PERTURBATIONS IN THE HUMAN GUT MICROBIOME WITH ANTIBIOTIC THERAPY AND INTESTINAL DISORDERS

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We hereby certify that the thesis entitled "*Perturbations in the human gut microbiome with antibiotic therapy and intestinal disorders*", submitted by **Suchita Panda**, presented in compliance for the degree Doctor of Philosophy, was carried out under the supervision of Dr. C. Manichanh, and tutorship of Dr. J. Vilaseca Momplet, and we authorise the submission to undertake its oral defence.

In witness whereof, we hereby sign this document.

Barcelona, November 2015

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"The role of the infinitely small in nature is infinitely great"

Louis Pasteur

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…And the journey has just begun!

ABSTRACT

The intestinal microbiota is a key determinant of gut homeostasis, thereby being an imperative agent of health status. Its composition could be altered as a consequence of external factors such as antibiotic treatment or as a possible cause of intestinal disorders such as irritable bowel syndrome (IBS). In this doctoral thesis we focused on understanding to which extent antibiotic treatment could modify the gut microbiome and how an alteration of its composition could be associated with IBS. Our results showed that the lower diversity occurring after antibiotic treatment was unexpectedly associated with an increase of the overall microbial load. Furthermore, subjects suffering from IBS, particularly diarrhoea predominant subtype, had lower bacterial diversity accompanied by a reduced relative abundance of butyrate-producing and methanogenic microbes compared to healthy subjects. Altogether the findings of this study are that uncontrolled intake of antibiotics may cause tremendous changes in the gut microbial community composition, favouring the overgrowth of resistant microbes; and this observation may explain that alterations of the microbial composition earlier in life could lead to intestinal disorders.

RESUMEN

La microbiota intestinal es un factor determinante de la homeostasis intestinal, siendo por lo tanto un agente imprescindible del estado de salud. Su composición podría estar alterada como consecuencia de factores externos tales como tratamientos con antibióticos o causada por enfermedades intestinales como el síndrome del intestino irritable (SII). Esta tesis doctoral se centró en la comprensión de la alteración de esta ecología microbiana con respecto a una terapia con antibióticos y la presencia de movimientos intestinales modificados en personas que sufren de síndrome de intestino irritable. Nuestros resultados mostraron que la baja diversidad surgida después del tratamiento con antibióticos

fue asociada, inesperadamente, con un aumento de la carga bacteriana total. Sin embargo, sujetos afectados con SII, particularmente del subtipo diarreico, tenían menor diversidad bacteriana acompañada de una reducción de las bacterias productoras de butirato y de las productoras de metano comparado con sujetos sanos. En general los hallazgos de este estudio son que una administración incontrolada de antibióticos puede causar cambios severos en la composición de la comunidad bacteriana intestinal, favorecer el sobrecrecimiento de microbios resistentes y esta observación puede explicar que las alteraciones microbianas en etapas iniciales de la vida podrían llegar a desarrollar enfermedades intestinales.

INDEX

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

16S rRNA	– 16S ribosomal RNA
A+T	– Adenine + Thymine content
AAD	– Antibiotics Associated Diarrhoea
ANOVA	– Analysis of variance
ATB	– Antibiotic
ATP	– Adenosine Triphosphate
BLAST	– Basic Local Alignment Search Tool
BMI	– Body Mass Index
CD	– Crohn's Disease
CDI	– *Clostridium difficile* Infection
cDNA	– complimentary DNA
CFU	– Colony Forming Units
DGGE	– Denaturing Gradient Gel Electrophoresis
ddNTP	– Dideoxynucleotide phosphate
DNA	– Deoxyribo Nucleic Acid
dNTP	– Deoxynucleotide phosphate
EDTA	– Ethylene Diamine Tetraacetic Acid
emPCR	– emulsion PCR
ENA_EMBL	– European Nucleotide Archive
FDR	– False Discovery Rate
FGID	– Functional Gastro Intestinal Disorder
FISH	– Fluorescence *In-Situ* Hybridisation
FMT	– Faecal Microbiota Transplant
FODMAP	– Fermentable Oligo-Di-Monosaccharides and Polyols
G+C	– Guanine + Cytosine content
GIT	– Gastro Intestinal Tract
HC	– Healthy Control
HITChip	– Human Intestinal Tract microarray Chip
HMP	– Human Microbiome Project
HPLC	– High Performance Liquid Chromatography
HTS	– High Throughput Sequencing

IBD	–	Inflammatory Bowel Disease
IBS	–	Irritable Bowel Syndrome
IBS-C	–	IBS subtype Constipation
IBS-D	–	IBS subtype Diarrhoea
IBS-M	–	Mixed type of IBS
IFN	–	Interferon
IL	–	Interlukin
MID	–	Multiplex Identifier
mRNA	–	messenger RNA
NGS	–	Next Generation Sequencing
OTU	–	Operational Taxonomic Unit
PBP	–	Penicillin Binding Protein
PCoA	–	Principal Coordinates Analysis
PCR	–	Polymerase Chain Reaction
PVPP	–	Poly Viny Poly-Pyrrolidone
QIIME	–	Quantitative Insights Into Microbial Ecology
qPCR	–	quantitative PCR
RDP	–	Ribosomal Database Project
RNA	–	Ribo Nucleic Acid
rRNA	–	ribosomal RNA
SCFA	–	Short Chain Fatty Acid
SD	–	Standard Deviation
SIBO	–	Small Intestinal Bacterial Overgrowth
TGGE	–	Temperature Gradient Gel Electrophoresis
T-RFLP	–	Terminal Restriction Fragment Length Polymorphism
UC	–	Ulcerative Colitis
UPGMA	–	Unweighted Pair Group Method with Arithmetic Mean

INTRODUCTION

1. Microbiota

The human body is host to more than 100 trillion microbes that reside peacefully in a symbiotic relationship [Savage, 1977; Gill *et al*., 2006; Qin *et al*., 2010]. Known as the human microbiota (previously known as the "normal flora"), the diverse bacterial, archaeal, fungal and viral communities, are estimated to be ten-fold the number of human cells [Backhed *et al*., 2005; Eckburg *et al*., 2005]. Each individual carries about 500,000 non-redundant genes [Qin *et al*. 2010].

1.1 Gut microbiome

The largest collection of the microbial population is colonised in the human gastro-intestinal tract (GIT). Starting from the oral cavity, through the oesophagus, stomach, small and large intestines till the rectum, the entire GIT is colonised by microbes, each section being inhabited by a certain population depending mainly on tissue structure, pH, oxygen availability and temperature of the section [Thadepalli *et al*., 1979; Pajecki *et al*., 2002; Pel *et al*., 2004; Jenkinson *et al*., 2005; Bik *et al*., 2006; Andersson *et al*., 2008; Zaura *et al*., 2009; Boojink *et al*., 2010b]. More than 70% of the entire microbiota is present in the human colon [Backhed *et al*., 2005; Eckburg *et al*., 2005] (**Figure 1**).
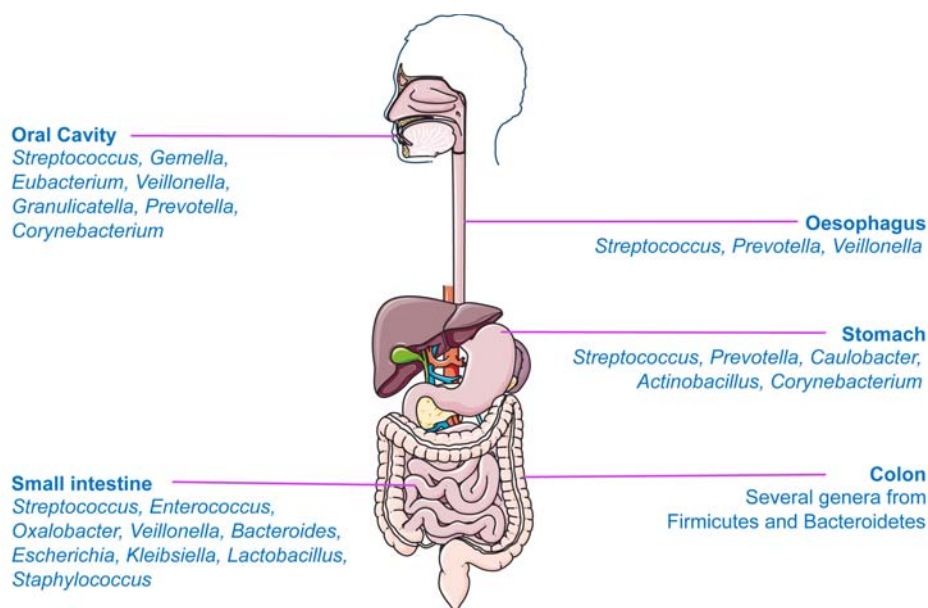
Figure 1: Human Gastro-Intestinal Tract – Showing dominant microbiota

The human gut microbial community is composed of 10^{13} to 10^{14} microbes, and their total genome (termed as the microbiome) contains 100 times more genes than the human genome [Backhed *et al*., 2005; Eckburg *et al*., 2005]. Firmicutes and Bacteroidetes constitute the dominating phyla (>90%) among the bacterial population [Hayashi *et al*., 2002; Hold *et al*., 2002; Wang *et al*., 2003; Eckburg *et al*., 2005; Qin *et al*., 2010; Arumugam *et al*., 2011). Actinobacteria, Proteobacteria, Fusobacteria, Verrumicrobia are the other phyla detected in lesser proportions.

The Firmicutes phylum is mostly composed of Gram-positive bacteria with a low G+C content, although some genera possess Gram-negative cell wall containing lipopolysaccharide. The members of the Bacteroidetes phylum are Gram-negative bacteria, having a DNA G+C content of 28–61%.

In healthy persons, the most abundant genera are *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Facecalibacterium*, *Prevotella*, and *Ruminococcus*, which belong to the families Bacteroidaceae, Bifidobacteriaceae Clostridiaceae, and Ruminococcaceae [Suau *et al*., 1999; Eckburg *et al*., 2005; Ley *et al*., 2006; Tap *et al*., 2009; Turnbaugh *et al*., 2009; Arumugam *et al*., 2011].

2. Investigation methods

In the 1600s when Anton van Leeuwenhoek first described "tiny living animalcules" through his self-made microscope, he used his own faeces, and gave the world a new perspective to follow.

Newly developed molecular techniques for the identification and/or enumeration of different microbial groups based on the 16S ribosomal RNA gene (16S rRNA) (**Figure 2**) has increased enormously during the past decade and brought henceforth a great amount of unprecedented knowledge (**Figure 3, Table 1**).
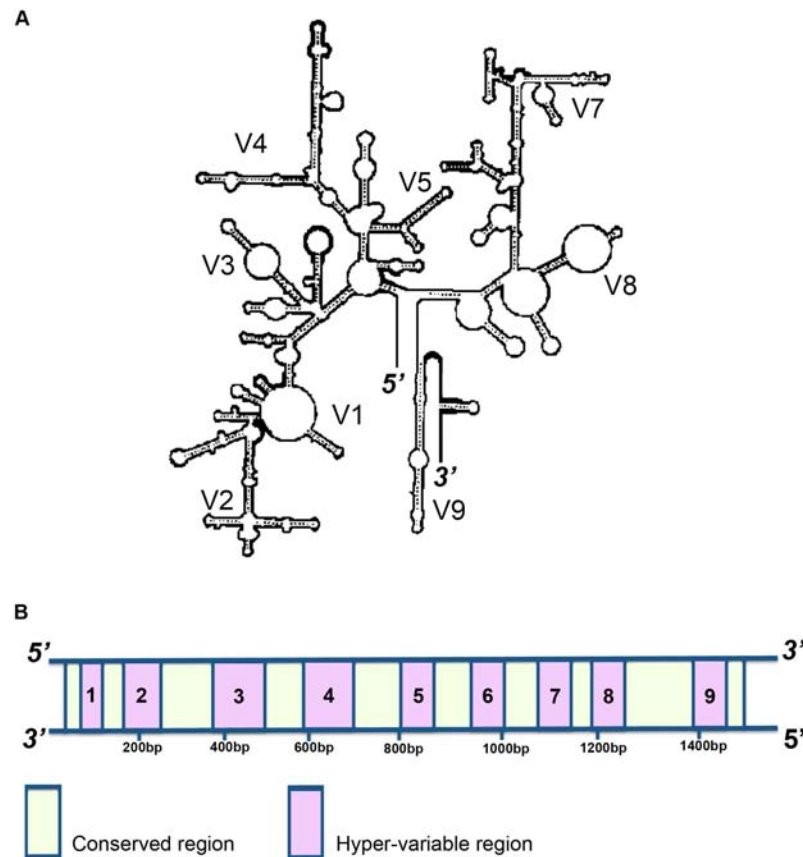
Figure 2: 16S rRNA gene – A) Structure, B) Conserved and hyper-variable regions

2.1 The use of faeces as samples

The advantage of simple, easy and non-invasive route of sample collection is the reason why faecal samples are used to study the colonic microbiome structure and function. Although the majority of bacteria are similar throughout the ileum to rectum, adherent microbiota residing on mucosal surfaces of the intestines may be widely different from those present in the luminal material. As shown by Eckburg *et al.*, 2005, the faecal microbiota had lesser Bacteroidetes, more Firmicutes and Proteobacteria as compared to the mucosal samples. These differences may be attributed to the flushing action of beverages and gastric juices, and the production of antimicrobial peptides by the intestinal mucosa [Wilson, 2008]. Distinct communities of bacteria may locally colonise different sections of the gut, which may be quantitatively and qualitatively different from the fresh stool [Eckburg *et al.*, 2005; Momozawa *et al.*, 2011].

Method	Material Used	Experimental Design	Type of Result
Culture	Tissue/ environmental sample	Diluted sample incubated in enriching medium followed by microscopy/ biochemical analysis	Physical/ biochemical details of different microbes
Dot-Blot hybridisation	DNA extracted from sample	DNA blotted in a membrane which is subjected to hybridisation with oligonucleotides	Semi-quantification and detection of certain sequences in sample
qPCR	DNA extracted from sample	Amplification of sequences using specific dye-labelled primers	Quantification as either an absolute number of copies or a relative amount when normalised to DNA input
DGGE	Total environmental DNA extracted from sample	PCR amplification of 16S rRNA (prokaryotic) or 18S rRNA (eukaryotic) gene from total extracted DNA. Amplicons are then subjected to changing chemical denaturing conditions in a gel. DNA strands denature completely within the gel depending on the sequence composition, which are visualised as bands	Possibility to discern differences in DNA sequences and similarity comparison giving an estimate of microbial richness
TGGE	Total environmental DNA extracted from sample	PCR amplification of 16S rRNA (prokaryotic) or 18S rRNA (eukaryotic) gene from total extracted DNA. Temperature gradient used to separate amplicon bands in a gel depending on sequence content (G+C vs. A+T content)	Gel band patterns used to visualise variations in microbial genetic diversity and provide a rough estimate of the richness of predominant microbial community members
FISH	DNA in tissues	Use of fluorescent probes to bind to DNA targets (in tissues) showing high degree of sequence complementarity after which fluorescence microscopy is used to locate the bound probes in tissues	Fluorescence probe hybridisation coupled with fluorescence microscopy enables the identification and enumeration of specific microbial groups within a sample
Microarray	Total environmental DNA extracted from sample	A solid substrate (chip) contains embedded labelled specific DNA sequences (probe) to which sample DNA (target) is added. Complementary strands undergo hybridisation that is detected and quantified to determine relative abundance of nucleic acid sequences in the target	Detection and measurement of specific sequences present in sample

Metagenomics	Total environmental DNA extracted from sample	Microbial genomic DNA is broken up randomly into numerous small segments that are then sequenced. Bioinformatic assembly methods are used to reconstruct overlapping ends of reads into a continuous sequence that are compared to databases	Comprehensive description of the microbial gene content and functions based on genes
16S rRNA gene survey	Total environmental DNA extracted from sample	PCR amplification of 16S rRNA (prokaryotic) gene from total extracted DNA. Amplicons are then sequenced, and compared to 16S rRNA gene databases	Composition and diversity of microbial groups present in a sample based on the 16S rRNA gene
Metatranscriptomics	Total environmental RNA / mRNA extracted from sample	Synthesis and further sequencing of cDNA from total RNA or mRNA (after rRNA removal from total RNA). Bioinformatic assembly methods are then used to reconstruct the sequences which are then compared to databases	Gene expression and functions carried out by microbes present in the sample
Metaproteomics	Total environmental proteins isolated from sample	Gel-based and non gel-based chromatography based separation of proteins with a further step of mass spectrometry based peptide identification	Quantification of protein expression, protein structure information
Metabolomics	Total environmental metabolites extracted from sample	Liquid / gas chromatography coupled to mass spectrometry. Metabolite identification and further pathway detection	Analyses of interactions and functions using molecular metabolic pathways

Table 1: Methods used to study the human gut microbiota (From Panda *et al*., 2014)
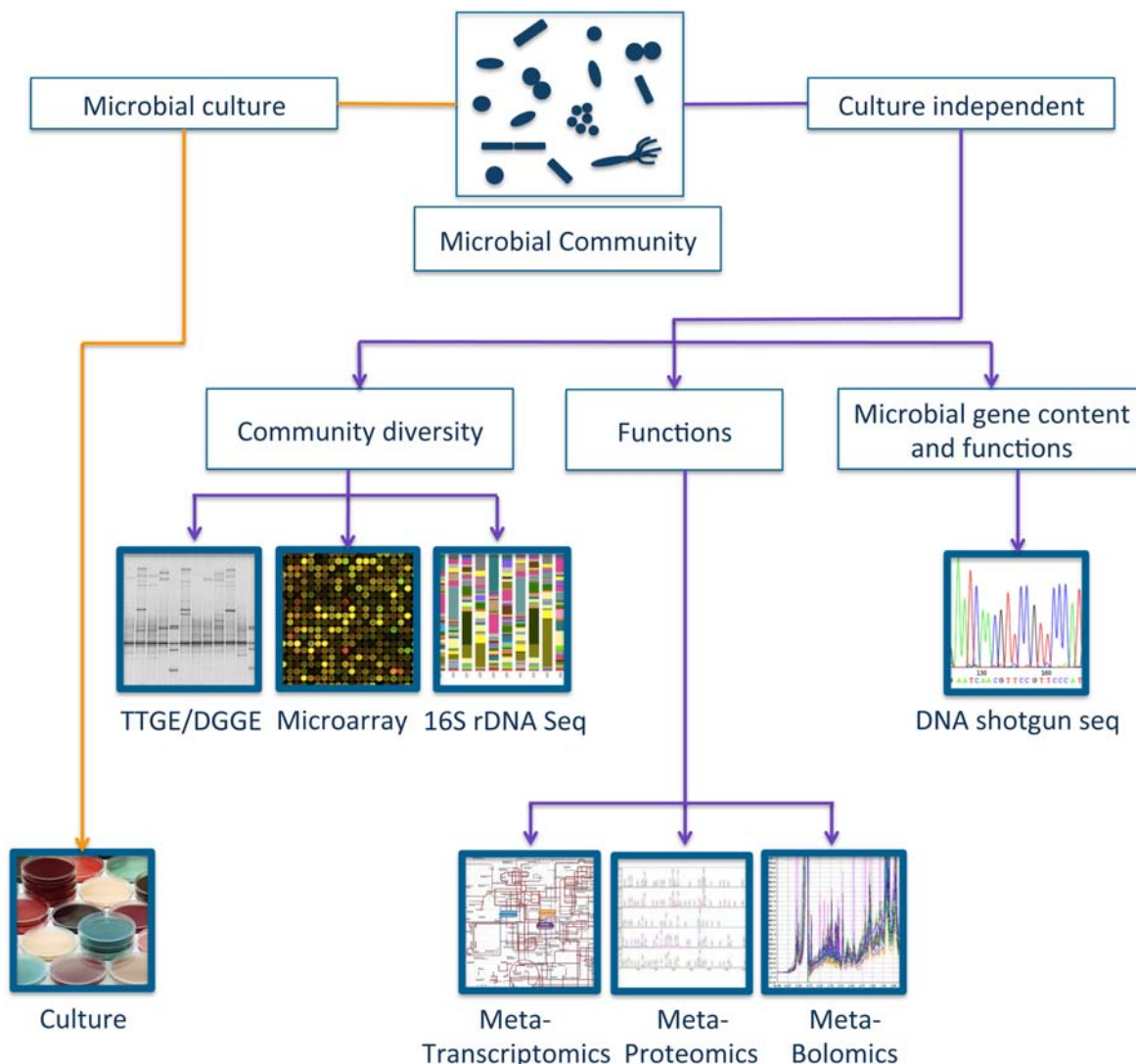
Figure 3: Methods used to study the microbiota – development of culture-independent techniques

2.2 Traditional culture methods

Traditionally, studies addressing microbiota were done using culture methods [Moore and Holdemann, 1974; Finegold *et al*., 1983], which rely on the biochemical and physical capacity of the microbes to grow and multiply in either liquid or solid medium infused with nutrients. Selective culture medium could be used for achieving the detection of certain bacteria of interest or groups that are present in lower numbers; while non- selective medium allows recovery of the dominant cultivable populations. Identification of bacterial isolates was based on colony characteristics on solid medium, microscopy and biochemical properties.

Throughout the decades, a great number of microorganisms have shown exceptional growth under specific nutrient, temperature and humidity conditions provided; thus making colony cultivation a standard method for microbial studies.

However, newer techniques demonstrated that more than 80% of the GIT microbiota had not been cultured. The incompetence of typical culture medium and lack of suitable atmospheric conditions could be the reason attributed for the inability of several bacteria to grow [Langendijk *et al*., 1995; Wilson *et al*., 1996; Zoetendal *et al*., 1998; Suau *et al*., 1999; Hayashi *et al*., 2002; Shanahan, 2002a]. Although culture techniques are still applied till date; especially when interaction between bacteria are studied (example: antibiotic resistance, production of growth factors). A recent study using anaerobic culturing techniques and high throughput sequencing has shown that about 50% species of the gut microbial community can now be cultivated and characterised [Goodman *et al*., 2011].

2.3 Culture independent molecular techniques

To overcome the limitation of culture based methods, culture-independent approaches were developed that mostly targeted the 16S rRNA gene. Ribosomes, two-subunit rRNA-protein complexes, are the protein synthesisers of the cell. In prokaryotes, the small subunit includes a 16S rRNA, whereas the large subunit includes 23S and 5S rRNAs. The 16S rRNA gene consisting of about 1,500 nucleotides is used for phylogenetic studies (with "universal" primers) as it is highly conserved between different species of bacteria and archaea throughout evolution, yet contains hyper-variable regions which are distinct among the specific species of bacteria and archaea. The conserved regions flank the hyper-variable regions, and are used for constructing primers that would complement these sequences. The primers are designed in a way that would subsequently allow the amplification of the hyper-variable regions (V1 → V9) (**Figure 2B**), which could provide information on the diversity of bacteria present in the sample [Fox *et al*., 1977; Pace and Waugh, 1991; Blaut *et al*., 2002]. This unique property of the 16S rRNA gene has been exploited, among various molecular techniques for bacterial identification, in Next Generation High Throughput Sequencing Techniques [Suau *et al*., 1999; Caporaso *et al*., 2012].

Dot-blot hybridisation: The nucleic acids are blotted onto a nitrocellulose or nylon membrane, where hybridisation is performed with universal or oligonucleotide probes specific to a certain group or species of bacteria. Semi-quantification of sequences present in a sample is based on the intensity of the blots as compared to a standard or a control [Bergmans and Gaastra, 1988; Stahl *et al*., 1988; Dore *et al*., 1998].

Real-Time quantitative PCR (qPCR): Amplification methods such as qPCR are used to detect and quantify a target DNA sequence using a small amount (as small as 10pg/μl DNA) of sample. The probes used for doing so could be universal primers to detect the total abundance of bacteria, or primers specific to a certain group or species that would allow to quantify that specific group or species in the entire sample and compare with other samples. Quantification is based on a standard curve established with known quantities of the probe sequence [Bartosch *et al*., 2004; Malinen *et al*., 2005; Carey *et al*., 2007].

Gradient Gel Electrophoreses: PCR-amplified DNA fragments of the same length but with different sequences can be differentiated based on the electrophoretic mobility of the partially melted double-stranded DNA molecule. Temporal Temperature Gradient Gel Electrophoresis (TTGE) and Denaturing Gradient Gel Electrophoresis (DGGE) are forms of polyacrylamide gel based electrophoretic methods where a gradient created by temperature or chemical (respectively) is used to denature the Nucleic Acid molecule as it moves within the gel. Partially melted DNA fragments are held together with a G+C-rich oligonucleotide, a GC-clamp. Therefore, each denaturing fragment generates only a single band in the gel (**Figure 3**). These analyses allow broad comparison of bacterial diversity and the opportunity for subsequent identification of community members by DNA fragment sequence analysis or hybridization with specific probes [Fischer and Lerman, 1979; Myers *et al*., 1985; Collins and Myers, 1987; Rosenbaun *et al*., 1987; Muyzer and Smalla, 1998; Zoetendal *et al*., 1998; Seksik *et al*., 2005].

Fluorescence In-Situ Hybridisation: Whole bacterial cells are detected via specific nucleic acids with fluorescently labelled oligonucleotide probes that hybridise to

DNA sequences inside microbial cells. The identification and enumeration of the microbial groups within a sample is based on the intensity of the fluorescence. The in-situ hybridisation technique is a much-used molecular tool in microbial ecology, since bacteria can be identified with minimal disturbance of their spatial distribution within the sample [Amann *et al*., 1995; Langendijk *et al*., 1995; Ames *et al*., 1999; Collins and Gibson, 1999; Harmsen *et al*., 2000].

Microarrays: Large number of target sequences (DNA or mRNA) within a sample are simultaneously hybridised to a high-density array of immobilised oligonucleotide probes on a solid support (**Figure 3**). This method is also used to uncover variations in the sequence or expression of a gene [Maskos and Southern, 1992; Schena *et al*., 1995; Shalon *et al*., 1996; Schena *et al*., 1996; Bates *et al*., 2002; Roy *et al*., 2002; Liu-Stratton *et al*., 2004; Larocque *et al*., 2005; Carey *et al*., 2007; Kovatcheva-Datchary *et al*., 2009; Tottey *et al*., 2013]. The Human Intestinal Tract Chip microarray (HITChip) is a phylogenetic microarray that contains a duplicate set of probes based on 16S rRNA gene sequences covering more than 1,100 intestinal bacterial phylotypes, as proposed by Rajilic-Stojanovic *et al*., 2009. This assay has been and continues to be extensively used for studying the gut microbiome [Biagi *et al*., 2010; Nikkila *et al*., 2010; Van den Abbeele *et al*., 2010; Rajilic-Stojanovic *et al*., 2012].

Sequence-based identification of bacteria relies on the postulate that two strains with matching sequences (97%) of housekeeping genes, such as the 16S rRNA gene, are likely to belong to the same species of bacteria. Sequencing of the 16S rRNA genes has resulted in more than one million small subunit rRNA entries, which are available through databases such as GeneBank [www.ncbi.nlm.nih.gov/genbank], ENA-EMBL [www.ebi.ac.uk/ena], Ribosomal Database Project (RDP) [https://rdp.cme.msu.edu/], SILVA Ribosomal RNA database [http://www.arb-silva.de/] and Greengenes [http://greengenes.lbl.gov].

2.4 Metagenomic approach

The study of the microbial ecosystem of the GI-tract changed dramatically upon the introduction of sequencing technologies. Jo Handelsman coined the term "metagenomics" after cloning and sequencing nucleic acid fragments from the entire microbial community of soil samples [Handelsman *et al*., 1998]. Metagenomics mainly defines the application of sequencing based methods to DNA molecules extracted directly from a sample, without cultivating the organisms individually (**Figure 4**). The sequence of nucleotides from different microorganisms can be used to identify a specific organism and its functions in a community sample. An example of such a sample is faeces, in which case, DNA is extracted and purified from the samples and then processed for sequencing. 16S rRNA gene based amplicon or shotgun sequencing of the entire metagenomic DNA is further executed (**Figure 5**). Shotgun sequencing was exploited in several recent high impact metagenomic studies, allowing a comprehensive description of the microbial gene content of the GI-tract in health and in disease, such as obesity [Qin *et al*., 2010; HMP Consortium, 2012; Le Chatelier *et al*., 2013].
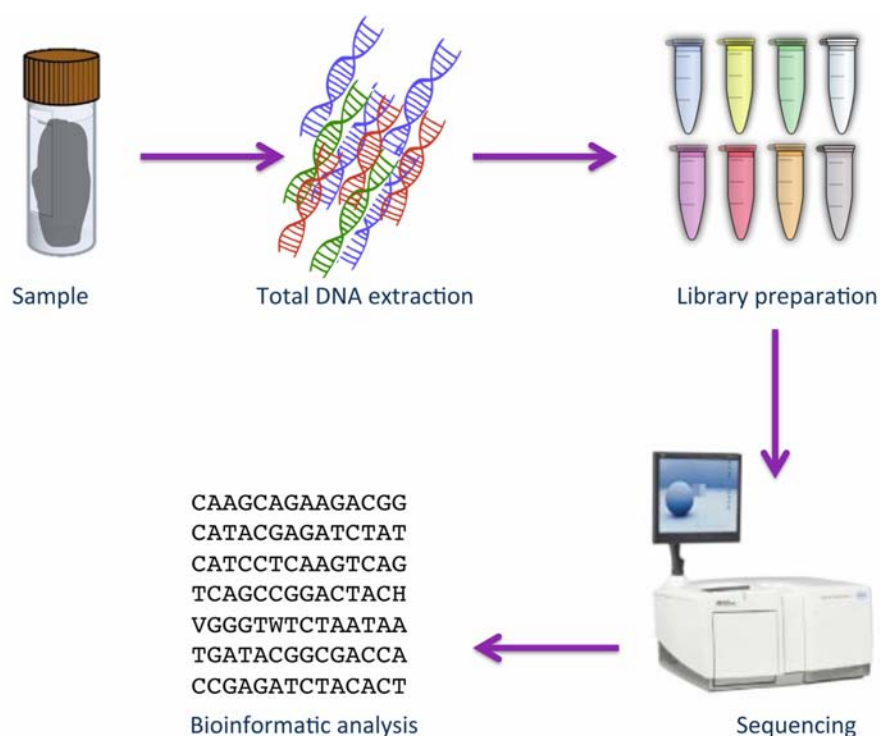
Figure 4: Metagenomic approach – overview of the metagenomic technique

2.4.1 Sanger sequencing

Frederick Sanger and colleagues developed Sanger DNA sequencing method in 1977 [Sanger *et al*., 1977]. This capillary- electrophoresis based technique relies on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase and primers during DNA replication. Sequence data are then analysed through various informatics tools based on ecological laws. To analyse microbial composition, the Sanger method involves either PCR amplification or cloning of each microbial fragment into a vector before it is sequenced. This method is laborious and expensive compared to Next-Generation Sequencing techniques (NGS).

2.4.2 Next Generation High Throughput Sequencing

Next Generation Sequencing technologies are sequencing methods that parallelise the sequencing process, producing several thousands to millions of sequence reads per sample. These high throughput methods could be applied on fragmented genomic DNA, on 16S rRNA based amplicon or even for transcriptome sequencing (**Figure 5**). Although several procedures have been developed and commercialised, two technologies have been mostly used over the last decade: pyrosequencing and Illumina, although pyrosequencing technology has now been replaced by newer technologies such as Illumina. Novel technologies also being commercialised include Ion Proton Sequencer and Oxford Nanopore technology [Eisenstein, 2012; Chen *et al*., 2014].
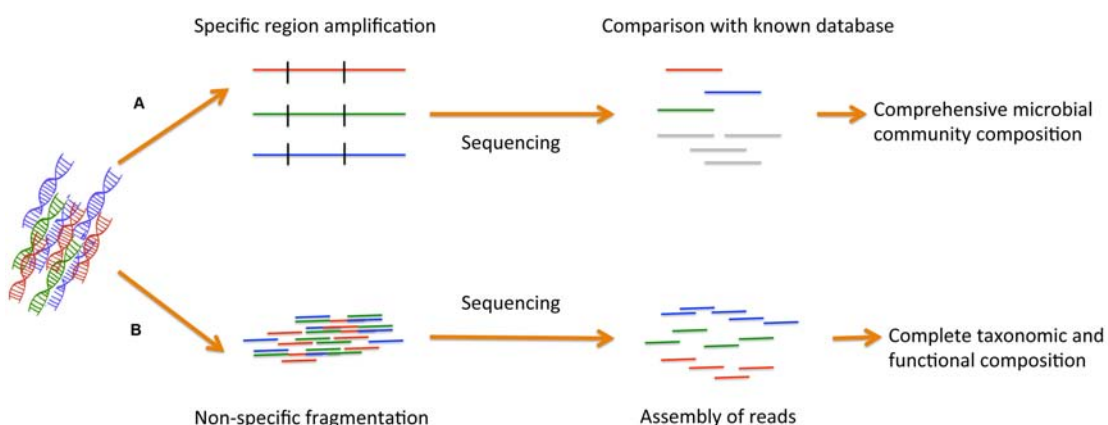
Figure 5: Metagenomic sequencing methods – A) Amplicon sequencing, B) Shotgun sequencing

NGS arrived with the technology of pyrosequencing developed by Mostafa Ronaghi and Pal Nyren in 1998 [Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998] and was later commercialised by 454 Life Sciences (Roche[©]). Pyrosequencing is a sequencing-by-synthesis method, based on the detection of the pyrophosphate release occurring at the nucleotide incorporation through emission of light during the sample amplification.
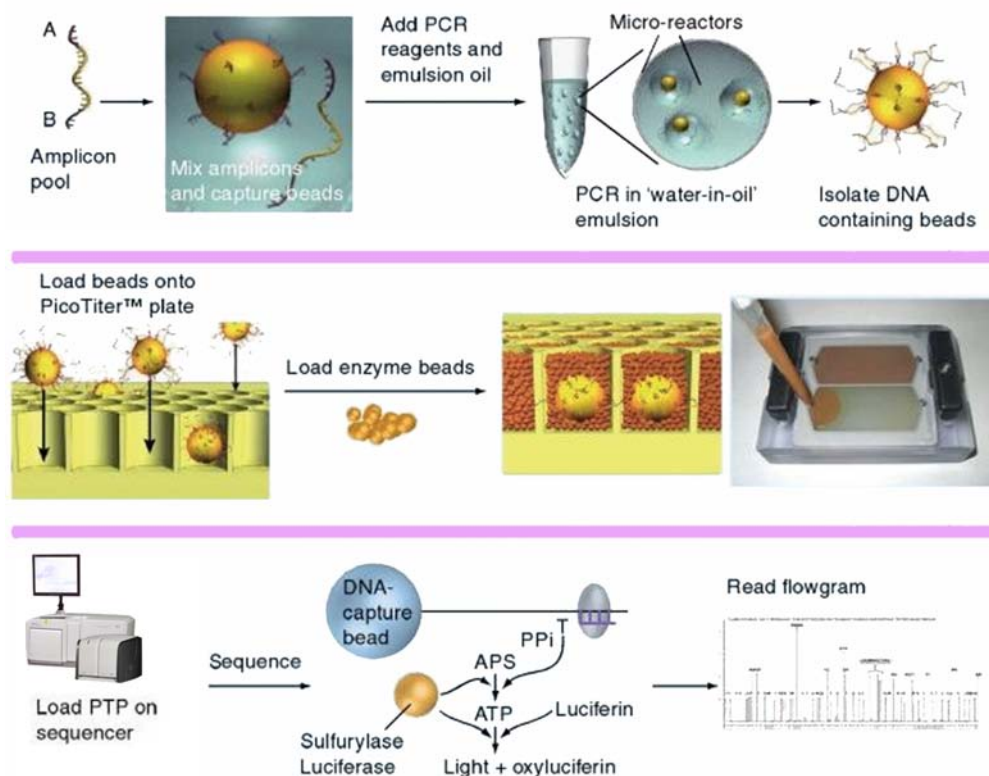
Figure 6: Pyrosequencing technology – Overview of the mechanism of amplicon sequencing using the pyrosequencing technology (Modified from Future Medicine Ltd. 2011)

"The GS FLX and GS Junior Systems support the sequencing of samples from genomic DNA and PCR products. For amplicon libraries, PCR products are created by amplifying the extracted metagenomic DNA with specific fusion primers, containing 454 Sequencing adaptor sequences. The resulting library is attached to DNA Capture Beads. Each bead carries a unique single-stranded library fragment. These beads are emulsified with amplification reagents in a

water-in-oil mixture to trap individual beads in amplification microreactors. The entire emulsion is amplified in parallel to create millions of clonally copies of each library fragment on each bead (emPCR). The emulsion is broken while the amplified fragments remain bound to their specific beads. The beads are then loaded onto the PicoTiterPlate device, where the surface design allows for only one bead per well. The PicoTiterPlate Device is then loaded in the instrument for sequencing. Individual nucleotides are flowed in sequence across the wells. Each incorporation of a nucleotide complementary to the template strand results in a chemiluminescent light signal recorded by the camera. Millions of copies of a single clonal fragment are contained on each DNA Capture Bead. 454 Sequencing Data Analysis software uses the signal intensity of each incorporation event at each well position to determine the sequence of all reads in parallel" [http://454.com/products/technology.asp] (**Figure 6**). Up to 1 million simultaneous sequence reads of 450–700bp could be obtained by using 454 FLX XL+.

Introduced in 1997 by S. Balasubramanium and D. Klenerman, the reversible dye-terminator based sequencing by synthesis technology was developed (Solexa), and was then bought by Illumina©. In 2008, the technology was used to sequence the whole human genome [Bentley *et al*., 2008]. Hundreds of millions of DNA clusters are generated on a flowcell slide in parallel, and sequencing is performed by using engineered polymerases and fluorescently labelled nucleotides. This technology currently allows 4000 million simultaneous sequence reads of 300–600bp (Illumina HiSeq 2500).

"Sequencing templates are immobilised on a proprietary flow cell surface designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface bound template and low non-specific binding of fluorescently labelled nucleotides. Solid-phase amplification creates up to 1,000 identical copies of each single template molecule in close proximity. Sequencing by synthesis technology uses four fluorescently labelled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labelled deoxyribonucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to

identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimises incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates" (**Figure 7**) [http://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf].

2.5 Other 'omics'

Although the techniques mentioned before provide an insight in the structure of the human gut microbiota that was unattainable with the use of traditional culturing methods, determining the function of all microbes continues to be a challenging task [Zoetendal *et al.*, 2008]. The detection of genes in a library constructed by the DNA fragment sequences (Shotgun metagenomic sequencing) does not necessarily mean that they are functionally active. Therefore, other meta-'omics' approaches have been developed which use RNA, proteins, and metabolites as a target to gain knowledge about the activity of the microbial community.

Metatranscriptomics is the study of the RNA pool of a community. This method links the microbiome's taxonomic structure and function, as the RNA is enriched not only in functionally but also taxonomically relevant molecules, i.e. mRNA and rRNA, respectively (**Figure 3**). This investigation of the expression of genes of a sample gives an idea of the microorganisms' *in situ* activity. cDNA is synthesised from RNA by reverse transcription and is then sequenced (RNA sequencing).
Unlike microarray or RT-qPCR data, metransriptomics is not influenced by parameters such as array composition, primer design, and hybridisation conditions, although however, bias may be introduced during the synthesis of cDNA from the RNA template [Moran, 2009]. This technique presents additional technical challenges (depletion of over 90% of ribosomal RNA), but has been used to describe faecal communities in samples [Booijink *et al.*, 2010a; Gosalbes *et al.*, 2011; Xiong *et al.*, 2012; Perez-Cobas *et al.*, 2013a].
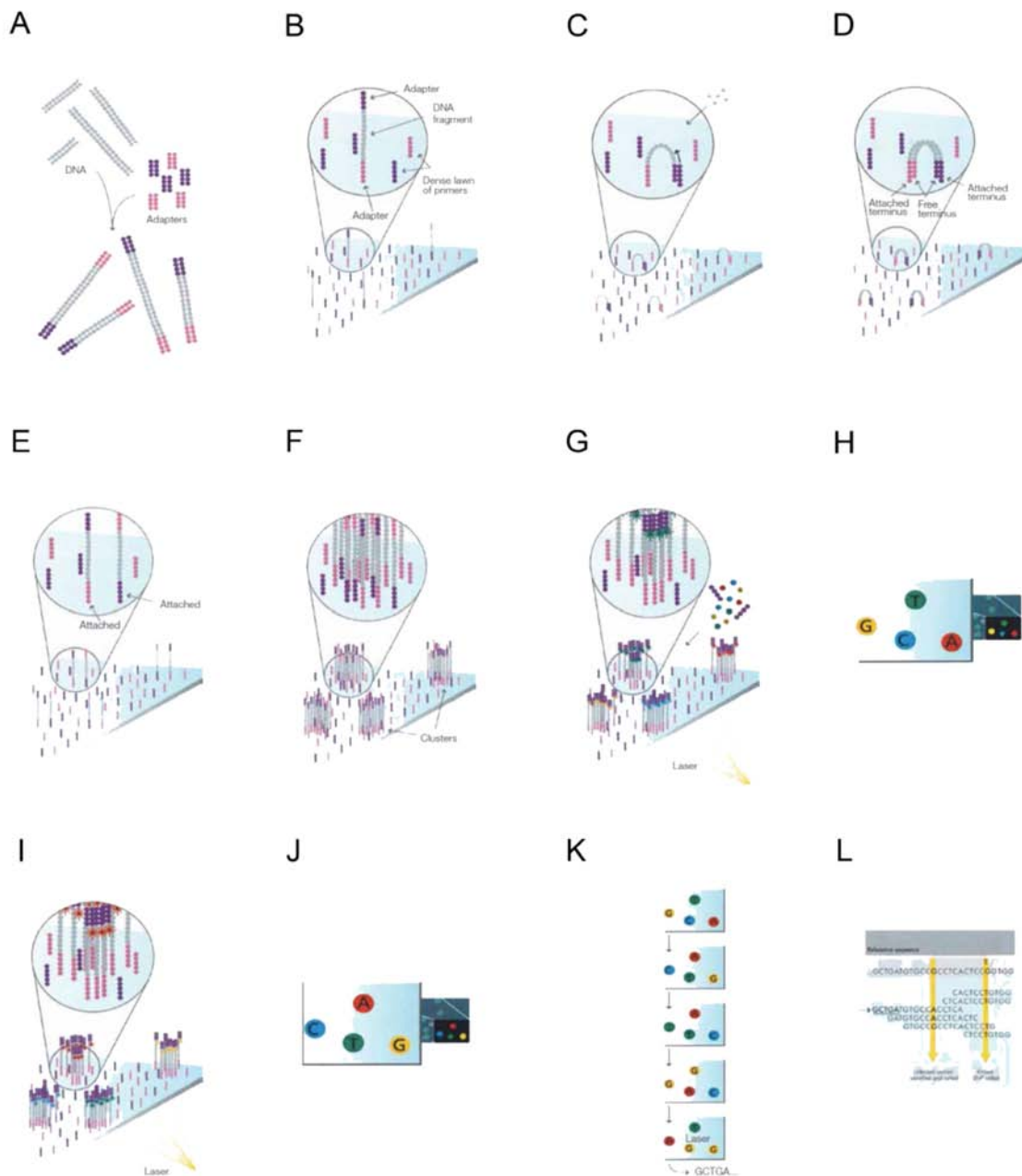
Figure 7: Illumina technology – (Adapted from www.illumina.com)
A) Randomly fragment genomic DNA and ligate adapters to both ends of the fragments
B) Bind single-stranded fragments randomly to the inside surface of the flow cell channels
C) Add unlabelled nucleotides and enzymes to initiate solid-phase bridge amplification
D) The enzyme incorporates nucleotides to build double stranded bridges on the solid-phase substrate
E) Denaturation leaves single-stranded templates anchored to the substrate
F) Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell
G) The first sequencing cycle begins by adding four labelled reversible terminators, primers, and DNA polymerase
H) After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified
I) The next cycle repeats the incorporation of four labelled reversible terminators, primers and DNA polymerase
J) After laser excitation, the image is captured as before, and the identity of the second base is recorded
K) The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time. This is repeated for several thousand clusters at the same time.
L) The data are aligned and compared to a reference, and sequencing differences are identified

The term "metaproteomics" was proposed by Francisco Rodríguez-Valera in 2004 to describe the genes and/or proteins most abundantly expressed in environmental samples (**Figure 3**). This technique, augmented by the recent improvements in protein and peptide separation efficiencies and highly accurate mass spectrometers, has shown that the proteins involved in translation, energy production, and carbohydrate metabolism are produced in part by the gut microbiome [Verberkmoes *et al*., 2009; Kolmeder and de Vos, 2014].

"Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind" [Bennett, 2005]. The global profiling of metabolites in faeces is a challenge since the samples are complex, non-homogenous and rich in macromolecules and particulate, non-digested matter. This may cause difficulties for analytical systems. Despite it, a recent study has revealed pathways differentiating patients suffering from Crohn's disease from healthy subjects, including pathways involved in the metabolism and/or synthesis of amino acids, fatty acids, bile acids, and arachidonic acid [Jansson *et al*., 2009].

3. Importance of standardisation

Metagenomic and metatranscriptomic projects involve numerous procedures starting from clinical induction of patients/ healthy volunteers, the sample collection and storage, transferring samples to the laboratory, nucleic acid extraction, processing for sequencing, sequencing, till data analysis and interpretation. Biases could originate at any of these given steps. The number and significance of metagenomic studies are continually increasing in the field of medical science, especially research on the human gut microbiome and its relevance to metabolic and other disorders. Thus it is vital that samples may be compared within and in-between studies without a bias.

3.1 Storage of faecal samples

Undamaged DNA fragments are imperative for metagenomic library construction [Bertrand *et al*., 2005; Liles *et al*., 2009] as degraded DNA reduces the potency of

shotgun sequencing [Qin *et al.*, 2010]. RNA molecules are prone to quick degradation due to enzymes present in the sample environment; thus, appropriate storage methods of the samples are a must to recover total RNA with high integrity for RNA sequencing [Zoetendal *et al.*, 2006; Gosalbes *et al.*, 2011; Wang *et al.*, 2011]. Sample storage is the first and most crucial step. It has been recommended that faecal samples may be stored at room temperature and be brought to the laboratory within 3 hours (maximum 24 hours) after collection or should be stored immediately after deposition at −20°C, transported to the laboratory in a freezer-pack where the samples must be stored at −80°C until further processing [Cardona *et al.*, 2012] (**Figure 8**).
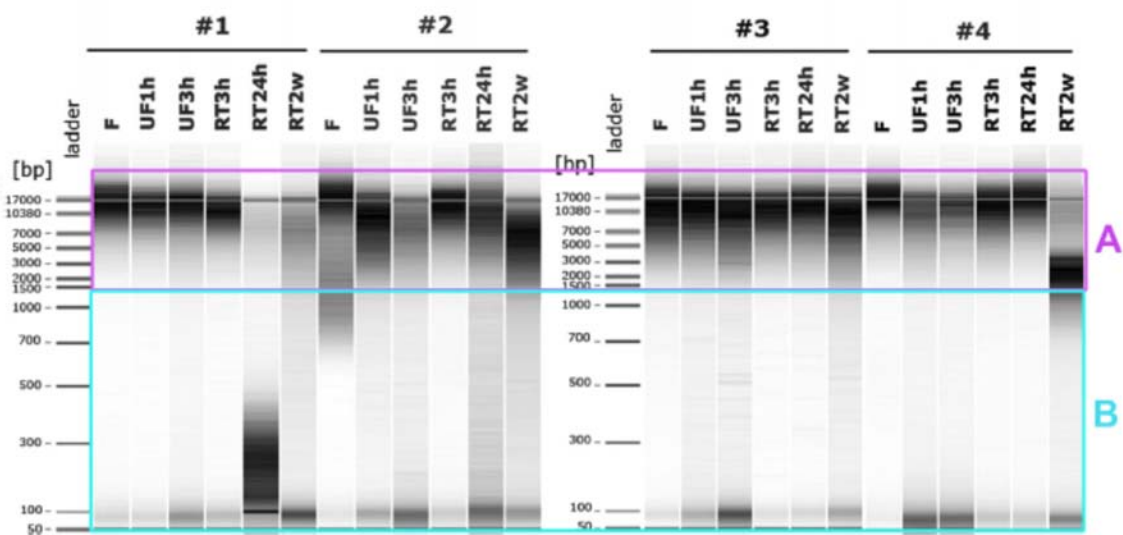
Figure 8: Optimum storage conditions for faecal matter – Micro-capillary electrophoresis patterns of genomic DNA extracted from faecal sample showed that optimum conditions for faecal matter storage was a maximum of 3 hours at Room Temperature, after which samples should be frozen until DNA extraction, without allowing sample to thaw before the actual extraction procedure – F: Frozen immediately; UF: Frozen and then Unrozen during 1 hour and 3 hours (UF1h, UF3h); RT: stored at room temperature during 3 hours, 24 hours and 2 weeks (RT3h, RT24h, RT2w). (From Cardona *et al.*, 2012)

3.2 Nucleic acid extraction

How the sample is processed for nucleic acid extraction is also a possible bias-inducing factor. The nucleic acids extracted should be representative of the

complete microbiota present in the sample, while retaining sufficient amounts of high-quality nucleic acids, which are a must for subsequent library production and sequencing. Nucleic acid extraction necessitates specific protocols for each sample type. Several methods for DNA extraction have been utilised including commercially available kits [Eckburg *et al*., 2005; Kennedy *et al*., 2014; Vervoort *et al*., 2015; Wagner Mackenzie *et al*., 2015], kits with modified protocols [Dethlefson *et al*., 2008; Abusleme *et al*., 2014; Starke *et al*., 2014; Plain *et al*., 2015] and manual extraction methods [Godon *et al*., 1997; Fujimoto *et al*., 2004; Cardona *et al*., 2013; Santiago *et al*., 2014].

In a study done by our group, it has been shown that homogenisation of the faecal matter during collection is recommended but not absolute. Furthermore, use of a mechanical lysis step (bead-beating) is essential to break the thick cell walls of Gram-positive bacteria, to prevent bias in the observed community structure [Santiago *et al*., 2014] (**Figure 9**).
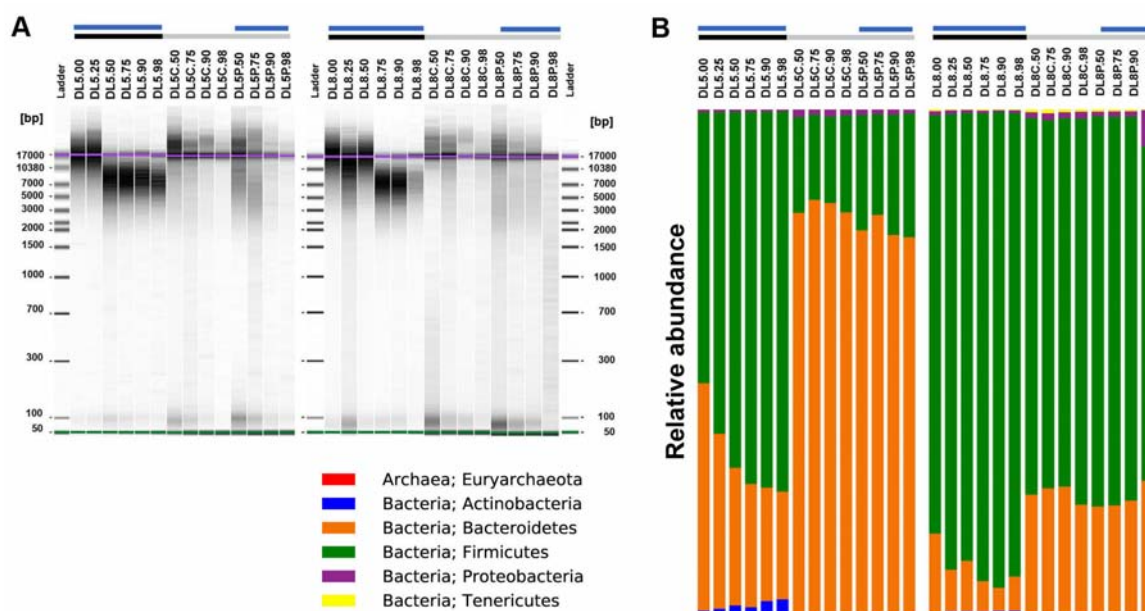
Figure 9: Effect of increased water content and bead-beating on genomic DNA integrity and on microbial community composition – A) Gel electrophoresis analysis, B) Microbial diversity profile at the phylum level showed that increased water content in faecal matter degraded genomic DNA, but did not cause differences in microbial composition, however, the absence of a mechanical bead-beating step did not allow some of the bacterial groups to be identified (From Santiago *et al*., 2014)

3.3 Library preparation by universal primers

The taxonomic information assimilated from high throughput sequencing (HTS) data needs to be accurately and rapidly assigned to each read, so that the composition of each community can be linked back to likely ecological roles played by members of each species, genus, family or phylum present in that sample. Thus, library preparation for amplicon based HTS depends crucially on the primers used for the amplification of the metagenomic DNA. The primers ought to amplify an appropriate region of DNA that is the right length for sequencing and also taxonomically informative, should not form secondary structures (primer dimers), and amplicon sequences should be abundantly available in public databases for appropriate taxonomy assignment. Based on these requirements, the V4 region of the 16S rRNA gene (**Figure 2B**) has been found to be the most appropriate segment to develop universal primers with lowest error rates [Wang *et al*., 2007; Liu *et al*., 2008; Walters *et al*., 2011]. 16S rRNA 515f/806r (and its slight modification 517f/805r) primer pair has been used extensively in many studies [Caporaso *et al*., 2010; Manichanh *et al*., 2010; Caporaso *et al*., 2011; Caporaso *et al*., 2012; Cardona *et al*., 2012; HMP Consortium, 2012; Yatsunenko *et al*., 2012; Cuenca *et al*., 2014; Manichanh *et al*., 2014; Santiago *et al*., 2014] although no one combination of primers works best in all environments [Soergel *et al*., 2012], and many large scale studies have used other primer pairs [Eckburg *et al*., 2005; Costello *et al*., 2009; Goodman *et al*., 2009; Turnbaugh *et al*., 2009; Goodman *et al*., 2011].

4. Variation of the human gut microbiome

The complexity and variability of microbial colonisation and development in a niche like the gastrointestinal tract is very high since the human hosts' environmental, biological and genetic backgrounds are significantly different between individuals, and may evolve within the same person over time.

4.1 Healthy core microbiome

Deep metagenomic sequencing has allowed exploring the possibility of an existence of a "human core gut microbiome", which is defined as those genes that are common to the gut microbiome of all or the majority of humans. Qin *et al*., 2010 identified 35% reads that were common in all the 124 samples sequenced. This has been hypothesised to be responsible for the functional stability of the gut microbiota [Turnbaugh *et al*., 2009].

Using shotgun sequencing and a 90% identity threshold, 13 species were found to be common in more than 90% of individuals and 35 species in more than 50% [Qin *et al*., 2010]. However a clear "human core gut microbiota", defined as a number of species that are common to all humans, would be difficult to define, as the diversity varies greatly based on several factors; as well as different combinations of species could fulfil the same functional roles, [Tschop *et al*., 2009; Turnbaugh *et al*., 2009]. Factors influencing the core microbiome are shown in **Figure 10**, which includes mainly host pathophysiology as well as the transient intestinal microbiota. Indeed, the genetics, health status and immunity, lifestyle and environment of the human host play a major role in the composition of the commensal bacteria and are a source of great variability in the relative abundances of the microbial composition. Furthermore, the size and nature of the
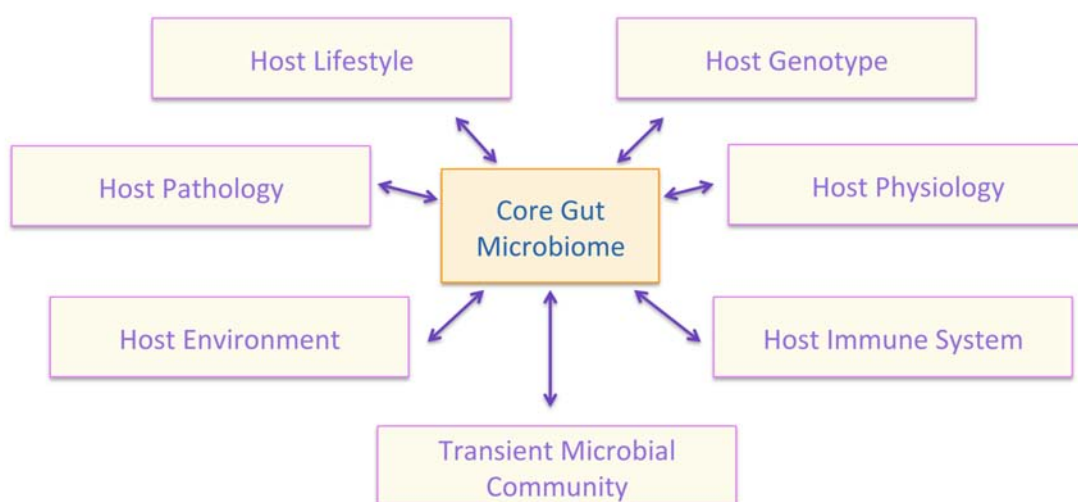
Figure 10: Host-related factors influencing the Core Gut Microbiome – Several factors are correlated to variations of the core gut microbiome; the relationships working both ways

selected cohort, parameter choices during the bioinformatic analyses also influence the outcome of the core microbiome. For example, the Human Microbiome Project (HMP) consortium estimated that the total human microbiome contained between 3,500 and 35,000 Operational Taxonomic Units (OTUs), and although several signature bacterial genera were observed across nearly all individuals, specific taxa were highly variable and almost never universal [HMP Consortium, 2012], thus resulting in the difficulty to define a clear core microbiome.

4.2 Modulating factors

Infant intestine was known to be devoid of any microbiota at birth, rapidly colonised by a wide array of microbes from a variety of sources, including maternal bacteria [Fanaro *et al*., 2003; Hallstrom *et al*., 2004; Palmer *et al*., 2007]. This knowledge, however, has been updated by a study carried out by Aagaard and colleagues, where they have observed the placental microbiome to be similar to the human oral microbiome [Aagaard *et al*., 2014].

The microbiome of the infant differs depending upon the type of delivery, the presence of vagina-dominating bacteria upon natural birth and dominance of skin-harbouring bacteria upon C-section delivery [Dominguez-Bello *et al*., 2010]. Apart from the delivery mode, feeding methods and even country of birth influence the faecal microbiome of infants [Fanaro *et al*., 2003; Fallani *et al*., 2010; Fan *et al*., 2013]. *Lactobacillus* and *Bifidobacteria sp*. prevail in breast milk–fed infants, whereas the Bacteroidetes dominate the gut upon intake of formula-food. By the age of 3, the gut microbial diversity is comparable to that of an adult [Koenig *et al*., 2011], the microbiome of children becoming more complex with age [Hopkins *et al*., 2002]. Throughout a healthy adulthood, microbial density and diversity in the gut remains moderately stable over time, indicating a high capacity to maintain homeostasis [Vanhoutte *et al*., 2004; Leser and Molback, 2009].

The adult microbiota shows an astonishing individual variability [Zoetendal *et al*., 1998; Suau *et al*., 1999; Eckburg *et al*., 2005; Ley *et al*., 2008a] from person to person, community to community, varying by factors such as age, diet and

lifestyle, and geographic origins (**Figure 11**). From the age of 3 when the microbiota mirrors an adult-like structure, the composition remains fairly stable. Advancing age associates with increased Bacteroidetes and decreased Firmicutes, Proteobacteria and Bifidobacteria [Hopkins *et al*., 2002; Costello *et al*., 2009; Koenig *et al*., 2011; Claesson *et al*., 2012]. Differences in the structure of food, such as starch content, lactic foods, meat proteins, high fat foods, are influencing factors in the variability of commensal microbiota. Indeed, the prevalence of consumption of processed, high calorie foods and diverse palates show a substantial difference in the composition of gut bacteria when compared to more rural diets that are rich in fibre and complex carbohydrates. Furthermore, lifestyle habits, including smoking, physical activity, are also attributed to variability of the gut microbiota [Mai, 2004; Ley *et al*., 2008a; Ley *et al*., 2008b; Wu *et al*., 2011; Schwartz *et al*., 2012; Yatsunenko *et al*., 2012; Biedermann *et al*., 2013; Albenberg and Wu, 2014]. As with the influence of diet and lifestyle, the corresponding geographic location, environment and economic status of the region also are determining features that may influence the intestinal ecosystem [De Filippo *et al*., 2010; Fallani *et al*., 2010; Yatsunenko *et al*., 2012].

Studies have also suggested that host genetics might not be the only cause of obesity, as the microbiota has an active role to play [Backhed *et al*., 2005; Ley *et al*., 2005; Turnbaugh *et al*., 2008; Turnbaugh and Godon, 2009]. Host – microbiota interactions are dependent on the host genotype, on which the host phenotype and the resident microbiota are dependent, but the exact degree of this dependence remains to be interpreted. The effect of host genetics on the gut microbiota is most profoundly observed in studies conducted on related individuals; microbial profile similarity was the lowest among unrelated persons, slightly higher among marital partners, significantly higher in dizygotic twins, and clearly the highest in monozygotic twins. This is a clear evidence of the effect of host genotype, since the twins did not live together anymore and yet, marital partners live in the same environment and generally have similar dietary habits [Erwin *et al*., 2001; Zoetendal *et al*, 2001; Ley *et al*., 2005; Stewart *et al*., 2005; Mueller *et al*., 2006; Khachatryan *et al*., 2008; Turnbaugh *et al*., 2009; Goodrich *et al*., 2014]. Although, there is greater sharing of microbiota among family members

than with non-members [De Filippo *et al*., 2010; Yatsunenko *et al*., 2012], each family member's gut microbial community varies; the percentage of each phylum differing from individual to individual [Ley *et al*., 2005; Turnbaugh and Godon, 2009; HMP Consortium 2012]. Furthermore, the microbiota of elderly people displays greater inter-individual variation than that of younger adults [Claesson *et al*., 2012].
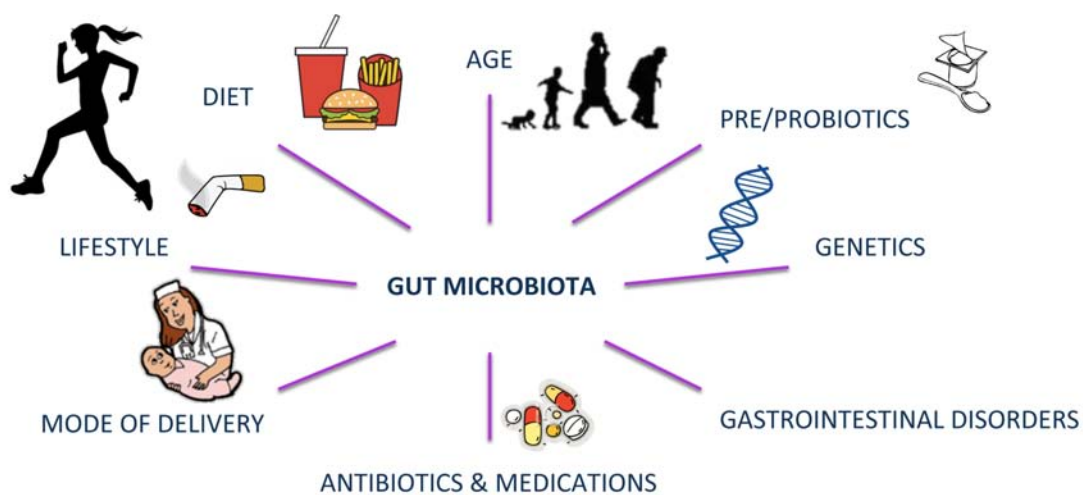
Figure 11: Modulators of the gut microbial community – From the time of birth, diet, lifestyle, medications, diseases till aging, and several factors affect the microbial community structure

5. Dysbiosis

Greater richness and diversity of bacterial species in the human intestine may be an indicator of health. Major alterations in the gut microbiota structure are called "dysbiosis", the functional implications of which are still poorly understood, but may affect the human physiology, health status and disease susceptibility.

A 'hygiene hypothesis' or 'microbiota hypothesis' has been suspected, suggesting that an increase in "western lifestyle" leads to a reduction of health-promoting symbionts in childhood, as a result of improved health measures. This has contributed to an immunological imbalance in the gut that could lead to dysbiosis [Round and Mazmanian, 2009; Singhal *et al*., 2011]. This imbalance may lead to a

restricted microbial heterogeneity and an augmented inflammatory response aggravated by an immature innate immune response that increases the risk of diseases.

5.1 Antibiotics and medications

The discovery of antibiotics has greatly affected the world we live in, with significant health benefits. Since the 1940's, together with penicillin, antimicrobials ushered in a new era of modern medicine saving millions of lives, and increasing the average life expectancy [Hayes *et al*., 1993]. Disruption of the microbial composition and structure has been linked to significant side effects such as antibiotic-associated diarrhoea, mal-absorption characterised by a celiac-like syndrome, impaired absorption of medications, altered metabolism and absorption of vitamins, overgrowth of or colonization by resistant organisms, and altered susceptibility to infections and increased susceptibility to subsequent disease [Levy, 2000; Sullivan *et al*., 2001; Sjolund *et al*., 2003; Keeny *et al*., 2014].

For several decades, research was focused on the effect of antibiotics on the growth of certain pathogenic species. With the emergence of drug resistance worldwide, efforts are now being made to study the reaction of antimicrobial intake on the overall gut microbial community and antibiotic resistance genes. Different types of antibiotics affect microbes in different ways based on their mode of action. However, what is still not known is the impact of commonly used antibiotic therapy on the community-wide properties of the human intestinal microbiota, since most studies have been based on culture techniques. Only recently, some studies have used HTS methods to determine the effects of antibiotics on the microbiota, many of which have found diminished levels of bacterial diversity with some degree of recovery. Few works demonstrated that resilience is not complete months after the cessation of antibiotic treatment, and the resulting microbial population may differ compared to before treatment [Jernberg *et al*., 2007; Dethlefson *et al*., 2008; Antonopoulos *et al*., 2009; Jakobsson *et al*., 2010; Jernberg *et al*., 2010; Dethlefson and Relman, 2011; Fouhy *et al*., 2012; Perez-Cobas *et al*., 2013b]. Clindamycin, a broad-spectrum antibiotic against anaerobes showed to have really long lasting effects [Jernberg *et al*., 2005; Jernberg *et al*., 2007; Jernberg *et al*.,

2010], as did a combination of clarithromycin, metronidazole, and omeprazole [Jakobsson *et al*., 2010] where the antibiotic effects were persistent up to 4 years. Ciprofloxacin decreased the microbial richness within the first few days of exposure [Dethlefson *et al*., 2008; Dethlefson *et al*., 2011], but recovery was observed later on. After a second antibiotic treatment; weeks later, the recovery was not as complete as the first time, demonstrating how the bacteria "get used to" the antibiotics, enabling the possibility of future resistance to the antibiotic.

As early as in 1956, the loss of colonisation resistance had been recorded [Miller *et al*., 1956]. Bacteria generally not prevalent in the gut (pathogens) were found to have colonised after a course of antibiotics [Bohnoff and Miller, 1962]. Competition for resources, direct interference by commensal bacteria, innate immune system, and effector molecules make sure that pathobiont levels are kept in check. But upon antibiotic intake, this homeostasis may get disrupted, allowing non-commensal or pathobiont levels to augment and may later cause multiple adverse effects [Buffie and Pamer, 2013].

Apart from reduction of bacterial diversity, antibiotics often set off other unwanted effects. One of the most common side effects observed immediately after administration of antibiotics was antibiotic-associated diarrhoea (AAD), which causes patients to have frequent watery bowel movements. Recent investigations show that effect of antibiotic intake may affect the host metabolic state, leading to obesity, diabetes mellitus and asthma (Cho *et al*., 2012; Keeney *et al*., 2014). Another major adverse effect of antibiotics such as fluoroquinolones, cephalosporins, clindamycin and penicillins is the emergence of *Clostridium difficile* infections (CDI). These antibiotics cause reduction of commensal microbiota, leading to overgrowth of the potential pathogenic bacterium *C. difficile*, which produces toxins that destroy intestinal cells and cause inflammation that often lead to watery diarrhoea [Kachrimanidou *et al*., 2011].

5.2 Intestinal disorders

Major alterations in the intestinal microbial structure, especially at the phylum level, have been linked to various diseases, including the major intestinal

disorders: Inflammatory Bowel Diseases (IBD) and Functional Gastro-intestinal Disorders (FGID).

Crohn's Disease (CD) and Ulcerative Colitis (UC) are two common forms of IBD. These are lifelong, idiopathic, inflammatory, destructive conditions of the GIT, and the patients undergo several cycles of relapse and remission throughout the course of the disease. CD is characterised by patchy, transmural inflammation, which may affect any part of the GI tract, right from the mouth to the anus; whereas UC is defined by a continuous mucosal inflammation limited only to the colon and rectal region [Ghosh *et al*., 2000; Farrel and Peppercorn, 2002; Podolsky, 2002; Shanahan, 2002b].

First described by Samuel Wilks in 1859 and Burrel Crohn in 1932, pathogens involved in the aetiology of IBD have been a subject of great interest and research. Among the microbes of interest that have been postulated to play a role in the pathogenesis of IBD are: *Mycobacterium avium* subspecies *paratuberculosis*, *Listeria*, *Pseudomonas* species, and *Helicobacter pylori* [Eckburg and Relman, 2007]. *Escherichia coli* has been reported to be 3-4 folds higher in the mucosal microbiota of IBD patients [Kotlowski *et al*., 2007]. However, exact pathogens are not yet identified with current molecular and culture methods.

Several studies using culture independent techniques have verified the involvement of intestinal microbiota in the pathogenesis of IBD [Manichanh *et al*., 2006; Frank *et al*., 2007; Sartor, 2008a; Sartor, 2008b; Cucchiara *et al*., 2009; Sokol *et al*., 2009; Joossens *et al*., 2011; Manichanh *et al*., 2012; Dey *et al*., 2013; Thorkildsen *et al*., 2013]. Still unresolved is whether tissue damage results from an abnormal immune response to a normal microbiota or from a normal immune response against an abnormal microbiota [Shanahan and Quigley, 2014]. Lowered microbial diversity, instability and a marked reduction of Firmicutes bacteria, especially *Faecalibacterium prausnitzii* have been associated with IBD [Frank *et al*., 2007; Martinez *et al*., 2008; Sokol *et al*., 2008a; Sokol *et al*., 2008b; Swidsinski *et al*., 2008; Sokol *et al*., 2009; Joossens *et al*., 2011; Rajilic-Stojanovic *et al*., 2013; Varela *et al*., 2013].

The most prevalent Functional Gastro-Intestinal Disorder (FGID) is Irritable Bowel Syndrome (IBS), affecting 10-20% worldwide [Longstreth, 2006]. IBS is a cause of notable discomfort for many and may be potentially disabling for some. Most common symptoms being abdominal pain, irregular bowel movements; and not infrequent symptoms include fatigue, anxiety and depression. So far, there is no validated and universally applicable biomarker, making the investigation to the pathophysiology of the disorder difficult. Furthermore, the symptoms are non-specific, non- homogenous and vary in the progression of the intensity. It is commonly subtyped as per the bowel habit – IBS-C (constipation), IBS-D (diarrhoea) and IBS-M (mixed type, alternating between constipation and diarrhoea) as per the Rome III criteria [Longstreth, 2006]. The diagnosis of this disorder in suffering persons is solely based on the experience of the clinician involved. There are no specific drugs for the treatment, and due to the symptoms being so varied; designing of any therapeutic measures continues to be a challenge [Shanahan and Quigley, 2014], for this reason alternative medicines, probiotics and prebiotics have been consumed by the patients. Prebiotics and probiotics promote the growth of beneficial bacteria, in part improving the symptoms, providing relief to the patients.

IBS is considered to be a multifactorial disorder. Host related factors such as abnormal stress response, psychiatric co-morbidity, cognitive dysfunctions and intestinal functions such as dysmotility, altered brain-gut function, visceral hypersensitivity, aberrant cerebral representation of visceral events, low-grade immune activation, altered barrier function are involved in the aetiology of the disorder. Previous history of frequent antibiotic intake and medications such as anti-inflammatory drugs and pain relievers have shown to make symptoms worse in persons having sensitive intestines as a result of IBS [Whitehead *et al*., 1980; Camilleri *et al*., 2012; Rajilic-Stojanovic *et al*., 2015]. Indeed, previous surveys have shown that prior long-term antibiotic therapy (particularly broad spectrum antibiotics) in persons correlated strongly to IBS symptoms [Mendall and Kumar, 1998; Maxwell *et al*., 2002; Villarreal *et al*., 2012].

Patients with altered intestinal anatomies, impaired motility or gastrointestinal symptoms have shown to have bacterial overgrowth in their small intestine (SIBO)

[Posserud, *et al.*, 2007], and the removal of this overgrowth alleviates the symptoms of IBS [Pimentel *et al.*, 2000; Pimentel *et al.*, 2003]. Few investigations have linked IBS to the variations of microbial populations or to the mucosal immune response [Bradely *et al.*, 1987; Si *et al.*, 2004; Malinen *et al.*, 2005; Matto *et al.*, 2005; Carroll *et al.*, 2011; Jeffery *et al.*, 2012; Quigley, 2013; Shukla *et al.*, 2015]. Although, no conclusive pattern of microbial dysbiosis has been determined, several aspects have been witnessed – in patients, Proteobacteria in the phylum level; Clostridial cluster XIVa and Ruminococcaceae in the family level; *Veillonella*, *Lactobacillus* and *Ruminococcus* at the genus level have been observed to be higher when compared to healthy subjects [Malinen *et al.*, 2005; Lyra *et al.*, 2009; Tana *et al.*, 2010; Carroll *et al.*, 2011; Rajilic-Stojanovic *et al.*, 2011; Saulnier *et al.*, 2011; Carroll *et al.*, 2012]. Adversely, lower diversity, higher instability and lower counts of *Bifidobacterium*, *Faecalibacterium* and methanogens have been observed in patients suffering from IBS [Matto *et al.*, 2005; Maukonen *et al.*, 2006; Krogius-Kurikka *et al.*, 2009; Salonen *et al.*, 2010; Rajilic-Stojanovic *et al.*, 2011; Jeffery *et al.*, 2012; Manichanh *et al.*, 2014]. According to a recent review, the hypothesis is that "an abnormal microbial composition activates mucosal innate immune responses, which increase epithelial permeability, activate nociceptive sensory pathways, and dysregulate the enteric nervous system" [Rajilic-Stojanovic *et al.*, 2015] (**Figure 12**).

Large-scale studies need to be conducted containing exhaustive metadata of patients to bring about a conclusive decision about the importance of microbiota with respect to IBS.
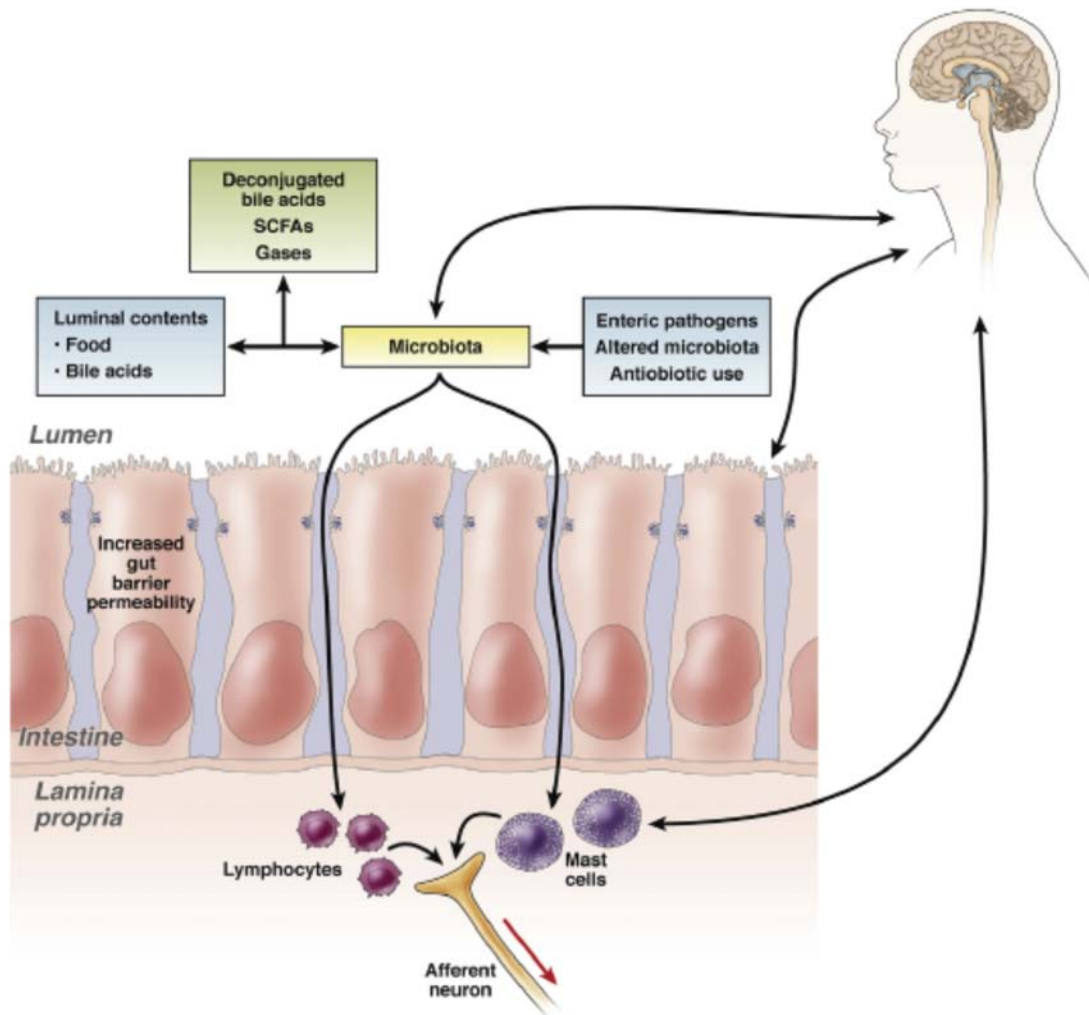
Figure 12: The microbiota in the pathogenesis of IBS – Interactions between microbes, food and bile acids could lead to production of de-conjugated bile acids, short-chain fatty acids and gases that could induce IBS symptoms. The microbiota could affect the epithelial barrier, allowing bacteria or their products to enter the sub-mucosa, leading to cytokine release which would ultimately activate sensory afferents initiating symptoms of IBS (From Shanahan and Quigley, 2014)

6. Manipulation of the gut microbiota for better health

The increased understanding of the impact of the gut microbiota, and the effects of its dysbiotic status on human health has resulted in endeavours to manipulate its composition.

6.1 Probiotics and prebiotics

Microorganisms have long been known to be causative agents of diseases as shown by the work of Pasteur and Koch. However, with the advent of probiotics as both a prophylactic and therapeutic agent, the beneficial effects of bacteria have been recognised.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf; Guarner and Malagelada, 2003]. This concept dates back over 100 years, when Metchnikoff suggested that the use of living bacteria in fermented milk products could improve health by detoxifying putrefactive substances [Metchnikoff, 1907]. Probiotics are generally marketed as functional foods or dietary supplements and consist of viable microorganisms, predominantly *Bifidobacterium* and *Lactobacillus spp.* [Steer *et al.*, 2000], although mixtures of various strains from these genera are also being used [Kadooka *et al.*, 2010; Chapman *et al.*, 2011; Park *et al.*, 2013]. Their main beneficial effects are to function as a fence to potential pathogenic organisms [Deshpande *et al.*, 2007], to improve intestinal permeability, and to stimulate the immune processes in the host [Floch and Montrose, 2005; Chermesh and Eliakim, 2006]. Probiotics such as *Lactobacillus* (*L. acidophilus*, *L. plantarum*, *L. bulgaricus*), *Bifidobacterium* (*B. lactis*, *B. longum*, *B. breve*, *B. bifidum*) and *Streptococcus thermophillus* have shown to alleviate symptoms of gastrointestinal disorders including IBD [Bibiloni *et al.*, 2005; Toumi *et al.*, 2014; Elian *et al.*, 2015] and IBS [Sinn *et al.*, 2008; Zeng *et al.*, 2008; Williams *et al.*, 2009].

A recent conjecture is, that probiotics need not be live microorganisms; instead, their cellular components, such as cell wall peptidoglycans, could be therapeutic by themselves, inducing similar benefits as those of live bacteria. [Collins, 2014]

Prebiotics, non-digestible short chain length carbohydrates stimulate the growth of *Bifidobacterium* and *Lactobacillus spp.* Defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the GI microflora that confer benefits upon host well-being and health" [Gibson *et*

al., 2004], the widely used prebiotics are oligosaccharides, including lactulose, galacto-oligosaccharides, fructo-oligosaccharides, malto-oligosaccharides, xylo-oligosaccharides and soya bean oligosaccharides. Prebiotic fermentation leads to increased short-chain fatty acid (SCFA) production that decreases the pH, increases mineral solubility and enlarges the enterocyte absorption surface, thus helping in micronutrient absorption. Several studies on both animal models and human subjects have been carried out, that show positive effects of prebiotic oligosaccharide consumption [Scholz-Ahrens *et al*., 2007; Whisner *et al*., 2013; Halmos *et al*., 2014; Sheridan *et al*., 2014]. When used in unison with probiotics, they are called synbiotics and have shown to have beneficial effects [Bartosch *et al*., 2005; Ouwehand *et al*., 2009].

6.2 Faecal Microbiota Transplantation

First reported in 4th-century China, transplantation of stool was performed for patients who had food poisoning or severe diarrhoea [Zhang *et al*., 2012], but was first scientifically published by Eiseman and colleagues in 1958 [Eiseman *et al*., 1958]. Faecal microbiota transplantation (FMT) is the process of introducing intestinal microbiota in the form of a faecal suspension from a healthy donor to a patient so as to reshape an altered gut microbiota. This method has been successfully used to treat recurrent cases of *Clostridium difficile* infection (CDI) when standard therapy fails [Brandt *et al*., 2012; Matilla *et al*., 2012; van Nood *et al*., 2013; Pierog *et al*., 2014; Walia *et al*., 2014a], and is also gaining popularity for the treatment of other types of disorders [Borody *et al*., 1989; Andrews *et al*., 1995; Anderson *et al*., 2012; Vrieze *et al*., 2012; Pinn *et al*., 2013; Peng *et al*., 2014; Walia *et al*., 2014b; Suskind *et al*., 2015]. FMT refurbishes the gut with normal "non pathogenic" microbes, but the specific action performed by the donor bacteria is not clear [Seekatz *et al*., 2014].

Adverse effects such as acute norovirus infection, bacteraemia and IBD relapse, have been reported following FMT [Aas *et al*., 2003; De Leon *et al*., 2013; Schwartz *et al*., 2013; Quera *et al*., 2014]. Therefore, there is a need to monitor the donor faeces, and to standardise the FMT procedure. Individuals with any history or signs of pathogen infections, parasites, metabolic syndrome,

autoimmunity, atopic disease, and neurologic and psychiatric disorders should be excluded [Khoruts and Weingarden, 2014].

Long-term effects of exposing the recipient to donor faecal microbiota need to be examined thoroughly [Grehan *et al*., 2010; Weingarden *et al*., 2015], and a complete understanding of how FMT promotes microbiota recovery is necessary for the advancement of bio-therapeutics.

6.3 Need of specific microbial cocktail

While FMT has shown promising results in *Clostridium difficile* infections, the inevitability of contracting unwanted microbes, including virus and fungal spores and the lack of data of long term effects, forces us to look for methods that could be similar but more favourable [Petrof *et al*., 2013]. A combination of the basic principles of FMT and probiotics; a "probiotic" cocktail of bacteria sourced from the faeces, containing microorganisms that are diminished in patients could be a new approach to re-colonise the gut. The cocktail would consist of microbial strains absent or found in extremely low relative abundance in patients, but found abundantly in healthy persons. Since most faecal bacteria are anaerobic, the bacteria used for preparing the cocktail would be anaerobically cultivated. The advantages to this approach are that the exact strains being transplanted to the patient would be known and monitored, and it would allow the possibility to use previously uncharacterised bacteria.

The other prerequisites to be kept in mind would be the effectiveness to pass through the upper GIT, unaffected by the acids and enzymes present in the stomach and small intestine, allowing the bacteria to colonise in the large intestine

7. Functions carried out by the gut microbiome

Apart from being a central force in the human pathophysiology due to the variations and modulations in its structural capacity, the gut microbiome is actively involved in carrying out additional functions that benefit the human host (**Figure 13**). The intestinal microbiota carry out metabolic and protective functions and are also actively involved in the host immune homeostasis.

7.1 Microbiome metabolic functions

Studies of the metagenomic data of the gut microbiota, has shown that several metabolic and functional pathways are ubiquitous among individuals, which mostly include ribosome and translational machinery, nucleotide charging, and ATP synthesis. Genes coding for carbohydrate metabolism, including glycolysis, have also been recorded in high abundance. These pathways, belonging to the core microbiome, are indicative of the microbial machinery required to thrive in the host [Booijink *et al.*, 2010a; Qin *et al.*, 2010; Gosalbes *et al.*, 2011; HMP Consortium, 2012].
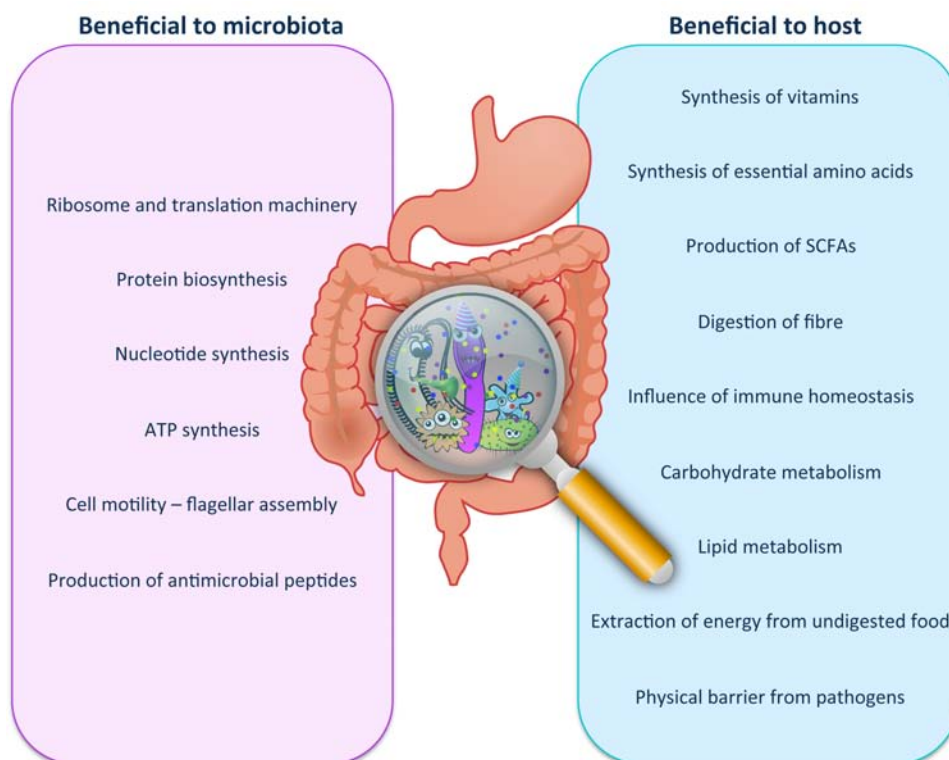
Figure 13: Functions of the human gut microbiome – Beneficial to the microbiota itself, as well as to the human host

Moreover, Bacteroidetes coded for carbohydrate metabolism pathways, while Firmicutes tend to have more genes for transport systems [Turnbaugh *et al.*, 2009]. Genes that encode cell motility, including for flagellar proteins, were found in lower abundance, which is expected since not all bacteria have flagella

[Turnbaugh *et al*., 2009; Qin *et al*., 2010]. During active colitis, there is an enrichment of genes for cell motility, signal transduction, xenobiotics biodegradation (including benzoate degradation) and metabolism, and lipid metabolism, while having reduced capacity for energy harvest, microbial signalling and cellular processing pathways [Steiner, 2007; Rooks *et al*., 2014]. During probiotic consumption, genes involved in flagellar motility, chemotaxis, and adhesion from *Bifidobacterium* and butyrate producing *Roseburia* and *Eubacterium* were increased [Eloe-Fadrosh *et al*., 2015].

Carbohydrate metabolism, phospho-transferase system, pyruvate metabolism, and energy metabolism are highly expressed during antibiotic intake [Perez-Cobas *et al*., 2013a]. Genes specific to resistance of antibiotics are also present in the microbiome as evidenced in few studies. In fact Clemente *et al*., 2015 found resistance genes to resistance to semisynthetic and synthetic antibiotics in a totally isolated Amerindian population of Yanomami. This is interesting as it indicates that antibiotic resistance could be transferred to the gut microbiota through soil microbes, presumably through food. In infants, microbial resistance to clinically important broad-spectrum β-lactam antibiotics were found to be transferred from their mothers [Moore *et al*., 2015]

7.2 Host metabolic functions

Throughout evolution, humans have developed mutually beneficial relationships with the gut microbiota. This microbial community is integral to host digestion and nutrition, and can generate energy and nutrients from substrates that are otherwise indigestible by the host.

The intestinal microbiota are involved in extraction of energy from indigestible dietary polysaccharides, and produce vitamins such as vitamin B complex and vitamin K as by-products of their metabolic cycles. Production of amino acids, certain gases, and Short Chain Fatty Acids (SCFA) is performed within the intestinal lumen through the fermentation of undigested dietary carbohydrates and fibres. SCFAs, especially butyrate, are important energy source for the intestinal mucosa and are critical for modulating immune responses in the gut [Wong *et al*.,

2006; Turnbaugh *et al*., 2009; Qin *et al*., 2010; Gosalbes *et al*., 2011; Belcheva *et al*., 2014; Clemente *et al*., 2015].

The microbiota participates in bile acid metabolism, and in turn, bile acids contribute to the suppression of unwanted bacterial colonization of the small intestine [Begley *et al*., 2005; Ridlon *et al*., 2006; Ridlon *et al*., 2015]. The enzyme pathway of transformation of primary bile acids to secondary bile acids influences cholesterol and glucose homeostatic pathways [Lefebvre *et al*., 2009]. Indeed, microbiota indirectly and directly influence cholesterol levels of the host [Wong *et al*., 2006; Hijova and Chmelarova, 2007].

Vegetables such as lettuce and onions contain xyloglucans, which are digested only by certain species of *Bacteroides*, which are found in about 92% of the population [Larsbrink *et al*., 2014]. Such findings could explain why some people have adverse digestive capabilities.

7.3 Protective functions

The human immune system plays a vital role in keeping the body healthy by providing equilibrium between the obstruction of invading pathogens and the sustenance of robust host. Interactions between the microbiota and the host immune system are numerous, complex, and bidirectional.

The microbial community prevents Infection by pathogen colonization through the production of antimicrobial peptides, as well as by the direct competition for metabolic niches [Prakash *et al*., 2011]. By increasing the epithelial barrier function through the modulation of the mucus layer, the microbiota provides a barrier to incoming inflammatory molecules [Kleessen and Blaut, 2005; Petersson *et al*., 2011].

Host innate and adaptive immune system development and regulation is also attributed to the gut microbes, who play a role in B-cell maturation, and in the evolvement of regulatory T-cells, T-helper type 1, 2 and 17 cells [Ivanov *et al*., 2009; Hapfelmeier *et al*., 2010; Atarashi *et al*., 2011]. Bacterial metabolites harmonise interaction between the commensal microbiota and the immune

system, affecting the balance between pro- and anti-inflammatory mechanisms, directly influencing immune homeostasis inside and outside the gut [Arpaia *et al*., 2013].

7.4 Functions influencing the host psychology

Over the years, the concept of 'microbiota–gut–brain axis' has unfolded. The microbiota of the gastro intestinal tract seems to play a role in brain chemistry and development, stress responses, behaviour and resulting gut functions [Carabotti *et al*., 2015]. Intestinal microbiota and the brain communicate through immune, endocrinal, neuronal and humoral pathways and modulate mood and behaviour – the brain influencing GI functions, while the gut interferes in brain signalling [Rhee *et al*., 2009; Cryan *et al*., 2011; Forsythe and Kunze, 2013]. Moreover, gut microbial colonization affects mammalian brain development through certain neuronal circuits involved in motor control and anxiety behaviour [Diaz Heijtz *et al*., 2011]. Much of the evidence of this interaction comes from studies of gut microbial dysbiosis in central nervous disorders including autism, anxiety, but also from IBS [Koloski *et al*., 2012; Mayer *et al*., 2014; Naseribafrouei *et al*., 2014].

HYPOTHESIS & OBJECTIVES

The human gut microbiome is now established as a key role player in the status of health, although there are several factors that influence the structure of this ecosystem. This doctoral thesis aimed to carry out studies pertaining to these factors in order to gain more knowledge as to how this "forgotten organ" affects our lives.

The hypothesis of this dissertation was that the composition, abundance and diversity of the gut microbial community undergo major changes when the human host is associated with functional disorders and undergoes antibiotic therapy.

The specific objectives were:

1. To study the immediate changes in the intestinal microbiome of persons who undergo antibiotic treatment for prescribed dosage.

2. To compare the gut microbial composition between healthy persons, and those suffering from Irritable Bowel Syndrome (IBS) to scan for certain groups of bacteria that may, in the future, serve as biomarkers for diagnostic methods.

The research work presented here resulted in the production of two research articles:

1   Panda, S *et al.* Short-term effect of antibiotics on human gut microbiota. *PLoS One* 9, e95476, doi:10.1371/journal.pone.0095476 (2014).

2   Pozuelo, M*, Panda, S* *et al*. Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. *Sci. Rep.* 5, 12693, doi:10.1038/srep12693 (2015).

Suchita Panda

METHODS

An overview of the methodology employed for the studies done in this doctoral thesis are shown below in **Figure 14**.
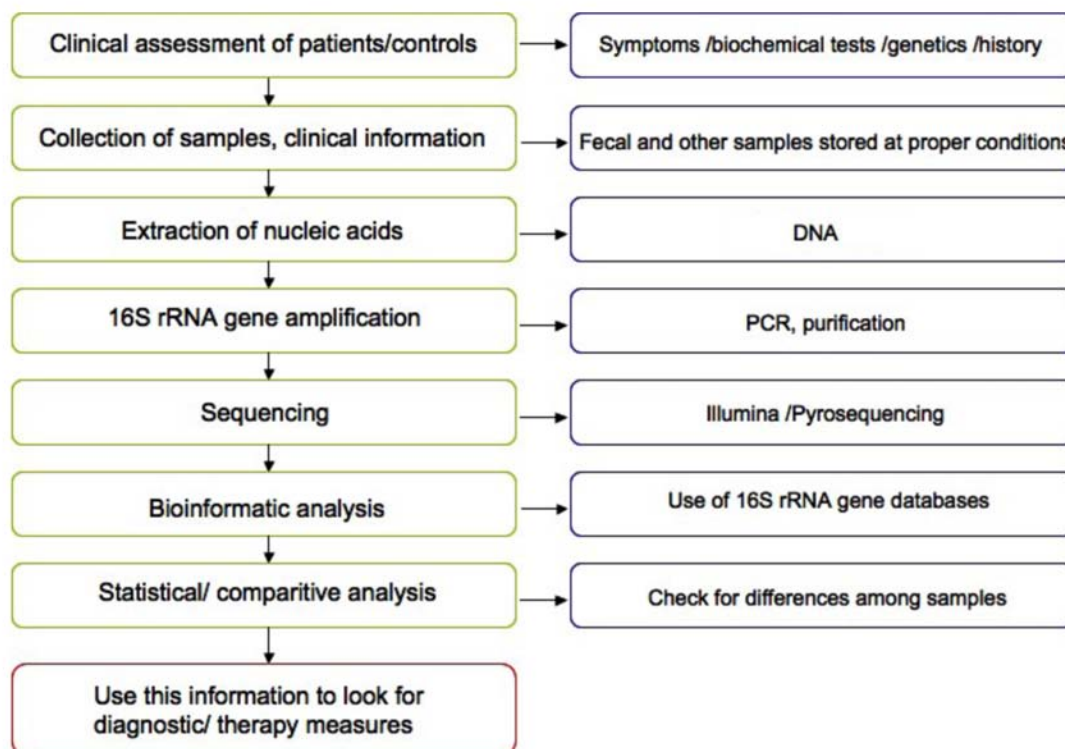
Figure 14: Overview of methodology employed – Starting from clinical assessment of subjects recruited for the studies to collection of samples and relevant clinical information is carried out in the hospital. Samples are further stored and processed, and subsequently sequenced for 16S rRNA gene amplicons. The QIIME bioinformatic pipeline is used for data analysis.

1. Subject recruitment and sample storage

All experiments were performed in accordance to ethical guidelines. The protocol was submitted and approved by the Ethical Committees (Capio Hospital General de Catalunya for the antibiotic study or Hospital Vall d'Hebron for the IBS study). All the subjects gave written informed consent to participate in the studies.

In total, 200 subjects were enrolled for both the studies - receiving 332 faecal samples. Details about the recruited subjects are given in the individual chapters.

After deposition of faecal matter, homogenization was done using a spatula by the participant and samples were immediately frozen in their home freezer at -20°C. Frozen samples were later brought to the laboratory in a freezer pack, where they

were stored at -80°C until further processing. Aliquots of 250mg were performed strictly on solid CO_2 (dry ice) to maintain frozen status of the sample and not allow degradation of nucleic acids (**Figure15**).
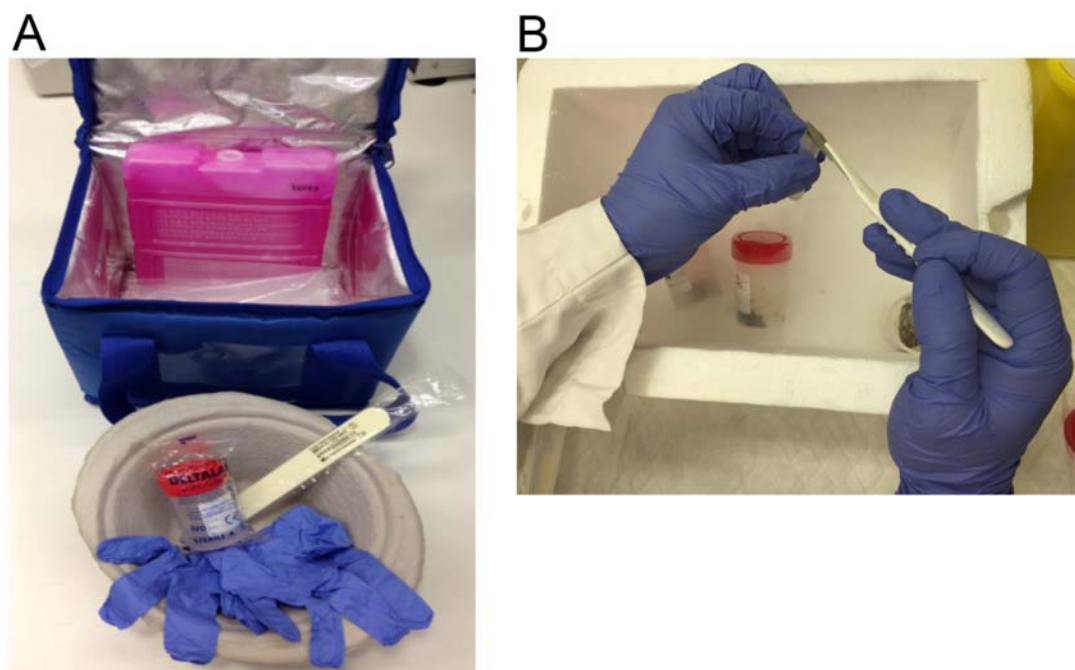
Figure 15: Sample collection and processing – A) Kit comprising of an emesis basin, gel ice pack, sterile spatula, sterile sampling bottle and gloves, B) Aliquots of 250mg faecal matter being carried out in solid CO_2 (dry-ice) without allowing the sample to de-freeze at any moment.

2. DNA extraction from faeces and quality check

250mg of frozen stool sample, added with commercially available zirconia silica beads, was suspended in 250µl of guanidine thiocyanate, 0.1 M Tris chloride (pH 8), 40 µl of 10% N-lauroyl sarcosine and 500µl 5% N-lauroyl sarcosine at 70°C for 1 hour to undergo chemical lysis. Further mechanical disruption was carried out using a Beadbeater (Biospec Products$^©$). Poly Viny Poly-Pyrrolidone (PVPP) was added in multiple washing steps to precipitate and discard aromatic molecules such as nuclei debris, cellular debris and proteins. To clear lysates, enzymatic digestion of RNA was performed, and resulting DNA was precipitated and further ethanol-purified. Pure DNA was re-suspended in 200µl Tris-EDTA buffer (**Figure 16**).
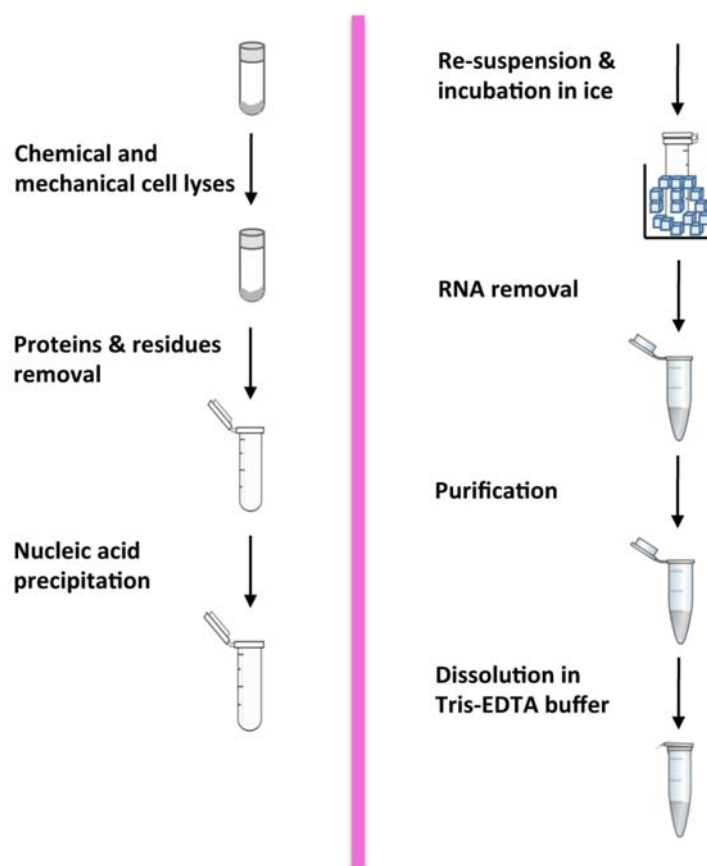
Figure 16: Overview of the DNA extraction method – 250mg of faecal matter is subjected to chemical and mechanical lyses, after which residues and proteins are removed. The resulting nucleic acid is precipitated, and RNA removal is carried out. The remaining nucleic acid (DNA) is purified by subjecting it to ethanol washes. The pure DNA is dried to remove traces of ethanol, and is dissolved in EDTA containing buffer (Based on Godon *et al*., 1997)

DNA quantification of an equivalent of 1 mg of sample was done using NanoDrop ND-1000 Spectrophotometer (Nucliber$^{©}$). DNA integrity was examined by micro-capillary gel electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies$^{©}$) with the DNA 12,000 kit, which resolves the distribution of double-stranded DNA fragments up to 17,000bp in length (**Figure 8**).

3. Quantitative PCR

Microbial load or abundance could be assessed by performing a real time quantitative Polymerase Chain Reaction (qPCR) with the amplification of 16S

Technology	Primer type	Sequence 5' → 3'
Pyrosequencing	Forward	CCATCTCATCCCTGCGTGTCTCCGACTCAG[10bp-barcode]GCCAGCAGCCGCGGTAA
	Reverse	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGACTACCAGGGTATCTAAT
Illumina sequencing	Forward	AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
	Reverse	CAAGCAGAAGACGGCATACGAGAT[12bp-barcode]AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
qPCR	Forward	GCCAGCAGCCGCGGTAA
	Reverse	GACTACCAGGGTATCTAAT

Table 2: Details of primers used -
V4 region of 16S rRNA gene specific sequence, adapter, linker, barcode specific to each sample, Sequencing machine flowcell specific sequence

rRNA gene, which is shared in all prokaryotic organisms. For this purpose, the V4 region of the 16S rRNA gene was amplified using universal primers V4F_517_17 (Forward primer) and V4R_805_19 (Reverse primer) (**Table 2**). A PCR reaction of 25µl in each well of optical-grade 96-well plates, containing 100nM of each primer and Power SYBR Green Master Mix (Applied Biosystems[©]) was used. A minimum of 5-log cycle standard was maintained to be able to plot the sample quantities against a known standard curve. An ideal qPCR standard curve is shown in **Figure 17**. Each sample, standards and negative controls were amplified in triplicates, from which mean values were calculated for final statistics. All reactions were performed with the 7500 Fast Real-Time PCR System (Applied Biosystems[©]). Amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analysed using Sequence Detection Software version 1.4, supplied by Applied Biosystems[©].
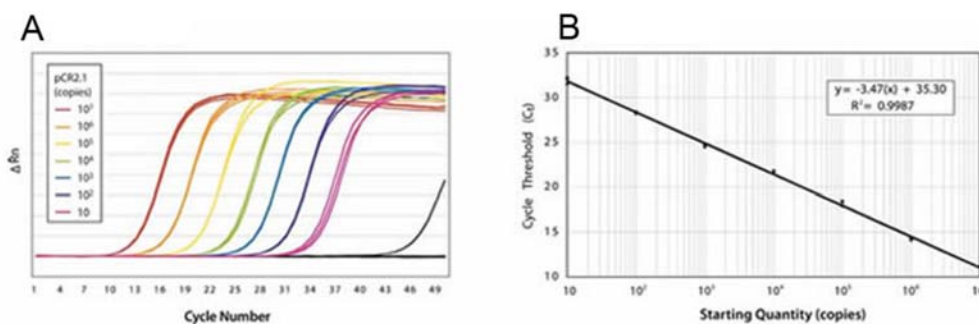
Figure 17: Standard curve for qPCR – A) Amplification plot shows the variation of log (ΔRn) with PCR cycle number, B) Samples are plotted against a standard curve to estimate the copy number of the bacteria in a sample

4. 16S rRNA gene amplification for

4.1 Pyrosequencing

The 5' ends of the forward (V4F_517_17) and reverse (V4R_805_19) primers targeting the V4 region of the 16S rRNA gene were tagged with specific sequences for pyrosequencing (**Table 2**). Multiplex identifiers (MIDs) of 10 bases provided by Roche[©], which were specified upstream of the forward primer sequence (V4F_517_17) help in serving as barcodes for each individual sample.

Standard PCR consisting of 0.75 units of AmpliTaq Gold (*Taq*) polymerase (Life Technologies©) and 20pmol/µl of the forward and reverse primers (IDT Technologies©) was run in a Mastercycler gradient (Eppendorf©) at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 72°C for 40 sec, and a final cycle of 72°C for 7 min (**Figure 18**).
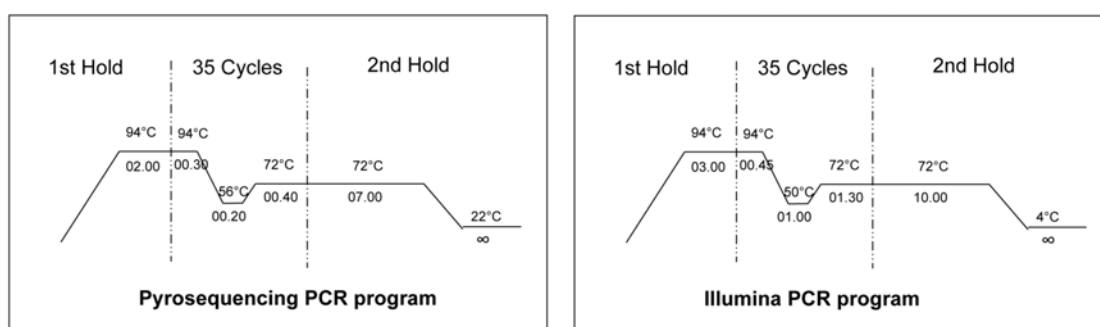
Figure 18: PCR program – Temperature and time cycles for the PCR duration.

4.2 Illumina sequencing

The 5' ends of the forward (V4F_515_19) and reverse (V4R_806_20) primers (containing toggle nucleotides) targeting the V4 region of the 16S rRNA gene were tagged with specific sequences for Illumina© MiSeq Technology (**Table 2**). 12 base paired Golay codes were specified downstream of the reverse primer sequence (V4R_806_20) to allow multiplex identification of individual samples. Standard PCR consisting of 0.75 units of AmpliTaq Gold (*Taq*) polymerase (Life Technologies©) and 20pmol/µl of the forward and reverse primers (IDT Technologies©) was run in a Mastercycler gradient (Eppendorf©) at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec, and a final cycle of 72°C for 10 min (**Figure 18**).

5. Agarose gel and purification

1% agarose gel stained with ethidium bromide was cast and run in 1x Tris Acetate EDTA (TAE) buffer. 5µl of PCR amplified DNA was mixed with 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and added to the wells of solidified agarose gel along with 100bp DNA Molecular Weight Marker XIV (Roche©). Electrophoresis was performed in an electrophoresis tank (Bio- Rad©) at 90V for 45 mins to visualise amplicon bands. The appearance of bands, visualised on a Gel Doc XR+ system (Bio-Rad©), confirmed that amplicons had been generated during PCR amplification. The absence of bands could refer to absence of sufficient bacterial DNA in the sample or presence of PCR inhibitors in the resulting DNA, and a diluted DNA sample would be necessary to achieve amplicons.
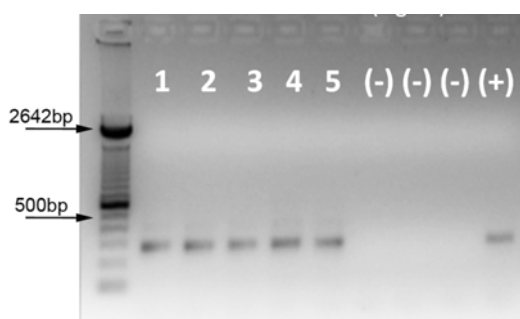
Figure 19: Typical agarose gel – 1.5% agarose gel, run at 90V for 30 minutes. Gel consists of 5 samples, 3 negative controls, 1 positive control, accompanied by 100bp DNA Molecular weight ladder. Significant bands are marked. Amplicon size of sample is about 300bp.

PCR products showing bands in agarose gel (**Figure 19**) were purified using the QIAquick PCR Purification Kit (Qiagen©) according to manufacturer's instructions, and further quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©).

6. Sequencing protocols for

6.1 Pyrosequencing

Equimolar quantities of the purified amplicons were subjected to an emPCR process to obtain about 10% bead enrichment. The resulting beads are then added to a PicoTitrePlate, where in each well of the plate allows only a single

bead to enter. Sequencing primers and reagents are added to perform the sequencing on a 454 Life Sciences Junior system (Roche[©]) (**Figure 6**).

6.2 Illumina sequencing

Amplicon pools of equal concentration diluted to 2nM, spiked with 15-30% denatured PhiX, and are further diluted to 5ρM and subsequently combined to give an 85% 16S rRNA gene amplicon library and 15% PhiX control pool. Sequencing is performed with the use of a MiSeq (Illumina Technologies[©]) Reagent Cartridge (300-cycle PE kit) where appropriate index and sequencing primers are added (**Figure 7**).

7. Sequence analysis

Quality of sequence files provided by the sequencing platform was checked, filtering out reads with Phred score less than 20 [Ewing *et al*., 1998]. Metadata containing sample identifiers, barcodes, primer sequence, time point, sample status, clinical information of the subjects involved in the studies and other additional information of the samples needed for the analyses were included in a file. The upstream analysis was performed with QIIME using the guidelines proposed [www.qiime.org] (**Figure 20**).

The pyrosequencing technology produces characteristic sequencing errors that may be classified as an additional Operational Taxonomical Unit (OTU). A denoising procedure is performed to reduce the amount of erroneous OTUs, increasing the accuracy of the pipeline.

Short-DNA sequences unique to each sample, known as barcodes, which were introduced during the PCR process, were removed from raw sequences and each read was assigned back to its sample. This demultiplexing step resulted in 16S rRNA gene amplicon sequences without primers and barcodes.

Using the pick-OTUs protocol (UCLUST), sequence reads were classified into OTUs on the basis of sequence similarity (97% similarity threshold) to Greengenes reference database [http://greengenes.secondgenome.com]. Chimeric sequences were removed using ChimeraSlayer.

Taxonomy was assigned using the Basic Local Alignment Search Tool (BLAST) with datasets of Greengenes using PyNAST that generated an OTU table containing all the OTUs with predicted taxonomy and abundances for each sample.
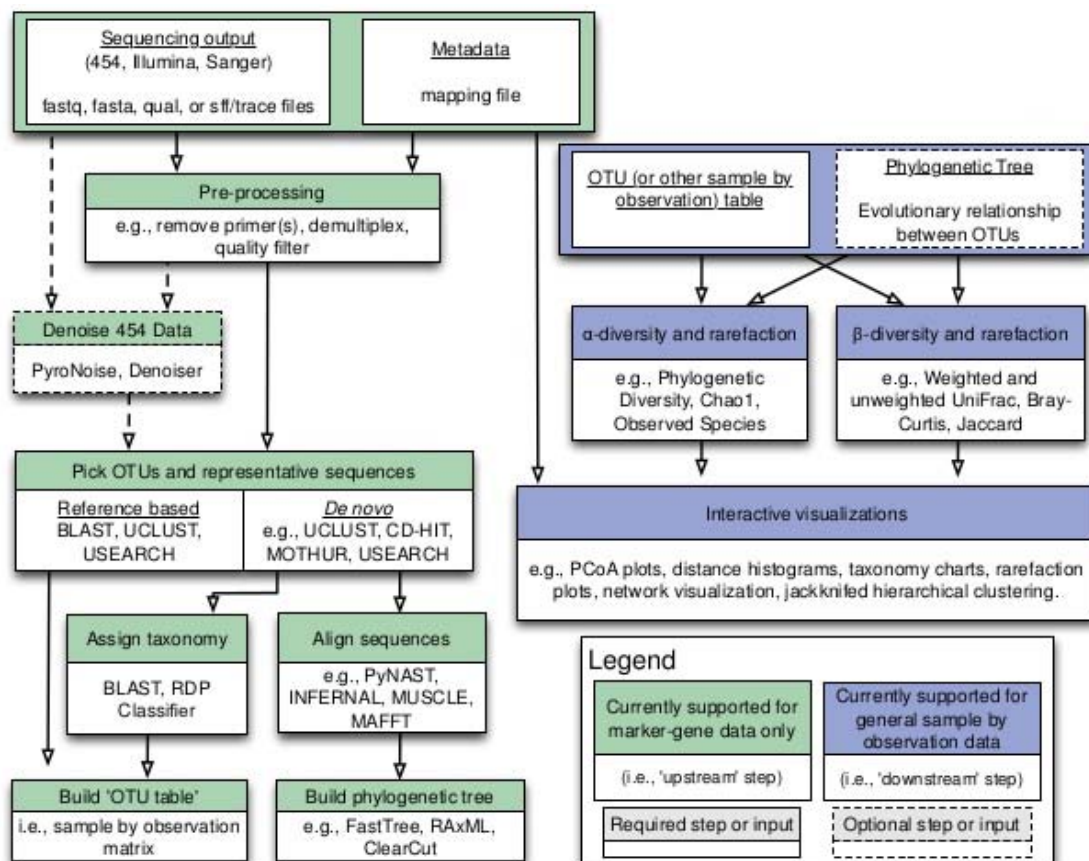
Figure 20: Data analysis using QIIME – Upstream and downstream analysis of Pyrosequencing-generated as well as Illumina sequencing-generated data is carried out using the proposed outline (From www.qiime.org)

Multiple rarefactions were applied and rarefaction tables were used to compute Chao1 statistic for estimating microbial richness (phylogenetic diversity metric) of the samples (alpha diversity). Between-samples diversity or beta diversity used weighted and unweighted UniFrac method to generate phylogenetic distance matrices that were later utilised for clustering samples in hierarchical cluster trees with Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Principal Coordinate Analysis representations (PCoA).

The summarize taxa feature was used to classify taxa from the Domain to the Species level that were subsequently used for study and sample specific statistical analyses.

8. Statistical tests

The Mann-Whitney test is a non-parametric analysis that compares two unpaired groups to examine the null hypothesis that these two groups have the same distribution [Mann and Whitney, 1947]. This test first ranks all the values from low to high, and then computes a p-value that depends on the comparison of the mean ranks of the two groups. However, the Mann-Whitney test only compares two groups; to compare three or more groups, the Kruskal-Wallis test is used.

The Kruskal-Wallis test is a non-parametric test that compares the medians of three or more unpaired or unmatched groups for testing whether the samples originate from the same distribution [Kruskal and Wallis, 1952]. It is used for comparing three or more samples that are independent, and that may have different sample sizes. The results of a Kruskal-Wallis test only make sense when the scatter is random – that a certain factor causing a value to be too high or too low affects only that one value. This test does not assume that the populations follow Gaussian distributions, but does assume that the shapes of the distributions are identical.

If the data are paired or matched, then Wilcoxon matched pairs test is used [Wilcoxon, 1945]. This is a non-parametric test that compares two paired groups where the factor that caused a difference between the paired values is too high or too low affecting only one pair. The Wilcoxon test first computes the difference between the two values in each row, and analyses only the list of differences, assuming that the differences are distributed symmetrically around their median.

A semi-parametric analyses of variance, the Shapiro–Wilk test is, a test of normality that tests the null hypothesis that the samples came from a normal

distribution [Shapiro and Wilk, 1965]. The test rejects the hypothesis of normality when the *p*-value is less than or equal to 0.05. Failing the normality test allows to state with 95% confidence the data does not fit the normal distribution. The Shapiro-Wilk test works very well if every value is unique, however, it does not work as well when several values are identical.

The D'Agostino-Pearson test is a versatile and powerful normality test based on descriptive statistics of the sample [D'Agostino and Pearson, 1973]. It first computes the skewness and kurtosis to quantify how far the distribution is from Gaussian in terms of asymmetry and shape, and then calculates how far each of these values differ from the value expected with a Gaussian distribution. A single *p*-value is computed from the sum of these discrepancies. The skewness and kurtosis tests can be combined to produce a single, global, "omnibus" statistic.

CHAPTER 1

Short-term Effect of Antibiotics on Human Gut Microbiota

ABSTRACT

From birth onwards, the human gut microbiota rapidly increases in diversity and reaches an adult-like stage at three years of age. After this age, the composition may fluctuate in response to external factors such as antibiotics. Previous studies have shown that resilience is not complete months after cessation of the antibiotic intake. However, little is known about the short-term effects of antibiotic intake on the gut microbial community.

Here we examined the load and composition of the faecal microbiota immediately after treatment in 21 patients, who received broad-spectrum antibiotics such as fluoroquinolones and β-lactams. A faecal sample was collected from all participants before treatment and one week after for microbial load and community composition analyses by quantitative PCR and pyrosequencing of the 16S rRNA gene, respectively.

Fluoroquinolones and β-lactams significantly decreased microbial diversity by 25% and reduced the core phylogenetic microbiota from 29 to 12 taxa. However, at the phylum level, these antibiotics increased the Bacteroidetes/Firmicutes ratio (p = 0.0007, FDR = 0.002). At the species level, our findings unexpectedly revealed that both antibiotic types increased the proportion of several unknown taxa belonging to the *Bacteroides* genus, a Gram-negative group of bacteria (p = 0.0003, FDR < 0.016). Furthermore, the average microbial load was affected by the treatment. Indeed, the β-lactams increased it significantly by two-fold (p = 0.04).

The maintenance of or possible increase detected in microbial load and the selection of Gram-negative over Gram-positive bacteria breaks the idea generally held about the effect of broad-spectrum antibiotics on gut microbiota.

Suchita Panda

INTRODUCTION

Clinicians commonly prescribe antibiotics to treat infections. The choice of antibiotic is well indicated in clinical guidelines for targeting specific pathogens, Gram-positive or Gram-negative bacteria [McNulty *et al*., 2012]. However, little is known about the effects of antibiotics on the whole composition and load of the gut microbiota immediately after treatment.

Human faecal microbiota is composed of four main groups of bacteria (phyla), namely Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria [Qin *et al*., 2010], the first two phyla accounting for more than 80% of the microbiota. Firmicutes comprise mostly Gram-positive bacteria with a DNA that has a low G+C content, but also include Gram-negative bacteria. Bacteroidetes include Gram-negative bacteria, which are represented mainly by the *Bacteroides* genus in the human gut. Proteobacteria consist of Gram-negative bacteria and include a wide variety of well-studied pathogens. Actinobacteria are a group of Gram-positive bacteria with a DNA that has a high G+C content.

Since the introduction of antibiotics in the 1940s, the short-term effect of these drugs on gut microbiota has been mainly documented on the basis of culture methods. However, given the difficulty in developing cultures for most gut bacteria [Goodman *et al*., 2011], the information gathered from this technique is insufficient to understand the full targets of antibiotics. A few recent studies have used high-throughput sequencing technology to deeply characterise the long-term effect of antibiotics [Dethlefson *et al*., 2008; Jakobsson *et al*., 2010; Fouhy *et al*., 2012; Perez-Cobas *et al*., 2013a]. These studies have shown that treatment is followed by a significant alteration of the gut microbiota composition and a decrease between one-fourth to one-third of the microbial diversity in the digestive tract [Jernberg *et al*., 2007; Dethlefson *et al*., 2008]. The microbiota is relatively resilient and returns to the pre-treatment state several weeks after drug cessation [De La Cochetiere *et al*., 2005].

However, other recent studies on the long-term effects of antibiotic intake have shown that microbiota does not show complete resilience three months after

treatment cessation [De La Cochetiere *et al*., 2005; Dethlefson *et al*., 2008; Jakobsson *et al*., 2010; Manichanh *et al*., 2010; Willing *et al*., 2011]. Variations in the resilience observed might be due to differences in the methodology used to analyse microbiota variability: TGGE [De La Cochetiere *et al*., 2005] versus high-throughput sequencing technique [Dethlefson *et al*., 2008; Jakobsson *et al*., 2010; Manichanh *et al*., 2010] (**Table 3).**

In experimental models, up to a 10-fold reduction in bacterial 16S rDNA was detected (qPCR) immediately after treatment with antibiotics [Hill *et al*., 2010; Manichanh *et al*., 2010]. Furthermore, Hill *et al*. showed that bacterial depletion was associated with anatomic, histologic, and immunologic changes characteristic of reduced microbial stimulation [Hill *et al*., 2010]. Indeed, for latter associated effect, the authors showed that transcript levels of ifng and il17a genes, coding for IFNγ or IL-17A, were significantly reduced in the small intestine of antibiotic-treated animals as compared to controls, thus demonstrating that microbial signals participate in the maintenance of normal intestinal effector T lymphocyte populations. However, as far as we know, no data in human adults has yet been reported regarding microbial load combined with microbial composition analysis before and immediately after antibiotic intake.

Using quantitative real-time PCR (qPCR) and high-throughput sequencing techniques, here we describe the short-term effect of antibiotics on the composition, structure and load of the gut microbial community of patients who received a seven-day treatment with commonly used antibiotics.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Institutional Review Board of the Capio Hospital General de Catalunya, Barcelona, Spain. Participants provided their written consent to participate in this study.

Ref	Subjects/animal	Antibiotic	Time points	Type of sample	Extraction technique	Technique	Average number of reads	Main results of the study	Results of microbial load/count
De LA Cochetiere *et al*., 2005	6 healthy	500mg amoxicillin (β-lactam) per day for 5 days	0,1, 30, 60 days for all after treatment, day 2, 3, 4 for some	Stool	Godon *et al*., 1997method	V6-V8 amplified 16S rRNA gene TTGE, sequencing by Sanger method of specific gel bands	No data provided	Dominant species markedly **decreased** within 2 to 3 days; These tended to return to initial profile within 60 days; Modifications persisted for at least 2 months	No qPCR, no culture
Noverr *et al*., 2005	Mice	0.5mg/ml cefoperazone (β-lactam) for 5 days	Day 5	Stool	Culture	CFU counts	Not applicable	Culturable anaerobic and enteric bacteria levels in the gut was **decreased** by 99.99% at day 4 of antibiotic treatment; When antibiotic treatment was discontinued, the numbers of both anaerobic and enteric bacteria increased	No qPCR, **decrease** in the bacterial counts at day 4 by culture
Jernberg *et al*., 2007	4 healthy controls/ 4 healthy received antibiotics	150mg clindamycin (lincosamide) 4 times a day for 7 days	0, 7, 14 days, 3, 6, 9, 12, 18, 24 months after treatment	Stool	FastDNA SPIN Kit for Soil (Q-BIOgene)	T-RFLP, RT-PCR	No data provided	*Bacteroides* showed sharp **decrease**; Samples at day 0 clearly separated from the rest of the samples; Microbiota stabilises to pre-clindamycin administration levels after three months post exposure	No culture, no qPCR for bacteria, but RT-PCR of *erm* genes showed **increase** at later time-points compared to basal
Dethlefson *et al*., 2008	3 healthy adults	500mg ciprofloxacin (fluoroquinolone) twice a day for 5 days	–60, –6, –2, –1 days (pre-treatment), days 3, 5, 33, 180 (post-treatment)	Stool	Bead-beating followed by QIAamp DNA Stool Mini Kit (Qiagen)	Near-complete 16S rDNA and V6 amplified 16S rDNA pyrosequencing	24,000 per sample	**Decrease** of one third of the taxonomic richness, diversity, and evenness of the community, inter-individual variation; Partial resilience after 4 weeks of treatment cessation	No qPCR, no culture
Ochoa-Reparez *et al*., 2009	8 mice	1g/ml ampicillin (β-lactam), 0.5g/ml vancomycin (glycopeptide), 1g/ml neomycin sulphate (aminoglycoside), and 1g/ml metronidazole (nitromidazole)	No data	Intestines, Stool	Culture in aerobic/anaerobic conditions	CFU counts	Not applicable	Oral treatment with antibiotics significantly **decreased** the bacterial counts present in faecal and intestinal samples; In aerobic and anaerobic conditions a significant **decrease** of bacterial counts was found after 1 week of treatment; antimicrobial treatment did not completely deplete bacterial presence, showing that certain bacterial populations remain viable despite antibiotic treatment	No qPCR, **Decrease** in the bacterial counts of faecal and intestinal samples by culture

Jakobsson *et al*., 2010	3 controls / 3 treated adults patients	400mg metronidazole (nitromidazole), 250mg clarithromycin (macrolide), and 20mg omeprazole (proton pump inhibitor)	8, 10, 12, 13 days (depending on patient), 1 year, 4 years after treatment	Throat swabs and stool	Throat: DNeasy Tissue Kit (Qiagen) Stool: Bead-beating followed by FastDNA SPIN Kit for Soil (BIO 101)	V6 amplified 16S rDNA T-RFLP and pyrosequencing	5000-11,000 per subject	Significant **decrease** in Actinobacteria in both throat and faeces immediately after treatment; Microbiota remained perturbed in some cases for up to four years post treatment	No qPCR, no culture
Manichanh *et al*., 2010	3 control rats/ 3 rats with antibiotics	50mg/kg/day vancomycin (glycopeptide) and 50mg/kg/day imipenem (β-lactam) for 3 days	-3 days (pre-treatment), 3 days, 1, 3 months post-treatment	Stool	Bead-beating followed by modified QIAamp DNA stool mini kit (Qiagen)	V4 amplified 16S rRNA gene Pyrosequencing, qPCR	546,230 reads total, 2000 reads per sample	**Decreased** bacterial phylotype richness controls clustered separately than treated; Major reduction of Bacteroides and Firmicutes; One month later, faecal samples regained similar bacterial load to the controls; Bacteroidetes and Firmicutes recovered as the two major phyla; Bacterial diversity was not resilient	No culture, using qPCR: 10-fold **decrease** in bacterial load of antibiotic treated rats compared with control rats
Ochoa-Reparez *et al*., 2010	Mice	1g/ml ampicillin (β-lactam), 0.5g/ml vancomycin (glycopeptide), 1g/ml neomycin sulphate (aminoglycoside), and 1g/ml metronidazole (nitromidazole) for 7 days	Day 7	Intestinal and stool samples	Culture	CFU counts	Not applicable	Oral antibiotic treatment significantly **decreased** the total bacterial numbers recovered	No qPCR, **decreased** bacterial colonies

Fouhy *et al.*, 2012	9 controls/ 9 treated infants (within 48 h of birth)	Ampicillin (β-lactam) and gentamicin (aminoglycoside) [no data on dosage]	4 and 8 weeks after treatment	Stool	QIAamp DNA stool mini kit (Qiagen)	V4 amplified 16S rDNA pyrosequencing and qPCR	No data provided	**Increase** of Proteobacteria at week 8 after cessation of treatment	No culture, using qPCR: No significant differences between antibiotic-treated infant samples compared to those for controls at week 4 or week 8; Significant **increase** at week 8, in total 16S rRNA values in the antibiotic-associated samples
Perez-Cobas *et al.*, 2013a	1 patient	Single dose of ampicillin/ sulbactam (β-lactam) and 14 days of cefazolin (β-lactam) treatment	0, 36, 11, 14 days after initiation of treatment and day 40 after cessation of treatment	Stool	QIAamp DNA Stool kit (Qiagen)	Total (16S rDNA) and active (16S rRNA) microbiota by V1-V3 amplified pyrosequencing, metagenome, metatranscriptome, metabolome and metaproteome	No data provided	**Decrease** of Gram-negative bacteria at day 6; Increase of active *Parabacteroides* at day 14; minimum richness at day 11; major metabolic changes at day 6	No qPCR, no culture

Table 3: Literature overview for studies related to effect of antibiotics on the gut microbiota

Patients and sample collection

Twenty-one participants (from 18 to 80 years old), who were admitted to the hospital for non-digestive diseases (bronchitis, urinary tract diseases, pneumonia, bacteraemia or prostatitis), were recruited to donate stool samples before and after seven-day course of antibiotics. Patients were treated with commonly used antibiotics (β-lactams (N = 11) and fluoroquinolones (N = 10)): amoxicillin-clavulanate (amoxiclav) (N = 7), levofloxacin alone (N = 8) or in combination with metronidazole (N = 1), ceftriaxone alone (N = 1) or in combination with azithromycin (N = 2), ciprofloxacin (N = 1), and piperacilin/tazobactam (N = 1). The dose of antibiotic was adjusted to the aetiology of the infection and patient characteristics, following current clinical guidelines. Participants had not received antibiotics during 2 months prior to the study. For microbial composition analyses, stool samples were collected before and on the seventh day of the antibiotic treatment and were stored immediately at −20°C in a home freezer and transported afterwards in a freezer pack to the laboratory.

Microbial community analyses

Genomic DNA extraction

A frozen aliquot (200mg) of each faecal sample was suspended in 250µl of guanidine thiocyanate, 0.1M Tris (pH 7.5) and 40µl of 10% N-lauryl sarcosine. Genomic DNA was extracted as described by Godon *et al*., 1997.

Pyrosequencing of the V4 variable region of the 16S rRNA gene

Extracted DNA was subjected to PCR-amplification of the V4 region of the bacterial and archaeal 16S rRNA gene (16S ribosomal RNA). On the basis of our analysis done using PrimerProspector software [Walters *et al*., 2011], the V4 primer pairs used in this study were expected to amplify almost 100% of the Archaea and Bacteria domains. The 5' ends of the forward (V4F_517_17: 5'-GCCAGCAGCCGCGGTAA -3') and reverse (V4R_805_19: 5'-GACTACCAGGGTATCTAAT -3') primers targeting the 16S gene were tagged with specific sequences for pyrosequencing as follows: 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG - {MID} - {GCCAGCAGCCGCGGTAA} -3' and 5'-

CCTATCCCCTGTGTGCCTTGGCAGTCTCAG - {GACTACCAGGGTATCTAAT} - 3'. Tag pyrosequencing was performed using multiplex identifiers (MIDs) of 10 bases provided by Roche©, which were specified upstream of the forward primer sequence (V4F_517_17). Standard PCR (1 unit of *Taq* polymerase (Roche©) and 20pmol/µL of the forward and reverse primers), was run in a Mastercycler gradient (Eppendorf©) at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 72°C for 40 sec, and a final cycle of 72°C for 7 min [Cardona *et al*., 2012]. The 16S rRNA V4 amplicons were subsequently sequenced on a 454 Life Sciences (Roche©) Junior system (Scientific and Technical Support Unit, Vall d'Hebron Research Institute, Barcelona, Spain), following standard 454 platform protocols.

Sequence analysis

The sequences were analysed using the QIIME pipeline [Caporaso *et al*., 2011] and have been deposited in the NIH Short Read Archive under accession number SRP035398. From the pyrosequencing, 159,536 high quality sequences of 290 base pairs (bp) on average were recovered from 42 samples (with an average of 3945 sequences per sample), after filtering high quality reads, as previously described [Manichanh *et al*., 2010]. From these 42 samples, we obtained 2171 taxa (or molecular species). After removing taxa with low abundance (i.e. in order to avoid false positive OTUs, we considered only taxa that representing at least 0.2% of the microbial community in at least one of the 42 samples), we recovered 427 microbial taxa.

Rarefaction analysis was done for all samples, with 10 repetitions using a step size of 100, from 100 to 2000 sequences per sample. For beta diversity analyses, which examine changes between microbial communities, sequence data were normalised at 2091 sequences per sample, excluding one sample that contained only 1331 sequences. The sequencing depth was evaluated by generating a rarefaction curve based on the number of estimated species (Chao1 estimator of species richness) [Chao, 1984] and the number of sequences per sample, and by applying Good's coverage formula [Esty, 1986]: $1-(n/N)]*100$, where n is the number of taxa in a sample represented by a singleton and N is the total number of sequences in the sample (N = 2091 sequence per sample). These estimators

allow researchers to gain insight into how the limited sampling relates to the entire community sampled. The rarefaction curves (**Figure 21**), which were calculated for each sequence data set (before and after antibiotic treatment), showed that richness almost reached a plateau with 2000 sequence reads. Good's coverage, calculated for each sample of this study, allowed us to recover an average value of 98.26%, indicating that any new sequence generated had only a 1.74% chance of corresponding to a new taxon. Caporaso *et al*., 2011 demonstrated that this depth of sequencing is sufficient to capture the same relationship among samples as with 3.1 million reads per sample. The principal coordinates analysis (PCoA) was performed on pairwise unweighted and weighted UniFrac distances [Lozupone and Knight, 2005].
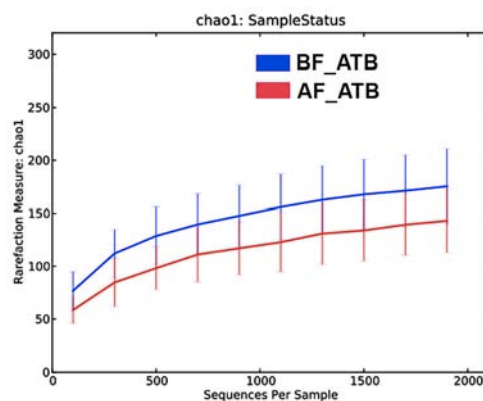
Figure 21. Rarefaction curves of OTU richness based on Chao1 estimation in faeces samples of patients before (BF_ATB) and after antibiotic treatment (AF_ATB).

Microbial load assessment

In order to assess the microbial load, the extracted DNA was used to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR) using the above-cited primers (V4F_517_17 and V4R_805_19). The qPCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems[©]) using optical-grade 96-well plates. The PCR reaction was performed in a total volume of 25µl using the Power SYBR Green PCR Master Mix (Applied Biosystems[©]), containing 100 nM of each of the universal forward and reverse primers. The reaction conditions for amplification of DNA were 50°C for 2 min, 95°C for 10 min, and 40

cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were performed in triplicate and mean values were calculated. This experiment was also duplicated to ensure accuracy. Mean values of both experiments were taken into account. Data were analysed using Sequence Detection Software version 1.4, supplied by Applied Biosystems©.

Statistical analysis

The D'Agostino-Pearson omnibus normality test was used to check the normality of data distribution. Comparisons of parametric normally distributed data were made by the paired t-test for intra-group comparisons; otherwise the Wilcoxon matched-pairs signed-rank test was used. p values < 0.05 were considered significant.

We used the otu_category_significance.py script from the QIIME pipeline and ANOVA to test which taxa were associated with the use of the antibiotics. This analysis provided the False Discovery Rate (*FDR*) value, which is defined to be the false discovery rate of the p value (corrected p value) and is considered significant when < 0.1 [Benjamini and Hochberg, 1995].

RESULTS

Microbiota composition before treatment

Twenty-one patients (18 men, 3 women; median age: 69 years), who were admitted to the hospital for bronchial infection (N = 15), urinary infection (N = 1) or other infections (pneumonia, bacteraemia or prostatitis; N = 5), were enrolled in the study. They took antibiotics (or antibiotic combinations) for seven days and provided faecal specimens just before and a week after the start of antibiotic treatment (i.e. on the seventh day).

The 16S rRNA gene sequence analysis on faecal samples taken before treatment identified 356 unique microbial taxa out of the 21 samples, with an average of 143 taxa per individual. As expected, gut microbiota was dominated by four bacterial phyla: Firmicutes (65%), Bacteroidetes (28%), Proteobacteria (5%) and

Actinobacteria (2%). The number of the detected groups, from phyla to species level, is given in **Table 4.**

BF_ATB	Number of microbial groups detected	Most abundant group
Phyla	7	Firmicutes
Class	12	Clostridia
Order	19	Clostridiales
Family	40	Ruminococcaceae
Genus	68	*Bacteroides*
Species	356	*Faecalibacterium prausnitzii*

Table 4: Number of microbial groups at different taxonomic levels. BF_ATB = Before antibiotic treatment

Twenty-nine bacterial taxa, which were shared by more than 80% of the subjects, constituted the core phylogenetic microbiota and accounted for 44% of the sequences, *Faecalibacterium prausnitzii* (7.2 %) being the most abundant (**Table 5**).

Consensus Lineage	Proportion sequences before antibiotics	Proportion sequences after antibiotics
Bacteroidetes;Bacteroidaceae;Bacteroides #46	2.87	10.74
Bacteroidetes;Bacteroidaceae;Bacteroides #569	1.41	9.86
Bacteroidetes;Bacteroidaceae;Bacteroides uniformis	3.06	4.58
Bacteroidetes;Rikenellaceae #1950	0.81	2.88
Firmicutes;Ruminococcaceae;Faecalibacterium prausnitzii	1.78	2.58
Bacteroidetes;Bacteroidaceae;Bacteroides #1010	ND	1.62
Bacteroidetes;Porphyromonadaceae;Parabacteroides	0.23	1.33

distasonis		
Firmicutes;Lachnospiraceae;Ruminococcus torques	2.18	1.03
Firmicutes;Lachnospiraceae;Blautia #1159	2.57	0.79
Bacteroidetes;Bacteroidaceae;Bacteroides #1698	ND	0.50
Firmicutes;Lachnospiraceae #1872	0.44	0.42
Firmicutes;Lachnospiraceae;Ruminococcus #789	0.34	0.18
Firmicutes;Ruminococcaceae;Faecalibacterium prausnitzii	5.42	ND
Bacteroidetes;Bacteroidaceae;Bacteroides #529	3.04	ND
Firmicutes;Ruminococcaceae;Oscillospira #1434	2.67	ND
Bacteroidetes;Rikenellaceae #531	2.30	ND
Firmicutes;Lachnospiraceae;Roseburia faecis	2.29	ND
Firmicutes;Lachnospiraceae;Coprococcus #11	1.88	ND
Firmicutes;Ruminococcaceae;Ruminococcus #1267	1.63	ND
Firmicutes;Ruminococcaceae #109	1.34	ND
Bacteroidetes;Bacteroidaceae;Bacteroides eggerthii	1.13	ND
Firmicutes;Lachnospiraceae;Lachnospira #153	1.08	ND
Firmicutes;Lachnospiraceae;Ruminococcus gnavus	1.01	ND
Firmicutes;Ruminococcaceae #1665	0.85	ND
Bacteroidetes;Bacteroidaceae;Bacteroides #1803	0.76	ND
Firmicutes;Ruminococcaceae;Oscillospira #1304	0.74	ND
Firmicutes;Ruminococcaceae #1470	0.73	ND
Firmicutes;Lachnospiraceae;Blautia #1299	0.67	ND
Firmicutes;Lachnospiraceae;Dorea formicigenerans	0.35	ND
Firmicutes;Ruminococcaceae #1866	0.21	ND
Firmicutes;Lachnospiraceae;Blautia #2036	0.20	ND

Table 5: Proportion of sequences for each OTU of the phylogenetic core before and after antibiotics. ND = Not detected; #number indicates an arbitrary identification for an OTU

Microbiota alteration after treatment

On the basis of the 16S rRNA sequence analysis and taking into account all the types of antibiotics, we observed that seven days of treatment caused a global change in microbial community structure, as attested by the separate clustering of

samples before and after antibiotic intake with both weighted and unweighted UniFrac methods (i.e. taking into account both composition and abundance of the species or only the composition, respectively, **Figure 22** and **Figure 23**). These methods measure similarity between microbial communities on the basis of the degree to which their component taxa share branch length on a bacterial tree of life. This observation indicates that microbial abundance and composition were affected by the antibiotic treatments. However, the clustering was stronger with weighted (38%) than unweighted (15%) UniFrac; implying that antibiotics affected both abundance and composition, and not only the latter.
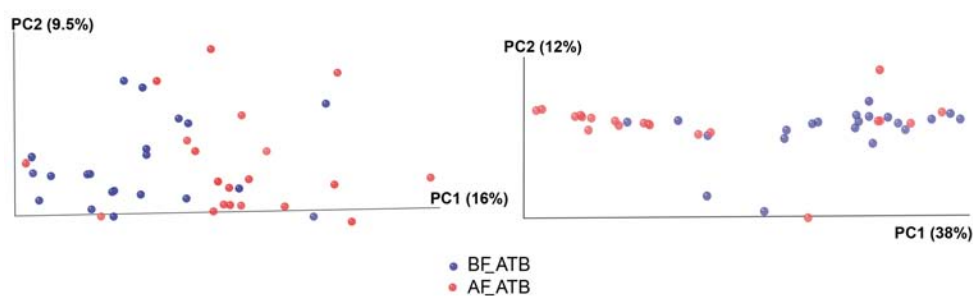
Figure 22. **Global effect of antibiotic treatment on faecal microbiome.** Communities clustered using PCoA of the unweighted (on the left) and weighted (on the right) UniFrac distance matrix. Only the two first principal components are shown. Each dot represents the whole microbiota of a faecal sample. BF_ATB = Before antibiotic treatment and AF_ATB = After antibiotic treatment (N = 21).
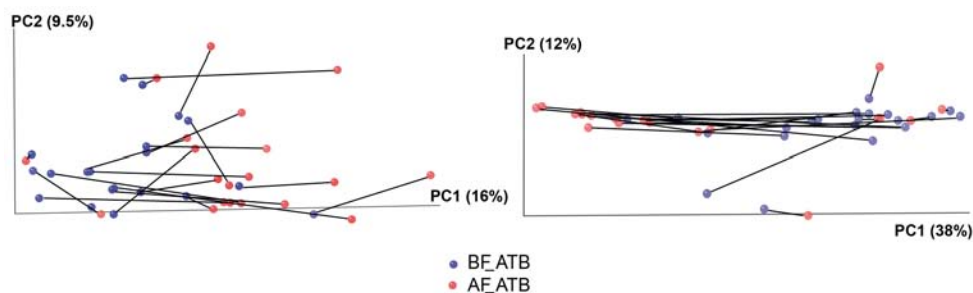
Figure 23. Global effect of antibiotic treatment on faecal microbiome. Communities clustered using PCoA of the unweighted (on the left) and weighted (on the right) UniFrac distance matrix. Only the two first principal components are shown. BF_ATB and AF_ATB = Before and after antibiotic treatment (N = 21). Each dot represents the microbial community of a sample, and dots representing samples from the same patient were connected by a line.

The core phylogenetic microbiota fell from 29 to 12 microbial taxa, these accounting for 36% of the sequences and shifting from *Faecalibacterium* to *Bacteroides* as the most dominant genus. From the 12 microbial taxa constituting

the core, two taxa from the *Bacteroides* genus were new compared to the core before treatment (**Table 5**). Indeed, *Bacteroides* genus increased by 2.5-fold ($p = 0.0003$, *FDR* = 0.016). At the phylum level, both types of antibiotics (β-lactams and fluoroquinolones) tested caused a decrease of Firmicutes and increase of Bacteroidetes ($p < 0.001$; *FDR* = 0.002) (**Figure 24**).
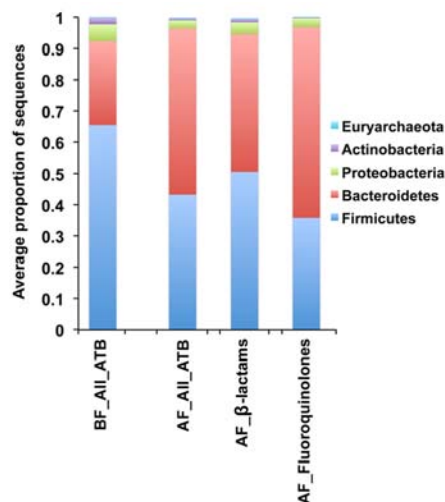
Figure 24. Microbial composition at the phylum level based on 16S rRNA gene sequences. BF = Before treatment; AF = After treatment; ATB = Antibiotics. For all antibiotics N = 21; for β-lactams N = 11; for fluoroquinolones N = 10.

Antibiotics alone or in combination, as depicted in **Figure 25,** also increased the Bacteroidetes/Firmicutes ratio except for two of the drug combinations: piperacilin/tazobactam and levofloxacin/metronidazole. Piperacilin/tazobactam is a combination of two drugs, which inhibits peptidoglycan subunit synthesis (piperacilin) and β-lactamase (tazobactam). Levofloxacin/metronidazole is a combination of two drugs, both inhibiting enzymes involved in nucleic acid synthesis.

Table 6 shows that antibiotics reduced microbial diversity, as evidenced by the significant decrease in the average number of taxa observed and the Chao1 metric of richness by approximately one fourth. Surprisingly, using quantitative PCR (qPCR) of the 16S rRNA gene, we observed that antibiotic intake did not decrease the microbial load, but instead showed a tendency to increase this

parameter, as indicated by the increase in the copy numbers of the 16S rRNA gene (**Figure 26A**, p = 0.082; Wilcoxon matched-paired test).
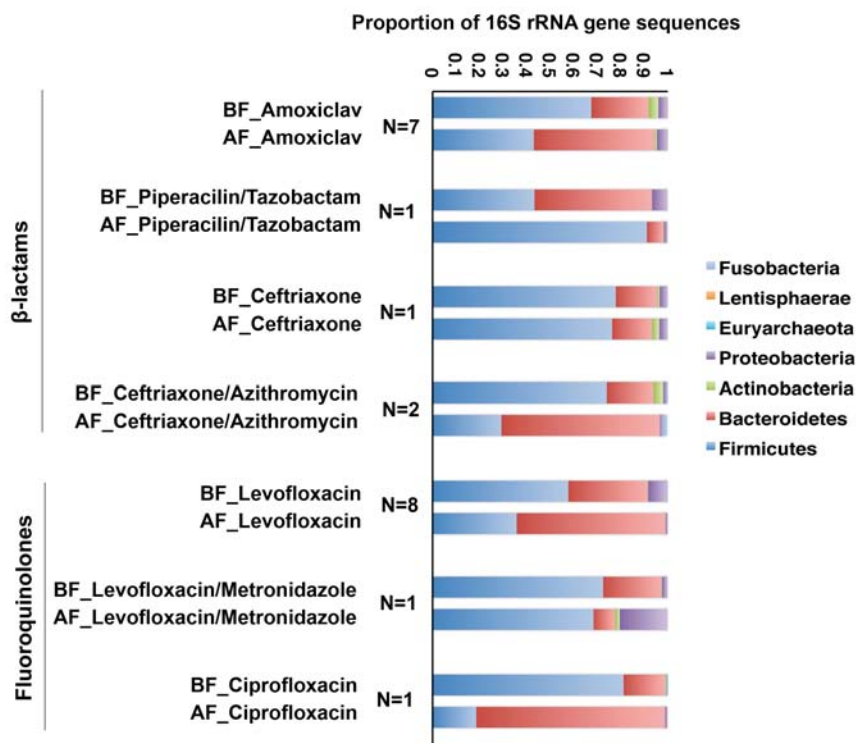
Figure 25. Microbial composition at the phylum level based on 16S rRNA gene sequences. BF and AF refer to before and after antibiotic treatment and N is the number of subjects.

	All subjects (N = 21)			Subjects treated with amoxiclav (N = 7)			Subjects treated with levofloxacin (N = 8)		
	BF_ATB	AF_ATB	p	BF_ATB	AF_ATB	p	BF_ATB	AF_ATB	p
No. of observed taxa	140	105	< 0.001	142	117	0.05	137	102	0.03
Chao1 index	179	143	< 0.001	188	160	0.04	173	140	0.009

Table 6. Microbial richness as assessed by the number of observed taxa and the Chao1 index. BF_ATB = Before antibiotic; AF_ATB = After antibiotic. p = p values.
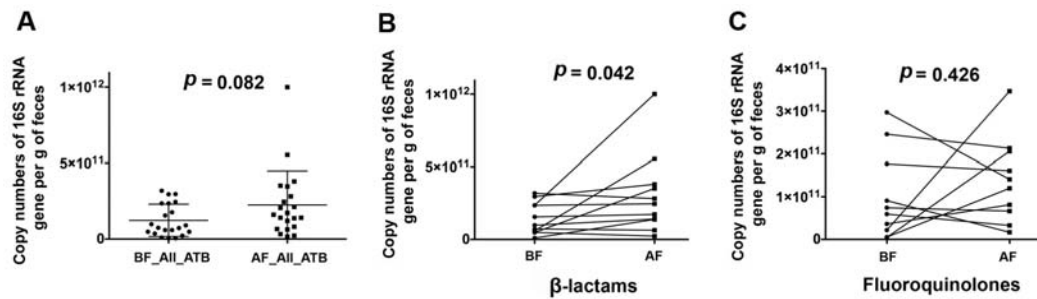
Figure 26. Microbial load as assessed by quantitative real-time PCR (qPCR) of the 16S rRNA gene. (A) Comparison of the microbial load between samples before (BF) and after (AF) treatment by both type of antibiotics (All_ATB). Data were compared using Wilcoxon matched-pairs test. **(B)** Comparison of the microbial load between before and after β-lactams treatment. Data were compared using Wilcoxon matched-pairs test. **(C)** Comparison of the microbial load before and after fluoroquinolone treatment. Data were compared using paired t-test. In all tests $p < 0.05$ is considered significant. For all antibiotics N = 21; for β-lactams N = 11; for fluoroquinolones N = 10.

Effect of β-lactams

β-lactam antibiotics interfere with cell wall synthesis by binding to penicillin-binding proteins (PBPs) located in bacterial cell walls. Inhibition of PBPs leads to suppression of peptidoglycan synthesis and finally to cell death [Mandell and Perti, 1996]. β-lactams show broad-spectrum activity against Gram-negative and Gram-positive bacteria and have been used for a wide range of indications in clinical practice [Holten and Onusko, 2000]. Sequence analysis revealed that this type of antibiotic significantly increased the proportion of Bacteroidetes 1.5 fold ($p = 0.019$; *FDR* = 0.095; **Figure 24**). No particular taxon from this phylum was significantly affected by the treatment. Quantitative PCR showed that β-lactams doubled the microbial load from 1.4E+11 to 3E+11 16S rRNA copy number per g of faeces (Wilcoxon matched-pairs test, $p = 0.042$; **Figure 26B**).

From the 11 patients with β-lactams treatment, 7 of them took amoxicillin-clavulanate (amoxiclav). This drug combines amoxicillin, a β-lactam antibiotic and clavulanic acid, a β-lactamase inhibitor. Clavulanic acid inactivates bacterial β-lactamase and is used to enhance the antibacterial action of β-lactam antibiotics [White *et al*., 2004]. Sequence analysis showed that amoxiclav, as with β-lactams, significantly decreased microbial diversity metrics (Chao1 and observed species) by around 20% (**Table 6**) while increasing the ratio of Bacteroidetes/Firmicutes. Unlike all β-lactams together, this antibiotic showed an effect at lower taxonomic

levels. It increased Bacteroidia (p = 0.0005, *FDR* = 0.004) and Bacteroidales groups of Gram-negative bacteria (p = 0.0005, *FDR* = 0.005). At the species level, it induced a 20-fold increase in the proportion of an unknown taxon from the *Bacteroides* group. As for β-lactams, qPCR showed that amoxiclav doubled the microbial load from 1.86E+11 to 3.68E+11 16S rRNA copy number per gram of faeces (Wilcoxon matched-paired test, p = 0.07).

Effect of fluoroquinolones

Fluoroquinolones are broad-spectrum antibacterial agents; however, they show limited activity against anaerobic bacteria [Stein and Goldstein, 2006]. They play a marked role in treatment of nosocomial bacterial infections. They are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae* [Schaumann and Rodloff, 2007]. They inhibit the bacterial DNA gyrase (Gram-negative) and topoisomerase IV (Gram-positive) [Suto *et al.*, 1992; Drlica and Malik, 2003]. The 10 patients who took fluoroquinolones presented a significant increased ratio of Bacteroidetes (p < 0.0001; *FDR* < 0.001, **Figure 24**). But, unlike β-lactams, fluoroquinolones did not significantly increase the microbial load (**Figure 26C**). It also affected the gut microbiota down to the species level, by increasing 3 unknown taxa from the *Bacteroides* genus (p < 0.001; *FDR* < 0.08).

Out of 10 patients who took fluoroquinolones, 8 of them took levofloxacin, which has activity against Gram-positive and Gram-negative aerobic bacteria and atypical respiratory pathogens [Croom and Goa, 2003]. It is used to treat respiratory, urinary tract, gastrointestinal, and abdominal infections [Solomkin *et al.*, 2010]. Levofloxacin also increased the Bacteroidetes/Firmicutes ratio and decreased bacterial diversity by 25%. Like all fluoroquinolones tested, levofloxacin did not cause a clear increase of the microbial load. However, it significantly affected 14 bacterial taxa, out of which 10 unknown *Bacteroides* and 1 unknown *Coproccocus* were 3 to 56-fold increased and 1 unknown *Blautia* was 2-fold decreased (p < 0.01; *FDR* < 0.09) (**Table 7**).

FDR corrected	Proportion of sequences		Ratio after:before Levofloxacin	Taxa
	Before Levofloxacin	After		
0.0024	0.0001	0.0033	30.42	Unknown Bacteroides
0.0253	3.2e-05	0.0018	56.72	Unknown Bacteroides
0.0206	0.0001	0.0049	34.79	Unknown Bacteroides
0.0364	0.0002	0.0048	28.72	Bacteroides plebeius
0.0322	6.4e-05	0.0017	27.50	Unknown Bacteroides
0.0329	0.0144	0.1651	11.49	Unknown Bacteroides
0.0386	0.005	0.0785	15.79	Unknown Bacteroideceae
0.0491	0.0002	0.001	5.66	Unknown Coprococcus
0.0507	0.0072	0.0855	11.82	Unknown Bacteroideceae
0.0582	0.0018	0.0097	5.32	Unknown Bacteroides
0.0582	0.0004	0.0015	3.39	Unknown Bacteroides
0.0695	0.0008	0.0067	8.49	Unknown Bacteroides
0.0654	0.0021	0.0001	0.05	Unknown Blautia
0.0899	0.0004	0.0016	4.08	Unknown Ruminococcaceae
0.0886	0.0015	0.0096	6.57	Unknown Bacteroides

Table 7. Microbial taxa affected by Levofloxacin

DISCUSSION

Here we used qPCR and 454 pyrosequencing of the 16S rRNA gene to analyse the short-term effect of fluoroquinolone and β-lactam antibiotics on gut microbiota. Our results show that seven days of treatment greatly and globally disturbed the composition and structure of the gut microbial community. Indeed, regardless of the antibiotic type, our results showed that a decrease in the number of microbial taxa by approximately 25% was associated with an increase in Bacteroidetes groups (Gram-negative bacteria). More specifically, although previous works have shown that species from the *Bacteroides* genus such as *B. fragilis* were relatively sensitive to both amoxiclav and levofloxacin [White *et al*., 2004; Blandino *et al*., 2007; Stein *et al*., 2008] using culture methods, our study revealed that both drugs significantly increased several taxa from this genus.

Not surprisingly, our results are concordant with previous studies regarding the reduction in gut microbiota diversity. This decrease appears to be a common trait, independent of the type or dosages of antibiotics or the experimental model used

(human/animals) [Antonopoulos *et al*., 2009; Hill *et al*., 2010; Jakobsson *et al*., 2010; Manichanh *et al*., 2010; Perez-Cobas *et al*., 2013a].

However, our results contradict the general opinion regarding the effect of broad-spectrum antibiotics on gut microbiota. Indeed, instead of causing a decrease in both Gram-positive and Gram-negative bacteria [Schaumann and Rodlof, 2007], these drugs induce a significant increase in the latter.

Moreover, our unexpected results, which did not show a decrease in microbial load but instead a tendency towards an increase, are discordant with previous studies using either qPCR [Hill *et al*., 2010; Manichanh *et al*., 2010] or culture methods [Noverr *et al*., 2005; Ochoa-Reparaz *et al*., 2009; Ochoa-Reparaz *et al*., 2010; Umenai *et al*., 2010] (**Table 3**). These authors reported, as expected, a significant decrease in microbial load after 3 to 7 days antibiotic intake. To the best of our knowledge, studies using qPCR to measure microbial load with respect to antibiotic studies in human adults have not been reported.

The discrepancy between our study in humans in terms of microbial diversity or load and those performed in animal models could be due to the differences in the type of antibiotics and in the relative dosage administered. Indeed, for instance, Antonopoulos *et al*., 2009, used around 2500 fold more concentrated antibiotics (amoxicillin/metronidazole/bismuth) in a mouse model than in the present study. Ourselves, in a previous work using a rat model, we used 17 fold more concentrated antibiotics (vancomycin/ imipenem) [Manichanh *et al*., 2010].

Our study, although using a very high-throughput technique to study the effect of β-lactams and fluoroquinolones on the human microbiome, presents several limitations. Indeed, considering that this work involved participants with specific criteria of recruitment, we used a relatively small cohort. Furthermore, the design for future studies should also include a questionnaire related to diet or probiotic intake, in order to exclude any external contributing factors. Moreover, by analysing only two samples per participant, we did not perform a longitudinal study, which were already published by previous research groups [Dethlefson *et al*., 2008; Jakobsson *et al*., 2010]. Finally, our study does not distinguish the presence of viable from non-viable bacteria, which could be solved, in the future, by using a PCR-based method using propidium monoazide [Nocker *et al*., 2006].

The maintenance of or possible increase in microbial load associated to a decrease in diversity suggest that eviction of microorganisms sensitive to these group of antibiotics provides space for resistant strains to overgrow and dominate the niche. This microbial reshaping due to differential sensitivity to antibiotics might explain why resilience is not complete long after treatment cessation. Therefore the systematic use of these antibiotics could reshape the microbiota in favour of resistant bacterial strains in the long-term. Future studies involving other types of antibiotic could help understanding if the effects in this study could be generalised to other antibiotics.

Suchita Panda

CHAPTER 2

Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome

ABSTRACT

The pathophysiology of irritable bowel syndrome (IBS) remains unclear. Here we investigated the microbiome of a large cohort of patients to identify specific signatures for IBS subtypes. We examined the microbiome of 113 patients with IBS and 66 healthy controls. A subset of these participants provided two samples one month apart. We analysed a total of 273 faecal samples, generating more than 20 million 16S rRNA sequences. In patients with IBS, a significantly lower microbial diversity was associated with a lower relative abundance of butyrate-producing bacteria ($p = 0.002$; $q < 0.06$). No differences were found between IBS-C (constipation) and healthy controls whereas four microbial groups differentiated both groups from IBS-M (mixed) and IBS-D (diarrhoea). IBS patients who did not receive any treatment harboured a lower abundance of Methanobacteria compared to healthy controls ($p = 0.005$; $q = 0.05$). Furthermore, significant correlations were observed between several bacterial taxa and sensation of flatulence and abdominal pain ($p < 0.05$). Altogether, our findings showed that IBS-M and IBS-D patients are characterised by a reduction of butyrate producing bacteria, known to improve intestinal barrier function, and a reduction of methane producing microorganisms a major mechanism of hydrogen disposal in the human colon, which could explain excess of abdominal gas in IBS.

Suchita Panda

INTRODUCTION

A recent review reported that irritable bowel syndrome (IBS) affects around 11% of the population worldwide, with the lowest prevalence occurring in South Asia (7%) and the highest in South America (21%) [Canavan *et al*., 2014]. However, it should be noted that prevalence-reporting rates are subject to the diagnostic criteria used. Although most clinicians use the Rome criteria for this purpose [Longstreth *et al*., 2006], the lack of biological markers leads them to frequently resort to other clinical findings, such as bloating and psychological stress.

The pathophysiological mechanisms underlying IBS are not fully known. Abnormal gastrointestinal (GI) motility, visceral hypersensitivity, altered brain-gut function, low-grade inflammation, and psychosocial disturbance, have been recognised in different subsets of patients. In addition, the onset of IBS following infective gastroenteritis and the involvement of small bowel bacterial overgrowth (SIBO), suggest that gut microbes play a role in at least some of the mechanisms leading to IBS. Fermentation of polysaccharides by colonic microorganisms can produce a number of by-products (gases- H_2 and CH_4 (methane) and short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate) that may have important implications in bowel movement and epithelial permeability [Carbonero *et al*., 2012; Zaleski *et al*., 2013]. The current working hypothesis is that an abnormal microbial composition activates mucosal innate immune responses, which increase epithelial permeability, activate nociceptive sensory pathways, and dysregulate the enteric nervous system [Simren *et al*., 2013].

Table 8 reports the methods and main results gathered from 24 studies on IBS and microbiome using culture-independent techniques. Over the past ten years, most of these studies have used 16S rRNA gene (16S) surveys through quantitative specific polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescent in situ hybridisation (FISH) or cloning, and Sanger sequencing to characterise the microbiome of patients with IBS.

IBS subtypes (N) and Healthy controls (N)	Gender in IBS	Type of study	Treatment during the study	Sample type	Technique used	Sequence depth for sequencing techniques	Results	References
IBS-D (12); IBS-C (9); IBS-A (6); HC (22)	ND	Longitudinal (3 months interval)	Regular IBS symptoms	Faeces	qPCR (16S rRNA)	ND	Lower amount of *Lactobacillus* in IBS-D; higher amount of *Veillonella* in IBS-C; higher *Ruminococcus productus* - *Clostridium coccoides* in IBS; lower *Bifidobacterium catenulatum* in IBS	Malinen *et al*., 2005
IBS-D (12); IBS-C (9); IBS-A (5); HC (25)	19 females; 7 males	Longitudinal (0, 3 and 6 months)	IBS symptoms, antibiotics	Faeces	PCR-DGGE	ND	Increase of coliforms; increase of aerobe/anaerobe ratio, temporal instability in IBS but explained by antibiotics intake	Matto *et al*., 2005
IBS-D (7); IBS-C (6); IBS-A (3); HC (16)	11 females; 5 males	Longitudinal (0, 6 months)	ND	Faeces	PCR-DGGE; quantitative hybridisation-based technique, transcript analysis	ND	*Clostridium coccoides* - Eubacterium rectale group lower in IBS-C; higher instability in IBS	Maukonen *et al*., 2006
IBS-D (10); IBS-C (8); IBS-M (6); HC (23)	19 females; 5 males	Cross-sectional	ND	Faeces	Cloning-sequencing and qPCR	3,753 for all samples	Different bacterial structure in IBS compared to HC; differences in genera *Coprococcus*, *Collinsella*, and *Coprobacillus* between IBS and HC	Kassinen *et al*., 2007
IBS-D (14); IBS-C (11); IBS-A (16); HC (26)	29 females; 12 males	Cross-sectional	No medication that could influence microbial composition	Faeces; duodenal mucosa	FISH; qPCR	ND	Decrease of bifidobacteria (in faeces) and *Bifidobacterium catenulatum* (in faeces and duodenal samples) of IBS	Kerckhoffs *et al*., 2009
IBS-D (10); HC (23)	6 females; 4 males	Cross-sectional	ND	Faeces	(%G+C)-based profiling and fractioning combined with 16S rRNA cloning/sequencing; qPCR	3,267 sequencing for all samples	IBS-D is enriched in Proteobacteria and Firmicutes (Lachnospiraceae) but reduced in Actinobacteria and Bacteroidetes	Krogius-Kurikka *et al*., 2009
IBS-D (8); IBS-C (8); IBS-M (4); HC (15)	14 females; 6 males	Longitudinal (0, 3, 6 months)	IBS medication (mainly commercial fibre analogues, laxatives, or antidiarrhoeals)	Faeces	qPCR	ND	Microbiota of the IBS-D patients differed from other sample groups (*Clostridium thermosuccinogenes*-like, *Ruminococcus torques*-like); a *Ruminococcus bromii* -like phylotype was associated with IBS-C patients in comparison to controls	Lyra *et al*., 2009
IBS-D (10); HC (10)	7 females; 3 males	Cross-sectional	No probiotic	Faeces; mucosal biopsy	qPCR; culture	ND	Decreased concentration of aerobic bacteria in IBS-D; increase in *Lactobacillus* in IBS-D	Carroll *et al*., 2010
IBS (47); HC (33)	47 female	Cross-sectional	No medication	Faeces; colonic biopsies	DGGE	ND	Different microbial community in faeces and colonic biopsies; difference in the gut microbiota between faeces from IBS and HC	Codling *et al*., 2010
IBS (44)	33 females; 11 males	Cross-sectional	No medication	Faeces	qPCR	ND	*Ruminococcus torques*-like was associated with severity of bowel symptoms	Malinen *et al*., 2010

IBS-D (8); IBS-C (11); IBS-M (7); HC (26)	13 females; 13 males	Cross-sectional	ND	Faeces	qPCR; culture; organic acids analysis (HPLC); quantification of bowel gas (X-ray film)	ND	Quantity of gas similar in IBS and HC; high acetic and propionic acid correlate with worse GI symptoms; high count of *Veillonella* and *Lactobacillus* in IBS	Tana *et al*., 2010
IBS-D (16); HC (21)	12 females; 4 males	Cross-sectional		Luminal and mucosal samples	T-RFLP	ND	Lower microbial diversity in IBS-D	Carroll *et al*., 2011
IBS-D (13); IBS-C (11); IBS-A (13); HC (12)	ND	Cross-sectional	No medication	Faeces; mucosa- associated small intestinal	16S DGGE; qPCR; cloning sequencing	ND	*Pseudomonas aeruginosa* more abundant in faeces and small intestine in IBS	Kerckhoffs *et al*., 2011
IBS-D (25); IBS-C (18); IBS-A (19); HC (46)	57 females; 5 males	Cross-sectional	ND	Faeces	qPCR; 16S phylogenetic microarray (129 genus-like groups)	ND	Increased Firmicutes / Bacteroidetes ratio in IBS; increased Dorea, *Ruminococcus* and *Clostridium*, but decreased *Bifidobacterium* and methanogens in IBS; decreased *Faecalibacterium* in IBS-A and IBS-C; more Archaea in HC	Rajilic-Stojanovic *et al*., 2011
IBS-D (1); IBS-C (13); IBS-U (7); HC (22)	8 females; 14 males paediatrics (7- 12y)	Longitudinal (6 months)	ND	Faeces	16S pyrosequencing; phylogenetic microarray (8000 species)	54,200 reads per sample	Higher abundance of Gammaproteobacteria (*Haemophilus parainfluenzae*) and *Ruminococcus* in IBS; pain correlates with *Alistipes* genus	Saulnier *et al*., 2011
IBS-D (23); HC (23)	17 females; 6 males	Cross-sectional	No probiotics 2 months prior to study; no anti- inflammatories.	Faeces	16S pyrosequencing	8,232 reads V1-3 and 6,591 reads V6 per sample	Higher proportion of Enterobacteriaceae, lower *Faecalibacterium* and lower diversity in IBS-D	Carroll *et al*., 2012
IBS-C (14); HC (12)	14 females	Cross-sectional	No laxatives, antidiarrhoeal, antispasmodic, analgesic	Faeces	Culture of anaerobes; FISH	ND	Lower numbers of lactate-producing, lactate- utilising bacteria, H2-consuming populations, methanogens and reductive acetogens, but higher number of lactate- and H2-utilising sulphate-reducing bacteria in IBS-C	Chassard *et al*., 2012
IBS-D (14); HC (18)	3 females; 11 males	Cross-sectional	No medication	Faeces	Faecal BA profiles; q-PCR	ND	Bile acids higher in IBS-D and correlated with stool consistency and frequency; increase of *Escherichia coli* and a significant decrease of leptum and *Bifidobacterium* in IBS-D	Duboc *et al*., 2012
IBS-D (13); IBS-C (3); HC (9)	ND	Cross-sectional	ND	Faeces; Mucosal biopsies	16S pyrosequencing	268,000 non- chimeric reads	Less diversity in IBS; larger differences in the microbiota composition between biopsies and faeces than between patients and controls	Durban *et al*., 2012
IBS-D (15); IBS-C (10); IBS-A (12); HC (20)	26 females; 11 males	Cross-sectional	No medication	Faeces	16S pyrosequencing	30,000 reads per sample	Associations detected between microbiota composition and clinical or physiological phenotypes; two subgroups of IBS: normal-like and abnormal-like. The latter showed increased Firmicutes / Bacteroidetes ratio.	Jeffery *et al*., 2012

(N)	Gender/Age	Study design	Medication	Sample	Method	Reads	Findings	Reference
IBS-D (27); IBS-C (20); HC (26)	ND	Cross-sectional	ND	Rectal biopsies	FISH	ND	Greater numbers of total mucosa-associated bacteria in patients; greater *Bacteroides* and *Eubacterium rectale - Clostridium coccoides* in IBS; bifidobacteria were lower in the IBS-D group than in the IBS-C group and controls	Parkes *et al.*, 2012
IBS-D (22); HC (22)	12 females; 10 males Paediatrics (12.6 y)	Cross-sectional	No medication	Faeces	16S Phylogenetic microarray (775 phylotypes); 16S pyrosequencing; FISH; qPCR	A total of 13,882 sequence reads from the four chosen samples	Increased levels of *Veillonella*, *Prevotella*, *Lactobacillus*, and *Parasporobacterium* in IBS-D; No difference in alpha-diversity between IBS and HC; larger phylotype core set in HC than in IBS-D; *Bifidobacterium* and Verrucomicrobium less abundant in IBS-D; positive correlations between *Veillonella* and both *Haemophilus* and *Streptococcus*, between *Anaerovorax* and *Verrucomicrobium* and between *Tannerella* and *Anaerophaga*	Rigsbee *et al.*, 2012
IBS-D (2); HC (1)	2 females	Longitudinal: less diarrhoea (days 1, 3, and 7 in Patient 1; day 35 in Patient 2); severe diarrhoea (days 14, 28, 37, and 42 in Patient 1; days 3 and 28 in Patient 2).	Hypertension, arthrosis, osteoporosis for patient 1; bowel spasms and contraceptives for patient 2	Faeces	Metagenomics and metatranscriptomics by pyrosequencing	2,013,366 reads for metagenomics (7 samples); 1,729,416 reads for metatranscriptomic (32 samples)	Higher temporal instability in the fraction of active microbiota related to the IBS condition and fluctuating symptoms	Durban *et al.*, 2013
PI-IBS (11); PI-nonBD (12); PI-BD (11); IBS-D (12) HC (11)	29 females; 17 males	Cross-sectional	ND	Biopsy; faeces	Host gene expression by microarray; phylogenetic microarray (130 genus-like groups)	ND	27 genus-like groups (IMD) separating HC and patients; more uncultured Clostridia in HC; more Bacteroidetes members in patients; correlation between IMD and host genes involved in epithelial barrier functions in IBS	Jalanka-Tuovinen *et al.*, 2014

Table 8: Summary of studies on gut microbiota and IBS using 16S rRNA gene survey – Abbreviations explained as:

(N) = Number of participants
ND = No data
HC = Healthy controls
IBS-D = Diarrhoea-predominant IBS
IBS-C = Constipation-predominant IBS
IBS-M = Mixed IBS
IBS-A = Alternating IBS
IBS-U = unsubtyped IBS

PI-IBS = Post-infectious IBS
PI-BD = Persistent bowel dysfunction
PI-non-BD = no bowel dysfunction
FISH = Fluorescent in situ hybridisation
IMD = Index of Microbial Dysbiosis
16S = 16S rRNA gene
DGGE = Denaturing Gradient Gel Electrophoresis

Only since 2011 have a few studies used high-throughput techniques, such as 16S phylogenetic microarray, 16S and shotgun pyrosequencing, and metatranscriptomics.

Results from those studies showed several common trends, as well as inconsistencies in the microbial signatures of patients with IBS or subtypes of this condition. Among the trends, patients show dysbiotic microbiota, which can be characterised at various phylogenetic levels. At the phylum level, a higher proportion of Proteobacteria [Krogius-Kurikka *et al*., 2009; Saulnier *et al*., 2011] has been reported in patients compared to healthy controls. At the genus level, a higher count or proportion of *Veillonella* [Malinen *et al*., 2005; Tana *et al*., 2010] *Lactobacillus* [Lyra *et al*., 2009; Tana *et al*., 2010; Carroll *et al*., 2011; Rigsbee *et al*., 2012] and *Ruminococcus* [Malinen *et al*., 2005; Lyra *et al*., 2009; Rajilic-Stojanovic *et al*., 2011; Saulnier *et al*., 2011], has been associated with IBS. In contrast, a lower count of *Bifidobacterium* [Kerckhoffs *et al*., 2009; Rajilic-Stojanovic *et al*., 2011; Duboc *et al*., 2012; Rigsbee *et al*., 2012], *Faecalibacterium* [Rajilic-Stojanovic *et al*., 2011; Carroll *et al*., 2012], and methanogens [Rajilic-Stojanovic *et al*., 2011; Chassard *et al*., 2012] was encountered in patients with this condition. Among the inconsistencies, three studies reported a higher ratio of Firmicutes/Bacteroidetes in patients [Krogius-Kurikka *et al*., 2009; Rajilic-Stojanovic *et al*., 2011; Jeffery *et al*., 2012], while the opposite was found in one study [Jalanka-Tuovinen *et al*., 2014]. Lower [Maukonen *et al*., 2006] and higher [Parkes *et al*., 2012] counts of *Eubacterium rectal* were found in two studies. Also, it has been proposed that IBS involves a higher count of *Clostridium coccoides* [Malinen *et al*., 2005; Maukonen *et al*., 2006], while other authors reported a lower count of this species. Despite the replacement of culture methods by more powerful molecular techniques, these studies described small sample sizes, ranging from 2 to 62 IBS patients and did not consider confounding factors such as medications for IBS symptoms.

Using a high number of healthy controls (n = 66) and IBS patients (n = 113), two-time points for 94 participants, and a deep sequencing coverage of the 16S rRNA gene, we sought to determine: (a) whether dysbiosis occurs in patients with IBS

and, if so, the phylogenetic level that defines it; (b) whether the three IBS subtypes can be distinguished by microbial community clustering; (c) whether microbial diversity is lower in patients compared to healthy controls and, if so, which bacteria are absent; (d) the temporal stability of the microbial community in IBS patients; (e) the effect of medication on the microbiota of IBS patients and (f) the correlation between microbiota and the patients' symptoms.

MATERIAL AND METHODS

Ethics Statement

All experiments were performed in accordance to ethical guidelines. The subjects gave written informed consent to participate in this study. The protocol was submitted and approved by the local Ethical Committee of the University Hospital Vall d'Hebron, Barcelona, Spain.

Participants

Outpatients fulfilling Rome III criteria for IBS and healthy subjects were prospectively enrolled over 2.5 years into the study [Longstreth *et al.*, 2006]. All participants filled out a clinical questionnaire specifying stool form according to a Bristol stool scale, symptoms and medications. The Rome III criteria include recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months with symptom onset at least 6 months prior to diagnosis associated with 2 or more of the following: improvement with defecation; onset associated with a change in frequency of stool; onset associated with a change in form of stool. IBS was subtyped by predominant stool pattern for each patient. IBS with constipation (IBS-C) was defined by hard or lumpy stools ≥ 25% and loose (mushy) or watery stools ≤ 25% of bowel movements. IBS with diarrhoea (IBS-D) was defined by loose (mushy) or watery stools ≥ 25% and hard or lumpy stool ≤ 25% of bowel movements. Mixed IBS (IBS-M) was defined by hard or lumpy stools ≥ 25% and loose (mushy) or watery stools ≥ 25% of bowel movements.

Sample ID	IBS Type	Proton pump inhibitors	Laxatives	Anti-diarrhoeics	Pre/probiotics	Food avoided by patients
MO.01	IBS_D	Omeprazole				Natural tomato paste
MO.12	IBS_D	Omeprazole				
MO.13	IBS_D	Omeprazole				Milk, yogurt, spicy food
MO.15	IBS_D				VSL3	
MO.17	IBS_D	Omeprazole	Psyillium husk			
MO.20	IBS_D		Psyillium husk			
MO.21	IBS_D	Omeprazole				Raw vegetables, coffee, fruits, eggs, cheese, spicy food
MO.27	IBS_D	Omeprazole				Legumes, soup, chocolate, coffee, lactic products, greasy food, excessive water intake
MO.32	IBS_D	Pantoprazole				
MO.35	IBS_D					Greasy food
MO.39	IBS_D	Omeprazole				Vegetables
MO.47	IBS_D					Milk, greasy food, capsicum
MO.49	IBS_D		Lactitol Mono-hydrate			
MO.53	IBS_D	Rabeprazole				
MO.60	IBS_D	Rabeprazole				
MO.62	IBS_D	Omeprazole				Blue fish, cucumber, melon
MO.71	IBS_D					Gluten
MO.74	IBS_D				Ferzym plus®	
MO.82	IBS_D				Probiotic	
MO.87	IBS_D					Lactic food, legumes, fizzy drinks, fried food
MO.88	IBS_D		Psyillium husk			Bread, pasta
MO.98	IBS_D	Omeprazole				
MO.106	IBS_D			Loperamide		
MO.108	IBS_D	Omeprazole				Lactic food
MO.115	IBS_D					Greasy food
MO.120	IBS_D					Flour products, potato, legumes, lactic food
MO.75	IBS_C					Oil, greasy and fried food
MO.77	IBS_C		Lactitol Mono-hydrate			
MO.79	IBS_C	Omeprazole				
MO.80	IBS_C	Omeprazole				
MO.89	IBS_C					

ID	Group	Medication	Laxative/Fibre	Probiotic	Special diet/food
MO.92	IBS_C	Omeprazole	Psyillium husk		
MO.101	IBS_C			Bifilax	
MO.112	IBS_C	Esomeprazole			
MO.127	IBS_C		Psyillium husk		
MO.04	IBS_M	Pantoprazole			
MO.05	IBS_M				Vegetables, tomato paste, salsa
MO.08	IBS_M	Esomeprazole			
MO.10	IBS_M				Vegetables, skimmed milk
MO.16	IBS_M	Omeprazole	Psyillium husk		
MO.23	IBS_M	Omeprazole			
MO.24	IBS_M				Chicken and eggs
MO.28	IBS_M			Probiotic	Fried food, artichoke, capsicum, salsa, lactic food
MO.34	IBS_M	Omeprazole	Psyillium husk		
MO.36	IBS_M		Psyillium husk		Fried food, desserts, coffee, alcohol, salsa, fizzy drinks, lactic food
MO.48	IBS_M	Pantoprazole			
MO.59	IBS_M		Movicol®		
MO.65	IBS_M		Psyillium husk		
MO.66	IBS_M		Psyillium husk		
MO.76	IBS_M	Pantoprazole			
MO.81	IBS_M				Milk, lactic food
MO.109	IBS_M			Ultra levura®	
MO.121	IBS_M	Omeprazole			

Table 9: Medications that could have a direct impact on gut microbiota – Subjects not having taken any form of medications/ special diet are not listed here

Individuals (non-related (91%) and among the research and hospital environment (9%)) without gastrointestinal symptoms and matching age, gender and BMI with patients were included as healthy controls. The participants did not fill out a specific questionnaire on their diet habit, but none of them (healthy controls and patients) had a coeliac disease and none of them were following an extreme diet intervention such as low FODMAP diet. Information on medications that could

have a direct impact on gut microbiota is provided in **Table 9**. Participants were included if they had not taken antibiotics in the previous three months. Information about group size, age, gender and BMI is given in **Table 10**.

	N	Second Time Point n	Average Age	Gender	BMI[a]
Healthy Controls	66	22	37.6 (18–63, SD = 13)	40F/26M	23.7 (SD = 3.4)
IBS[b]	113	72	42.6 (20–86, SD = 13)	80F/33M	23.7 (SD = 4)
IBS-D[c]	54	33	41.9 (SD = 13)	29F/25M	25 (SD = 4.6)
IBS-C[d]	32	26	39.4 (SD = 10.8)	31F/2M	23.3 (SD = 3.8)
IBS-M[e]	27	13	48.2 (SD = 16.4)	32F/8M	23.9 (SD = 3.6)

Table 10. Clinical data from healthy controls and patients with IBS or other functional disorders - [a]Body Mass Index, [b]Irritable Bowel Syndrome, [c]Irritable Bowel Syndrome with diarrhoea, [d]Irritable Bowel Syndrome with constipation, [e]Mixed Irritable Bowel Syndrome

Sample collection and genomic DNA extraction

Faecal samples were collected from the 179 participants (66 healthy controls and 113 IBS patients). In order to study the variability of the microbiome over time, 22 healthy controls and 72 IBS patients (IBS-D, n = 33; IBS-C, n = 26 and IBS-M, n = 13) provided a second stool sample one month after the first. After collection and homogenization, samples were immediately frozen by the participants in their home freezer at -20ºC and later brought to the laboratory in a freezer pack, where they were stored at -80°C. A frozen aliquot (250mg) of each sample was suspended in 250µl of guanidine thiocyanate, 0.1M Tris (pH 7.5), 40µl of 10% N-lauroyl sarcosine and 500µl 5% N-lauroyl sarcosine. DNA was extracted by mechanical disruption of the microbial cells with beads, and recovery of nucleic acids from clear lysates was achieved by alcohol precipitation, as previously

described [Godon *et al*., 1997; Santiago *et al*., 2014]. An equivalent of 1mg of each sample was used for DNA quantification using a NanoDrop ND-1000 Spectrophotometer (Nucliber©). DNA integrity was examined by micro-capillary electrophoresis using an Agilent 2100 Bioanalyzer (Applied Biosystem©) with the DNA 12,000 kit, which resolves the distribution of double-stranded DNA fragments up to 17,000bp in length.

High-throughput DNA sequencing

For profiling microbiome composition, the hyper-variable region (V4) of the bacterial and archaeal 16S rRNA gene was amplified by PCR. On the basis of our analysis done using Primer Prospector software [Walters *et al*., 2011], the V4 primer pairs used in this study were expected to amplify almost 100% of the bacterial and archaeal domains. The 5' ends of the forward (V4F_515_19: 5'-GTGCCAGCMGCCGCGGTAA -3') and reverse (V4R_806_20: 5'-GGACTACHVGGGTWTCTAAT -3') primers targeting the 16S gene were tagged with specific sequences as follows: 5'-{AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGT} {GTGCCAGCMGCCGCGGTAA}-3' and 5'-{CAAGCAGAAGACGGCATACGAGAT} {Golay barcode} {AGTCAGTCAGCC} {GGACTACHVGGGTWTCTAAT}-3'. Multiplex identifiers, known as Golay codes, had 12 bases and were specified downstream of the reverse primer sequence (V4R_806_20) [Caporaso *et al*., 2012; Navas-Molina *et al*., 2013].

Standard PCR (0.75 units of Taq polymerase (Roche©) and 20pmol/µL of the forward and reverse primers) was run in a Mastercycler gradient (Eppendorf©) at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec, and a final cycle of 72°C for 10 min. Amplicons were first purified using the QIAquick PCR Purification Kit (Qiagen©), quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber©), and then pooled in equal concentration. The pooled amplicons (2nM) were then subjected to sequencing using Illumina© MiSeq technology at the technical support unit of the Autonomous University of Barcelona (UAB, Spain) following standard Illumina platform protocols.

Sequence data analysis

We loaded the raw sequences into the QIIME 1.8.0 pipeline for analysis, as described by Navas-Molina *et al*., 2013. The first step was to filter out quality sequence reads by applying default settings and a minimum acceptable Phred score of 20. Correct primer and proper barcode sequences were also checked. After filtering, from 290 faecal samples we obtained a total of 12,494,196 high-quality sequences with a read length ranging from 237 to 307 nucleotides; 73% of the reads had a length of 306 nucleotides.

We used the UCLUST [Edgar, 2010] algorithm to cluster similar filtered sequences into OTUs based on a 97% similarity threshold. Then, we identified and removed chimeric sequences using ChimeraSlayer [Haas *et al*., 2011]. Since each OTU can comprise many related sequences, we picked a representative sequence from each one. Representative sequences were aligned using PyNAST against Greengenes template alignment (gg_13_8 release), and taxonomy was assigned to the detected OTUs using the basic local alignment search tool (BLAST) reference database and the Greengenes taxonomy-mapping file. The script make_phylogeny.py was used to create phylogenetic trees using the FastTree program [Price *et al*., 2009]. To correctly define species richness for the analysis of between-sample diversity, known as beta diversity, the OTU table was rarefied at 16,800 sequences per sample. Rarefaction is used to overcome cases in which read counts were not similar in numbers between samples. Next, the OTU table was split into the different groups (Diarrhoea (IBS-D), Constipation (IBS-C), Mixed (IBS-M) IBS, and Healthy Controls (HC)). In order to avoid false positive taxa, OTUs that did not represent at least 0.2% of sequences for any given sample were removed from the resulting OTU table. The summarize taxa feature was used to classify taxa from the Domain to the Species level. To provide community alpha diversity estimates, we calculated Chao1 [Chao, 1984]. To calculate between-sample diversity, weighted and unweighted Unifrac metrics were applied to build phylogenetic distance matrices, which were then used to construct hierarchical cluster trees using unweighted pair group method with arithmetic mean (UPGMA) and PCoA representations. Finally, distance-based redundancy analyses were performed using the eigenvalues obtained in the PCoA.

Statistical and correlation analyses

Statistical analyses were carried out in QIIME. To work with normalised data, we analysed an equal number of sequences from both healthy and patient groups. The Shapiro-Wilk test [Shapiro and Wilk, 1965] was used to check the normality of data distribution. The Kruskal Wallis one way analysis of variance [Kruskal and Wallis, 1952], a non-parametric test, was used to compare the mean number of sequences of the groups, i.e. that of different groups of patients based on distinct parameters with that of healthy subjects, in various taxonomic levels. Since we used nonparametric correlations, significance was determined through permutations. The analysis provided false discovery rate (*FDR*) corrected *p*-values (*q*).

Statistical significance of observed differences between sample groups was measured by the t-test, with *p*-values < 0.05 considered significant for all tests. Non-parametric Spearman's correlation coefficient was used to calculate possible relationships among microbial genera and variables in clinical data such as abdominal pain and sensation of flatulence.

RESULTS

Sequence data description

Faecal samples were collected from 66 healthy controls and from 113 IBS patients. Of the 179 participants, 94 provided two faecal samples 1-month apart. We therefore analysed the microbiome of a total of 273 samples, from which we produced 20.5 million high quality sequence reads of 314bp in length, using the default Quantitative Insights Into Microbial Ecology (QIIME) pipeline parameters and a quality Phred score > 20. Additionally, we removed singletons and taxa with very low abundance that could represent false positive taxa, as described in the method section. We finally recovered a total of 14 million sequences with a mean of 48,409 sequences per sample. In order to compare microbial community between samples, we normalised the sequences at 16,800 per sample (fitting to a sample with the lowest sequence number).
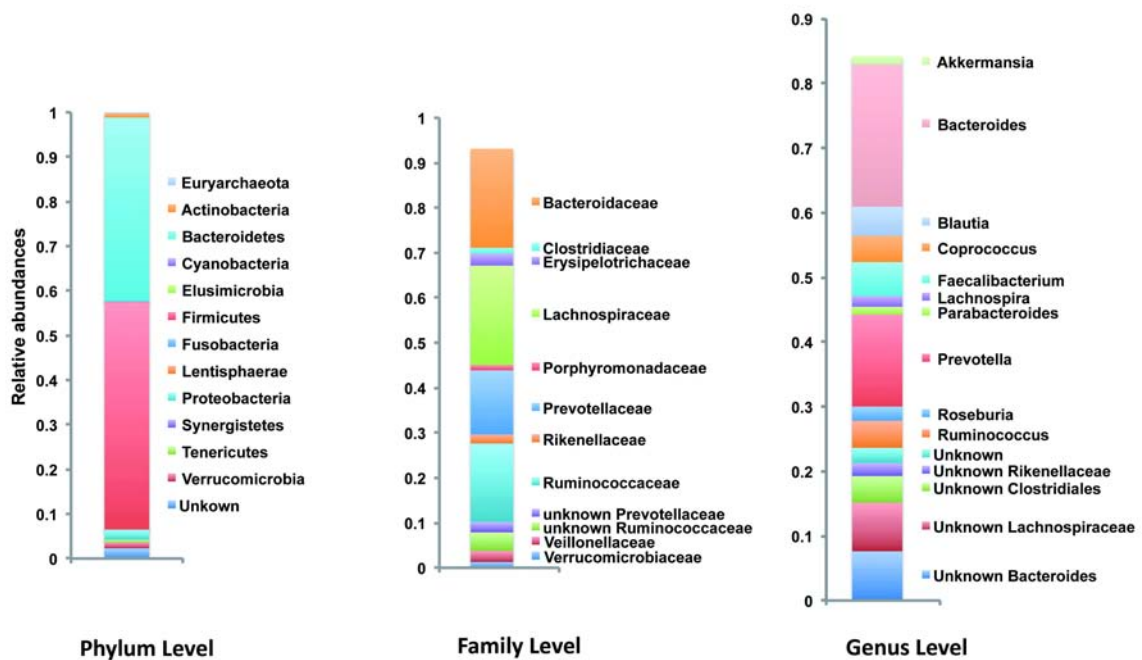
Figure 27: Diversity of the human microbiome at various phylogenetic scales – Using the 16S rRNA sequence data of 66 healthy controls, the average relative abundance of each microbial group is represented at phylum, family and genus level.

Microbiota of the healthy controls

Of the 66 healthy controls, we detected 12 known phyla, 43 families, 84 genera (**Figure 27**) and 2,126 operational taxonomic units (OTUs), with a mean of 629 OTUs (SD = 132) per sample. Firmicutes and Bacteroidetes accounted for 91% of the sequences, and Proteobacteria, Verrucomicrobia and Actinobacteria 4.9%. At the family level, 85% of the sequences were assigned, 65% at the genus level and only 0.6 % at the species level. This observation indicates that although databases of 16S rRNA sequences are increasing exponentially, the lack of annotation continues to be a bottleneck for the scientific community. Our findings thus revealed the predominance of Firmicutes in the proportion of sequences per subject and in the proportion of the healthy population. Two OTUs, *Faecalibacterium prausnitzii* and an unknown Ruminococcaceae, were detected in all individuals and at the two sampling points (88 samples in total), thereby indicating that these OTUs were not only highly prevalent but also stable over time. They were found at the average proportions of 5.3% and 0.23%, respectively.
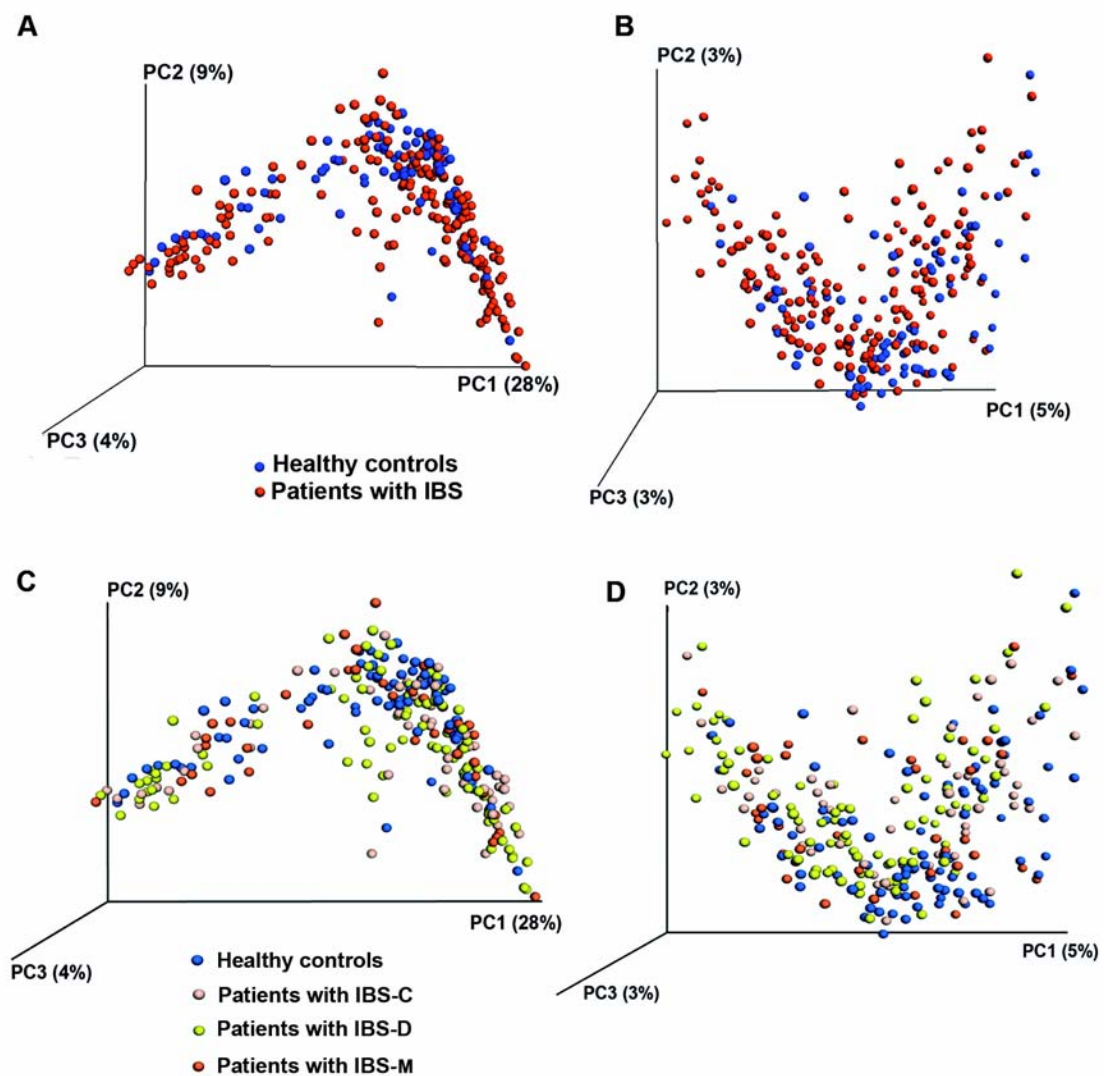
Figure 28: Absence of sample clustering by health status at the first time point – A) Weighted UniFrac PCoA plot of the whole dataset and, B) unweighted UniFrac PCoA plot of the whole dataset, C) weighted UniFrac PCoA plot of the controls and three IBS subtypes and, D) unweighted UniFrac PCoA plot of the controls and the three IBS subtypes - illustrate that samples from healthy controls and patients with IBS did not cluster separately.

Level of dysbiosis among IBS patients

At a global level, the microbial communities of healthy controls and patients did not cluster separately; according to Unifrac metrics in a principal coordinate analysis (PCoA, **Figure 28**). However, distance-based redundancy analysis showed that the microbiome of all IBS patients, as well as that of particular IBS subtype patients, clustered separately from that of healthy controls (p = 0.002 and p =

0.001, respectively) (**Figure 29**), although only 1 and 3% of the data explained the variation observed in IBS and IBS subtypes, respectively.
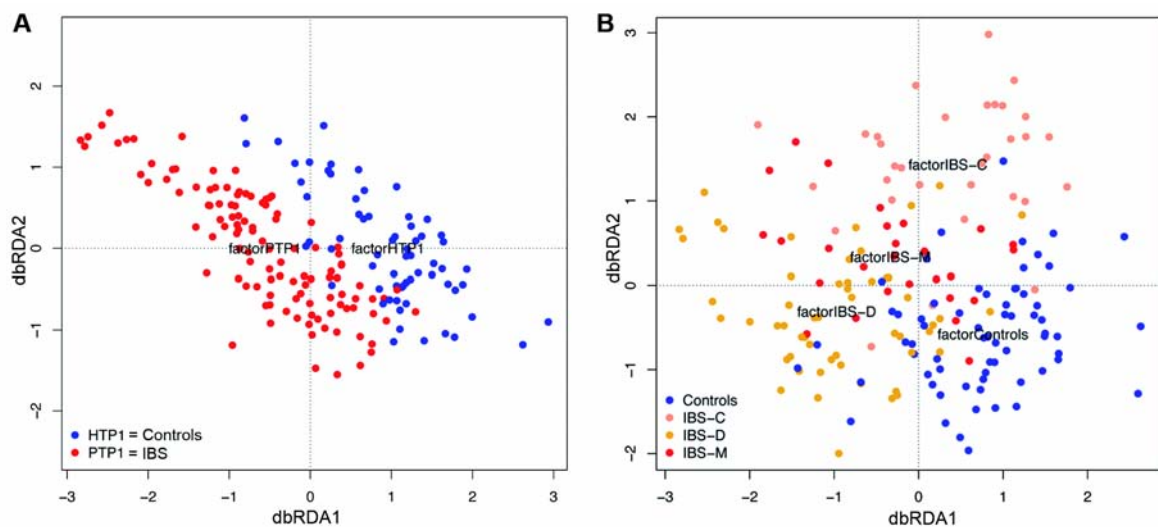
Figure 29: Unweighted UniFrac data redundancy analysis on the first time point samples constrained by A) controls and IBS patients groups, and B) constrained by the four groups of participants: controls (n = 66), IBS-C (n = 32), IBS-D (n = 54) and IBS-M (n = 27).

Using the Kruskal Wallis test to compare healthy controls (n = 66) with IBS patients (n = 133), dysbiosis was indeed present at various phylogenetic levels. At the phylum level, there is a tendency for IBS patients to harbour a higher average count of Bacteroidetes compared to healthy controls (52.6% versus 42.7%; *p* = 0.02, *q* = 0.09) and a lower count of Firmicutes (39.8% versus 49%; *p* = 0.02, *q* = 0.09). Furthermore, 41% of IBS patients (versus 50% in healthy subjects) harboured a higher relative abundance of Firmicutes than Bacteroidetes (**Figure 30**). Compared to healthy controls, IBS patients also showed a lower count of Tenericutes (*p* = 0.004; *q* = 0.05). At the family level, two Firmicutes groups, Erysipelotrichaceae and Ruminococcaceae, were found significantly in a higher proportion in healthy controls than in patients (**Figure 31A**) (*p* = 4.7 e-5, *q* = 0.002 and *p* = 0.002, *q* = 0.06, respectively). At the species level, one OTU from the *Lachnobacterium* genus (Lachnospiraceae, Firmicutes) differentiated healthy subjects from IBS patients (*p* = 5.9 e-6, *q* = 0.04). This OTU, which belongs to the Lachnospiraceae family and the Firmicutes phylum, was detected in 15 out of 66

controls (22%) and in only 2 out of 113 patients (1.7%) at a very low proportion of sequences, 0.0001 and 0.00002 respectively. *Faecalibacterium prausnitzii* present in all 66 healthy controls, were also detected in at least 95% of the 113 patients and did not show significant differences in abundance. This observation indicates that this bacterium is highly prevalent but probably not involved in IBS, as proposed by others [Rajilic-Stojanovic *et al*., 2011].
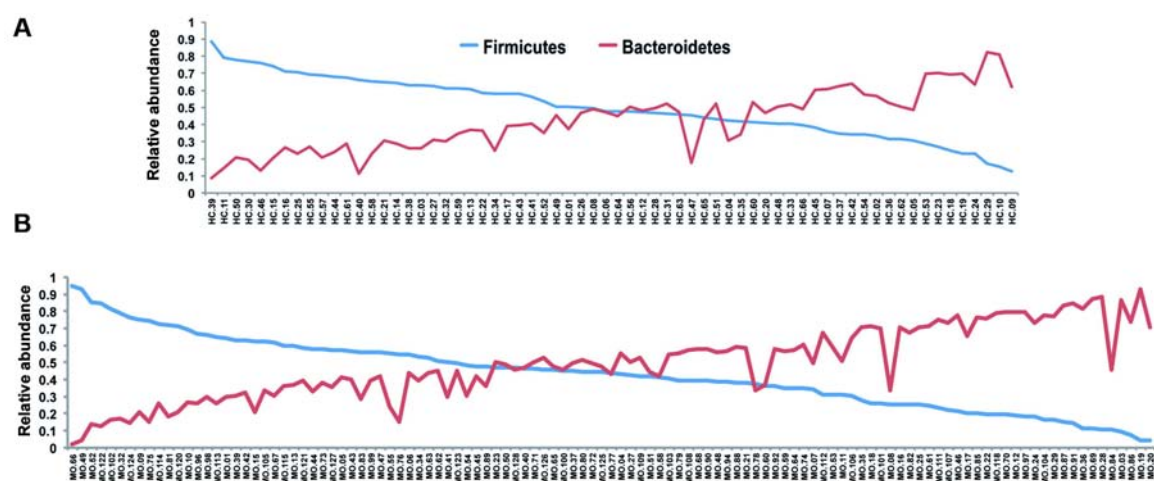
Figure 30: Higher relative abundance of Bacteroidetes - Proportion of Firmicutes and Bacteroidetes are plotted for A) each healthy subject and, B) for each IBS patient. Patients are characterised by a relatively higher proportion of Bacteroidetes than healthy controls (p = 0.02, q = 0.09)

Microbiota and IBS subtypes

The diversity analysis showed that, overall, patients presented a lower diversity of gut microbiota than healthy controls (p < 0.01), especially as a result of a reduction in the diversity of the IBS-D subtype (p < 0.05; **Figure 31B**). This result suggests that the microbial species that contribute to maintaining homeostasis may be missing in patients with a diarrhoeic symptom. Therefore, we compared the microbial communities of healthy participants (n = 66) with those of each IBS subtype using the Kruskal Wallis test. Patients with IBS-D (n = 54) showed dysbiosis at various phylogenetic levels. **Figure 32A** shows the lower abundant groups at the family (Ruminococcaceae, unknown Clostridiales,

Erysipelotrichaceae, Methanobacteriaceae; $p < 0.006$; $q <0.06$) and genus levels (unknown Ruminococcocaceae; $p = 0.0001$, $q = 0.009$), respectively.
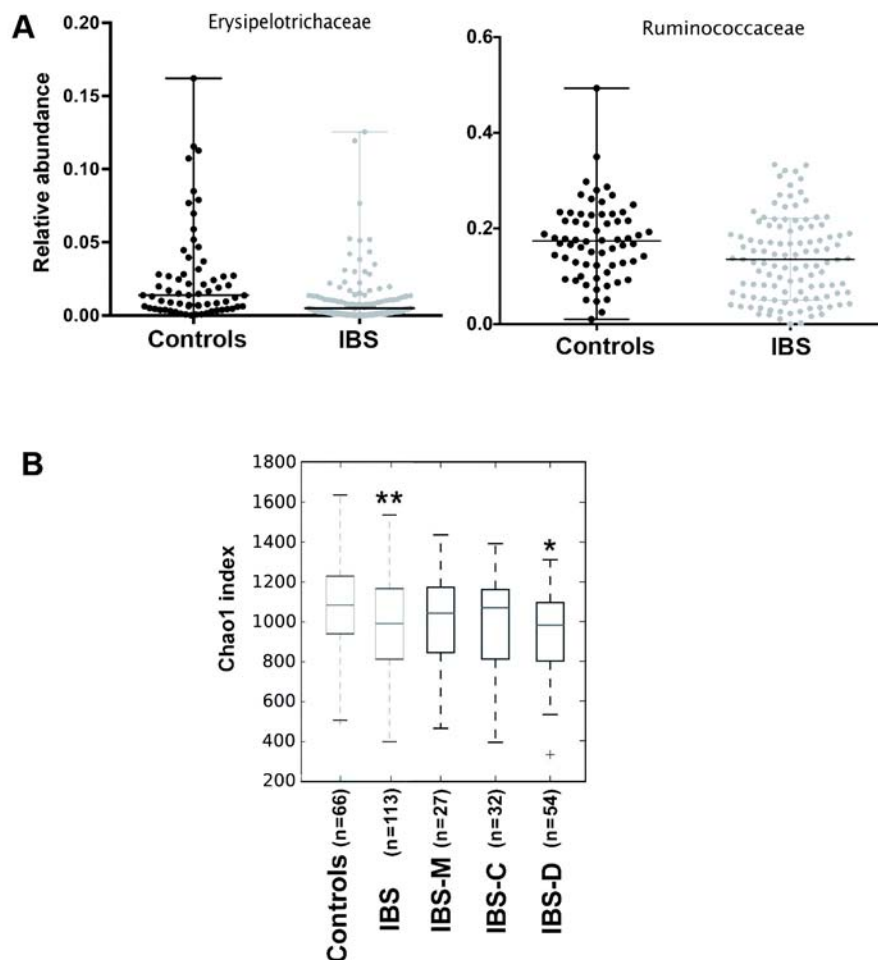
Figure 31: Higher relative abundance of two bacterial families and higher alpha-diversity in healthy controls compared to IBS patients – A) Erysipelotrichaceae and Ruminococcaceae were found in significantly higher abundance in healthy subjects (n = 66) compared to IBS patients, regardless of their IBS subtype (Kruskal Wallis test, $p = 4.7$ e-5, $q = 0.002$ and $p = 0.002$, $q = 0.06$, respectively). The two bacterial families belong to the Firmicutes phylum. B) The Chao1 index based on species-level OTUs was estimated for healthy controls, IBS, IBS-M, IBS-C and IBS-D. Significance (*$p = 0.04$, ** $p < 0.003$) was determined by Monte Carlo permutations, a non-parametric test.

The microbiome of IBS-C patients did not show significant differences at any phylogenetic level with that of healthy controls. However, IBS-M patients, who

alternate diarrhoea and constipation, were associated with a 4.7-fold lower abundance of Erysipelotrichaceae (p = 0.0001, q = 0.006; **Figure 32B**), sharing this difference with IBS-D patients. Our findings suggest that this bacterial group, which is absent in IBS-M and IBS-D, is associated with a diarrhoeic phenotype.

Comparing the healthy control group with the three IBS subtypes, we found that four genera were significantly different between healthy subjects/IBS-C patients and IBS-M/IBS-D patients. Indeed, an unknown Ruminococcaceae, an unknown Christensenellaceae, *Akkermansia*, and *Methanobrevibacter* were present in a higher proportion in healthy controls and IBS-C patients (p < 0.003, q ≤ 0.05; **Figure 32C**).

IBS patients and treatment

Regarding treatment, the inclusion criteria for all participants was only that they had not taken any antibiotics during the 3 months prior to stool collection. However, among the 113 patients with IBS, 69 of them reported following a treatment for IBS symptoms during the study. These treatments included laxatives (n = 13), proton pump inhibitors (n = 25), pre/probiotics (n = 6) (**Table 9**), and other medications, such as anti-depressants or anxiolytic drugs (n = 29). Since medications could have an effect, we explored their potential role on modulating the gut microbiome. For this, we re-analysed the whole dataset, on the one hand, separating the patients who were under treatment that could affect the microbiome composition and on the other hand, grouping them according to the treatment type that could directly affect the microbial composition (laxative, proton pump inhibitors, or probiotics/prebiotics) or indirectly (anti-depressants or anxiolytic drugs) affect the microbial composition. We were unable to take into account the effect of other drugs, since the number of patients for each of the drugs was too small and did not allow statistical analysis.
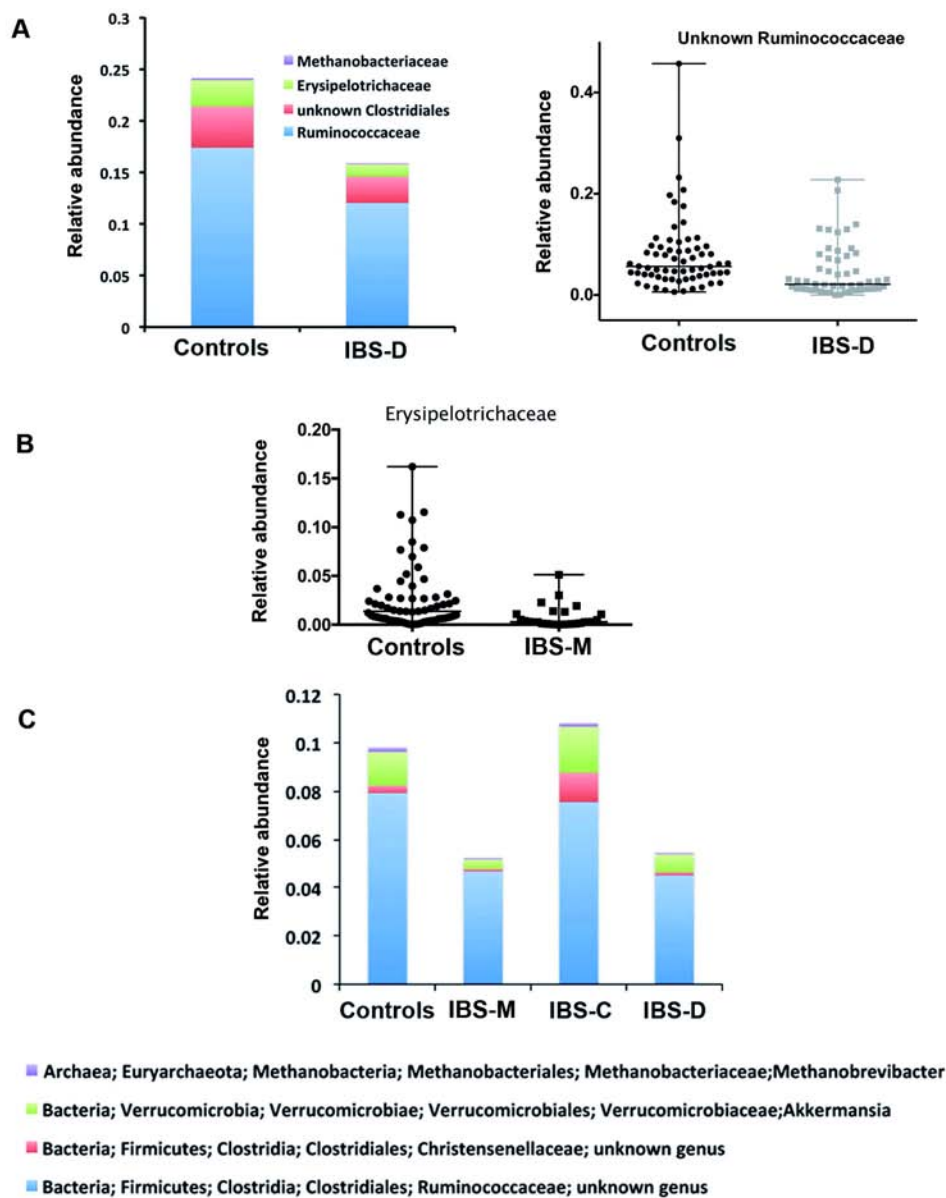
Figure 32: Dysbiosis at the family and genus level in IBS subtypes – A) Four microbial families and one genus discriminate the 66 healthy controls from the 54 patients with IBS-D (Kruskal Wallis test, $p < 0.006$; $q < 0.06$). B) One bacterial family was found in a lower proportion in the 27 IBS-M patients compared to the 66 controls ($p = 0.0001$, $q = 0.006$). C) Comparing the healthy control group with the three IBS subtypes, four genera were enriched in healthy subjects and IBS-C patients compared to IBS-M and IBS-D patients (Kruskal Wallis test, $p < 0.003$, $q \leq 0.05$).

Removing patients who were under treatment that could affect the gut microbiome composition such as laxative, proton pump inhibitors, or probiotics/prebiotics, we re-analysed a cohort of 73 patients (25 patients with IBS-C, 35 patients with IBS-D, 13 patients with IBS-M). Alpha-diversity analysis using the chao1 index showed that IBS patients without treatment and without classification in subtypes continued

to present a lower diversity of microbes compared to healthy controls (p = 0.002) (**Figure 33**). However, significance was not reached when comparing healthy controls with each of the IBS subtypes and only IBS-D showed a trend towards a lower diversity (p = 0.09). Stability analysis also confirmed that neither the microbiome of healthy controls nor that of the IBS patients as a whole group or as subtypes differed significantly over the one-month sampling period. Distance-based redundancy analysis showed that, although only 3% of the data explained the variation, the microbiome of patients still clustered separately on the basis of their subtypes and also separately from that of the healthy controls (p = 0.001) (**Figure 34**). Also, as shown in the data without discarding patients under treatment, we confirmed that Erysipelotrichaceae was in lower relative abundance in patients (p = 0.001; q = 0.04). Ruminococcaceae was also found in lower relative abundance in patients, although the difference was not significant (p = 0.03; q = 0.4). Interestingly, Methanobacteriaceae was in lower abundance (10 fold) in patients with IBS-D and IBS-M compared to healthy controls (p = 0.004; q = 0.05).
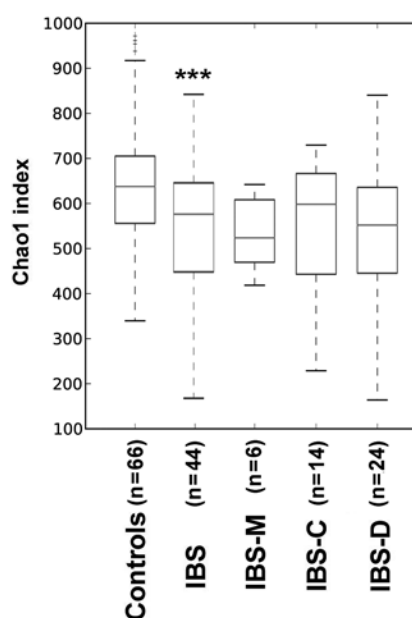
Figure 33. The Chao1 index based on species-level OTUs was estimated for healthy controls, IBS, IBS-M, IBS-C and IBS-D patients, taking into account only participants not receiving treatment. Significance (***p = 0.002) was determined by Monte Carlo permutations, a non-parametric test.
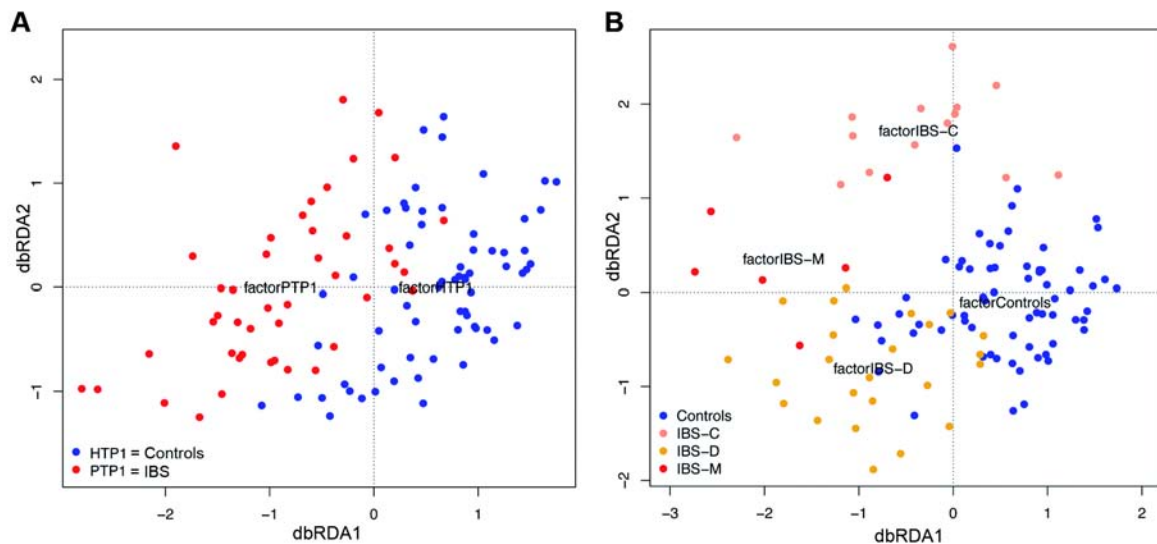
Figure 34: Unweighted UniFrac data redundancy analysis (dbRDA) on the first time point samples constrained by A) the controls and IBS patients, and constrained by B) the four groups of participants: controls (n = 66), IBS-C (n = 14), IBS-D (n = 24) and IBS-M (n = 6), discarding patients under treatment

Among the medications, we uncovered a significant effect only for the proton pump inhibitors. Indeed, patients taking proton pump inhibitors such as omeprazole (n = 25), during the study presented a 37-fold higher proportion of Pasteurellales (p = 0.002, q = 0.05), a Gammaproteobacteria group, compared to those who did not receive any treatment. At the genus level, *Haemophilus* (belonging to Pasteurellaceae) maintained this effect (p = 0.001, q = 0.08). When we compared controls (n = 66) with patients without treatment (n = 44), Methanobacteriales at the class level was also enriched in healthy controls by 10-fold (p = 0.005; q = 0.05) (**Figure 35**). This group of microbes remains significantly in higher abundance in controls when compared with IBS-D patients without treatment (n = 24; p = 0.0019; q = 0.038). In the same group of patients, microbes from the Erysipelotrichi also showed a tendency of decrease (p = 0.0019; q = 0.13). IBS-C patients without treatment (n = 14) presented a higher level of Methanomassiliicoccaceae, belonging to methanogenic Archeae (p = 0.0001; q = 0.015). No significant differences were found between healthy subjects and IBS-M patients (n = 6), probably due to a too small sample size.
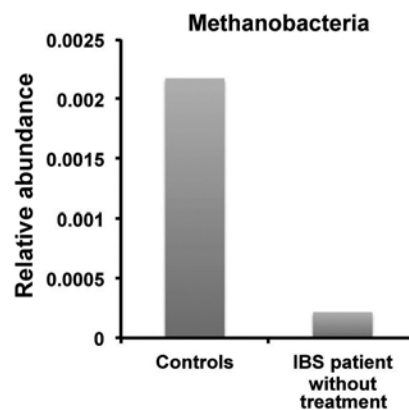
Figure 35: Abundance of methanobacteria - Methanobacteria from the Euryarchaeota phylum is enriched in controls (n = 66) compared to IBS patients (n = 44) who did not follow any medical treatment (Kruskal Wallis test, *p* = 0.005, *q* = 0.05)

Stability of the microbiota of IBS patients

Previous studies have reported the instability of the gut microbiome of IBS patients as a signature of the disease. Comparing faecal samples from patients (the whole cohort (n = 77) or without patients under treatment (n = 47)) taken one month apart, we did not detect significant differences in the microbial composition between the two time points for the IBS subtypes and as for the 22 healthy controls and no changes in microbiome associated with changes in symptoms, suggesting a stable gut microbiota over a relatively short time, which is in agreement with a previous comprehensive study by Faith *et al*., 2013. Indeed, in a previous work, we showed that instability of the gut microbiota was only conditioned by a challenge with diet enriched in fibres [Manichanh *et al*., 2014].

Correlation with symptoms

Taking into account only patients who did not take any medication (n = 44), we examined the correlation between microbial composition and IBS symptoms, such as sensation of flatulence and abdominal pain. Subjective sensations of flatulence are defined as anal gas evacuation, abdominal bloating (pressure/fullness), abdominal distension (girth increment), borborygmi and abdominal discomfort/pain, as described in our previous work [Manichanh *et al*., 2014]. The highest level of sensation of flatulence (level 6 in a scale of 0 to 6) was positively correlated with three OTUs from Lachnospiraceae, one belonging to the *Blautia*

genus (r from 0.40 to 0.48, *p* < 0.006). The highest level of abdominal pain (level 6 in a scale of 0 to 6) was moderately and positively correlated with three bacterial genera, specifically *Bacteroides* (r = 0.46, *p* = 0.002) and *Ruminococcus* (r = 0.42, *p* = 0.004), and an unknown Barnesiellaceae (r = 0.30, *p* = 0.041). Abdominal pain was also moderately and negatively correlated with three other genera, namely *Prevotella* (r = -0.44, *p* = 0.003) and *Catenibacterium* (r = -0.35, *p* = 0.019), and a genus from the Erysipelotrichaceae family. The latter was also observed in a higher proportion in healthy controls compared to IBS patients.

DISCUSSION

To explore the gut microbiota in IBS, we compared the microbial communities of IBS patients with those of healthy controls using the 16S gene survey and Illumina technology. Our findings revealed that IBS patients based on their subtype clustered separately from healthy controls at a global level. Indeed, at various phylogenetic levels we showed that major differences were found between controls and IBS patients, and revealed differences between IBS subtypes, correlation of groups of bacteria with sensation of flatulence and abdominal pain, and an effect of medication on gut microbiota.

At the phylum level, our findings corroborate the results reported by Jalanka *et al*., 2014 but differ from those of Krogius-Kurikka *et al*., 2009, Rajilic-Stojanovic *et al*., 2011 and Jeffery *et al*., 2012 who related higher Firmicutes and lower Bacteroidetes in IBS patients. The differences in the proportion of Gram-positive bacteria (Firmicutes) versus Gram-negative bacteria (Bacteroidetes) could be related to various factors, such as small sample size, an absence of mechanical disruption of the microbial cell wall during the DNA extraction procedure, and the DNA extraction method itself or the sequencing platform, as demonstrated by Lozupone *et al*., 2013 and by our recent work [Santiago *et al*., 2014]. In this previous study, by freezing the faecal samples immediately after collection, using mechanical disruption during DNA extraction, performing deep sequencing using Illumina technology, and normalizing the sequences per sample, we optimised all

the steps from stool collection to sequence analysis. Moreover, we previously showed that the percentage of water typically found in diarrhoeic samples does not affect the microbial composition of samples from the same subjects [Santiago *et al*., 2014].

Our results indicate that dysbiosis in IBS-D involved dominant microbial groups such as Ruminococcaceae and unknown Clostridiales, as well as much less dominant ones such as Erysipelotrichaceae and Methanobacteriaceae. Regarding Methanobacteriaceae (Archaebacteria), our finding is in agreement with previous studies showing a lower count of methanogens in IBS patients [Rajilic-Stojanovic *et al*., 2011; Chassard *et al*., 2012]. Interestingly, methane levels were reported to be higher in patients with slow-transit constipation compared with normal-transit constipation and non-constipated controls [Attaluri *et al*., 2010; Sahakian *et al*., 2010]. Our results, showing that only patients with a constipation phenotype presented higher abundance of methanogenic Archaea, confirmed a link between low transit time and methane production capacity. In the colon, methanogens and sulphate- reducing bacteria are the primary hydrogen-consuming microbes, converting this gas into methane or hydrogen sulphide, respectively [Pimental *et al*., 2006a]. However, we did not find any increase in sulphate-reducing bacteria associated with the decrease in methanogens. Furthermore, a recent metatranscriptomic study [Franzosa *et al*., 2014] showed that *Methanobrevibacter smithii* tends to be highly transcriptionally active relative to other species in the gut, suggesting that IBS patients compared to healthy subjects lack the functions for hydrogen removal.

Regarding Ruminococcaceae, unknown Clostridiales and Erysipelotrichaceae, known as being butyrate-producing bacteria, our results suggest that a reduction of these bacteria could decrease availability of butyrate and therefore to increase the epithelial permeability, which has been previously associated with patients with IBS-D [Dunlop *et al*., 2006]. An impaired epithelial barrier function has been proposed as a potential mechanism causing passage of microbes or their products through the barrier to other body sites, which might affect symptoms through interaction with immune and nerve cells in the gut wall.

We did not find significant differences between healthy controls and IBS-C patients. Thus, our results did not confirm those obtained previously showing a higher abundance of *Veillonella* and *Ruminococcus* in this IBS subtype [Malinen *et al.*, 2005; Lyra *et al.*, 2009; Tana *et al.*, 2010; Rajilic-Stojanovic *et al.*, 2011; Saulnier *et al.*, 2011; Rigsbee *et al.*, 2012], but showed that a group of IBS patients may harbour normal-like microbiota. Altogether, this finding suggests that any attempt to modulate the gut microbiome composition of IBS patients should take into account that all IBS patients may not respond equally to the treatment. On the basis of our results, we propose a new monitoring approach for at least two of the IBS subtypes, as a combination of four genera (an unknown Ruminococcaceae, an unknown Christensenellaceae, *Akkermansia* and *Methanobrevibacter*), which may have the capacity to discriminate healthy controls and IBS-C patients from IBS-M and IBS-D patients.

To our knowledge, our study is the first to take into account the effect of medication and proposes a cohort size large enough to perform analyses taking into account patients under treatments, which could be a confounding factor for microbiome analysis. Indeed, removing patients receiving a treatment allowed us to uncover a lower relative abundance of Methanobacteriales in IBS-D and IBS-M but a higher relative abundance of Methanomassiliicoccaceae in IBS-C. Furthermore, our results indicate that patients taking proton pump inhibitors show a significant increase in *Haemophilus*. This genus belongs to the Pasteurellaceae family and the Gammaproteobacteria class. A higher count of Gammaproteobacteria has also been reported by Krogius-Kurikka *et al.*, 2009 and Saulnier *et al.*, 2011, but it has not been associated with the administration of proton pump inhibitors. Our result is also in line with the findings of a previous study in which the authors isolated more *Haemophilus* sp. by culture from subjects under omeprazole treatment [Klinkenberg-Knol *et al.*, 2000]. The potential adverse effects of this medication should, therefore, be addressed in greater depth in future studies. Finally, our study revealed a group of bacteria that correlates with IBS symptoms. Indeed, sensation of flatulence correlated with a high relative abundance of Lachnospiraceae and *Blautia*, while abdominal pain correlated with

a high relative abundance of *Bacteroides* and *Ruminococcus*, a low relative abundance of *Prevotella* and *Catenibacterium* and a genus belonging to Erysipelotrichaceae. Interestingly, the association of a high abundance of *Ruminococcus* with severity of abdominal symptoms is in agreement with several studies [Malinen *et al*., 2005; Lyra *et al*., 2009; Rajilic-Stojanovic *et al*., 2011; Saulnier *et al*., 2011], thus confirming that this genus is involved in abdominal discomfort.

Although none of the participants underwent a strict diet intervention such as low fermentable oligo-, di-, mono-saccharides and polyols (FODMAP) diet, our study did not include a specific questionnaire regarding participants' type of diet. We are aware that, as for treatment, diet could act as another confounding variable that may affect the microbiome composition. However, previous works have shown that, unless an extreme switch in diet is undertaken, short-term interventions have only a modest effect on the microbiome composition [Wu *et al*., 2011; Coitillard *et al*., 2013; Rajilic-Stojanovic *et al*., 2015]. Our recommendation for future studies would be to collect diet information such as type of excluded food or type of diet intervention if any.

In conclusion, our study proposes a comprehensive characterisation of the microbiome in IBS, as summarised in **Figure 36**. Our results showed that each IBS subtype could be differentiated from healthy controls. Patients with diarrhoeic phenotype shared more-common microbial profiles. In contrast, IBS patients with constipation presented a microbiome similar to healthy controls, but with one exception, they harboured a greater abundance of methanogenic Archaea. The lower microbial diversity in patients, in particular in the diarrhoeic phenotype, could be explained by the loss of a few groups of microbes, such as methanogens, which are involved in H_2 removal, and loss of butyrate-producing bacteria, such as Erysipelotrichaceae. In this regard, the development of a method to reduce the microbe populations involved in low transit, such as methanogens in IBS-C, to increase those missing in IBS-D and IBS-M, or to increase those negatively correlated with abdominal pain, by bacteriotherapy for instance, may offer a therapeutic strategy for the management of these patients. Furthermore, our study

stresses the need to include patients not receiving treatment, and psychological data in microbiome-IBS studies.
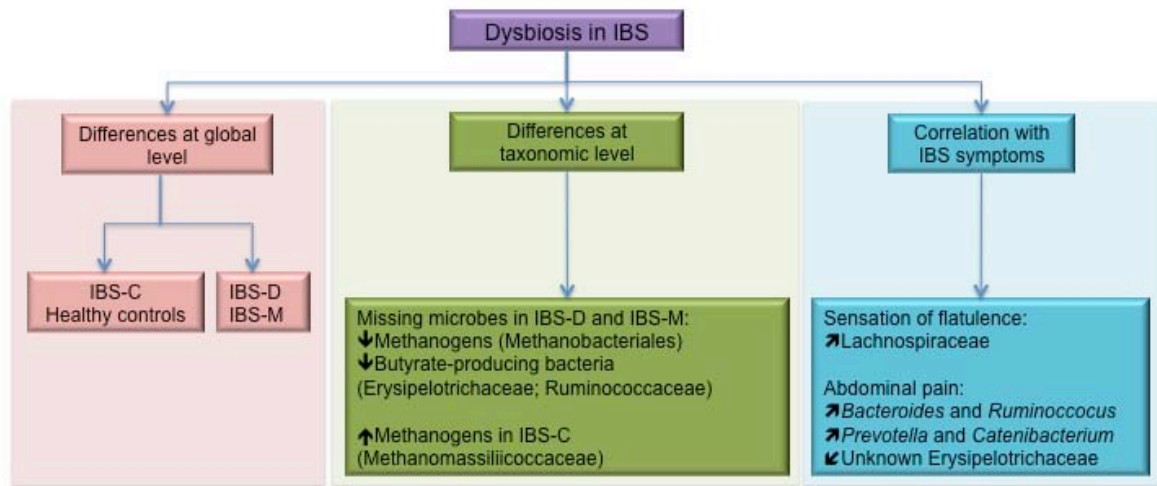

Figure 36: Summary of the findings of this study – ↑ = Higher abundance of; ↓ = Lower abundance of; ↗ = positive correlation with; ↙ = negative correlation with

Suchita Panda

DISCUSSION

This doctoral thesis aimed at understanding the presence of a gut microbial dysbiosis, and its impact on the health status. The project comprised of two studies: effect of antibiotics, and difference of microbial structure between healthy controls and patients suffering from Irritable Bowel Syndrome.

From the results of both undertaken projects, it is evident that the gut microbiota is altered (i) upon antibiotic therapy, and (ii) in between healthy and individuals with a functional bowel disorder. Although pervious studies had demonstrated dysbiosis in regard to these themes, our projects were novel in their own way.

In the case of the first work, we were aware of the long-term effects of antibiotic intake [Jernberg *et al*., 2007; Jakobsson *et al*., 2010], but no research had shown the immediate effects, upon termination of the prescribed dosage. We sought to determine the instantaneous changes in the gut microbial community composition and structure.

Regarding the second study, past investigations on IBS-microbiota association, had used small cohort sizes, and had been unable to clearly define dysbiosis for this functional disorder [Rajilic-Stojanovic et *al*., 2011; Jeffery *et al*., 2012; Rigsbee *et al*., 2012]. We intended to study the possible differences in the structure of the intestinal microbiome when comparing healthy subjects, to those suffering from Irritable Bowel Syndrome using improved sequencing methods (Illumina Technology©). The cohort size used by us was extensive as compared to previous studies using sequencing methods [Saulnier *et al*., 2011; Carroll *et al*., 2012; Durban *et al*., 2012; Jeffery *et al*., 2012; Rigsbee *et al*., 2012; Durban *et al*., 2013]. Besides accounting for the different subtypes of IBS in regard to altered motility, we took into account any treatment that the patients may have been undergoing, and also looked at the stability of the microbiome in one month interval.

In the first study, we compared the gut microbial profile of 21 persons before and after undergoing antibiotic therapy of seven days for either respiratory or urinary tract infections. Antimicrobials prescribed were broad-spectrum antibiotics, belonging to β-lactam group or fluoroquinolones.

16S rRNA amplicon pyrosequencing showed that the composition and structure of the microbial community altered at a global level, leading to a decrease in the

microbial taxa after antibiotic intake. These results were expected as shown by other reports [Antonopoulos *et al*., 2009; Hill *et al*., 2010; Jakobsson *et al*., 2010; Manichanh *et al*., 2010; Perez-Cobas *et al*., 2013a].

Challenging the general notion that broad-spectrum antibiotics reduce both Gram-positive and Gram-negative bacteria [Schaumann and Rodlof, 2007], we observed an increase in Bacteroidetes groups that are Gram-negative bacteria.

Regarding the individual antibiotic groups, β-lactams and fluoroquinolones, we saw that similar to the global level, both antibiotic groups showed an increase in the Bacteroidetes/Firmicutes ratio.

To test the microbial load in the samples, we used qPCR methods; amplifying, in real time, the same 16S rRNA gene as used for sequencing. The results showed an un-expected increase in global microbial load, which is not concordant with previous studies [Noverr *et al*., 2005; Ochoa-Reparaz *et al*., 2009; Hill *et al*., 2010; Manichanh *et al*., 2010; Ochoa-Reparaz *et al*., 2010; Umenai *et al*., 2010].

Individually, fluoroquinolones showed increase in the load of 3 unknown taxa belonging to the *Bacteroides* genus, while β-lactams showing a 2.5 fold increase.

The different types of antibiotics and dosage administered could be a reason the results of this study were different from previous studies [Antonopoulos *et al*., 2009; Manichanh *et al*., 2010]. The reduction of diversity complied with the increase of bacterial load after antibiotic therapy, implies that microbes sensitive to these antibiotics are killed, but the niche is seized by antibiotic-resistant strains. This may also be a possible reason that altered gut microbial profile continues to be seen long after the therapy is stopped [Jakobsson *et al*., 2010].

The second study focuses on comparing the faecal microbial community of 113 subjects with IBS with 66 healthy controls. Previous studies have not always been able to exhibit a clear distinction between IBS patients, their subtypes, and healthy controls [Rajilic-Stojanovic *et al*., 2011; Jeffery *et al*., 2012].

Illumina amplicon sequencing of the 16S rRNA gene demonstrated separate clustering between the healthy subjects and patients using distance-based tests and showed that healthy subjects presented a higher microbial diversity and higher amounts of Firmicutes, with Erysipelotrichaceae and Ruminococcaceae being the more representative. The results displayed that IBS patients had higher

Bacteroidetes and lower Firmicutes which contrast from the studies by Krogius-Kurikka *et al*., 2009, Rajilic-Stojanovic *et al*., 2011 and Jeffery *et al*., 2012, and possibly due to dissimilar process for DNA extraction or sequencing methods. We have previously shown the importance of proper sample storage and processing in the outcome of sequencing data analysis [Cardona *et al*., 2012; Santiago *et al*., 2014].

Major dissimilarities were seen when comparing the healthy controls to IBS patients, especially in the IBS-D subtype group, where clear differences were visible at various phylogenetic levels. Lesser Methanobacteriaceae was associated with dysbiosis in IBS-D, which is in accordance with the works of Rajilic-Stojanovic *et al*., 2011 and Chassard *et al*., 2012. Butyrate-producing bacteria such as Ruminococcaceae, unknown Clostridiales and Erysipelotrichaceae were decreased in the IBS-D subtype persons, and these bacteria have previously been related to this subtype of patients [Dunlop *et al*., 2006], IBS-C subtype group was similar in structure to the healthy controls, although having higher methanogenic bacteria. This group of methane-generating bacteria is involved in utilising high volume of H_2 gas in the intestine to convert it to lesser volume of methane gas (CH_4) [Pimental *et al*., 2006a], and has been previously reported to be higher in persons suffering from constipation [Attaluri *et al*., 2010; Sahakian *et al*., 2010]. A 4.7 fold decrease was observed in the Erysipelotrichaceae family in the case of IBS-M subtype.

Persons having IBS have many discomforts, and generally take some form of treatment for it, including laxatives, pre/probiotics, proton-pump inhibitors (omeprazole), antidepressants or anxiolytic drugs; that could have been confounding factors for the analyses. Analysis of the patients, who had not taken any form of treatment, showed that their microbiota had less diversity and less abundance of Methanobacteria (especially for IBS-D and IBS-M cohorts). Individuals who consumed Omeprazole and other proton pump inhibitors, had higher amounts of Pasteurellaceae. Studies by Krogius-Kurikka *et al*., 2009 and Saulnier *et al*., 2011, revealed higher prevalence of Gamma-Proteobacteria, but they had no information regarding the use of medications.

When samples collected a month later than the first, were analysed, the microbial composition for both healthy and patient subjects remained the same, demonstrating that the microbiota remains stable over time.

Symptoms such as flatulence, bloating and abdominal pain are common in IBS patients [Manichanh *et al*., 2014]. In our study, high relative abundance of Lachnospiraceae and *Blautia* correlated with flatulence; while *Bacteroides* and *Ruminococcus*, were in high abundance in the microbiome of persons having abdominal pain. These persons also had lower relative abundance of *Prevotella* and *Catenibacterium* and a genus belonging to Erysipelotrichaceae.

The involvement of the Ruminococcaceae group has been evidenced throughout this study, the relative high abundance in IBS-C subtype individuals, and those complaining of abdominal pain. The positive correlation of this group to the constipation subtype of IBS patients has been previously described by Malinen *et al*., 2005; Lyra *et al*., 2009; Tana *et al*., 2010; Rajilic-Stojanovic *et al*., 2011; Saulnier *et al*., 2011 and Rigsbee *et al*., 2012, while Malinen *et al*., 2005; Lyra *et al*., 2009; Rajilic-Stojanovic *et al*., 2011 and Saulnier *et al*., 2011, described positive association of Ruminococcaceae to symptoms of abdominal pain. This family of microbes are normally abundant butyrate-producing anaerobic bacteria in the intestine. Butyric acid has anti-inflammatory effects, and is known to decrease the permeability of the intestinal epithelial lining. Colonic defence barriers are reinforced by butyric acid, via increasing antimicrobial peptide levels and mucin production. Thus the depletion of such bacteria, as seen in our study in the IBS-D group, may amount to epithelial dysfunction and increased osmotic load within the intestine, resulting in diarrhoea [Cook and Sellin, 1998; Wong *et al*., 2006; Antharam *et al*., 2013].

Undoubtedly, changes in the gut microbial environment with respect to antibiotic intake and IBS, has been established by previous, and by our studies
Antibiotics are designed to stall the growth of or kill microbes, for which the medical world continues to depend on them to combat the rising number of infectious diseases that lead to higher worldwide morbidity and premature mortality. Regarding the microbiome alteration seen upon antibiotic therapy, it is clear that the xenobiotic compounds are causing these perturbations [Jernberg *et*

al., 2007; Dethlefson *et al*., 2008; Jakobsson *et al*., 2010; Fouhy *et al*., 2012; Perez-Cobas *et al*., 2013a], and as established in our study, they eliminate microbes, causing reduced diversity. Over the years, antibiotics are becoming decreasingly effective due to antimicrobial resistance, whereby the designated target microbes incorporate resistance methods to the specific *modus operandi* of the chemical involved. In fact, several bacteria are known to have developed resistance to more than a single type of antibiotic which would allow their overgrowth and increase in relative abundance, thereby increasing the overall microbial load. Moreover, increased intake of such medications could lead to even higher resistance by the microbes, not only in a single individual patient, but in the community as well; as demonstrated clearly by a meta-analysis performed by Bell *et al*., 2014. Regular consumption of these drugs would allow continuous disruption of the native microbial homeostasis which would cause further complications in the maintenance of health status, probably leading to diseases [Levy, 2000; Sullivan *et al*., 2001; Sjolund *et al*., 2003; Keeney *et al*., 2014]. Indeed, heavy antibiotic treatment has been linked to obesity, asthma, necrotizing enterocolitis and arrested immune development [Cox *et al*., 2014; Gibson *et al*., 2015].

Although non-absorbable antibiotics such as neomycin and rifaximin are prescribed for relief to IBS patients [Pimental *et al*., 2006b; Pimental *et al*., 2011], antibiotics such as tetracyclines and macrolides have shown to be associated with the development of IBS [Villarreal *et al*., 2012]. Furthermore, following antimicrobial therapy, *Candida* overgrowth has shown to occur. *Candida* releases alcohol and glycoproteins that stimulate mast cells to release histamine and prostaglandins which are inflammation-causing substances that could cause IBS like symptoms [Santelmann and Howard, 2005]. Clinical surveys have also reported that many patients had undergone antibiotic therapy in the months prior to being diagnosed with IBS [Mendall and Kumar, 1998; Maxwell *et al*., 2002].

If the un-prescribed, excessive use of such drugs could be reduced, it may prevent the development of resistance by such bacteria. Thus the guarded use of these antimicrobials is absolutely vital.

Irritable bowel syndrome is a type of functional gastro-Intestinal disorder, for which there are no established biomarkers for diagnostic purposes. Clinicians rely on expertise and symptoms to deduce the disorder [Longstreth *et al*., 2006]. The aetiology is not understood, and is considered a multi-factorial malady. Stress, diet, psychiatric ailment such as depression and anxiety, altered intestinal barrier function, and gut microbiota have been associated as contributing factors to this infirmity [De Palma *et al*., 2014; Rajilic-Stojanovic *et al*., 2015]. However, in this circumstance, the role of the microbiota is not established as being the causative agent of the disorder. Although accumulating data suggest that gut microbiota influences central nervous system function and host's behaviour, it is unclear whether the brain abnormalities drive the gut symptoms or the changes in the gut alter brain function. Any alteration seen in the gut microbial structure could be a consequence of IBS [De Palma *et al*., 2014]. The main symptoms are changed bowel habits, abdominal pain, flatulence, visceral hypersensitivity and bloating. Through the results of our study, we see that IBS patients have lower diversity of microbes, and each subtype is distinguishable from healthy controls. Butyrate-producing bacteria, that are essential to maintain intestinal barrier function [Zaleski *et al*., 2013], were depleted, especially in the IBS-D subtype, probably being the root of the diarrhoeic conditions. Upon comparison of the microbial community between healthy subjects, patients having taken some form of treatment, and those not having taken any medication, it was observed that the relative abundance of methanogenic bacteria was highly reduced in persons who had not taken any therapeutics. Current therapeutic methods help to alleviate symptoms, but do not target the causative factors (since these are not well established). It is therefore, a possibility that the microbes that have been reduced or eliminated from the niche are bacteria correlated to better health. Future studies should attempt to administer these potentially beneficial bacteria to patients to note the health benefits, and the likelihood of an altered gut microbiota as a cause of IBS.

Future projects should recruit subjects including a questionnaire related to intake of therapeutics, daily lifestyle and in-depth knowledge of diet. All these factors should be taken into account while analysing the data, as each has the possibility to be a contributing actor to dysbiosis of the gut microbiota.

16S rRNA amplicon sequencing has some limitations which include mainly PCR bias and probability of environmental contamination. We recommend the use of chemicals like Propidium MonoAzide [Nocker *et al*., 2006] to help distinguish viable bacteria from dead cells, resulting in a more accurate analysis of the abundance of bacterial population.

Experimental batch effects were observed by Leek *et al*., 2010 in a microarray based study, where different results were observed from the work performed on the different days dues to technical artefacts. After normalisation of data, this variability was reduced, but not completely removed. Salter *et al*., 2014 tested several kits and reagents, and found contaminating DNA to be ubiquitous that affect both PCR and non PCR-based sequencing methods. They used a pure culture of *Salmonella bongori* as a control: in sequentially serially diluted samples, the resulting DNA sequences showed lower relative proportion of *S. bongori* sequences, along with increased proportion of contaminating background bacterial sequences. When samples were amplified using lesser PCR cycles the resulting amplicon quantity was not sufficient for sequencing, while increased PCR cycles resulted in more proportion of contaminating bacterial sequences. Weiss *et al*., 2014, demonstrated that in case of faecal or high microbial biomass samples, the inherent microbiota overshadows the contamination, but in case of lower biomass samples such as placenta or blood/serum as the true biomass decreases, proportion of contaminating bacterial sequences increase. Batch differences coupled with background contaminants lead to inaccurate results and erroneous result interpretation. Since microbial DNA may be present even in sterile environments, methods to improve credibility of sequencing based studies need to be settled. Some suggestions include:

a) A careful study design that includes equal proportion of different time points or healthy and patient samples in single batch of extraction/PCR

b) Inclusion of all reagents and personnel responsible in metadata

c) Use of clean, new, bleach and UV sterilised equipment, reagents etc.

d) Use of positive (pure culture) and negative (blanks) controls

e) Removal of contaminating OTUs during bioinformatic analysis

f) Use of appropriate statistical tests

Prospective studies on the gut microbiome should be devised with a multi-dimensional approach, including methods to study the function of the microbes apart from only the composition and structure. New techniques such as metatranscriptomics, metaproteomics and metabolomics should be supplemented with 16S rRNA based studies to procure cohesive data on which microbial groups or species are actually involved in the diseases. Also, the fungal and viral microbiomes should be studied along with the bacterial microbiome, to attain a better understanding of the structure and functions of the entire gut microbial population.

In cases where a clear dysbiosis is evidenced, the next step would be to find a method to repeal the changes in the microbial structure. Probiotics, often in association with prebiotics, have been used, for this purpose [Bibiloni *et al*., 2005; Sinn *et al*., 2008; Zeng *et al*., 2008; Williams *et al*., 2009; Toumi *et al*., 2014; Elian *et al*., 2015]. In the recent years, a form of bacteriotherapy has gained much recognition: Faecal Microbiota Transplant. Especially beneficial in restoring health status in persons with incurable *Clostridium difficile* Infection (CDI) [Andrews *et al*., 1995; Walia *et al*., 2014a], this method has been attempted to overcome dysbiosis in other disorders as well [Singh *et al*., 2014; Pinn *et al*., 2015]. In systematic reviews of FMT for IBD patients, reduction of IBD symptoms following FMT was observed in 77% patients, while a complete remission of 3-36 months was noted in 63% patients [Anderson *et al*., 2012; Sha *et al*., 2014; Rossen *et al*., 2015]. FMT is seen to be more beneficial in UC (almost 90% as indicated by reduction in Ulcerative Colitis Activity Index) rather than in CD. The microbiota, initially being altered, is restored to baseline by the 8th week following FMT. In IBD patients also having CDI, a marked reduction or absence of diarrhoea has been seen.

89% of IBS patients suffering from chronic constipation, who were treated with FMT, reported relief in defecation, bloating, and abdominal pain [Andrews *et al*., 1995]. In another study [Pinn *et al*., 2013], 70% patients suffering from various subtypes of IBS, stated relief in terms of abdominal pain, dyspepsia, bowel habits, bloating, and flatus.

Other methods of bacteriotherapy, such as a stool-substitute mixture [Petrof *et al*., 2013] are being developed for this purpose, and need to be encouraged as an

alternative method of remedy. Petrof and colleagues developed a stool substitute preparation that was made from intestinal bacterial cultures. Faecal matter from a healthy donor was cultivated for bacteria, from which 33 isolates were blended to prepare the infusion, which were further administered to 2 patients suffering from severe, antibiotic resistant, recurrent *C. difficile* infection. Normal bowel pattern was restored within 3 days and the patients remained healthy beyond 6 months after the treatment. Similar stool-substitute mixtures comprising of multi-species community of bacteria could eventually replace FMT procedures in the future.

Suchita Panda

CONCLUSION

The results of this PhD work offer valuable insights to the structure of the gut microbiome, demonstrating differences between healthy status and non-healthy status in regard to antibiotic therapy and Irritable Bowel Syndrome. Although several studies have been done on these themes, from this work we are able to conclude the following:

1) The gut microbial structure in healthy persons consists of high diversity of bacteria which is correlated to good health status

2) Upon antibiotic intake, there is a global change in the microbial composition, and diversity is reduced

3) Microbial load is unexpectedly increased, suggesting the dominance of resistant bacteria

4) Microbial composition is altered in IBS patients, diversity being reduced

5) Type of dominant bowel movement is associated with increase or decrease of certain microbial groups or species, which may form a good target for bacteriotherapy

6) In patients of IBS-D, butyrate-producing bacteria are reduced probably causing intestinal barrier dysfunction, leading to diarrhoea

7) Methanogenic bacteria is reduced in patients who did not undergo any therapeutic treatment, signifying the cause of bloating and flatulence

Future work should comprise of investigating therapeutic methods to recover beneficial bacteria to relieve patient discomfort and disease. This theme constitutes the foundation for further on-going work.

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