominal pain, bloating, flatulence, constipation, and diarrhea–symptoms that overlap with IBS[256]. Recently, a large study from Italy on 200 IBS patients reported nearly twice the incidence of SIBO as assessed using the glucose breath test among patients using PPIs compared to IBS patients (50% vs. 24.5%), although the frequency in both groups was higher than in healthy controls (6%)[259]. Moreover, Compare et al. performed a prospective study in patients with reflux disease receiving PPI therapy, and found that 43% developed de novo bloating after 8 weeks of therapy[256]. After 6 months of PPI treatment, nearly 1 in 5 PPI users had developed new IBS symptoms. Data also indicate that, among patients with a positive glucose hydrogen breath test who received rifaximin for eradication, return of a positive breath test is independently predicted by use of concurrent PPI therapy[262]. Thus, not only might PPI therapy lead to SIBO in some patients with IBS, but the recurrence of SIBO following antibiotic therapy might be accelerated in the setting of PPI therapy. In contrast to these various studies in which SIBO was diagnosed using the glucose breath test, Law and Pimentel reported that PPI therapy did not significantly alter hydrogen production on lactulose breath tests in IBS patients[111]. This may well be because while PPIs alter small bowel bacteria they do not alter colonic bacteria which is what lactulose breath is largely assessing[114]. A recent survey of duodenal aspirate and culture in 675 patients in a tertiary care setting did find about 2% of IBS had SIBO. They also found a positive relationship between PPIs exposure and indeterminate bacterial growth but not with $>10^5$ orgs/ml[252].

### b.iii. Could IBS be Linked to SIBO through Underlying Dysmotility?

The above studies suggest that around 2-4% of IBS have SIBO. It is likely that IBS symptoms are related, in part, to abnormal intestinal motility which can cause SIBO. Perhaps the most powerful example is scleroderma, where hypomotility and stagnation lead to SIBO, with attendant defecatory symptoms and bloating[263], some of which can be improved by antibiotic therapy[264]. Recent data suggests that IBS-D is associated with fast small bowel transit and that this accounts for the early rise in breath hydrogen after lactulose as it enters the colon[114, 265]. The observation that antibiotics help some IBS patients[266] is not necessarily evidence that SIBO *causes* IBS, since it is more likely that the antibiotics alter colonic fermentation and that this accounts for the improvement in bloating and flatulence. As shown in Figure 6.1, when the lactulose hydrogen breath test (LHBT) was combined with technician scintigraphy to evaluate oro-caecal transit time, it was apparent that 63% of IBS patients had an abnormal LHBT at 180 minutes, and 35% were abnormal at 180 minutes[114]. However, in almost every case of a positive LHBT (88%), the technician arrived in the cecum *before* the LHBT became abnormal, indicating that the early rise in breath hydrogen was due to rapid transit to the cecum with ensuing fermentation by colonic bacteria, not SIBO. This study is important, because it demonstrates that the test used to develop the SIBO hypothesis was probably not

measuring SIBO in the first place, but was simply reflecting fast oro-caecal transit in many IBS patients. There are other compelling data to support this hypothesis. Posserud and colleagues performed jejunal aspirates in 126 patients with Rome III IBS, and measured LHBT results in a subset of 80 patients[115]. The investigators tracked the relationship between these biomarkers and IBS symptoms. They found that only 3% of IBS subjects met the traditional $\geq 10^5$ CFU/mL criterion for SIBO, and only 9% met the less stringent $\geq 10^3$ criterion. Similarly, only 6% demonstrated a "double peak" on lactulose hydrogen breath testing, but 43% had a detectable peak before 90 minutes. For purposes of this discussion, the most important finding was that patients with a 90-minute rise were more likely to have severe diarrhoea and loose stools compared to those without a 90-minute rise. In contrast, the other definitions of SIBO did not correlate IBS symptoms at all, as also found by Grover and colleagues in a separate study[125].
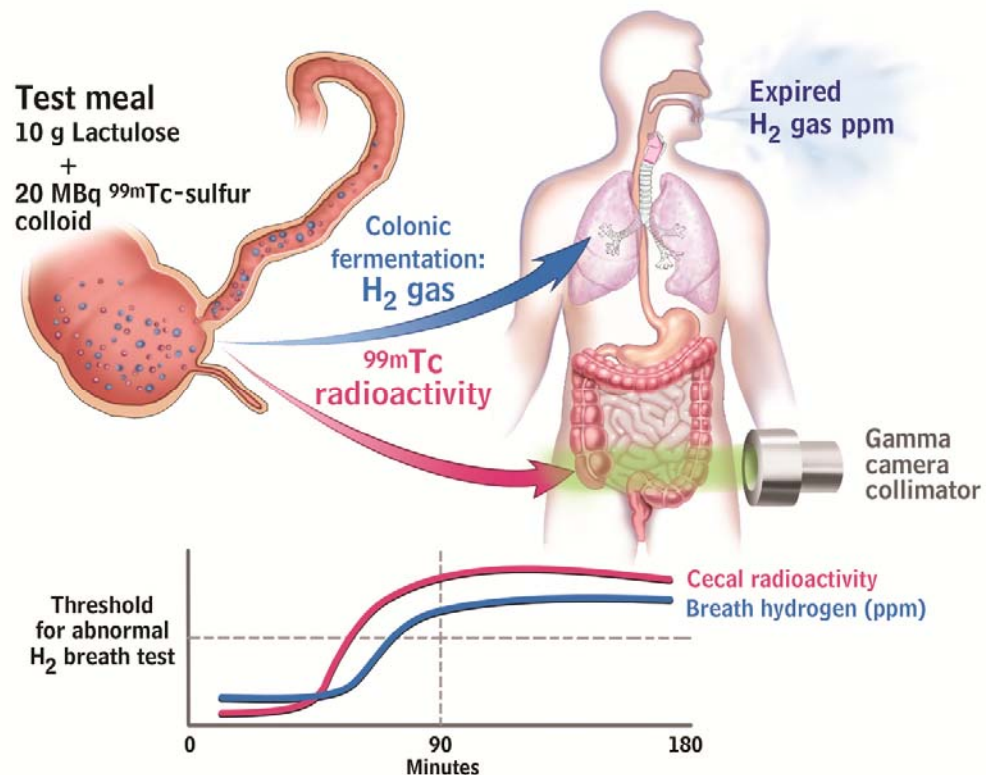


**Fig 6.1.** The LHBT measures small intestinal transit rather than SIBO in IBS patients. Upper schematic shows ingestion of test meal with subsequent serial measurement of both $H_2$ gas, resulting from fermentation of the lactulose by intestinal bacteria, and Tc99 scanning in the cecum. This latter measurement detects when the test meal has reached the cecum. The stylized drawing below shows a representative result from an IBS patient with serial measurements over time. The Tc99 had already reached the cecum in large quantities before the $H_2$ PPM level has reached the threshold for an abnormal test (see Yu et al. 2011 for details). This demonstrates that the increased $H_2$ production results from fermentation by colonic bacteria, not by abnormal bacteria small intestine (i.e. SIBO).

***b.iv. Other Potential Confounders***

There are a range of other confounders that might undermine the causal link between alterations in small bowel microbiota and IBS. These include variations in antibiotic use, probiotic, prebiotic, or other dietary ingestions (e.g. fermentable oligo-, di- and mono-saccharides, and polyols [FODMAP][267, 268]) in IBS patients vs. controls. There are several studies indicating that antibiotic consumption is commoner in IBS than controls[269] and that their use is a risk factor for developing IBS[270]. Furthermore their use can be followed by persistent abnormalities in the faecal microbiota even after 60 days[271]. Other factors could involve the effects of low grade inflammation in the small intestine or even altered immune function[272, 273].

### c. Large Bowel Microbiome in Healthy Controls and IBS patients

The human GI tract microbiota is very complex in composition, can be influenced many factors (Figure 6.2), is individual specific and consists of numerous uncultured microbes, as discussed in Chapter 4. Ten bacterial phyla have been detected in fecal samples from which the Firmicutes, Bacteroidetes and Actinobacteria dominate[21] . Despite the individual variation, a recent paper suggested that the human GI tract microbiota can be divided in three robust clusters called enterotypes that are indicated to be driven by groups of species that together contribute to their respective preferred community composition[274]. Remarkably, these enterotypes do not appear to be dependent on features, such as nation, gender, age or body mass index, although these findings are based on relatively small numbers of subjects.

The GI tract microbiota may have an important role in the onset and maintenance of IBS, particularly postinfectious IBS. Several studies have already described the microbiota composition in IBS patients (Table 6.2) and although differences from controls have been described these are inconsistent and sometimes contradictory with respect to identification of species associated with IBS.

One of the problems with a meta-analysis of the different IBS studies is the fact that a limited number of subjects is used. In addition, samples are in general single snapshots from the individuals and since IBS symptoms are characteristically erratic, analysis of a single sample may be misleading when it is not linked to symptoms at the time when it is taken.

Another major concern that hampers a detailed analysis is the different methods that have been used to identify the microbiota as they include selective cultivation, specific targeted qPCR and FISH, DGGE, G+C - and microarray profiling. In addition, most studies focused on composition analysis of the microbiota whereas what may be more important is the microbiota function and metabolic and immunomodulatory activity.

Besides microbiota, detailed description of the patients, such as IBS typing beyond the Rome III criteria, IBS symptom scores and diet recordings are often not complete and comparable between

different studies. In addition, the number of studies itself is very limited considering that more than half of the studies originate from a single country in which the same cohorts have been used in multiple studies. Temporal fluctuations in microbiota compositions have been observed in IBS[275] and IBD[180], which contrasts to the rather stable community composition in the GI tract of healthy adults . However, different enterotypes of the gut microbiota exists[274] and it would be worth determining whether IBS features might be linked to enterotype-specific microbes. A recent paper by Rajilic-Stojanovic et al described that enterotype 3 is significantly more represented in IBS patients compared to healthy subjects [276] REF IBS is a very heterogeneous disorder, which means that the correlation with the microbiota might also be heterogeneous, and another recent study found one subgroup of IBS patients to have a microbiota composition similar to healthy individuals, whereas another IBS subgroup demonstrated clear differences in their microbiota composition compared to healthy controls and also an association with the clinical profile[277]. Moreover, it has been demonstrated that the microbiota composition represented in the feces does not necessarily represent other locations in the GI tract and this adds to the complexity of the ecosystem[23, 184, 185, 223]. It should be noted that elevated BMI is associated with accelerated colonic transit in IBS[265, 278] and this may account for the differences in microbiota noted in IBS patients with BMI >25 $kg/m^2$ [279].

Based on the current studies and the difficulty in comparing them, future studies are needed that are longitudinal, with multiple measurements at regular time intervals. These measurements should at least include detailed description of IBS patients and their symptoms, have sufficient power with respect to numbers of IBS patients and matched controls, and include culture-independent high throughput phylogenetic and functional characterization of the microbiota.
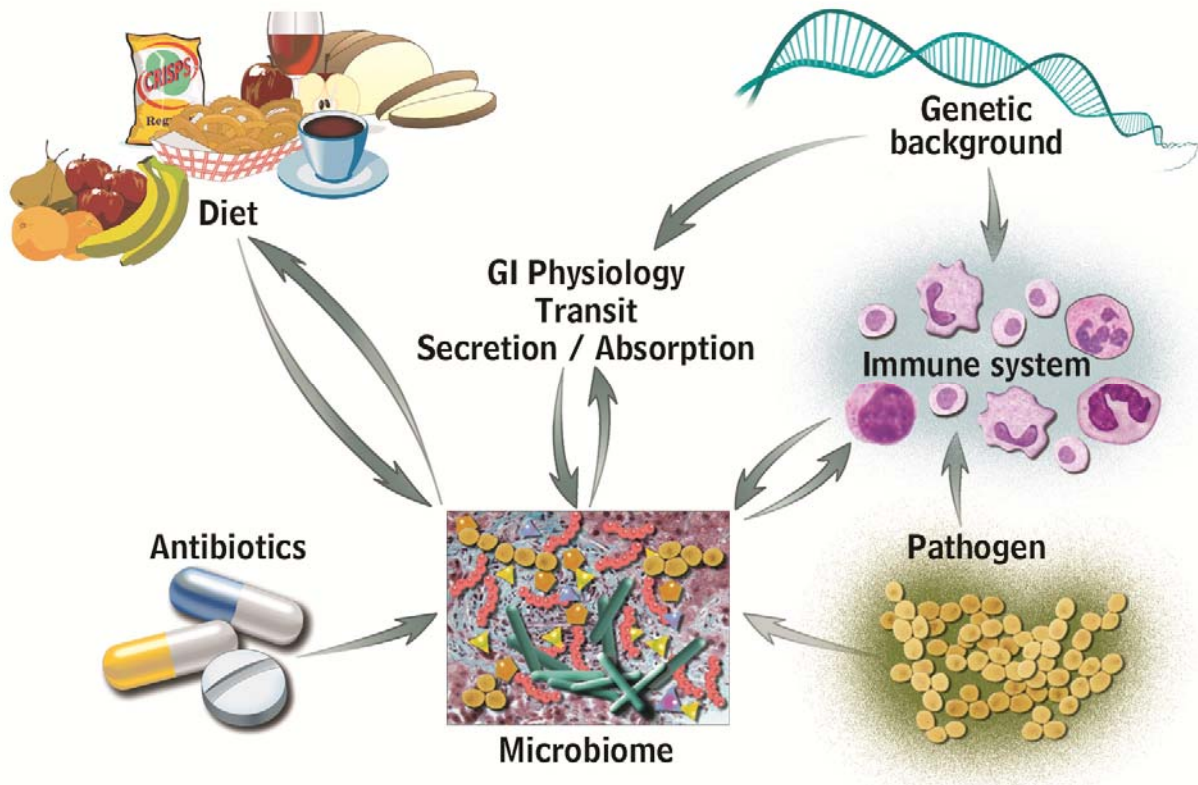
**Fig.6.2.** Multiple factors influence the composition of the intestinal microbiota.

**Table 6.2.** Summary of Culture and Molecular Studies of Colonic Microbiome[33, 178, 179, 275-277, 279-294]

| Study | Subject | Sample | Method | Main finding | Country of study |
|---|---|---|---|---|---|
| Balsari et al Microbiologica 1982[280] | IBS (n=20) Ctrls (n=20) | Faeces | Culture | IBS: ↓ Coliform bacteria ↓ *Lactobacillus spp.* ↓ *Bifidobacterium spp.* | Italy |
| Si et al WJG 2004[291] | IBS (n=25) Ctrls (n=25) | Faeces | Culture | IBS: ↓*Bifidobacterium* ↑ *Enterobacteriaceae* ↓ *C. perfringens* | China |
| Malinen et al AJG 2005[275] | IBS (n=27) Ctrls (n=22) | Faeces | qPCR | IBS: ↓ *B.catenulatum* ↓ *Cl. coccoides* group IBS-D: ↓ *Lactobacillus spp.* IBS-C: ↑ *Veillonella spp.* ↑ *Lactobacillus spp.* | Finland |
| Mättö et al FEMS Immunol Med Microbiol 2005[179] | IBS (n=26) Ctrls (n=25) | Faeces | Culture PCR-DGGE | IBS: ↑ Coliform bacteria ↑ aerob/anaerob ratio ↓ temporal stability | Finland |
| Maukonen et al J Med Microbiol 2006[178] | IBS (n=24) Ctrls (n=16) | Faeces | PCR-DGGE, Affinity capture | IBS: ↓ temporal stability IBS-C: ↓ *Cl. coccoides* group | Finland |
| Kassinen et al Gastroenterology 2007[283] | IBS (n=24) Ctrls (n=23) | Faeces | GC-profiling + sequencing of 16S rRNA genes qPCR | IBS: ↓ *Collinsella aerofaciens* ↓ *Cl. cocleatum* ↓ *Coprococcus eutactus* Subgroup-diff (D,C,M) | Finland |
| Rajilić-Stojanović PhD thesis[289] | IBS (n=20) Ctrls (n=20) | Faeces | Microarray | IBS: ↓ *Bacteroides spp* ↑ *Bacillaceae* | Finland |

| | | | | | |
|---|---|---|---|---|---|
| Kerckhoffs et al WJG 2009[284] | IBS (n=41) Ctrls (n=26) | Faeces Duodenal mucosa | FISH qPCR | IBS: ↓ *Bifidobacterium spp.* ↓ *B. catenulatum* | Netherlands |
| Krogius-Kurikka et al BMC Gastro 2009[285] | IBS-D (n=10) Ctrls (n=23) | Faeces | GC-profiling + sequencing of 16S rRNA genes | IBS-D: ↑ *Proteobacteria* ↑ *Firmicutes* ↓ *Actinobacteria* ↓ *Bacteroidetes* | Finland |
| Lyra et al WJG 2009[286] | IBS (n=20) Ctrls (n=15) | Faeces | qPCR | IBS-D: ↑ *R. torques* 94% ↓*Cl.thermosuccinogenes* 85% IBS-C: ↑ *R. bromii*-like IBS-A: ↓*R. torques* 93% ↑ *Cl. thermosuccinogenes* 85% | Finland |
| Tana et al NGM 2010[293] | IBS (n=26) Ctrls (n=26) | Faeces | Culture qPCR | IBS: ↑ *Veillonella spp.* ↑ *Lactobacillus spp.* | Japan |
| Codling Dig Dis Sci 2010[282] | IBS (n=41) Ctrls (n=33) | Faeces Colonic mucosa | PCR-DGGE | IBS: ↑ temporal stability no sign diff fecal/mucosal | Ireland |
| Carroll Gut Pathogens 2010[281] | IBS-D (n=10) Ctrls (n=10) | Faeces Colonic biopsies | Culture qPCR | IBS-D: ↓ aerobic bacteria *Lactobacillus spp.* | USA |
| Noor BMC Gastro 2010[287] | IBS (n=11) Ctrls (n=22) UC (n=13) | Faeces | PCR-DGGE + sequencing of 16S rRNA genes | IBS: ↓ bacterial species ↓ biodiversity ↑ biological variability of predominant bacteria | UK |
| Malinen WJG 2010[279] | IBS (n=44) | Faeces | qPCR | *R. torques* 94% - symptom severity Other phylotypes neg assoc. | Finland |
| Ponnusamy et al JMed Microb 2011[288] | IBS (n=11) Ctrls (n=8) | Faeces | DGGE + qPCR of 16S rRNA genes | ↑ diversity in Bacteroidetes & Lactobacilli ↑ alanine & pyroglutamic acid & phenolic compounds | Korea |
| Rinttila et al 2011 Gut Pathogens[290] | IBS (n=96) Ctrls (n=23) | Faeces | qPCR | IBS: *S. aureus* (17%) | Finland |
| Saulnier et al Gastroenterology 2011[291] | IBS (n = 22) Ctrls (n = 22) (Children) | Faeces | 16S metagenomic sequencing and DNA microarray | IBS: ↑ Gammaproteobacteria Classified IBS subtypes using sets of discriminant bacterial species | United States |
| Rajilic-Stojanovic et al Gastroenterology 2011[276] | IBS (n = 62) Ctrls (n = 42) | Faeces | Phylogenetic 16S rRNA microarray and qPCR | IBS: Proteobacteria and specific Firmicutes ↑ Other Firmicutes, Bacteroidetes, and bifidobacteria ↓ | Finland |
| Carroll et al AJP 2011[33] | IBS-D (n=16) Ctrls (n=21) | Faeces Colonic mucosa | T-RFLP fingerprinting of 16S rRNA - PCR | IBS-D: Diminished microbial biodiversity in fecal samples | USA |
| Parkes et al NMO 2012[294] | IBS-D (n=27) IBS-C (n=26) Ctrls (n=26) | Colonic mucosa | FISH Confocal microscopy | IBS: Expansion of mucosa-associated microbiota; mainly bacteroides and clostridia; association with IBS subgroups and symptoms | UK |
| Jeffery et al Gut 2012[277] | IBS (n=37) Ctrls (n=20) | Faeces | Pyrosequencing 16S rRNA | Clustering of IBS patients – normal-like vs. abnormal microbiota composition (increased ratio of Firmicutes to Bacteroidetes) ; association with symptom profile | Sweden |

**d. Post infectious IBS and the effect of infections on the gut microbiome**

*d.i Postinfectious IBS*

A healthy commensal microbiota resists pathogen colonisation by occupying the different niches in the GI tract and producing a range of antibacterial products including both antibiotics, as well as metabolites such as short chain fatty acids. Animal studies clearly show that depleting the microbiota by giving antibiotics can break this colonisation resistance[295]. If the normal resistance is overcome, the resulting infective gastroenteritis produces a profound depletion of the commensal microbiota[296], though for how long this disruption lasts and how completely recovery occurs, is unclear. The incidence of infective gastroenteritis in the UK is 19/100 person years[297], around 1/3rd of episodes are viral (Norovirus / Rotavirus being commonest), the commonest bacterial infection, *Campylobacter* and *Salmonella* accounting for 10% and 3% respectively. Recovery from viral infection is usually rapid with minimal tissue destruction[298] while bacterial enteritis breaks the epithelial barrier exposing TLRs and other bacterial recognition molecules to both pathogen and commensal bacterial products. This causes acute inflammation and ulceration which may last weeks and often leaves a prolonged legacy of increased T lymphocytes and enteroendocrine cells[299].

Animal studies using *Salmonella typhimurium* and *Citrobacter rodentium* infection show that inflammation, induced by either infection or chemical colitis deplete Bacteroidetes and allow overgrowth of enterobacteria[296]. Indeed inducing inflammation may be regarded as a strategy evolved by pathogens to manipulate the host immune system to inhibit other microbiota and so create an environment favourable for their own proliferation.

Onset of new IBS symptoms after a bout of infective gastroenteritis is relatively common reported by 6% - 17% of IBS patients[300], while a recent internet survey reported 18%[301], with around 40% beginning while travelling. The clinical features of PI-IBS are predominantly those of IBS–D[302, 303]. A recent meta-analysis pooling 18 studies indicated a relative increased risk of developing IBS 1 year after bacterial gastroenteritis (mostly *Shigella, Campylobacter* and *Salmonella*) Relative Risk (RR)= 6.5 CI (2.6-15.4), an effect still apparent at 36 months, RR=3.9(3.0–5.0)[304]. Viral gastroenteritis, the commonest cause of acute gastroenteritis[297] shows a reduced incidence of PI-IBS compared to bacterial infections[305, 306]. The strongest risk factors are bacterial toxicity[307], prolonged duration of diarrhoea[303], rectal bleeding[308] and fever[304]. Acute enteritis is associated with a prolonged increase in mucosal cytotoxic T lymphocytes and increase in enteroendocrine cells[302] along with accelerated gut transit and visceral hypersensitivity at 3 months[309]. Also a gastrointestinal infection caused by the non-invasive protozoan *Giardia lamblia* has recently been found to be a risk factor for the development of IBS [310].

### d.ii Effects of GI infections on the microbiome

It is likely that in addition to these effects on gut physiology there will also be changes in the gut microbiota though detailed microbiological studies of the immediate post infectious period are limited. An early study of children with acute gastroenteritis demonstrated alkalinisation of stool pH associated with a fall in amount and variety of bacterial metabolites (short chain fatty acids) and a fall in numbers of *Bacteroides, Bifidobacterium, Lactobacillus* and *Eubacterium*[311]. Conventional enumeration of fecal bacteria showed a 10 fold fall in anaerobes (Bacteroidaceae and *Eubacterium*), little change in aerobes but $10^9$ cfu/gm of pathogens. Another study using conventional culture methods showed a reversal of the normal anaerobe/ aerobe dominance during acute infection[312].

More recent human studies using modern culture-independent methods have tended to confirm these findings[313, 314]. PCR-DGGE profiling of 16S rRNA genes showed a reduced diversity, often associated with a dominant band suggesting overgrowth of one subtype which may not always be the original pathogen. A recent clinical trial of an oral rehydration solution containing a prebiotic, amylase resistant starch, in acute diarrhoea in India children aged 3 months to 5 years, used PCR primers directed at selected bacteria including *Eubacterium spp*. and *Faecalibacterium prausnitzii*, key bacteria involved in starch fermentation. These studies showed a decline in some anaerobes (*Bacteroides spp*.,*Eubacterium spp*. and *F. prausnitzii)* while other genera including *Bifidobacterium spp.* were unchanged[313].

This depletion of anaerobes could be due to acceleration of transit which could lead to a loss of the anaerobic niche. Since these are the key bacteria involved in colonic salvage of unabsorbed carbohydrate[315] this may also contribute to the diarrhoea phenotype by preventing fermentation to SCFAs. These are known to stimulate colonic salt and water absorption, both directly and by inducing increased expression of transporters[316-318]. Previous earlier studies in IBS-D suggest impaired SCFA concentrations and production rates in ex vivo incubation which may also reflect reduced anaerobes[319].

Another cause of depletion of anaerobes is broad spectrum antibiotics which by inhibiting certain bacteria reduce colonization resistance to a range of pathogens including *Clostridium difficile* and *Klebsiella occitoca* and are known risk factors for IBS[269, 270]. There are no RCTs but epidemiological studies show an association between antibiotic use and an increased the risk of PI-IBS. A study of children showed that 3 months after *Salmonella* infection vomiting, abdominal pain and diarrhoea were reported by 9.5% of those treated with antibiotics but only 2.9% of those received no antibiotics[320]. Another study showed that antibiotic treatment for Travellers diarrhoea was associated with a relative risk of 4.1(1.1-15.3)[321], though in neither case can one exclude the possibility of confounding by indication. Thus obtaining antibiotic treatment could be a marker of

health anxiety or severity of initial illness which would increase the risk of developing PI-IBS rather than the antibiotic treatment per se.

**e. Pathophysiological mechanisms**

***e.i. Gut neuromotor-sensory dysfunction***

The selective modulation of gut motility and other aspects of gut physiology by components of the intestinal microbiome (reviewed in Chapter 4) raise therapeutic possibilities to restore altered bowel habits and visceral sensitivity using probiotic bacteria (Fig 6.3.) Studies in animal models of IBS[98] have demonstrated that the probiotic bacterium *Lactobacillus paracasei* NCC2461, its secreted products, or metabolites, modulate contractility of intestinal smooth muscle. The probiotics *L. rhamnosus* R0011 and *L. helveticus* R0052 improved gastric emptying in a model of post-infectious gastric dysmotility[96]. Conditioned media from *E. coli* Nissle 1917 was shown to modulate contractility of muscle strips isolated from humans (Bar 09). In animal models, co-administration of conditioned media from *L. paracasei* NCC2461 with antibiotics reduced visceral hypersensitivity associated with the antibiotic treatment and normalized sensory neurotransmitter expression in the myenteric and submucosal plexuses[98] . *L. acidophilus* NCFM and *L. paracasei* NCC2461 have also shown capacity to modulate visceral and pain perception in other models of visceral hypersensitivity[99, 100]. Thus, certain probiotic bacteria, or their products, can directly affect gut motor function in both animals and humans. It is likely that the pathways affected by these specific probiotics differ according to the strains and model used, and their effectiveness in attenuating visceral pain in humans remains to be determined.
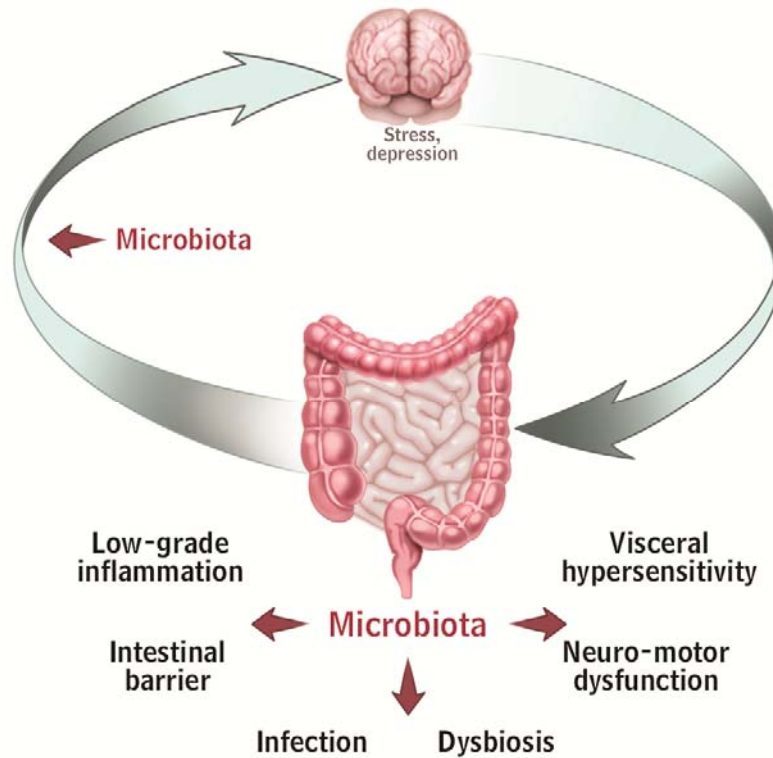
**Fig 6.3.** Integrated conceptual framework of the pathophysiology of functional disorders.

### e.ii Intestinal barrier dysfunction

In addition to being a selective physical barrier, extrinsic factors in the intestinal mucosa such as mucin production[322] and secretory immunogloubulin A (sIgA)[323] protect the host from potential noxious stimuli present in the gut lumen. Intestinal epithelial cells and Paneth cells also secrete a broad range of antimicrobial peptides including defensins, cathelicidins and calprotectins[322]. On one hand, the secretion of these agents provides defense against infections[324], but they may also contribute to shape microbial colonization of the gut. Gnotobiotic studies have revealed that intestinal bacteria are required for expression of C-type lectins and of functional α-defensins[325, 326]. This highlights that bidirectional pathways exist between the host epithelium and its microbiota (Chapter 4). In a study that included patients with IBD, IBS and healthy controls and investigated mucosal and faecal bacteria using FISH, adherent bacterial biofilms were detected in a majority of IBD patients compared to IBS and healthy controls[19]. Mucosal biofilms were also observed in healthy volunteers and IBS patients, but bacterial concentrations in both groups were lower than those in patients with overt inflammation and IBD. No clear bacterial infiltration of the mucosa was observed in any of the patients, except for areas with mucosal breaks[19]. In another study in patients with idiopathic diarrhoea, faecal analysis showed increased mucus strands and reduction in concentrations of *Eu. rectale, Bacteroides*, and *F. prausnitzii* groups [327]. Fecal levels of Human beta-

defensin-2 (HBD-2) were increased in IBS compared with healthy controls and were similar to those in the patients with ulcerative colitis[73]. Moreover, increased beta-defensin 2 peptide expression was detected by immunohistochemistry technique in colonic epithelial enterocytes of IBS patients[73]. Overall the results suggest that bacterial-host interactions may be initiated by components of the microbiota that can cross the mucus and adhere to epithelial cells, inducing activation of the mucosal innate defense system even in the absence of mucosal destruction.

### *e.iii Alterations in Gut-Brain Axis*

Psychological co-morbidity is common in gut functional disorders[328]. Animal studies have shown that psychological stress induced by maternal separation induces a shift in the bacterial composition in the gut and this is accompanied by a systemic cytokine response and increased intestinal permeability[329]. Recent studies reported behavioural changes in mice in which the microbiota had been perturbed by dietary alterations[330] or antibiotic treatment[331]. Animal models of acute[332-334] and chronic gut infection[335] have also shown induction of anxiety-like behaviour. Interestingly, in a model of anxiety-like behaviour induced by chronic parasitic infection, altered behaviour was reversed by administration of the probiotic *B. longum* NCC3001. The probiotic normalized BDNF in the hippocampus but did not influence the immune response or kynurenine levels[335]. In a model of non-infectious colitis, the same probiotic decreased excitability of enteric neurons, suggesting that in this model it may signal to the CNS by activating vagal pathways at the level of the enteric nervous system[336]. Similarly, administration of *L.rhamnosus* (JB-1) in healthy mice promoted exploratory behaviour and altered central GABA mRNA in a vagal-dependent fashion[337]. The results support the concept that components of the intestinal microbiota can affect the brain biochemistry and behaviour in adult mice and raise the hypothesis that modulation of the microbiota is a potential therapeutic approach in psychiatric co-morbidity in IBS. To date, no direct evidence of brain to microbiome interplay has been reported in humans.

There is, however, indirect evidence that the gut microbiome can affect CNS function in humans. Oral antibiotics and laxatives, that affect the microbiota, have long been used in clinical practice to treat hepatic encephalopathy[338]. The mechanism underlying this dramatic improvement in brain function in patients is unclear, but animal studies have shown that bacteria can produce a ligand for the benzodiazepine receptor[339]. On the other hand, patients with depression have altered profiles of breath hydrogen excretion after ingestion of fructose, and one uncontrolled trial showed that bowel symptoms and depression scores improved after an elimination diet but whether this is secondary to improved bowel symptoms or a direct effect is unclear[340]. Moreover, fructose malabsorption, which modifies gut physiology and the composition of the microbiome[341], has been associated with decreased plasma tryptophan levels[342].

7. THE RELATIONSHIP BETWEEN IBS AND OTHER CHRONIC GASTROINTETSINAL DISORDERS.

**Key points:**

- Although diverticulitis, IBD or coeliac disease can co-exist with IBS, an "IBS" diagnosis in the presence of an organic disease may be challenging.

- Symptomatic uncomplicated diverticular disease, celiac disease, and IBD can mimic IBS symptoms, which should encourage active case finding in people with IBS. Once the diagnosis of an organic disease is made, "IBS" is generally ruled out, even though coexistence of IBS and organic diseases seems to be relatively common.

- Chronic GI disorders share common pathogenic factors, and the insight gained from coeliac disease and IBD pathogenesis will help us unravel novel mechanisms involved in symptom generation in IBS. For instance, gluten, the storage protein in wheat that causes celiac disease in genetically susceptible people, has recently been shown to cause gut dysfunction in mice and IBS symptoms in the absence of coeliac disease.

**a. Introduction.**

The most common chronic disorders of the gastrointestinal (GI) tract, such as IBS, IBD and coeliac disease share common pathophysiological factors: genetic susceptibility that predisposes to abnormal or dysregulated host responses to a number of environmental triggers (Fig 7.1). For most disorders, such as IBS and IBD, it has been difficult to link precisely how these factors interact to increase disease susceptibility. In the case of coeliac disease we have gained tremendous understanding on how the triggering agent (gluten in wheat) interacts with genetic factors (HLA-DQ2/8) to induce a T-cell mediated immune response that causes villous atrophy.

Co-existence of more than one chronic GI disorder or development of a second illness after diagnosis of a first one can occur. Moreover, in some cases IBD and celiac disease may initially mimic IBS symptoms and it may be challenging to distinguish clinically between these disorders. Not surprisingly, proposed underlying mechanisms such as abnormal immune, barrier or neuro-motor responses to microbial or dietary antigens are shared between these disorders. (Fig 7.1)
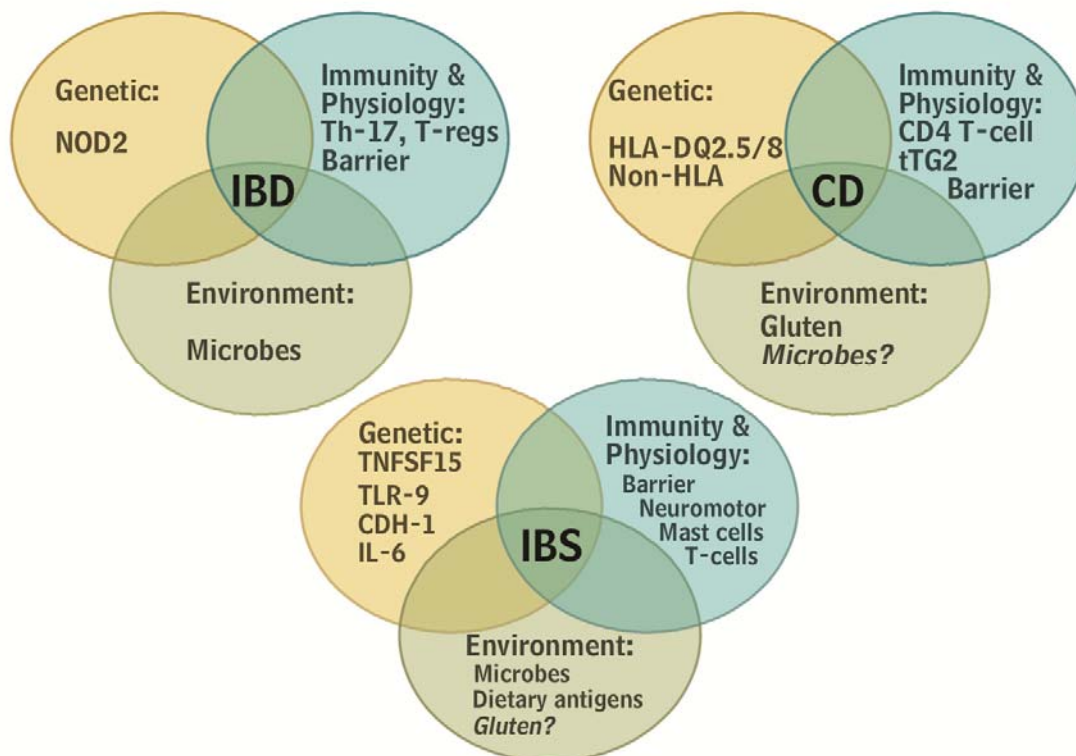
**Fig 7.1.** Common factors in the pathogenesis of chronic gut disorders. Chronic disorders of the GI tract, including inflammatory bowel disease (IBD), celiac disease (CD) and irritable bowel syndrome (IBS), share three main pathogenetic pathways: genetic predisposition, abnormal host responses and exposure to environmental triggers. In CD, the environmental trigger, gliadin, the toxic protein fraction in gluten, has been identified, as well as the mechanisms of interaction between immune (T-cell) and genetic factors (HLA DQ2/8). Progress has been made in the understanding of IBD pathogenesis. The best characterized genetic mutation associated with IBD (NOD2) may predispose to abnormal host immune responses to the intestinal microbiota. No characteristic genetic mutation has been associated with IBS, but a variety of genes that regulate innate immune function (TNFSF15, IL-6), bacterial recognition (TLR-9) and barrier function (CDH-1) have been detected. These genetic defects may predispose the host to react inappropriately to a variety of environmental triggers such as the intestinal microbiota or specific food antigens such as gluten.

**b. IBS and co-morbidities: Symptom mimicking, overlap or both?**

*b.i. IBS, Coeliac Disease and Gluten Sensitivity*

There is discussion as to whether (a) IBS symptoms and full-blown coeliac disease coexist as two separate entities, (b) coeliac disease mimics IBS symptoms ruling out "IBS" as a functional entity when coeliac disease is diagnosed or (c) milder forms of gluten intolerance that may belong to the spectrum of coeliac disease ("potential celiac disease") or not (innate or allergic immune responses

to gluten that will never develop into full blown celiac disease), can cause IBS symptoms (Fig 7.2).

Coeliac disease is a chronic inflammatory disorder of the small intestine in genetically susceptible individuals, and the only one in which the exact trigger, gluten, has been identified. Its prevalence has increased substantially in recent years, and this includes both European and North American populations[343, 344]. The increase in prevalence may not only be explained by improved diagnostic accuracy, and, consequently, environmental factors, some of which may involve alterations in the intestinal microbiota, have been proposed[344, 345]. Clinically, coeliac disease can present at any age, with a variety of gastrointestinal (GI) and non-GI manifestations[346, 347]. Some patients have symptoms of IBS that respond well to a gluten-free diet but they do not have tissue translgutaminase auto-antibodies or histological markers (mucosal atrophy) of celiac disease. This entity termed "non-coeliac gluten intolerance" or "gluten sensitive IBS" is believed to be very common[348-351]. A recent paper by Biesiekierski et al provides evidence that gluten can induce functional symptoms in subjects without coeliac disease[352]. However, the mechanisms through which gluten induces gut dysfunction in the absence of coeliac disease remain unclear. Using humanized animal models, based on mice that express the human HLA-DQ8 transgene and are deficient of endogenous mouse MHC class II molecules[353] (HLA-DQ8 and NOD-DQ8 mice), a direct link between gluten-induced activation of innate immunity and neuro-muscular dysfunction, was established[354], and these changes were exacerbated in the presence of dysbiosis[355]. The mechanistic link between gluten as a contributor of gut dysfunction in humans remains to be investigated, but could involve several pathogenetic mechanisms that have been proposed for the unselected IBS population. These may include altered microbiota composition[345], induction of low grade inflammation that leads to neuromuscular dysfunction and symptom generation, without the development of frank autoimmunity[354].
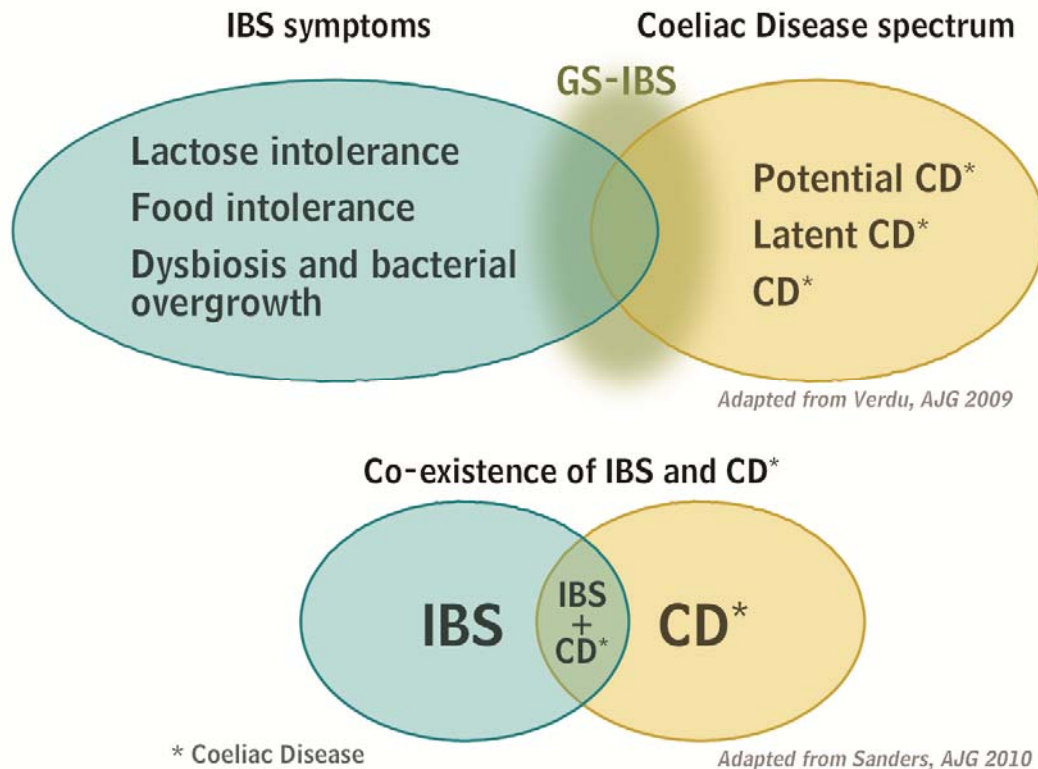
**Fig 7.2.** The association between IBS and coeliac disease. IBS symptoms and full-blown CD can coexist. However, milder forms of gluten sensitivity that may or may not belong to the celiac spectrum, can cause IBS-like symptoms. Thus, coexistence of both diseases is possible, but because active CD can cause IBS-like symptoms, this, in itself, rules out "IBS," as the definition of the syndrome requires the absence of organic pathology. Similarly as low-grade inflammation and infectious gastroenteritis, this model proposes mild gluten sensitivity as yet another possible cause of IBS symptoms.

*b.ii. IBS and Inflammatory Bowel Disease*

Recently several lines of evidence support some commonalities in the pathogenesis of IBS and IBD. The role of the intestinal microbiota in IBD has long been recognized. It is generally accepted that IBD results from a dysregulated immune response to intestinal microbiota, in genetically susceptible hosts. Consequently, several studies have investigated bacterial communities in IBD. The intestinal microbiota is altered in patients with IBD compared to healthy controls. Faecal samples from CD patients display greater temporal instability [11] and decreased number of commensal bacteria with reduction in the Firmicutes phylum, particularly *Clostridium leptum* group[356]. A significant decrease of bifidobacteria and lactobacilli has also been reported[357, 358]. On the contrary, patients with active CD had larger *Escherichia coli* faecal populations than did patients with quiescent disease or normal

controls[358], with specific increase in enteroadherent *E. coli* [359]. Studies investigating mucosa-associated microbiota showed larger numbers of bacteria in biopsies from CD patients compared to controls[357, 360-365] but their biodiversity was reduced with less prevalent members of Firmicutes[360, 366]. In particular, decreases in members of Firmicutes with anti-inflammatory capacity, such as *Faecalibacterium prausnitzii* [366] were observed. There are controversial reports on the Bacteroidetes in CD patients, with most studies reporting increase[360, 364, 365] while others show decrease in their counts[18]. Moreover, bacterial populations seem to differ in abundance depending on the different clinical different phenotypes of CD[367]. Studies in UC patients also showed smaller diversity of faecal microbiota and decrease in total lactobacilli compared to controls[177, 358, 368]. UC patients were also reported to have higher concentrations of bacteria, particularly anaerobes, but lower bifidobacteria counts in colonic biopsies than in healthy controls[361, 363-365, 369]. Other studies have detected an increase in the mucolytic bacteria *Ruminococcus gnavus* and *Ruminococcus torques* in macroscopically and histologically normal intestinal epithelium of both CD and UC. In contrast, the mucin utilizer *Akkermansia mucinphila* is frequently found to be decreased in numbers in patients suffering from inflammatory disorders, such as IBD and appendicitis compared to healthy controls [370, 371].

It has been suggested that IBS-like symptoms are common in IBD patients in long-standing remission[372, 373]. There is also clinical overlap between IBS and IBD, with IBS-like symptoms frequently reported in patients before the diagnosis of IBD[374]. It is possible that IBS and IBD coexist with a higher than expected frequency, or may exist on a continuum, with IBS and IBD at different ends of the inflammatory spectrum. In fact, a study investigating IBS symptoms in IBD patients who were thought to be in clinical remission, demonstrated high levels of calprotectin levels, suggesting that in most cases, IBS symptoms are the result of undetected ongoing inflammation[375]. Underlying mechanistic links are lacking but the intestinal microbiota may be an important factor in both diseases[376].

### *b.iii.* IBS-like symptoms following Diverticulitis

While for many patients diverticulosis is an asymptomatic condition, around 1 in 5 have complications including acute diverticulitis, perforation, abscess/ fistula formation and stricturing[377, 378]. Complications are thought to originate from microabscess formation resulting from ingress of faecal bacteria into the lamina propria via breaches in the epithelial lining of the diverticulum, possibly due to mechanical factors like impaction of faecal pellets. Risk factors for developing diverticulitis include lack of exercise[379], obesity[380] and low fibre/ fruit and vegetable intake[381]. The dietary risk factors, as well as obesity, would certainly alter the faecal microbiota as well as the consistency of stool, though which is more important in pathogenesis is unknown. Most attention so

far has been on evidence that fibre softens stool with no studies describing the changes in faecal microbiota in diverticular disease.

A high proportion of those hospitalised with acute diverticulitis continue to have persistent symptoms[382]. The precise mechanism is unclear since many do not have any of the above complications yet have recurrent short lived pain which in some features mimics IBS[383]. There is evidence that inflammation induces prolonged changes in neurochemical coding in experimental colitis[384] and diverticular disease[385] with upregulation of tachykinins which in animals can be linked to visceral hypersensitivity. An alternative explanation involving changes in the microbiota has been suggested based on some flawed uncontrolled studies claiming benefit from antibiotics and/or mesalazine but better studies are needed before any definitive conclusion can be drawn[377].

8. TREATMENT IMPLICATIONS – ANTIBIOTICS, PROBIOTICS, PREBIOTICS AND SYMBIOTICS

**Key points**

- A short course of a non-absorbable antibiotic such as rifaximin has been shown to improve the symptoms of IBS, particularly bloating and flatulence. Improvement persists after the cessation of treatment but the exact duration of this effect remains uncertain.

- The majority of trials of probiotics in IBS show some degree of efficacy although it has to be acknowledged that some of the early studies were of very poor quality. It should also be noted that different symptoms respond to different probiotics and some appear to be much more effective than others.

- Prebiotics and synbiotics should theoretically have potential in treating functional gastrointestinal disorders but there is as yet no reliable data to support this view.

As a consequence of the emerging evidence that has already been presented, one potential treatment approach is to alter the microbiota towards normal, where possible. This goal might be achieved either by administering an antibiotic or some form of "beneficial" bacteria, usually referred to as a probiotic. In addition, prebiotics and synbiotics are attracting attention as treatments for patients with functional GI disorders.

**a. Antibiotics and functional bowel disorders**

Despite evidence that previous antibiotic use may be related to the development of IBS [269, 270], as well as the fact that antibiotic treatment does not reduce digestive symptoms after a bacterial gastroenteritis [320], some nonetheless support use of antibiotics in IBS. The antibiotic approach is based on the contention that a high proportion of patients might exhibit small intestinal bacterial overgrowth, as measured by lactulose hydrogen breath testing [107]. In an attempt to avoid absorption and side effects, neomycin was the original choice and the clinical response was favourable. Neomycin led to a 35% improvement in a composite GI symptom score compared to 11% with placebo; the effect was most pronounced in those who normalized their lactulose hydrogen breath test, according to the definition used by the investigators[107, 108]. There have also been a number of mainly small and uncontrolled studies using metronidazole to treat functional gut symptoms, with variable success[386-389]. However, more recently interest has focused on a non-absorbable derivative of rifampicin, called rifaximin [390].

Rifaximin, which is approved in several counties for treatment of travellers' diarrhoea caused by non-invasive strains of *Escherichia coli,* and to reduce the risk of recurrent hepatic encephalopathy, has an excellent safety profile and minimal drug interactions [390]. To date, there are 3 fully-published, double blind, placebo controlled trials of rifaximin in functional bowel disorders[266, 391, 392]. The single-centre study by Shahara and co-workers included a mixed group of patients with abdominal bloating and flatulence as their predominant symptom, and it is not entirely clear how carefully this group of individuals was screened for other conditions that could lead to these symptoms. Of the patients included, 70/124 (56%) fulfilled the Rome II criteria for IBS [392]. Rifaximin was found to be clearly superior to placebo in reducing bloating and flatulence (symptom relief in 41% vs. 23%), even in the IBS subgroup, and the symptom improvement was correlated with a reduction in $H_2$ breath excretion. The other placebo-controlled trials where undertaken exclusively in IBS and included patients fulfilling the Rome I [391] or Rome II criteria [266] for the condition. The most recent of these only included patients with IBS without constipation [266], whereas all IBS subgroups were included in the previous study, which was mainly performed at one site [391, 393]. The data suggests an improvement in symptoms, especially bloating and flatus over the 10 weeks following cessation of treatment[266, 391]. In the most recent large study reported in 2011, the therapeutic gain for the primary (i.e. adequate relief of global IBS symptoms) and key secondary endpoint (i.e. adequate relief of bloating) relative to placebo was 9-11%, which is at the lower spectrum of what is usually considered to be clinically relevant [394]. However, there was diminished efficacy over the ten-week observation period [266], and at this stage the efficacy and safety of re-treatment is unclear, even though a recent single-centre chart review suggested that re-treatment with rifaximin in IBS was successful (as defined using subjective assessment of retrospective chart review) up to five times without any decrease in duration or effect [395]; these limited data suggest that clinicians will not experience diminishing returns with recurrent cycles of therapy, but require confirmation in larger, prospective, multi-centre studies. There has also been a controlled trial comparing the effect of rifaximin to that of activated charcoal in patients with functional bowel disorders [396], as well as a number of other uncontrolled studies and retrospective chart reviews, reporting mainly positive results with the treatment [388, 397-400]. The doses of rifaximin used in these studies vary between 600 and 2400mg/day for 7-14 days. There are important concerns regarding the widespread use of antibiotics for large groups of patients with functional bowel disorders, not only because of the potential for antibiotic resistance to develop, but also because of the question whether such treatments could lead to *C. difficile* infection. However, resistance does not appear to be a major problem so far [401], and rifaximin actually appears to have activity against *C. difficile* [402, 403], although there are reports of the organism developing resistance to this antibiotic [404, 405].

To conclude, at this stage there are promising results suggesting that a subgroup of patients with functional bowel disorders may respond favourably to a short course of gut-specific antibiotics. Among the symptoms of which patients with functional bowel disorders, bloating and flatulence appear to be especially responsive to non-absorbable antibiotics. However, in order to safely direct these treatment options to the appropriate patients, we need to know more about predictors of treatment responsiveness, the risk of development of antibiotic resistance, the efficacy and safety of re-treatment schedules and the optimal dosing regimen [393, 394].

**b.Probiotics and functional bowel disorders**

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host [406]. The most common examples are strains belonging to *Lactobacillus spp.* and *Bifidobacterium spp.*, although strains belonging to other species such as non-pathogenic *E. coli* or *Streptococcus spp.* have been used, as well as yeasts. Probiotics can be offered as preparations containing just one organism or a mixture, and they can be delivered in different formulations. In order to qualify as a probiotic there must be good scientific evidence that the preparation produces beneficial effects in humans, as laboratory or animal studies are not sufficient proof of efficacy in man. Moreover, it is important to realise that the health benefits demonstrated for a specific microbial strain cannot be extended to other strains of the same species, and the evidence accumulated for one population group cannot necessarily be extrapolated to other populations of different age or physiological state [406].

Probiotic bacteria can have a wide range of activities (Table 8.1), and from animal studies there is evidence supporting a direct effect on at least some of the putative pathophysiological mechanisms implicated in IBS, such as visceral hypersensitivity [98, 100, 407, 408], GI dysmotility [95, 409], and altered intestinal permeability [407, 410]. There are also a small number of human studies, exploring the potential mechanisms underlying a positive clinical effect, such as effects on gut motility [411-413], intestinal permeability [414], composition as well as the stability of intestinal microbiota [415, 416] and immune modulation [417]. However these activities can differ considerably between one organism and another even if, for example, they both belong to the same species. Thus, just because one organism has a beneficial effect in a condition it does not mean that a similar organism will have similar activity. Obviously, for use in the field of gastrointestinal disease a particular bacterium needs to be acid- and enzyme resistant and should survive transit through the gastrointestinal tract maintaining its beneficial activity until the desired anatomical location is reached. For instance, if activity in the small bowel is desirable, then the propensity for good adherence to the mucosa seems to be an advantage and should be maintained. Furthermore, it is also critically important that products

contain a sufficient quantity of microorganisms needed to provide benefit to the host, which for some products is questionable [418-420].

In the light of our understanding of the pathophysiology of IBS, many of the activities attributable to probiotics listed in Table 8.1, suggest potential in the treatment in IBS; this has resulted in thirty fully-published placebo-controlled clinical trials to date, which are listed in Table 8.2 [411-414, 416, 417, 421-445], although it should be noted that in one study two different probiotics were compared with placebo[417]. Unfortunately the design of these trials has varied considerably and some of the older studies do not fully meet the standards that would be demanded today. Furthermore, few of these studies attempted to define the mechanism of action of probiotics or assessed whether any symptomatic change was accompanied by an effect on the microbiota of the gut itself. However, it is possible to draw some conclusions. Of the thirty-one studies reported, twenty-three revealed some degree of symptom improvement, although the individual symptoms varied from study to study, and the therapeutic gain over placebo was generally modest, at best. In line with this reasoning, the general conclusion drawn from systematic reviews is that probiotics appear to be efficacious in IBS, but the magnitude of the benefit remains uncertain and it also remains unclear which strains and species are most effective. Furthermore, there are important limitations in the existing studies, including methodological shortcoming and concerns regarding validity of the endpoints employed in these studies[446-449]. Moreover, there is heterogeneity in the available data and possible evidence of funnel plot asymmetry, suggesting there may be publication bias, with an over-representation of small positive studies in the published literature, and the higher quality studies reported a more modest treatment effect compared with lower quality trials[449]. Thus probiotic bacteria do appear to have a tangible effect in IBS, although it would seem that the choice of organism may depend on which symptom is being targeted. For instance, some products mainly affect bloating and flatulence [412, 413, 437], whereas others improve bowel frequency [422], and some have a positive effect on a global symptom score [416, 417, 424, 428, 432, 443, 444].

When evaluating the individual trials, including large number of subjects and relevant endpoints, the results seems to be somewhat better for a number of *Bifidobacterium spp*., including *B. infantis* 35624 [417, 443, 446], *B. lactis* DN 173010 [411, 429] and *B. bifidum* MIMBb75 [428], than for some of the other probiotics. In addition, there are studies of high quality undertaken on sufficient numbers that have demonstrated a convincingly positive effect for some of the probiotic mixtures[416, 424, 432]. On the other hand, it has to be acknowledged that some large, high quality trials with negative results have also been reported[423, 434, 440, 442]. To date there have only been four published trials of the effects of probiotic products in children, but all of these have demonstrated a positive result [414, 421, 426, 427]. Most published studies included all subgroups of IBS patients, but some investigations targeted specific subgroups of IBS patients, such as *B. lactis* DN 173010 for constipation predominant IBS [411, 429], the

probiotic mixture VSL#3 for patients with diarrhoea predominant IBS [412] or IBS with bloating [413], and *Bacillus coagulans* GBI-30, 6086 for diarrhoea predominant IBS [422].

However there are many questions surrounding this therapeutic approach that still need answering:

- Are single organisms better than mixtures or vice versa?

- Are there any problems associated with taking multiple probiotic preparations?

- What are the best delivery systems in terms of liquids or capsules?

- How can viability and bioavailability be ensured?

- How should optimal doses be determined?

- How long do these preparations have to be given for?

- How often does colonisation of the host occur?

- Given that probiotics are not going to be as potent as pharmacological agents, at what patient group should they best be targeted?

- Are there any potential drawbacks to this treatment approach – it should be noted that one study reported an apparent deterioration of symptoms with active treatment compared to placebo [434].

- Should different probiotics be given to specific subgroups of IBS patients?

- Which symptoms of IBS should be the main target for therapy with probiotics?

- How should doctors and patients currently be guided regarding advice about their administration?

To conclude, probiotics in general seem to have a positive, albeit modest, effect in both children and adults with functional bowel disorders, especially IBS. However, head-to-head comparisons between different probiotic products would be useful and future trials need to be large scale, high-quality and use valid endpoints. It would also be useful if such trials could include an exploration of the possible mechanisms behind symptom improvement.

**c. Pre- and synbiotics in functional bowel disorders**

Prebiotics were defined in 1995 as "…nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health…" [267]. Recently, this definition was revisited, and modified to "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" [450]. A variety of oligosaccharides serve this function and when a prebiotic is combined with a probiotic it is called a symbiotic.

So far as prebiotics are concerned, there has only been one double blind placebo-controlled trial in IBS and this involved the use of a trans-galactooligosaccharide mixture, B-GOS [451]. Compared to placebo, both doses of this prebiotic (3.5g/day and 7g/day) resulted in symptom reduction, as well as specifically stimulating the growth of *Bifidobacterium spp..* This is an encouraging observation, but more research is required on dosing and the relative merits of different compounds. With regard to synbiotics, there are many studies of these preparations but their design has not been sufficiently robust to draw firm conclusions [452-456]. However, combining a prebiotic and a probiotic could, at least from a theoretical standpoint, increase the 'potency' of such a product.

**d. Conclusion**

From the results of these studies aimed at modifying the microbiota it is possible to draw two tentative conclusions: Firstly that this approach has therapeutic potential and secondly that if attempting to change the microbiota can improve symptoms, then this supports the view that there might be a microbial imbalance in the first place. However, future trials more explicitly address the question of how symptomatic improvement is achieved. Is it mirrored by a change in the microbiota of the gut or is some other mechanism involved?

**Table 8.1. Some effects of probiotics**

- Stimulation of anti-inflammatory and immune responses

- Enhancement of epithelial barrier and reduction of bacterial translocation

- Inhibition of growth of pathogens such as *Salmonella spp.*

- Inhibition of adhesion of viruses such as rotavirus

- Elaboration of active proteins and metabolites with toxin binding, immune modulatory and active bactericidal activity

**Table 8.2.** Placebo-controlled clinical trials of single or mixed probiotic preparations in irritable bowel syndrome [411-414, 416, 417, 421-445]

| Studies in adult patients | | | |
|---|---|---|---|
| Organism | n | Outcome | Reference |
| S.faecium | 54 | ↓ global score | Gade et al 1989[425] |
| L.acidophilus | 18 | ↓ global score | Halpern et al 1996[430] |
| L.plantarum 299V | 60 | ↓ flatulence | Nobaek et al 2000[437] |
| L.plantarum 299V | 20 | ↓ pain, "all IBS symptoms" | Niedzielin et al 2001[435] |
| L.plantarum 299V | 12 | negative | Sen et al 2002[439] |
| L.plantarum MF1298 | 16 | deterioration of symptoms | Ligaarden et al 2010[434] |
| L.rhamnosus GG | 25 | negative | O'Sullivan et al 2000[438] |
| L.reuterii ATCC 55730 | 54 | negative | Niv et al 2005[436] |
| L.salivarius UCC4331 | 75 | negative | O'Mahony et al 2005[417] |
| B.infantis 35624 | 75 | ↓ pain and composite score | O'Mahony et al 2005[417] |
| B.infantis 35624 | 362 | ↓ pain and composite score | Whorwell et al 2006[443] |
| B.lactis DN-173-010 | 274 | ↓ digestive discomfort | Guyonnet et al 2007[429] |
| B.lactis DN-173-010 | 34 | ↓ maximum distension & pain | Agrawal et al 2008[411] |
| B.bifidum MIMBb75 | 122 | ↓ global score | Gugliemetti et al 2011[428] |
| Bacillus coagulans GBI-30, 6086 | 52 | ↓ bowel movements | Dolin 2009[422] |
| E.coli Nissle 1917 | 120 | ↑ treatment satisfaction | Kruis et al 2011[445] |
| VSL#3® (x8)* | 25 | ↓ bloating | Kim et al 2003[412] |
| VSL#3® (x8)* | 48 | ↓ flatulence | Kim et al 2005[413] |
| medilac DS® (x2)* | 40 | ↓ pain | Kim et al 2006[433] |
| Mixture (x4)* | 103 | ↓ global score | Kajander et al 2005[432] |
| Mixture (x4)* | 86 | ↓ global score | Kajander et al 2008[416] |
| LAB4 (x4)* | 52 | ↓ global score | Williams et al 2009[444] |
| Mixture (x4)* | 106 | negative | Drouault-Holowacz et al 2008[423] |
| Mixture (x2)* | 40 | ↓ pain | Sinn et al 2008[441] |
| ProSymbioFlor® (x2)* | 297 | ↓ global score | Enck et al 2008[424] |
| Cultura® (x3)* | 74 | negative | Simrén et al 2010[440] |
| Cultura® (x3)* | 52 | negative | Sondergaard et al 2011[435] |
| Mixture (x4)* | 70 | ↓ pain | Hong et al 2009[431] |
| | | | |
| Studies in pediatric patients | | | |
| Organism | n | Outcome | Reference |
| L.rhamnosus GG | 50 | ↓ abdominal distension | Bausserman et al 2005[421] |
| L.rhamnosus GG | 104 | ↓ pain | Gawronska et al 2007[419] |

| | | | |
|---|---|---|---|
| *L.rhamnosus GG* | 141 | ↓ pain | Francavilla et al 2010[414] |
| VSL#3® (x8)* | 59 | ↓ global score | Guandalini et al 2010[427] |

S. = *Streptococcus*; L. = *Lactobacillus*; B. = *Bifidobacterium*
* = Number of organisms in a mixture

9. CLINICAL GUIDANCE REGRADING MODULATION OF INTESTINAL MICROBIOTA IN IBS.

**Key points**

| Summary of Clinical Guidance Regarding Modulation of Intestinal Microbiome in IBS (Fig.9.2) |
| --- |
| • There is currently no clinically useful way of identifying whether the microbiota is disturbed in a particular patient with IBS. As a consequence, any approach that aims to alter the gut microbiota has, at present, to be undertaken on an empirical basis . |
| • Attempts to modify the microbiota should start with the safest and most economical options. |
| • Dietary evaluation and exclusion of possible sources of unabsorbable carbohydrate including FODMAPs and excessive fibre should be performed first. |
| • Prebiotics which stimulate the growth of certain bacteria have possible benefit in constipation (e.g. lactulose) |
| • Probiotics have a reasonable evidence base and should be tried, for a period of at least one month, at adequate doses before a judgment is made about response to treatment. Since they do not generally colonise the gut, if effective they will probably have to be administered long term. |
| • Not all probiotic preparations are necessarily of a standard that would be demanded for pharmaceuticals and therefore it is essential to ensure that the source of a particular preparation is completely reliable. |
| • Probiotics are unlikely to exhibit the potency of pharmacological agents and consequently are likely to be effective in the less severe cases of IBS. |
| • The utility of testing for SIBO in the setting of IBS remains an area of uncertainty but the lactulose breath test is unreliable and should be abandoned. |
| • If there is good reason to suspect SIBO then the glucose breath test or jejunal aspirate should be performed. |
| • Consideration should be given to discontinuing PPIs in those with SIBO. |
| • There is emerging evidence that non-absorbable antibiotics may have the potential to reduce symptoms in some patients with IBS. |
| • The non-absorbable antibiotic with the most evidence is rifaxamin with a number needed to treat of 11. However, questions remain about duration of treatment, how long the beneficial effects last, the development of resistance and the long term safety for the population as a whole. Thus antibiotic therapy should be considered only for those failing other treatments. |

### a. Overview of Clinical Considerations

Although the science regarding the role of microbiota in FGIDs is fascinating it remains in its infancy; there is much left to learn.  Despite our ignorance, clinicians faced with a patient still have to make decisions.  This section aims to help clinicians decide on whether to suggest modulators of intestinal

microbiota to benefit patients with FGIDs, particularly IBS to which most of the limited evidence applies.

### b. Diet

As described in Chapter 4, diet profoundly alters the microbiota since unabsorbed dietary components provide the nutrients which drive microbial growth. Bloating and diarrhoea have been shown to respond to reducing fibre intake[387] or reducing intake of fructose, fructans and other poorly absorbable but fermentable dietary items, the basis of the "FODMAP diet" [457]. The great advantage of such treatments is their undoubted safety and prolonged effect[458]. Detailed description of how these diets alter gut microbiota are lacking but the change in metabolism of lactulose observed with a low residue diet[459] is a marker that these diets do alter colonic fermentation, which can be considered a marker of altered microbiome. Systematic exclusion diets may help[458] though they are laborious; it may be more practical to enact targeted exclusion of likely suspects like regularly consumed dairy, wheat, fruit and vegetables. There are few proper randomized, placebo controlled trials because of the difficulty in controlling for the placebo effect. One RCT showed bran aggravated symptoms[460] and hence it would be logical that excluding it would help. Many patients exclude lactose, which in lactose malabsorbers will act as a prebiotic and undoubtedly alter the microbiota.

### c. Prebiotics

Lactulose was once widely used as a laxative. It is a potent prebiotic which causes a substantial increase in beta-galactosidase activity and corresponding increased efficiency in metabolising lactulose during chronic dosing[54]. More recently studies assessing directly the changes in fecal bacteria have confirmed that lactulose induces a significant increase in faecal *Bifidobacterium spp*. counts and b-galactosidase activity[58]. A novel prebiotic trans-galactooligosaccharide designed to be preferentially metabolised by bifidobacteria has been shown to increase their number in IBS and although the study very small, there was a reduction in bloating and flatulence[451]. However inulin also increases Bifidobacteria but increases flatulence[61] as most other studies of prebiotics have shown. This is likely to be a major limitation of prebiotic therapy in IBS.

### d. Probiotics

While a meta-analysis has suggested these do have benefit in IBS, the variability in study design and probiotic used suggests that this group of very differing treatments is not well suited to a meta-analytical approach. The studies are often small and poorly designed and since they used unvalidated endpoints are hard to compare with other established treatments. Furthermore there are many different probiotics which vary in their effectiveness and mode of action. Many studies include less than 50 subjects and are obviously underpowered to assess symptoms, which typically need 100-200 subjects per treatment. A recent systematic review reported that studies with poorer quality scores

tended to show larger effects and published data indicates a publication bias, with non-reporting of negative effects in small trials[449].

Table 9.1. shows studies with >50 subjects per treatment. Only one small study in mixed IBS reported unfavourable effects with exacerbation of diarrhoea and worsening symptoms even in those with constipation[434]. The evidence suggests that other small studies with negative effects have never been published. Few studies have assessed the impact on faecal bacteria and hence shown a direct link between changes in bacteria and symptoms. One study showed a multispecies probiotic containing *Lactobacillus spp., Bifidobacterium spp.,* and *Proprionobacterium spp.* showed a fall in a phylotype with 94% similarity to *Ruminococcus torques* and increase in certain *Clostridium spp.*[461]. More studies which demonstrate the effect of probiotics on faecal microbes are needed to see if these changes can be reliably linked to symptom improvement. This would help in determining the true mechanism of action. Several studies have shown a beneficial effect of probiotics on abnormal gut permeability[414, 462, 463]. However although others have linked abnormal permeability to severity of pain[464], the study in children found no relationship between the effect on permeability and the effect on symptoms[414]. However gut permeability may be a worthwhile objective biomarker of probiotic effect in future studies.

Thus the evidence base is weak and this should be acknowledged when discussing with a patient whether to try probiotics. Safety in the setting of IBS seems good but at least one study found symptoms got worse so the patients should be warned about this and told to discontinue if this happens. At present the strongest evidence is for *Bifidobacterium infantis* 35624 at a dose of $1 \times 10^8$ cfu/day[443]. The evidence suggests that this should be taken for at least 4 weeks since the benefit did appear greater towards the end of the 4 week trial. Much work needs to be done to define who benefits, what is the best variety of probiotic and what are the best endpoints to determine efficacy.

**Table 9.1.** Larger randomized controlled trials of probiotics in IBS.

| RCTs probiotics in IBS with >50 subjects per treatment | | | | | |
|---|---|---|---|---|---|
| **Probiotic dose** | **Reference** | **Subjects** | **Duration of treatment** | **Outcome Significant differences** | **Comment** |
| *L. rhamnosus GG & LC705, B breve Bb99 & Proprionobacterium Freudenreichii ssp.Shermanii* $0.9 \times 10^{10}$ *cfu/day* | Kajander et al 2005[432] | 103 subjects 37% IBS-D | 24 weeks | Improvement in composite score | No difference in change in quality of life (QOL) |
| *L. rhamnosus GG & LC705, B breve Bb99 & Proprionobacterium Freudenreichii ssp.Shermanii* $0.48 \times 10^{10}$ *cfu/day* | Kajander et al 2008[416] | 86 unselected | 20 weeks treatment + 3 weeks follow-up | Composite IBS score decreased | |
| *Bifidobacterium infantis* $1 \times 10^6$, $10^8$ & $10^{10}$ *cfu/day* | Whorwell et al 2006[443] | 362 unselected 58% IBS-D | 4 weeks treatment + 2 weeks follow-up | Decrease in composite symptom score + pain discomfort with $10^8$ cfu/day dose only | Formulation problem with $10^{10}$ cfu/day dose |
| Fermented milk containing *Bifidobacterium animalis* $1.25 \times 10^{10}$, cfu/day *Streptococcus thermophilus & L. bulgaircus* $1.2 \times 10^9$, cfu/day compared to heat killed yoghurt | Guyonnet et al 2007[429] | 274 IBS-C | 6 weeks | Significant difference in "responder" rate at 3 but not 6 weeks | Subgroup with <3 BM/wk responded better with BM/wk & responder rate |
| *Bifidobacterium Longum, Lactobacillus acidophilus, Lactobacillus lactis, Streptococcus thermophilus* $13 \times 10^{10}$ *in total* | Drouault-Holowacz et al 2008[423] | 116 IBS Rome II | 4 weeks | No difference in symptoms of IBS | Increased stool frequency in IBS-C |
| *Escherichia coli* lysate | Enck et al 2009[424] | 298 IBS | 8 weeks | Abdominal pain score fell Responder rate 18% active vs. 5% placebo | Responder = absence of abdominal pain on 1 or more weeks |
| Yoghurt (*Lactobacillus paracaseii ssp. paracasei, Lactobacillus acidophilus & Bifidobacterium lactis*) All $5 \times 10^7$ cfu daily | Simren et al 2010[440] | 74 IBS Rome II | 8 weeks | No difference in responder rate | Benefit appeared transient, maximum in weeks 1-3 |
| *Lactobacillus rhamnosus* GG $3 \times 10^9$ twice daily | Francavilla et al 2010[414] | 141 children with abdominal pain | 8 weeks | "Significant reduction in pain" 72% active vs. 53% placebo | Active reduced % with abnormal permeability by 40% vs. 21% on placebo |
| *Bifidobacterium bifidum MIMBb75* | Guglielmetti et al 2011[428] | 122 mild/moderate IBS unselected (59% mixed) | 8 weeks | Composite score (0-6) fell 0.8 on active versus 0.2 on placebo | Responder= decrease of 1 point on 0-6 global scale for 50% of weeks |

| | | | | | 57% active versus 21% placebo |
|---|---|---|---|---|---|
| *Escherichia coli* Nissle 1917 | Kruis et al 2011[445] | 120 IBS Rome II | 12 weeks | Higher treatment satisfaction weeks 11 & 12 | |

### e. Treatments altering Motility

An alternative approach to manipulating the microbiota would be to alter intestinal transit which can itself alter intestinal microbiota, as previously discussed in Section 4. It is well-established that motility is abnormal in many IBS patients and therapies that address motility improve IBS symptoms. Thus alosetron slows transit in IBS[465], while linaclotide[466], lubiprostone[467], and tegaserod[468] all accelerate transit in IBS-C, and all have high quality randomized controlled trials supporting their efficacy in IBS. However only one study using loperamide has examined the impact on the microbiome[63]. Future research should evaluate whether these effective treatments alter the gut microbiota and whether these changes correlate with symptom improvement.

### f. Discontinuing Proton Pump Inhibitor (PPI) Therapy

Over 40% of reflux patients starting PPI therapy develop bloating after 8 weeks of therapy, and that 1 in 5 meet Rome III criteria for IBS after 6 months of PPI use (without meeting criteria at baseline)[256]. Furthermore, data also reveal that return of an abnormal glucose breath test in IBS following rifaximin therapy is independently predicted by use of concurrent PPI therapy[262]. Thus clinicians should, where possible, consider discontinuing PPI therapy in IBS patients and perhaps replacing with less potent acid suppression such as $H_2$ blockers or antacids.

### g. Antibiotics Therapies in IBS

Given the rapid development of antibiotic resistance whenever antibiotics are widely used the use of antibiotics in a condition as ubiquitous as IBS seems problematic, particularly as the condition is chronic and the treatment seems likely to be given repeatedly since the benefit appears to be wearing off by 12 weeks[266]. However if intestinal microbiota are an important cause of IBS (or FGID) symptoms, then it would seem reasonable to employ gut-directed antibiotics in a carefully selected group of IBS patients. Various antibiotics have been employed in IBS but the early trials were poorly designed and underpowered. The best evidence comes from two large RCTs of the poorly absorbed, broad spectrum antibiotic, rifaximin 550 mg, thrice daily for 2 weeks[266]. More patients in the rifaximin group achieved adequate relief of IBS symptoms during the 4 week period after treatment

compared to those receiving placebo (40.7% versus 31.7%, P<0.001); treated patients also had a higher response rate for bloating (40.2% versus 30.3%, P<0.001). Of note, the investigators did not test patients for small intestinal bacterial overgrowth (SIBO) in advance of treatment. The rationale for empiric treatment was not described by the authors and indeed the evidence discussed above (Chapter 6) suggests that the antibiotic effect was most likely mediated via effects on colonic bacteria rather than those in the small bowel. Although rifaximin was superior to placebo, the number-needed-to-treat (NNT) was 11 which is on par or worse than most other potential treatments. Table 9.2. shows the NNT from recent trials in which the methodology is comparable suggesting that at 11 the NNT for rifaximin is at the high end of treatments shown to be better than placebo, while  fig. 9.1. shows the trade-off between effectiveness versus invasiveness/safety of treatments that modulate intestinal microbiota [266, 469-474].

**Table 9.2.** Number Needed to Treat (NNT) for Irritable Bowel Syndrome (IBS).

| IBS Treatment | NNT vs. Placebo[*] | References |
|---|---|---|
| "Placebo without deception"[***] | 4 | [474] |
| Alosetron | 8 | [469] |
| Linaclotide[**] | 8 | [473] |
| Rifaximin | 11 | [266] |
| Lubiprostone | 12 | [470,472] |
| Tegaserod | 14 | [471] |

***"Placebo without deception" involves giving a placebo and actively informing the patient that it is an inactive agent. Patients were informed that they received "placebo pills made of an inert substance, like sugar pills, that have been shown in clinical studies to produce significant improvement in IBS symptoms through mind-body self-healing processes." Compared to no treatment, this approach was highly effective in a well-documented randomized controlled trial[474].
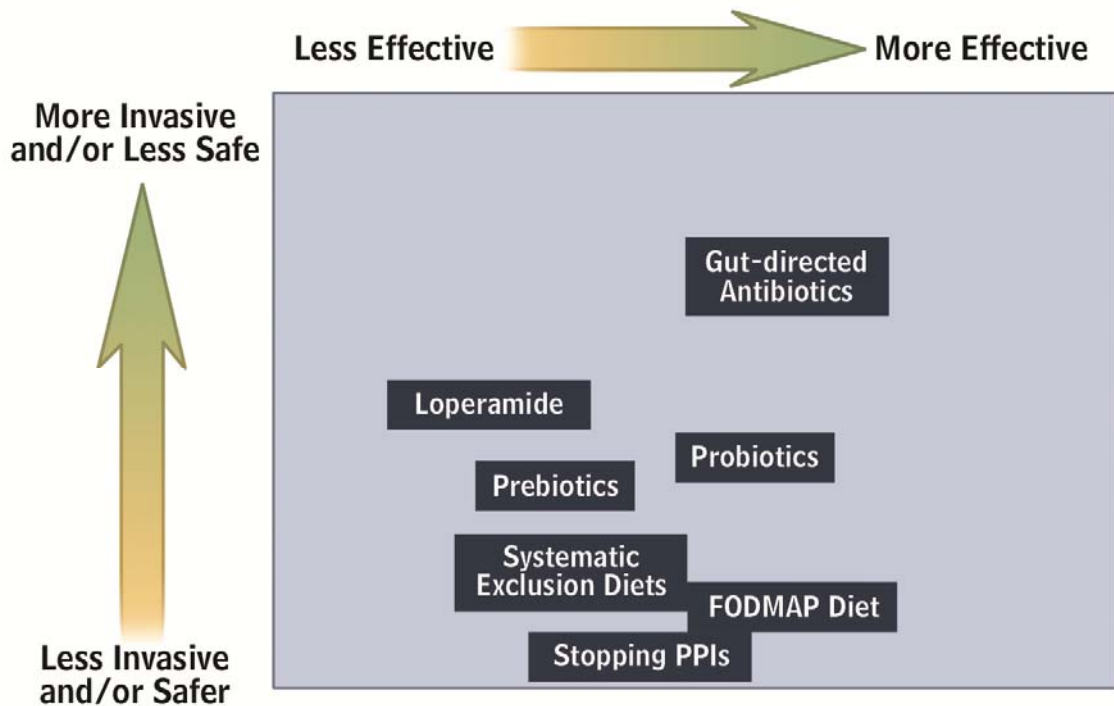
**Fig 9.1.** Effectiveness vs. invasiveness/safety of treatments that modulate intestinal microbiota in irritable bowel syndrome (IBS)

**h. Potential Risks of Gut-Directed Antibiotic Use in Clinical Practice**

Although rifaximin appears to be well tolerated and safe, given its relatively low potency it is important to consider its downside. Rifaximin use can promote rifampin-resistant strains of staphylococci which remained resistant for at least 9 weeks implying that there is no fitness cost to the resistance mutation that emerges[405]. This is potentially problematic, because rifampin is a vital treatment for management of staphyloccal foreign body infections such as prosthetic valve endocarditis or prosthetic joint infections. Although IBS patients are themselves unlikely to develop staphylococci foreign body infections, they could potentially harbour and transmit resistant strains, though further research is needed to reproduce these initial findings and establish the clinical importance of the results.
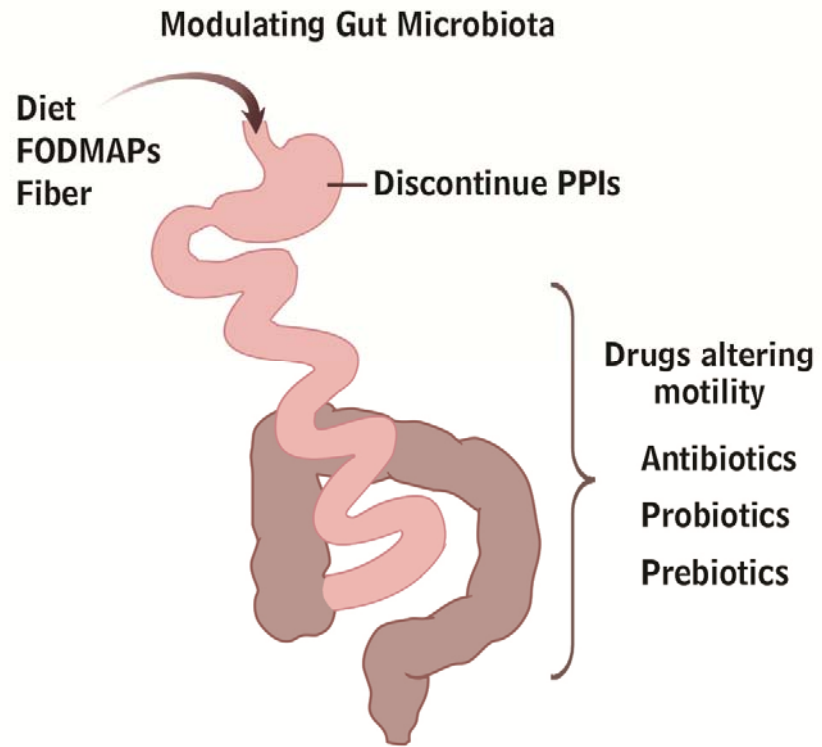
**Fig 9.2.** Different ways to modulate gut microbiota.

10. CONCLUSIONS & RECOMMENDATIONS FOR FUTURE RESEARCH AND DEVELOPMENT

In this review we critically discussed the latest developments in the understanding of the role of intestinal ecosystem and the efficacy of microbiota directed therapies in functional bowel disorders. Several observations suggest that gut bacteria are important in the pathophysiology of functional bowel disorders in some patients. These include: 1) evidence of both qualitative and quantitative changes in intestinal microbiota in IBS; 2) the well-established role enteric infection in the development of IBS symptoms in predisposed individuals and 3) data supporting the evidence that microbiota modulation with probiotics and non-absorbable antibiotics may provide some beneficial effects in subgroups of patients. Nonetheless, as we acknowledge the importance of these recent developments, we also realize that the field is in its infancy and a number of both basic and clinical key issues remain to be addressed in the future.

Since the diversity of gut bacteria is under-represented by characterized cultured isolates, the introduction of modern molecular techniques has offered the opportunity to obtain novel information on the phylogenetic and functional properties of microbial ecosystems in health and disease states[274]. However, this enormous technological potential has been so far only applied to the field of functional bowel disorders in a limited way. Thus, a deeper phylogenetic characterization of microbiota through high throughput sequencing or phylogenetic microarraying is crucial in future studies. We need to know more about mucosal ecological niches in patients with functional bowel disorders since the vast majority of studies are currently based on faecal samples, which may not representative of the microbiota throughout the intestine. Microbes embedded in the mucous layer likely form a different microbial ecosystem retaining important properties in the regulation expression of host genes (eg, TLR) and epithelial, endocrine and immune physiological functions of the intestine[33, 184]. The controversial SIBO hypothesis in patients with IBS could also take advantage of modern molecular techniques. In fact, currently the SIBO hypothesis is largely based on findings obtained with the lactulose breath test which has poor sensitivity and limited ability to discriminate the detection of ileal as opposed to caecal bacteria[113] The study by Posserud et al. provided no evidence of clear-cut jejunal bacterial overgrowth in patients with IBS compared with controls. However, interestingly enough this study showed sub-threshold increases in cultivable microbes[115] which are worth further characterization with high throughput culture-independent technologies.

Despite the need of a deeper characterization of qualitative and quantitative microbiota changes in functional bowel disorders, a phylogenetic approach alone may turn out to be insufficient to elucidate if microbiota changes are cause or consequence of altered bowel physiology. Time has come to move on and direct research to the characterization of functional properties of microbiota in functional bowel disorders. This type of research can take advantage now of a wide range of novel tools including metagenomic, metatranscriptomics, metaproteomics, metabonomic techniques[274]. These studies should be also extended to the identification of the impact of regulatory signals produced by intestinal bacteria to the epithelium, immune system and enteric nerves. In turn, these mechanistic as opposed to descriptive studies could provide novel insights on the role of intestinal bacteria in the pathophysiology of functional bowel disorders. One field of particular interest is related to gut immunity. Microbiota is a driving force for the mucosal immune system and several recent studies have pointed out the presence of low grade immune activation in patients with IBS [13]. It has been hypothesized that low grade inflammation in IBS may occur as a consequence of exaggerated exposure of the immune system to luminal bacterial antigens through a leaky epithelial barrier[475]. Data showing increased expression of TLR recognising pathogen associated molecular patterns in patients with IBS as compared with healthy controls, is in line with this concept and suggest the existence of altered host-microbiota and the innate immune system interactions. Mucosal release of antimicrobial substances such as defensins produced by Paneth cells represent another extremely exciting field of study deserving further attention[476].

Since host-bacterial interactions is a dynamic process, particularly in diseases states, there is also a need for longitudinal or interventional studies assessing the role of microbiota and diet, the relationship of changes in microbiotia to remission and symptom flare-ups, and stress, infection or therapeutic modulation (e.g. probiotics, prebiotics, antibiotics). The demonstration of a bidirectional brain-gut-microbial axis[477] and the existence of a systemic immune response to microbial luminal antigens (anti-flagellin antibodies)[478] points out that microbial homeostasis may be perturbed beyond the gastrointestinal tract and opens the field to novel avenues which could not be even imaginable only few years ago.

The wide heterogeneity of functional bowel disorders and the inter-individual variability of microbiota profiles suggest that larger sample size studies will be of key importance in the future. In addition, a recent study demonstrated that the microbiota in humans can be divided into enterotypes [274] and hence, typing patients based on their enterotype could be as important as typing them for other phenotypic and genotypic characteristics in relation to health and disease.

Moreover, microbiota signatures can be developed to contribute to IBS diagnostics and segmentation. When cause-effects have been established then they offer the potential to develop therapeutic avenues. Attention should also be directed to accurate characterization of patient's symptom and, whenever possible, studies should be stratified by factors known to affect intestinal microbiota. As detailed in Chapter 4, this should include primarily the effect of age and diet. In particular, diet has powerful influences on gut microbiota and dietary manipulation is often employed by both patients and clinicians in the attempt to improve symptoms. Researchers should also consider the potential confounding effect of previous or concomitant use of drugs with potential interference on intestinal microbiota including antibiotics, probiotics, laxatives, prokinetcs, proton pump inhibitors and mesalazine.

Currently, there are promising results suggesting that a subgroup of patients with functional bowel disorders may respond favourably to a short course of gut-specific antibiotics. Among the symptoms, bloating and flatulence appear to be especially responsive to non-absorbable antibiotics. However, in order to safely direct these treatment options to the appropriate patients, we need to know more about, predictors of treatment responsiveness, the risk of development of antibiotic resistance, the efficacy and safety of re-treatment schedules and the optimal dosing regimen [393,394]. Further studies should also investigate the mechanism and site of action of non-absorbable antibiotics since amelioration of gas-related symptoms in patients occurred also in patients with no evidence of SIBO [392]. Probiotics in general seem to have a positive, albeit modest, effect in both children and adults with functional bowel disorders, especially IBS. However, head-to-head comparisons between different probiotic products would be useful and future trials need to be large scale, high-quality and use valid endpoints. It would also be useful if such trials could include an exploration of the possible mechanisms behind symptom improvement.

To conclude, a better definition of the role of intestinal microbiota in the pathogenesis, pathophysiology of functional bowel diseases represents a challenge for the future. Although promising, therapeutic implications will need to be better defined in well conducted large trials. A strict cooperation of experienced clinical researchers with microbial ecologists should be considered an important factor for the success of these future studies.

## 11. REFERENCES

1.  Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology 2006;130:1377-90.
2.  Koloski NA, Talley NJ, Boyce PM. Epidemiology and health care seeking in the functional GI disorders: a population-based study. Am J Gastroenterol 2002;97:2290-9.
3.  Belsey J, Greenfield S, Candy D, Geraint M. Systematic review: impact of constipation on quality of life in adults and children. Aliment Pharmacol Ther 2010;31:938-49.
4.  Gralnek IM, Hays RD, Kilbourne A, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. Gastroenterology 2000;119:654-60.
5.  Simren M, Svedlund J, Posserud I, Bjornsson ES, Abrahamsson H. Health-related quality of life in patients attending a gastroenterology outpatient clinic: functional disorders versus organic diseases. Clin Gastroenterol Hepatol 2006;4:187-95.
6.  Spiegel BM. The burden of IBS: looking at metrics. Curr Gastroenterol Rep 2009;11:265-9.
7.  Hillila MT, Farkkila NJ, Farkkila MA. Societal costs for irritable bowel syndrome--a population based study. Scand J Gastroenterol 2010;45:582-91.
8.  Jiang X, Locke GR, Zinsmeister AR, Schleck CD, Talley NJ. Health care seeking for abdominal bloating and visible distention. Aliment Pharmacol Ther 2009;30:775-83.
9.  Singh G, Lingala V, Wang H, Vadhavkar S, Kahler KH, Mithal A, Triadafilopoulos G. Use of health care resources and cost of care for adults with constipation. Clin Gastroenterol Hepatol 2007;5:1053-8.
10. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology 2006;130:1480-91.
11. Gunnarsson J, Simren M. Peripheral factors in the pathophysiology of irritable bowel syndrome. Dig Liver Dis 2009;41:788-93.
12. Ohman L, Simren M. New insights into the pathogenesis and pathophysiology of irritable bowel syndrome. Dig Liver Dis 2007;39:201-15.
13. Ohman L, Simren M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. Nat Rev Gastroenterol Hepatol 2010;7:163-73.
14. Clouse RE, Mayer EA, Aziz Q, Drossman DA, Dumitrascu DL, Monnikes H, Naliboff BD. Functional abdominal pain syndrome. Gastroenterology 2006;130:1492-7.
15. Young VB, Schmidt TM. Overview of the gastrointestinal microbiota. Adv Exp Med Biol 2008;635:29-40.
16. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiol Rev 2010;90:859-904.
17. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep 2006;7:688-93.
18. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007;104:13780-5.
19. Swidsinski A, Loening-Baucke V, Lochs H, Hale LP. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World J Gastroenterol 2005;11:1131-40.
20. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 2007;449:811-8.
21. Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut 2008;57:1605-15.
22. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307:1915-20.
23. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005;308:1635-8.

24. Parkes GC, Brostoff J, Whelan K, Sanderson JD. Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. Am J Gastroenterol 2008;103:1557-67.

25. Spiller R, Garsed K. Postinfectious irritable bowel syndrome. Gastroenterology 2009;136:1979-88.

26. Quigley EM. Therapies aimed at the gut microbiota and inflammation: antibiotics, prebiotics, probiotics, synbiotics, anti-inflammatory therapies. Gastroenterol Clin North Am 2011;40:207-22.

27. Schmulson M, Chang L. Review article: the treatment of functional abdominal bloating and distension. Aliment Pharmacol Ther 2011;33:1071-86.

28. Ford AC, Spiegel BM, Talley NJ, Moayyedi P. Small intestinal bacterial overgrowth in irritable bowel syndrome: systematic review and meta-analysis. Clin Gastroenterol Hepatol 2009;7:1279-86.

29. Salonen A, de Vos WM, Palva A. Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. Microbiology 2010;156:3205-15.

30. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. Science 2008;320:1647-51.

31. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837-48.

32. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102:11070-5.

33. Carroll IM, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, Ringel Y. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2011;301:G799-807.

34. Gronlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr 1999;28:19-25.

35. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511-21.

36. Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol 1982;15:189-203.

37. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007;5:e177.

38. Schwiertz A, Gruhl B, Lobnitz M, Michel P, Radke M, Blaut M. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. Pediatr Res 2003;54:393-9.

39. Balamurugan R, Janardhan HP, George S, Chittaranjan SP, Ramakrishna BS. Bacterial succession in the colon during childhood and adolescence: molecular studies in a southern Indian village. Am J Clin Nutr 2008;88:1643-7.

40. Enck P, Zimmermann K, Rusch K, Schwiertz A, Klosterhalfen S, Frick JS. The effects of ageing on the colonic bacterial microflora in adults. Z Gastroenterol 2009;47:653-8.

41. Martin R, Jimenez E, Heilig H, Fernandez L, Marin ML, Zoetendal EG, Rodriguez JM. Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol 2009;75:965-9.

42. Martin R, Heilig GH, Zoetendal EG, Smidt H, Rodriguez JM. Diversity of the Lactobacillus group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J Appl Microbiol 2007;103:2638-44.

43. Coppa GV, Bruni S, Morelli L, Soldi S, Gabrielli O. The first prebiotics in humans: human milk oligosaccharides. J Clin Gastroenterol 2004;38:S80-3.

44. Marzotto M, Maffeis C, Paternoster T, Ferrario R, Rizzotti L, Pellegrino M, Dellaglio F, Torriani S. Lactobacillus paracasei A survives gastrointestinal passage and affects the fecal microbiota of healthy infants. Res Microbiol 2006;157:857-66.

45. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 2010;107:14691-6.

46. Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR, Tuohy KM. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. Br J Nutr 2008;99:110-20.

47. Leach ST, Mitchell HM, Eng WR, Zhang L, Day AS. Sustained modulation of intestinal bacteria by exclusive enteral nutrition used to treat children with Crohn's disease. Aliment Pharmacol Ther 2008;28:724-33.

48. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. Nature 2009;457:480-4.

49. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 1999;65:4799-807.

50. Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. Appl Environ Microbiol 2007;73:1073-8.

51. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 2009;1:6ra14.

52. Humblot C, Bruneau A, Sutren M, Lhoste EF, Dore J, Andrieux C, Rabot S. Brussels sprouts, inulin and fermented milk alter the faecal microbiota of human microbiota-associated rats as shown by PCR-temporal temperature gradient gel electrophoresis using universal, Lactobacillus and Bifidobacterium 16S rRNA gene primers. Br J Nutr 2005;93:677-84.

53. Lewis S, Burmeister S, Brazier J. Effect of the prebiotic oligofructose on relapse of Clostridium difficile-associated diarrhea: a randomized, controlled study. Clin Gastroenterol Hepatol 2005;3:442-8.

54. Florent C, Flourie B, Leblond A, Rautureau M, Bernier JJ, Rambaud JC. Influence of chronic lactulose ingestion on the colonic metabolism of lactulose in man (an in vivo study). J Clin Invest 1985;75:608-13.

55. Jouet P, Sabate JM, Cuillerier E, Coffin B, Lemann M, Jian R, Flourie B. Low-dose lactulose produces a tonic contraction in the human colon. Neurogastroenterol Motil 2006;18:45-52.

56. Ballongue J, Schumann C, Quignon P. Effects of lactulose and lactitol on colonic microflora and enzymatic activity. Scand J Gastroenterol Suppl 1997;222:41-4.

57. Bouhnik Y, Attar A, Joly FA, Riottot M, Dyard F, Flourie B. Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. Eur J Clin Nutr 2004;58:462-6.

58. Bouhnik Y, Neut C, Raskine L, Michel C, Riottot M, Andrieux C, Guillemot F, Dyard F, Flourie B. Prospective, randomized, parallel-group trial to evaluate the effects of lactulose and polyethylene glycol-4000 on colonic flora in chronic idiopathic constipation. Aliment Pharmacol Ther 2004;19:889-99.

59. Attar A, Lemann M, Ferguson A, Halphen M, Boutron MC, Flourie B, Alix E, Salmeron M, Guillemot F, Chaussade S, Menard AM, Moreau J, Naudin G, Barthet M. Comparison of a low dose polyethylene glycol electrolyte solution with lactulose for treatment of chronic constipation. Gut 1999;44:226-30.

60.     Smith SC, Choy R, Johnson SK, Hall RS, Wildeboer-Veloo AC, Welling GW. Lupin kernel fiber consumption modifies fecal microbiota in healthy men as determined by rRNA gene fluorescent in situ hybridization. Eur J Nutr 2006;45:335-41.

61.     Ramnani P, Gaudier E, Bingham M, van Bruggen P, Tuohy KM, Gibson GR. Prebiotic effect of fruit and vegetable shots containing Jerusalem artichoke inulin: a human intervention study. Br J Nutr 2010;104:233-40.

62.     Cloetens L, Broekaert WF, Delaedt Y, Ollevier F, Courtin CM, Delcour JA, Rutgeerts P, Verbeke K. Tolerance of arabinoxylan-oligosaccharides and their prebiotic activity in healthy subjects: a randomised, placebo-controlled cross-over study. Br J Nutr 2010;103:703-13.

63.     Lewis S, Cochrane S. Alteration of sulfate and hydrogen metabolism in the human colon by changing intestinal transit rate. Am J Gastroenterol 2007;102:624-33.

64.     Oufir LE, Barry JL, Flourie B, Cherbut C, Cloarec D, Bornet F, Galmiche JP. Relationships between transit time in man and in vitro fermentation of dietary fiber by fecal bacteria. Eur J Clin Nutr 2000;54:603-9.

65.     Kamath PS, Hoepfner MT, Phillips SF. Short-chain fatty acids stimulate motility of the canine ileum. Am J Physiol Gastrointest Liver Physiol 1987;253:G427-33.

66.     Kamath PS, Phillips SF, Zinsmeister AR. Short-chain fatty acids stimulate ileal motility in humans. Gastroenterology 1988;95:1496-502.

67.     Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki SI, Kuwahara A. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol 2008;59 Suppl 2:251-62.

68.     Soret R, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. Gastroenterology 2010;138:1772-82.

69.     Zoetendal EG, Akkermans AD, Akkermans-van Vliet WM, de Visser AJGM, de Vos WM. The host genotype affects the bacterial community in the human gastrointestinal tract. . Microb Ecol Health Dis 2001;13:129-134.

70.     Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L. Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci U S A 2008;105:2117-22.

71.     Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, Kobayashi KS. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A 2009;106:15813-8.

72.     Villani AC, Lemire M, Thabane M, Belisle A, Geneau G, Garg AX, Clark WF, Moayyedi P, Collins SM, Franchimont D, Marshall JK. Genetic risk factors for post-infectious irritable bowel syndrome following a waterborne outbreak of gastroenteritis. Gastroenterology 2010;138:1502-13.

73.     Langhorst J, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. Am J Gastroenterol 2009;104:404-10.

74.     Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, Teggatz P, Barman M, Hayward M, Eastwood D, Stoel M, Zhou Y, Sodergren E, Weinstock GM, Bevins CL, Williams CB, Bos NA. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76-83.

75.     Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.

76.     Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, Geuking MB, Beutler B, Tedder TF, Hardt WD, Bercik P, Verdu EF, McCoy KD, Macpherson AJ. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science 2009;325:617-20.

77.  Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, Fagarasan S. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A 2004;101:1981-6.

78.  Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004;303:1662-5.

79.  Johansen FE, Pekna M, Norderhaug IN, Haneberg B, Hietala MA, Krajci P, Betsholtz C, Brandtzaeg P. Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med 1999;190:915-22.

80.  Philpott DJ, Girardin SE. The role of Toll-like receptors and Nod proteins in bacterial infection. Mol Immunol 2004;41:1099-108.

81.  Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 1998;95:588-93.

82.  Zhang S, Li J, Jia X, Wu Y. The expression of toll-like receptor 2 and 4 mRNA in local tissues of model of oropharyngeal candidiasis in mice. J Huazhong Univ Sci Technolog Med Sci 2004;24:639-41.

83.  Girardin SE, Hugot JP, Sansonetti PJ. Lessons from Nod2 studies: towards a link between Crohn's disease and bacterial sensing. Trends Immunol 2003;24:652-8.

84.  Freitas M, Axelsson LG, Cayuela C, Midtvedt T, Trugnan G. Indigenous microbes and their soluble factors differentially modulate intestinal glycosylation steps in vivo. Use of a "lectin assay" to survey in vivo glycosylation changes. Histochem Cell Biol 2005;124:423-33.

85.  Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol 2003;4:269-73.

86.  Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A 2008;105:20858-63.

87.  Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, Kozakova H, Rossmann P, Bartova J, Sokol D, Funda DP, Borovska D, Rehakova Z, Sinkora J, Hofman J, Drastich P, Kokesova A. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol Lett 2004;93:97-108.

88.  Crabbe PA, Nash DR, Bazin H, Eyssen H, Heremans JF. Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. Lab Invest 1970;22:448-57.

89.  Helgeland L, Dissen E, Dai KZ, Midtvedt T, Brandtzaeg P, Vaage JT. Microbial colonization induces oligoclonal expansions of intraepithelial CD8 T cells in the gut. Eur J Immunol 2004;34:3389-400.

90.  Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect Immun 1995;63:3904-13.

91.  Caenepeel P, Janssens J, Vantrappen G, Eyssen H, Coremans G. Interdigestive myoelectric complex in germ-free rats. Dig Dis Sci 1989;34:1180-4.

92.  Husebye E, Hellstrom PM, Midtvedt T. Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. Dig Dis Sci 1994;39:946-56.

93.  Husebye E, Hellstrom PM, Sundler F, Chen J, Midtvedt T. Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. Am J Physiol Gastrointest Liver Physiol 2001;280:G368-80.

94.  Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881-4.

95.  Verdu EF, Bercik P, Bergonzelli GE, Huang XX, Blennerhasset P, Rochat F, Fiaux M, Mansourian R, Corthesy-Theulaz I, Collins SM. Lactobacillus paracasei normalizes muscle

hypercontractility in a murine model of postinfective gut dysfunction. Gastroenterology 2004;127:826-37.

96. Verdu EF, Bercik P, Huang XX, Lu J, Al-Mutawaly N, Sakai H, Tompkins TA, Croitoru K, Tsuchida E, Perdue M, Collins SM. The role of luminal factors in the recovery of gastric function and behavioral changes after chronic Helicobacter pylori infection. Am J Physiol Gastrointest Liver Physiol 2008;295:G664-70.

97. Bar F, Von Koschitzky H, Roblick U, Bruch HP, Schulze L, Sonnenborn U, Bottner M, Wedel T. Cell-free supernatants of Escherichia coli Nissle 1917 modulate human colonic motility: evidence from an in vitro organ bath study. Neurogastroenterol Motil 2009;21:559-66, e16-7.

98. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182-90.

99. Eutamene H, Lamine F, Chabo C, Theodorou V, Rochat F, Bergonzelli GE, Corthesy-Theulaz I, Fioramonti J, Bueno L. Synergy between Lactobacillus paracasei and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. J Nutr 2007;137:1901-7.

100. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13:35-7.

101. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM. The Intestinal Microbiota Affect Central Levels of Brain-Derived Neurotropic Factor and Behavior in Mice. Gastroenterology 2011.

102. Vanner S. The small intestinal bacterial overgrowth. Irritable bowel syndrome hypothesis: implications for treatment. Gut 2008;57:1315-21.

103. Read NW, Krejs GJ, Read MG, Santa Ana CA, Morawski SG, Fordtran JS. Chronic diarrhea of unknown origin. Gastroenterology 1980;78:264-71.

104. Shah ED, Basseri RJ, Chong K, Pimentel M. Abnormal breath testing in IBS: a meta-analysis. Dig Dis Sci 2010;55:2441-9.

105. Gasbarrini A, Corazza GR, Gasbarrini G, Montalto M, Di Stefano M, Basilisco G, Parodi A, Usai-Satta P, Vernia P, Anania C, Astegiano M, Barbara G, Benini L, Bonazzi P, Capurso G, Certo M, Colecchia A, Cuoco L, Di Sario A, Festi D, Lauritano C, Miceli E, Nardone G, Perri F, Portincasa P, Risicato R, Sorge M, Tursi A. Methodology and indications of H2-breath testing in gastrointestinal diseases: the Rome Consensus Conference. Aliment Pharmacol Ther 2009;29 Suppl 1:1-49.

106. Khoshini R, Dai SC, Lezcano S, Pimentel M. A systematic review of diagnostic tests for small intestinal bacterial overgrowth. Dig Dis Sci 2008;53:1443-54.

107. Pimentel M, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 2000;95:3503-6.

108. Pimentel M, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. Am J Gastroenterol 2003;98:412-9.

109. Lin HC. Small intestinal bacterial overgrowth: a framework for understanding irritable bowel syndrome. JAMA 2004;292:852-8.

110. Yamini D, Pimentel M. Irritable bowel syndrome and small intestinal bacterial overgrowth. J Clin Gastroenterol 2010;44:672-5.

111. Law D, Pimentel M. Proton pump inhibitor therapy does not affect hydrogen production on lactulose breath test in subjects with IBS. Dig Dis Sci 2010;55:2302-8.

112. Pimentel M. An evidence-based treatment algorithm for IBS based on a bacterial/SIBO hypothesis: Part 2. Am J Gastroenterol 2010;105:1227-30.

113. Vanner S. The lactulose breath test for diagnosing SIBO in IBS patients: another nail in the coffin. Am J Gastroenterol 2008;103:964-5.

114. Yu D, Cheeseman F, Vanner S. Combined oro-caecal scintigraphy and lactulose hydrogen breath testing demonstrate that breath testing detects oro-caecal transit, not small intestinal bacterial overgrowth in patients with IBS. Gut 2011;60:334-40.

115. Posserud I, Stotzer PO, Bjornsson ES, Abrahamsson H, Simren M. Small intestinal bacterial overgrowth in patients with irritable bowel syndrome. Gut 2007;56:802-8.

116. Bratten JR, Spanier J, Jones MP. Lactulose breath testing does not discriminate patients with irritable bowel syndrome from healthy controls. Am J Gastroenterol 2008;103:958-63.

117. Simren M, Stotzer PO. Use and abuse of hydrogen breath tests. Gut 2006;55:297-303.

118. Bond JH, Jr., Levitt MD, Prentiss R. Investigation of small bowel transit time in man utilizing pulmonary hydrogen (H2) measurements. J Lab Clin Med 1975;85:546-55.

119. Read NW, Al-Janabi MN, Bates TE, Holgate AM, Cann PA, Kinsman RI, McFarlane A, Brown C. Interpretation of the breath hydrogen profile obtained after ingesting a solid meal containing unabsorbable carbohydrate. Gut 1985;26:834-42.

120. Mishkin D, Mishkin S. Re: Pimentel et al.--Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 2001;96:2505-6.

121. Pimentel M, Mayer AG, Park S, Chow EJ, Hasan A, Kong Y. Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Dig Dis Sci 2003;48:86-92.

122. Pimentel M, Lin HC, Enayati P, van den Burg B, Lee HR, Chen JH, Park S, Kong Y, Conklin J. Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. Am J Physiol Gastrointest Liver Physiol 2006;290:G1089-95.

123. Pimentel M. Evaluating a bacterial hypothesis in IBS using a modification of Koch's postulates: part 1. Am J Gastroenterol 2010;105:718-21.

124. Walters B, Vanner SJ. Detection of bacterial overgrowth in IBS using the lactulose H2 breath test: comparison with 14C-D-xylose and healthy controls. Am J Gastroenterol 2005;100:1566-70.

125. Grover M, Kanazawa M, Palsson OS, Chitkara DK, Gangarosa LM, Drossman DA, Whitehead WE. Small intestinal bacterial overgrowth in irritable bowel syndrome: association with colon motility, bowel symptoms, and psychological distress. Neurogastroenterol Motil 2008;20:998-1008.

126. Hold GL, Pryde SE, Russell VJ, Furrie E, Flint HJ. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol Ecol 2002;39:33-9.

127. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, Louis P, McIntosh F, Johnstone AM, Lobley GE, Parkhill J, Flint HJ. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J 2011;5:220-30.

128. Moore WE, Moore LH. Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol 1995;61:3202-7.

129. Finegold SC, Sutter VL, Mathisen GE. Normal indigenous intestinal flora. In: Hentges DJ, ed. Human intestinal microflora in health and disease. New York: Academic Press, 1983:3-31.

130. Benno Y, Endo K, Mizutani T, Namba Y, Komori T, Mitsuoka T. Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. Appl Environ Microbiol 1989;55:1100-5.

131. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol 2008;74:2461-70.

132. Chassard C, Scott KP, Marquet P, Martin JC, Del'homme C, Dapoigny M, Flint HJ, Bernalier-Donadille A. Assessment of metabolic diversity within the intestinal microbiota from healthy humans using combined molecular and cultural approaches. FEMS Microbiol Ecol 2008;66:496-504.

133. Woodmansey EJ, McMurdo ME, Macfarlane GT, Macfarlane S. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl Environ Microbiol 2004;70:6113-22.

134.    Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 1998;64:3336-45.

135.    Delgado S, Suarez A, Mayo B. Bifidobacterial diversity determined by culturing and by 16S rDNA sequence analysis in feces and mucosa from ten healthy Spanish adults. Dig Dis Sci 2006;51:1878-85.

136.    Hopkins MJ, Sharp R, Macfarlane GT. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut 2001;48:198-205.

137.    Duncan SH, Scott KP, Ramsay AG, Harmsen HJ, Welling GW, Stewart CS, Flint HJ. Effects of alternative dietary substrates on competition between human colonic bacteria in an anaerobic fermentor system. Appl Environ Microbiol 2003;69:1136-42.

138.    Tu S-I, Reed S, Gehring AYH, Paoli G. Capture of Escherichia coli O157:H7 using immunomagnetic beads of different size and antibody conjugating chemistry. Sensors 2009;9:717-730.

139.    Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol 2007;9:1101-11.

140.    Louis P, Flint HJ. Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl Environ Microbiol 2007;73:2009-12.

141.    Simmering R, Pforte H, Jacobasch G, Blaut M. The growth of the flavonoid-degrading intestinal bacterium, Eubacterium ramulus, is stimulated by dietary flavonoids in vivo. FEMS Microbiol Ecol 2002;40:243-8.

142.    Duncan SH, Louis P, Flint HJ. Cultivable bacterial diversity from the human colon. Lett Appl Microbiol 2007;44:343-50.

143.    Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr 2009;101:541-50.

144.    Chassard C, Goumy V, Leclerc M, Del'homme C, Bernalier-Donadille A. Characterization of the xylan-degrading microbial community from human faeces. FEMS Microbiol Ecol 2007;61:121-31.

145.    Dore J, Morvan B, Rieu-Lesme F, Goderel I, Gouet P, Pochart P. Most probable number enumeration of H2-utilizing acetogenic bacteria from the digestive tract of animals and man. FEMS Microbiol Lett 1995;130:7-12.

146.    Duncan SH, Louis P, Flint HJ. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 2004;70:5810-7.

147.    Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 2004;54:1469-76.

148.    Robert C, Bernalier-Donadille A. The cellulolytic microflora of the human colon: evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiol Ecol 2003;46:81-9.

149.    Barcenilla A, Pryde SE, Martin JC, Duncan SH, Stewart CS, Henderson C, Flint HJ. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl Environ Microbiol 2000;66:1654-61.

150.    Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 2002;22:283-307.

151.    Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilic-Stojanovic M, de Graaf AA, Smidt H, de Vos WM, Venema K. Linking phylogenetic identities of bacteria to starch fermentation in an in vitro model of the large intestine by RNA-based stable isotope probing. Environ Microbiol 2009;11:914-26.

152. Leitch EC, Walker AW, Duncan SH, Holtrop G, Flint HJ. Selective colonization of insoluble substrates by human faecal bacteria. Environ Microbiol 2007;9:667-79.

153. Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. J Bacteriol 2004;186:2099-106.

154. Charrier C, Duncan GJ, Reid MD, Rucklidge GJ, Henderson D, Young P, Russell VJ, Aminov RI, Flint HJ, Louis P. A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology 2006;152:179-85.

155. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol 2010;12:304-14.

156. Deplancke B, Hristova KR, Oakley HA, McCracken VJ, Aminov R, Mackie RI, Gaskins HR. Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. Appl Environ Microbiol 2000;66:2166-74.

157. Mihajlovski A, Alric M, Brugere JF. A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the mcrA gene. Res Microbiol 2008;159:516-21.

158. Ingham CJ, Sprenkels A, Bomer J, Molenaar D, van den Berg A, van Hylckama Vlieg JE, de Vos WM. The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proc Natl Acad Sci U S A 2007;104:18217-22.

159. Woese CR. Bacterial evolution. Microbiol Rev 1987;51:221-71.

160. Woese CR, Fox GE. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci U S A 1977;74:5088-90.

161. Fox GE, Pechman KR, Woese CR. Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to procaryotic systematics. Int J Syst Bacteriol 1977;57:44-57.

162. Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A 1990;87:4576-9.

163. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141-5.

164. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR. Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr 2004;134:465-72.

165. Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM. Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 2002;68:114-23.

166. Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 2001;67:2578-85.

167. Satokari RM, Vaughan EE, Akkermans AD, Saarela M, de Vos WM. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 2001;67:504-13.

168. Maukonen J, Matto J, Satokari R, Soderlund H, Mattila-Sandholm T, Saarela M. PCR DGGE and RT-PCR DGGE show diversity and short-term temporal stability in the Clostridium coccoides-Eubacterium rectale group in the human intestinal microbiota. FEMS Microbiol Ecol 2006;58:517-28.

169. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 2002;68:5445-51.

170. Matsuda K, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Appl Environ Microbiol 2007;73:32-9.

171. Harmsen HJ, Raangs GC, He T, Degener JE, Welling GW. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl Environ Microbiol 2002;68:2982-90.

172. Zoetendal EG, Ben-Amor K, Harmsen HJ, Schut F, Akkermans AD, de Vos WM. Quantification of uncultured Ruminococcus obeum-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. Appl Environ Microbiol 2002;68:4225-32.

173. Rigottier-Gois L, Bourhis AG, Gramet G, Rochet V, Dore J. Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. FEMS Microbiol Ecol 2003;43:237-45.

174. Lay C, Rigottier-Gois L, Holmstrom K, Rajilic M, Vaughan EE, de Vos WM, Collins MD, Thiel R, Namsolleck P, Blaut M, Dore J. Colonic microbiota signatures across five northern European countries. Appl Environ Microbiol 2005;71:4153-5.

175. Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998;64:3854-9.

176. Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P, Jian R, Dore J. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut 2003;52:237-42.

177. Andoh A, Sakata S, Koizumi Y, Mitsuyama K, Fujiyama Y, Benno Y. Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. Inflamm Bowel Dis 2007;13:955-62.

178. Maukonen J, Satokari R, Matto J, Soderlund H, Mattila-Sandholm T, Saarela M. Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. J Med Microbiol 2006;55:625-33.

179. Matto J, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, Saarela M. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. FEMS Immunol Med Microbiol 2005;43:213-22.

180. Martinez C, Antolin M, Santos J, Torrejon A, Casellas F, Borruel N, Guarner F, Malagelada JR. Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. Am J Gastroenterol 2008;103:643-8.

181. Wang X, Heazlewood SP, Krause DO, Florin TH. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J Appl Microbiol 2003;95:508-20.

182. Wang M, Ahrne S, Jeppsson B, Molin G. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol Ecol 2005;54:219-31.

183. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. J Med Microbiol 2005;54:1093-101.

184. Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 2002;68:3401-7.

185. Lepage P, Seksik P, Sutren M, de la Cochetiere MF, Jian R, Marteau P, Dore J. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. Inflamm Bowel Dis 2005;11:473-80.

186. Hartman AL, Lough DM, Barupal DK, Fiehn O, Fishbein T, Zasloff M, Eisen JA. Human gut microbiome adopts an alternative state following small bowel transplantation. Proc Natl Acad Sci U S A 2009;106:17187-92.

187. Booijink CC, El-Aidy S, Rajilic-Stojanovic M, Heilig HG, Troost FJ, Smidt H, Kleerebezem M, De Vos WM, Zoetendal EG. High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 2010;12:3213-27.

188. Guschin DY, Mobarry BK, Proudnikov D, Stahl DA, Rittmann BE, Mirzabekov AD. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl Environ Microbiol 1997;63:2397-402.

189. Loy A. DNA Microarray technology for biodiversity inventories of sulfate reducing prokaryotes. Vienna, Austria.: Universität Wien, 2003:35.

190. DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 2007;53:371-83.

191. Wang RF, Beggs ML, Robertson LH, Cerniglia CE. Design and evaluation of oligonucleotide-microarray method for the detection of human intestinal bacteria in fecal samples. FEMS Microbiol Lett 2002;213:175-82.

192. Palmer C, Bik EM, Eisen MB, Eckburg PB, Sana TR, Wolber PK, Relman DA, Brown PO. Rapid quantitative profiling of complex microbial populations. Nucleic Acids Res 2006;34:e5.

193. Rajilic-Stojanovic M, Heilig HG, Molenaar D, Kajander K, Surakka A, Smidt H, de Vos WM. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ Microbiol 2009;11:1736-51.

194. Paliy O, Kenche H, Abernathy F, Michail S. High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. Appl Environ Microbiol 2009;75:3572-9.

195. Kang S, Denman SE, Morrison M, Yu Z, Dore J, Leclerc M, McSweeney CS. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. Inflamm Bowel Dis 2010;16:2034-42.

196. Nikkila J, de Vos WM. Advanced approaches to characterize the human intestinal microbiota by computational meta-analysis. J Clin Gastroenterol 2010;44 Suppl 1:S2-5.

197. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 2008;3:e2836.

198. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4586-91.

199. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4554-61.

200. Quince C, Lanzen A, Curtis TP, Davenport RJ, Hall N, Head IM, Read LF, Sloan WT. Accurate determination of microbial diversity from 454 pyrosequencing data. Nat Methods 2009;6:639-41.

201. Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, MacPhee R, Reid G. Microbiome profiling by illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 2010;5:e15406.

202. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4516-22.

203. Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 2009;4:e6669.

204. Van den Bogert B, De Vos WM, Zoetendal EG, Kleerebezem M. Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples. In preparation 2011.
205. Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 2004;68:669-85.
206. Henne A, Schmitz RA, Bomeke M, Gottschalk G, Daniel R. Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. Appl Environ Microbiol 2000;66:3113-6.
207. Handelsman J, Liles M, Mann D, Riesenfeld C, Goodman RM. Cloning the metagenome: Culture-independent access to the diversity and functions of the uncultivated microbial world. In: Nick WA, ed. Methods in Microbiology: Academic Press, 2002:241-255.
208. Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM. Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2007;2:285-95.
209. Raes J, Bork P. Molecular eco-systems biology: towards an understanding of community function. Nat Rev Microbiol 2008;6:693-9.
210. Nelson KE, Weinstock GM, Highlander SK, Worley KC, Creasy HH, Wortman JR, Rusch DB, Mitreva M, Sodergren E, Chinwalla AT, Feldgarden M, Gevers D, Haas BJ, Madupu R, Ward DV, Birren BW, Gibbs RA, Methe B, Petrosino JF, Strausberg RL, Sutton GG, White OR, Wilson RK, Durkin S, Giglio MG, Gujja S, Howarth C, Kodira CD, Kyrpides N, Mehta T, Muzny DM, Pearson M, Pepin K, Pati A, Qin X, Yandava C, Zeng Q, Zhang L, Berlin AM, Chen L, Hepburn TA, Johnson J, McCorrison J, Miller J, Minx P, Nusbaum C, Russ C, Sykes SM, Tomlinson CM, Young S, Warren WC, Badger J, Crabtree J, Markowitz VM, Orvis J, Cree A, Ferriera S, Fulton LL, Fulton RS, Gillis M, Hemphill LD, Joshi V, Kovar C, Torralba M, Wetterstrand KA, Abouellleil A, Wollam AM, Buhay CJ, Ding Y, Dugan S, FitzGerald MG, Holder M, Hostetler J, Clifton SW, Allen-Vercoe E, Earl AM, Farmer CN, Liolios K, Surette MG, Xu Q, Pohl C, Wilczek-Boney K, Zhu D. A catalog of reference genomes from the human microbiome. Science 2010;328:994-9.
211. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. Science 2006;312:1355-9.
212. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, Takami H, Morita H, Sharma VK, Srivastava TP, Taylor TD, Noguchi H, Mori H, Ogura Y, Ehrlich DS, Itoh K, Takagi T, Sakaki Y, Hayashi T, Hattori M. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res 2007;14:169-81.
213. Jones BV, Marchesi JR. Transposon-aided capture (TRACA) of plasmids resident in the human gut mobile metagenome. Nat Methods 2007;4:55-61.
214. Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 2010;466:334-8.
215. Jones BV, Begley M, Hill C, Gahan CG, Marchesi JR. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc Natl Acad Sci U S A 2008;105:13580-5.
216. Booijink CCGM. Analysis of diversity and function of the human small intestinal microbiota. Wageningen, the Netherlands: Wageningen University, 2009:144.
217. Uchiyama T, Abe T, Ikemura T, Watanabe K. Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat Biotechnol 2005;23:88-93.
218. Williamson LL, Borlee BR, Schloss PD, Guan C, Allen HK, Handelsman J. Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. Appl Environ Microbiol 2005;71:6335-44.
219. Gloux K, Leclerc M, Iliozer H, L'Haridon R, Manichanh C, Corthier G, Nalin R, Blottiere HM, Dore J. Development of high-throughput phenotyping of metagenomic clones from the

human gut microbiome for modulation of eukaryotic cell growth. Appl Environ Microbiol 2007;73:3734-7.

220. Lakhdari O, Cultrone A, Tap J, Gloux K, Bernard F, Ehrlich SD, Lefevre F, Dore J, Blottiere HM. Functional metagenomics: a high throughput screening method to decipher microbiota-driven NF-kappaB modulation in the human gut. PLoS One 2010;5.

221. Warnecke F, Hess M. A perspective: metatranscriptomics as a tool for the discovery of novel biocatalysts. J Biotechnol 2009;142:91-5.

222. Zoetendal EG, Booijink CC, Klaassens ES, Heilig HG, Kleerebezem M, Smidt H, de Vos WM. Isolation of RNA from bacterial samples of the human gastrointestinal tract. Nat Protoc 2006;1:954-9.

223. Booijink CC, Boekhorst J, Zoetendal EG, Smidt H, Kleerebezem M, de Vos WM. Metatranscriptome analysis of the human fecal microbiota reveals subject-specific expression profiles, with genes encoding proteins involved in carbohydrate metabolism being dominantly expressed. Appl Environ Microbiol 2010;76:5533-40.

224. Klaassens ES, Boesten RJ, Haarman M, Knol J, Schuren FH, Vaughan EE, de Vos WM. Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol 2009;75:2668-76.

225. Passalacqua KD, Varadarajan A, Ondov BD, Okou DT, Zwick ME, Bergman NH. Structure and complexity of a bacterial transcriptome. J Bacteriol 2009;191:3203-11.

226. Yoder-Himes DR, Chain PS, Zhu Y, Wurtzel O, Rubin EM, Tiedje JM, Sorek R. Mapping the Burkholderia cenocepacia niche response via high-throughput sequencing. Proc Natl Acad Sci U S A 2009;106:3976-81.

227. Mane SP, Evans C, Cooper KL, Crasta OR, Folkerts O, Hutchison SK, Harkins TT, Thierry-Mieg D, Thierry-Mieg J, Jensen RV. Transcriptome sequencing of the Microarray Quality Control (MAQC) RNA reference samples using next generation sequencing. BMC Genomics 2009;10:264.

228. Turnbaugh PJ, Quince C, Faith JJ, McHardy AC, Yatsunenko T, Niazi F, Affourtit J, Egholm M, Henrissat B, Knight R, Gordon JI. Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. Proc Natl Acad Sci U S A 2010;107:7503-8.

229. Gosalbes MJ, Durban A, Pignatelli M, Abellan JJ, Jimenez-Hernandez N, Perez-Cobas AE, Latorre A, Moya A. Metatranscriptomic approach to analyze the functional human gut microbiota. PLoS One 2011;6:e17447.

230. Ozsolak F, Platt AR, Jones DR, Reifenberger JG, Sass LE, McInerney P, Thompson JF, Bowers J, Jarosz M, Milos PM. Direct RNA sequencing. Nature 2009;461:814-8.

231. Wilmes P, Bond PL. Microbial community proteomics: elucidating the catalysts and metabolic mechanisms that drive the Earth's biogeochemical cycles. Curr Opin Microbiol 2009;12:310-7.

232. Klaassens ES, de Vos WM, Vaughan EE. Metaproteomics approach to study the functionality of the microbiota in the human infant gastrointestinal tract. Appl Environ Microbiol 2007;73:1388-92.

233. Verberkmoes NC, Russell AL, Shah M, Godzik A, Rosenquist M, Halfvarson J, Lefsrud MG, Apajalahti J, Tysk C, Hettich RL, Jansson JK. Shotgun metaproteomics of the human distal gut microbiota. ISME J 2009;3:179-89.

234. Rooijers K, Kolmeder C, Juste C, Dore J, de Been M, Boeren S, Galan P, Beauvallet C, de Vos WM, Schaap PJ. An iterative workflow for mining the human intestinal metaproteome. BMC Genomics 2011;12:6.

235. Nicholson JK, Lindon JC. Systems biology: Metabonomics. Nature 2008;455:1054-6.

236. Oresic M. Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction. Nutr Metab Cardiovasc Dis 2009;19:816-24.

237. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, Wilson ID, Wang Y. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res 2007;6:546-51.

238. Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, Duncan G, Johnstone AM, Lobley GE, Wallace RJ, Duthie GG, Flint HJ. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr 2011;93:1062-72.

239. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57-63.

240. Corrodi P, Wideman PA, Sutter VL, Drenick EJ, Passaro E, Jr., Finegold SM. Bacterial flora of the small bowel before and after bypass procedure for morbid obesity. J Infect Dis 1978;137:1-6.

241. Drasar BS, Shiner M, McLeod GM. Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. Gastroenterology 1969;56:71-9.

242. Gorbach SL. Intestinal microflora. Gastroenterology 1971;60:1110-29.

243. Ahmed S, Macfarlane GT, Fite A, McBain AJ, Gilbert P, Macfarlane S. Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. Appl Environ Microbiol 2007;73:7435-42.

244. Kerckhoffs AP, Visser MR, Samsom M, van der Rest ME, de Vogel J, Harmsen W, Akkermans LM. Critical evaluation of diagnosing bacterial overgrowth in the proximal small intestine. J Clin Gastroenterol 2008;42:1095-102.

245. Lin HC, Pimentel M. Bacterial concepts in irritable bowel syndrome. Rev Gastroenterol Disord 2005;5 Suppl 3:S3-9.

246. Corazza GR, Menozzi MG, Strocchi A, Rasciti L, Vaira D, Lecchini R, Avanzini P, Chezzi C, Gasbarrini G. The diagnosis of small bowel bacterial overgrowth. Reliability of jejunal culture and inadequacy of breath hydrogen testing. Gastroenterology 1990;98:302-9.

247. Drasar BS, Shiner M. Studies on the intestinal flora. II. Bacterial flora of the small intestine in patients with gastrointestinal disorders. Gut 1969;10:812-9.

248. Lewis SJ, Young G, Mann M, Franco S, O'Keefe SJ. Improvement in specificity of [14C]d-xylose breath test for bacterial overgrowth. Dig Dis Sci 1997;42:1587-92.

249. Sullivan A, Tornblom H, Lindberg G, Hammarlund B, Palmgren AC, Einarsson C, Nord CE. The micro-flora of the small bowel in health and disease. Anaerobe 2003;9:11-4.

250. Pyleris M, Giamarellos-Bourboulis EJ, Koussoulas B, Barbatzas C, Pimentel M. Small Bowel Culture Confirms the Presence of Small Intestinal Bacterial Overgrowth in a Subset of IBS Subjects. Gastroenterology 2011;140:S-152.

251. Bardhan PK, Gyr K, Beglinger C, Vogtlin J, Frey R, Vischer W. Diagnosis of bacterial overgrowth after culturing proximal small-bowel aspirate obtained during routine upper gastrointestinal endoscopy. Scand J Gastroenterol 1992;27:253-6.

252. Choung RS, Ruff KC, Malhotra A, Herrick L, Locke GR, 3rd, Harmsen WS, Zinsmeister AR, Talley NJ, Saito YA. Clinical predictors of small intestinal bacterial overgrowth by duodenal aspirate culture. Aliment Pharmacol Ther 2011.

253. Rumessen JJ, Gudmand-Hoyer E, Bachmann E, Justesen T. Diagnosis of bacterial overgrowth of the small intestine. Comparison of the 14C-D-xylose breath test and jejunal cultures in 60 patients. Scand J Gastroenterol 1985;20:1267-75.

254. Spiegel BM, Chey WD, Chang L. Bacterial overgrowth and irritable bowel syndrome: unifying hypothesis or a spurious consequence of proton pump inhibitors? Am J Gastroenterol 2008;103:2972-6.

255. Verdu E, Viani F, Armstrong D, Fraser R, Siegrist HH, Pignatelli B, Idstrom JP, Cederberg C, Blum AL, Fried M. Effect of omeprazole on intragastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. Gut 1994;35:455-60.

256. Compare D, Pica L, Rocco A, De Giorgi F, Cuomo R, Sarnelli G, Romano M, Nardone G. Effects of long-term PPI treatment on producing bowel symptoms and SIBO. Eur J Clin Invest 2011;41:380-6.

257. Fried M, Siegrist H, Frei R, Froehlich F, Duroux P, Thorens J, Blum A, Bille J, Gonvers JJ, Gyr K. Duodenal bacterial overgrowth during treatment in outpatients with omeprazole. Gut 1994;35:23-6.

258. Lewis SJ, Franco S, Young G, O'Keefe SJ. Altered bowel function and duodenal bacterial overgrowth in patients treated with omeprazole. Aliment Pharmacol Ther 1996;10:557-61.

259. Lombardo L, Foti M, Ruggia O, Chiecchio A. Increased incidence of small intestinal bacterial overgrowth during proton pump inhibitor therapy. Clin Gastroenterol Hepatol 2010;8:504-8.

260. Theisen J, Nehra D, Citron D, Johansson J, Hagen JA, Crookes PF, DeMeester SR, Bremner CG, DeMeester TR, Peters JH. Suppression of gastric acid secretion in patients with gastroesophageal reflux disease results in gastric bacterial overgrowth and deconjugation of bile acids. J Gastrointest Surg 2000;4:50-4.

261. Thorens J, Froehlich F, Schwizer W, Saraga E, Bille J, Gyr K, Duroux P, Nicolet M, Pignatelli B, Blum AL, Gonvers JJ, Fried M. Bacterial overgrowth during treatment with omeprazole compared with cimetidine: a prospective randomised double blind study. Gut 1996;39:54-9.

262. Lauritano EC, Gabrielli M, Scarpellini E, Lupascu A, Novi M, Sottili S, Vitale G, Cesario V, Serricchio M, Cammarota G, Gasbarrini G, Gasbarrini A. Small intestinal bacterial overgrowth recurrence after antibiotic therapy. Am J Gastroenterol 2008;103:2031-5.

263. Khanna D, Hays RD, Park GS, Braun-Moscovici Y, Mayes MD, McNearney TA, Hsu V, Clements PJ, Furst DE. Development of a preliminary scleroderma gastrointestinal tract 1.0 quality of life instrument. Arthritis Rheum 2007;57:1280-6.

264. Kaye SA, Lim SG, Taylor M, Patel S, Gillespie S, Black CM. Small bowel bacterial overgrowth in systemic sclerosis: detection using direct and indirect methods and treatment outcome. Br J Rheumatol 1995;34:265-9.

265. Sadik R, Bjornsson E, Simren M. The relationship between symptoms, body mass index, gastrointestinal transit and stool frequency in patients with irritable bowel syndrome. Eur J Gastroenterol Hepatol 2010;22:102-8.

266. Pimentel M, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. N Engl J Med 2011;364:22-32.

267. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of nutrition 1995;125:1401-12.

268. Rangnekar AS, Chey WD. The FODMAP diet for irritable bowel syndrome: food fad or roadmap to a new treatment paradigm? Gastroenterology 2009;137:383-6.

269. Mendall MA, Kumar D. Antibiotic use, childhood affluence and irritable bowel syndrome (IBS). Eur J Gastroenterol Hepatol 1998;10:59-62.

270. Maxwell PR, Rink E, Kumar D, Mendall MA. Antibiotics increase functional abdominal symptoms. Am J Gastroenterol 2002;97:104-8.

271. De La Cochetiere MF, Durand T, Lepage P, Bourreille A, Galmiche JP, Dore J. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. J Clin Microbiol 2005;43:5588-92.

272. Brint EK, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. Am J Gastroenterol 2011;106:329-36.

273. De Giorgio R, Barbara G. Is irritable bowel syndrome an inflammatory disorder? Curr Gastroenterol Rep 2008;10:385-90.

274. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Dore J, Consortium M,

Weissenbach J, Ehrlich SD, Bork P, Antolin M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Merieux A, Melo Minardi R, M'Rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G. Enterotypes of the human gut microbiome. Nature 2011.

275.   Malinen E, Rinttila T, Kajander K, Matto J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol 2005;100:373-82.

276.   Rajilic-Stojanovic M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 2011;141:1792-801.

277.   Jeffery IB, O'Toole PW, Ohman L, Claesson MJ, Deane J, Quigley EM, Simren M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 2011.

278.   Manabe N, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. Neurogastroenterol Motil 2010;22:293-e82.

279.   Malinen E, Krogius-Kurikka L, Lyra A, Nikkila J, Jaaskelainen A, Rinttila T, Vilpponen-Salmela T, von Wright AJ, Palva A. Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. World J Gastroenterol 2010;16:4532-40.

280.   Balsari A, Ceccarelli A, Dubini F, Fesce E, Poli G. The fecal microbial population in the irritable bowel syndrome. Microbiologica 1982;5:185-94.

281.   Carroll IM, Chang YH, Park J, Sartor RB, Ringel Y. Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Gut Pathog 2010;2:19.

282.   Codling C, O'Mahony L, Shanahan F, Quigley EM, Marchesi JR. A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. Dig Dis Sci 2010;55:392-7.

283.   Kassinen A, Krogius-Kurikka L, Makivuokko H, Rinttila T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology 2007;133:24-33.

284.   Kerckhoffs AP, Samsom M, van der Rest ME, de Vogel J, Knol J, Ben-Amor K, Akkermans LM. Lower Bifidobacteria counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients. World J Gastroenterol 2009;15:2887-92.

285.   Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Makivuokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 2009;9:95.

286.   Lyra A, Rinttila T, Nikkila J, Krogius-Kurikka L, Kajander K, Malinen E, Matto J, Makela L, Palva A. Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. World J Gastroenterol 2009;15:5936-45.

287.   Noor SO, Ridgway K, Scovell L, Kemsley EK, Lund EK, Jamieson C, Johnson IT, Narbad A. Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota. BMC Gastroenterol 2010;10:134.

288.   Ponnusamy K, Choi JN, Kim J, Lee SY, Lee CH. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. J Med Microbiol 2011;60:817-27.

289.   Rajilic-Stojanovic M. Diversity of the human gastrointestinal microbiota: novel perspectives from high throughput analyses. Wageningen, The Netherlands: Wageningen University, 2007.

290.   Rinttila T, Lyra A, Krogius-Kurikka L, Palva A. Real-time PCR analysis of enteric pathogens from fecal samples of irritable bowel syndrome subjects. Gut Pathog 2011;3:6.

291.  Saulnier DM, Riehle K, Mistretta TA, Diaz MA, Mandal D, Raza S, Weidler EM, Qin X, Coarfa C, Milosavljevic A, Petrosino JF, Highlander S, Gibbs R, Lynch SV, Shulman RJ, Versalovic J. Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. Gastroenterology 2011;141:1782-91.

292.  Si JM, Yu YC, Fan YJ, Chen SJ. Intestinal microecology and quality of life in irritable bowel syndrome patients. World J Gastroenterol 2004;10:1802-5.

293.  Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. Neurogastroenterol Motil 2010;22:512-9, e114-5.

294.  Parkes GC, Rayment NB, Hudspith BN, Petrovska L, Lomer MC, Brostoff J, Whelan K, Sanderson JD. Distinct microbial populations exist in the mucosa-associated microbiota of sub-groups of irritable bowel syndrome. Neurogastroenterol Motil 2012;24:31-9.

295.  Barthel M, Hapfelmeier S, Quintanilla-Martinez L, Kremer M, Rohde M, Hogardt M, Pfeffer K, Russmann H, Hardt WD. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun 2003;71:2839-58.

296.  Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2007;2:204.

297.  Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, Hudson MJ, Roderick PJ. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The Infectious Intestinal Disease Study Executive. BMJ 1999;318:1046-50.

298.  Troeger H, Loddenkemper C, Schneider T, Schreier E, Epple HJ, Zeitz M, Fromm M, Schulzke JD. Structural and functional changes of the duodenum in human norovirus infection. Gut 2009;58:1070-7.

299.  Spiller RC, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. Gut 2000;47:804-11.

300.  Longstreth GF, Hawkey CJ, Mayer EA, Jones RH, Naesdal J, Wilson IK, Peacock RA, Wiklund IK. Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials. Aliment Pharmacol Ther 2001;15:959-64.

301.  Spiller R, Card T, Mearin F, Azpiroz F, Barbara G, Marshall JK, Aziz Q, Santos J, Boeckxstaens G, Enck P. Incidence and characteristics of Postinfectious IBS (PI-IBS): a multinational internet survey. . Gut 2010;59:A32.

302.  Dunlop SP, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. Gastroenterology 2003;125:1651-9.

303.  Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. BMJ 1997;314:779-82.

304.  Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. Aliment Pharmacol Ther 2007;26:535-44.

305.  Marshall JK, Thabane M, Borgaonkar MR, James C. Postinfectious irritable bowel syndrome after a food-borne outbreak of acute gastroenteritis attributed to a viral pathogen. Clin Gastroenterol Hepatol 2007;5:457-60.

306.  Porter CK, Gormley R, Tribble DR, Cash BD, Riddle MS. The Incidence and gastrointestinal infectious risk of functional gastrointestinal disorders in a healthy US adult population. Am J Gastroenterol 2011;106:130-8.

307.   Thornley JP, Jenkins D, Neal K, Wright T, Brough J, Spiller RC. Relationship of Campylobacter toxigenicity in vitro to the development of postinfectious irritable bowel syndrome. J Infect Dis 2001;184:606-9.

308.   Marshall JK, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. Gastroenterology 2006;131:445-50; quiz 660.

309.   Gwee KA, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW. The role of psychological and biological factors in postinfective gut dysfunction. Gut 1999;44:400-6.

310.   Wensaas KA, Langeland N, Hanevik K, Morch K, Eide GE, Rortveit G. Irritable bowel syndrome and chronic fatigue 3 years after acute giardiasis: historic cohort study. Gut 2012;61:214-9.

311.   Fujita K, Kaku M, Yanagase Y, Ezaki T, Furuse K, Ozawa A, Saidi SM, Sang WK, Waiyaki PG. Physicochemical characteristics and flora of diarrhoeal and recovery faeces in children with acute gastro-enteritis in Kenya. Ann Trop Paediatr 1990;10:339-45.

312.   Albert MJ, Bhat P, Rajan D, Maiya PP, Pereira SM, Baker SJ. Faecal flora of South Indian infants and young children in health and with acute gastroenteritis. J Med Microbiol 1978;11:137-43.

313.   Balamurugan R, Janardhan HP, George S, Raghava MV, Muliyil J, Ramakrishna BS. Molecular studies of fecal anaerobic commensal bacteria in acute diarrhea in children. J Pediatr Gastroenterol Nutr 2008;46:514-9.

314.   Mai V, Braden CR, Heckendorf J, Pironis B, Hirshon JM. Monitoring of stool microbiota in subjects with diarrhea indicates distortions in composition. J Clin Microbiol 2006;44:4550-2.

315.   Rao SS, Edwards CA, Austen CJ, Bruce C, Read NW. Impaired colonic fermentation of carbohydrate after ampicillin. Gastroenterology 1988;94:928-32.

316.   Musch MW, Bookstein C, Xie Y, Sellin JH, Chang EB. SCFA increase intestinal Na absorption by induction of NHE3 in rat colon and human intestinal C2/bbe cells. Am J Physiol Gastrointest Liver Physiol 2001;280:G687-93.

317.   Ruppin H, Bar-Meir S, Soergel KH, Wood CM, Schmitt MG, Jr. Absorption of short-chain fatty acids by the colon. Gastroenterology 1980;78:1500-7.

318.   Zeissig S, Fromm A, Mankertz J, Weiske J, Zeitz M, Fromm M, Schulzke JD. Butyrate induces intestinal sodium absorption via Sp3-mediated transcriptional up-regulation of epithelial sodium channels. Gastroenterology 2007;132:236-48.

319.   Treem WR, Ahsan N, Kastoff G, Hyams JS. Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation. J Pediatr Gastroenterol Nutr 1996;23:280-6.

320.   Barbara G, Stanghellini V, Berti-Ceroni C, De Giorgio R, Salvioli B, Corradi F, Cremon C, Corinaldesi R. Role of antibiotic therapy on long-term germ excretion in faeces and digestive symptoms after Salmonella infection. Aliment Pharmacol Ther 2000;14:1127-31.

321.   Stermer E, Lubezky A, Potasman I, Paster E, Lavy A. Is traveler's diarrhea a significant risk factor for the development of irritable bowel syndrome? A prospective study. Clin Infect Dis 2006;43:898-901.

322.   Artis D, Grencis RK. The intestinal epithelium: sensors to effectors in nematode infection. Mucosal Immunol 2008;1:252-64.

323.   Fagarasan S. Evolution, development, mechanism and function of IgA in the gut. Curr Opin Immunol 2008;20:170-7.

324.   Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 1999;286:113-7.

325.   Ayabe T, Satchell DP, Pesendorfer P, Tanabe H, Wilson CL, Hagen SJ, Ouellette AJ. Activation of Paneth cell alpha-defensins in mouse small intestine. J Biol Chem 2002;277:5219-28.

326.   Mukherjee S, Vaishnava S, Hooper LV. Multi-layered regulation of intestinal antimicrobial defense. Cell Mol Life Sci 2008;65:3019-27.

327.  Swidsinski A, Loening-Baucke V, Verstraelen H, Osowska S, Doerffel Y. Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. Gastroenterology 2008;135:568-79.

328.  Locke GR, 3rd, Weaver AL, Melton LJ, 3rd, Talley NJ. Psychosocial factors are linked to functional gastrointestinal disorders: a population based nested case-control study. Am J Gastroenterol 2004;99:350-7.

329.  Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 2007;56:1522-8.

330.  Li W, Dowd SE, Scurlock B, Acosta-Martinez V, Lyte M. Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. Physiol Behav 2009;96:557-67.

331.  Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 2011;141:599-609, 609 e1-3.

332.  Gaykema RP, Goehler LE, Lyte M. Brain response to cecal infection with Campylobacter jejuni: analysis with Fos immunohistochemistry. Brain Behav Immun 2004;18:238-45.

333.  Goehler LE, Park SM, Opitz N, Lyte M, Gaykema RP. Campylobacter jejuni infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. Brain Behav Immun 2008;22:354-66.

334.  Lyte M, Varcoe JJ, Bailey MT. Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. Physiol Behav 1998;65:63-8.

335.  Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, Malinowski P, Jackson W, Blennerhassett P, Neufeld KA, Lu J, Khan WI, Corthesy-Theulaz I, Cherbut C, Bergonzelli GE, Collins SM. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 2010;139:2102-2112 e1.

336.  Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM, Verdu EF. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 2011;23:1132-9.

337.  Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 2011;108:16050-5.

338.  Morgan MY, Blei A, Grungreiff K, Jalan R, Kircheis G, Marchesini G, Riggio O, Weissenborn K. The treatment of hepatic encephalopathy. Metab Brain Dis 2007;22:389-405.

339.  Yurdaydin C, Walsh TJ, Engler HD, Ha JH, Li Y, Jones EA, Basile AS. Gut bacteria provide precursors of benzodiazepine receptor ligands in a rat model of hepatic encephalopathy. Brain res 1995;679:42-8.

340.  Ledochowski M, Widner B, Bair H, Probst T, Fuchs D. Fructose- and sorbitol-reduced diet improves mood and gastrointestinal disturbances in fructose malabsorbers. Scand J Gastroenterol 2000;35:1048-52.

341.  Gibson PR, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. J Gastroenterol Hepatol 2010;25:252-8.

342.  Ledochowski M, Widner B, Murr C, Sperner-Unterweger B, Fuchs D. Fructose malabsorption is associated with decreased plasma tryptophan. Scand J Gastroenterol 2001;36:367-71.

343.  Lohi S, Mustalahti K, Kaukinen K, Laurila K, Collin P, Rissanen H, Lohi O, Bravi E, Gasparin M, Reunanen A, Maki M. Increasing prevalence of coeliac disease over time. Aliment Pharmacol Ther 2007;26:1217-25.

344.  Rubio-Tapia A, Kyle RA, Kaplan EL, Johnson DR, Page W, Erdtmann F, Brantner TL, Kim WR, Phelps TK, Lahr BD, Zinsmeister AR, Melton LJ, 3rd, Murray JA. Increased prevalence and mortality in undiagnosed celiac disease. Gastroenterology 2009;137:88-93.

345. De Palma G, Nadal I, Collado MC, Sanz Y. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. Br J Nutr 2009;102:1154-60.

346. Ludvigsson JF, Montgomery SM, Ekbom A, Brandt L, Granath F. Small-intestinal histopathology and mortality risk in celiac disease. JAMA 2009;302:1171-8.

347. Tack GJ, Verbeek WH, Schreurs MW, Mulder CJ. The spectrum of celiac disease: epidemiology, clinical aspects and treatment. Nat Rev Gastroenterol Hepatol 2010;7:204-13.

348. Sapone A, Lammers KM, Casolaro V, Cammarota M, Giuliano MT, De Rosa M, Stefanile R, Mazzarella G, Tolone C, Russo MI, Esposito P, Ferraraccio F, Carteni M, Riegler G, de Magistris L, Fasano A. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. BMC Med 2011;9:23.

349. Wahnschaffe U, Ullrich R, Riecken EO, Schulzke JD. Celiac disease-like abnormalities in a subgroup of patients with irritable bowel syndrome. Gastroenterology 2001;121:1329-38.

350. Verdu EF. Editorial: Can gluten contribute to irritable bowel syndrome? Am J Gastroenterol 2011;106:516-8.

351. Verdu EF, Armstrong D, Murray JA. Between celiac disease and irritable bowel syndrome: the "no man's land" of gluten sensitivity. Am J Gastroenterol 2009;104:1587-94.

352. Biesiekierski JR, Newnham ED, Irving PM, Barrett JS, Haines M, Doecke JD, Shepherd SJ, Muir JG, Gibson PR. Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. Am J Gastroenterol 2011;106:508-14; quiz 515.

353. Black KE, Murray JA, David CS. HLA-DQ determines the response to exogenous wheat proteins: a model of gluten sensitivity in transgenic knockout mice. J Immunol 2002;169:5595-600.

354. Verdu EF, Huang X, Natividad J, Lu J, Blennerhassett PA, David CS, McKay DM, Murray JA. Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. Am J Physiol Gastrointest Liver Physiol 2008;294:G217-25.

355. Natividad JM, Huang X, Slack E, Jury J, Sanz Y, David C, Denou E, Yang P, Murray J, McCoy KD, Verdu EF. Host responses to intestinal microbial antigens in gluten-sensitive mice. PLoS One 2009;4:e6472.

356. Scanlan PD, Shanahan F, O'Mahony C, Marchesi JR. Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. J Clin Microbiol 2006;44:3980-8.

357. Conte MP, Schippa S, Zamboni I, Penta M, Chiarini F, Seganti L, Osborn J, Falconieri P, Borrelli O, Cucchiara S. Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. Gut 2006;55:1760-7.

358. Giaffer MH, Holdsworth CD, Duerden BI. The assessment of faecal flora in patients with inflammatory bowel disease by a simplified bacteriological technique. J Med Microbiol 1991;35:238-43.

359. Giaffer MH, Holdsworth CD, Duerden BI. Virulence properties of Escherichia coli strains isolated from patients with inflammatory bowel disease. Gut 1992;33:646-50.

360. Gophna U, Sommerfeld K, Gophna S, Doolittle WF, Veldhuyzen van Zanten SJ. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. J Clin Microbiol 2006;44:4136-41.

361. Kleessen B, Kroesen AJ, Buhr HJ, Blaut M. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. Scand J Gastroenterol 2002;37:1034-41.

362. Prindiville T, Cantrell M, Wilson KH. Ribosomal DNA sequence analysis of mucosa-associated bacteria in Crohn's disease. Inflamm Bowel Dis 2004;10:824-33.

363. Schultsz C, Van Den Berg FM, Ten Kate FW, Tytgat GN, Dankert J. The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls. Gastroenterology 1999;117:1089-97.

364. Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002;122:44-54.

365. Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. J Clin Microbiol 2005;43:3380-9.

366. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 2008;105:16731-6.

367. Willing BP, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, Jarnerot G, Tysk C, Jansson JK, Engstrand L. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. Gastroenterology 2010;139:1844-1854 e1.

368. Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskas L, Dore J, Raedler A, Schreiber S. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology 2011;141:227-36.

369. Mylonaki M, Rayment NB, Rampton DS, Hudspith BN, Brostoff J. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. Inflamm Bowel Dis 2005;11:481-7.

370. Png CW, Linden SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, McGuckin MA, Florin TH. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 2010;105:2420-8.

371. Swidsinski A, Dorffel Y, Loening-Baucke V, Theissig F, Ruckert JC, Ismail M, Rau WA, Gaschler D, Weizenegger M, Kuhn S, Schilling J, Dorffel WV. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum. Gut 2011;60:34-40.

372. Simren M, Axelsson J, Gillberg R, Abrahamsson H, Svedlund J, Bjornsson ES. Quality of life in inflammatory bowel disease in remission: the impact of IBS-like symptoms and associated psychological factors. Am J Gastroenterol 2002;97:389-96.

373. Isgar B, Harman M, Kaye MD, Whorwell PJ. Symptoms of irritable bowel syndrome in ulcerative colitis in remission. Gut 1983;24:190-2.

374. Pimentel M, Chang M, Chow EJ, Tabibzadeh S, Kirit-Kiriak V, Targan SR, Lin HC. Identification of a prodromal period in Crohn's disease but not ulcerative colitis. Am J Gastroenterol 2000;95:3458-62.

375. Keohane J, O'Mahony C, O'Mahony L, O'Mahony S, Quigley EM, Shanahan F. Irritable bowel syndrome-type symptoms in patients with inflammatory bowel disease: a real association or reflection of occult inflammation? Am J Gastroenterol 2010;105:1788, 1789-94; quiz 1795.

376. Quigley EM. Commensal bacteria: the link between IBS and IBD? Curr Opin Clin Nutr Metab Care 2011;14:497-503.

377. Humes D, Smith JK, Spiller RC. Colonic diverticular disease. Clin Evid (Online) 2011;2011.

378. Humes DJ, Solaymani-Dodaran M, Fleming KM, Simpson J, Spiller RC, West J. A population-based study of perforated diverticular disease incidence and associated mortality. Gastroenterology 2009;136:1198-205.

379. Strate LL, Liu YL, Aldoori WH, Giovannucci EL. Physical activity decreases diverticular complications. Am J Gastroenterol 2009;104:1221-30.

380. Strate LL, Liu YL, Aldoori WH, Syngal S, Giovannucci EL. Obesity increases the risks of diverticulitis and diverticular bleeding. Gastroenterology 2009;136:115-122 e1.

381. Aldoori WH, Giovannucci EL, Rimm EB, Wing AL, Trichopoulos DV, Willett WC. A prospective study of diet and the risk of symptomatic diverticular disease in men. Am J Clin Nutr 1994;60:757-64.

382. Simpson J, Neal KR, Scholefield JH, Spiller RC. Patterns of pain in diverticular disease and the influence of acute diverticulitis. Eur J Gastroenterol Hepatol 2003;15:1005-10.

383. Humes DJ, Simpson J, Neal KR, Scholefield JH, Spiller RC. Psychological and colonic factors in painful diverticulosis. Br J Surg 2008;95:195-8.

384. Simpson J, Sundler F, Humes DJ, Jenkins D, Wakelin D, Scholefield JH, Spiller RC. Prolonged elevation of galanin and tachykinin expression in mucosal and myenteric enteric nerves in trinitrobenzene sulphonic acid colitis. Neurogastroenterol Motil 2008;20:392-406.

385. Simpson J, Sundler F, Humes DJ, Jenkins D, Scholefield JH, Spiller RC. Post inflammatory damage to the enteric nervous system in diverticular disease and its relationship to symptoms. Neurogastroenterol Motil 2009;21:847-e58.

386. D'Anchino M, Orlando D, De Feudis L. Giardia lamblia infections become clinically evident by eliciting symptoms of irritable bowel syndrome. The Journal of infection 2002;45:169-72.

387. Dear KL, Elia M, Hunter JO. Do interventions which reduce colonic bacterial fermentation improve symptoms of irritable bowel syndrome? Dig Dis Sci 2005;50:758-66.

388. Morken MH, Valeur J, Norin E, Midtvedt T, Nysaeter G, Berstad A. Antibiotic or bacterial therapy in post-giardiasis irritable bowel syndrome. Scand J Gastroenterol 2009;44:1296-303.

389. Nayak AK, Karnad DR, Abraham P, Mistry FP. Metronidazole relieves symptoms in irritable bowel syndrome: the confusion with so-called 'chronic amebiasis'. Indian journal of gastroenterology : official journal of the Indian Society of Gastroenterology 1997;16:137-9.

390. Koo HL, DuPont HL. Rifaximin: a unique gastrointestinal-selective antibiotic for enteric diseases. Curr Opin Gastroenterol 2010;26:17-25.

391. Pimentel M, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. Ann Intern Med 2006;145:557-63.

392. Sharara AI, Aoun E, Abdul-Baki H, Mounzer R, Sidani S, Elhajj I. A randomized double-blind placebo-controlled trial of rifaximin in patients with abdominal bloating and flatulence. Am J Gastroenterol 2006;101:326-33.

393. Drossman DA. Treatment for bacterial overgrowth in the irritable bowel syndrome. Ann Intern Med 2006;145:626-8.

394. Tack J. Antibiotic therapy for the irritable bowel syndrome. N Engl J Med 2011;364:81-2.

395. Pimentel M, Morales W, Chua K, Barlow G, Weitsman S, Kim G, Amichai MM, Pokkunuri V, Rook E, Mathur R, Marsh Z. Effects of Rifaximin Treatment and Retreatment in Nonconstipated IBS Subjects. Dig Dis Sci 2011;56:2067-72.

396. Di Stefano M, Strocchi A, Malservisi S, Veneto G, Ferrieri A, Corazza GR. Non-absorbable antibiotics for managing intestinal gas production and gas-related symptoms. Aliment Pharmacol Ther 2000;14:1001-8.

397. Cuoco L, Salvagnini M. Small intestine bacterial overgrowth in irritable bowel syndrome: a retrospective study with rifaximin. Minerva Gastroenterol Dietol 2006;52:89-95.

398. Jolley J. High-dose rifaximin treatment alleviates global symptoms of irritable bowel syndrome. Clinical and experimental gastroenterology 2011;4:43-8.

399. Peralta S, Cottone C, Doveri T, Almasio PL, Craxi A. Small intestine bacterial overgrowth and irritable bowel syndrome-related symptoms: experience with Rifaximin. World J Gastroenterol 2009;15:2628-31.

400. Yang J, Lee HR, Low K, Chatterjee S, Pimentel M. Rifaximin versus other antibiotics in the primary treatment and retreatment of bacterial overgrowth in IBS. Dig Dis Sci 2008;53:169-74.

401. Koo HL, DuPont HL. Current and future developments in travelers' diarrhea therapy. Expert Rev Anti Infect Ther 2006;4:417-27.

402. Jiang ZD, DuPont HL, La Rocco M, Garey KW. In vitro susceptibility of Clostridium difficile to rifaximin and rifampin in 359 consecutive isolates at a university hospital in Houston, Texas. J Clin Pathol 2010;63:355-8.

403.    Shah D, Dang MD, Hasbun R, Koo HL, Jiang ZD, DuPont HL, Garey KW. Clostridium difficile infection: update on emerging antibiotic treatment options and antibiotic resistance. Expert Rev Anti Infect Ther 2010;8:555-64.

404.    Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, Pasculle AW, Harrison LH. High frequency of rifampin resistance identified in an epidemic Clostridium difficile clone from a large teaching hospital. Clin Infect Dis 2009;48:425-9.

405.    Valentin T, Leitner E, Rohn A, Zollner-Schwetz I, Hoenigl M, Salzer HJ, Krause R. Rifaximin intake leads to emergence of rifampin-resistant staphylococci. J Infect 2011;62:34-8.

406.    Guarner F, Requena T, Marcos A. Consensus statements from the Workshop "Probiotics and Health: Scientific evidence". Nutricion hospitalaria : organo oficial de la Sociedad Espanola de Nutricion Parenteral y Enteral 2010;25:700-4.

407.    Ait-Belgnaoui A, Han W, Lamine F, Eutamene H, Fioramonti J, Bueno L, Theodorou V. Lactobacillus farciminis treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. Gut 2006;55:1090-4.

408.    Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J. Inhibitory effects of Lactobacillus reuteri on visceral pain induced by colorectal distension in Sprague-Dawley rats. Gut 2006;55:191-6.

409.    Wang B, Mao YK, Diorio C, Pasyk M, Wu RY, Bienenstock J, Kunze WA. Luminal administration ex vivo of a live Lactobacillus species moderates mouse jejunal motility within minutes. FASEB j 2010;24:4078-88.

410.    Zareie M, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM, Soderholm JD, Perdue MH, Sherman PM. Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. Gut 2006;55:1553-60.

411.    Agrawal A, Houghton LA, Morris J, Reilly B, Guyonnet D, Goupil Feuillerat N, Schlumberger A, Jakob S, Whorwell PJ. Clinical trial: the effects of a fermented milk product containing Bifidobacterium lactis DN-173-010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. Aliment Pharmacol Ther 2008;29:104-114.

412.    Kim HJ, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, Zinsmeister AR. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2003;17:895-904.

413.    Kim HJ, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. Neurogastroenterol Motil 2005;17:687-96.

414.    Francavilla R, Miniello V, Magista AM, De Canio A, Bucci N, Gagliardi F, Lionetti E, Castellaneta S, Polimeno L, Peccarisi L, Indrio F, Cavallo L. A randomized controlled trial of Lactobacillus GG in children with functional abdominal pain. Pediatrics 2010;126:e1445-52.

415.    Kajander K, Krogius-Kurikka L, Rinttila T, Karjalainen H, Palva A, Korpela R. Effects of multispecies probiotic supplementation on intestinal microbiota in irritable bowel syndrome. Aliment Pharmacol Ther 2007;26:463-73.

416.    Kajander K, Myllyluoma E, Rajilic-Stojanovic M, Kyronpalo S, Rasmussen M, Jarvenpaa S, Zoetendal EG, de Vos WM, Vapaatalo H, Korpela R. Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. Aliment Pharmacol Ther 2008;27:48-57.

417.    O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128:541-51.

418.    De Vecchi E, Nicola L, Zanini S, Drago L. In vitro screening of probiotic characteristics of some italian products. J Chemother 2008;20:341-7.

419.    Drago L, De Vecchi E, Nicola L, Colombo A, Gismondo MR. Microbiological evaluation of commercial probiotic products available in Italy. J Chemother 2004;16:463-7.

420.    Drago L, Rodighiero V, Celeste T, Rovetto L, De Vecchi E. Microbiological evaluation of commercial probiotic products available in the USA in 2009. J Chemother 2010;22:373-7.

421.    Bausserman M, Michail S. The use of Lactobacillus GG in irritable bowel syndrome in children: a double-blind randomized control trial. J Pediatr 2005;147:197-201.

422.    Dolin BJ. Effects of a proprietary Bacillus coagulans preparation on symptoms of diarrhea-predominant irritable bowel syndrome. Methods exp clin pharmacol 2009;31:655-9.

423.    Drouault-Holowacz S, Bieuvelet S, Burckel A, Cazaubiel M, Dray X, Marteau P. A double blind randomized controlled trial of a probiotic combination in 100 patients with irritable bowel syndrome. Gastroenterol Clin Biol 2008;32:147-52.

424.    Enck P, Zimmermann K, Menke G, Muller-Lissner S, Martens U, Klosterhalfen S. A mixture of Escherichia coli (DSM 17252) and Enterococcus faecalis (DSM 16440) for treatment of the irritable bowel syndrome--a randomized controlled trial with primary care physicians. Neurogastroenterol Motil 2008;20:1103-9.

425.    Gade J, Thorn P. Paraghurt for patients with irritable bowel syndrome. A controlled clinical investigation from general practice. Scand J Prim Health Care 1989;7:23-6.

426.    Gawronska A, Dziechciarz P, Horvath A, Szajewska H. A randomized double-blind placebo-controlled trial of Lactobacillus GG for abdominal pain disorders in children. Aliment Pharmacol Ther 2007;25:177-84.

427.    Guandalini S, Magazzu G, Chiaro A, La Balestra V, Di Nardo G, Gopalan S, Sibal A, Romano C, Canani RB, Lionetti P, Setty M. VSL#3 improves symptoms in children with irritable bowel syndrome: a multicenter, randomized, placebo-controlled, double-blind, crossover study. J Pediatr Gastroenterol Nutr 2010;51:24-30.

428.    Guglielmetti S, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. Aliment Pharmacol Ther 2011;33:1123-32.

429.    Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier CH, Matuchansky C. Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 2007;26:475-86.

430.    Halpern GM, Prindiville T, Blankenburg M, Hsia T, Gershwin ME. Treatment of irritable bowel syndrome with Lacteol Fort: a randomized, double-blind, cross-over trial. Am J Gastroenterol 1996;91:1579-85.

431.    Hong KS, Kang HW, Im JP, Ji GE, Kim SG, Jung HC, Song IS, Kim JS. Effect of probiotics on symptoms in korean adults with irritable bowel syndrome. Gut and liver 2009;3:101-7.

432.    Kajander K, Hatakka K, Poussa T, Farkkila M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther 2005;22:387-94.

433.    Kim YG, Moon JT, Lee KM, Chon NR, Park H. [The effects of probiotics on symptoms of irritable bowel syndrome]. Korean J Gastroenterol 2006;47:413-9.

434.    Ligaarden SC, Axelsson L, Naterstad K, Lydersen S, Farup PG. A candidate probiotic with unfavourable effects in subjects with irritable bowel syndrome: a randomised controlled trial. BMC Gastroenterol 2010;10:16.

435.    Niedzielin K, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. Eur J Gastroenterol Hepatol 2001;13:1143-7.

436.    Niv E, Naftali T, Hallak R, Vaisman N. The efficacy of Lactobacillus reuteri ATCC 55730 in the treatment of patients with irritable bowel syndrome--a double blind, placebo-controlled, randomized study. Clin Nutr 2005;24:925-31.

437.    Nobaek S, Johansson ML, Molin G, Ahrne S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. Am J Gastroenterol 2000;95:1231-8.

438. O'Sullivan MA, O'Morain CA. Bacterial supplementation in the irritable bowel syndrome. A randomised double-blind placebo-controlled crossover study. Dig Liver Dis 2000;32:294-301.

439. Sen S, Mullan MM, Parker TJ, Woolner JT, Tarry SA, Hunter JO. Effect of Lactobacillus plantarum 299v on colonic fermentation and symptoms of irritable bowel syndrome. Dig Dis Sci 2002;47:2615-20.

440. Simren M, Ohman L, Olsson J, Svensson U, Ohlson K, Posserud I, Strid H. Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome - a randomized, double-blind, controlled study. Aliment Pharmacol Ther 2010;31:218-27.

441. Sinn DH, Song JH, Kim HJ, Lee JH, Son HJ, Chang DK, Kim YH, Kim JJ, Rhee JC, Rhee PL. Therapeutic effect of Lactobacillus acidophilus-SDC 2012, 2013 in patients with irritable bowel syndrome. Dig Dis Sci 2008;53:2714-8.

442. Sondergaard B, Olsson J, Ohlson K, Svensson U, Bytzer P, Ekesbo R. Effects of probiotic fermented milk on symptoms and intestinal flora in patients with irritable bowel syndrome: A randomized, placebo-controlled trial. Scand J Gastroenterol 2011;46:663-72.

443. Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol 2006;101:1581-90.

444. Williams E, Stimpson J, Wang D, Plummer S, Garaiova I, Barker M, Corfe B. Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. Aliment Pharmacol Ther 2009;29:97-103.

445. Kruis W, Chrubasik S, Boehm S, Stange C, Schulze J. A double-blind placebo-controlled trial to study therapeutic effects of probiotic Escherichia coli Nissle 1917 in subgroups of patients with irritable bowel syndrome. Int J Colorectal Dis 2011.

446. Brenner DM, Moeller MJ, Chey WD, Schoenfeld PS. The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. Am J Gastroenterol 2009;104:1033-49; quiz 1050.

447. Hoveyda N, Heneghan C, Mahtani KR, Perera R, Roberts N, Glasziou P. A systematic review and meta-analysis: probiotics in the treatment of irritable bowel syndrome. BMC gastroenterology 2009;9:15.

448. McFarland LV, Dublin S. Meta-analysis of probiotics for the treatment of irritable bowel syndrome. World J Gastroenterol 2008;14:2650-61.

449. Moayyedi P, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 2010;59:325-32.

450. Roberfroid M. Prebiotics: the concept revisited. The Journal of nutrition 2007;137:830S-7S.

451. Silk DB, Davis A, Vulevic J, Tzortzis G, Gibson GR. Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome. Aliment Pharmacol Ther 2009;29:508-18.

452. Andriulli A, Neri M, Loguercio C, Terreni N, Merla A, Cardarella MP, Federico A, Chilovi F, Milandri GL, De Bona M, Cavenati S, Gullini S, Abbiati R, Garbagna N, Cerutti R, Grossi E. Clinical trial on the efficacy of a new symbiotic formulation, Flortec, in patients with irritable bowel syndrome: a multicenter, randomized study. J Clin Gastroenterol 2008;42 Suppl 3 Pt 2:S218-23.

453. Bittner AC, Croffut RM, Stranahan MC. Prescript-Assist probiotic-prebiotic treatment for irritable bowel syndrome: a methodologically oriented, 2-week, randomized, placebo-controlled, double-blind clinical study. Clin Ther 2005;27:755-61.

454. Bittner AC, Croffut RM, Stranahan MC, Yokelson TN. Prescript-assist probiotic-prebiotic treatment for irritable bowel syndrome: an open-label, partially controlled, 1-year extension of a previously published controlled clinical trial. Clin Ther 2007;29:1153-60.

455. Colecchia A, Vestito A, La Rocca A, Pasqui F, Nikiforaki A, Festi D. Effect of a symbiotic preparation on the clinical manifestations of irritable bowel syndrome, constipation-variant.

Results of an open, uncontrolled multicenter study. Minerva Gastroenterol Dietol 2006;52:349-58.

456.    Tsuchiya J, Barreto R, Okura R, Kawakita S, Fesce E, Marotta F. Single-blind follow-up study on the effectiveness of a symbiotic preparation in irritable bowel syndrome. Chin J Dig Dis 2004;5:169-74.

457.    Shepherd SJ, Gibson PR. Fructose malabsorption and symptoms of irritable bowel syndrome: guidelines for effective dietary management. J Am Diet Assoc 2006;106:1631-9.

458.    Nanda R, James R, Smith H, Dudley CR, Jewell DP. Food intolerance and the irritable bowel syndrome. Gut 1989;30:1099-104.

459.    King TS, Elia M, Hunter JO. Abnormal colonic fermentation in irritable bowel syndrome. Lancet 1998;352:1187-9.

460.    Snook J, Shepherd HA. Bran supplementation in the treatment of irritable bowel syndrome. Aliment Pharmacol Ther 1994;8:511-4.

461.    Lyra A, Krogius-Kurikka L, Nikkila J, Malinen E, Kajander K, Kurikka K, Korpela R, Palva A. Effect of a multispecies probiotic supplement on quantity of irritable bowel syndrome-related intestinal microbial phylotypes. BMC Gastroenterol 2010;10:110.

462.    Liu Z, Qin H, Yang Z, Xia Y, Liu W, Yang J, Jiang Y, Zhang H, Wang Y, Zheng Q. Randomised clinical trial: the effects of perioperative probiotic treatment on barrier function and post-operative infectious complications in colorectal cancer surgery - a double-blind study. Aliment Pharmacol Ther 2011;33:50-63.

463.    Zeng J, Li YQ, Zuo XL, Zhen YB, Yang J, Liu CH. Clinical trial: effect of active lactic acid bacteria on mucosal barrier function in patients with diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2008;28:994-1002.

464.    Zhou Q, Zhang B, Verne GN. Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome. Pain 2009;146:41-6.

465.    Houghton LA, Foster JM, Whorwell PJ. Alosetron, a 5-HT3 receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers. Aliment Pharmacol Ther 2000;14:775-82.

466.    Andresen V, Camilleri M. Irritable bowel syndrome: recent and novel therapeutic approaches. Drugs 2006;66:1073-88.

467.    Camilleri M, Bharucha AE, Ueno R, Burton D, Thomforde GM, Baxter K, McKinzie S, Zinsmeister AR. Effect of a selective chloride channel activator, lubiprostone, on gastrointestinal transit, gastric sensory, and motor functions in healthy volunteers. Am J Physiol Gastrointest Liver Physiol 2006;290:G942-7.

468.    Prather CM, Camilleri M, Zinsmeister AR, McKinzie S, Thomforde G. Tegaserod accelerates orocecal transit in patients with constipation-predominant irritable bowel syndrome. Gastroenterology 2000;118:463-8.

469.    Cremonini F, Delgado-Aros S, Camilleri M. Efficacy of alosetron in irritable bowel syndrome: a meta-analysis of randomized controlled trials. Neurogastroenterol Motil 2003;15:79-86.

470.    Drossman DA, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. Aliment Pharmacol Ther 2009;29:329-41.

471.    Evans BW, Clark WK, Moore DJ, Whorwell PJ. Tegaserod for the treatment of irritable bowel syndrome. Cochrane Database Syst Rev 2004:CD003960.

472.    Johanson JF, Drossman DA, Panas R, Wahle A, Ueno R. Clinical trial: phase 2 study of lubiprostone for irritable bowel syndrome with constipation. Aliment Pharmacol Ther 2008;27:685-96.

473.    Johnston JM, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, O'Dea C, Baird M, Lembo AJ. Linaclotide improves abdominal pain and bowel habits in a phase IIb study of patients with irritable bowel syndrome with constipation. Gastroenterology 2010;139:1877-1886 e2.

474.    Kaptchuk TJ, Friedlander E, Kelley JM, Sanchez MN, Kokkotou E, Singer JP, Kowalczykowski M, Miller FG, Kirsch I, Lembo AJ. Placebos without deception: a randomized controlled trial in irritable bowel syndrome. PLoS One 2010;5:e15591.

475.    Barbara G. Mucosal barrier defects in irritable bowel syndrome. Who left the door open? Am J Gastroenterol 2006;101:1295-8.

476.    Barbara G. Editorial: toll-like receptor expression in irritable bowel syndrome: on the alert for a microbial threat? Am J Gastroenterol 2011;106:337-9.

477.    Collins SM, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 2009;136:2003-14.

478.    Schoepfer AM, Schaffer T, Seibold-Schmid B, Muller S, Seibold F. Antibodies to flagellin indicate reactivity to bacterial antigens in IBS patients. Neurogastroenterol Motil 2008;20:1110-8.