Diet:microbiota interaction in the gut

focus on amino acid metabolism

Alma Mater Studiorum - Università di Bologna

Dottorato di Ricerca in Scienze Biochimiche e Biotecnologiche Ciclo XXVIII

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Settore Concorsuale di afferenza: 03/D1

Settore Scientifico disciplinare: CHIM/11

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Esame finale anno 2016

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This thesis concludes research carried out mainly at the Research and Innovation Centre - Fondazione Edmund Mach Via E. Mach 1, 38010 - S. Michele all'Adige (TN), Italia from 2013 until 2016.

The studies were supervised by Dr. Kieran Tuohy, research manager of the Nutrition and Nutrigenomics Food Quality Nutrition & Health Department Research and Innovation Centre - Fondazione Edmund Mach and Prof. Patrizia Brigidi, Director of the Institute for Advanced Studies and Coordinator Master of Science in Pharmaceutical Biotechnology, Department of Pharmacy and Biotechnology.

The work presented here is the result of a multidisciplinary project, which allowed me to work in the Metabolomics technological platform at FEM, headed by Dr. Urska Vrhovsek and in the School of Chemistry, Food & Pharmacy at University of Reading, Food and Nutritional Sciences Deparment, United Kingdom, where NMR-based metabonomics analysis were corried out under the supervising of Dr. Jordi Mayneris-Perxachs and Dr. Jonathan Swann.

SCIENTIFIC ENVIRONMENT

The Department of Pharmacy and Biotechnology (FaBiT), at the University of Bologna (founded in 1088), promotes and coordinates research and education in the chemical-pharmaceutical-technological, biomedical, biotechnology, organic and biological molecules.
Prof. Brigidi's research group is involved in the study of the gut microbiota by the most advanced molecular methods, in the perspective of the modulation and the promotion of human gut health and are pioneers in the use of probiotics for the treatment of gastrointestinal functional disorders and have contributed to the development of new probiotic strains utilized in clinical trials.

The Fondazione Edmund Mach in San Michele all'Adige is an institution with a history dating back more than 140 years, specialising in research, training and the transfer of technology in the agricultural, food and environmental sectors. The Fondazione Edmund Mach (FEM) is a private research center established by the government of the Autonomous Province of Trento (PAT). The Center of Research Institute (CRI) is organised in five Departments (divided in Research Groups and Technological Platforms) and one Research Consortium. Its goals are sharing knoledge and to contribute to economic growth, social development and the overall improvement of quality of life. The Nutrition and Nutrigenomics (NN) group, where I worked, is part of the Research and Innovation Centre (CRI), Food Quality and Nutrition Department (DQAN). The aim of NN group is to measure how food and diet modulates host health and protects against chronic disease through microbial interactions.

This study aims to measure the impact of protein and amino acid fermentation on the composition and metabolic output of gut microbiota. Although dissimilatory pathways have been described for most amino acids, microbial degradation routes within the gut microbiota are relatively unexplored. The objectives were (1) to characterize amino acid breakdown by the colonic microbiota, (2) to determine the fermentation products formed from individual amino acids/protein (3) to examine how amino acid metabolism is impacted by the presence of a fermentable fiber (prebiotic inulin) and finally (4) to evaluate with an in vivo model (trout fish) diet:-microbe interactions and the development of gut microbiota during fish farming. Interactions between the healthy human intestinal microbiota of the distal colon and different combinations of nutrients were simulated using in vitro pH-controlled anaerobic batch cultures of human faeces. Combining high-throughput sequencing of 16S rRNA amplicons, with high-throughput ^{1}H NMR, changes in faecal microbiota composition and metabolic output were measured. During exogenous substrate microbial fermentation (e.g. beef, Trp or fish feed) in the large bowel bioactive compounds (harmful or beneficial) are produced. Many factors affect the gut-microbial metabolism including pH, type and quantity of growth substrate (e.g. protein/carbohydrate) and make up of the gut microbiota. Considerable interindividual variation was observed in response to different digested substrates but over all, the beneficial impact of prebiotic fiber fermentation on production of bioactive compounds from amino acids/proteins was confirmed in this study. In trout, although our dietary intervention with essential oils had little impact on the gut microbiota, the study showed for the first time a dramatic shift in the composition and diversity of the gut microbiota in juvenile compared to adult fish. These observations may have relevance in designing dietary strategies to reduce chronic diseases like colon cancer and heart disease and for fish farming respectively.

ABBREVIATIONS

ID	Name
AA	Amino Acids
AAA	Aromatic Amino Acids
AD	Alzheimer's Disease
ANS	Autonomic Nervous System
ASD	Autism Spectrum Disorder
ASF	Altered Schaedler Flora
BALT	Brown Adipose Tissue
BBB	Blood Brain Barrier
BCFA	Branched Chain Fatty Acids
BDNF	Brain Derived Neurotrophic Factor
BM	Basal Medium
bp	base pairs
BSCFA	Brached Short Chain Fatty Acids
CA	Catecholamines
CD	Crohn's Disease
CNS	Central Nervous System
CRFR	Corticotropin Release Factor
CVD	Cardio Vascular Disease
EAA	Essential Amino Acids
EC	Enterochromaffin Cells
ENS	Enteric Nervous System
FISH	Fluorescent In Situ Hybridization
FOS	Fructo Oligo Saccharides
GABA	Gamma Amino Butyric Acid
GALT	Gut Associated Lymphoid Tissue
GF	Germ Free
GIT	Gastro Intestinal tract

ID	Name
GLP	Glucagon Like Peptide
GOS	Galacto Oligo Saccharides
HPA	Hypothalamic Pituitary Adrenal (axis)
IARC	International Agency for Research on Cancer
IBD	IInflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
LC	Locus Coeruleus
LNAA	Large Neutral Amino Acids
LPS	Lipo Poly Saccharides
MICCA	Microbial Comunity Analysis
MO	Mix Oil
MSA	Multiple Sequence Allignment
NAFLD	Non Alcoholic Fatty Liver
NASH	Non Alcoholic Steato Hepatitis
NEAA	Non Essential Amino Acids
NGS	Next Generation Sequencing
NOC	Nitroso Compounds
NSP	Non Starch Polysaccharides
OPLS	Orthogonal Partial Least Squares
OSAS	Obstructive Sleep Apnea Syndrome
OTU	Operational Taxonomic Units
PCA	Principal Component Analysis
QIIME	Quantitative Insights Into Microbial Ecology
SAA	Sulfur-containing Amino Acid
SCFA	Short Chain Fatty Acids
SCN	Supra Chiasmatic Nucleus
SCP	Short Chain Proteins

ID	Name
SNS	Sympathetic Nervous System
SPF	Specific Pathogen Free
TILs	Tumor Infiltrating Lymphocytes
UC	Ulcerative Colitis
UL	Tolerable Upper Intake Level
WAT	White Adipose Tissue
WHC	Water Holding Capacity

Introduction

1.1 The gut microbiota

Human beings have communities of microorganisms in different parts of the body, such as in the surface or deep layers of the skin (skin microbiota), the mouth (oral microbiota), the vagina (vaginal microbiota) and so on, but the majority of micro-organisms, an estimated 100 trillion microorganisms, reside in the gut (Backhed, 2005). The gut microbiota is the global microbial community inhabiting the gastro-intestinal tract (GIT) of all animals with an organised intestine. It is composed of a wide variety of microbial species and strains that interact with one another and with the host. Total numbers of microbial cells in the body outnumber the human cells by a factor of ten (Abbott, 2016; Savage et al., 1977). What is really interesting is that the genetic diversity and functional capabilities of all these micoorganisms together is massive. Bacteria add about 8 million genes to the 22 000 genes of the human genome (Ravel et al., 2014). This "metagenome" therefore has a great influence on host metabolic capabilities and it is not surprising that it is now recognised to play an important role in host health and disease.
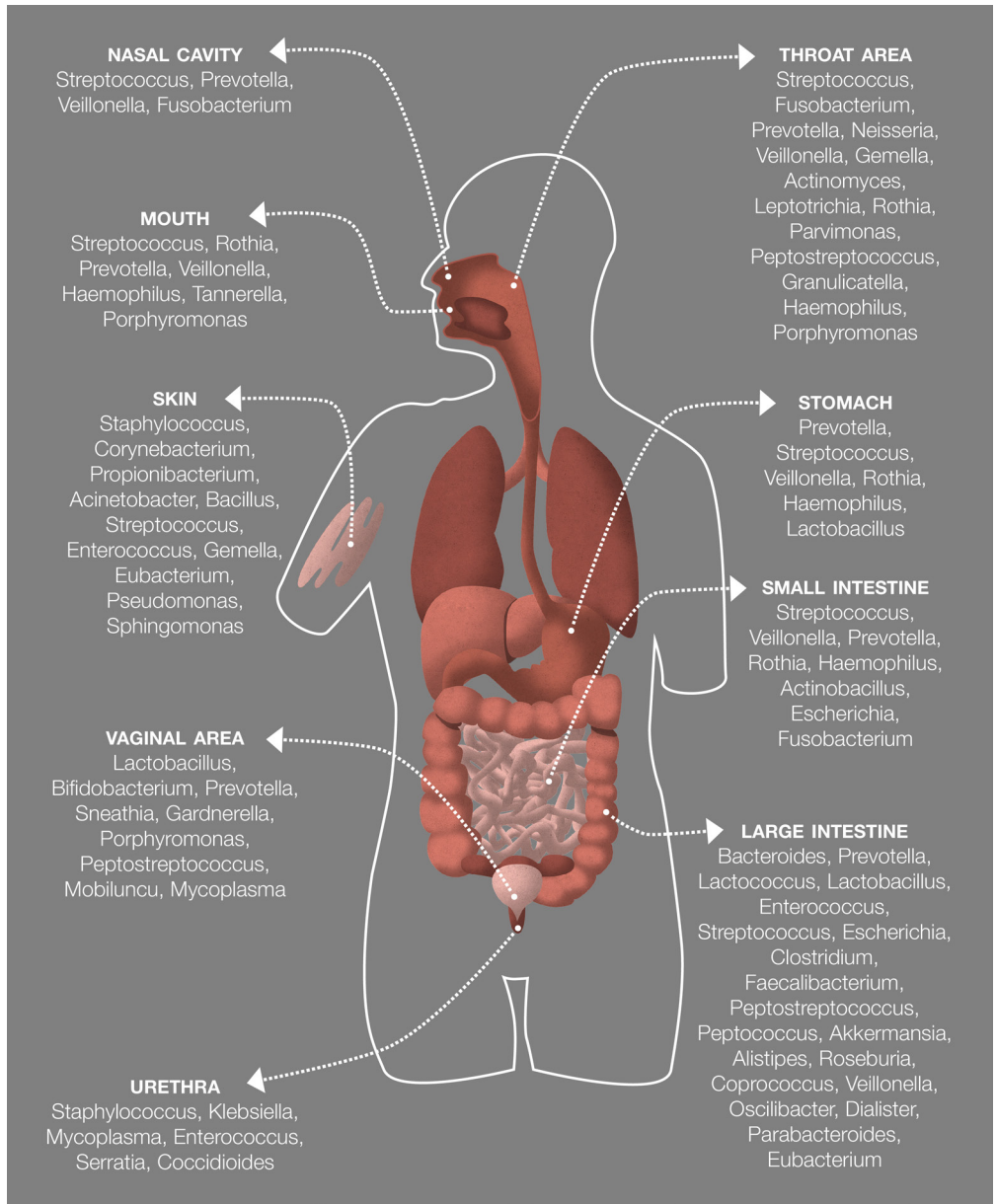
NASAL CAVITY
Streptococcus, Prevotella,
Veillonella, Fusobacterium

THROAT AREA
Streptococcus,
Fusobacterium,
Prevotella, Neisseria,
Veillonella, Gemella,
Actinomyces,
Leptotrichia, Rothia,
Parvimonas,
Peptostreptococcus,
Granulicatella,
Haemophilus,
Porphyromonas

MOUTH
Streptococcus, Rothia,
Prevotella, Veillonella,
Haemophilus, Tannerella,
Porphyromonas

SKIN
Staphylococcus,
Corynebacterium,
Propionibacterium,
Acinetobacter, Bacillus,
Streptococcus,
Enterococcus, Gemella,
Eubacterium,
Pseudomonas,
Sphingomonas

STOMACH
Prevotella,
Streptococcus,
Veillonella, Rothia,
Haemophilus,
Lactobacillus

SMALL INTESTINE
Streptococcus,
Veillonella, Prevotella,
Rothia, Haemophilus,
Actinobacillus,
Escherichia,
Fusobacterium

VAGINAL AREA
Lactobacillus,
Bifidobacterium, Prevotella,
Sneathia, Gardnerella,
Porphyromonas,
Peptostreptococcus,
Mobiluncu, Mycoplasma

LARGE INTESTINE
Bacteroides, Prevotella,
Lactococcus, Lactobacillus,
Enterococcus,
Streptococcus, Escherichia,
Clostridium,
Faecalibacterium,
Peptostreptococcus,
Peptococcus, Akkermansia,
Alistipes, Roseburia,
Coprococcus, Veillonella,
Oscilibacter, Dialister,
Parabacteroides,
Eubacterium

URETHRA
Staphylococcus, Klebsiella,
Mycoplasma, Enterococcus,
Serratia, Coccidioides

Fig. 1 Human microbiome

1.1.1 Gut microbiota development during early life

It appears that closely related individuals have more similar microbiota composition than unrelated people and both genetic and environmental factors play a role in the acquisition and maintenance of the gut microbiota (Turnbaugh et al., 2009). All of our bacteria come from the environment initially (with other people included as part of our environment), so a mother and daughter are likely to be exposed to the similar microorganisms both via contact with each other and contact with their shared environment (Faith et al., 2013). The genetic influences impacting on these interactions are highly complex and have not been fully worked out.

Microorganisms are everywhere. Even the statement that fetuses remain sterile in the womb has recently come under scrutiny (Funkhouser and Bordenstein, 2013). Early studies found low numbers of mainly facultative anaerobes in amniotic fluid, especially from mothers with membrane disruption, but were limited by a reliance on culture based methodologies, which only detect a tiny fraction of microbial diversity under natural conditions (Miller et al., 1980). Umbilical cord blood from healthy neonates, born by elective cesarean section, was investigated and found to harbour bacteria belonging to the genus *Enterococcus*, *Streptococcus*, *Staphylococcus*, or *Propionibacterium* (Jiménez et al., 2005). More recently, meconium, which is the earliest "faeces-like excretion" composed of materials ingested during the gestation, was shown to contain a complex microbiota, predominantly of genera *Enterococcus* and *Staphylococcus* (Jiménez et al., 2008; Moles et al., 2013). Meconium appears to contain a specific microbiota from which you can infer specific condition of the baby (e.g. *Serratia* - strongly associated with neonatal immaturity), as well as hospital-related parameters (e.g. antibiotherapy and mechanical ventilation) (Moles et al., 2013). Finally, fetal membranes analyzed by broad-range 16S rDNA PCR, resulted positive for bacteria known to reside in the vaginal, gastrointestinal or respiratory tract including *Ureaplasma parvum*, *Lactobacillus crispatus*, *Fusobacterium spp.*, *Pantoea spp.* and *Eubacterium rectale* (Jones et al., 2009). Although microorganisms have been observed associated with these habitats even before or very shortly after birth, indicating that the gastrointestinal tract might not be sterile in utero, all studies agree numbers are very low and it is not until birth that a significant colonization of the gastrointestinal tract takes place.

Almost immediately upon exiting the uterus, babies are exposed to a number of microorganisms from their mother (from vaginal and intestinal tracts) and also microorganisms from their immediate environment (handling physicians, surfaces, etc.) which rapidly colonize their skin and intestine (Gilbert, 2014). Generally, due

to the abundance of oxygen in the neonatal gut, facultative anaerobes, mainly *Enterobacteriaceae*, *Enterococcus*, and *Streptococcus* species, represent the first colonizers. The *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium* are commonly found, followed by *Klebsiella* and *Enterobacter*, and more rarely and transiently, *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *α-hemolytic Streptococcus*, and *coagulase-negative Staphylococcus*. The gradual consumption of oxygen in the gut promotes the proliferation of obligately anaerobic bacteria, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium*, followed by *Veillonella*, *Eubacterium*, and *Ruminococcus* species. Many factors determine the make up of the infant gut microbiota including, mode of delivery (vaginal delivery or caesarian section) (Bäckhed et al., 2015; Jakobsson et al., 2014), full-term or pre-term birth (Groer et al., 2014), maternal diet (Paul et al., 2016; Gohir et al., 2015), health status (including autoimmune diseases, perinatal antibiotic exposure, diabetes and stress) (Soderborg et al., 2016; Persaud et al., 2015) and even body weight (Santacruz et al., 2010). Babies born vaginally have been shown to be colonized predominantly by *Lactobacillus* in the first few days, whereas in cesarean delivered babies potentially pathogenic bacteria typically found on the skin and in hospitals, such as *Staphylococcus* and *Acinetobacter* appear to be more commonly encountered (Neu and Rushing, 2011). Infant diet also plays a critical role in shaping the composition of the infant gut microbiota both in terms of species present and their relative abundance. Infants fed human breast milk rapidly develop a microbiota dominated by species of bifidobacteria. Breast milk contains many different factors which influence the gut microbiota, not least immune molecules (like leukocytes, immunoglobulins and lactoferrin) (Hassiotou et al., 2013) and non-digestible oligosaccharides, which escape digestion in the upper gut and act as specific growth substrates for bifidobacteria further along the alimentary canal (Ballard and Morrow, 2013). Commonly, infants fed formula milk have significantly higher counts of faecal *Clostridium* and *Streptococcus* species, *Bacillus subtilis*, *Bacteroides vulgatus*, *Veillonella parvula*, *Lactobacillus acidophilus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Atopobium*, but lesser amounts of *Lactobacillus rhamnosus* and staphylococci, compared to breast fed infants (Guaraldi and Salvatori, 2012). Such differences between breast fed and formula fed infants, are particularly evident in earlier studies, while more recent studies, especially with modern feed formulations, which often contain prebiotics, show these differences in microbiota composition and relative abundance to be less obvious.

Recently, breast milk too has been shown to possess a distinct microbiota itself made up predominantly of species of *Bifidobacterium* (Wickramasinghe et al., 2015) and also species of staphylococci, streptococci and lactic acid bacteria (Fernández et al., 2013). This breast milk microbiota is suggested to act in the manner of an inoculum for the developing infant, assisting the successional development of the gut microbiota and possibly mediating vertical transmission of certain species from mother to infant. It remains debated, however, where these bacteria come from, with data supporting both maternal origin, via the blood stream possibly in immune cells, or via colonisation of the mothers breast from the infant itself during suckling (Fernández et al., 2013; Martín et al., 2004). In either case, viable bacteria (usually bifidobacteria and lactobacilli), have been isolated from breast milk during breast feeding, highlighting the importance of breast feeding, in the successional development of the gut microbiota and infant health.

Colonization early in life may have lasting implications for health, especially in terms of immune function, metabolic diseases risk and even for proper brain development. The American Academy of Pediatrics in 2013 noted that prematurity and hospitalization were associated with higher prevalence of *Clostridium difficile* an opportunistic gut pathogen and that antibiotic treatment in early life has been associated with decreased numbers of bifidobacteria and *Bacteroides*. Finally, infants with older siblings have slightly higher numbers of bifidobacteria compared with infants without siblings (Committee on infectious diseases, 2013; Penders et al., 2006). Similarly, close contact with animals, both farm animals (Sjögren et al., 2009) and companion animals (dogs especially), has been shown to impact on the composition of the gut microbiota in children and is suggested to help train the immune system to respond appropriately to new immunological stimuli (Azad et al., 2013).

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1.1.2 Microbiota distribution along the GI tract

The microorganisms that inhabit the intestine have a complex and sometimes dichotomous relationship with the host. Through their activities they influence many aspects of human physiology and disease risk. In fact, one could view the human intestine as an organ that was largely shaped by its microbiota during evolution. This theory is supported by findings that some members of the intestinal microbiota at least, e.g. *Bacteroides thetaiotaomicron*, occupy stable ecological niches within the gut, despite dietary changes and attack by the human immune system (Comstock and Coyne, 2003). Furthermore, *Bacteroides thetaiotaomicron* is also able to modulate the expression of several human genes implicated in host metabolic pathways, illustrating direct microbiota regulation of host physiology (Zocco et al., 2007). Moreover, the microbiota performs functions which the host is not been able to carry out alone, such as polysaccharide break down, which represents the main energy source for the intestinal microbiota, about 50% of the energy required for the gut wall and about 10% of human daily energy intake (Cummings and Macfarlane, 1997). This production of energy, mainly from the microbial fermentation of non-digestible carbohydrates or fibers, may be especially important where diets are low in calories and high in fiber, such as traditional Mediterranean diets and rural African diet (De Filippo et al., 2010).

The human body influences its microbial community creating different habitats and niches along the gut by modulating levels of moisture, oxygen, bile acids, pH, digestive secretions and interactions with the immune system (Boon et al., 2014). The intestinal microbiota changes, both in number and in species composition, along the alimentary canal from mouth to anus (Goktepe et al., 2006). The mouth microbiota shows predominance of Gram-positive aerobic and anaerobic cocci (Neut et al., 1985). In the oral cavity of newborns, *Streptococcus* are the pioneer organisms, followed by colonization by *Actinomyces*, *Veillonella*, *Fusobacterium*, *Porphromonas*, *Prevotella*, *Treponema*, *Nisseria*, *Haemophilis*, *Eubacteria*, *Lactobacterium*, *Capnocytophaga*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, and *Propionibacterium* (Avila et al., 2009). Low abundances and transitory microorganisms populate the esophagus (pH < 4.0) including *Bacteroides*, *Gemella*, *Megasphaera*, *Pseudomonas*, *Prevotella*, *Rothia spp.*, *Streptococcus*, *Veillonella* (Liu et al., 2013). The predominant phyla found in the stomach (pH 2) are: *Streptococcus*, *Lactobacillus*, *Prevotella*, *Enterococcus* and *Helicobacter pylori* (Andersson et al., 2008; Bik et al., 2006). Duodenal is the first section of the small intestine and the most abundant phyla encountered are: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*,

Actinobacteria, and *Fusobacteria* (Li et al., 2015). The small intestine is designed to promote a fast flow of contents a feature that has the effect of discouraging microbial growth by washing microorganisms through before they can establish themselves. From duodenum towards ileum numbers, microbial diversity and the proportion of anaerobes all increase. In the jejunal and ileum microbial communities mainly comprise streptococci, lactobacilli, *γ-Proteobacteria*, *Enterococcus* and *Bacteroides* group. Most of the species are facultative anaerobes and acid-tolerant (Hayashi et al., 2005). Towards the end of the ileum, the lumen is colonized prevalently by streptococci, enterococci and coliforms, while in the mucosa, obligate anaerobes, such as *Bacteroides spp.*, *Clostridium spp.*, *Bifidobacterium spp.*, increase in relative abundance (Booijink et al., 2010).

The flow of contents through the colon is slow and microorganisms can easily establish themselves and reach concentrations that are high enough to make up at least 30 percent of human colonic contents by weight. In the colon, bacteria perform an important role in metabolising food components which escape digestion in the stomach and small intestine. They also metabolise material such as mucin and enzymes secreted into the lumen by the host and the host epithelial cells from the gut wall, which slough off continuously into the intestinal lumen. The colon contains a vast and complex microbial ecosystem. In humans, the vast majority of microorganisms inhabiting the large intestine are bacteria. Protozoa are seldom detected in healthy people but yeasts and other fungi can sometimes be recovered, although their cell population densities are low (Macfarlane and Macfarlane, 2011; Rizzetto et al. 2015). More than 90% of the species belong to the bacterial phyla *Firmicutes* and *Bacteroidetes*. Although, there is a high inter-individual variability in gut microbiota composition, a conserved set of gut colonizers or at least in gene/metabolic functions, appears to be shared amongst individuals and may be required for the correct functioning of the gut (Tremaroli and Bäckhed, 2012). In the proximal colon the pH ranges from 5-5.7 and the most abundant bacteria are obligate anaerobes mainly made up of species from the genera *Bacteroides*, *Clostridium*, *Prevotella*, *Porphyromonas*, *Eubacterium*, *Ruminococcus*, *Lachnospira*, *Roseburia*, Butyrivibrio, *Streptococcus*, *Enterobacterium*, *Enterococcus*, *Lactobacillus*, *Peptostreptococcus* and *Fusobacterium*. The pH changes across the proximal (pH 5.5), transverse colon (pH 6.6) and to the distal colon pH close to neutrality, with some change in species composition but major changes occur in metabolic activity of the resident microbiota due to changes in substrate availability (Gibson and Roberfroid, 1999).

Recent studies using next generation sequencing and statistical models to describe community structure and species co-occurance suggest the existance of different phylogenetic clusters or "enterotypes" that distinguish an individuals gut micro-biota. Each enterotypes is identified by variation in the levels of one of three main genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Siezen and Kleerebezem, 2011). The concept of these enterotypes is based on statistical probabilities and co-variance within large sequence based da-tasets and both their biological relevance and indeed their very biological existance outside computational models remains hotly debated.

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1.1.3 The physiological role of the gut microbiota

The gut microbiota protects against enteropathogens, extracts nutrients and energy from our diet and contributes to normal physiological and immune function (Maslowski and Mackay, 2011). A dense resident community of commensal bacteria prevent overgrowth of invading pathogens through several strategies such as, producing bacteriocins (proteinaceous toxins), which inhibit members of the same or similar bacterial species, producing short chain fatty acids (SCFA), which alter host environmental conditions (e.g. pH) and also down-regulate the expression of several virulence genes. Moreover, commensal bacteria can consume common limited resources (e.g. oxygen, specific nutrients, iron), causing the starvation of competing pathogens, and they may also induce indirectly development of host's Th17 cells in the intestine which are important for protection against pathogens (Kamada et al., 2013). Dominant colonic organisms such as *Bacteroides*, *Roseburia*, *Fecalibacterium*, *Bifidobacterium* and *Enterobacteria*, are able to carry out the fermentation of non-digestible oligosaccharides and fibers, resulting in the synthesis of short chain fatty acids (SCFA), mainly acetate, propionate and butyrate. These organic acids, are rich sources of energy both for certain species of gut bacteria and for the host. Similarly, they play an important role in modulating the immune system, as mentioned above. Cross-feeding between gut bacteria on SCFA, released by primary fermenting bacteria occurs with both, acetate and lactate, shown to be converted into butyrate, during cross-feeding (Duncan et al., 2004).

The intestinal microbiota not only modulates, but orchestrates the formation of the host's immune system, supporting the "hygiene hypothesis", which aims to explain the peculiar generational rise in immune dysregulation – the incidence of autoimmune and allergic disease has reached epidemic proportions in the developed world since the 1950's (El Aidy et al., 2015). During early life, the intestinal microbiota plays an important role in gut-associated lymphoid tissue (GALT) formation and education. The GALT is a complex immune organ designed to recognise pathogenic microorganisms and mediate an appropriate defensive response (Forchielli and Walker, 2005). GALT produces IgA which can modulate innate immune responses when fragments of capsular polysaccharide of commensal bacteria encounter Intestinal dendritic cells (DCs). These IgAs can also play a direct clearance role, by binding to specific unwanted microorganisms in the lumen of the intestine and clearing them in faeces, while allowing commensals to persist and colonise the GI tract (Lebeer et al., 2010). Gut microorganisms are also responsible of production of anti-inflammatory metabolites, mainly SCFA from carbohydrate fermentation,

such as propionate and butyrate which increase the differentiation of regulatory T (Treg) cells, belonging to the class of CD4+ T cells. Tregs appear to play a key role in preventing autoimmune diseases by maintaining self-tolerance, suppression of allergy, asthma and pathogen induced immunopathology, feto-maternal tolerance and oral tolerance. Some probiotics (e.g. *Lactobacillus sakei*, *L. reuteri*, *L. paracasei*, *L. plantarum*, *L. acidophilus*, *L. salivarius* and *Bifidobacterium breve*), although their mechanism of action is not yet clear, appear to down regulate proinflammatory cytokines IL-8 and interferon-γ, while up regulating anti-inflammatory cytokine IL-10, and inhibiting TNF-α (Ganguli et al., 2013; Thomas and Versalovic, 2010; Pena et al., 2005).

In humans, the immune system and host metabolism appear to be intricately related and both are regulated by the activities of the gut microbiota. Recent studies have shown a clear difference between the gut microbiota of lean individuals and that of obese subjects (Ley et al., 2006; Kasai et al., 2015). Diets which support a "healthy" and diverse gut microbiota, e.g. high in fiber and whole plant foods, also are protective against obesity and metabolic disease. The pathophysiology of obesity is still not completely understood, probably due to its multifactorial aetiology. Some evidence links obesity with a low-grade systemic chronic inflammation status, suggesting that inflammation could be a potential mechanism by which obesity leads to insulin resistance (Shoelson et al., 2007). Studies have shown that the gut microbiota and their metabolic activities, play an important role in regulating gut permeability and the flow of inflammatory molecules from the intestinal lumen (e.g. lipopolysaccharide, LPS), which have been shown to trigger the systemic inflammation which goes on to cause insulin resistance and metabolic disease (Cani et al., 2007). Indeed, in animals with high fat diet induced obesity and insulin resistance, prebiotic microbiota modulation and fortification of the gut wall reduces systemic inflammation, cause by intestinal LPS and amelioriates markers of metabolic disease (Cani et al., 2007). Nevertheless, several studies confirm the modulatory action of some probiotics (belonging to the genus *Lactobacillus* and *Bifidobacterium*) in the control and reduction of body weight (Minami et al., 2015; Yoo et al., 2013). For example, *B. breve* seems to prevent intestinal inflammation through stimulation of the development of IL-10, producing Treg1 cells in the large bowel (Jeon et al., 2012). Comparative meta-analysis of *Lactobacillus* species has associated *Lactobacillus plantarum* and *Lactobacillus gasseri* with weight loss in animals and humans (Million et al., 2012). Different studies suggest that these

bacteria protect against obesity through multiple mechanisms of action, such as hydrolyzing bile salt, reducing cholesterol, and inhibiting the accumulation of lipid in pre-adipocytes, reducing plasma leptin levels, down-regulating adipogenesis and strengthening intestinal integrity (Wu et al., 2015). Disruptions to the normal balance between the gut microbiota and the host have been associated also with type 2 diabetes, characterized by an altered gut microbiota composition with an elevated *Firmicutes*/*Bacteroidetes* ratio compared with healthy people which leads to a progressive insulin resistance (pre-diabetes condition) (Zhang and Zhang, 2013). Many other pathological conditions were also ascribed to a dismicrobism or dysbiosis within the gut microbiota, including malnutrition (Gordon et al., 2012), inflammatory bowel disease (IBD) (Sartor and Mazmanian, 2012), neurological disorders (Perlmutter and Loberg, 2015) and bowel cancer (Poutahidis et al., 2014). However, in most cases direct cause an deffect between either microbiota composition or dietary supplementation with probiotics remains to be clearly demonstrated in well controlled human trials, limiting our ability to effectively treat obesity through microbiota modulation.

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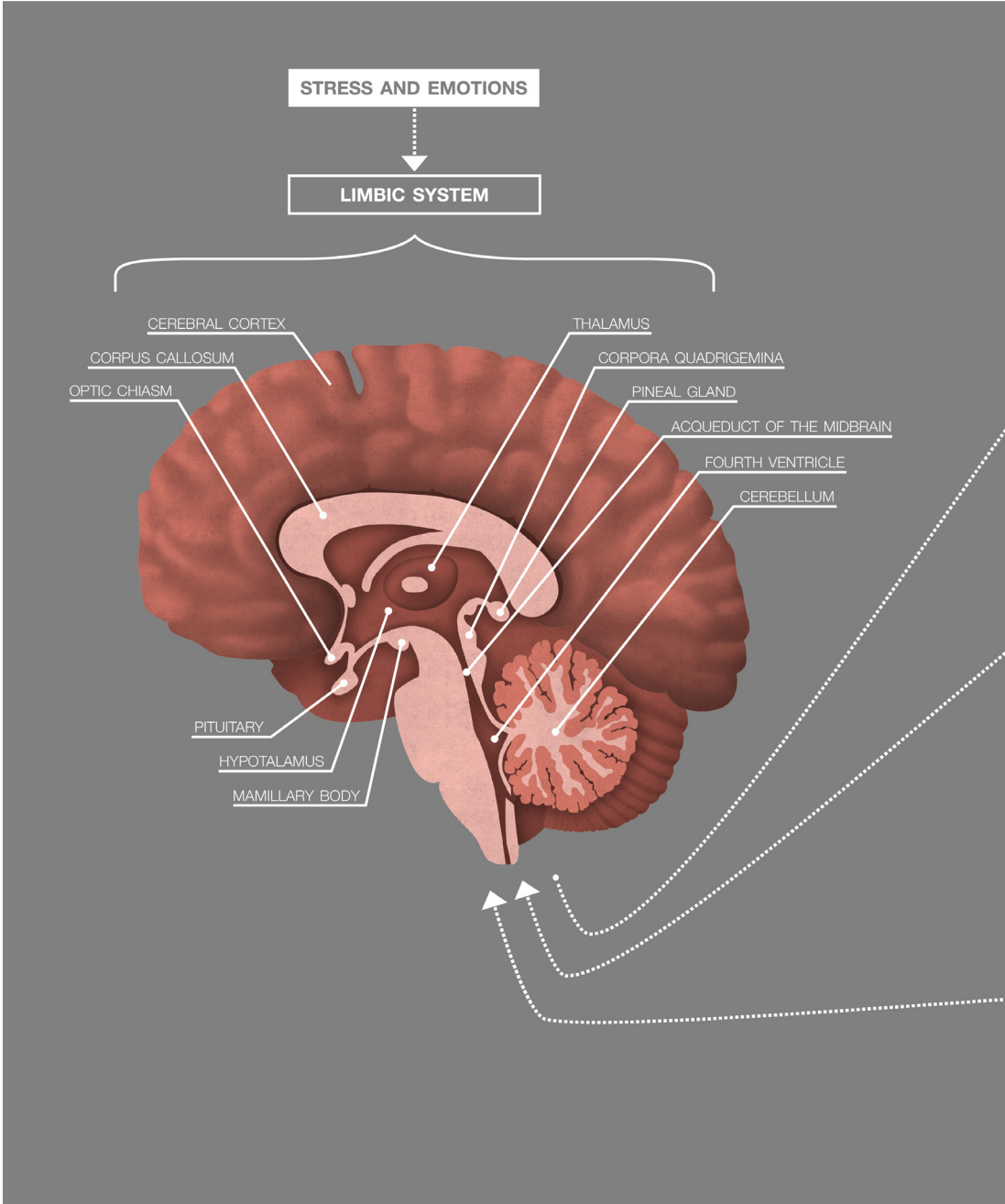
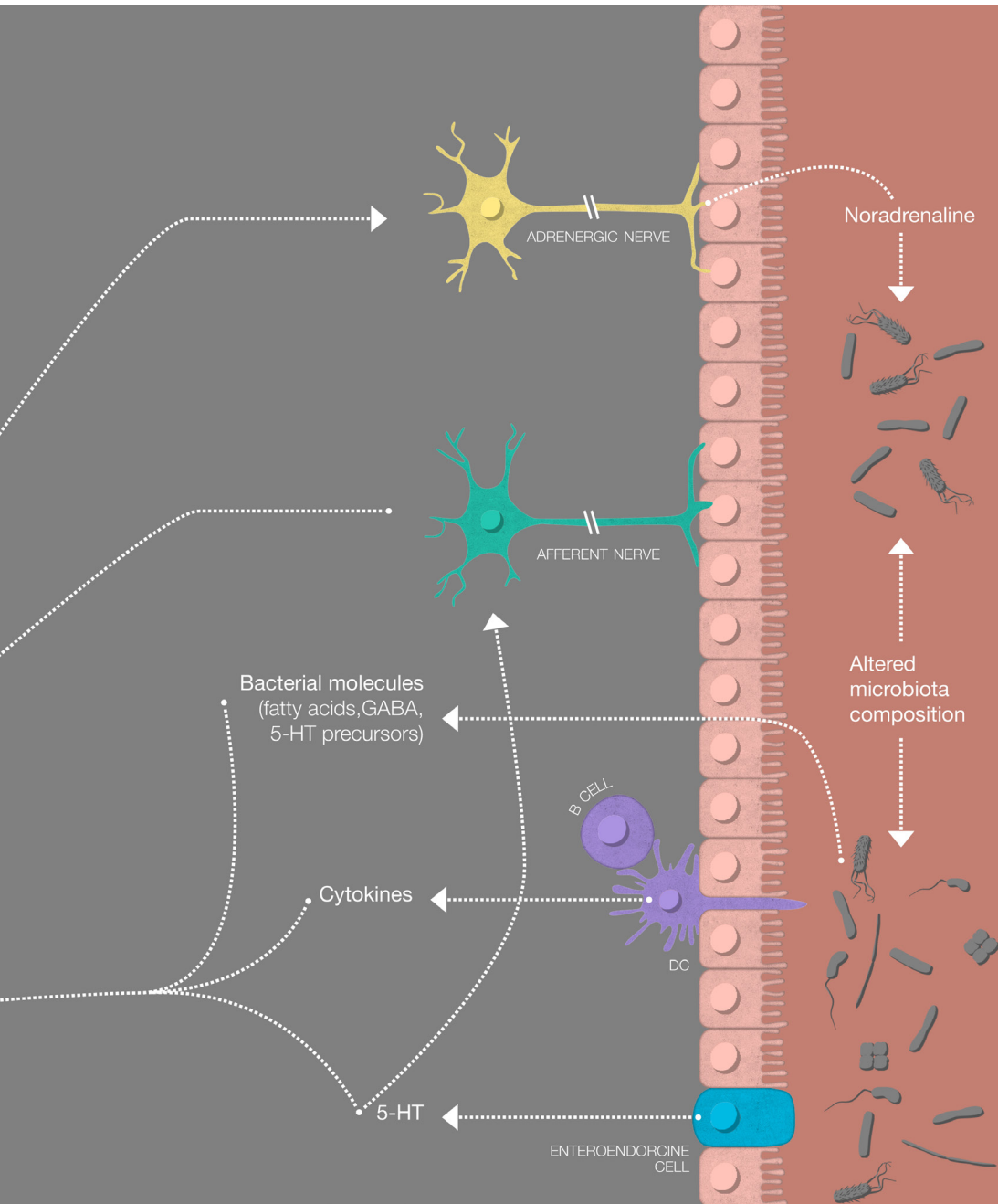# 1.2 Gut – microbiota – brain axis

The gut-brain axis includes the central nervous system (CNS), the neuroendocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS) and the intestinal microbiota (Grenham et al., 2011). Visceral messages from the gut can influence brain function and the possibility of regulating the gut microbiota is opening up a new therapeutic target for a host of brain disorders. Brain levels of serotonin, the major chemical involved in the regulation of mood and emotion, are regulated by the bacteria in the gut. Work in germ-free mouse models has shown how the absence of bacteria can affect brain serotonin concentrations and has highlighted that, this appears to occur in a gender-dependent component, with male brain serotonin levels more liable to alteration than that of females. Indeed, colonization with the gut microbiota in early live, and even the sequential pattern of microbiota colonization or the successional development of the microbiota, appears to shape neonatal brain bevelopmental processes. The brain circuitry laid down in germ-free mice, in the absence of a gut microbiota, appears to be permanent and different from conventional animals. This indicates the important and long lasting effects of appropriate microbial colonisation in infancy in programming brain development and function throughout life (Dinan and Cryan, 2012). There are many potential signaling mechanisms by which gut microbiota could influence brain activity including changes in microbiota-produced signaling molecules, such as SCFA and ketone bodies (Cummings and Macfarlane, 1991; Kimura et al., 2011), phenolic acids (Gasperotti et al., 2015), steroids (Gérard, 2013), amino acids and their derivatives (Fernstrom, 2012; Dai et al., 2011), catecholamines (Rhee et al., 2009), incretins – gut hormones (Cani, 2015; Cani et al., 2009), mucosal immune mechanisms (Sherman et al., 2015; El Aidy et al., 2014), and enterochromaffin cell-mediated vagal activation (Rhee et al., 2009; Raybould, 2010), as summarized in Figure 2. However, it is still unclear which specific metabolites may be involved in the cerebral mechanism and what the contribution of the microbiota per se is to systemic levels of these metabolites and any subsequent impact on brain function.

The gut microbiota, appears to be involved in the development of both central and peripheral neural processes (Tuohy et al., 2015; Sherman et al., 2015). Most of the studies exploring intestinal microbiota interactions with the CNS have been performed using animal models, typically mice. The gut microbiota has been shown to influence the development of the hypothalamic–pituitary–adrenal (HPA) axis, with exaggerated release of corticosterone and adrenocorticotropic hormone in germ-

free (GF) mice in response to stress compared to mice carrying a specific pathogen free (SPF) microbiota. This situation could be reversed in a time dependant manner by colonization with the probiotic *B. infantis* in the post natal period. However, colonization with *E. coli* led to an exaggerated response to the stress in the ex-GF mice, indicating early life exposure of microorganisms is required for the HPA system to become fully susceptible to inhibitory neuronal activity (Sudo et al., 2004). Importantly, it also shows that specific microbial encounters in early life or the sequence of such encounters, can have a dramatic effect on host physiological devemopment and brain function in later life. Neuromodulators, such as corticotropin release factor 1 (CRFR1) in the colon, seem to play an important role in stress-induced permeability dysfunction and modulation of mucosal immune and inflammatory responses (Larauche et al., 2009). Intestinal permeability is linked to systemic inflammation, a trigger for both inflammatory and metabolic diseases and possibly also responsible for increasing inflammation in the brain. Conversely, social stress has been tested in mice and shown to impact on gut microbiota composition, with a reduction in the relative abundance of *Bacteroides* and a concommitant incresase in caecal clostridia (Lyte, 2013). Stressor exposure significantly changed the community structure of the gut microbiota at genus level, modulating *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea*, with a significant correlatation with increased circulating levels of IL-6 and MCP-1 (Bailey et al., 2011). GF mice showed increased motor activity and reduced anxiety compared to SPF mice, together with altered expression of secondary messenger pathway and synaptic long-term potentiation genes in brain regions involved in motor control and anxiety-like behaviour (Diaz-Heijtz et al., 2011). These effects could be reversed by early colonization with specific-pathogen-free microbiota in the neonatal period. Intervention with various probiotic microorganisms, both lactobacilli and bifidobacteria, has been shown in animal models to alleviate anxiety-like behaviour to standard animal stress challenges. Rats fed *B. infantis* showed a significant reduction of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-6, following mitogen activation; elevated concentrations of tryptophan; kynurenic acid; 5-hydroxyindole acetic acid – serotonine metabolite (in the frontal cortex); 3,4-dihydroxyphenylacetic acid – dopamine metabolite (in the amygdaloid cortex), compared to control animals (Desbonnet et al., 2008).
B. infantis could counteract the stress effects. In mice, who had been subjected to maternal separation, a recognised model of depression, *B. infantis* decreased noradrenaline in the brain, elevated systemic IL-6 and amygdala corticotropin-releasing

STRESS AND EMOTIONS

LIMBIC SYSTEM

CEREBRAL CORTEX

CORPUS CALLOSUM

OPTIC CHIASM

THALAMUS

CORPORA QUADRIGEMINA

PINEAL GLAND

ACQUEDUCT OF THE MIDBRAIN

FOURTH VENTRICLE

CEREBELLUM

PITUITARY

HYPOTALAMUS

MAMILLARY BODY

Fig. 1 Gut-microbiota-brain axis: neural, immunological, endocrine and metabolic pathways by which the microbiota influences the brain.

ADRENERGIC NERVE

Noradrenaline

AFFERENT NERVE

Altered
microbiota
composition

Bacterial molecules
(fatty acids,GABA,
5-HT precursors)

B CELL

Cytokines

DC

5-HT

ENTEROENDORCINE
CELL

factor mRNA. In rats fed a probiotic mix (*Lactobacillus helveticus R0052* and *B. longum R0175*), reduced anxiety-like behaviour was observed and the same probiotic mix in humans alleviated psychological distress (Messaoudi et al. 2011). The probiotic *L. rhamnosus JB-1* has been shown to reduce stress induced by corticosterone, anxiety and depression related behaviour and concomitantly, induced changes in γ-aminobutyric acid (GABA) receptor expression in different regions of the brain. In the probiotic fed animals, GABA B1b receptor was upregulated in cingulate and limbic cortical regions and down regulated in the hippocampus, amygdala and locus coeruleus, compared to control animals (Bravo et al., 2011). Similarly, GABA Aα2 receptor expression was reduced in the prefrontal cortex and amygdala, while it increased in the hippocampus. Importantly, neither behavioural changes nor changes in GABA-receptor expression induced by the probiotic occured in vagotomized mice, indicating the central role of the vagus nerve in the gut:brain communication highway. The mRNA expression of GABA B1b receptor after treatment with *Lactobacillus rhamnosus*, was increased in cortical regions, and decreased in the hippocampus, amygdala, and locus coeruleus; while mRNA expression of GABA Aa2 receptor, was reduced in the prefrontal cortex and amygdala, and increased in the hippocampus (Bravo et al., 2011). Similarly, the expression of m-opioid and cannabinoid receptors in intestinal epithelial cells could be induced by *Lactobacillus* strains, which mimic the effects of morphine in promoting analgesia (Rousseaux et al., 2007). It may be therefore that the successional development of the gut microbiota, relative exposure to probiotic or pathogenic microorganisms in early life, plays a role in brain development at a critical point in neurological development that can have far reaching consequences for behaviour and disease risk throughout life. However, there is a clear lack of human data describing the contribution of gut microbiota successional development to either immunological or metabolic communication along the information highways linking gut and brain.

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1.3 Diet:microbe interactions

The digestive system is responsible for the breakdown and modification of complex food macromolecules into smaller components and usable nutrients, electrolytes, and fluids. In addition, it excretes unabsorbed residues in faeces, provides a protective barrier against the entry of toxic substances and infectious agents, serves as the largest endocrine organ in the body and interacts with other endocrine organs, the nervous system, circulatory system and the immune system (Yaqoob and Childs, 2016; Furness, 2000). Chemical digestion of food, begins in the mouth and continues in the stomach, small intestine and finally the colon. Enzymes secreted by the salivary glands, pancreas, stomach, and small intestine all play a role in the digestion of the carbohydrates, proteins, and fats found in our food.

Compounds which escape digestion and absorption in the stomach and small intestine reach the colon where they come in contact with the majority of the gut microbiota. As an anaerobic environment, fermentation is an important activity within the gut microbiota and indeed can be viewed as a key form of metabolism, providing both energy and carbon for the fermentative microorganisms themselves and other organisms which cross-feed off their metabolic end-products (Macfarlane and Macfarlane, 2011). Two major forms of fermentation predominate within the gut microbiota, saccharolytic or carbohydrate fermentation, and protein/amino acid fermentation. When and where these two forms of fermentation occur appears to change with pH and substrate availability in different regions of the colon and may have both metabolic and health related consequences for the host (Macfarlane et al., 1992).

1.3.1 Carbohydrate fermentation

Carbohydrate metabolism begins in the mouth with the secretion of the enzyme salivary amylase, which breaks down digestible starch and glycogen into disaccharides. Chyme, the product obtained by gastric activities (consists of bolus and gastric juices), empties into the small bowel where monosaccharides (e.g. glucose) are absorbed into the bloodstream. Gastric emptying is regulated by incretins, especially glucagon-like peptide-1 (GLP-1), a hormone produced in the upper intestine but which appears to be under direct regulation of SCFA concentrations in the large bowel, via the neuro-endocrine system (Kasubuchi et al., 2015). However, not all dietary carbohydrates are digested efficiently or equally in the stomach and small intestine. Many studies in the last decades have endeavoured to identify, classify and estimate, the amount of carbohydrate which escapes digestion and enters in the colon. The exact amounts and types of carbohydrate that reach the colon and are available for fermentation remains uncertain due to several factors, such as the complexity of the anatomical area under study, several different methodological approaches, food processing, maturity stage of plant foods tested, fluctuations in food intake and individual differences in the intestinal physiology/functions. However, FAO estimate that about 20-40 g carbohydrates reach the colon each day in populations following a typical "westers-style" diet, rich in refined carbohydrates/sugars, fat, red and processed meats, while they estimate that at least 50 g carbohydrate per day reaches the colon in people following more "traditional" diets, rich in whole grains, fruit and vegetables (e.g the Mediterranean or Okinawan diets). This carbohydrate not digested in the stomach or small intestine is mainly made up of non-starch polysaccharides, resistant starch, oligosaccharides, polyols and lactose (Mann et al., 2007). Carbohydrates that reach the large intestine enter into a very different type of metabolism, determined by the anaerobic microbiota and mainly driven by anaerobic fermentation. Intestinal bacteria secrete extracellular enzymes or express membrane bound enzymes, which hydrolyse polysaccharides into oligosaccharides and monosaccharides which in turn enter into the bacterial cells where they are fermented, producing energy (ATP), SCFA and gases (mainly carbon dioxide - CO_2, hydrogen - H_2, and methane - CH_4) (see equation 1). Microbial carbohydrate fermentation in the colon is a relatively efficient process and almost completely catabolizes starch, lactose, alcohol-sugars and fructans. Few plant derived polysaccharides escape colonic fermentation completely untouched even cellulose. Human colonic microbiota metabolize available carbohydrate to obtain energy for their own growth and maintenance (e.g. motility, enzyme synthe-

sis, maintenance of ionic and osmotic gradients, and active ion transport) (Williams et al., 2001).

$$57.5\ C_6H_{12}O_6 + 45\ H_2O \rightarrow 65\ Ac + 20\ PPA + 15\ BTR + 140\ H_2 + 95\ CO_2 + 288\ ATP$$
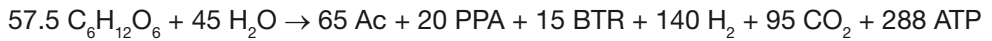
Eq. 1 General equation of anaerobic fermentation. Acetate (Ac), propionate (PPA), butyrate (BTR), adenosine triphosphate (ATP).

Not all carbohydrates are, however, fermented in the same way. One of the first characteristics affecting the fermentation susceptibility is their solubility. The more soluble substrates being more accessible to hydrolytic enzymes, are likely to be degraded more rapidly. Nevertheless, some soluble fibres, such as alginates or carrageenans are poorly fermented, because of their chemical structure. Other factors involved in carbohydrate susceptability to fermentation include chemical structure and binding, the individual differences in the intestinal microbiota and the environmental conditions (e.g. pH, others nutrients presents in the medium, amount of material to be digest) (Krajmalnik-Brown et al., 2012). Although a part of the products of fermentation (as reported in table 3), are utilized by microorganisms, the majority of SCFAs produced are rapidly absorbed by the colonic mucosa. The remaining "waste food" and microbial biomass are eliminated via rectum as feces and gases. Interestingly, not all gases are excreted as waste. Acetogenic bacteria produce acetate from CO_2 and H_2, while methanogenic bacteria produce CH_4 by reduction of CO_2 with H_2 and finally, sulfate reducing bacteria utilise H_2 to reduce sulfates and produce sulfites or hydrogen sulfide (Gibson et al., 1990). Absorption of SCFA is rapid and leads to accumulation of bicarbonates and to an increase of the pH of the lumen, especially in the distal colon, where carbohydrate availability, at least for people who follow a Western diet, becomes limiting. SCFA stimulate proliferation of colonic epithelial cells. Butyrate is the preferred substrate of the colonocytes, the epithelial cells lining the colon, providing about 50% of intestinal energy supply. Absorbed SCFA which are not metabolized in the mucosa, are oxidized in the liver or metabolised in the peripheral tissues. The majority of the butyrate is metabolised in the intestine, unlike the propionate, which is eliminated mainly through the liver, while about 40% of acetate absorbed in the intestine passes the liver and eventually enters the circulatory system (Pomare et al. 1985; Cummings et al. 1987; Cummings et al. 1997). Acetate may, under certain conditions, be used as a substrate for cholesterol in the liver and also in the brain, which harbours 80% of the bodies

cholesterol which must be synthesised in situ as it does not pass through the blood brain barrier (BBB).

Phyla	Families	Metabolites Produced
Bacteroidetes	*Bacteroides* *Prevotellae* *Prophyromonadaceae* *Rikenellaceae*	acetate, propionate, succinate
Firmicutes	*Clostridiaceae* *Lactobacillaceae* *Leuconostocaceae* *Bacillaceae*	acetate, formate, L,D-lactate, butyrate, succinate, propionate
	Streotococcaceae *Eubacteriaceae* *Staphylococcaceae* *Peptococcaceae* *Peptostreptococcaceae*	BCFA, indoles, sulphides, phenols, amines, NH_3, H_2, CO_2, CH_4
Actinobacteria	*Bifidobacteriaceae* *Actinomycetaceae* *Coriobacteriaceae* *Corynebacteriaceae* *Propionibacteriaceae*	lactate, acetate, formate
Protobacteria	*Micrococcaceae* *Enterobacteraceae* *Oxalobacteriaceae* *Pseudomonadaceae*	lactate, acetate, succinate, formate
	Desulfovibrionaceae *Helicobacteraceae*	sulphide, sulphate, H_2S, mercaptans
Euryarchaeota	*Methanobacteriaceae*	CH_4
Fusobacteria	*Fusobacteriaceae*	acetate, butyrate, NH_3, formate, lactate
Verrucomicrobia	*Verrucomicrobiaceae*	N.R.
Lentisphaerae	*Victivallaceae* (e.g. *Victivallis vadensis*)	acetate, ethanol, H_2

Tab. 1 Human gut fermenting bacteria with important families and fermentation products. Green indicates the metabolites produced mainly from protein sources, yellow from carbohydrate sources, while no color from both. N.R. means "Not Rated".

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1.3.2 Prebiotic concept

Observations that different dietary fibers appear to be fermented differently or with different preferences by the intestinal microbiota and that the human milk oligosaccharides selectively stimulate the growth of bifidobacteria within the infant gut led to the development of the "prebiotic" concept. A prebiotic was first defined as, "a non-digestible food ingredient, that beneficially affects the host, by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health" (Roberfriod et al 1998). The most widely accepted current definition describes a prebiotic as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2010). The list of the compounds recognized as prebiotic, has grown from compounds like inulin, lactulose, fructooligosaccharides (FOS), and galacto-oligosaccharides (GOS) to include new potential prebiotics, including certain resistant starches, arabinoxylans, and some whole grain products or fractions thereof (Bindels et al., 2015). However, to be classed a prebiotic these compounds must be evaluated in human studies and shown to have selective modulatory activities on the composition of the intestinal microbiota, preferably in a dose response manner and reproducibly, and also to generate one (or more) measurable benificial effects on human health.

Inulin, which was the first dietary fiber defined as a prebiotic, is still one of the most studied. It is a naturally occurring oligosaccharide (DP2-60) belonging to a group of carbohydrates known as fructans. It occurs naturally in foods such as leek, asparagus, chicory, Jerusalem artichokes, garlic, onions, wheat, oats, and in the soybeans (van Loo et al., 1995). Now considered the "gold standard" prebiotic, inulin is undigestible for human enzymes, passing intact through the stomach and small intestine, and arrives in the colon where it is extensively fermented by the resident microbiota. The selective action on the growth of some bacterial populations by inulin intake has been demonstrated repeatably by different researchers and using different microbiological approaches. Within the human gut microbiota inulin stimulates the growth of bifidobacteria, and also some commensal species including *Roseburia* and *Faecalibacterium prausnitzii* (Scott et al., 2011; Ramirez-Farias et al., 2009). Both these bacteria are prominent butyrate producing bacteria within the human commensal microbiota and *F. prausnitzii* appears to be reduced in patients with inflammatory bowel disease (IBD) and is considered to have anti-inflammatory activities in models of IBD (Scott et al., 2015). Table no. 2, shows the scientific evi

FUNCTIONS	ACTIVITIES	REFERENCES
Enhances mineral (Mg^{2+} and Ca^{2+}) uptake by acidification of the intestinal lumen	Remineralizing	(Hess et al., 2015; Abrams et al., 2005)
Immunomodulation (attenuates Cr-induced intestinal inflammation through mechanisms affecting NF-κB and Smad 7 signaling); human vaccine adjuvants (increases production of activated C3 and thereby activate macrophages, stimulates cellular (Th1, Th2) immunity)	Immunomodulant, protective (GIT and systemic)	(Lomax et al., 2015; Franco-Robles and López, 2015; Saade et al., 2013; Kuo, 2013; Foye et al., 2012; Layton et al., 2011; Lobigs et al., 2010; Guarner, 2005)
Epigenetic effect by enhanced production of SCFA in the colon (mainly butyrate), acting on leucocytes, intestinal cells, and endothelial cells through inhibition of histone deacetylase		(Jung et al., 2015; Canani et al., 2012 and 2011; Gao et al., 2009;)
Bifidogenic activity (*Bifidobacterium longum* and *Bifidobacterium adolescentis*)		(Joossens et al., 2011; Falony et al., 2009; Kolida et al., 2002; Guigoz et al., 2002: Kruse et al., 1999; Gibson et al., 1995)
Stimulate the growth of *Lactobacillus* strains	Anti-pathogenetic	(Jung et al., 2015)
Lowering of plasma triglycerides and LDL cholesterol	Lipid-lowering	(Daubioul et al., 2005; Causey et al., 2000; van Dokkum et al., 1999; Pedersen et al., 1997)
Modulates hyperglycemic and insulin resistance (inhibition of glucose absorption from the intestine, stimulated glucose uptake into the skeletal muscle cells)	Antihyperglycemic	(Nishimura et al., 2015; Yun et al., 2009)
Lowers postprandial appetite, but not energy intake	Weight control	(Heap et al., 2016)
Lowers Plasma p-Cresol in Chronic Kidney Disease	Antitoxic	(Salmean et al., 2015)

Tab. 2 Physiological or health effects of dietary inulin and other fructans

dence support this fructan fiber as a prebiotic.

The main role of prebiotics in the diet is to promote the growth and proliferation of beneficial bacteria within the intestinal tract and to improve host health. Since our understanding on which bacteria are actually beneficial for the host is still in its infancy, most of the studies have focused on bifidobacteria because of their occurrence in breast fed infants and their beneficial host-health effects at least confirmed by animal models (Conlon and Bird, 2014). Prebiotics, have also been shown to increase the absorption of certain minerals (e.g. calcium, magnesium and zinc) (Scholz-Ahrens et al., 2007), to inhibit the growth of adenomas and carcinomas in the intestine, and thus reduce the risk factors involved in colorectal diseases (Liong, 2008). They may also result in changes in the production of cytokines and chemokines and exercise anti-inflammatory effects, promote cell growth and differentiation, reinforce intestinal barrier function, enhance intestinal motility, inhibit intestinal cholesterol synthesis, control body weight by enhancing mitochondrial function, and increase insulin sensitivity in type 2 diabetics (Hardy et al., 2013; Tremaroli and Bäckhed, 2012) (Table 3). However, most of these beneficial health effects have only been demonstrated convincingly in animal models and await confirmation in appropriately designed human intervention studies.

Prebiotics	Function	Source
Arabic gum	↑ SCFAs	acacia, carrageenan, guar, locust bean and xanthan
Fructooligosaccharide	→ Bifidobacterium and Lactobacillus strains, ↑ Ca and Mg absorption, ↓ triglycerides and has anticarcinogenic effects	jerusalem artichokes, onions, leeks, grains and honey
Inulin	→ Bifidobacterium sp. (colon), osmotic regulator, has immunomodulatory properties ↓ serum cholesterol and triglycerides and ↓ calorie value	liliaceae, amaryllidaceae, chicory, onions, leeks, garlic, bananas, asparagus, and artichokes
Isomalto-oligosaccharide isomalto tetrarose	→ Bifidobacterium and Lactobacillus strains	isomaltose, panose, and other higher branched oligosaccharides
Lactitol	Releases constipation and hepatic encephalopathy	produced by catalytic hydrogenation of lactose
Lactosucrose	↑ Bifidobacterium sp.	produced from lactose and sucrose by p-fructofuranosidase from Arthrobacter sp.
Pyrodextrin	→ Bifidobacterium sp. (colon)	produced by heat-treating of starch
Soy oligosaccharide	→ Bifidobacterium sp. (colon)	soybeans and peas
Trans Galacto-oligosaccharide	→ Bifidobacterium sp. (colon)	produced from lactose by α-galactosidases from Lactobacillus reuteri
Xylo oligosaccharide	↑ Bifidobacterium sp., ↓ blood sugar levels and ↑ fat metabolism, restore normal intestinal flora, ↑ mineral absorption and vitamin B production and ↓ intestinal putrification	produced from lignocellulosic materials by enzymatic hydrolysis of xylan

Tab 3. Prebiotics – putative physiological effects and sources

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1.3.3 Protein fermentation

Protein is the major nitrogen-containing component of the diet. It is composed of long chains of the 20 different amino acids (AA) linked together by peptide bonds. Proteins are essential constituents of receptors and cell signaling molecules, structural building blocks, enzymes, ion channel pumps, O_2 and CO_2 transporters (hemoglobin), hormones, etc.. Dietary proteins are digested to their constituent amino acids prior to their absorption in the small intestine. In the gastric and pancreatic juices, peptidase enzymes hydrolyze peptide bonds to generate small peptides and free amino acids. Others enzymes located on the mucosal surface of the small bowel, continue protein and polypeptide digestion (Webb, 2012). Like fat and carbohydrates, amino acids are also metabolized to form ATP (see equation 2).

$-NH_2CHRCOOH$ (protein) $+ H_2O \rightarrow H_2NCHRCOOH$ (AA) $+ NH_3 + CO_2 \rightarrow$ ATP
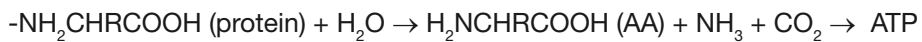
Eq. 2 General equation of proteolytic anaerobic fermentation.

Amino acids have a similar oxidation state to that of glucose and undergo microbial fermentation and their breakdown fosters ATP synthesis. Via the citric acid cycle, amino acids, can be oxidised and converted into urea, pyruvate and other keto acids, which are used in the anabolic synthesis of other compounds. Amino acid degradation follows several pathways, but the Stickland reaction remains the most efficient one. Stickland couples the oxidation of one amino acid with the reduction of another. Keto acids obtained from amino acid oxidised by deamination (amino acid dehydrogenases), leads to ATP synthesis, through loss of CO_2 and subsequent phosphorylation of the substrate. This reaction is coupled with reductive deamination (reductase), of the other amino acid. The total amount of C atoms are equal in both donor and acceptor amino acid. Usually, the second reaction does not produce ATP, except if it involves two molecules of glycine. The produced ATP, thus depends on the specific pair of amino acids involved in and to the stoichiometry of the reaction (e.g. 4 moles of glycine produce 3 moles of ATP) (de Vladar, 2012).

1 glycine + 1 alanine $\rightarrow$ 1.7 ATP

Fermentation of protein remains with a lower energetic yield compared to the fermentation of carbohydrates:

2 glucose $\rightarrow$ 5 ATP
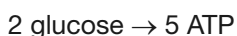
The amino acids play a vital role in maintaining the integrity and mass of the intestinal mucosal, as well as being involved in the synthesis of glutathione, nitric oxide, polyamines, purine and pyrimidine nucleotides, and small amino acids (alanine, citrulline and proline) (Wu, 1998). Amino acids can be directly incorporated into bacterial cells, as building blocks of proteins and some amino acids can also be synthesized de novo by the gut microbiota (Neis et al., 2015). The intestinal microbiota plays an important role in the synthesis and/or bioavailability of metabolites involved in neurotransmission, many of which are derived from amino acidic metabolic pathways (Neis et al., 2015). Competition for peptides and free amino acids between actively growing bacteria in the intestinal lumen and colonocytes would seem to preclude uptake by the colonic mucosa, which has no specific transport systems for them (Bergen and Wu, 2009). Furthermore, the exchange of nutrients between the intestinal mucosa/microbiota and host, can proceed in both directions making flow prediction difficult. High protein diets and resistant protein intake (e.g. glycated protein, formed during food cooking) (Tuohy et al., 2006), could determine a buildup of undigested protein in the colon skipping previous catabolism (see figure no. 4). It has been estimated that about 12–18 g of dietary protein reaches the colon each day (Scott et al., 2013). There is also an important contribution of endogenous protein sources (e.g. mucin, human/bacterial protein from cell turnover, enzymes, etc.) to colonic protein availability (Moughan et al., 2005). Studies in ileostomy patients suggest that endogenous protein can reach about 1852 mg N/day, thus representing an important contribution to the N economy of the colonic microbiota (Moughan and Rutherfurd, 2012).

The microbial catabolism of amino acids may result in competition between different microorganisms as well as on the production of biologically active compounds including SCFA, Branched-Chain Fatty Acids (BCFA), phenols, indoles and ammonia (Scott et al., 2013). The main metabolites involved in this pathway are the SCFA and BCFA, indicating that dissimilatory reactions are primarily affected through reductive deamination (Macferlane et al., 1986). SCFA are important end-products, formed during AA fermentation by clostridia and anaerobic gram-positive cocci. The ratio of SCFA formation is dependent on the chemical composition of the AA involved. Moreover, the time taken for the catabolic reactions changes from a rapid fermentation for the basic amino acids, such as lysine and arginine, to a slow and sometime incomplete fermentation for the sulfur amino acids (Macfarlane, 1992). Acetate and butyrate are formed from glutamate, while acetate and propionate are

mainly produced from aspartate (Smith and Macfarlane, 1997). Acetate, is required for optimal growth of *F. prausnitzii* and *Roseburia intestinalis / Eubacterium rectale*. Acetate is also re-absorbed by certain microorganisms and diverted into the butyrate production pathway (Duncan et al., 2004). Besides the most studied organic fatty acids, acetate, propionate, and butyrate, BCFA, such as isobutyrate and isovalerate, are produced by degradation of AA and have been shown to possess antimicrobial effects against pathogenic species by decreasing the pH in the intestine (Williams et al. 2001). Clostridia largely use Stickland reaction for energy production from amino acids, which consists as mentioned before of coupled deamination between two amino acids, acting as donor and acceptor of hydrogen respectively (Nisman, 1954). The principal H-donors involved in this reaction are alanine, leucine, isoleucine, valine and histidine, while H-acceptors are glycine, proline, ornithine, arginine and tryptophan (Davila et al., 2013).

The main gases produced during protein fermentation include, hydrogen (H_2) and carbon dioxide (CO_2), resulting mainly from urea hydrolysis (Cone and van Gelder, 1999). Hydrogenotrophic microorganisms, such as sulfate reducing bacteria, methanogenic archaea and acetogenic bacteria, may consume these gases to generate hydrogen sulfide (H_2S), methane (CH_4) and acetate (Bernalier et al., 1996). Methane is a stable end product that is excreted from the gut (Triantafyllou et al., 2014). H_2S is another gas produced by cysteine and methionine breakdown and induces hyperproliferation of crypt cells and is therefore thought to predispose to colonic carcinomas and ulcerative colitis (Pitcher et al., 2000). Fortunately, it may also be further metabolized and thus, detoxified by colonocytes (Barton et al., 2014). The metabolites derived from commensal microorganisms can have various effects on the host immune system (Jacobs et al., 2009). Some of these components are generally considered toxic and it is thought they can cause adverse effects on the colonic epithelium. The best characterized of these protein endproducts include phenols and indoles (González-Parra et al., 2013; Evenepoel et al., 2009), which result from deamination, decarboxylation, fermentation and α or β elimination reactions of protein degradation by colonic bacteria (Hughes et al., 2000). Ammonia is a toxic waste product, produced by the deamination of amino acids or from hydrolysis of urea via microbial urease activity. It is a very volatile compound which can be rapidly absorbed from the intestinal lumen and its concentration in faeces also appears to be directly proportional to the consumption of protein in the diet. Ammonia causes damage in the intestine and, in patients with end-stage liver
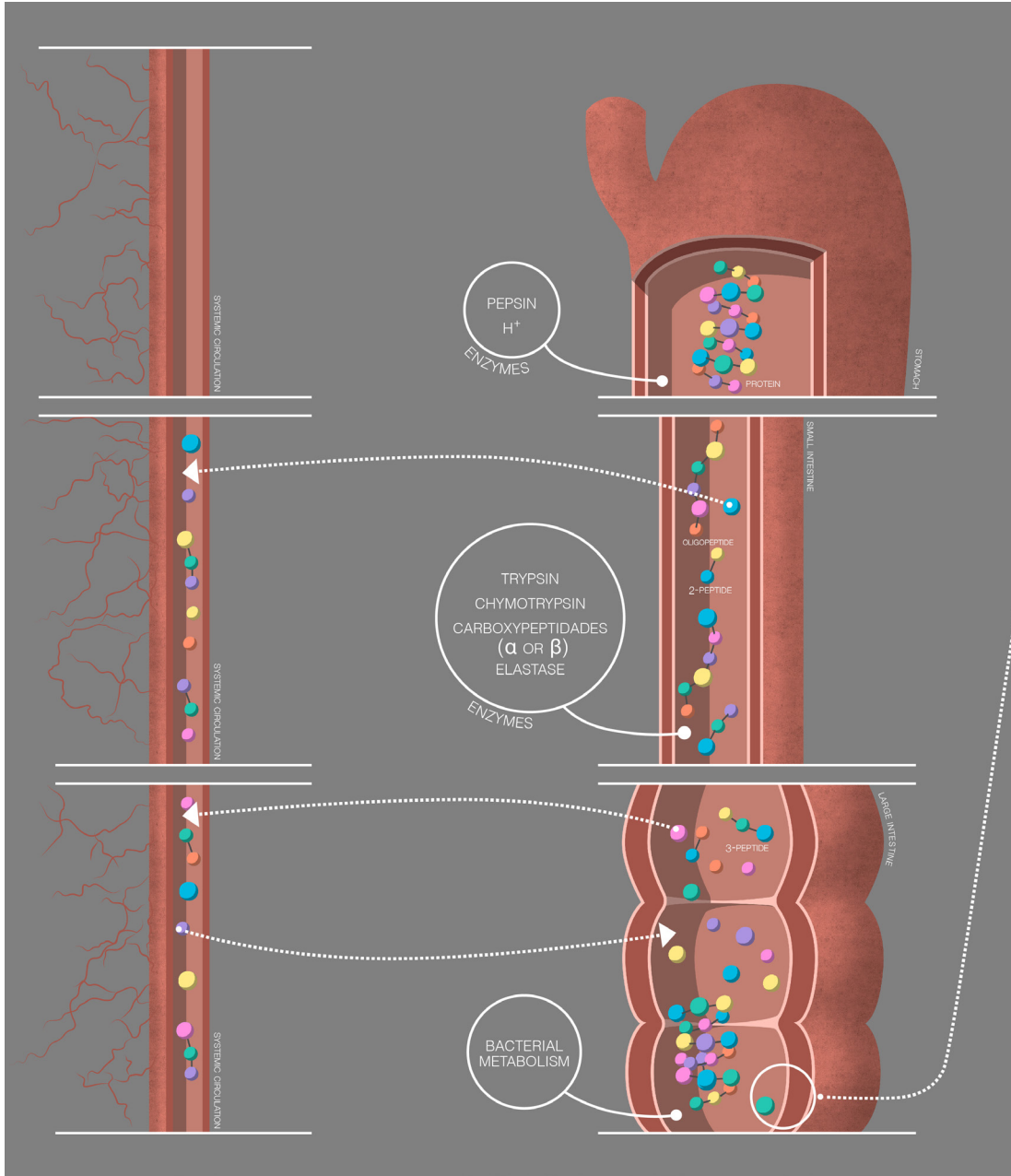
disease, it may cause hepatic encephalopathy (Butterworth et al., 1987). This brain intoxication can lead to cognitive impairment marking the key role the liver plays in the detoxification of compounds absorbed fr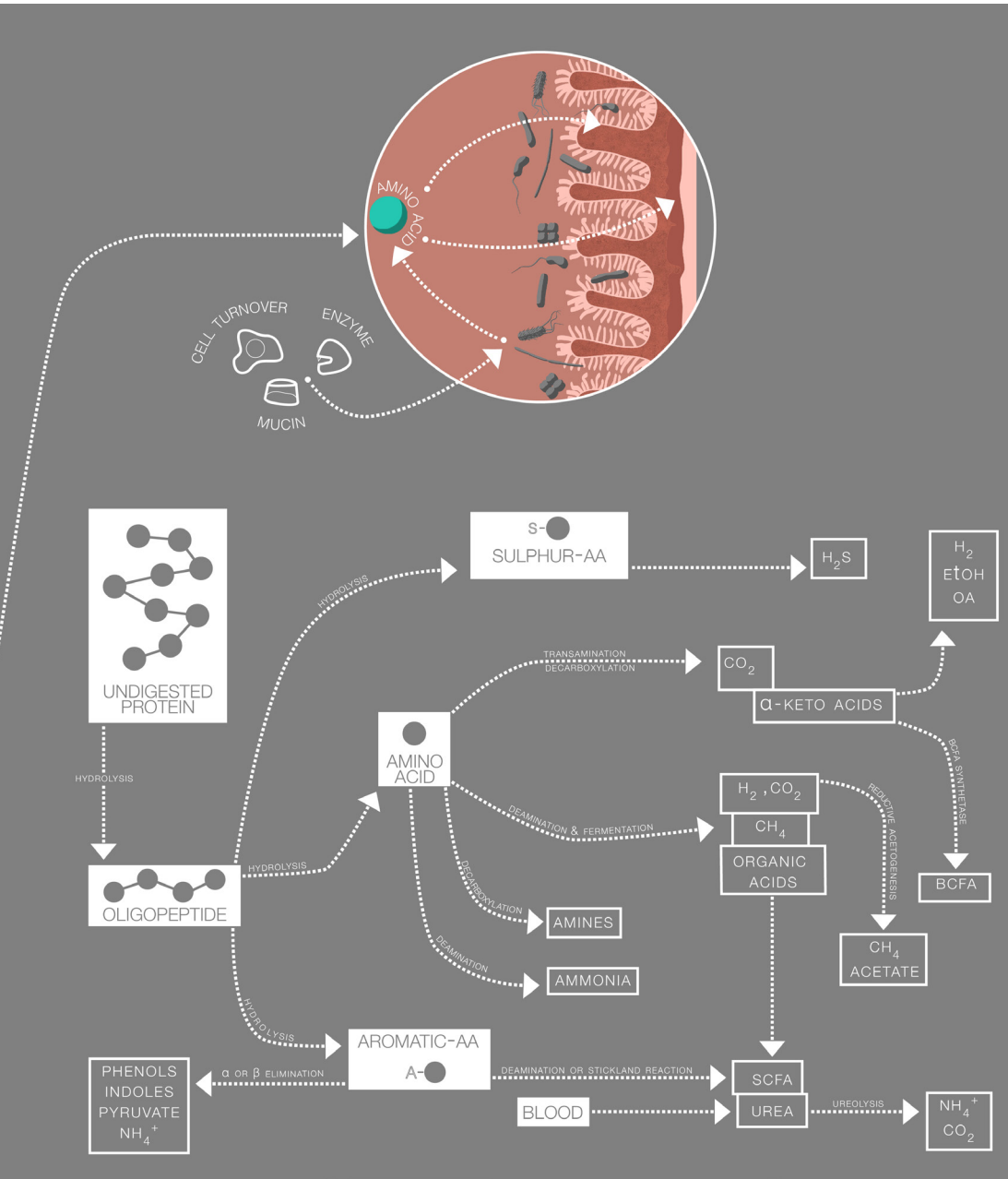om the gut (Macfarlane et al., 1986). The synthesis of lysine by bacteria using free ammonia from urea hydrolysis or amino acid fermentation is also possible (Metges, 2000). However, again its significance to host nutritional, needs remains to be determined.

Amino acid metabolism plays an important role in the biosynthesis of metabolites involved in neurotransmission and this process, may be influenced by diet and the activities of the gut microbiota (Clarke et al., 2014). Also amino acids themselves can modify the ability of gut bacteria to utilize other dietary amino acids, as shown for L-arginine and L-glutamine, which appear to interfere with the transport, metabolism and utilization of other AA, by gut bacteria (Dai et al., 2013; Dai et al., 2012). Additionally, glutamate, glutamine, and aspartate are major metabolic fuels for enterocytes and also regulate intestinal and neurological development and function (Wu, 2010). Glutamine is essential for ATP production, synthesis of nucleotides, expression of anti-oxidative genes, and redox signaling in enterocytes. While glutamate, activates chemical sensing in the gastrointestinal tract and may inhibit degradation of both Essential Amino Acids (EAA) and Non- Essential Amino Acids (NEAA) by intestinal microorganisms. Moreover, proline and arginine, which are major sources of ornithine for intestinal and placental synthesis of polyamines, are essential for DNA and protein synthesis and also participate in protein and DNA methylation, and, thus to the genetic and epigenetic regulation of cell growth and development (Wu, 2014). Similarly, beta-alanine a common amino acid in meat, inhibits the uptake of γ-amino butyric acid (GABA) by intestinal cells, such as Caco2 (Aanesen et al., 1996).

and peroxidation of dietary fats, which may explain the increased risk of colon cancer with diets high in red and processed meats (Bastide et al., 2011). High meat consumption seems to be linked to an increased bioavailability of the amino acid arginine, which induces nitric oxide synthase to produce nitric oxide (NO) (Rath et al., 2014). However, there was no effect on faecal nitrite on changing from a low to high white meat and fish diet in rats (Parnaud et al., 2000). This suggests that, the increase in faecal NOC and nitrosating agents, may be due to iron content, which is the main difference between red and white meat and links red meat with an increased risk of colon cancer characterized by elevated endogenous deamination mechanisms (Bingham et al., 1996).

PEPSIN
H$^+$
ENZYMES

STOMACH
PROTEIN

SMALL INTESTINE
OLIGOPEPTIDE
2-PEPTIDE

TRYPSIN
CHYMOTRYPSIN
CARBOXYPEPTIDADES
(α OR β)
ELASTASE
ENZYMES

SYSTEMIC CIRCULATION

LARGE INTESTINE
3-PEPTIDE

BACTERIAL
METABOLISM

Fig. 4 Undigested protein chart and biochemical pathway

UNDIGESTED
PROTEIN

OLIGOPEPTIDE

AMINO
ACID

S-●
SULPHUR-AA

H_2S

H_2
EtOH
OA

TRANSAMINATION
DECARBOXYLATION

CO_2

α-KETO ACIDS

DEAMINATION & FERMENTATION

H_2, CO_2

CH_4

ORGANIC
ACIDS

BCFA SYNTHETASE

BCFA

REDUCTIVE ACETOGENESIS

CH_4
ACETATE

DECARBOXYLATION

AMINES

DEAMINATION

AMMONIA

HYDROLYSIS

AROMATIC-AA

A-●

α OR β ELIMINATION

PHENOLS
INDOLES
PYRUVATE
NH_4^+

DEAMINATION OR STICKLAND REACTION

SCFA

BLOOD

UREA

UREOLYSIS

NH_4^+
CO_2

HYDROLYSIS

CELL TURNOVER

ENZYME

MUCIN

AMINO ACID

37

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1.3.4 Pathophysiology implications of protein metabolism

Currently, we know little about the microbial species or indeed metabolic processes involved in amino acid bioavailability and biotransformation by the gut microbiota as a community and less about the possible implications for neurological function. We also do not know much about how different foods and food components interact to regulate amino acid uptake or metabolism by the gut microbiota. Early in vitro studies by Smith and Macfarlane showed that amino acid fermentation by the human gut microbiota is inhibited both by low pH and by the presence of fermentable fiber/carbohydrate (Smith and Macfarlane, 1997). However, we have little information about how this process translates in vivo or how it changes in disease states, upon antibiotic therapy or upon the over-nutrition typical of the Western-style diet and likely to radically impact on fermentation in the colon.

Many products of the fermentation of amino acids in the colon are considered harmful to the host (Fig. 5), if availability of organic-N containing compounds is high and/or if the supply of carbohydrates, that hinder the putrefactive process, is too low. A recent hypothesis proposes heme iron in the form of hemin (a ferric form of heme), as catalyst in the endogenous formation of N-nitroso compounds (NOC) and peroxidation of dietary fats, which may explain the increased risk of colon cancer with diets high in red and processed meats (Bastide et al., 2011). High meat consumption seems to be linked to an increased bioavailability of the amino acid arginine, which induces nitric oxide synthase to produce nitric oxide (NO) (Rath et al., 2014). However, there was no effect on faecal nitrite on changing from a low to high white meat and fish diet in rats (Parnaud et al., 2000). This suggests that, the increase in faecal NOC and nitrosating agents, may be due to iron content, which is the main difference between red and white meat and links red meat with an increased risk of colon cancer characterized by elevated endogenous deamination mechanisms (Bingham et al., 1996). Recentrly, it has been reported that almost 90% of the gastrointestinal cancers could be attributed to dietary habits (Anand et al., 2008). The association between red and processed meat consumption, as well as the low fiber, calcium, vitamin D and folate intake, with the higher colon cancer risk (CRC) is now well established (Cancer Research UK). Metagenomic studies have now identified a specific oncogenic microbiome, implicating *Strepto-*

coccus gallolyticus (previously *S. bovis*), which was one of the first microorganisms associated with tumors, although the causative link has not been established. Other microorganisms subsequently associated with this disease are: *Fusobacterium necrophorum*, *Enterococcus faecalis*, *Fusobacteria*, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia spp.* and *Methanobacteriales*, while *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium spp.*, *Roseburia*, and *Treponema*, are reduced. Nevertheless, whether this represents cause or effect remains unknown. A potential causal mechanism proposed lies in the ability of these microorganisms to adhere to the colonic epithelium through FadA adhesion and by stimulation of E-cadherin/β-catenin signaling to promote carcinogenesis. Significant higher abundance of *Bacteroides–Prevotella* group in CRC patients, with an increased levels of IL-17 immunoreactive cells, suggests the involvement of immunological functions in the development of microbiota dysbiosis. The bacterial butyrate producers, who are amongst those most reduced during the intestinal dysbiosis, play an important role in the prevention and modulation of the tumor process. Butyrate, produced predominantly by *Clostridium clusters XIVa* and *IV*, exhibits anti-proliferative activity and can induce apoptosis, or programmed cell death, in cancer cells. Butyrate, as a histone deacetylase (HDAC) inhibitor activates the apoptotic cascade, arresting the growth of tumors via histone hyperacetylation. Moreover, butyrate can suppress tumors by potentiating p53 gene expression and transforming growth factor-β (TGF-β) signaling, and increase the immunogenicity of cancer cells. Proteolytic fermentation of undigested meat by microrganism in the gut (mainly *Bacteroides spp.*, and *Clostridium spp.*), can produce inflammatory and procarcinogenic end-products, such as nitrosamines, branched-chain fatty acids, phenolic (phenols and p-cresol) and indolic compounds from aromatic amino acids catabolism. Red meat moreover, is also rich in sulfur containing amino acids, which promote the growth of sulfur-reducing bacteria (SRB) (e.g. *Desulfovibrio vulgaris*) that convert the H_2 gas produced, during saccharolytic fermentation, into an inflammatory and genotoxic end-product, hydrogen sulfide (H_2S). This compound is able to impair cytochrome oxidase, inhibit mucin synthesis, suppressing butyrate utilization, and promotes methylation of DNA by generating free radicals. Finally, SRB can also suppress the growth of methanogens, such as the methanogenic Archaea (e.g. *Methanobrevibacter smithii*), which is involved in the metabolism of H_2 through its conversion into non-toxic methane, which may be exhaled in the breath (Vipperla and O'Keefe, 2016).

BEEF

Ch
PCh
CAR

HEME

CATALYSIS

LIPID PEROXIDATION

ATNC FORMATION

MDA

4-HNE

DNA DAMAGE/
ADDUCT

DNA ADDUCT

MUTAGENIC/
GENOTOXIC

CYTOTOXIC/
GENOTOXIC

APC$^{+/+}$ CELLS

K-RAS, p53

PROMOTION/
PROGRESSION

INITIATION/
PROMOTION

ADENOCARCINOMA

INITIATION

PROMOTION

NORMAL
CRYPT

ABERRANT
CRYPT

LARGE INTESTINE
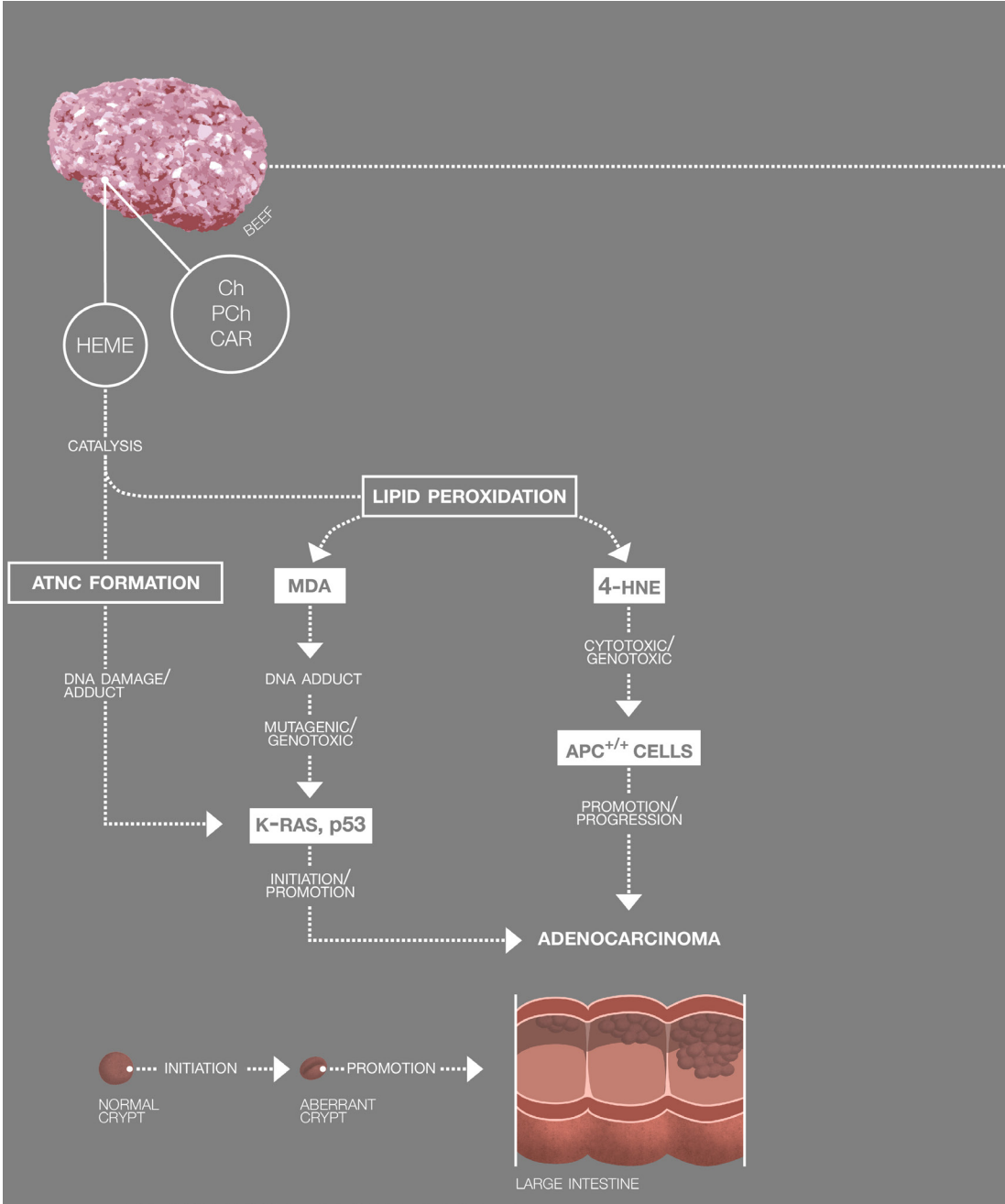
42   Fig. 5 Potentially toxic pathways of red meat metabolism

LARGE BOWEL

TMA

NICOTINIC AGONIST?

BRAIN

TMA $\xrightarrow{FMO3}$ TMAO

TMAO

LIVER

INSULIN

−

HDL

SYSTEMIC CIRCULATION

+

CVD

Foam cell

43
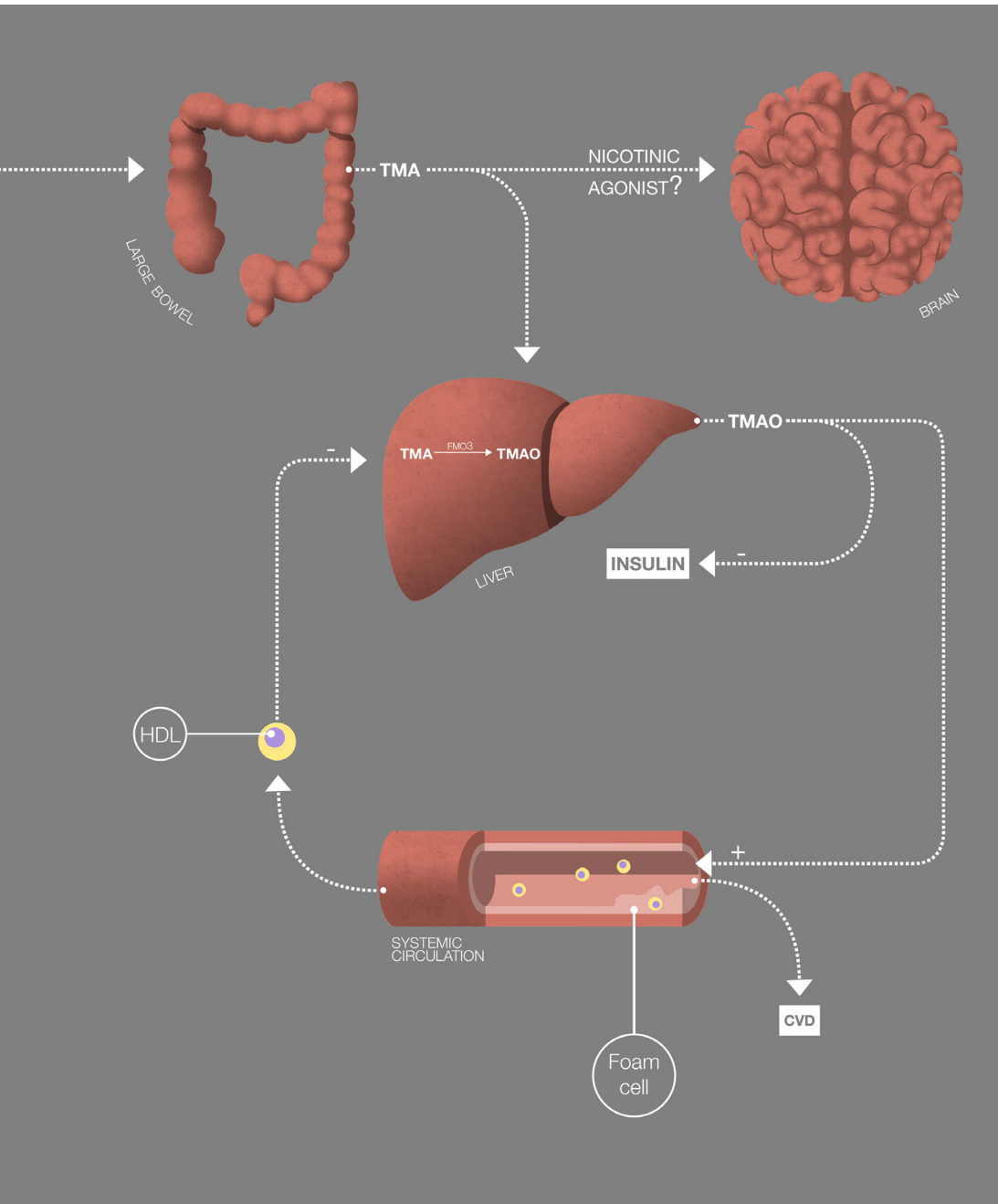
A number of recent studies have focused also on the role of certain metabolites derived from protein fermentation in cardiovascular disease (CVD). Studies examining microbial metabolism of phosphatidylcholine, choline and betaine, have linked microbial metabolic processes with production of a cardiotoxicant trimethylamine-N oxide (TMAO). Indeed TMAO is becoming recognised as a putative new target for the prevention of cardiovascular disease (CVD) risk or at least an early biomarker of CVD (Koeth et al., 2013). Pro-atherogenic TMAO is formed by a two step process: the first step starts with the gut microbiota, dependent cleavage of a TMA molecule from PC/choline/betaine and the second step, takes place in the liver, with the enzyme FMO3 mediated oxidation producing TMAO (Brugère et al., 2014). TMAO not only promotes atherogenesis through foam cell formation and interferes with macrophage reverse cholesterol transport in atherosclerotic plaques, but recent studies in mice also suggest that dietary TMA may exacerbate impaired glucose tolerance, obstruct hepatic insulin signaling, and promote adipose tissue inflammation in animals fed a high-fat diet (Tang and Hazen, 2014). Many of the bacteria involved in undigested-protein metabolism, such as clostridia, *Bacteroides*, sulphate-reducing bacteria, *Escherichia coli*, *Peptostreptococcus*, *Fusobacterium* species, and species belonging to the Actinobacteria are recocnized as capable of producing TMA (Richardson et al., 2013; Le Leu et al., 2006). Usually *Prevotella*–dominated microbiota, or "enterotype", is associated with fiber intake, while *Bacteroides*-dominated microbiota is correlated with protein intake (Wu et al., 2011). Nevertheless, starvation response of species that derive energy from carbohydrate in the colon, is often to switch to AA as a substrate for generating metabolic energy, indicating that microbial metabolism may be more relevant than microbioal phylogenetic identity under an given environmental condition (Rinttila and Apajalahti, 2013).

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1.3.5 TRP in neurogastroenterology

In the rumen, microorganisms play an important role in making dietary amino acids bioavailable for the host and also synthesising amino acids, including the essential amino acid tryptophan. Although the same metabolic pathways exist within the human gut microbiota, colonic TRP production does not appear to contribute significantly to hosts needs (estimated dietary requirement of 3.5 mg/kg per day – FAO/WHO/UNU Expert Consultation, 1991). Tryptophan rich foods include nuts, seeds, tofu, cheese, red meat, chicken, turkey, fish, oats, beans, lentils, and eggs. The diet-dependent tryptophan, which is a precursor to many neuroactive compounds, can follow three different metabolic pathways (Fig. 6) in the human body: (1) kynurenine, (2) serotonin, (3) indole ways.

Fig. 6 Main tryptophan metabolic pathways.
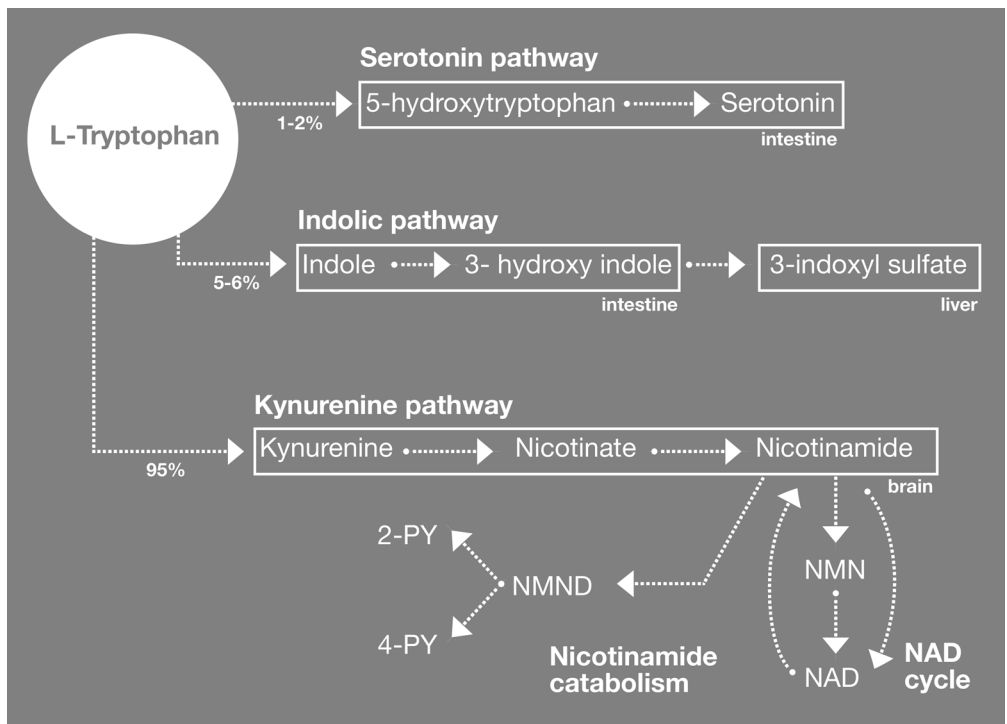
Kynurenine pathway is the main metabolic route, which involves almost 95% of free tryptophan. The enzymes responsible for the initial conversion of tryptophan to kynurenine, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), are immuno (IFN-γ, TNF and IL-1β) and stress (cortisol and HPA) responsive, respectively. This too highlights the important interconnections between the immune system, the brain and host:microbiota co-metabolic processes. While TDO is mainly located in the liver, IDO is an extra-hepatic enzyme found in numerous cells including macrophages, microglia, neurons and astrocytes (Hwu et al., 2000). Activation of both enzymes may impact, by limiting the availability of tryptophan, on serotonin synthesis and increase the downstream production of neurotoxic/neuroprotective metabolites. For exemple, IL-17A and IL-22 cytokines, are regulated by the intestinal microbiota, as demonstrated in an in vivo study, where colonization of the small intestine of mice with *Arthromitus* (related to the genus *Clostridium*), segmented filamentous bacterium (SFB), induced the appearance of CD4^{+} T helper cells (Th17 cells) that produce IL-17 and IL-22 in the lamina propria (Ivanov et al., 2009). Moreover, bacterially produced-ATP can augment Th17 differentiation, whereas commensal microbiota seems to inhibit this pathway via IL-25. How intestinal microbiota control RORgt and IL-22 in gut NKp46^{+} cells remains to be defined, but both direct and indirect mechanisms could be operative (Satoh-Takayama et al., 2008). In the brain, kynurenine (KYN) can be converted to two other components of the pathway: the neurotoxic quinolinic acid (QUIN) and the neuroprotective kynurenic acid (KYNA). Kynurenic acid, one of the main products of the kynurenine pathway, exerts neuroprotective effects via its antagonistic action at glutamate receptors (NMDA and AMPA), but also at cholinergic α7nAChR nicotinic receptors, that mediate presynaptic release of glutamate (Wu et al., 2010). Moreover through its ability to scavenge hydroxyl, superoxide anion and other free radicals, KYNA, is also an antioxidant (Lugo-Huitrón et al., 2011). Its potential use in neuroprotection has been suggested against complex neurodegenerative processes (e.g. Alzheimer's disease, lateral amyotrophic sclerosis, Huntington disease and Parkinson disease) (Tajti et al., 2015). This action is attributed to its beneficial influence against neuroinflammatory processes, cerebral β amyloid peptides (Aβ) accumulation, excitotoxicity and apoptosis leading to irreversible histopathological alterations of the brain (Klein et al., 2013). Conversely, quinolinic acid, is a neurotoxic metabolite which is normally present in small concentrations (nmol) in the human brain and cerebrospinal fluid, but can be increased in patological conditions (Heyes et al., 1996).

Macrophages, infected or stimulated with IFN-γ, TNF-α, and IFN-α, have a particularly high capacity to synthesize quinolinic acid in the brain (Pemberton et al., 1997). Overactivation of the N-methyl-D-aspartate (NMDA) receptor, a glutamate receptor, by quinolinic acid, leads an enhanced recruitment of cytoplasmic Ca^{2+}, as well as a mitochondrial dysfunction, massive use of ATP and cytochrome c release. Moreover, also the loss of GABAergic synapses and cholinergic neurons, as well as the oxidative stress, maybe due to the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively) are recognized as toxic effects of quinolinic acid (Santamaria et al., 2012).

Serotonin (5-hydroxytryptophan, 5-HT), is an alternative route-metabolite (around 1-2%) of tryptophan metabolism. Serotonin has been linked with several functions in the human body, including mood, appetite, systemic hemodynamics and gastrointestinal motility, in addition to being the precursor for melatonin synthesis in pinealocytes. Serotonin is synthesized from tryptophan through hydroxylation – tryptophan hydroxylase (TPH) and decarboxylation – aromatic acid decarboxylase (AADC), which also catalyzes the synthesis of dopamine from tyrosine (Walther, 2003; Zhu and Juorio, 1995). AADC enzyme needs vitamin B_6 (pyridoxine), naturally present in many foods (e.g. rice bran, pistachio nuts, fish – tuna and salmon and Liver – Beef), as a cofactor to carryout its decarboxylation activity. These processes take place in the intestine at enterochromaffin cells mucosal layer, where it has been calculated that about 90% of the bodies serotonin is produced. While the remaining 10% is largely attributed to serotonergic neurons of the enteric nervous system (ENS) (Kim, 2000). Importantly, significant modulatory effect of gut microbiota upon 5-HT biosynthesis and GI-activities has been reported. Fecal metabolites may increase 5-HT in chromaffin cell cultures (Yano et al., 2015). More recently, Reigstad et al (2015) have confirmed that gut microbiota promote serotonin production by enterochromaffin cells. Microbial-derived SCFA promotes EC-Tph1 (the rate-limiting enzyme for serotonin biosynthesis in EC cells) expression in a concentration-dependent manner, and so 5-HT production. They are also able to increases mRNA transcript levels for Tph1 and ChgA (a biomarker of neuroendocrine secretion) in the proximal colon (Reigstad et al., 2015). Also α-tocopherol, tyramine, and para-aminobenzoic acid (PABA) induce 5-HT release. Some bacteria (*Streptococcus faecalis*, *Streptococcus lactis*, *Micrococcus luteus*, *Leuconostoc cremoris strains*) can induce in the gut decarboxylation of tyrosine in tyramine, which stimulates fast ileal contractions and neuropeptide Y release (Voigt & Eitenmiller,1977; Gale, 1940).

PABA is an intermediate of folic acid synthesis and essential nutrient for some bacteria (genus *Lactobacillus*). The physiological roles for PABA in the GI tract are unclear (Rossi et al., 2011).

The indole pathway of tryptophan degradation is mainly dependent on the anaerobic intestinal bacteria with tryptophanase activity. Lactobacilli (e.g. *L. reuteri*), are typically auxotrophic (unable to synthesize a particular organic compound required for its growth), for most of the amino acids. Under carbohydrate starvation conditions, they are able to shift their metabolic activities and produce bioactive indole derivatives from tryptophan breakdown, as a energy source (Zelante et al., 2013). Many indole-metabolites are able to bind to the aryl hydrocarbon receptor (AhR), a ligand–activated transcription factor that controls the expression of a diverse set of genes (Beischlag et al., 2008). Indole 3-aldehyde (IAld) is synthesized through indole pyruvate (IPyA) route. This compound is a tryptophan metabolite, produced by aromatic amino acid aminotransferase (ArAT), a phylogenetically conserved enzyme in many bacterial species, including the lactobacilli, which plays a major role in the conversion of aromatic amino acids to aromatic compounds (Rijnen et al., 1999). Quorum–sensing (QS) autoinducer–molecules are produced and released by bacteria in response to fluctuations in cell-population density (Miller and Bassler, 2001). Indole, is able to modulate intercellular signals in bacteria as a QS molecule. It has also been shown (*in vitro*) to act on the expression of several human genes enhancing barrier functions of intestinal epithelial cells (EC) (Shimada et al., 2013). Co-metabolite indoxyl sulfate, on the other hand, has been shown to be involved in both cardiovascular and renal disease progression. It is an endproduct of bacterial tryptophan fermentation in the colon (Viaene et al., 2014).

A new tryptophan catabolic pathway which passes over the formation of kynurenine and leads to the production of pyruvate, acetaldehyde and acetate as end-metabolites has been proposed. The main tryptophan-microbial users are *Bacillus megaterium*, *Rhodococcus erythropolis*, many pseudomonads and *Burkholderia cepacia*. In this pathway, tryptophan is converted to 3-hydroxyanthranilate, using the same enzymatic steps for NAD biosynthesis. 3-hydroxyanthranilate, is cleaved to 2-amino-3-carboxymuconate semialdehyde (ACMS) and then further degraded enzymatically to 2-aminomuconate and then 4-oxalocrotonate. The same enzymatic activities are involved in the degradation of catechols and anthranilatene and lead to the production of pyruvate and acetate (Colabroy and Begley, 2005). Metabolites derived from microbial catabolism of tryptophan, arise from complex

processes of co-metabolism which probably stem from the involvement of the different TRP pathways described above. In addition, the metabolic potential of the intestinal microbiota - the metabotype - as well as genetics, gender and lifestyle (eg. stress, sleep) of the host and environmental factors within the intestine, in particular the amount and the proportions between amino acids, the pH and the presence of carbohydrates or other exogenous substrates, can modulate the metabolism of tryptophan.

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1.3.6 Gut bacteria-derived bioactive compounds

Many recent studies have shown that the intestinal microbiota may be emitting and receiving a multiplicity of signals to and from the brain. These signals, are modulating or regulating a variety of GI functions. It has become increasingly evident that this microbe-related bidirectional gut-brain signalling, plays a role in human cognitive function and brain development and also brain pathophysiology (Collins and Bercik, 2009). Diet has an important role in shaping the gut microbiota and also the flux of neurochemicals they produce. As discussed above, certain fibers and prebiotics in particular, support a butyrogenic gut microbiota characterised by increased relative abundance of *Bifidobacterium* and sometimes *Lactobacillus* and possibly butyrate producing bacteria, like *Roseburia inulinivorans* and *F. prausnitzii*. These bacteria appear to be important members of the beneficial gut microbiota and induce beneficial host immune effects, improve mucosal integrity and some species also produce bioactive compounds other than SCFA, such as folate, serotonin, dopamine and γ-aminobutyric acid (GABA) (Selhub et al., 2014; Asano et al. 2012). GABA is the main inhibitory neurotransmitter in the brain and regulates many physiological and psychological processes. Dysfunction in the GABA system is implicated in anxiety and depression (Cryan, 2005; Schousboe, 2007). Certain strains of *Lactobacillus* and *Bifidobacterium* can produce GABA from monosodium glutamate and it has been suggested that the microbially produced GABA in the gut may have an effect on the gut-brain axis. Screening a number of gut bacteria for GABA production has identified *L. brevis* and *B. Dentium* as efficient GABA producer species (Barrett, 2012). Another GABA producer tested in rats, *Lactobacillus brevis FPA3709*, was shown to have a similar effect as Fluoxetine, a common antidepressant drug, but without the drug side-effects which include loss of appetite and body weight (Patterson, et al., 2014). At the level of gene expression, the ingestion of *L. rhamnosus JB-1*, was shown to alter the mRNA expression of both GABA A and B receptors, which have been implicated in anxiety and depression (Bravo, 2011). Strains of both genera are commonly used as probiotics or are present in traditional fermented foods, especially dairy products (Selhub et al., 2014). Since the early 1950s serotonin has been the subject of intense biological research both for its activities, as a neurotransmitter, but also more recently as an immunomodulatory in the periphery. It is also involved in the regulation of gastrointestinal motility, secretion and sensation, and has served as a basis for development of novel treatments for gastrointestinal disorders, such as IBS, besides its involvement in mood, appetite and hemodynamics. As previously discussed, almost 90% of the

human body's serotonin is produced from TRP by enterochromaffin cells in the gut (Oh et al., 2015; Kashyap et al., 2013). The gut microbiota, appears to influence serotonin levels, with germ-free mice showing significantly lower 5-HT plasma concentrations compared to conventional mice (Wickoff, 2009). In addition, oral ingestion of the probiotic *B. infantis 35624*, increased levels of the serotonin precursor, TRP, in the plasma of rats, suggesting that this bacterial strain, may have potential modulatory activities on TRP metabolism in the gut and could possibly be investigated as a novel therapy for depression (Desbonnet, 2008). However, the ability to produce serotonin or induce serotonin production does not seem to be restricted to bifidobacteria and lactobacilli, with strains of the yeast Candida, *Streptococcus*, *Escherichia*, and *Enterococcus*, all shown to produce serotonin (Lyte, 2011). As mentioned before, intestinal microbiota can modulate 5-HT levels through the production of SCFA (particularly butyrate and acetate), which significantly affects TPH1 expression in a concentration-dependent manner (Reigstad et al., 2015). Clearly, further studies are urgently needed to explore regulation of TRP metabolism by the gut microbiota.

Catecholamines (CA), such as dopamine (DA), norepinephrine (NE) and epinephrine (E), are the major neurotransmitters in the brain. They can modulate a variety of functions of nervous system, such as motor control, cognition, emotion, memory processing and the endocrine secretions (Kobayashi, 2001). CA might be involved not only in pro-absorptive functions and alteration in bacterial pathogenicity, but also in a variety of physiological functions, such as gut motility and modulation of immune reactions. Substantial levels of free DA and NE were identified in the gut lumen of germ free mice after inoculation of with *E. coli* (Asano et al., 2012). Also *Bacillus* and *Saccharomyces* strains appear to be involved in the production of endogenous CA, while *Bacillus* and *Serratia* have the potential to produce dopamine, which plays a role in reward-motivated behaviour (Lyte, 2011). Acetylcholine, is a neurotransmitter located both in the central and peripheral nervous systems. It plays a critical role in cognitive function, particularly in memory and learning. Previous studies have shown that acetylcholine is both a component of bacterial strains, including *L. plantarum* and *Bacillus* subtilis and a microbial metabolite (Girvin, 1954; Rowatt, 1948; Horiuchi, 2003).

Endocannabinoids are lipid molecules that act as neurotransmitters/neuromodulators in the brain which contain specific endocannabinoid receptors (Piomelli, 2003). These receptors also engage with Δ^9-tetrahydrocannabinol, the active constituent

of cannabis. The endocannabinoid system and the gut microbiota can impact on the development of obesity and related disorders (Muccioli, 2010). In addition, a *L. acidophilus* strain has been shown to modulate expression of cannabinoid receptors in the spinal cord (Rousseaux, 2007).

Diet can have a marked impact on the gut, including gut transit time and pH, while the main dietary macronutrients (carbohydrates, proteins and fats) can significantly affect the composition of the gut microbiota. It has been suggested that food intake could have important effects on behaviour (Bellisle, 2003). Nutritional components, such as prebiotics (eg. inulin, GOS, FOS), might play a neuroprotective role against white matter injury, through modulation of inflammation and infection, and may influence the microbiome-gut-brain axis (Keunen et al., 2015). A dietary supplementation for 3 weeks with GOS/FOS explored the neuroendocrine effects on 45 healthy human volunteers. Prebiotic GOS intake was associated with decreased cortisol levels and altered attentional bias compared to the placebo, while no effects were found after the administration of FOS (Schmidt et al., 2015). Anxiety and depression are highly comorbid in individuals with IBS. Daily administration of prebiotic GOS mixture, in patients with chronic fatigue syndrome, decreased anxiety scores and had a significant positive impact on quality of life. GOS intake was previously shown to increase the numbers of bifidobacteria and lactobacilli (Gopal et al., 2001), as well as SCFA (Rodriguez-Colinas et al., 2013), which are well know to be associated with an increase of epithelial barrier against potential pathogens and toxins, and are also able to modulate the immune system (Vulevic et al., 2008). These combined effects could be responsible of the modulation and improvement of symptoms related to a state of chronic stress (Schmidt et al., 2015; Silk et al., 2009). Furter studies are needed to test the ability of prebiotics in the treatment of stress-related disorders through modulation of the gut:brain axis.

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Aims of the study

2. Aims of the study

The microorganisms which inhabit the colon have a complex and sometimes dichotomous relationship with the host. Through their activities, they influence many aspects of human physiology. In fact, one could view the human intestine as an organ that was largely shaped by the microbiota during evolution and also be diet:microbe interactions from birth to old age. The human body influences its microbial community by imposing different environments, regulating levels of moisture, oxygen, bile acids, pH, digestive secretions and interactions with the immune system (Boon et al., 2014). The gut microbiota protects against enteropathogens, extracts nutrients and energy from our diets and contributes to normal immune function (Maslowski and Mackay, 2011). Disruptions to the normal balance between the gut microbiota and the host have been associated with obesity and type 2 diabetes (Joost, 2012), malnutrition (Gordon et al., 2012), inflammatory bowel disease (Sartor and Mazmanian, 2012), neurological disorders (Perlmutter and Loberg, 2015) and cancer (Poutahidis et al., 2014). Diet can have a marked impact on the gut, including gut transit time and pH; the main dietary macronutrients (carbohydrates, proteins and fats), can significantly affect the composition of the gut microbiota and importantly, their metabolic output. Furthermore, it has been suggested that food intake could have important effects on brain development and cognitive function (Bellisle, 2003). The objectives of this PhD project were to (a) examine how protein/amino acid metabolism is impacted by the presence of a fermentable fiber, the prebiotic inulin; (b) measure the impact of inulin on microbiota tryptophan metabolism *in vitro*; (c) measure *in vitro* metabolism of different amino acids by the gut microbiota, including amino acid precursors of human neurotransmitters; and (d) measure the impact of diet on the gut microbiota over the life-course of farmed fish *in vivo*. This study represents an integrated multidisciplinary approach, using *in vitro* model of the human gut microbiota, a dietary intervention in an animal model (trout), culture independent microbiology (next generation sequencing and fluorescent in situ hybridization - FISH), metabolomic (NMR) analysis, bioinformatic (MICCA) analysis and data integration.

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2.1 Valorisation and dissemination of results

Journal Article

Ceppa F. A., Martelli R., Marconi P., Faccenda F., Albanese D., De Filippo C., Pindo M., Lunelli F., Fava F., Parisi G. (xxx). Growth, gut microbiota and fillet quality of rainbow trout (Oncorhynchus mykiss) influence of diet and life-stage. Pre-submitted
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Ceppa F. A., Mayneris-Perxachs J., Albanese D., Mancini A., Fava F., Pindo M., Swann J., Tuohy K. (xxx). Measuring the impact of the prebiotic inulin on gut microbiota tryptophan metabolism. Draft
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Ceppa, F.A., Tuohy, K.M. (xxx) Diet:microbe interactions in shaping the gut:brain axis – a review. Draft

Book chapter

Tuohy, K.M.; Venuti, P.; Cuva, S.; Furlanello, C.; Gasperotti, M.; Mancini, A.; Ceppa, F.; Cavalieri, D.; de Filippo, C.; Vrhovsek, U.; Mena, P.; Del Rio, D.; Fava, F. (2014). Diet and the gut microbiota: how the gut:brain axis impacts on autism. In: Diet-microbe interactions in the gut: effects on human health and disease (editor(s) Tuohy, K.M.; Del Rio, D.). Amsterdam ... [et al.]: Elsevier: 225-245. ISBN: 978-0-12-407825-3 doi: 10.1016/B978-0-12-407825-3.00015-0. handle: http://hdl.handle.net/10449/23938net/10449/23331

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Inulin modulates microbial metabolism of digested red meat

3. Inulin modulates microbial metabolism of digested red meat

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Abstract

Certain metabolites produced by the colonic microbiota, like the biogenic amine trimethylamine (TMA) appear to increase the risk of cardiovascular disease upon colonic fermentation of choline, L-carnitine and perhaps other compounds of red meat. Anaerobic batch cultures of human faeces (n = 5), were used to measure the microbial ecology of the distal colon upon the fermentation of beef (red meat grinded, roasted and predigested simulating gastric and duodenal activities). Prebiotic inulinwas included in the study, fermented alone or added to beef, to evaluate its ability to down-regulate toxic metabolite production during protein fermentation. Combined high-throughput pyrosequencing with biofluid NMR-based metabolic profiling allowed the measurement of changes in microbial ecology both in terms of diversity measures and changes in relative abundance, and also shifts in metabolites produced or consumed during the fermentations over a 24 hours period. Inulin improved protein metabolism by the distal colonic microbiota by increasing the consumption of amino acids, such as tryptophan and phenylalanine, but at the same time reducing the production of potentially toxic metabolites such as TMA, 5-Aminopentanoic acid and phenol. Moreover, inulin had an impact on the structure of the distal colonic microbiota, shifting relative abundance in favour of *Butyricicoc-*

cus, *Butyricimonas*, *Coprococcus* and *Dorea* in detriment of other genera such as *Blautia* and *Lachnospiraceae incertae sedis* and *Clostridium cluster XVIII*. However, there was considerable inter-individual variation in response to either digested meat or inulin between faecal donors limiting statistical strength of observations. Similarly, inulin did not appear to increase the relative abundance of bifidobacteria suggesting a greater effect of pH than substrate availability in determining the relative abundance of different bacteria within the distal colon. However, taken together, this study supports the notion that increased consumption of fermentable fiber or prebiotics like inulin may be one means of reducing the production of harmful metabolites upon protein fermentation in the distal colon.

Keywords

3.1 Introduction

Diets high in protein, especially red meat, have been associated with increased risk of cardiovascular disease and colorectal cancer (Lippi et al., 2015; Aykan, 2015). It is estimated that about 12–18 g of dietary protein reach the colon each day, having escaped digestion by host enzymes and absorption in the stomach and small intestine. In the large intestine, this protein comes in contact with the colonic microbiota, the single largest collection of microorganisms inhabiting the intestinal tract. Dietary protein in the colon serves as a source of amino acids both for the microbiota itself (and converted into microbial biomass) and for the host. However, dietary protein and derived amino acids can also be fermented by the gut microbiota to derive energy, resulting in a range of metabolites including short chain fatty acids (acetate, butyrate and propionate), branched chain fatty acids (mainly isobutyrate, isovalerate and isocaproate), indoles, sulphides, ammonium, phenols, histamine, oxaloacetate and biogenic amines like trimethylamine (TMA) (Scott et al., 2013). Some of these metabolites are potentially harmful to the host and have been suggested to play a role both in colon cancer risk and in cardiovascular disease. Some of these compounds have been implicated in the development, function and homeostasis of the immune system (Brestoff and Artis, 2013; Jacobs et al., 2009), e.g. SCFA, but many also appear to harmful effects on the bowel epithelium (e.g. nitrosamines, nitrosamides, and nitrosoguanidines) and/or at systemic level (e.g. Trimethylamine N-Oxide – TMAO). Protein fermentation by the colonic microbiota is less efficient in terms of energy production (13.4 kJ/g) than carbohydrate (15.7 kJ/g) fermentation (Elia and Cummings, 2007). Early observations, recorded a shift in colonic microbiota fermentation patterns, followed the substrate and pH gradient, with saccharolytic fermentation predominating in the proximal colon and protein/amino acid fermentation in the distal colon. (Cummings and Macfarlane, 1991). The pH of the colon changes from proximal (pH 5.5), through the transverse colon (pH 6.2) to the distal colon (pH 6.8-7.2). Recent studies have shown that pH can have a significant impact on the relative abundance of different bacteria under simulated colonic conditions, as for example, bifidobacteria which grow rapidly in the presence of prebiotics, like inulin, at pH 5.5, while they are poorly represented, in the presence of the same substrate, at pH 6.9 or higher (Chung et al. 2016). Few studies have however, examined the impact of protein and/or carbohydrate fermentation on both production of fermentation end products and structure of the microbial community in the distal colon.

TMA can derives from choline (Ch) and phosphatidylcholine (PCh), which are found

in many different foods, including red meat, fish/sea-food and soy products or L-carnitine derived mainly from red meat. Ch is recognized as an essential nutrient since it is needed for the synthesis of the neurotransmitter acetylcholine, for cell-membrane signaling (phospholipids), lipid transport (lipoproteins), and for its role as in methyl-group metabolism (homocysteine reduction) (Zeisel and da Costa, 2009). It is also the major dietary source of methyl groups via the synthesis of S-adenosylmethionine (SAMe) (Penry and Manore, 2008). SAMe, which works with vitamin B_{12} and B_9 (folate), plays a important role in the immune system, maintains cell membranes, and helps produce and break down brain chemicals, such as serotonin, melatonin, and dopamine (Bottiglieri, 2013). Furthermore, choline deficiency may cause diseases such as nonalcoholic fatty liver disease, atherosclerosis (via lipoprotein secretion), and neurological disorders (Dumas et al 2006; Zeisel and da Costa, 2009; Alpert et al., 2004). L-carnitine is a quaternary ammonium compound demonstrated to be involved in the β-oxidation of fatty acids and in the transport of long-chain fatty acids through the inner mitochondrial membrane of mammals (Krämer et al., 2001). Beef is the third most widely eaten meat in the world representing over 22% of meat intake (FAO World Food Outlook 2014). For healthy adults - with a default body weight of 70 kg for the european adult population - the average requirement (AR) is 0.66 g protein/kg body weight for day based on nitrogen required data. In european countries the main dietary protein intake come from meat and meat products, grains and grain-based products, and milk and dairy products, with a protein intake of 0.8 to 1.25 g/kg body weight for day (EFSA, 2012). Although the data are not sufficient to establish a tolerable upper intake level (UL) for protein, the over amount of daily animal/resistant protein, can be responsible for the production/increase of toxic compounds along bowel, including nitroso compounds (amines and amides), hemin, volatile phenols and indoles (Bastide et al., 2011). The International Agency for Research on Cancer (IARC), and the cancer agency of the World Health Organization (WHO), have evaluated the carcinogenicity of the consumption of red meat and processed meat last October (2015). Pro-atherogenic TMAO is formed by two steps process: the first one starts with the bowel microbiota dependent cleavage of a TMA species from PCh/Ch/betaine/L-carnitine generating the precursor TMA, and the second one in the liver with oxidation by FMO3 enzyme to produce TMAO (Brugère et al., 2014). TMAO not only promotes atherogenesis, but recent studies in mice also suggest that dietary TMAO may exacerbate impaired glucose tolerance, obstruct hepatic insulin signa-

ling, and promote adipose tissue inflammation when fed a high-fat diet (Tang and Hazen, 2014). Nevertheless, TMAO also plays important biological role of slowing urea-induced dissociation of multimeric intracellular proteins by diminishing the rate of lactate dehydrogenase activity with significant protection of the enzyme against urea-induced time-dependent inactivation (Baskakov and Bolen, 1998) and is an important osmolyte in the body. A number of recent studies have focused on the role of microbial metabolites of phosphatidylcholine, choline and betaine into TMA and its oxide form (TMAO) in cardio vascular diseases (CVD) risk. Underlining how vegans or vegitarians have much lower levels of TMAO (Koeth et al., 2013). Moreover, has been acknowledged a partial agonist activity of TMA upon muscle nicotinic receptors (Lape et al., 2009). Although TMA administration at high doses has not shown to act on locus coeruleus (LC) neurons (Engberg and Hajos, 1994), it is able to simulate the effect of nicotine on LC (Hajós and Engberg, 1988). This could be another action of TMA, with the opportunity to new studies to confirming the link between bowel and neural system.

Although the link between high red meat intake and both CVD and colon cancer is now well accepted, much debate remains concerning the relative contribution of TMAO or TMA production in the colon, especially since foods like soy beans and fish (both high in PCh and Ch) have been associated with protection from these same diseases in epidemiological studies (Rajaie and Esmaillzadeh, 2011) and also L-carnitine is a common dietary supplement used to lower the risk of heart disease (Lee et al. 2015; DiNicolantonio, 2014; Lee et al. 2014). Host genetics, especially polymorphism in the TMAO producing enzyme FMO3 have been suggested to play an important role in regulating circulating TMAO concentrations, but few studies have examined the impact of different food matrices, or the relative impact of protein vs carbohydrate fermentation, on TMA production by the gut microbiota, which for any given host impacts on circulating TMAO levels (Hartiala et al. 2015).

In this study, the effects of the prebiotic inulin on colonic dissimilatory protein metabolism was examined, using anaerobic faecal batch cultures. The pH set between 6.8 and 7.2, represent the environment conditions in the distal colon. Prime ground beef was chosen as a PCh, Ch and L-carnitine rich substrate and was cooked (roasted) and subjected to simulated gastric and small intestinal digestion, prior to use as substrate for faecal batch cultures. Inulin, a polymer (2-60 units) of fructose units, with a glucose molecule at the end of the chain, is present in many vegetables and fruits including, onions, leeks, garlic, bananas, asparagus, chicory, and

Jerusalem artichokes. It is undigestible by human intestinal enzymes and reaches the colon intact, where is totally fermented by colonic microbiota (Niness, 1999). Inulin is also called the "gold-standard" prebiotic, defined as a selectively fermented ingredient, that results in specific changes in the composition and/or activities of gastrointestinal microbiota, thus conferring benefit(s) upon host health (Gibson et al., 2010). Changes in faecal microbial ecology and metabolic output, were studied using a combination of 16S rRNA gene sequencing (meta-taxonomics), on the Illumina MiSeq platform, fluorescent in situ hybridization with 16S rRNA targeted probes, for selected bacteria and a non-targeted ^{1}H NMR-based metabonomics, to profile metabolites produced during fermentation experiments.

3.2 Materials & Methods

In vitro digestion

The homemade hamburger, with selecting beef of high-grade, directly from a butcher and freshly ground. For simulating all steps followed in a daily meal, the hamburger was roasted (10' for each side) and grinded, as after mastication, and then pre-digested by *in vitro* method, with all enzyme and activities to which the food is subjected, during the passage from the oral channel to the duodenal tract. Cargill Oliggo-Fiber® inulin DS2 5LB (dietary fiber – 98% and free fructose; glucose and saccharose – 2%) (Warcoing Industries S.A. Belgium), composes of short and medium length chains with a degree of polymerization average (DPA) of 12 (Gibson and Roberfroid, 1995), was pre-digested in the same way of beef.

The substrates used for batch fermentations was subject to *in vitro* upper gastrointestinal digestion consisting of an initial gastric phase, followed by a duodenal-intestinal tract, which simulates the chemical and enzymatic conditions of relevant portions of the stomach and small intestine. The protocol was drawn from Mandalari (2013) and adapted. The below description of the protocol is considering the digestion of 1.5 g starting material.

Preparation of phospholipid vesicles. The lipid vesicles were prepared by removing the solvent by 0.28 ml of a stock solution of L-α-phosphatidylcholine (PCh) from egg in chloroform (0.235 ml in solution of 100 mg/ml of chloroform) (Grade 1, Lipid Products, UK). The organic solvent was removed overnight using a Rota vacuum evaporation system, thus producing a thin lipid film on a round bottom flask. To avoid oxidation, the lipid film was covered with Argon. The PCh was rehydrated with 7.6 ml of 0.15 M NaCl pH 2.5, then 5 2 mm diameter glass beads were added and the flask was incubated for 1 hour at 37 °C on a horizontal shaker. The suspension was then sonicated (Branson mod.) at 30% power for 30 minutes, non-continuous mode, in order to avoid the excessive increase of the temperature and the overheating of the probe. The suspension of liposomes in single layer was finally filtered with a 0.22 μm Nalgene filter to remove any deposit of titanium resulting from sonication.

Gastric phase. Each test substrate was re-suspended in 6.3 ml of 0.15 M NaCl pH 2.5 and the pH was adjusted to 2.5 with HCl (0.5 M) or NaOH (0.5 M). A 6.1 ml suspension of lipid vesicles was then added to the solution. The gastric enzymes, pepsin and lipase (Sigma-Aldrich) were added at 146 and 60 U/ml concentration of reaction respectively, and the samples were incubated at 37 °C for 2 hours on a

horizontal shaker.

Duodenal phase. The pH was brought to 6.5 and the following reagents were added in such quantities as to reach the respective final concentration: $CaCl_2$ - 11.7 mmol/l, BIS-TRIS buffer - 0.73 mmol/l and bile acid sodium taurocholate and sodium glycodeossicholate - 4 mmol/l. The chemicals were purcased from AppliChem. The pH of 6.5 was checked again and the enzymes were then added at the respective final concentrations: α-chymotrypsin - 5.9 U/ml, trypsin - 104 U/ml, colipase - 3.2 U/ml, pancreatic lipase - 54 U/ml and α-amylase - 25 U/ml. The chemicals were purchased from Sigma. The digestion was performed by incubating the samples at 37 °C for 1 hour on a horizontal shaker.

Dialysis. The digested sample was subjected to dialysis, according to the indications of Millis et al. (2008). The dialysis membrane, Spectra/Por (Spectrum Labs) with a cut-off molecular weight of 1 kDa, which had been previously washed in distilled water, was filled with the digested material and closed on both sides. The membrane was then placed in dialysis solution (10 mM NaCl), at a volume about 100 times greater than the volume of the material to be dialyzed and incubated at 4 °C overnight with slow stirring.

Lyophilization. The dialyzed retentate material were transferred to a round bottom flask and placed at -80 °C until frozen. The material was then lyophilized and the obtained powder was weighed and stored at -80 °C.

Fecal batch cultures

Faecal fermentations were conducted using the basal nutrient medium, prepared as from the following formula (g/l): starch – 8.0; mucin (porcine gastric) – 4.0; casein – 3.0; peptone water – 5.0; tryptone water – 5.0; bile #3 – 0.4; yeast – 4.5; $FeSO_4$ – 0.005; NaCl – 4.5; KCl – 4.5; KH_2PO_4 – 0.5; $MgSO_4$ $7H_2O$ – 1.25; $CaCl_2$ $6H_2O$ – 0.15; $NaHCO_3$ – 1.5; tween 80 – 1; hemin – 0.05; cysteine HCl – 0.8. All chemicals were purchased from Sigma. The final, working volume of all fermentations was 150 ml. Anaerobic conditions were maintained by O_2-free N_2 (15 ml/min) flow overnight. Temperature was held at 37 °C using a circulating water bath, and pH was controlled between 6.8 and 7.2 using an automated pH FerMac 260 controller (Gloucester, England-GL208JH, United Kingdom), which added acid and alkali as required (0.5 M HCl and 0.5 M NaOH). Five healthy human donors (2 male,

3 female), who had taken no prebiotics, probiotics, or antibiotics within 3 months of the study were recruited to provide faecal samples as inocula. Faecal inocula (10% w/v) were prepared using pre-reduced phosphate-buffered saline (PBS 0.1 mol/l) and used to inoculate each fermentation vessel to a final concentration of 1% faeces w/v. The substrates tested in fermentation, were basal medium (Blank), Inulin at 1% (w/v) (Cargill Oliggo-Fiber® inulin DS2 5LB), as positive control, tryptophan (5 mmol/l) (Sigma-Aldrich, Germany) and tryptophan added Inulin, with the same their respective concentrations. Each fermentation was conducted once with faecal inocula from each of the 5 faecal donors (n = 5). Batch cultures were run for 24 hour and samples obtained from each vessel at 0, 5, 10, 24 h, were centrifuged at 13 000 rpm, where the supernatants and pellets were stored at -80 °C for metabolomics and metagenomics analysis respectively. Samples for FISH were prepared as described below and stored at -20 °C.

Fluorescence In Situ Hybridization-Flow Cytometry (FISH/FC)

Sample fixing and hybridization, were performed as previously described (Rochet et al., 2004; Saulnier et al., 2008). Briefly, 375 µl of culture fluid was diluted in 1125 µl of 4% (w/v) paraformaldehyde and fixed overnight at 4 °C. Bacterial cells were pelleted by centrifugation at 13 000 rpm for 5 min and washed twice in filter-sterilized PBS. Cells were then resuspended in 150 µl PBS and 150 µl ethanol. Samples were stored at –20 °C for a minimum of 3 h before further processing. Genus-specific 16S rRNA oligonucleotide probes, labelled with the fluorescent dye Cy5 (Sigma Aldrich, Italy), were utilized for selected bacterial groups, the nucleic acid stain 2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium - SYBR Green I - (Sigma Aldrich, Italy), for total cell counts. The probes used were as follows: Bif 164, specific for *Bifidobacterium* (Langendijk et al., 1995) and Enterobac D, for *Enterobacteriaceae* (Ootsubo et al., 2002). In 96 well plate, 10 µl of fixed suspension was mixed into 190 µl of PBS (pH 7.2) and then centrifuged at 4000 rpm for 15 min at 4 °C. After one wash in Tris EDTA buffer (TrisHCl – 100 mM; EDTA – 50 mM (pH 8)), when requested, pellets were suspended in Tris EDTA buffer, containing 1 mg/ml of lysozyme (Sigma Aldrich, Italy) and incubated for 10 min at room temperature. Cells were washed in PBS (pH 7.2), to remove lysozyme and were suspended in the hybridization solution (NaCl – 900 mM; TrisHCl –20 mM (pH 8.0); Sodium Dodecyl

Sulfate – 0.01%; formamide as requested), containing 5 ng/μl of specific probes, for a total volume of 55 μl. The hybridization step, was performed overnight at the appropriate labelled probe temperature. Following hybridization, a volume of 145 μl of hybridization solution, was added in each well, and cells were pelleted at 4000 rpm for 15 min, at 4 °C. Nonspecific binding of the probe, was removed by incubating the bacterial cell suspension, at the appropriate temperature for 20 min in 200 μl, of a washing solution (NaCl – 64 mM; TrisHCl – 20 mM (pH 8.0)). Cells were suspended in 50 μl of PBS (pH 7.2), containing 1x SYBR Green I and incubated at room temperature for 10 min. Following hybridization, a volume of 150 μl of PBS (pH 7.2) was added in each well, and cells were centrifuged at 4000 rpm for 15 min, at 4 °C. Final pellet was suspended in 100 μl of PBS (pH 7.2) and then subsequently analyzed.

Data acquisition by flow cytometry (FCM)

The acquisition threshold was set in the side scatter channel using a Guava easyCyte 8T flow cytometer (Millipore, Italy). For each sample, a total of 10 000 events were stored in list mode files. Analyses were made using the inCyte software (Millipore, Italy). Cells were enumerated by combining in the same hybridization tube, one group Cy5-probe with the SYBR green. An FLG histogram (green fluorescence), was used to evaluate the total number of bacteria stained, with SYBR green. A gate was designed, in this histogram representing the total number of bacterial cells, in the sample and was used to build an FLR histogram (red fluorescence), to estimate the proportion of cells targeted, by the group Cy5-probe in the sample. The proportion of group cells, was corrected by eliminating background fluorescence. Results were expressed as cells hybridizing with the groupCy5 probe, as a proportion of total bacteria stained with SYBR green.

Real-Time qPCR

Bacterial groups in fecal samples were quantified by qPCR using the LightCycler® 480 Real-Time PCR System (Roche, Germany) and the primers as shown in the table below:

Bifidobacterium spp.
Forward TCGCGTC(C/T)GGTGTGAAA
Reverse CCACATCCAGC(A/G)TCCAC
Reference (Rinttila et al., 2004)

Enterobacteriaceae
Forward CATGCCGCGTGTATGAAGAA
Reverse CGGGTAACGTCAATGAGCAAA
Reference (Furet et al., 2009)

Primers for amplification of *Bifidobacterium spp.* and *Enterobacteriaceae* were designed using 16S rDNA gene sequences, as indicate in references. The qPCR reaction mixture (20 µl), was composed of 0.25 µM of each universal primer, 2x SensiFAST™ HRM Kit (Cat. No. BIO-32005; Bioline Reagents Ltd.), and 0.25 ng of DNA from 80 samples collected during 24 h fermentation. Nuclease free water was add to reach the final volume defined. The Standards (STD), were prepared by 10-fold serial dilutions starting from the DNA extract of a pure culture of *Lactobacillus adolescentis* and *Escherichia coli DH 5α*, which cells/ml were counted by plate count on MRS (LB for *E. Coli*). The amplification program consisted of one cycle of 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, and 60 °C for 30 sec. Standard curves were constructed for each experiment using six point of 10- fold serial dilutions of bacterial genomic DNA of known concentration. Standard curves were created according to LightCycler® 480 software and normalized to the copy number of the 16S rRNA gene for each species.

¹H NMR Spectroscopy

For spectroscopic analysis, 400 µL of fermented slurry were diluted with 200 µL of phosphate buffer (pH 7.4; 100% D_2O), containing 1mM of TSP (3-trimethylsilyl-1-[2,2,3,3-2H4] propionate) as an external standard and 2mM sodium azide as a bacteriocide. Samples were mixed by vortex and centrifuged at 13 000 g for 10

min, prior to transferring 550 μL to a 5 mm NMR tube. Spectroscopic analysis were carried out on a 500 MHz Bruker NMR spectrometer. For each sample, 8 dummy scans were followed by 128 scans and collected into 64 000 data points. A spectral widths of 20 ppm was used. Spectra were manually phased, corrected for baseline distortions and referenced to the TSP signal at δ 0.00. ^{1}H-NMR spectra (0.2 to 10.0 ppm) were digitized into consecutive integrated spectral regions (~20.000) of equal widths (0.00055 ppm). The regions containing the residual signal from water (4.50 to 5.00 ppm), TSP (0.00 ppm) and the large resonance arising from basal medium, were removed to minimize the effects of baseline distortion. Each spectrum was then normalized to unit area.

NMR data analysis and metabolites identification

Multivariate modeling, including Principal Component Analysis (PCA) and Orthogonal Partial Least Squares analysis (OPLS), was performed in MatLab (The Mathworks, Inc., Natwick, MA), using scripts provided by Korrigan Sciences Ltd., United Kingdom. The ^{1}H-NMR spectroscopic profiles were used as the descriptor matrix (X), and donors, sampling time and treatments were used individually as response variables (Y). Loading coefficient plots were generated by back-scaling transformation to display the covariance between the Y-response matrix and the signal intensity of the metabolites in the NMR data (X). Colors projected onto the coefficient plot indicate the correlation coefficient (R^2) between each metabolite and the Y-response variable, with red indicating strong significance and blue indicating weak significance. The predictive performance of the model (Q^2Y) was calculated using a seven-fold cross validation approach. Model validity was established by permutation (1000 permutations) and the results are given as p values. Metabolite identification was performed using HMDB database (http://www.hmdb.ca/), KEGG (Kyoto Encyclopedia of Genes and Genomes), Chenomix profiler 8.1 software (Chenomx Inc., Edmonton, Canada).

DNA extraction

The genomic DNA extraction was obtained by FastDNA™ SPIN Kit for Fecesn (MP Biomedicals, Santa Ana,CA), following the manufacturer's protocols. The final product was 100 μl of application-ready DNA. Nucleic acid concentration and purity were measured using the Nanodrop 8000 spectrophotometer. DNA concentrations were in the range 18.2 – 96.6 ng/μL.

16S rRNA amplicon library preparation and MiSeq sequencing

Using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') (Klindworth et al. 2013) and 806R (5' GACTACNVGGGTWTCTAATCC 3') (Apprill et al. 2015) with overhang Illumina adapters, total genomic DNA was subjected to PCR amplification, by targeting a ~460-bp fragment of the 16S rRNA variable region V3-V4. PCR amplification of each sample, was carried out using 25 µl reactions with 1 µM of each primer. Specifically 12.5 µl of 2x KAPA HiFi HotStart ReadyMix, 5 µl forward primer, 5 µl reverse primer, were used in combination with 2,5 µl of template DNA (5 ng/ul). All PCR amplification was carried out, using a GeneAmp PCR System 9700 (Thermo Fisher Scientific) and the following steps: melting step – 95 °C for 3 minutes (one cycle); annealing step – 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds (25 cycles); extension step – 72 °C for 5 minutes (1 cycle). The PCR products were checked on 1.5 % agarose gel and cleaned from free primers and primer dimer, using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. Subsequently, dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina), were attached by 7 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina). The final libraries, after purification by the Agencourt AMPure XP system (Beckman), were analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA) and quantified, using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific), by the Synergy2 microplate reader (Biotek). Finally, all the libraries were pooled in an equimolar way, in a final amplicon library and analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Barcoded library were sequenced on an llumina® MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18).

Metagenomic data analysis

The sequences were assigned to samples, according to sample-specific barcodes. This allowed to collect FASTQ formatted files. Raw data fastq files were merged using micca-mergepairs tool. Raw data will be submitted to the http://www.ebi. ac.uk/ena/submit/sequence-submission. Reads were processed using the MICCA pipeline (version 1.0, http://micca.org/) (Albanese et al., 2015). Forward and reverse primers trimming, were performed using micca trim (parameters 5' CCTACGG-

GNGGCWGCAG 3' - 5' GACTACNVGGGTWTCTAATCC 3'). Quality filtering, were performed using micca filter (parameters -e 0.5 -m 400 -t), truncating reads shorter than 400 nt and discarding reads, with an expected error (EE) rate > 0.5%. De-novo sequence clustering, chimera filtering and taxonomy assignment, were performed by micca otu (parameters -d 0.97 -c): operational taxonomic units (OTU) were assigned by clustering the sequences, with a threshold of 97% pairwise identity, and their representative sequences were classified, using micca classify with the RDP classifier version 2.8 (Wang et al., 2007). Template-guided multiple sequence alignment (MSA), was performed using micca msa (parameters -m nast), against the multiple alignment of the Greengenes (DeSantis et al., 2006) database (release 13_05) clustered at 97% similarity. Finally, phylogenetic tree was inferred, using micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013).

3.3 Results

Metabolomic fingerprint by ^{1}H-NMR and Multivariate Data Analysis (MVDA) analysis

OPLS models with one predictive component and no orthogonal components were calculated to identify metabolites associated with gut microbial fermentation time (0, 5, 10 and 24 h) with Inulin, beef and beef+Inulin, respectively.

A significant model for beef fermentation (Tab. 1), showed an increase over time of phenol, TMA, methylamine (MA), 5-aminopentanoic acid, propionate, butyrate and acetate (Fig. 2). When combined with inulin (Tab. 1), it seems to inhibit the formation of 5-aminopentanoic acid (Fig. 3).

Figure 4a shows a PCA of metabolite profiles describing the Beef vs Beef+Inulin fermentations after 24h. The first two principle components (PCs) explain 38% of the total variance within the data comparing Beef and Beef+Inulin, and although each donor can be clearly distinguished, the PCA shows a clear and consistent separation between the treatments (Fig. 4a). Interestingly, faecal donors 3, 4 and 5, appear to group or cluster together indicating that there is a degree of similarity in their metabolite profiles at 24 h compared to donor 2 and 1 which show difference in metabolite profiles after 24 hours of fermentation. The corresponding OPLS model (Fig. 4b, Tab. 1) shows that butyrate and ethanol are increased in Beef+Inulin treatment compared to Beef alone, while phenylalanine (Phe), tyrosine (Tyr), Betaine, 5-aminopentanoic acid, TMA, and leucine are found in lower amounts, confirming the positive effect of the prebiotic inulin in the reduction of potentially toxic compounds generated from meat consumption.

Responses at the OTU level

A total of 80 samples were collected from four treatments (basal medium (Blank), Inulin (1% w/v), Beef (0.5% w/v) and Beef+Inulin (1.5% w/v)), four sampling time (0, 5, 10, 24 hours) and five healthy faecal human donors (No. 1-5), for DNA extraction, PCR and sequencing. Using a distance-based similarity of $\geq 97\%$ for operational taxonomic units (OTU) assignment, a total of 204.56 ± 53.48 OTUs were identified per sample. For taxonomic alpha diversity (accounting for relative abundances of OTUs at each time point), all fermentations showed decrease at 5, 10 and 24 hours compared to baseline (0 hours), except for Beef+Inulin, which showed a slight increase (Figure 5). The increase in phylotype richness in the Beef+Inulin fermentation, is significantly from Beef ($P = 1.1e-05$) and Inulin ($P = 0.0047$) alone and with the blank ($P = 1.9e-05$) (Tab. 2). There was no statistically difference in α-diversity

between fermentation of Beef, Inulin or Blank.

UniFrac is a phylogenetically aware measure of beta diversity that can be used to compare OTU structure and community diversity, in high resolution 16S rRNA gene inventories, of microbial communities. The weighted quantitative measure, detects changes present in many sequences from each lineage, as well as changes in which taxa present. For describing community changes, the relative abundance of different bacteria is critical and here, the scenario appears opposite to that seen for α-diversity. All fermentations showed an increase in Weighted Unifrac distance beta-diversity over time, compared to baseline (Fig. 6). Beef fermentation, slightly increased in β-diversity after 5 hours of fermentation, decreased at time 10 hours and increased again by 24 h. This means that although the species or pylotype richness decreased over time, the inter-group differences in diversity (e.g. relative abundances of different microorganisms), actually increased from starting levels, during fermentation of all substrates. Beef+Inulin follows the same trend of Inulin, but the magnitude of change in β-diversity appears less, showing a lower degree of microbial community change with compared to Inulin alone (Figure 6).

In Figure 7, individual responses or change in β-diversity, are shown for each faecal donor at each time point, relative to the starting level. Weighted UniFrac analysis of distance between donors, was calculated for evaluate the inter-individual variability, which led to a wide data spread. A lower UniFrac score, indicates more similarity between the compared microbiota with a score of 0, representing microbiota profiles exactly the same in bacterial presence and abundance. A score of 1 represents profiles with no overlapping in bacterial response. Independent of the sex, some donors seems to have similar behavior (3,4, 5), while others differ moderately (1, 2) both between one another and with donors 3, 4 and 5 (Fig. 7).

The change in OTU relative abundance from time 0, for the different treatments are shown in figure 8. Comparing the treatment effects, Weighted UniFrac distances, no significant differences were found, at each sampling time for Beef+Inulin, compared to Beef alone or the Blank fermentation While Inulin appears different between Beef and Beef+Inulin, for most sampling times (Fig. 8). Significant differences in the relative abundance of different bacteria at genus level were observed both between treatments and over the time course of the fermentations.

3.4 Conclusion

Alterations in dietary composition result in quantitative and qualitative changes in the supply of substrates to the colonic microbiota. In particular, the type and quantity of dietary carbohydrates reaching the colon regulates microbiome energy production (through fermentation) and carbon economy (biomass generation). Between 12 and 18 g dietary protein also reaches the colon every day, although this figure depends greatly on the type of dietary protein, the food matrix, how it is processed (e.g. cooked, glycated to sugars, fried) and the quantity of protein in the diet (Tuohy et al., 2006). Fermentation patterns change along the colon in response to changing substrate gradients and resultant changes in pH (Macfarlane et al., 1992). In the proximal colon, carbohydrate is plentiful and since its fermentation generates more energy than amino acid fermentation (Gest, 1980), it is used preferentially by the resident microbiota. The SCFA produced as a consequence lower pH in the proximal colon, typically to about 5.5. However, as the availability of carbohydrate decreases along the colon, so to does saccharolytic fermentation giving way to protein or amino acid fermentation in the distal colon, with concommittant production of potentially toxic metabolites like BCFA, amines, phenols, indoles and ammonia. Fermentable non-digestible carbohydrates (such as inulin), may decrease the concentration of putrefactive compounds that are generated during proteolytic fermentation, by providing the microbiota an alternative source of energy and carbon (Macfarlane and Macfarlane, 2012; Williams et al. 2001; Swanson et al. 2002). Indeed, non-digestible carbohydrates, fibers and prebiotics, have been shown to reduce production of toxic endproducts from proteolytic fermentation in laboraroy animals (Fujiwara et al 1991) and in farm animals (Lluis et al. 2014). Few studies have examined how supplementation with a prebiotics, such as inulin, impacts on protein fermentation, subsequent metabolite production and also impacts on microbial community structure, in the human distal colon. In this study, we have employed an *in vitro* systems, anaerobic, stirred, faecal batch cultures with pH set at 6.8 to 7.2 to model the fermentative environment of the human distal colon. Using fresh faecal samples collected from 5 different health donors (male and female), we used a combined metabolomics and 16S rRNA profiling approach, in order to track changes both in microbiota composition and metabolic output during protein (pre-digested red meat/beef) fermentation in the presence or absence of inulin.

Multivariate statistical analysis of ^{1}H-NMR metabolic profiles was used to identify main changes in metabolites over a 24 h fermentation period. Amino acids were consumed during inulin fermentation, probably reflecting a conversion into micro-

bial biomass, as microorganisms grew, during the fermentation. Beef fermentation, lead to a significant increases, in the NMR signals, for methylamine (MA) and TMA. TMA can also be demethylated into dimethylamine (DMA) and methylamine (MA), as well known is putrescine (diamines), a chemical analogue to TMA (Wallraben-stein et al., 2013). The Beef+Inulin fermentation gave a profile of metabolites with NMR signals typical of metabolites form both proteolytic and saccharolytic fermen-tations. Importantly, TMA production was reduced upon proteolytic fermentation in the presence of the prebiotic. Similarly, the BCAA, valine, leucine and isoleucone, appeared to be more depleted during the Beef+Inulin fermentation, than in the Beef alone. These three amino acids, are important for human physiology because they are involved in stress, energy and muscle metabolism (Burrill et al., 2015). Valine, has been established as a useful supplemental therapy to liver diseases (Taniguchi et al., 1996), while leucine, decreases brain levels of tyrosine, serotonin, but not do-pamine (Le Masurier et al., 2006). Leucine also stimulates insulin release, which in turn stimulates protein synthesis and inhibits protein breakdown. BCAA decreased in patients with liver disease, such as hepatitis, hepatic coma, cirrhosis, extrahe-patic biliary atresia or portacaval shunt (Tajiri, 2013; Weisdorf et al., 1987; James, 2002). Aromatic amino acids (AAA) such as tyrosine, tryptophan and phenylalani-ne, are more present in Beef alone, perhaps because in the presence of fiber, the metabolism is moved to carbohydrate component at the expense of that protein which remains undigested. Since BCAA and AAA pass into the brain by a similar mechanism, their compete for the brain absorption (Fernstrom, 2005). 5-aminopen-tanoic acid, whose formation is inhibited in the presence of Inulin, is a lysine degra-dation product, and can be produced both endogenously, or through *Clostridium* species catabolism (Rodionov et al., 1988). The multi-step reaction, expected the production of cadaverine from L-lysine, which is metabolized in L-piperideine and then, 5-aminopentanoic acid (Fothergill and Guest, 1977). High levels of 5-amino-pentanoic acid in biofluids, may indicate bacterial overgrowth or endogenous tissue necrosis (Callery and Geelhaar, 1984). Besides being a waste product, 5-amino-pentanoic acid may act as a methylene homologue of γ-aminobutyric acid (GABA) and functions as a weak GABA agonist (Callery and Geelhaar, 1985). Furthermore, it shows a weak inhibitory action of route of blood clotting by acting as a similar amino acid antifibrinolytic activities (Cole and Castellino, 1984).

TMA presents a clear decreased profile in the combined metabolism between red meat and prebiotic fiber, indicating the possible antibacterial effect, charged to the

species involved in the production of these harmful compounds, of highly fermentable Inulin. For many compounds, enterohepatic cycling has the effect of rendering the molecules, or its metabolites, more difficult to eliminate from the body, contributing or prolonging their toxic effects (Hill, 1995). Importantly, PCA analysis showed that although each faecal donor showed different metabolic signatures, the combination of Inulin to the Beef fermentations moved these metabolic profiles in a consistent manner in the faecal microbiota of all subjects. Moreover, although in the same direction, three of the faecal microbiota, behave in a very similar manner, clustering closely together in terms of NMR spectra (Fig. 4a), while the faecal microbiota of donors 1 and 2 were distinct albeit responding in the same direction as donors 3, 4 and 5. The present study, while limited in size and number of subjects, therefore suggests that although microbiota metabolite production in response to different dietary substrates may be unique, certain groupings of "metabotypes" may exist, regulated by microbial composition or metagenomic makeup, as suggested previously (Stella et al. 2006; Vázquez-Fresno et al. 2016).

Fermentation of Beef, Inulin or Beef+Inulin, had dramatic effects on the architecture of the faecal microbiota, under simulated distal colonic conditions. In terms of α-diversity, species or phylotype richness, decreased for the blank fermentation, Inulin and Beef fermentations, over the 24 hours, while species richness of Beef+Inulin increased significantly, albeit with a small magnitude and a large apparent variation between faecal donors. Inulin appeared to have about 10% higher number of OTU, compared to the Beef fermentation. The fact that species richness was maintained in the Beef+Inulin fermentation, may be due to a more complex and diverse substrate availability, including increased metabolic cross-feeding, between different bacteria and of course the increased substrate availability in this fermentation compared to all other fermentations examined. However, the fact that α-diversity decreased even 5 hours, after inoculation, suggests that changes in microbiota diversity were likely, due to the composition of the test substrates rather than lack of overall energy availability, as previous studies using the same model systems, substrates and substrate concentrations showed strong fermentation, as measured accumulation of SCFA up to at least 10 hours post inoculation (Gomez et al. 2010; Khalil et al. 2014).

Beta-diversity is a measure of dissimilarity between two communities, it gives a quantitive measure of the taxonomic differences between two communities for example, and it is usually measured as the amount of species change between the

communities (Jiang et al. 2012; Morgan and Huttenhower, 2012; Zinger et al. 2012). In this study, using Weighted Unifrac distance of β-diversity from time 0 h, significantly increased biodiversity, over the 24 h fermentation, both in the Blank and treatments (Beef, Inulin and Beef+Inulin) were observed (Fig. 6). In terms of response of the faecal microbiota from each of the 5 different healthy donors, weighted unifrac distances, showed increased disimmilarity and inter-individual variation, in species diversity over the course of the fermentation (Fig. 7). Interestingly, and in congruence with the ^{1}H-NMR data, the response of the faecal microbiota of donors 3, 4 and 5 were more similar one to the other, than that of donor 1 or 2, which showed more dissimilar microbial diversity. Over the course of the fermentation, the response to Inulin, was more dissimilar to Beef and Beef+Inulin, and likewise, Beef appeared to be more dissimilar to Inulin than Beef+Inulin. Our hypothesis is that these results could be due to the modulatory effect of Inulin on protein metabolism, providing a more suitable substrate to the growth of certain bacterial genus (e.g. *Butyricicoccus*, *Butyricimonas*, *Coprococcus* and *Dorea* – with butyrogenic activity, Fig. 9) to the detriment of others. Finally, Beef is the only one that shows a decrease around time 10 h, with an upswing at the end of fermentation (24 h) (Fig. 6). This "inverted parabola" trend, hired by the fermentation of Beef, is maybe due to the formation of compounds ranging to inhibit bacterial growth, which is then raised again, perhaps by the ability of some bacteria to use and metabolize them, making the growth environment more accessible. Substantial difference in the biodiversity associated with each substrate intake (Beef, Inulin, Beef+Inulin), varied among individuals (Fig. 5, 6), as confirmed by others studies (Wu et al., 2010; Li et al., 2009). Contrary to the recently study published on the effect of resistant fiber (maltodextrin) (He et al., 2015) and to the recognized increase of microorganisms tolerant to bile, in an animal-based diet (David et al., 2013), all dietary treatments with Inulin (alone or added to Beef) showed an increase in relative abundance of the genus *Alistipes*, normally considered degraders of fiber (e.g. fructans) and glucosinolates, producing succinic acid as principal metabolic endproduct of glucose fermentation (Koropatkin et al., 2012; Li et al., 2009; Song, 2006). Butyrate-producing bacteria from the human large bowel such as *Butyricicoccus* (belonging to *Clostridial cluster IV*) were more abundant in pure Inulin fermentation. *Butyricicoccus* are generally mucosa-associated bacteria, are underrepresented in colonic mucosa of patients with, active ulcerative colitis (UC) and Crohn's disease (CD); *B. pullicaecorum* has been investigated as a marker in inflammatory bowel disease (Eeckhaut et al.,

2013). Enterobacteriaceae, such as Shigella and Escherichia genus, were significantly under-represented in samples of human faecal fermentation of protein source (Beef). While, *Odoribacter* and *Parabacteroides* genus, increased in relative abundance when Inulin was fermented. *Odoribacter* belongs to the Porphyromonadaceae family and to the Bacteroidetes phylum; *O. splanchnus* is known to produce acetate, propionate, and butyrate, therefore, its growth has been linked to protective effects against inflammation in the host via increased SCFA availability (Morgan et al., 2012). A shift from Bacteroides to Parabacteroides, can often be observed, after fiber intake (Graf et al., 2015). In the inulin fermentations, Parabacteroides appeared to increase in relative abundance, while in our experiments no signficiant differences in relative abundance of Bacteroides genus was observed, under any of the test fermentation conditions. This is consistent with recent studies by Chung et al (2016), showing few species of Bacteroides respond in mixed culture, to the presence of inulin and in a pH dependant manner, suggesting differences in Bacteroides species microbiota composition, between individuals, could explain apparent lack of response of these prevalent and dominant member, of the gut microbiota to the different fermentation substrates, used in our study.

Inulin is recognized as the "gold standard" prebiotic and has been shown in many *in vitro*, animal and human studies, to increase the relative abundance of bifidobacteria, within the gut microbiota. However, most of these in vitro studies, were conducted under conditions simulating the proximal or transverse colon, and few studies have examined the response of bifidobacteria to inulin, in mixed culture, under the environmental conditions, of the distal colon. Similarly, studies in faeces may not faithfully represent microbiota profiles, in any given region of the gut, but are seen more as a summation of microbiota profiles, over the combined intestinal tract (Tuohy and Scott, 2015). In this current study, small changes were observed in relative abundance of bifidobacteria, with significant changes in relative abundance for Beef, Inulin and Beef+Inulin over the 24 h fermentation, compared to Blank. Data from qPCR and FISH (Fig. 10, 11), confirmed high interindiviaul variation, in response to the different substrates, and that although bifidobacteria tended to increase in 16S rRNa copy number, or relative abundance, according to FISH (Fig. 10), these changes were small due to interindividual variation. Indeed, very similar results were observed by Chung et al (2016), who showed that bifidobacteria respond poorly to fermentable substrates, like inulin or pectin, at near neutral pH ranges, in mixed faecal culture. During Beef fermentation, commensal members of the

intestinal community, such as *Blautia* and *Lachnospiraceae incertae sedis* (belong Lachnospiraceae family), increased significantly, with respect to Beef+Inulin and the Blank fermentation. Lachnospiraceae, received in the last years the most attention because they are very abundant in the human colon, comprising 10 to 20% of the total bacteria (Meehan and Beiko, 2014). Members of this family have been linked to obesity, with positive correlation with cholesterol and LDL cholesterol levels (Koren et al., 2011), but also to an anticancer action at the level of the colon in human (Wang et al., 2012), perhaps thanks to the butyrogenic activities of some species. *Butyricimonas*, a member of the family 'Porphyromonadaceae', appears stifled by the presence of Inulin. The strains, isolated from rat faeces, were related to *Odoribacter splanchnicus* and not growth on medium containing 20 % bile (Sakamoto et al., 2009).

Several factors are involved in the onset of a predominant macronutrient-utilizing organisms, such as compounds related to partial degradation products, fermentation products and growth or inhibitory factors, which arise not only by the presence of specific compounds, but also competitive or synergistic interaction between two or more bacterial populations. Many microbial communities, show significant division in metabolic work. Endproducts of some organisms are metabolites for others; working together they turn raw resources into fully metabolized nutrients. Recent studies have confirmed the important role that pH plays, in determining not just relative abundance of different bacteria in the different regions of the gut, but also their metabolic activity (Chung et al. 2016). This work has confirmed that the presence of a fermentable fiber or prebiotics like inulin, can radically modify metabolic activities, of the gut microbiota in the distal colon, specifically reducing the production of potentially harmful metabolites, including TMA, during fermentation of digested red meat. Moreover, we have observed that both proteolytic and saccharolytic fermentation in the distal gut, can dramatically remodel microbiota archecture, both in terms of α- and β-diversity, and in the relative abundance of different bacteria at the genus level. Some of these microbiota modulations, differ from those reported previously for different regions of the colon, and are consistent with recent studies, showing the dramatic impact of pH on microbial ecology within the colon. Taken together, this current study highlights the value of *in vitro* models for studying diet:microbe interactions in the gut and the importance of using combined high-resolution post-genomics methodologies, to derive information on the metabolic activity of the gut microbiota.

3.5 Figure & Table
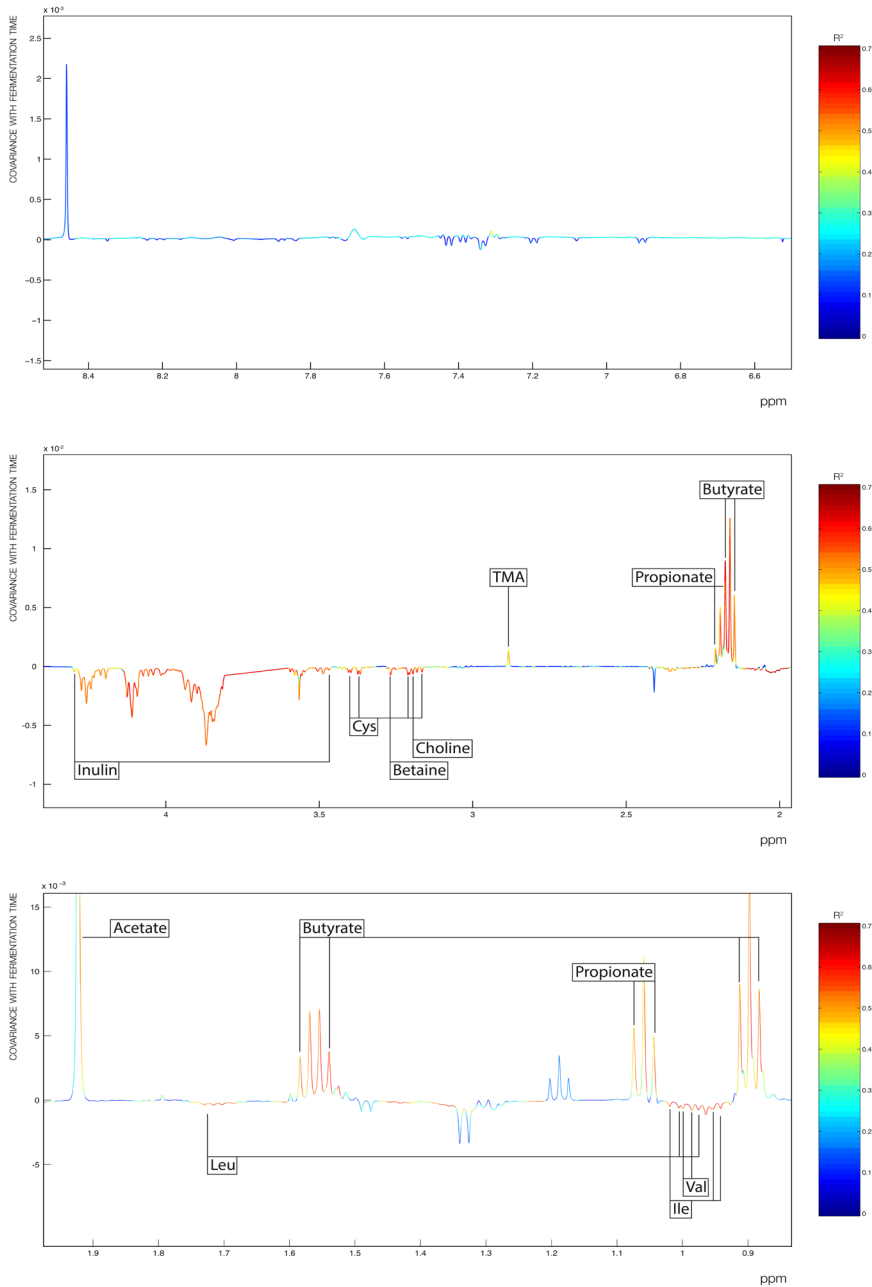
Fig. 1 Coefficient plots extracted from the OPLS model constructed from the ^{1}H-NMR profiles of Inulin (1% w/v) *in vitro* fermentation supernatant of five human faecal donor and the corresponding fermentation time (0 - 24 h).
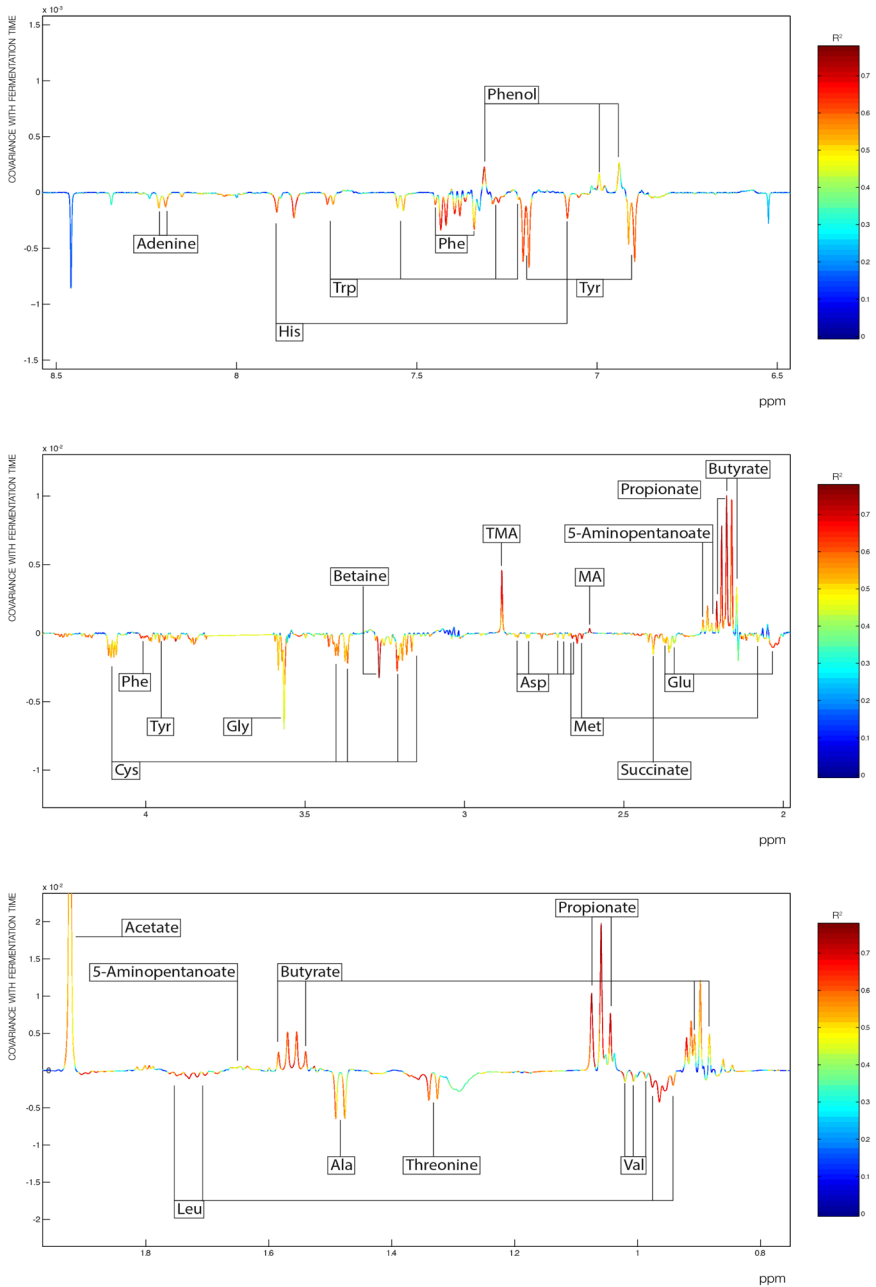
Fig. 2 OPLS coefficient-coded loading plot derived from the ^{1}H-NMR spectra of Beef (0.5% w/v) *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).
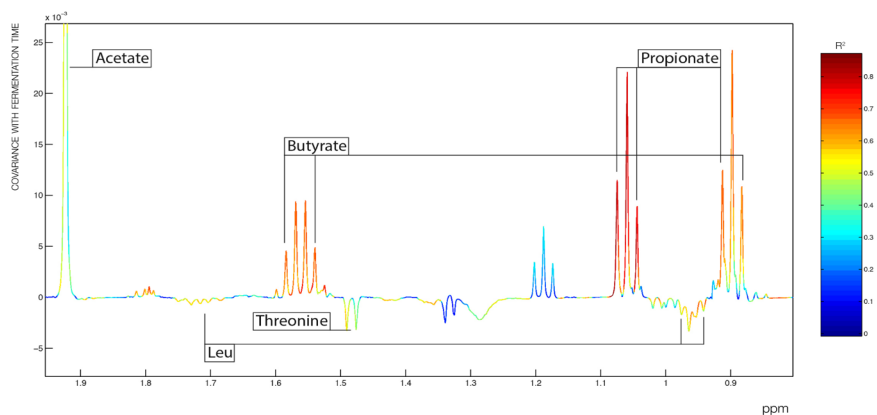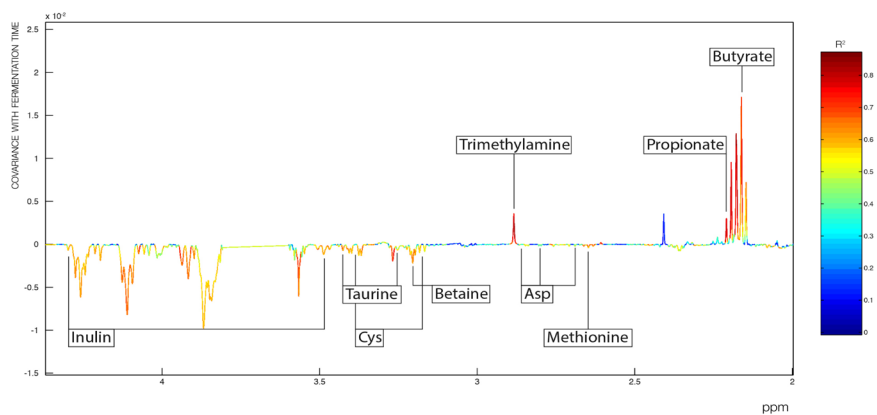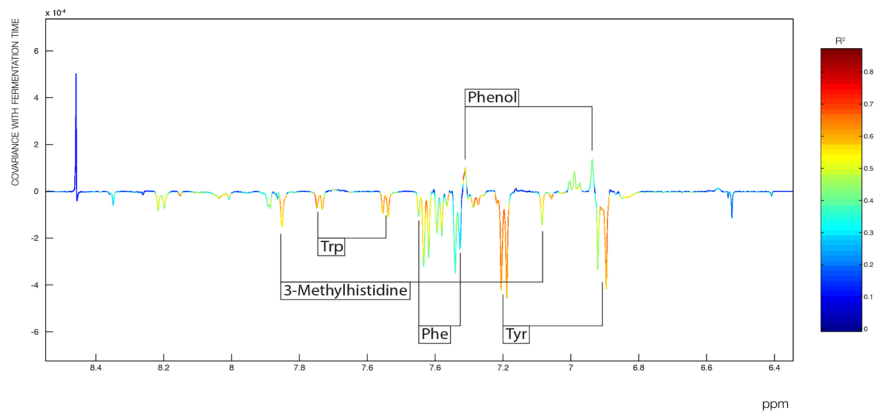
Fig. 3 OPLS coefficient-coded loading plot derived from the ¹H-NMR spectra of Beef+Inulin (1.5% w/v) *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).
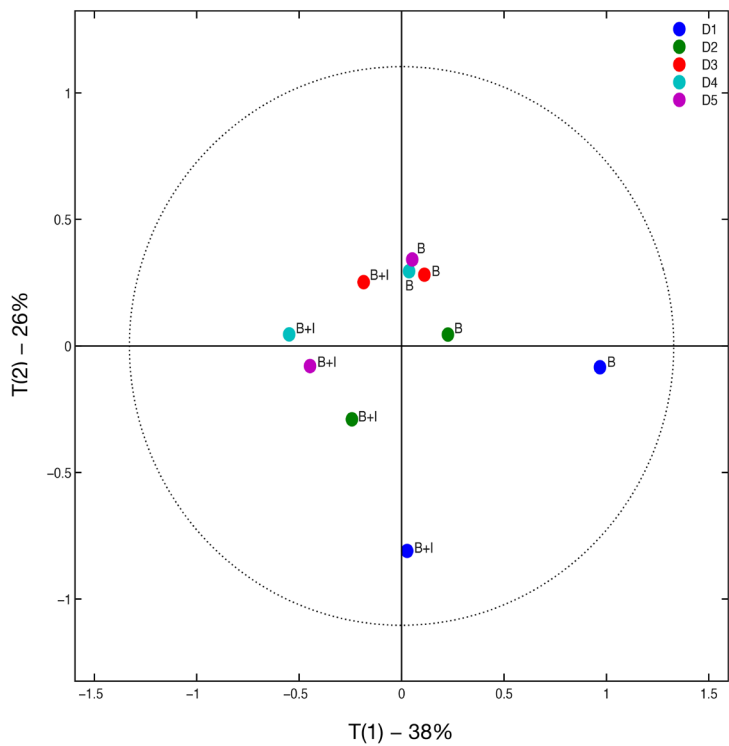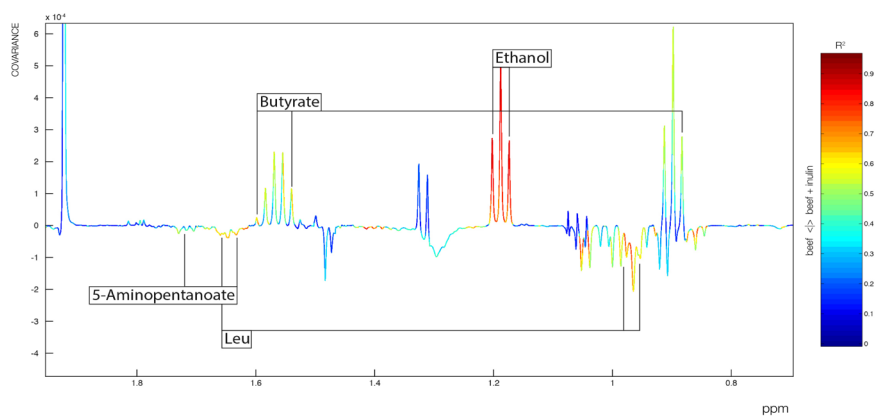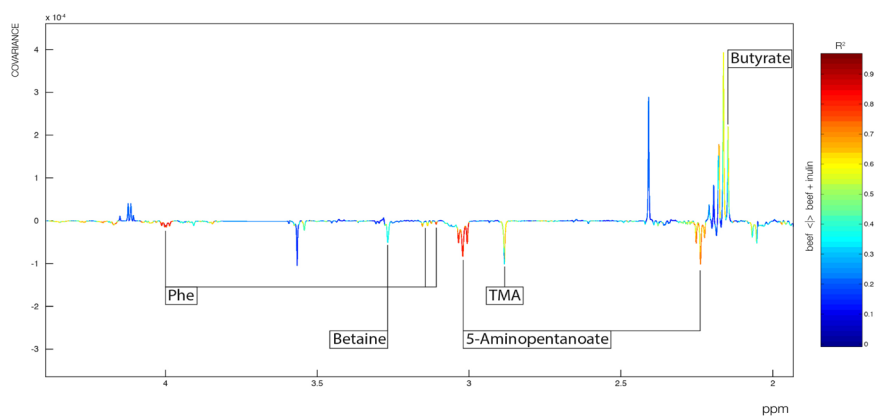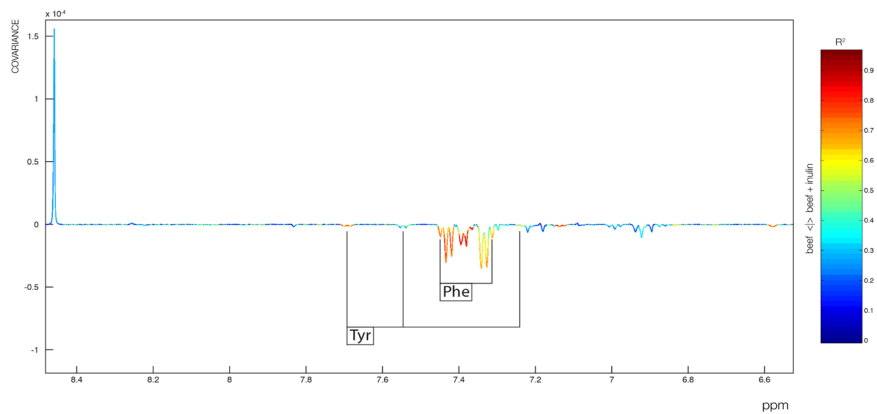
Fig. 4a Principal component analysis (PCA) score plot of ¹H-NMR spectra of Beef and Beef+Inulin *in vitro* fermentations after 24 h of 5 human healthy fecal donors (D1 - D5).

Fig. 4b OPLS coefficient-coded loading plot derived from comparison of ^{1}H-NMR spectra of Beef (0.5% w/v) and Beef+Inulin (1.5% w/v) *in vitro* fermentation by five human faecal donor, at 24 h.

	BLANK	INULIN	BEEF	BEEF+INULIN	BEEF VS BEEF+INULIN
R^2X	0,19	0,37	0,38	0,34	0,33
Q^2Y	0,55	0,67	0,66	0,70	0,54
p value	0,02	0,01	0,01	0,01	0,01

Tab. 1 Summary of the parameter values (R^2X, Q^2Y and p-value) describing the OPLS model.
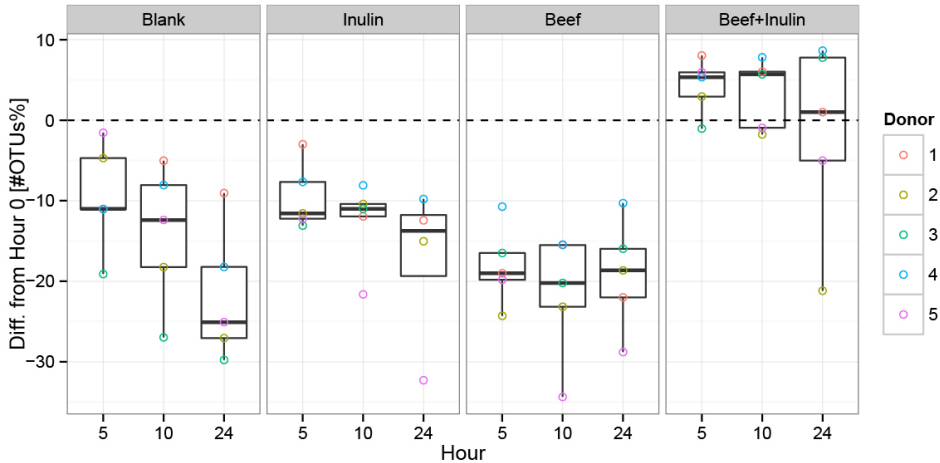
Fig. 5 Alpha-diversity, ranges from time 0 of each treatments and sampling time. Repeated measure for five different human faecal donors (1 - 5), are marked with colored dots.

	Beef	Beef+Inulin	Inulin
Beef+Inulin	1.1e-05	-	-
Inulin	0.0047	1.9e-05	-
Blank	0.2083	1.9e-05	0.6516

Tab. 2 Observed OTU: pairwise comparisons using Wilcoxon rank sum test. P value were adjusted by False Discovery Rate (FDR) method.
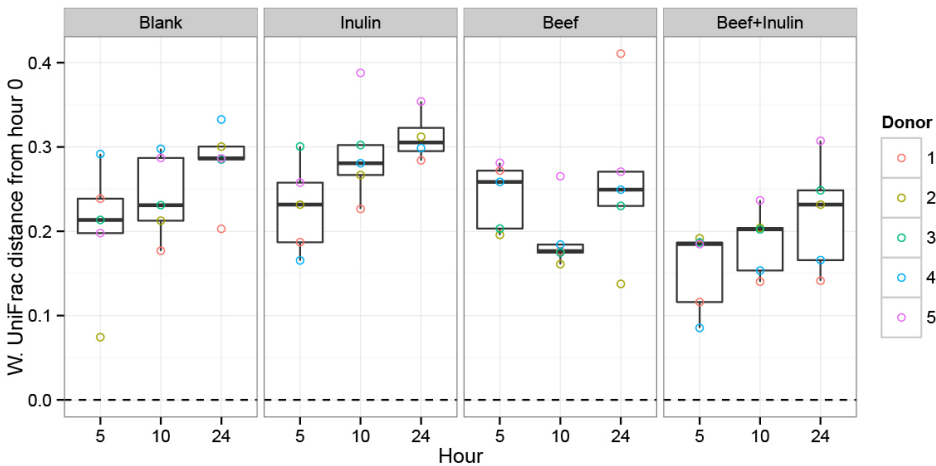
Fig. 6 Weighed UniFrac distances from hour 0 for hours 5, 10 and 24. Only intra-donor distances were computed.
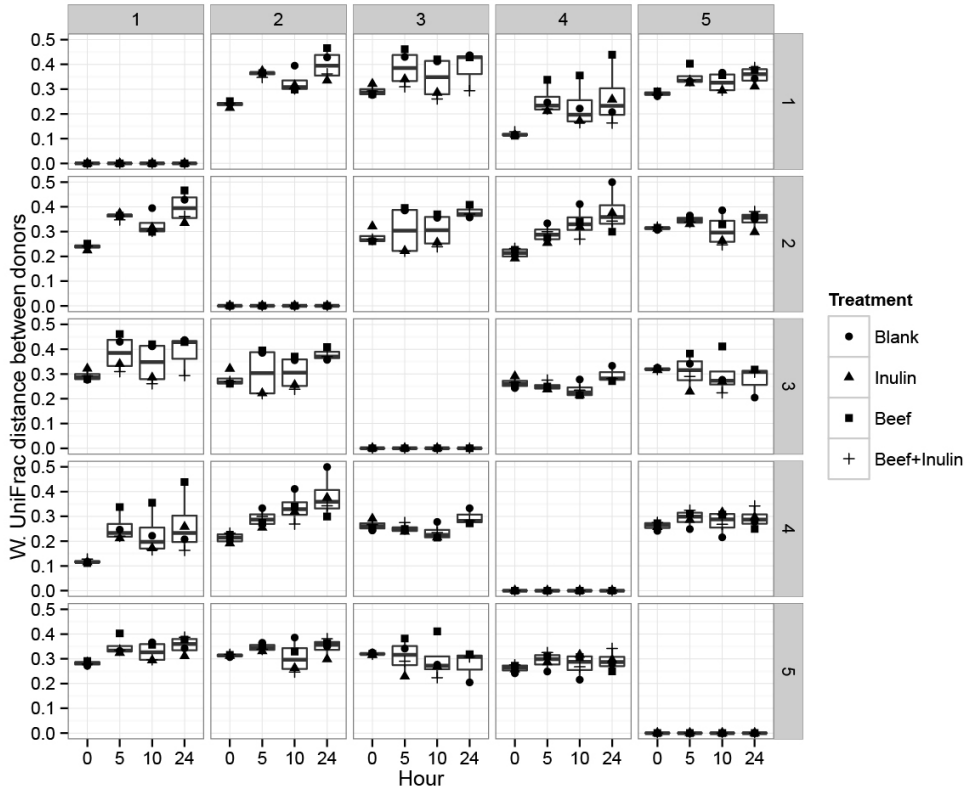
Fig. 7 Weighted unifrac distances between donors (1 - 5), at time 0, 5, 10 and 24 h. The donors are shown to the side of the frame, while treatments are indicated with a shape.
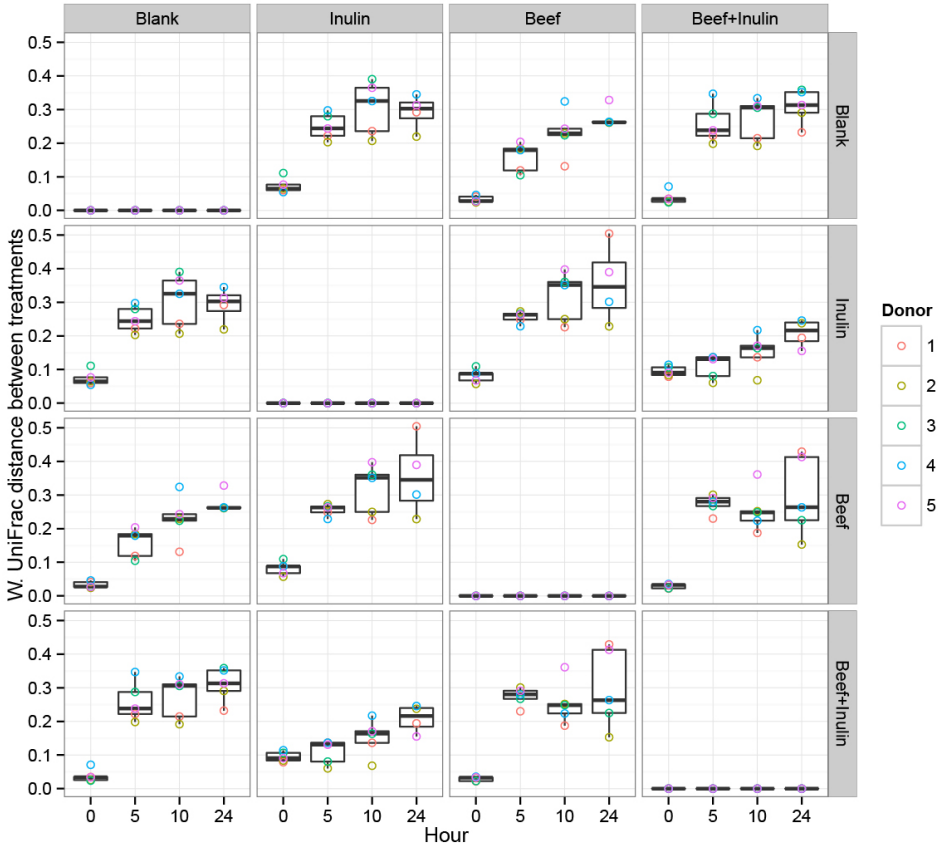
Fig. 8 Weighted unifrac distances between treatments (n = 4), at time 0, 5 , 10 and 24 h. The treatments are shown to the side of the frame, while donors (1 - 5) are marked with a color – code dots.
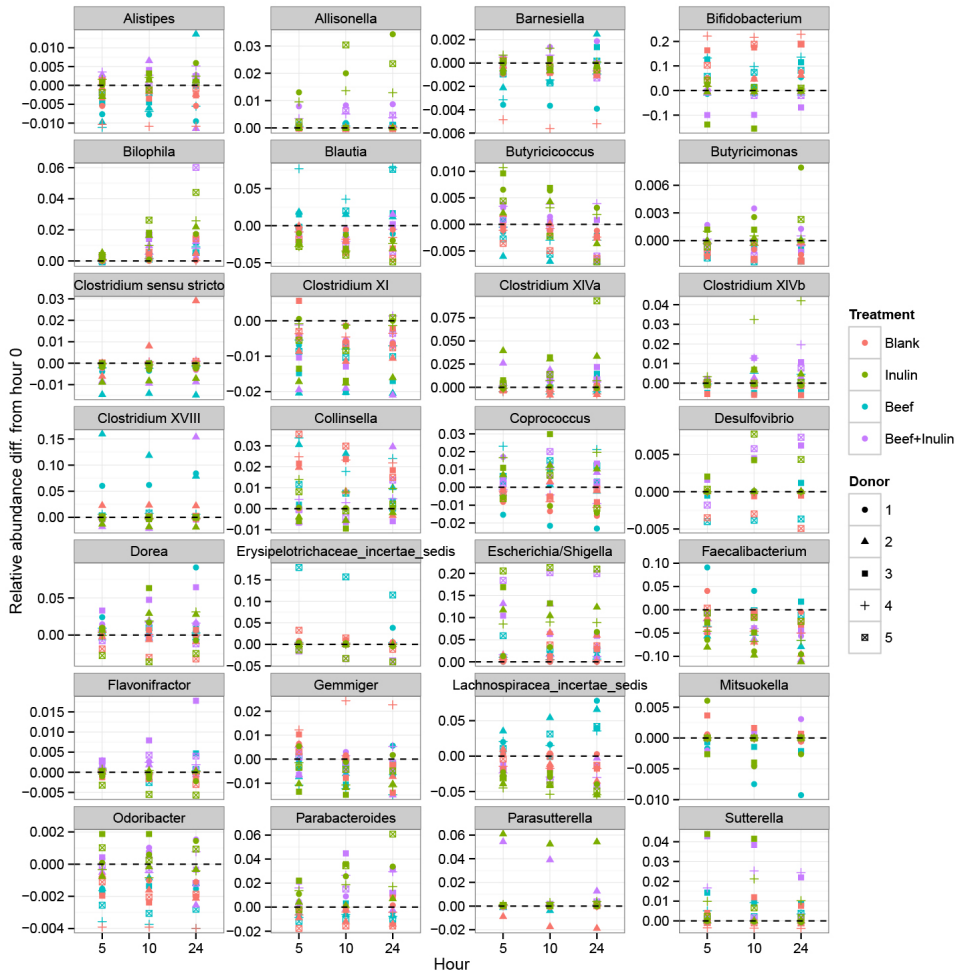
Fig. 9 Relative abundance differences from hour 0, of each time points (5, 10, 24 h), estimated at the genus level. Only genera with at least one significant treatment different from the blank are shown in table 2.

	BACTERIA ENRICHED	BACTERIA DEPLETED
INULIN	*Alistipes*	*Bifidobacterium*
	Allisonella	*Clostridium XVIII*
	Barnesiella	*Collinsella*
	Bilophila	*Erysipelotrichaceae incertae sedis*
	Butyricicoccus	*Faecalibacterium*
	Butyricimonas	*Lachnospiracea incertae sedis*
	Clostridium sensu stricto	*Parabacteroides*
	Clostridium XIVa	
	Clostridium XIVb	
	Desulfovibrio	
	Escherichia/Shigella	
	Gemmiger	
	Odoribacter	
	Parasutterella	
	Sutterella	
BEEF	*Allisonella*	*Bifidobacterium*
	Blautia	*Clostridium sensu stricto*
	Clostridium XVIII	*Clostridium XI*
	Mitsuokella	*Gemmiger*
BEEF+INULIN	*Alistipes*	*Bifidobacterium*
	Allisonella	*Collinsella*
	Barnesiella	*Erysipelotrichaceae incertae sedis*
	Butyricicoccus	*Lachnospiracea incertae sedis*
	Butyricimonas	*Parabacteroides*
	Clostridium XIVa	
	Clostridium XIVb	
	Clostridium XVIII	

	BACTERIA ENRICHED	BACTERIA DEPLETED
BEEF+INULIN	*Coprococcus*	
	Desulfovibrio	
	Dorea	
	Escherichia/Shigella	
	Flavonifractor	
	Odoribacter	
	Parasutterella	
	Sutterella	

Tab. 3 Statisticly significant changes at genus level comparing treatmnets and Blank, over 24h.
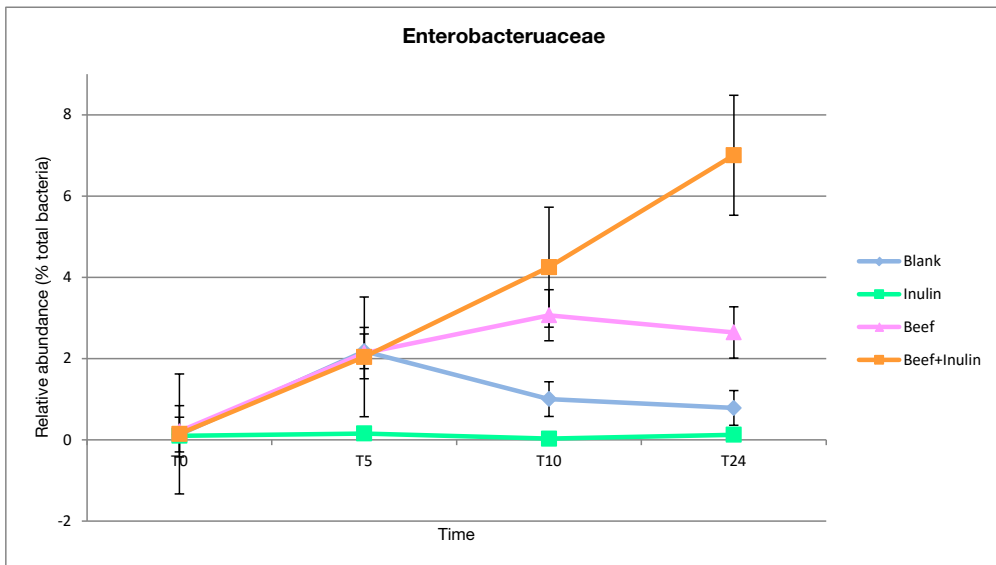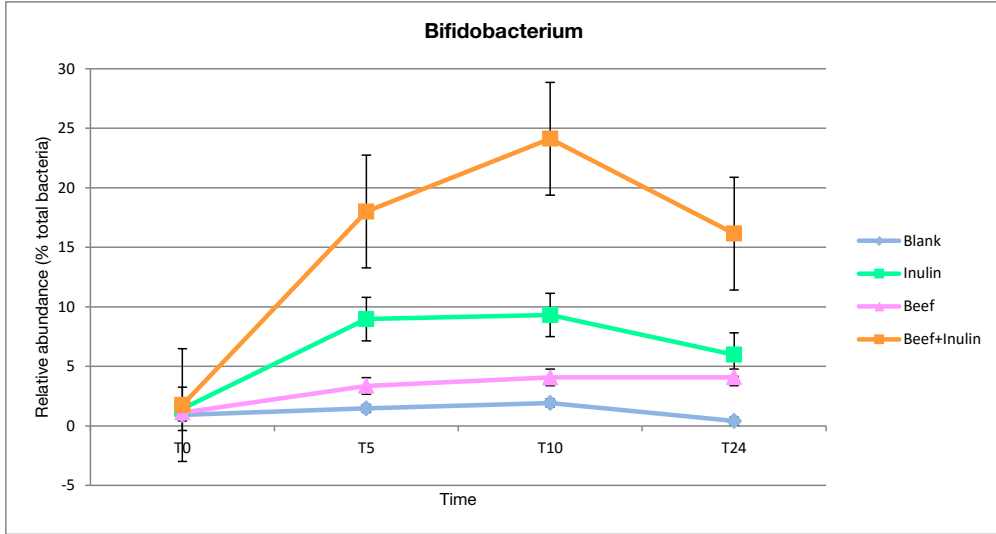
Fig. 10 Distribution of the proportion of **Bifidobacteria** and Enterobacteriaceae in human faeces assessed, over the time (0, 5, 10, 24 h), by genus-specific 16S rRNA oligonucleotide probes and by relative cell enumeration by FISH combined with flow cytometry. Treatments are color – coded: basal medium (blank) - blue, Inulin (1% w/v) - green, Beef (0.5% w/v) - pink and Beef+Inulin (1.5% w/v) – orange.
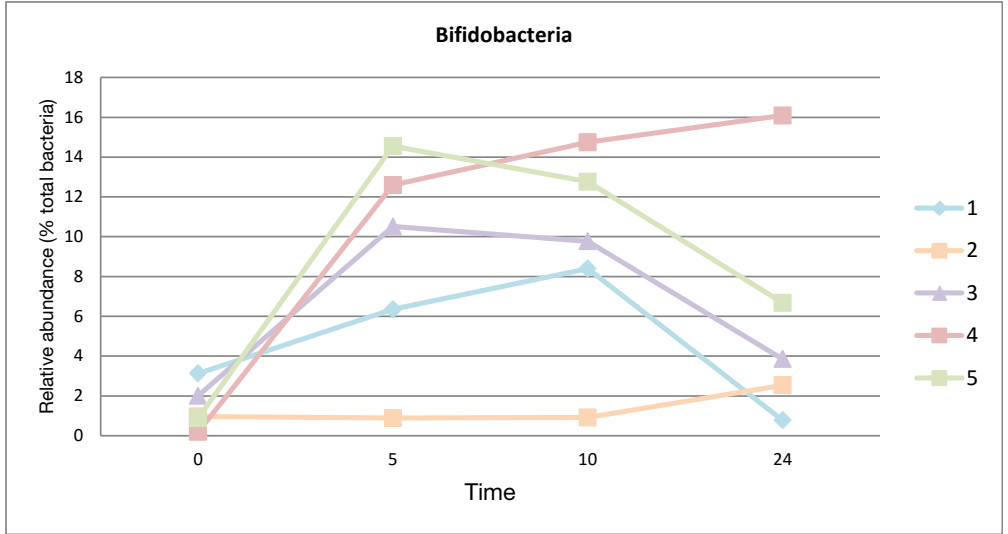
Fig. 11 Distribution of the proportion of Bifidobacteria on Inulin fermentation, between 5 human faecal donors, during the time (0, 5, 10, 24 h), by genus-specific 16S rRNA oligonucleotide probes and by relative cell enumeration by FISH combined with flow cytometry. Donors are color – coded.

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Measuring the impact of the prebiotic inulin on gut microbiota tryptophan metabolism

4. Measuring the impact of the prebiotic inulin on gut microbiota TRP metabolism

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Abstract

Recent studies have highlighted that different tryptophan metabolic pathways, such as the serotonin and kynurenine pathways, may regulate host health/disease, via the immune modulation and the gut:brain axis. Little is known about its interactions with the gut microbiota and how food compounds, such as fermentable fibers or prebiotics, might impact on microbiota TRP interactions. Using anaerobic, stirred batch cultures of human faeces, we measured if tryptophan metabolism by gut microbiota changes in the presence of inulin, using a combination of ^{1}H-NMR based metabolomics and 16S rRNA community profiling. Orthogonal projection to latent structures (OPLS) analysis showed that during TRP fermentation there was an increase in indole and indole acetic acid, along with tyrosine and small amounts of the short chain fatty acids, particularly acetate. In the presence of inulin, indole metabolites were depleted and SCFA enriched with concomitant significant depletion of both inulin and TRP over the fermentation. Both TRP and TRP+Inulin fermentation modulated the microbial diversity within the fermentations over time, and at the genus level *Alistipes*, *Barnesiella*, *Clostridium IV*, *Desulfovibrio*, *Odoribacter*, *Oscillibacter*, *Phascolarctobacterium* had significantly lower relative abundance (p < 0.0011) in the presence of Inulin compared to TRP alone. This *in vitro* study confirms that the presence of a fermentable carbohydrate, in this case the prebiotic

inulin, reduces microbial metabolism of TRP into indole metabolites re-directing it towards microbial biomass formation. Diet:microbe interactions in the distal colon may therefore play an important role in the bioavailability of TRP and its indole derivatives possibly impacting on TRP pathways linked to host health in the gut, periphery and along the gut:brain axis.

Keywords

Tryptophan metabolism contributes to a variety of important (patho)physiological processes, including mood and cognitive function, metabolic syndrome and immune function, but it is still not fully understood which environmental factors regulate its metabolic pathways and how this may impact human health or disease risk (Steinmeyer et al., 2015; Hattersley et al., 2014). Tryptamine, produced by decarboxylation of this essential amino acid, is a monoamine compound that is a common precursor molecule to many hormones and neurotransmitters (Young and Gauthier, 1981). The most well-known tryptamines are, serotonin (5-HT), an important neurotransmitter (Fernstrom and Wurtman, 1971), and melatonin, a hormone involved in regulating the sleep-wake cycle (Borjigin, 2000). Serotonergic system, is involved in a wide range of central and periferal processes. 5-HT, modulates (non-)photic input to the suprachiasmatic nucleus (SCN), a small group of brain cells located in the hypothalamus, that controls mammalian clock, which in turn affect cognition and mood, including psychiatric conditions, such as anxiety, autism, up to more serious conditions (e.g schizophrenia, bipolar disorders, depression and suicidal behavior) (O'Mahony et al., 2015). Acting directly on cardiomyocytes and stimulating chemosensitive nerves in the heart, 5-HT regulates also cardiovascular system (Côté et al., 2004). Over the years, an increasing number of biological functions, have been associated to the serotonergic system. This include modulation of appetite, through cross actions on different nuclei within the brain stem and hypothalamic regions, as well as on cells containing functional peptides (e.g. neuropeptide Y, cholecystokinin) (Lee and Clifton, 2010). Besides being directly involved in the regulation of adipose tissues homeostasis. 5-HT, increases glucose uptake into brown adipose tissue (BALT), while reduces the diameter of adipocytes in white adipose tissue (WAT), suggesting the involvement of serotonin on lipogenesis and thermogenesis mechanisms (Oh et al., 2015). In addition to the well recognized role of melatonin in regulating sleep patterns, the serotonergic system appears to be also involved in the obstructive sleep apnea syndrome (OSAS) (Kokturk and Kanbay, 2015). Interesting observation given that an estimated 90% of the bodies serotonin is produced in the gut, and its production is directly regulate from enterochromaffin cells on the gut wall, via short chain fatty acids (Reigstad et al., 2015). *In vitro* ^{3}H-tryptophan binding to proteins (maybe glycoproteins) of cytosols or nuclei synthesis, especially in the liver, where activities of nuclear NTPase and protein phosphokinase are increase, also in *in vivo* experimental rats (Cortamira, 1991; Sidransky, 2002). Nevertheless, the main way of tryptophan catabolism, consists of

the kynurenine pathway; where kynurenine (KYN), is recognized as the first stable and the main metabolite of tryptophan (more than 95 %) in mammals (Oxenkrug, 2010). This degradation pathway includes also the production of quinolinic acid, an NMDA antagonist, hypothesized as intermediate in the conversion of tryptophan to niacin (St'astný et al., 2004); 3-hydroxyanthranilic acid, a pro-convulsive excitotoxin (Luthman, 2000); N-methyl-D-aspartic acid (NMDA) receptor agonist; picolinic acid, with neuroprotective activity; finishing the route with the synthesis of Nicotinamide Adenosine Dinucleotide (NAD), the biologically active coenzyme form of vitamin B_3 (niacinamide) (Chen, 2009). Metabolites of tryptophan including kynurenine, quino-linic acid, and picolinic acid, are directly toxic to CD4^{+} Th1 cells, which promotes CD8^{+} cell survival, memory response, tumor localization and therapy, and also to Tumor Infiltrating Lymphocytes (TILs), immunological biomarker, recruited into the tumour in an attempt to control its growth (Soliman et al., 2010). Significant cor-relation between intestinal and portal amino acid concentrations, was found for tryptophan. Timing and measured levels of an amino acid in portal plasma reflect their concentration in the dietary source, well as its liberation during digestion, and the rate and extent of its absorption (Goldberg and Guggenheim, 1962). TRP levels in the brain are determined by competitive interactions at the Blood Brain Barrier (BBB) (Choi et al., 2009). The transporter responsible for TRP uptake is located on capillary endothelial cells of the BBB, and it is saturable and competitive, with shared affinities for TRP and several other amino acids, such as Large Neutral Amino Acids – LNAA (e.g. tyrosine and phenylalanine), or Branched-Chain Amino Acids – BCAA (e.g. leucine, isoleucine and valine). Brain uptake of tryptophan de-pends therefore on its blood concentration but also on its relative concentration to other amino acids (Fernstrom, 2012). Recently, Hattersley et al (2015) showed that in obese individuals, both metabolic risk and plasma BCAA concentrations could be altered with different isoenergetic diets, differing in protein, cereal fiber and fat content.

Almost 5% of total protein intake from food survives the small intestine, and rea-ches the colon where it falls under the activities of the colonic microbiota, the most metabolically active collection of microorganisms within the human microbiome (Sniffen, et al., 1992). In the colon, the main environmental parameters affecting tryptophan metabolism, as other aromatic amino acids, are intestinal pH (favored at neutral pH), the availability of others nitrogenous compounds, which determine the amino acid requirements, and the amount of carbohydrate present and the wide va-

riability in terms of microbial abundance and biodiversity between subjects (Davila et al., 2013). Carbohydrates intake stimulates insulin secretion, which causes a reduction of amino acids plasma levels, due to the dissociation of non-esterified fatty acid molecules from albumin, with the entrance of the firsts in adipocyte cells and an increase bioavailability of the seconds, which can bind to the free amino acids in the blood. (Felig et al., 1969).

Anaerobic degradation of L-tryptophan, leads mainly to indole, pyruvate, and ammonia, via pyridoxal phosphate-dependent tryptophanase (Kurnasov et al., 2003). In the colon, asaccharolytic obligately amino acid-fermenting bacteria, include *Clostridium spp.* (e.g. *C. perfringens*), *Enterococcus*, *Shigella* and *Escherichia coli* (Richardson et al., 2013). The proteolytic activity differs between the dependent protease bound to cell membranes, described for *Bacteroides fragilis*-type organisms, and extra-cytosol proteases, for *Streptococcus faecalis ST6*, *Propionibacterium acnes P6*, *Clostridium perfringens C16*, *C. bifermentans C21* and *C. sporogenes C25* (Macfarlane et al., 1988). However, not all of these organisms are able to metabolize tryptophan; Macfarlane by direct plate counting methods, enumerated the fermenting bacteria in faeces obtained from three healthy donors, highlighting the predominance of *Peptostreptococcus indolicus*, *Bacteroides putredinis*, *Clostridium sporogenes*, *Peptostreptococcus asaccharolyticus*, as TRP consumers (Macfarlane et al., 1988). Other reserchers report *Peptostreptococcus indolicus*, *Bacteroides putredinis*, *Clostridium sporogenes*, *Peptostreptococcus asaccharolyticus*, *Xenorhabdus nematophilus* and *Bacillus atrophaeus*, as producers of tryptamine, and *Lactobacillus bulgaricus*, which is able to excrete tryptamine (Williams et al., 2014).

Evidence on the brain availability change of TRP in the cross-feed interaction, was well described by Choi in 2009. The transporter located on capillary endothelial cells, of the blood–brain barrier, is saturable and competitive, well as shares with several other amino acids, such as LNAA - tyrosine and phenylalanine, and the BCAA - leucine, isoleucine and valine. The brain uptake of tryptophan, depends therefore on its blood concentration. Recently, Hattersley et al (2015) showed that in obese individuals, both metabolic risk and plasma BCAA concentrations, could be altered with different isoenergetic diets, differing in protein, cereal fiber and fat content (Hattersley et al., 2015). Moreover, using a combined metabolomics and metagenomics approach, Zhang et al. (2015) showed at a radical dietary shift to a high fiber, high polyphenol and low fat/sugar diet, improved metabolic health in

both genetic and diet-lifestyle associated obesity in children (Zhang et al., 2015). Together with reduced body weight, improved BMI, insulin sensitivity and markers of liver and immune function, microbiota re-modelling, including a reduction in microbiota TRP metabolic pathways, was observed together with reduced production of TRP metabolites, such as indoxyl sulfate, which had been previously linked to heart disease. Similarly, other cell based work has recently shown that, the indole, a microbial metabolite of dissimilatory TRP metabolism, regulates production of glucagon-like peptide-1 (Glp-1), an important anoretic incretin produced by intestinal enteroendocrine L cells (Chimerel et al., 2014). The following experiment, was designed to measure the impact of the prebiotic inulin on microbial TRP metabolism, in the distal colon, and its modulation on the TRP availability and absorption, and then subsequent conversion into neurotransmitters (like serotonin), or catabolism into metabolites like indole and indoxyl-sulfate, which appear to play a pathophysiological role in metabolic disease, and may have important consequences for understanding the role of the gut microbiota in obesity and the metabolic diseases.

Tryptophan stock solution

Tryptophan (Sigma-Aldrich), was added to the basal medium in the form of a stock solution as described by Smith and Macfarlane (1997), with a concentration of 5 mmol/l in the final volume (150 ml). Wait the restoration of pH, before procededing with the faecal inoculum.

In vitro digestion

Cargill Oliggo-Fiber® inulin DS2 5LB (dietary fiber – 98% and free fructose, gluco-se and saccharose – 2%) (Warcoing Industries S.A. Belgium), underwent *in vitro* upper gastrointestinal digestion, consisting of an initial gastric phase, followed by a duodenal-intestinal phase, which simulated the chemical and enzymatic conditions of relevant portions of the stomach and small intestine. The protocol was drawn from Mandalari et al. (2013) and adapted. The below description of the protocol is relative the digestion of 1.5 g starting material.

Preparation of phospholipid vesicles. The lipid vesicles were prepared by remo-ving the solvent by 0.28 ml of a stock solution of L-α-phosphatidylcholine (PCh) from egg in chloroform (0.235 ml in solution of 100 mg/ml of chloroform) (Grade 1, Lipid Products, UK). The organic solvent was removed overnight using a Rota va-cuum evaporation system, thus producing a thin lipid film on a round bottom flask. To avoid oxidation, the lipid film was covered with Argon. The PCh was rehydrated with 7.6 ml of 0.15 M NaCl pH 2.5, then 5 2 mm diameter glass beads were added and the flask was incubated for 1 hour at 37 °C on a horizontal shaker. The suspen-sion was then sonicated (Branson mod.) at 30% power for 30 minutes, non-conti-nuous mode, in order to avoid the excessive increase of the temperature and the overheating of the probe. The suspension of liposomes in single layer was finally filtered with a 0.22 μm Nalgene filter to remove any deposit of titanium resulting from sonication.

Gastric phase. Each test substrate was re-suspended in 6.3 ml of 0.15 M NaCl pH 2.5 and the pH was adjusted to 2.5 with HCl (0.5 M) or NaOH (0.5 M). A 6.1 ml suspension of lipid vesicles was then added to the solution. The gastric enzymes, pepsin and lipase (Sigma-Aldrich) were added at 146 and 60 U/ml concentration of reaction respectively, and the samples were incubated at 37 °C for 2 hours on a horizontal shaker.

Duodenal phase. The pH was brought to 6.5 and the following reagents were

added in such quantities as to reach the respective final concentration: $CaCl_2$ - 11.7 mmol/l, BIS-TRIS buffer - 0.73 mmol/l and bile acid sodium taurocholate and sodium glycodeossicholate - 4 mmol/l. The chemicals were purcased from AppliChem. The pH of 6.5 was checked again and the enzymes were then added at the respective final concentrations: α-chymotrypsin - 5.9 U/ml, trypsin - 104 U/ml, colipase - 3.2 U/ml, pancreatic lipase - 54 U/ml and α-amylase - 25 U/ml. The chemicals were purchased from Sigma. The digestion was performed by incubating the samples at 37 °C for 1 hour on a horizontal shaker.

Dialysis. The digested sample was subjected to dialysis, according to the indications of Millis et al. (2008). The dialysis membrane, Spectra/Por (Spectrum Labs) with a cut-off molecular weight of 1 kDa, which had been previously washed in distilled water, was filled with the digested material and closed on both sides. The membrane was then placed in dialysis solution (10 mM NaCl), at a volume about 100 times greater than the volume of the material to be dialyzed and incubated at 4 °C overnight with slow stirring.

Lyophilization. The dialyzed retentate material were transferred to a round bottom flask and placed at -80 °C until frozen. The material was then lyophilized and the obtained powder was weighed and stored at -80 °C.

Fecal batch cultures

Faecal fermentations were conducted using the basal nutrient medium, prepared as from the following formula (g/l): starch – 8.0; mucin (porcine gastric) – 4.0; casein – 3.0; peptone water – 5.0; tryptone water – 5.0; bile #3 – 0.4; yeast – 4.5; FeSO4 – 0.005; NaCl – 4.5; KCl – 4.5; KH^2PO4 – 0.5; $MgSO_4$ $7H_2O$ – 1.25; $CaCl_2$ $6H_2O$ – 0.15; $NaHCO_3$ – 1.5; tween 80 – 1; hemin – 0.05; cysteine HCl – 0.8. All chemicals were purchased from Sigma. The final, working volume of all fermentations was 150 ml. Anaerobic conditions were maintained by O_2-free N_2 (15 ml/min) flow overnight. Temperature was held at 37 °C using a circulating water bath, and pH was controlled between 6.8 and 7.2 using an automated pH FerMac 260 controller (Gloucester, England-GL208JH, United Kingdom), which added acid and alkali as required (0.5 M HCl and 0.5 M NaOH). Five healthy human donors (2 male, 3 female), who had taken no prebiotics, probiotics, or antibiotics within 3 months of the study were recruited to provide faecal samples as inocula. Faecal inocula (10% w/v) were prepared using pre-reduced phosphate-buffered saline (PBS 0.1 mol/l)

and used to inoculate each fermentation vessel to a final concentration of 1% fae-ces w/v. The substrates tested in fermentation, were basal medium (Blank), Inulin at 1% (w/v) (Cargill Oliggo-Fiber® inulin DS2 5LB), as positive control, tryptophan (5 mmol/l) (Sigma-Aldrich, Germany) and tryptophan added Inulin, with the same their respective concentrations. Each fermentation was conducted once with faecal inocula from each of the 5 faecal donors (n = 5). Batch cultures were run for 24 hour and samples obtained from each vessel at 0, 5, 10, 24 h, were centrifuged at 13 000 rpm, where the supernatants and pellets were stored at -80 °C for metabolo-mics and metagenomics analysis respectively. Samples for FISH were prepared as described below and stored at -20 °C.

Fluorescence In Situ Hybridization-Flow Cytometry (FISH/FC)

Sample fixing and hybridization, were performed as previously described (Rochet et al., 2004; Saulnier et al., 2008). Briefly, 375 µl of culture fluid was diluted in 1125 µl of 4% (w/v) paraformaldehyde and fixed overnight at 4 °C. Bacterial cells were pelleted by centrifugation at 13 000 rpm for 5 min and washed twice in filter-sterili-zed PBS. Cells were then resuspended in 150 µl PBS and 150 µl ethanol. Samples were stored at –20 °C for a minimum of 3 h before further processing. Genus-spe-cific 16S rRNA oligonucleotide probes, labelled with the fluorescent dye Cy5 (Sigma Aldrich, Italy), were utilized for selected bacterial groups, the nucleic acid stain 2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium - SYBR Green I - (Sigma Aldrich, Italy), for total cell counts. The probes used were as follows: Bif 164, specific for *Bifidobacterium* (Langendijk et al., 1995) and Enterobac D, for *Enterobacterua-ceae* (Ootsubo et al., 2002). In 96 well plate, 10 µl of fixed suspension was mixed into 190 µl of PBS (pH 7.2) and then centrifuged at 4000 rpm for 15 min at 4 °C. After one wash in Tris EDTA buffer (TrisHCl – 100 mM; EDTA – 50 mM (pH 8)), when requested, pellets were suspended in Tris EDTA buffer, containing 1 mg/ml of lysozyme (Sigma Aldrich, Italy) and incubated for 10 min at room temperature. Cells were washed in PBS (pH 7.2), to remove lysozyme and were suspended in the hybridization solution (NaCl – 900 mM; TrisHCl – 20 mM (pH 8.0); Sodium Dodecyl Sulfate – 0.01%; formamide as requested), containing 5 ng/µl of specific probes, for a total volume of 55 µl. The hybridization step, was performed overnight at the appropriate labelled probe temperature. Following hybridization, a volume of 145 µl

of hybridization solution, was added in each well, and cells were pelleted at 4000 rpm for 15 min, at 4 °C. Nonspecific binding of the probe, was removed by incubating the bacterial cell suspension, at the appropriate temperature for 20 min in 200 µl, of a washing solution (NaCl – 64 mM; TrisHCl – 20 mM (pH 8.0)). Cells were suspended in 50 µl of PBS (pH 7.2), containing 1x SYBR Green I and incubated at room temperature for 10 min. Following hybridization, a volume of 150 µl of PBS (pH 7.2) was added in each well, and cells were centrifuged at 4000 rpm for 15 min, at 4 °C. Final pellet was suspended in 100 µl of PBS (pH 7.2) and then subsequently analyzed.

Data acquisition by flow cytometry (FCM)

The acquisition threshold was set in the side scatter channel using a Guava easyCyte 8T flow cytometer (Millipore, Italy). For each sample, a total of 10 000 events were stored in list mode files. Analyses were made using the inCyte software (Millipore, Italy). Cells were enumerated by combining in the same hybridization tube, one group Cy5-probe with the SYBR green. An FLG histogram (green fluorescence), was used to evaluate the total number of bacteria stained, with SYBR green. A gate was designed, in this histogram representing the total number of bacterial cells, in the sample and was used to build an FLR histogram (red fluorescence), to estimate the proportion of cells targeted, by the group Cy5-probe in the sample. The proportion of group cells, was corrected by eliminating background fluorescence. Results were expressed as cells hybridizing with the groupCy5 probe, as a proportion of total bacteria stained with SYBR green.

¹H NMR Spectroscopy

For spectroscopic analysis, 400 µL of fermented slurry were diluted with 200 µL of phosphate buffer (pH 7.4; 100% D_2O), containing 1 mM of TSP (3-trimethylsilyl-1-[2,2,3,3-2H4] propionate) as an external standard and 2 mM sodium azide as a bacteriocide. Samples were mixed by vortex and centrifuged at 13 000 g for 10 min, prior to transferring 550 µL to a 5 mm NMR tube. Spectroscopic analysis were carried out on a 500 MHz Bruker NMR spectrometer. For each sample, 8 dummy scans were followed by 128 scans and collected into 64 000 data points. A spectral widths of 20 ppm was used. Spectra were manually phased, corrected for baseline distortions and referenced to the TSP signal at δ 0.00. ¹H-NMR spectra (0.2 to 10.0 ppm) were digitized into consecutive integrated spectral regions (~20.000) of equal

widths (0.00055 ppm). The regions containing the residual signal from water (4.50 to 5.00 ppm), TSP (0.00 ppm) and the large resonance arising from basal medium, were removed to minimize the effects of baseline distortion. Each spectrum was then normalized to unit area.

NMR data analysis and metabolites identification

Multivariate modeling, including Principal Component Analysis (PCA) and Orthogonal Partial Least Squares analysis (OPLS), was performed in MatLab (The Mathworks, Inc., Natwick, MA), using scripts provided by Korrigan Sciences Ltd., United Kingdom. The ^{1}H-NMR spectroscopic profiles were used as the descriptor matrix (X), and donors, sampling time and treatments were used individually as response variables (Y). Loading coefficient plots were generated by back-scaling transformation to display the covariance between the Y-response matrix and the signal intensity of the metabolites in the NMR data (X). Colors projected onto the coefficient plot indicate the correlation coefficient (R^2) between each metabolite and the Y-response variable, with red indicating strong significance and blue indicating weak significance. The predictive performance of the model (Q^2Y) was calculated using a seven-fold cross validation approach. Model validity was established by permutation (1000 permutations) and the results are given as p values. Metabolite identification was performed using HMDB database (http://www.hmdb.ca/), KEGG (Kyoto Encyclopedia of Genes and Genomes), Chenomix profiler 8.1 software (Chenomx Inc., Edmonton, Canada).

DNA extraction

The genomic DNA extraction was obtained by FastDNA™ SPIN Kit for Fecesn (MP Biomedicals, Santa Ana,CA), following the manufacturer's protocols. The final product was 100 µl of application-ready DNA. Nucleic acid concentration and purity were measured using the Nanodrop 8000 spectrophotometer. DNA concentrations were in the range 25.03 ± 11.01ng/µL.

16S rRNA amplicon library preparation and MiSeq sequencing

Using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') (Klindworth et al. 2013) and 806R (5' GACTACNVGGGTWTCTAATCC 3') (Apprill et al. 2015) with overhang Illumina adapters, total genomic DNA was subjected to PCR

amplification, by targeting a ~460-bp fragment of the 16S rRNA variable region V3-V4. PCR amplification of each sample, was carried out using 25 µl reactions with 1 µM of each primer. Specifically 12.5 µl of 2x KAPA HiFi HotStart ReadyMix, 5 µl forward primer, 5 µl reverse primer, were used in combination with 2,5 µl of template DNA (5 ng/ul). All PCR amplification was carried out, using a GeneAmp PCR System 9700 (Thermo Fisher Scientific) and the following steps: melting step – 95 °C for 3 minutes (one cycle); annealing step – 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds (25 cycles); extension step – 72 °C for 5 minutes (1 cycle). The PCR products were checked on 1.5 % agarose gel and cleaned from free primers and primer dimer, using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. Subsequently, dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina), were attached by 7 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina). The final libraries, after purification by the Agencourt AMPure XP system (Beckman), were analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA) and quantified, using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific), by the Synergy2 microplate reader (Biotek). Finally, all the libraries were pooled in an equimolar way, in a final amplicon library and analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Barcoded library were sequenced on an llumina® MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18).

Metagenomic data analysis

The sequences were assigned to samples, according to sample-specific barcodes. This allowed to collect FASTQ formatted files. Raw data fastq files were merged using micca-mergepairs tool. Raw data will be submitted to the http://www.ebi.ac.uk/ena/submit/sequence-submission. Reads were processed using the MICCA pipeline (version 1.0, http://micca.org/) (Albanese et al., 2015). Forward and reverse primers trimming, were performed using micca trim (parameters -w CCTACGG-GNGGCWGCAG -r GACTACNVGGGTWTCTAATCC -W -c). Quality filtering, were performed using micca filter (parameters -e 0.5 -m 400 -t), truncating reads shorter than 400 nt and discarding reads, with an expected error (EE) rate > 0.5%. De-novo sequence clustering, chimera filtering and taxonomy assignment, were performed by micca otu (parameters -d 0.97 -c): operational taxonomic units (OTU) were assigned by clustering the sequences, with a threshold of 97% pairwise identity,

and their representative sequences were classified, using micca classify with the RDP classifier version 2.8 (Wang et al., 2007). Template-guided multiple sequence alignment (MSA), was performed using micca msa (parameters -m nast), against the multiple alignment of the Greengenes (DeSantis et al., 2006) database (release 13_05) clustered at 97% similarity. Finally, phylogenetic tree was inferred, using micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013). micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013).

4.3 Results

¹H-NMR-based Inulin-changed metabolites of tryptophan metabolism

Differences in faecal slurry metabolites between time 0 and time 24 of Inulin fermentation were detected by principal component analysis (PCA) of ¹H-NMR profiles (Fig. 1a). The two first principal components accounted for 74% of the total variation in the data. The corresponding loading plots results, in the prebiotic fiber consumption, with the simultaneous formation of SCFA, such as butyrate, propionate and acetate, in addition to ethanol production and small amount of valerate. Lactate appers with a high peaks, but no significant as a target of fermentation (Fig. 1b).

The first two PCs, obtained from TRP samples, explain 49% of the total variance within the data and shows a similar, but independent metabolic trend for each donor, indicating that the TRP fermentation followed a similar metabolic pattern for all faecal fermentations but that the metabolites produced by colonic microbiota is subject to interindividual variation. Over the 24 hours, a small but significant increase in indole, indole acetic acid and acetate was observed during TRP fermentation by OPLS model (Tab. 1, red colour-code in Fig. 2b). Also tyrosine, propionate, acetate and isoleucine tended to increase, but these changes were not statistically significant over the 24 hour fermentation. In the TRP+Inulin fermentations, two different metabolic trajectories were observed, one for faecal donor 1 and 5, and the second for donors 3 and 4. These trends were observed in the first 2 PC and explained 63% of the total variance within the data (Fig. 3a). Corresponding loading plot (Tab. 1), shows an increase of Indole-3-lactate, 3-indoxyl sulphate, indole-3-acetate, propionate, butyrate, acetate, lactate, ethanol and a decreased of tryptophan (Fig. 3b).

Finally both PCA (Fig. 4a) (explained 48% of the total variance) and OPLS (Fig. 4b; Tab. 1) discriminated analysis of TRP from TRP+Inulin treatments over the time, showed a marked difference in tryptophan, BCAA (valine, leucine, isoleucine), indole-3-lactate and tyrosine for TRP treatment alone, while inulin, acetate, ethanol and lactate (although not significant) characterize TRP+Inulin fermentated substrate. Investigating on the metabolic profiles of these two groups, it was possible highlight, a few differences (Fig. 5a,b). As shown in the two spectra related to the TRP + Inulin treatment at 24 h of fermentation, the main difference, lies of the one hand, in the formation of lactate for group 2 (donor 3 and 4), compared to group 1 (donor 1 and 5), whose spectrum shows a weak signal for this compound, while on the other

hand the group 1 presents a more intense signal for propionate and in the aromatic region, only this group shows the formation of 3-indoxyl sulfate in a small amount.

Microbial diversity of bowel microbiota

A total of 80 samples were collected from four treatments: basal medium (Blank), Inulin, TRP and TRP+Inulin, four sampling time (0, 5, 10, 24 hours) and five healthy human faecal donors, for 16R rRNA gene community profiling, using the Illumina sequencing platform. Using a distance-based similarity of ≥ 97% for operational taxonomic units (OTU) assignment, a total of 15087 OTUs were identified. Taxonomic alpha diversity (accounting for relative abundances of OTU), was significantly different between TRP and TRP+Inulin treatments (p < 0.0011) (Fig. 6, Tab. 2). TRP and Blank fermentation, seem to have a similar trend, with no changes over the course of the fermentations, except for donor 5 in the TRP fermentation, which showed exceptionally high increase in α-diversity. Although the α-diversity of the Blank and TRP alone fermentations remained more or less constant over the 24 hour fermentation period, during fermentations of TRP+Inulin and Inulin alone showed a reduction in species richness of about 30% (OTU %) between time 5 h to time 24 h (Fig. 7). Bacterial community composition was analyzed by statistical comparisons, as indicated in the methods section, of each sampling time, respect to time 0 h. Figure 10, shows the change, with respect to time 0 h, in relative abundance of bacteria at genus level over the course of the fermentation. Only genus level changes which showed statistically significant differences between TRP and TRP+Inulin fermentations compared to the control Blank fermentation are shown. Since data are presented as relative abundances, an increase in any given genus for example, may be due either to an increase in its actual population level or cell number or a decrease on other bacteria within the same community. According to these preliminary considerations, we can say that although with a strong interindividual variability, Inulin (alone or in addition to tryptophan), seems enriched of *Escherichia/Shigella* (about 5%), and slightly above the zero appears the genus *Phascolarctobacterium*. While with greater homogeneity among donors, a slight positive trend was found for *Dorea*. Instead, during fermentation of pure TRP, an increase of about 1% and 2% in *Alistipes*, compared with Inulin and TRP+Inulin respectively, were found. Finally TRP shows for *Desulfovibrio*, *Oscillibacter* and *Phascolarctobacterium* a slight positive stimulation. Compare with the other treatments, a negative trend (over the time) of the genus *Clostridium XVIa* (about -3%), was measured when Inulin was

present in the medium. The high variability between donors and over the time, prevents an understanding of how the genus *Ruminococcus*, behaves during Inulin fermentation. Inulin alone, leads to a reduction of *Barnesiella* genus (about -1%), *Clostridium IV* (about - 0.5%), *Clostridium XVIa* (about -3%), and even shows a slight negative trend for *Flavonifractor* (about -0.5%) and *Odoribacter* (about -0.25%). A fluorescence in situ hybridization-flow cytometry (FISH/FC)-based method, was also used to enumerate the relative abundance of Enterobacteriaceae and Bifido-bacteria, of all 80 samples collected during this experiment. As shown in figure 11, TRP is characterized with the higher level of Enterobacteriaceae, while not allowing a suitable medium for the growth of *Bifidobacterium*. Moreover Inulin, as expected, has been an ideal substrate for the growth of *Bifidobacteria* and also in association with TRP, showed a remarkable growth of this bacterial population.

4.4 Discussion

This study was designed firstly, to measure the impact of TRP metabolism on metabolite profiles produced by the human faecal microbiota using anaerobic pH controlled and stirred batch cultures simulating the fermentation conditions of the distal colon. A range of known TRP metabolites were formed during TRP fermentation over 24 h. We also examined whether the presence of a fermentable fiber, the prebiotic inulin, could modulate both microbiota composition and their metabolic output during TRP metabolism. Metabolism of TRP by the faecal microbiota resulted in the production of a range of known TRP metabolites including indole, indole acetic acid (IAA) and Branched-Chain Amino Acids (BCAA), such as valine, leucine and isoleucine. Low correlation of acetate, was also found over the course of the fermentation, although this is likely to have been derived from either residual carbohydrate in the growth medium or organic matter delivered with the faecal inoculum (e.g. residual dietary fiber).

Similarly, acetate and lactate are the main end products of carbohydrate fermentation by *Bifidobacterium* and *Lactobacillus* (Pokusaeva et al., 2011); while lactobacilli and other Firmicutes such as certain clostridia produce ethanol during fermentation of carbohydrates (Krajmalnik-Brown et al., 2012).

The results obtained through high-throughput sequencing, have showed a reduction of bifidobacteria in terms of relative abundance, during fermentation of Inulin. Being well known the bifidogenic activity of inulin, reported in many *in vitro* and *in vivo* studies, quantification fluorescent in situ hybridization have been integrate. The results obtained through high-throughput sequencing, have showed a reduction of bifidobacteria in terms of relative abundance, during fermentation of Inulin. Being well known the bifidogenic activity of inulin, fluorescent in situ hybridization quantitative method, has been integrated. Measure of microbial absolute abundance, have confirmed, as aspected, the ability of Inulin to stimulate the growth of bifidobacteria. However, the relative abundance may give an important contribution to understanding the dynamics that regulate the growth of more taxa in a specific substrate. During fermentation of prebiotic fiber, the absolute increase of bifidobacteria, may have been joined the growth of other populations, in relative terms, which have grown more and then led to the results observed for metagenomics. Moreover, early work by McBain and Mcfarlane (1997), showed by three stage continuous *in vitro* gut model system, that prebiotic inulin has a specific bifidogenic stimulation in the proximal colon stage (with a pH 5.5), while no significant effects were reported for the other two tract (transverse and distal colon, with more neutral

pH). Furthermore in all conditions, nitroreductase and azoreductase activities were increased and at the same time it was stimulate the growth of Enterobacteria and *C. perfringens* (McBain and Macfarlane, 1997). More recently, Chung and colleagues, described the observed pH effect on bifidobacteria relative abundance by four stage continuous *in vitro* gut model. The best result, in terms of promotion of bifidobacteria by inulin, was observed at pH 5.5, while at higher pH it was inhibited, maybe due to a competition between several co-use of this fiber microorganisms as an energy source (Chung et al., 2016).

In this paper Illumina MiSeq sequencing and quantitative FISH and real-time PCR were used to monitoring the specific effect of inulin on the bacterial growth and the impact of colonic pH on this modulatory activity of prebiotic fiber. An hypothesis upon investigation limits of microbial changes during nutritional intervention, lies in the possible conversion of some phyla that, once phylogenetically distant, find themselves to share more characteristics, gained during the evolution, as the use of the same substrates, as dietary sources, for the their survival within a given environment.

Prebiotic addition of Inulin during faecal microbiota metabolism of TRP appears to have had a positive impact on the consumption of tryptophan, which is more closely related to TRP treatment alone, after 24 h. This is in good agreement with the know metabolism of inulin, especially by bifidobacteria, and also the suggests growth of the microbiota in response to the fermentable carbohydrate resulted in TRP being incorporated into microbial biomass. Decreased levels of tryptophan occurs without the formation of characteristic breakdown metabolites such as the indoles, suggesting that the presence of inulin, microbial growth increases the demand for this essential amino acid. This is in contrast to the TRP fermentation, where NMR signals for indole-3-lactate, BCAA valine, leucine and isoleucine increased over the 24 h fermentation period. In the TRP+Inulin fermentations, BCAA, may have been consumed by a more metabolically active microbiota in the presence of the fermentable substrate Inulin (Neis et al., 2015).

As shown in figure 3a, each donor appear to have the same start condition (score plot overlap in one point), however, as confirmed by weighted UniFrac phylogenetic distances between donors (Fig. 8), two groups have split up along diverging trajectories, during fermentation.

OPLS and PCA modeling were performed on all 80 samples collected during this experiment. The metabolic profiles observed, have inevitably influenced the re-

sults obtained and have made more difficult the interpretation of the data, due to the overlap of several peaks of metabolites linked to different donor-response at the same dietary intervention (like inulin substrate) interfering with the subsequent multivariate data analysis. Nevertheless, we were able to identify and interpret the differences emerged between donors in response to the tested substrates, confirming previously published findings, as that inulin stimulates the growth of bifidobacteria via formation of SCFA and that carbohydrate availability may reduced the aromatic amino acid metabolism by some microorganisms, but may stimulated this process by others.

Thus, even if genera *Alistipes* and *Barnesiella*, are defined saccharolytic, while species belonging to *Odoribacter* are predominantly asaccharolytic (Rajilić-Stojanović and de Vos, 2014), only *Alistipes* results more abundant in tryptophan metabolism, while the others appear not affected by the treatments (*Barnesiella* decrease slightly over the time in all treatments except for the Blank). *Barnesiella*, which is a member of the family Porphyromonadaceae, order Bacteroidales, is a common members of the human intestinal and oral microbiota (Morotomi et al., 2009) and it contributes to carbohydrate degradation and prevents the colonisation of the intestine by pathogenic bacteria (Kulagina et al., 2012). Although it is difficult to precisely define the preferred substrates of fermentation, as shown in figure 10, *Oscillibacter*, includes bacteria starch degraders, which increase both on the resistant starch that carbohydrate starvation, suggesting that other factors must be involved in their response to diet. Summing, Inulin (alone or in addition to tryptophan), seems increase the abundance of *Escherichia/Shigella*, *Dorea* and in a less amount *Phascolarctobacterium*; TRP shows a positive influence on *Alistipes*, *Desulfovibrio*, *Oscillibacter* and *Phascolarctobacterium*; *Clostridium XVIa* was inhibited by Inulin substrate (alone or added to TRP), while alone, inulin leads to a reduction of *Barnesiella* genus, *Clostridium IV*, *Clostridium XVIa* and even shows a slight negative trend for *Flavonifractor* and *Odoribacter*.

4.5 Conclusions

TRP bioavailability and conversion into indole metabolites in particular appears to have important consequences for human health, impacting on the pathophysiology of immune and metabolic diseases, including obesity and on brain function. Although 90% of the bodies serotonin is produced at the gut wall from dietary TRP, little is know about how the gut microbiota impact on TRP metabolism and subsequent systemic TRP pathways involving kynurenine, neurotransmitters like serotonin and indole compounds. Similarly, little is known about how diet, especially the presence or absence of fermentable carbohydrates and prebiotics, known to modulate the gut microbiota, impacts on these interactions. In this present study, focusing on the distal colon as a typical proteolytic environment in the gut, we examined the ability of the faecal microbiota of 5 health individuals to metabolise TRP in the presence or absence of inulin. Inulin had a dramatic impact not only on the production of indole TRP catabolites, but also apparently on the bioavailability of TRP itself. Both were reduced in the presence of the fermentable carbohydrate, indicating that diet:microbta interactions might play an important role in TRP availability and associated biological activities in vivo. Similarly, this study also supports the recent findings that pH as well as the type and quantity of growth substrate can have a dramatic impact on both the composition and metabolic activity or growth of bacteria as observed by Chung et al. (2016) and in this case illustrating that the bifidogenic effect of inulin may not be as obvious in the distal colon as it is further up the colon.
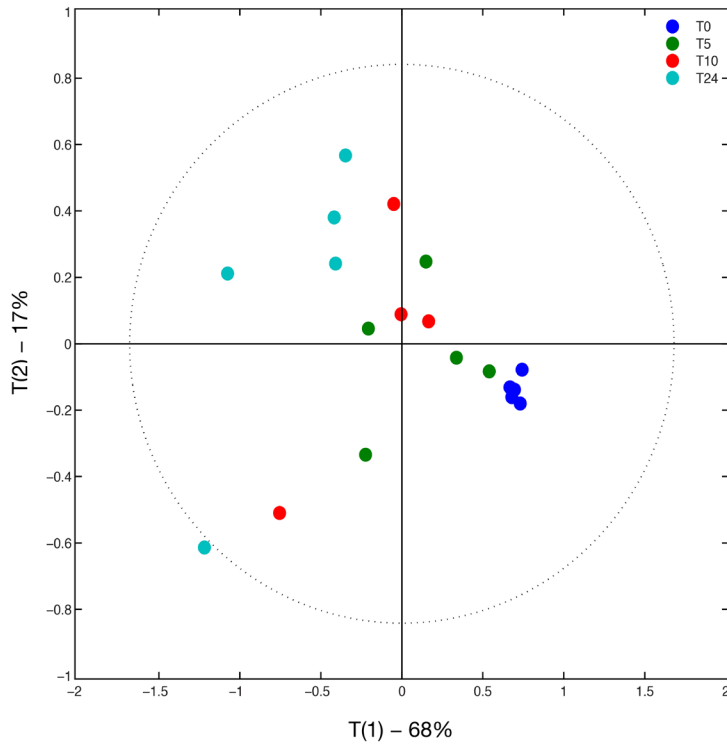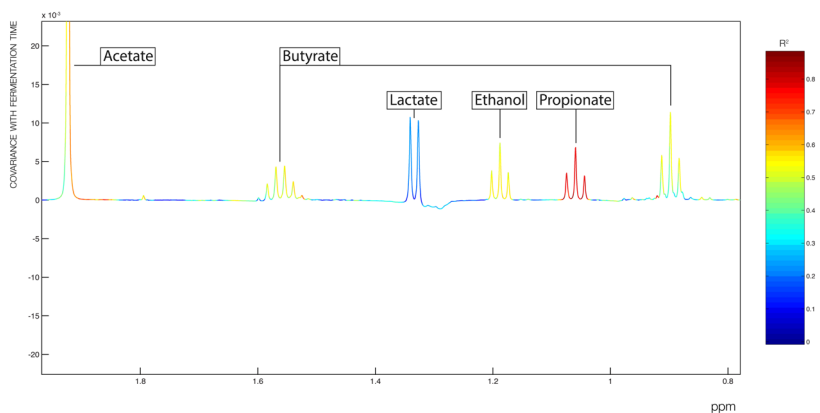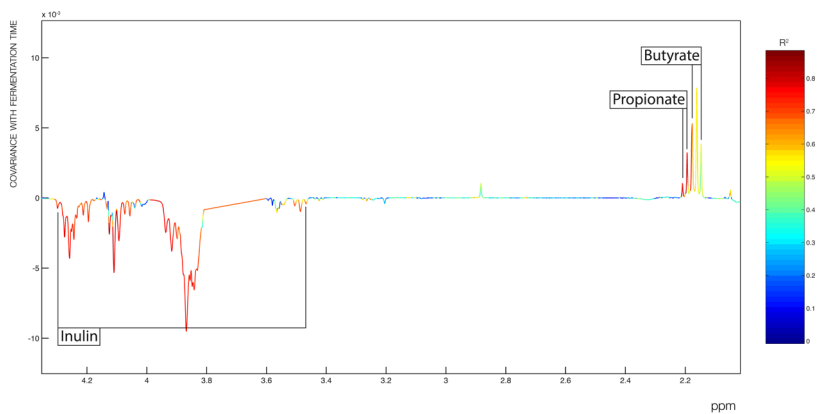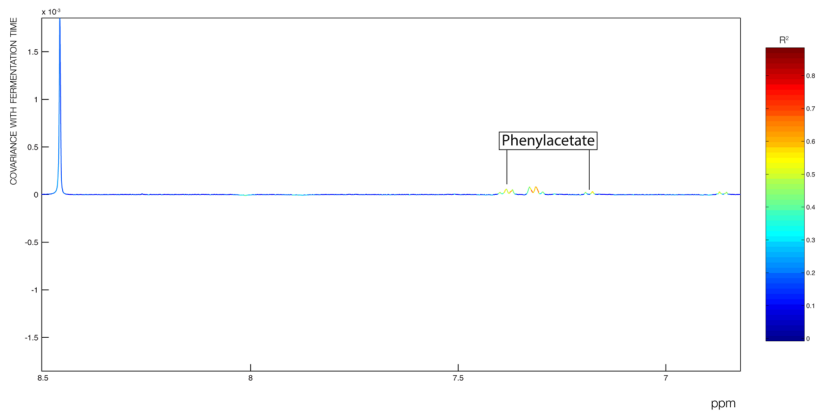
Fig. 1a Principal component analysis (PCA) score plot of ^{1}H NMR spectra of Inulin *in vitro* fermentation, over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).

Fig. 1b Coefficient plots extracted from the OPLS model constructed from the ^{1}H-NMR profiles of Inulin (1% w/v) *in vitro* fermentation supernatant of five human faecal donor and the corresponding fermentation time (0 - 24 h).

Fig. 2a Principal component analysis (PCA) score plot of ¹H NMR spectra of TRP *in vitro* fermentation, over the time (0, 5, 10, 24h) and for 4 human healthy faecal donors (D1 - D4).
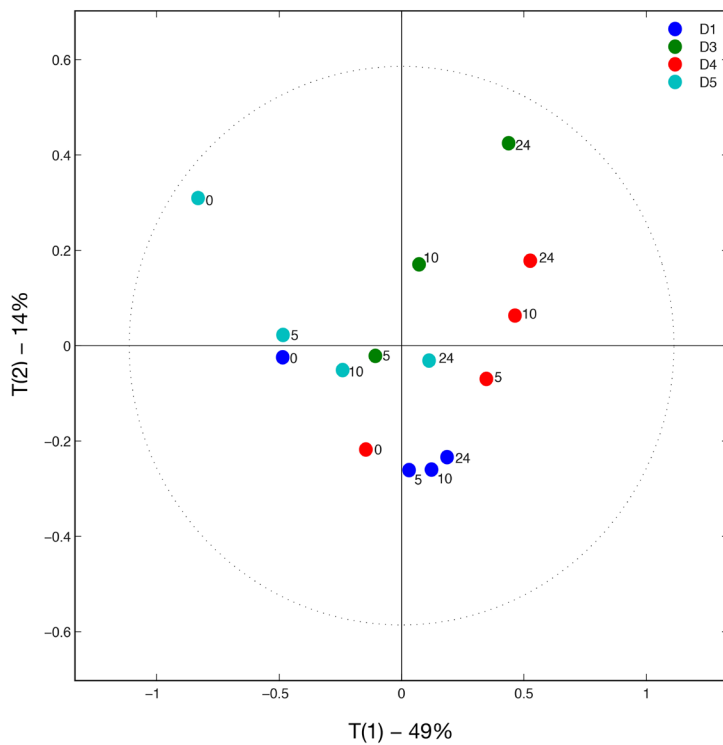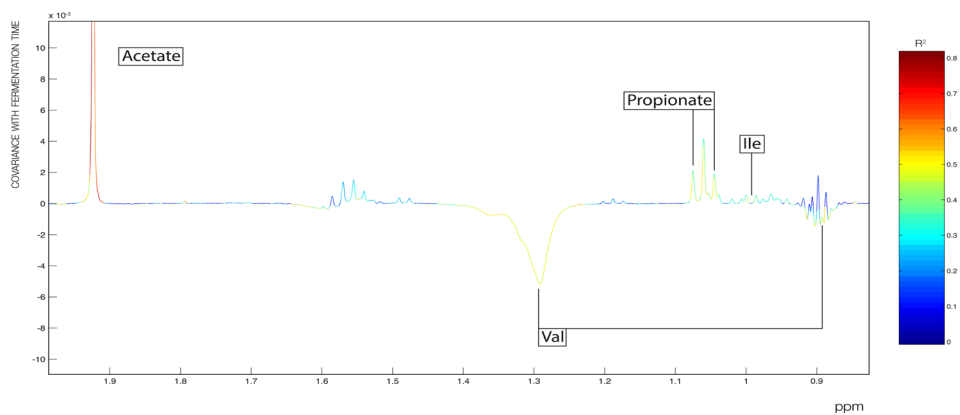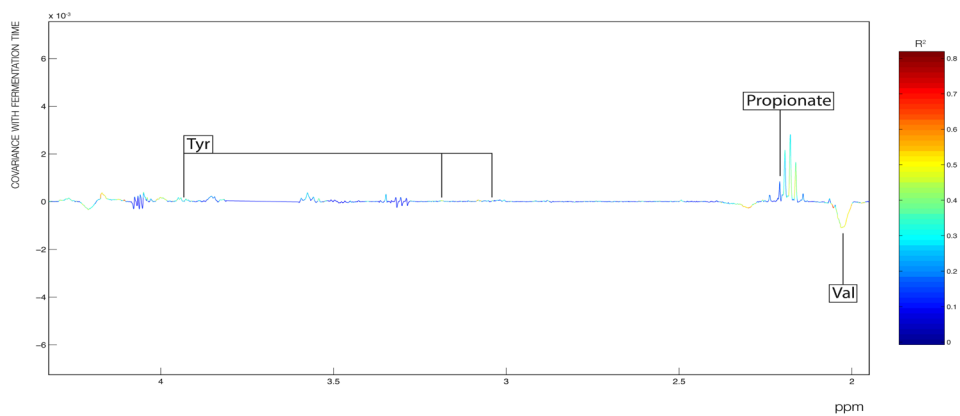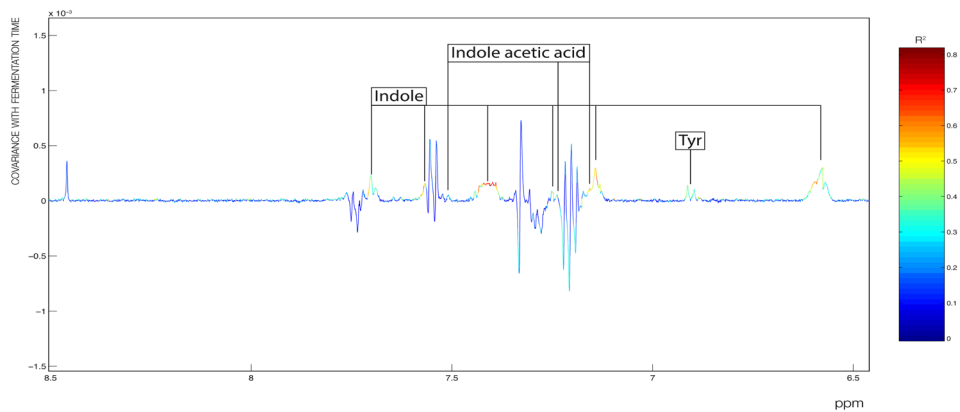
Fig. 2b Coefficient plots extracted from the OPLS model constructed from the ^{1}H-NMR profiles of TRP (5 mmol/l) *in vitro* fermentation supernatant of five human faecal donor and the corresponding fermentation time (0 - 24 h).

Fig. 3a Principal component analysis (PCA) score plot of TRP+Inulin *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
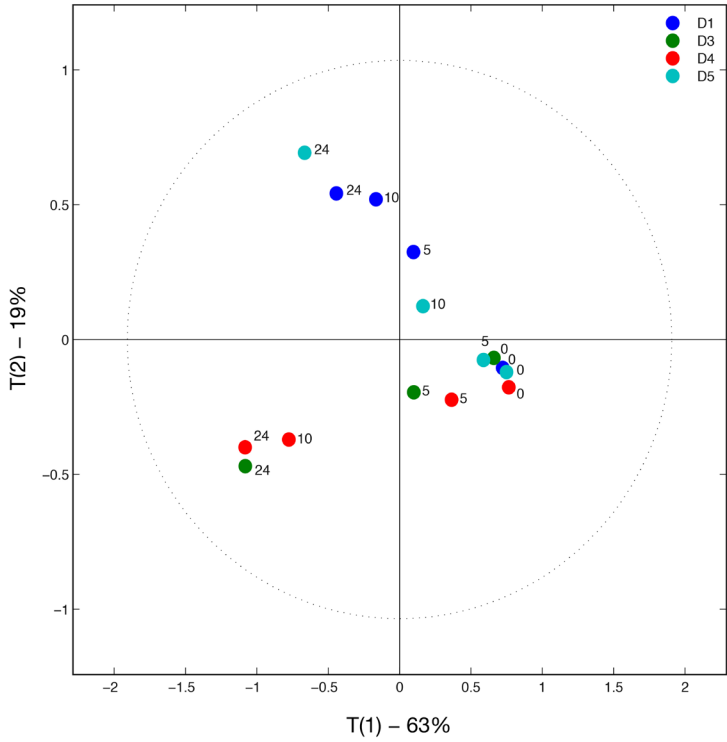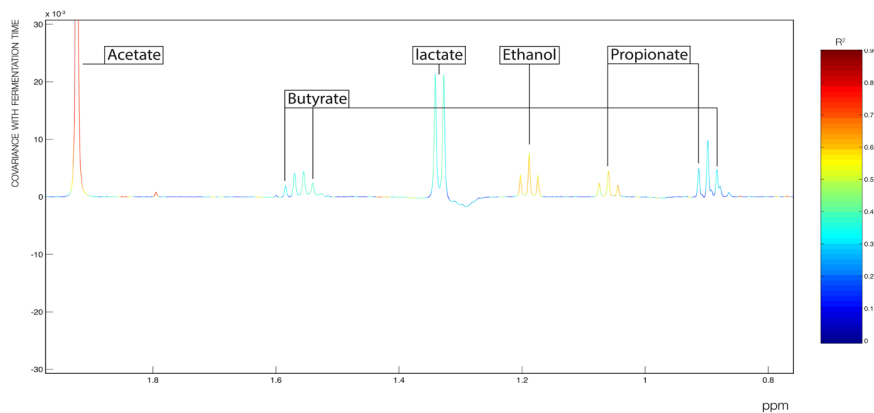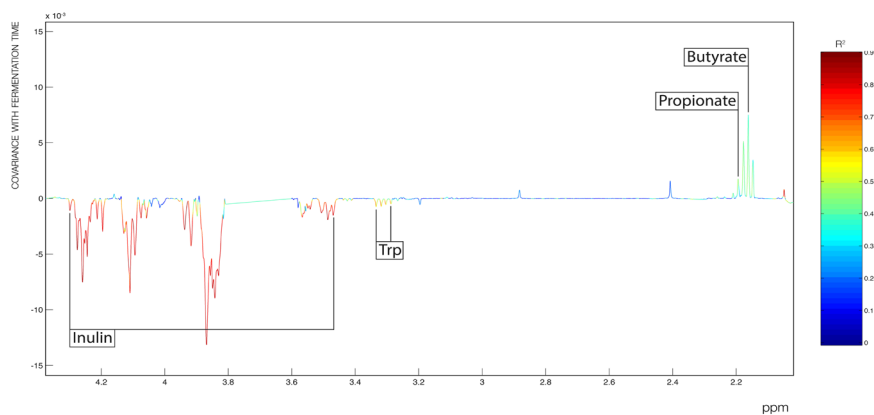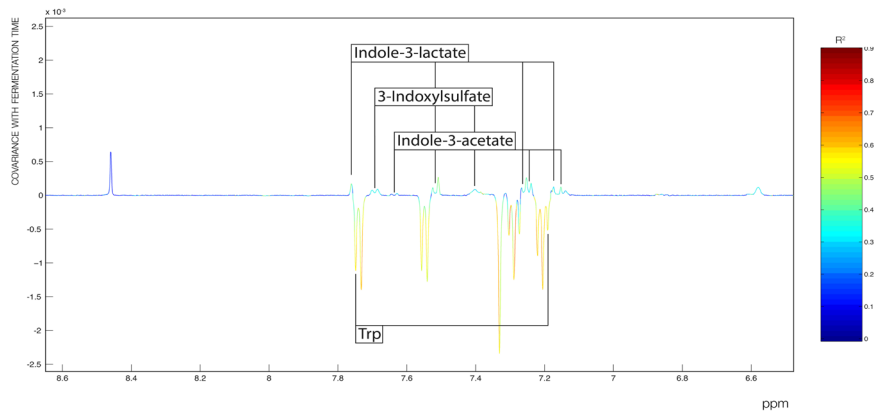
Fig. 3b Coefficient plots extracted from the OPLS model constructed from the ^{1}H-NMR profiles of TRP+Inulin *in vitro* fermentation supernatant of five human faecal donor and the corresponding fermentation time (0 - 24 h).

Fig. 4a Principal component analysis (PCA) score plot of ¹H NMR spectra of TRP vs TRP+Inulin *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors.
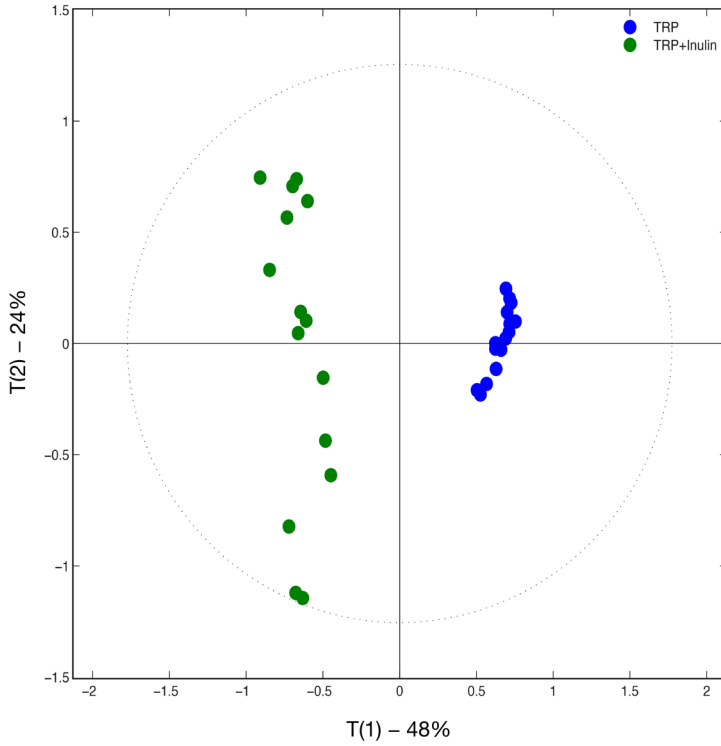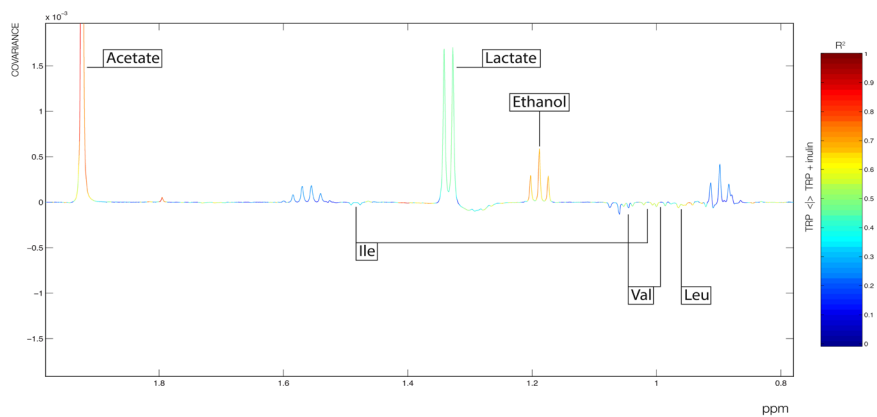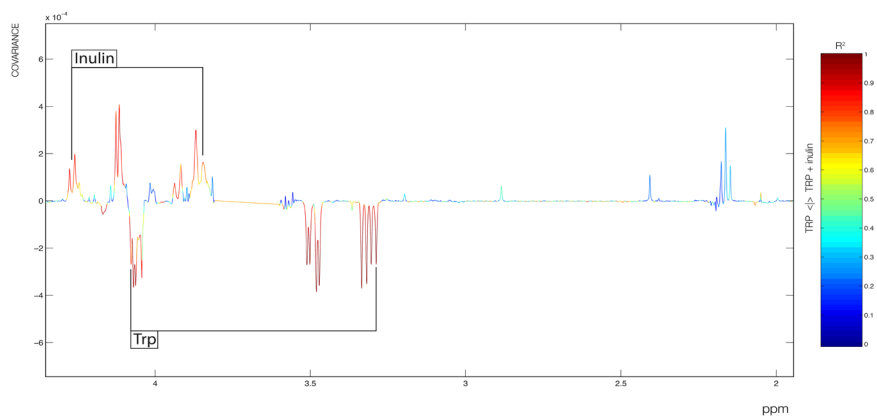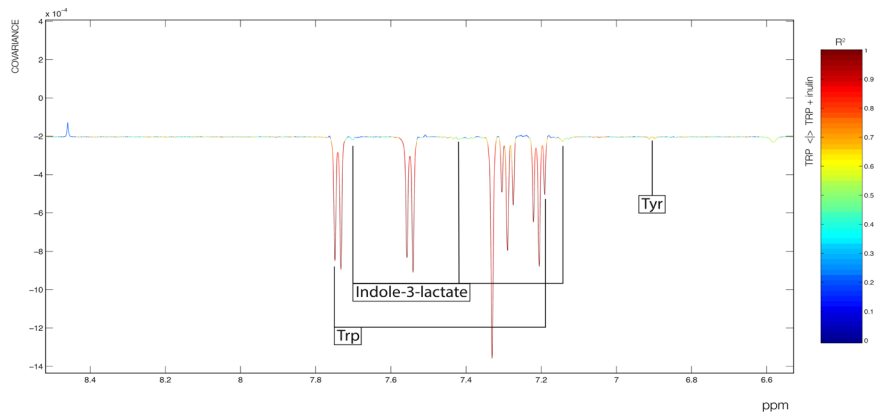
Fig. 4b OPLS coefficient-coded loading plot derived from comparison of ¹H-NMR spectra of TRP (5 mmol/l) and TRP+Inulin (1% w/v) *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

Signal intensity

x 10^{-3}

Indole-3-lactate

3-Indoxylsulfate

TRP

INULIN

TRP

Fig. 5a ¹H-NMR spectrum of TRP+Inulin of donors 1 and 5

ACETATE

PROPIONATE

BUTYRATE

ETHANOL

LACTATE

3   2   1   ppm

Fig. 5b ¹H-NMR spectrum of TRP+Inulin of donors 3 and 4
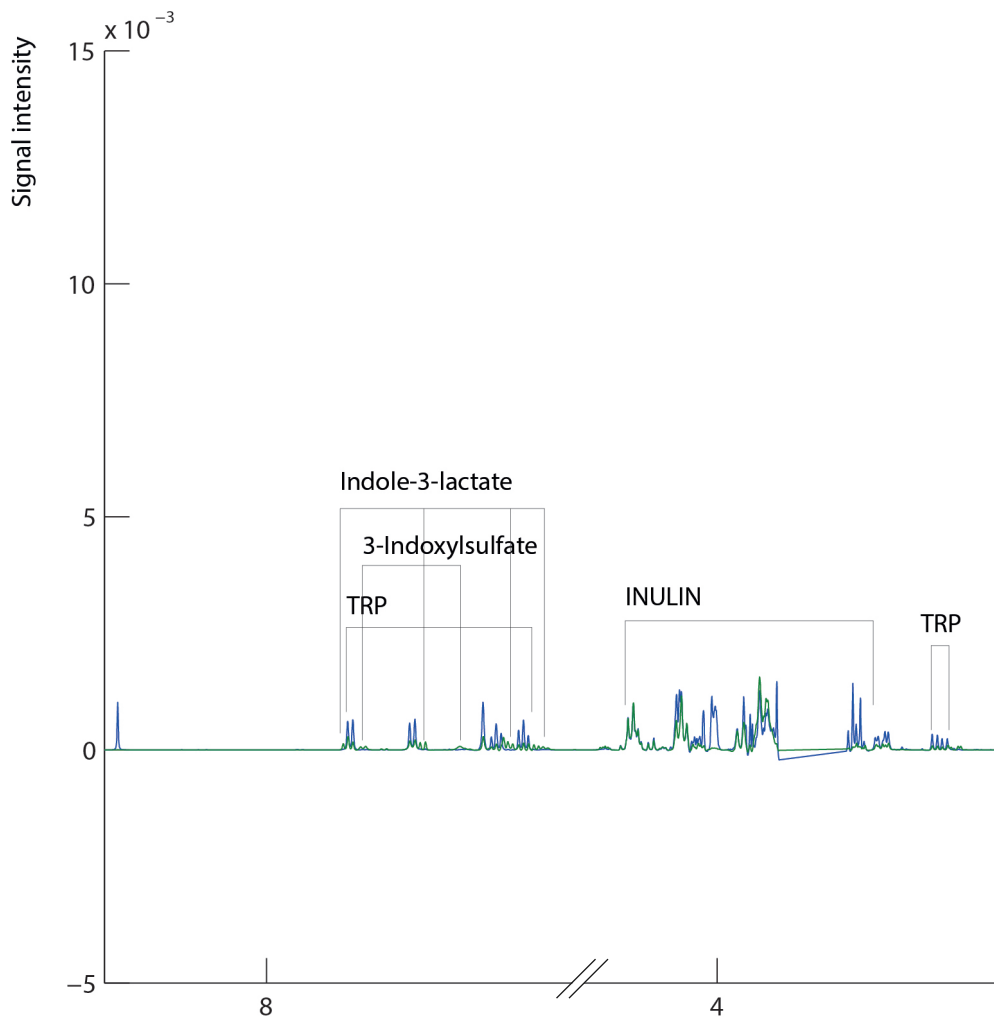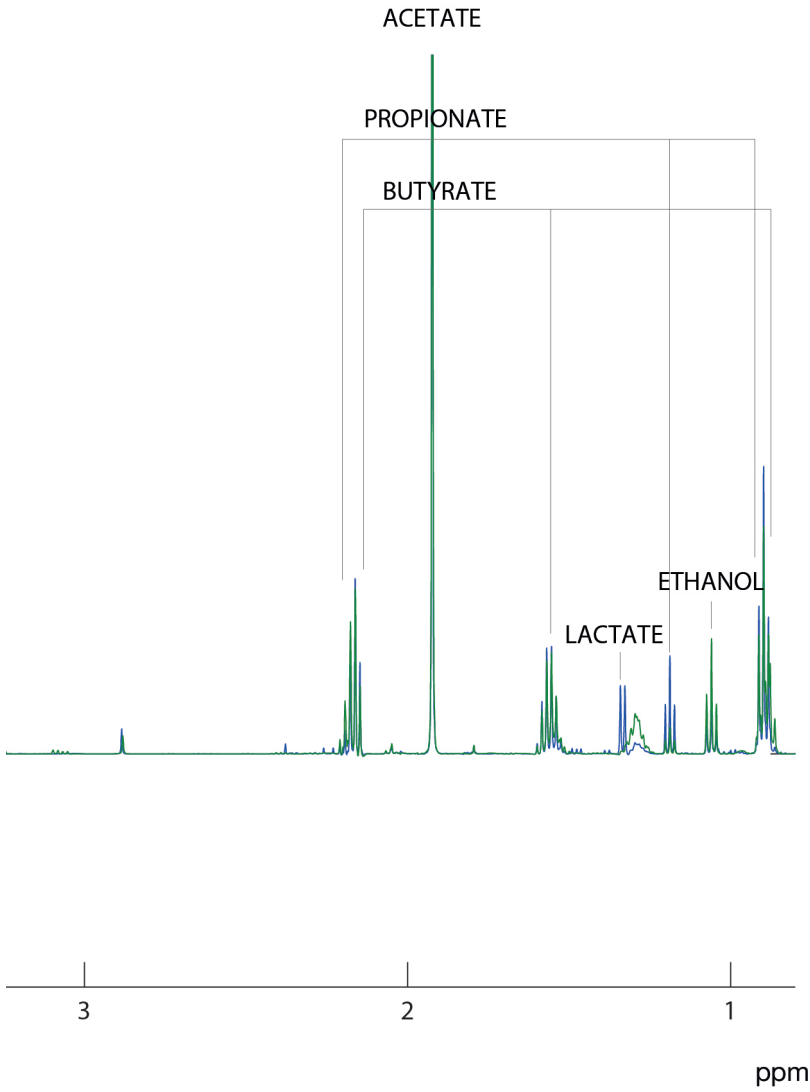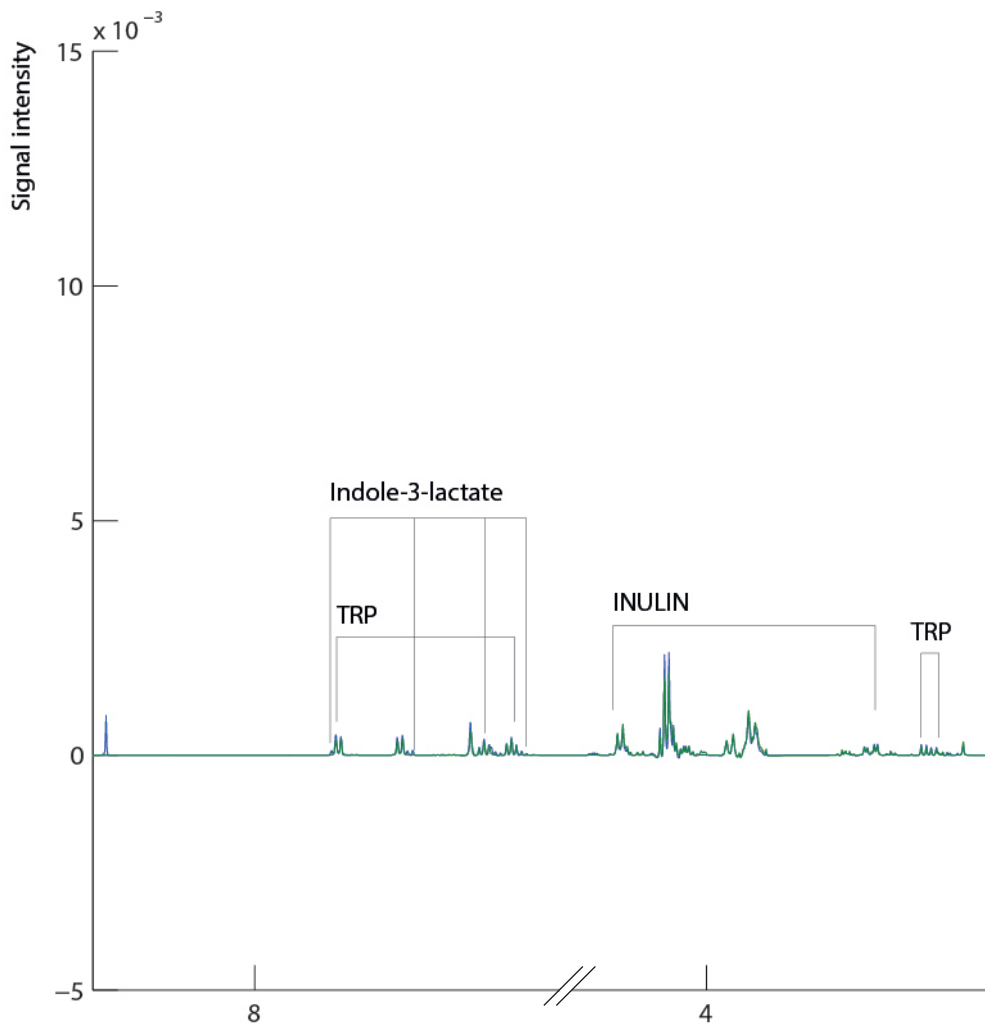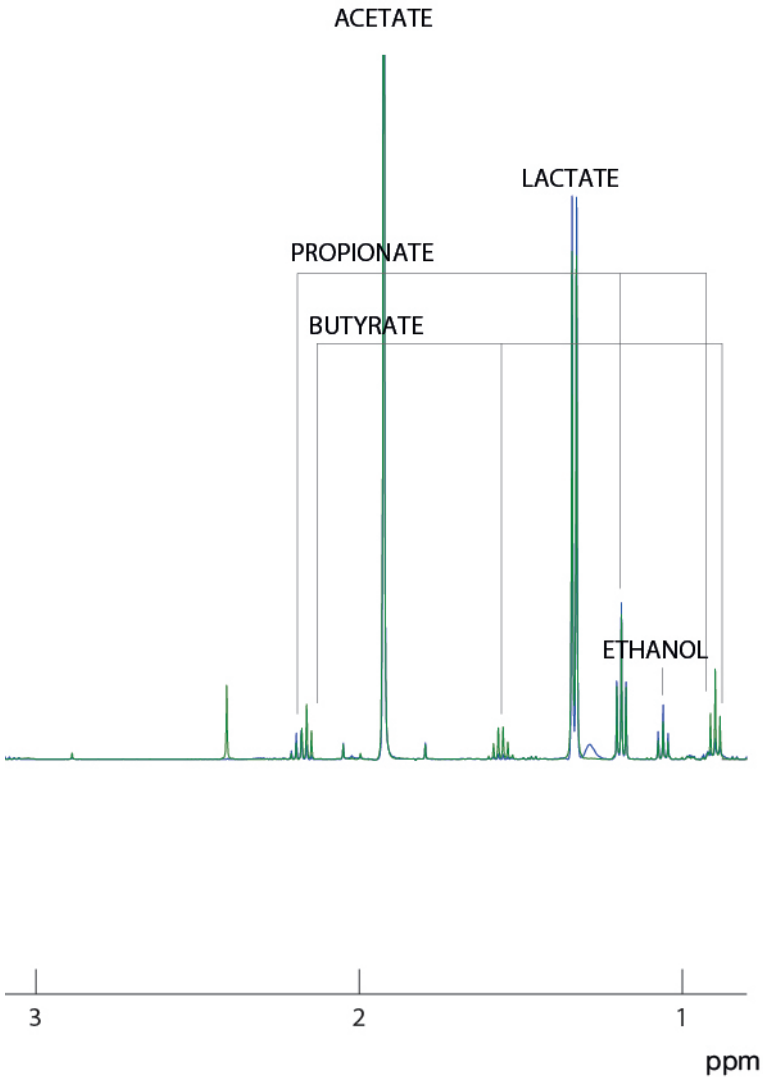
	BLANK	INULIN	TRP	TRP+INULIN	TRP VS TRP+INULIN
R²X	-	0,19	0,16	0,21	0,45
Q²Y	-	0,75	0,44	0,73	0,71
p value	> 0.05	0,01	0,01	0,01	0,01

Tab. 1 Summary of the parameter values (R²X, Q²Y and p-value) describing the OPLS model.

Fig. 6 Alpha-diversity, ranges from time 0 of each treatments and sampling time. Repeated measure for five different human faecal donors (1 - 5), are marked with colored dots.
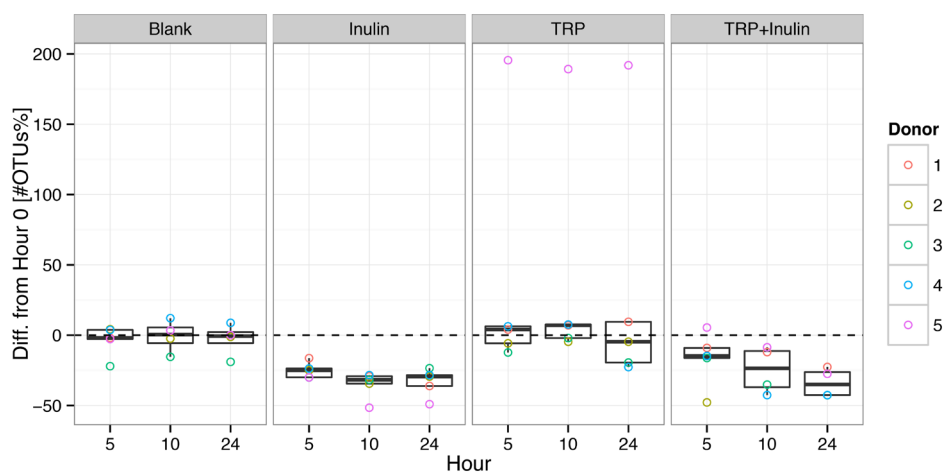
	BLANK	INULIN	TRP
TRP+INULIN	0.0037	0.2364	0.0011
TRP	0.5189	3.0e-05	-
BLANK	-	3.6e-05	-

Tab. 2 Observed OTU: pairwise comparisons using Wilcoxon rank sum test. P value were adjusted by False Discovery Rate (FDR) method.
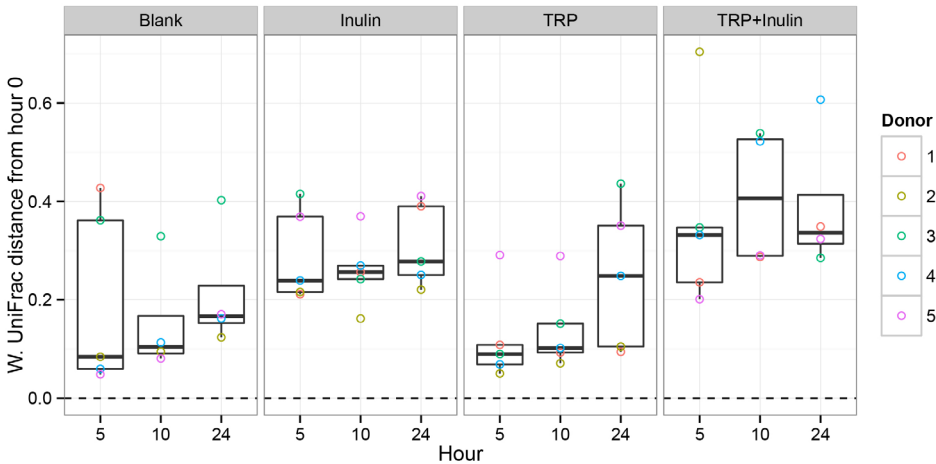
Fig. 7 Weighed UniFrac distances from hour 0 of each time points (5, 10, 24 h). Only intra-donor distances were computed. **137**
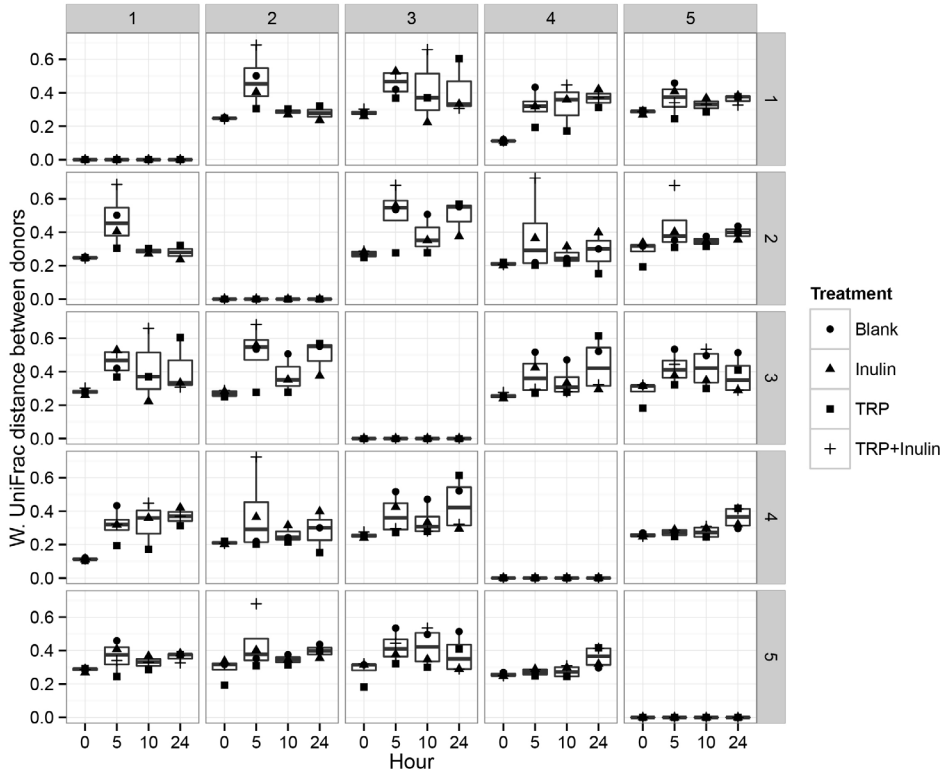
Fig. 8 Weighted unifrac distances between donors (1 - 5), at time 0, 5, 10 and 24 h. The donors are shown to the side of the frame, while treatments are indicated with a shape.
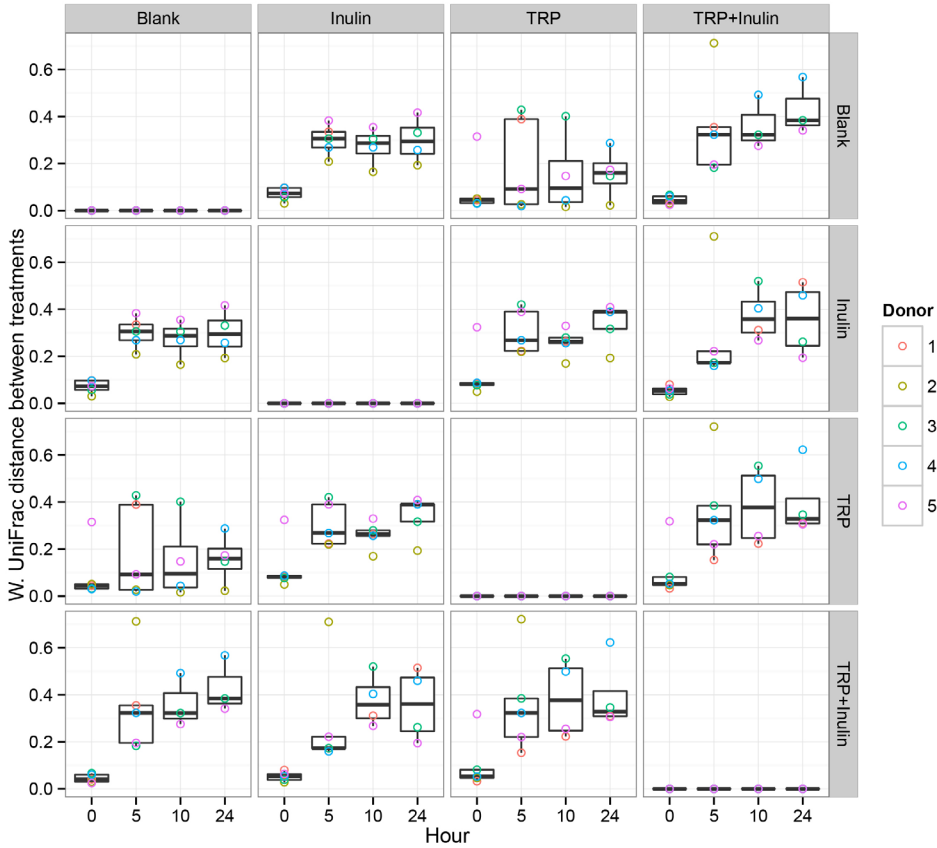
Fig. 9 Weighted unifrac distances between treatments (n = 4), at time 0, 5, 10 and 24 h. The treatments are shown to the side of the frame, while donors (1 - 5) are marked with a color – code dots.
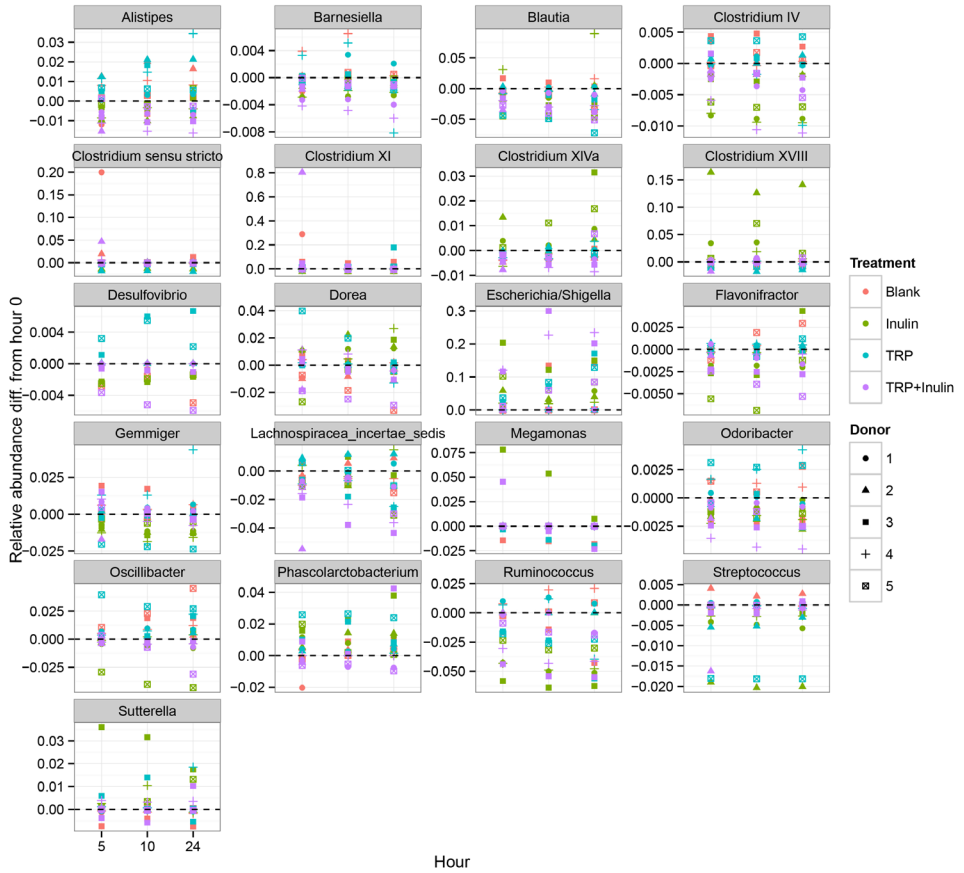
Fig. 10 Relative abundance differences from hour 0, of each time points (5, 10, 24 h), estimated at the genus level. Only genera with at least one significant treatment different from the blank are shown in table 2.

	BACTERIA ENRICHED	BACTERIA DEPLETED
INULIN	Clostridium XIVa	Barnesiella
	Clostridium XVIII	Clostridium IV
	Dorea	Clostridium sensu stricto
	Escherichia/Shigella	Clostridium XI
	Megamonas	Flavonifractor
	Phascolarctobacterium	Gemmiger
	Sutterella	Oscillibacter
		Ruminococcus
TRP	Desulfovibrio	Streptococcus
TRP+INULIN		Clostridium sensu stricto
		Alistipes
		Barnesiella
		Blautia
		Clostridium IV
		Flavonifractor
		Lachnospiracea incertae sedis
		Odoribacter
		Oscillibacter
		Ruminococcus

Tab. 3 Statisticly significant changes at genus level comparing treatmnets and Blank, over 24 h.
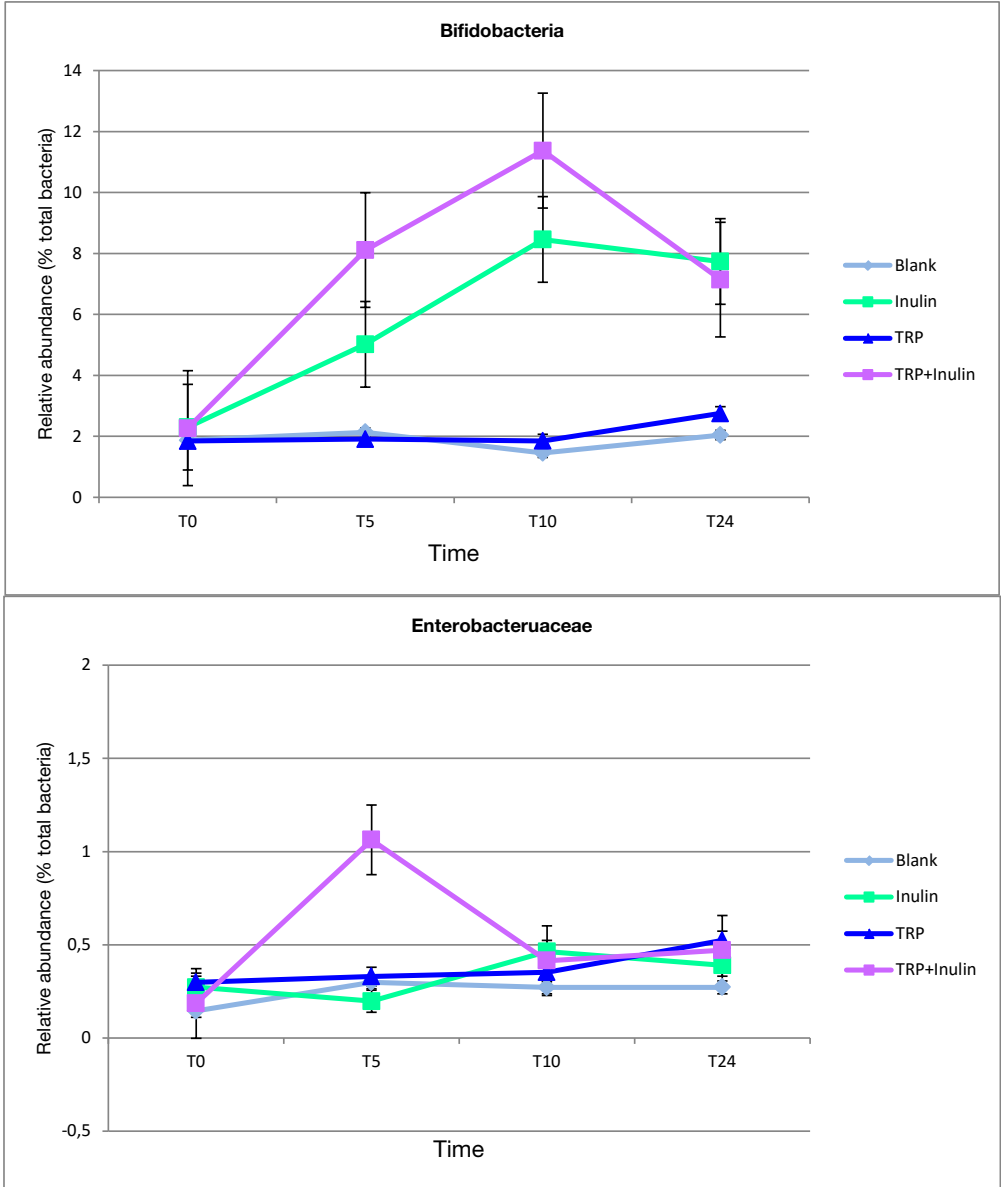
Fig. 11 Distribution of the proportion of bifidobacteria and Enterobacteriaceae in batch cultures assessed, over the time (0, 5, 10, 24 h), by genus-specific 16S rRNA oligonucleotide probes and by relative cell enumeration by FISH combined with flow cytometry. Treatments are colour – coded: basal medium (Blank) - blue, Inulin - green, TRP - pink and TRP+Inulin - purple.

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Measuring gut microbiota metabolism of neuroactive compounds

5. Measuring gut microbiota metabolism of neuroactive compounds

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Abstract

There are many potential signaling mechanisms by which the gut microbiota could influence brain activity, including changes in microbiota-produced signaling molecules, such as neurotransmitters, neuroactive phenolic compounds, short chain fatty acids and amino acid (AA)-metabolites. This current work tested the fermentation of selected neuroactive compounds (dopamine, tyrosine, tryptophan, phenylalanine, glutamate and methionine), using *in vitro* anaerobic batch culture, designed to simulate fermentation in the human distal colon. Using ^{1}H-NMR spectroscopy based metabolic profiling, we measured changes in levels of metabolites associated to the selected neuroactive compound and identified recognized metabolic derivatives which might potentially impact on human physiology through the gut:brain axis. Significant changes over time in bacterial populations were observed using fluorescence in situ hybridization (FISH) with 16S rRNA targeted oligonucleotide probes for bifidobatteria, *Lattobacillus/Enterococcus*, *Bacteroides*, *Desulfuvibrio* and total bacteria. No statistically significant changes, in alpha – or beta-diversity based on OTU obtained from Illumina 16S sequencing over the course of any of the fermentations were observed. However, considerable interindividual variation in response to different fermentation substrates between the faecal microbiota of different donors may account for the lack of statistical significance. Indeed, for

the aromatic amino acids and dopamine, changes in metabolite profiles, appeared to cluster into two distinct groups, suggesting the existence of metabotypes or signature metabolic responses dependent on microbiome structure. Intriguingly, comparative analysis of 16S rRNA community structures, between these two "metabotypes", revealed statistically significant differences, between important groups of gut bacteria, at the family level. Including organism already suspected, of forming alternative community structures, within the human microbiome, specifically the Prevotellaceae and Bacteroidaceae.

Keywords

5.1 Introduction

Nutrients and gut microbiota, can activate physiological pathways and modulate the central nervous system, by acting on flow of neuroactive compounds, between gastrointestinal environment and neural system. Nutrients derived from food and followed digestion by gut microbiota, by regulating the flow of neuroactive compounds, between the gut and neuronal system, could modulate the physiological activities of the central nervous system, including the brain. Similarly, signals from the brain regulate important gut functions, including food intake (Farr et al., 2016), peristalsis (Schemann, 2005), enzyme/chemical secretions (Konturek, 2003) and maybe the enterohepatic circulation of bile acids (Hofmann, 2011). It is estimated that there are between 200 and 600 million neurons in the human enteric nervous system (ENS) (Mayer, 2011), which has led to it being defined, as a second brain. This "gut brain" receives information and sends impulses, records emotional experiences and responds to them. Its nerve cells, are signaling routes and are stimulated by the same brain-neurotransmitters. ENS and enterochromaffin (EC) cells on the gut wall, produces about 10% and 80% of human body's total serotonin, respectively (Resnick and Gray, 1961), while about 50% of the dopamine measured in human body, is produced by mesenteric organs (Eisenhofer et al., 1997). The much-studied gut-brain axis includes the central nervous system (CNS), the neuro-endocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS) and the intestinal microbiota (Grenham et al., 2011). Visceral messages from the gut, can influence brain function and, the possibility of regulating the gut microbiota, is opening up as a tractable therapeutic target, for a host health disorders. There are many potential signaling mechanisms, by which gut microbiota could influence brain activity. Including changes in microbiota-produced signaling molecules, such as short chain fatty acids and ketone bodies (Cummings and Macfarlane, 1991; Kimura et al., 2011), phenolic acids (Russell et al., 2013), steroids (Gérard, 2013), amino acids and their derivatives (Fernstrom, 2012; Dai et al., 2011), catecholamines (Rhee et al., 2009), incretins (Cani, 2015; Cani et al., 2009), mucosal immune mechanisms (Sherman et al., 2015; El Aidy et al., 2014), and enterochromaffin cell-mediated vagal activation (Rhee et al., 2009; Raybould, 2010). Food intake, changes the composition of the blood plasma, which affects the release of amine neurotransmitters. For example, changes in plasma levels of choline, lead to changes in brain levels of the neurotransmitter acetylcholine; tryptophan levels absorbed from the gut, lead to serotonin; and tyrosine, is used to produce catecholamines.

The relationship between precursors and neurotransmitters, in addition to controlling food intake, can of course influence neurotransmission and thus mental and physical performance, with possible physiological and medical implications, which are only now coming to light (Marriott, 1994).

Phenylalanine (PHE), is an essential amino acid and given its hydrophobicity, it is mainly found within protein (Institute of Medicine (U.S.), 1999). Although the metabolic pathway for PHE, is well recognized and many gut bacteria, possess genes involved in this pathway (especially the Proteobacteria), we still do not fully understand either, the biochemistry or microbial ecology of PHE metabolism, by the gut microbiota (Teufel et al., 2010). Phenylalanine plays a key role in the biosynthesis of protein and nonessential amino acid tyrosine.

Tyrosine (TYR), is in turn responsible for the synthesis of monoamine neurotransmitters dopamine, norepinephrine, and epinephrine, as well as the skin pigment, melanin (Daubner et al., 2011). The peptide tyrosine–tyrosine, better know as peptide PYY, plays an important role in regulating appetite and energy metabolism, in modulating gut motility and in reducing harvest of energy, from the diet. Recently, homologous AA sequences, between these neuropeptides and proteins belonging to certain commensal bacteria, suggest that the gut microbiota may have the capability to influence production of gut hormones, like PYY and, therefore regulate human eating behaviour (Samuel et al., 2008; Wynne and Bloom, 2006).

Meals with high concentration of tyrosine, stimulate tyrosine hydroxylation rate, the rate-limiting enzyme for the synthesis of dopamine (Fernstrom, 2012). An alternative dopamine biosynthesis route, involves tyramine oxidization by Cyp2D. Tyramine comes in turn directly from decarboxylation, by aromatic amino acid decarboxylase (AADC), of L-tyrosine, or from phenylethylamine, which derives from decarboxylation of L-phenylalanine, by the same AADC enzyme (Meiser et al., 2013).

Dopamine (DA), is now recognized as an important neurotransmitter and not just as a precursor of epinephrine or, an intermediate of tyrosine degradation. It stimulates exocrine secretions, modulates sodium absorption and gut mucosal blood flow, inhibits gut motility and is protective against gastroduodenal ulcer (Eisenhofer et al., 1997). In the gastrointestinal tract, the extent of dopaminergic innervation, is not well documented and there is still much debate, concerning the relative importance, of neuronal and/or non-neuronal sources, of enteric dopamine.

Trypthophan (TRP), on the other hand, is probably one of the most studied essential amino acids of recent years, is abundantly present in dietary proteins and is the

main precursur of serotonin. Serotonin, is a monoamine neurotransmitter and a key signalling molecule in both the enteric and central nervous systems. Tryptophan hydroxylase (TPH), the rate limiting step, uses tetrahydrobiopterin cofactor to convert tryptophan to 5-hydroxytryptophan (5-HTP); while amino acid decarboxylase, the second enzyme involved in serotonin biosynthesis, converts 5-HTP into serotonin (Nakamura and Hasegawa, 2009). Approximately 4–6% of total amount of TRP, undergoes bacterial degradation, which leads to the release of the metabolites including indole, indican, and indole acid derivatives, which have an important role in host gut immune homeostasis (Keszthelyi et al., 2009; Zelante et al., 2013). The impact of a meal, can indirectly affect brain TRP uptake (and serotonin synthesis), depending on blood concentration of large neutral amino acids (LNAA) and tryptophan, which are transport competitors in the BBB (Fernstrom, 2012).

Glutamate (GLU), is one of the most abundant amino acids, isolated from protein matrices (Giacometti,1979). It is converted to glutamine, by cells lining the intestinal tract. Studies show that glutamate, is an energy source for enterocytes and drives our digestion, sending signals to the stomach, small intestine, and liver. Besides its role in digestive health and function, glutamate is the major excitatory neurotransmitter in the brain. It plays an essential role in learning and memory (Uneyama et al., 2008; Reeds et al., 2000). Decarboxylation of the C-1 carboxylic group of glutamate, leads to the biosynthesis of GABA (gamma-aminobutyric acid), which is the main inhibitory or calming neurotransmitter in adult human brain (Roberts and Frankel, 1950). A build-up of glutamate with a deficiency of GABA, has been linked to serious brain disorders, like autism (Horder et al., 2013), alzheimer's disease (Lanctôt et al., 2004), stiff man syndrome (Manto et al., 2015) and diabetes (Wang et al., 2015).

Methionine (MET), is required during brain development and for mantaining brain function throughout life. Classified as sulfur-containing amino acid (SAA), it supplies sulfur and other compounds required by the body for normal metabolism and growth and it constitutes one of the nine essential amino acids. In addition to dietary sources, methionine is commonly found in sulfated polysaccharides, including mucin and chondroitin sulfate produced endogenously by the gastrointestinal tract (Magee et al., 2000). Methionine was discovered and isolated in 1922 from casein and belongs to a group of compounds called lipotropics, which includes choline, inositol, and betaine (trimethylglycine), all of which assist lipid breakdown in the liver. Physiological increases in brain methionine, are directly related to its serum

concentration and thus, factors regulating methionine levels in the circulation, will also regulate methionine in the brain. Brain methionine appears to be utilized, mainly for protein synthesis and increases the rate of S-adenosyl methionine (SAM) synthesis (Rubin et al., 1974). SAM, is involved in the biosynthesis of serotonin, melatonin, and dopamine in the brain, as well as plays an important role in the immune system, has a protective effect on cellular and mitochondrial membranes and is able to inhibit of inflammatory response (Baldessarini and Kopin, 1966). As for other sulfur-containing amino acids, methionin metabolism in the gut has been shown to be influenced by the presence of specific lactobacilli and other closely related Gram-positive bacteria, including *Bacillus subtilis*, *Lactococcus lactis*, *Streptococcus mutans* (Liu et al., 2012). Methionine and cysteine are involved, as precursors, in the biosynthesis of numerous bioactive compounds, including glutathione, polyamines, and taurine (Brosnan and Brosnan, 2006). In numerous cells within the body, methionine, is metabolized via transamethylation to homocysteine and in the process produces S-adenosylmethionine, which donates an aminopropyl moiety, in the formation of the polyamines, spermidine and spermine. Homocysteine is converted to cysteine via transsulfuration. Cysteine, is one of three constituent amino acids of glutathione (GSH), along with glutamate and glycine. Moreover, cysteine is metabolized to form taurine. Glutathione is a major cellular antioxidant in cells and forms, an important part of the bodies defence mechanism against oxidative stress. Cysteine and taurine can also function as cellular antioxidants. The quantitative significance of these functional endproducts to splanchnic methionine, cysteine and glutamate utilization, is unknown however. Early studies in humans, suggested that splanchnic tissues, are an important site of transsulfuration. Studies in piglets, indicate that first-pass utilization of dietary methionine ranged from 30% to 40% (Bauchart-Thevret et al., 2009).

However, we still know little about what regulates the flux of amino acids or their bioactive derivatives, including neurotransmitters from the gut. In particular, few studies have examined how amino acids are handled by the gut microbiota and what influence amino acids have in turn on gut microbiota composition and metabolic activity. Although direct absorption of amino acids from the distal colon, may not contribute significantly to circulating amino acid concentrations, their metabolism and production of bioactive derivatives, not least neurotransmitters may have a direct effect on the ENS, colonic mucosal metabolism and health, and the microbial ecology of this region of the colon. Here, using *in vitro* anaerobic model of human

distal colon, with pH set at 6.8-7.2 and repeted by five human faecal donor, we measured the influence of different amino acids (dopamine, tyrosine, tryptophan, phenylalanine, glutamate and methionine), on metabolites production and microbial populations, using metabolomics and culture-independent microbiological approaches.

Neuroactive compound stock solution

Dopamine, tyrosine, tryptophan, phenylalanine, glutamate and methionine were added to the basal medium in the form of a stock solution, made in a weakly acid or basic solution (to increase their solubility) to give final concentrations, in the batch culture vessels (200 ml) of 10 mmol/l for the linear compounds (glutamate and methionine), or 5 mmol/l for the aromatic compounds (dopamine, tyrosine, tryptophan and phenylalanine) as described by Smith and Macfarlane (1997).

Fecal batch cultures

Faecal fermentations were conducted using the basal nutrient medium of Smith and Macfarlane, (1997) which contained no added amino acids. The medium, was prepared as from the following formula (g/l): $CaCl_2$ $2H_2O$ – 0.02; $MgSO_4$ $7H_2O$ – 0.02; K_2HPO_4 – 0.08; KH_2PO_4 – 0.08; $NaHCO_3$ – 0.8; NaCl – 0.2; haemin – 0.01; Tween 80 – 2.0. Moreover, trace element solution and vitamin solutions, were added at 8 and 20 ml/l respectively. Mineral solution contained the following amounts (g/l) of salts: $C_2H_3NO_4$ – 1.5; $MgSO_4$ – 3.0; $MnSO_4$ – 0.5; NaCl – 1.0; $FeSO_4$ – 0.1; $CaCl_2$ –0.1; CoC_{12} – 0.1; $ZnSO_4$ – 0.1; $CuSO_4$ – 0.01; $AlK(SO_4)_2$ – 0.01; H_3BO_3 – 0.01; Na_2MoO_4 – 0.01. While Vitamin solution contained (mg/l): biotin – 2.0; folic acid – 2.0; pyridoxine hydrocgloride – 10.0; riboflavin – 5.0; thiamine – 5.0; nicotinic acid – 5; pantothenic acid – 5.0; vitamin B_{12} – 0.1; p-aminobenzoic acid – 5.0; thioctic acid – 5.0 (Wolin et al., 1963). All chemicals were purchased from Sigma. The final, working volume of all fermentations was 150 ml. Anaerobic conditions were maintained by O_2-free N_2 (15 ml/min) flow overnight. Temperature was held at 37 °C using a circulating water bath, and pH was controlled between 6.8 and 7.2 using an automated pH FerMac 260 controller (Gloucester, England-GL208JH, United Kingdom), which added acid and alkali as required (0.5 M HCl and 0.5 M NaOH). Five healthy human donors (2 male, 3 female), who had taken no prebiotics, probiotics, or antibiotics within 3 months of the study were recruited to provide faecal samples as inocula. Faecal inocula (10% w/v) were prepared using pre-reduced phosphate-buffered saline (PBS 0,1 mol/l) and used to inoculate each fermentation vessel to a final concentration of 1% faeces w/v. The test fermentations were basal medium (Blank), Inulin at 1% (w/v) (Cargill Oliggo-Fiber® inulin DS2 5LB), as positive control, TRP (Sigma-Aldrich, Germany) (5 mmol/l) and TRP (5 mmol/l) with Inulin (1% w/v). Each fermentation was conducted once with faecal inocula from each of the 5 faecal donors (n = 5). Batch cultures were run for 24 hour and samples

obtained from each vessel at 0, 5, 10, 24 h, were centrifuged at 13 000 rpm, where the supernatants and pellets were stored at -80 °C for metabolomics and metagenomics analysis respectively. Samples for FISH were prepared as described below and stored at -20oC.

Fluorescence In Situ Hybridization (FISH)

Sample fixing and hybridization were performed as previously described (Rochet et al., 2004; Saulnier et al., 2008). Briefly, 375 µl of culture fluid was diluted in 1125 µl of 4% (w/v) paraformaldehyde and fixed overnight at 4 °C. Bacterial cells were pelleted by centrifugation at 13 000 rpm for 5 min and washed twice in filter-sterilized PBS. Cells were then resuspended in 150 µl PBS and 150 µl ethanol. Samples were stored at −20 °C for a minimum of 3 h before further processing. The probes used were as follows: Eub 338 (I, II, III) – specific for the total bacteria (Daims et al., 1999); Bif 164 – specific for *Bifidobacterium* (Langendijk et al., 1995); Bac 303 – specific for the *Bacteroides* and *Prevotella* group (Manz et al., 1996); Lab 158 – specific for *Lactobacillus* and *Enterococcus* (Harmsen et al., 1999); DSV 687 – for *Desulfovibrionales* and *Desulfomonales* (Devereux et al., 1992). Probes sequences and their respective hybridization temperatures were previously described (Saulnier et al., 2008). To permeabilize the cells for use with probe Lab158, samples were treated with 50 µl of lysozyme (1 mg/ml in 100 mM TrisHCl, pH 8.0) at 37°C for 15 min before being washed (2–3 s) in water and were finally dehydrated in the ethanol series (50%, 80% and 96% v/v ethanol, 3 min each). A probe/hybridization buffer mixture (5 ng/5 µl of a 50 ng/µl probe in stock solution plus 45 µl of hybridization buffer), was applied onto the surface of each well. Hybridizations were performed for 4 h in an oven. For the washing step, slides were placed in 50 ml of wash buffer containing 20 µl of 4, 6-diamidino-2-phenylindole di-hydrochloride (DAPI; 50 ng/µl; Sigma, UK) for 15 min. They were then washed (2–3 s) in ice-cold water and dried under a stream of compressed air. Five µl of antifade reagent (polyvinyl alcohol mounting medium, with DABCOTM antifading, Sigma), was added to each well and a coverslip was applied. Slides were stored in the dark at 4°C (for a maximum of 3 days) until cells were counted under a microscope. DAPI slides were visualized with the aid of a DM 400 filter and probe slides with the aid of a DM 575 filter.

NMR spectra acquisition and processing

Batch culture samples (400 µl) were combined with 200 µl of phosphate buffer (pH 7.4; 100 % D_2O) containing 1 mM of the internal standard, TSP (3-trimethylsilyl-1-[2,2,3,3-2H4] propionate). Samples were mixed by vortex and centrifuged (10 000 g for 10 min) before transfer to a 5 mm diameter NMR tube. Spectroscopic analysis was carried out on a 500 MHz Bruker NMR spectrometer. Standard one dimensional ^{1}H-NMR spectra of the batch culture samples were acquired with water, TSP and basal medium peacks suppression using a standard pulse sequence. For each sample, 8 dummy scans were followed by 128 scans and collected into 64 000 data points. A spectral widths of 20 ppm was used. Chemical shifts in the spectra were referenced to the TSP singlet at 0.0 ppm. Spectra were manually phased and corrected for baseline distortions. The ^{1}H-NMR spectra (0.2 to 10.0 ppm) were digitized into consecutive integrated spectral regions (~20 000) of equal widths (0.00055 ppm). The regions containing the residual signal from water (4.50 to 5.00 ppm) and the large resonance arising from polyethylene glycol (3.70 ppm) were removed to minimize the effects of baseline distortion. Each spectrum was then normalized using a total option. Multivariate modeling, including Principal Component Analysis (PCA) and Orthogonal projection to latent structes (OPLS) analysis, was performed in MatLab (The Mathworks, Inc., Natwick, MA) using scripts provided by Korrigan Sciences Ltd., United Kingdom. The predictive performance of the model (Q^2Y) was calculated using a seven-fold cross validation approach. Model validity was established by permutation (1000 permutations) and the results are given as p values.

Metabolite identification was performed using HMDB database (http://www.hmdb.ca/), KEGG (Kyoto Encyclopedia of Genes and Genomes), Chenomix profiler 8.1 software (Chenomx Inc., Edmonton, Canada).

DNA extraction

The genomic DNA extraction was obtained by FastDNA™ SPIN Kit for Feces (MP Biomedicals, Santa Ana,CA), following the manufacturer's protocols. The final product was 100 µl of application-ready DNA. Nucleic acid concentration and purity were measured using the Nanodrop 8000 spectrophotometer. Nucleic acid concentration and purity were measured using the Nanodrop 8000 spectrophotometer. DNA concentrations were in the range 25,77 ± 15,97 ng/µL.

16S rRNA amplicon library preparation and MiSeq sequencing

Using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') (Klindworth et al. 2013) and 806R (5' GACTACNVGGGTWTCTAATCC 3') (Apprill et al. 2015), with overhang Illumina adapters, total genomic DNA was subjected to PCR amplification, by targeting a ~460-bp fragment of the 16S rRNA variable region V3-V4. PCR amplification of each sample, was carried out using 25 µl reactions with 1 µM of each primer. Specifically 12.5 µl of 2x KAPA HiFi HotStart ReadyMix, 5 µl forward primer, 5 µl reverse primer, were used in combination with 2.5 µl of template DNA (5 ng/ul). All PCR amplification was carried out, using a GeneAmp PCR System 9700 (Thermo Fisher Scientific) and the following steps: melting step – 95 °C for 3 minutes (one cycle); annealing step – 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds (25 cycles); extension step – 72 °C for 5 minutes (1 cycle). The PCR products were checked on 1.5 % agarose gel and cleaned from free primers and primer dimer, using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. Subsequently, dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina), were attached by 7 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina). The final libraries, after purification by the Agencourt AMPure XP system (Beckman), were analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA) and quantified, using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific), by the Synergy2 microplate reader (Biotek). Finally, all the libraries were pooled in an equimolar way, in a final amplicon library and analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Barcoded library were sequenced on an llumina® MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18).

Data analysis

The sequences were assigned to samples, according to sample-specific barcodes. This allowed to collect FASTQ formatted files. Raw data fastq files were merged using micca-mergepairs tool. Raw data will be submitted to the http://www.ebi.ac.uk/ena/submit/sequence-submission. Reads were processed using the MICCA pipeline (version 1.0, http://micca.org/) (Albanese et al., 2015). Forward and reverse primers trimming, were performed using micca trim (parameters -w CCTACGG-GNGGCWGCAG -r GACTACNVGGGTWTCTAATCC -W -c). Quality filtering, were

performed using micca filter (parameters -e 0.5 -m 400 -t), truncating reads shorter than 400 nt and discarding reads, with an expected error (EE) rate > 0.5%. De-novo sequence clustering, chimera filtering and taxonomy assignment, were performed by micca otu (parameters -d 0.97 -c): operational taxonomic units (OTU) were assigned by clustering the sequences, with a threshold of 97% pairwise identity, and their representative sequences were classified, using micca classify with the RDP classifier version 2.8 (Wang et al., 2007). Template-guided multiple sequence alignment (MSA), was performed using micca msa (parameters -m nast), against the multiple alignment of the Greengenes (DeSantis et al., 2006) database (release 13_05) clustered at 97% similarity. Finally, phylogenetic tree was inferred, using micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013). micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013).

5.3 Results

NMR-based metabolites profiling data

Phenylalanine (PHE). During PHE fermentation, two individuals-class were observed, by PC2 (with 15% of the total variance within the data) and described as group A = donor 1 and 2; group B = donor 3, 4, 5. Wide spread of samples scores were obtained only for group A , while more closed was the trajectory of group B (Fig. 1a). Fermentation of phenylalanine, yields mainly phenylacetate after 24 h. Other compounds identified included hydroxypropyl derivatives, like hydroxypropyl pyruvate (HPPA), propionate, butyrate, acetate, valerate and isovalerate (all statistically significant, except for the last one) (Fig. 1b).

Tyrosine (TYR). OPLS analysis during the time, shows a reduction of tyrosine concentration and an increase of acetate as the main product of fermentation (Fig. 2b). The lack of recognition of other secondary metabolites is confirmed by the PCA, which sees a shorter path along time, compared with DA (Fig. 2a). Other metabolites produced by human colonic bacteria, but not statistically significant, are: m-hydroxyphenyl propionyl sulfate (m-HPPS), p-hydroxyphenylacetate (4-HPA), p-hydroxyphenylacetate (2-HPA), phenol, in the aromatic region and cresol, propionate, butyrate, isovalerate, acetate and ethanol, in the aliphatic region, as shown in figure 2b.

Dopamine (DA). PCA score plot of ^{1}H-NMR spectra of DA *in vitro* fermentation (Fig. 3a), shows a logical evolutive trend, over the time, of metabolite profile originated from each donors. However, samples at time 0 have a dissimilarity origin and class-separation by PC1 for groups A and B, were observed too. The corresponding loading plots (Fig. 3b), shows that the donors are distinguishable by independent metabolic patterns, but follow the same trend, during the fermentation time. The values of the parameters (R^2X, Q^2Y and permutation test) describing the OPLS model are statistically significant, as reported in table 1. Metabolites identified after 24 h from DA fermentation are SCFA, such as propionate, acetate, butyrate and isovalerate. Other compounds identified, but not statistically significant are: phenylacetate, m-hydroxyphenylacetate (3-HPA), o-hydroxyphenylacetate (2-HPA), p-hydroxyphenylacetate (4-HPA). Significant microbial metabolism was hypnotized by depletion of initial DA concentrations during the time. Dopamine spectra, in the region of δ 6.9–3.0, show a different profile respect to the chemical shift reported from HMBC database. Proton NMR chemical shift found from by us includes doublet of doublet at δ 3.06, doublet of doublet at δ 3.16, doublet of doublet at δ 3.93,

doublet at δ 6.74, singlet at δ 6.84, and doublet at δ 6.91. While, triplet at δ 2.88, triplet at δ 3.23, doublet at δ 6.76, singlet at δ 6.87, and doublet at δ 6.91 is reported in HMBC database.

Tryptophan (TRP). The first PC explain 53% of the total variance within the data, showing a separation between samples at time 0 and 24, but with independent metabolic trend for each donor (Fig. 4a). Two donor groups (A and B), as from DA results, clusterized respects to PC2, which explains 13% of the variation across all samples. Tryptophan, as expected, was converted to Indole, indole-3-acetate, indoleacetate (IAA), propionate, butyrate and acetate (Fig. 4b). Nevertheless, production of indole, propionate, butyrate and acetate showed a strong statistical significance over 24 h, corresponding with a significant depletion of TRP (Fig. 4b).

Glutamate (GLU). Differently from the fermentation of all aromatic AA and DA, the principal analysis obtained from GLU fermentation, shows a clear separation between time 0 and 24 of all donors (PC1 with 69% of variation). Moreover a dissimilarity responses for only donor 5 was observed (PC2 with 10% of variation), compare with the other donors (Fig. 5a). As shown in figure 5b, SCFA, as propionate, butyrate and acetate have been significantly produced, while no N-containing compounds were observed after 24 h.

Methionine (MET). Changes in metabolites over time for the MET fermentations are visualized in PCA (Figure 6a). A wide spread of score was observed, despite ^{1}H-NMR spectral profiling was not possible for faecal microbiota derived from donors 4 and 5 because of poor spectral resolution, and these data were excluded from analyses. The metabolite profiles for the remaining three faecal microbiota (D1-3) behaved in a similar manner, clustering closely together (Fig. 6a). 24 h-fermentation of methionine resulted in the formation of metabolites including phenylalanine, tyrosine, 4-hydroxyphenylacetate (4-HPA), scyllo-inositol, 5-aminolevulinate, propionate, butyrate, propionate, acetate, alanine, leucine and valine. Despite this, only few of them were recognised to make a significant contribution towards fermentation, as SCFA propionate and butyrate, BCAA leucine and valine and scyllo-inositol (Fig. 6b).

Faecal microbiota composition and community structure.

A total of 60 samples were collected from four treatments (Blank, DA, TYR, TRP, PHE, GLU, MET), two sampling time (0 and 24 hours) and five healthy human faecal

donors, for 16R rRNA gene community profiling using the Illumina sequencing platform. Using a distance-based similarity of ≥ 97% for operational taxonomic units (OTU) assignment, a total of 12 303 OTU were identified. Taxonomic alpha diversity (accounting for relative abundances of OTU) shows no significant differences between treatments (Fig. 7). The same result was obtained calculating weighted UniFrac distance, related to time 0, of each treatments, despite all of them are significantly distant from time 0 (Fig. 9).

Exempt for methionine, the treatments appeared reduce the biodiversity, from the start of fermentation to the end. However, considerable variations in response, were observed between donors and there were no statistically significant overall change in alpha-diversity. Similarly, for beta-diversity, measuring how different types or bacterial sequence are distributed amongst different fermentations, no statistically significant changes were observed after 24 h fermentation compared to 0 hours, probably due to large variation in response to amino acid/dopamine fermentations of the microbiota, form the 5 different faecal donors (Fig. 8). The weighted UniFrac analysis of distance between each donor, before (0 h) and after (24 h) fermentation, is shown in figure 9. Although, clear differences between 0 h and 24 h can be seen for each substrate, these differences were not statistically significant, compared to the blank/control, due to inter-individual variation. In contrast, there were consistent differences between bacterial counts, obtained by FISH for total bacteria, TRP from T10-T24 (p = 0,04), PHE from T0-T10 (p = 0,002) and Blank from T0-T10 (p = 0,002); bifidobacteria, only for TYR from T0-T10 (p = 0,016), Bacteroides/Prevotella, GLU from T5-T24 (p= 0,010), Blank from T5-T24 (p = 0,041) and MET from T0-T5 (p = 0,016); lactobacilli/enterococci, PHE from T0-T24 (p = 0,031) and TYR from T0-T24 (p = 0,039) and finally Desulfobacteraceae, with only GLU from T5-T24 (p = 0,039) (Fig. 12). However, clear trends in response of given groups of bacteria, e.g. between similar substrates, like the aromatic amino acids, were not evident and high interindividual variation in bacterial counts, as illustrated by large standard errors around the mean, hinder useful interpretation of the data.

In order to visualize better the interindividual variation in microbiota composition between each donor, Figure 9 shows the weighted UniFrac distance between microbiota of faecal donors. Donors 1 and 2, although presenting with different microbiota compositions at time 0, showed very similar response to the different substrates at time 24 hours. The microbiota of these two donors changed in a similar manner at time 24 h. Similarly, the change in weighted UniFrac distances of the

microbiota of donors 3, 4 and 5, appeared to behave in a more similar manner, one another than to the microbiota of donors 1 and 2. However, inter-substrate differences meant these trends were not statistically significant.

These trends however, do appear to support the clear separation obtained with respect to the second principal component in the PCA of ^{1}H NMR metabolomic fingerprint for the aromatic amino acids and dopamine (Fig. 1a, 2a, 3a and 4a). Dividing these donors according to NMR "metabotype" or differences in aromatic amino acid metabolic profiles we formed two groups, Group A (D1 and D2) and Group B (D3, D4 and D5). Using one-way ANOSIM, Bray-Curtis distance measure, 9999 permutations, and pair-wise comparisons, with Bonferroni correction (using PAST software, http://nhm2.uio.no/norlex/past/download.html), we observed a significant difference between Group A and B in terms of microbiota relative abundance. Examining individual differences in relative abundance at the family level Desulfovibrionaceae, Enterobacteriaceae, Bifidobacteriaceae, Prevotellaceae, Bacteroidaceae, Hyphomicrobiaceae, Victivallaceae, Lachnospiraceae, Peptococcaceae 1, Acidaminococcaceae, Actinomycetaceae, Porphyromonadaceae, were all statistically different (P value < 0.01) as was Comamonadaceae (P = 0.02) (Table 3).

5.4 Discussion

Unexpected mechanisms are involved in the complex two-way communication that links the brain and intestine. Despite considerable progress for identify and follow the microbial metabolic pathways and for characterize their roles upon human physiology, we are only at the beginning of them understanding. The semi-permeability of the enteric epithelium, allows the huge interchange of metabolite, many of whom come from dependent metabolism of the microbiota, that inhabits the last part of the large bowel. Interactions within the supraorganism (mibrobioma-host), are largely caused by the circulating level of neurotransmitters and other neuroactive substances. Many of these substances come directly from the diet, or indirectly by the bacterial metabolism in the intestine. Through studies on the metabolism of these compounds (e.g. dopamine, tryptophan and glutamate), we shall be able to predict the formation and thus bioavailability levels, of secondary metabolites, able to modulate peripheral and central neuronal functions. To this end, we used an *in vivo* model, capable of simulating the environment of the human colon (37 °C, pH 6.8–7.2, basal medium mimic–intestinal content) and thanks to healthy fecal donors of both genders, we carried out 5 different fermentations containing the same, selected, substrates (DA, TYR, TRP, PHE, GLU and MET). Through the metabolic (by ^{1}H NMR analysis), and microbial (by FISH and Illumina sequencing) characterizations, we have tried to shed light on the dynamics that develop in the colon, during fermentation of these biologically active compounds.

The mainly phenylalanine degradation product, is phenylacetate. Aerobic phenylacetate-metabolizing bacteria pathway, is still under scrutiny, due to accumulation of active intermediate (e.g. CoA thioesters, ring epoxy, and possibly oxepin intermediates as well as a hydrolytic ring cleavage), of phenylacetate, which could be toxic for the host. It was hypotized a connection between expression of genes leads, with reactive pathway intermediates of phenylacetate and virulence of some bacteria, such as *Burkholderia cenocepacia* and *Mycobacterium abscessus*. These microorganisms can cause serious infections and immune responses, compromising the life of the host (Teufel et al., 2010). Many gut bacteria are involved in this pathway, such as members of Proteobacteria, (e.g *Escherichia coli*, *Pseudomonas putida*), pathogens (e.g. *Bordetella pertussis*, *Shigella dysenteriae*), or opportunistic pathogens (e.g. *Burkholderia cenocepacia*), as well as, gram positive (e.g. *Rhodococcus spp.*) and members of the *Deinococcus/Thermus* phylum (Teufel et al., 2010).

Amino acid tyrosine, is the precursor for the catecholamines (CA) (dopamine, epine-

phrine and norepinephrine) biosynthesis, which take their name from the catechol moiety coming from the tyrosine. Local tyrosine concentrations, after food ingestion, could stimulate DOPA synthesis (Fernstrom and Faller, 1978). Nevertheless, no catecholamine production was recorded by our in vitro gut model; other in vivo studies instead have measured, in a biologically active- free form, an high level of dopamine at the cecum level (the higher among the three CA) (Asano et al., 2012). On the other hand, secondary metabolites, without statistical significances, were identified: m-HPPS, phenol, cresol, 4 and 2-HPA. These compounds are recognized as discriminating key metabolites of human healthy gut and reflects the alterations on gut microbial co-metabolic activities (Garcia-Perez et al., 2012; Windey et al., 2012; Bone et al., 1976). *Clostridium* species, has been defined as bacteria-derived alpha-glucuronidase activity (GUS), as necessary for the production of free CA in the large bowel (Asano et al., 2012). Various species of *Clostridium*, *Bifidobacterium*, and *Bacteroides fragilis* has been attributed to the production of cresols from tyrosine; while other bacteria such as *E. coli* are associated with phenol production (Nicholson et al., 2012).

Sample preparation, with change of pH and/or interaction between chemicals, may affect chemical shifts and the mathematical alignment of peaks detected by NMR (Defernez and Colquhoun, 2003), as happened for dopamine (Fig. 3b). In our case dopamine stock solution, was prepared by dissolving DA powder (Sigma Aldrich), in HCl 1N, to increase the solubility of the compound. This preparation step perhaps, caused this change in the DA spectra. The general distribution of proton chemical shifts, associated to the main compounds identified in this study, are reported in table 1. Spread of dopamine samples score at time 0, suggests two loose clusters of metabolite profiles based on faecal donor at the beginning of fermentation. Metabolites produced by colonic microbiota are also subject to interindividual variation, as observed for all treatments after 24 h. Propionate, butyrate, and acetate are the most important SCFA, which were identified, as key compounds produced, after 24 h DA fermentation. In the last years, SCFA have been shown to have neuroactive properties. SCFA are able to activate intestinal gluconeogenesis (IGN gene) expression, via a gut-brain neural circuit and through the activation of the fatty acid receptor FFAR3 (De Vadder et al., 2014) and/or their blood circulation, are able to acting on the brain regions, where activate G-protein-coupled receptors (GPCRs), which are linked to the regulation of metabolism, inflammation, but also pathological conditions (den Besten et al., 2013).

Tryptophan feed intake, may be involved in mood and behavior regulations (Jenkins et al., 2016), and in the modulation of immune response (Moffett and Namboodiri, 2003). In the gastrointestinal tract, tryptophan can takes several metabolic pathways as, synthesis of protein or serotonin, or bacterial degradation, even if kynurenine pathway, remains the preferred route (Palego et al., 2016). Bacterial tryptophanase enzyme, is the principal responsable of tryptophan catabolism in the gut (DeMoss and Moser, 1969). Indole and their metabolites, who are the main metabolites from this metabolic activity, would seem to be indirectly linked to the kynurenine pathway, and able to modify neuronal function and animal behaviour by interacting with voltage-dependent Na^+ channels (Moroni, 1999).

Compared to just discussed aromatic compounds, the main raprasentative metabolites originated from the glutamate and methionine fermentations are SCFA, propionate, butyrate and acetate. Acetate compare with the other SCFA, shows a less change during the fermentation process of methionine. The absence of detection of nitrogen compounds, afer 24 h fermentation of glutamate and methionine, may be due to their incorporated into microbial biomass without accumulating in the medium, or more likely formed small, with similar NMR chemical shifts like water (Fig. 5b). However this result, despite require further confirmation, it remains in line with preceding literature reported that no dietary glutamate appeared in the portal circulation after protein (or other source of glutamate) intake (Bauchart-Thevret et al., 2013; Reeds et al., 1996). Glutamate, which about 95% derives from food intake, is a metabolic intermediates and key links between carbon (from both carbohydrate and protein surces) and nitrogen metabolism and it is used as energy source by the enterocytes of the intestinal mucosa (Beyreuther et al., 2007). Furthermore methionine, a sulfur-containing amino acid, is a dietary indispensable amino acid, which leads to the formation of scyllo-inositol (Fig. 5b), one of the promising inositol isomers tested, as a treatment in Alzheimer's disease (AD) (Choi et al., 2010).

The weighted UniFrac analysis of distance for microbiota from each donor before (0 h) and after (24 h) fermentation of the different substrates is shown in figure 9. Although, clear differences between 0 h and 24 h can be seen for each substrate, these differences were not statistically significant, compared to the Blank, due to interindividual variation. All the aromatic amino acid fermentations and DA, lead to different metabolite patterns (more or less evident), formed depending on the gut microbiota. Unexpectedly PCA analysis, as well as weighted UniFrac distance between donors, showed a distinct donor-dependent clustering, indicating the

effect of donor upon amino acids and dopamine fermentations. Figure 11a,b show the distribution of the main families in each sample and the % value calculated from the global community composition. It can be seen a certain level of between-sample variability, which gives rise to two groups (A and B). The main dissimilarity its rapresents by the Prevotellaceae family, which is dominant in the group A (Fig. 11a), while appears almost missing in the second one (Fig. 11b). This family, belonging to the phylum Bacteroidetes, contains carbohydrate-fermenting and H_2-producing bacteria implicated in energy production (Kovatcheva-Datchary et al., 2009). On the other hand the group B, shows richness in terms of relative abundance of Bacteroidaceae, which growth well in the neutral pH of colonic environment, compared to the low pH required for the growth of both bifidobacteria and lactobacilli (Dalland and Hofstad, 1974). The family Hyphomicrobiaceae, with a lower abundance, it is more represented in the group B as well. It was associated to omnivorous feeding in non invasive fish model by metabolic and microbial analysis after nutritional intervention (Asakura et al., 2014). A recent work identified, as member of this family, the genera *Gemmiger*, which shows high sequence homology of 16S rRNA gene, with some of the bacteria included in Ruminococcaceae family (Firmicutes), that could reveal an incorrect taxonomic assignment (Garrity et al., 2015). The Lachnospiraceae family, which was closely related to the genus *Blautia*, and it is one of the most abundant human fecal bacterial phylotype (Newton et al., 2011). It appears 6% more representative in the second group. Lachnospiraceae are able to produce butyric acid, and then, through environmental changes (e.g. pH, availables nutrients), might influence the evolution of closely related organisms (Meehan and Beiko, 2014).

5.5 Conclusions

Metabolites arising from the microbial metabolism in the intestine, have been recently recognized to have a key role in the inter-communication between brains (located in the head and belly). The changes in these processes are underlie of important brain disorders, and it is increasingly clear that the answer to some of most feared brain disorders, may actually reside outside of the brain. Diet is an often overwhelming decision that people face every meal of every day. Intestinal bacteria modulate several metabolic reactions of the host and through digestion and fermentation of food metabolize and in turn synthesize many neurotransmitters and neuromodulators. This metabolites released through gut lumen may induce epithelial cells and modulate neural signalling acting directly on primary afferent axons or indirectly within the enteric nervous system. Physiologic factors that influence brain pools of these amino acids, notably diet, influence their rates of conversion to neurotransmitter products, with functional consequences. Using NMR based metabonomics, as well as 16S metagenomic sequencing, with multivariate data analysis, this study want to remark the importance of omics methodologies to examining questions related to gut microbiota metabolism and their roles in the host (pato) physiology. Although this is an *in vitro* study, with its natural limitations, it offered food for thought on how gut microbioma could be involved in the metabolism and production of several compounds, which could be involved in several pathwey. Furthermore the division, both in terms of metabolomic and metagenomic profile, of 5 subjects here examined, revealed an association between specific metabolic profile and gut microbiota composition. Future studies should be conducted to confirm the possibility to classify "metabotype", shared between subjects, able to respond in a manner similar to the metabolic processes in the colon.

Fig. 1a Principal component analysis (PCA) score plot of PHE *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
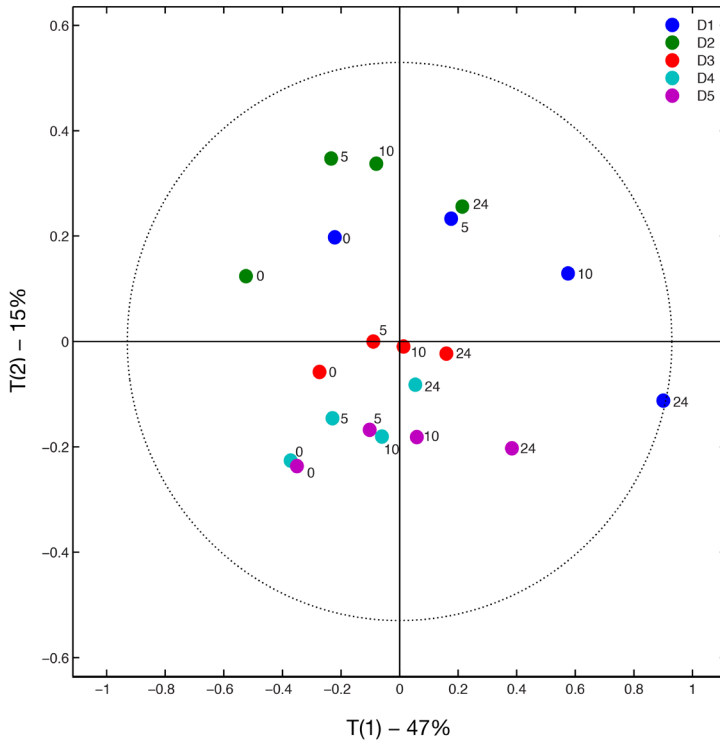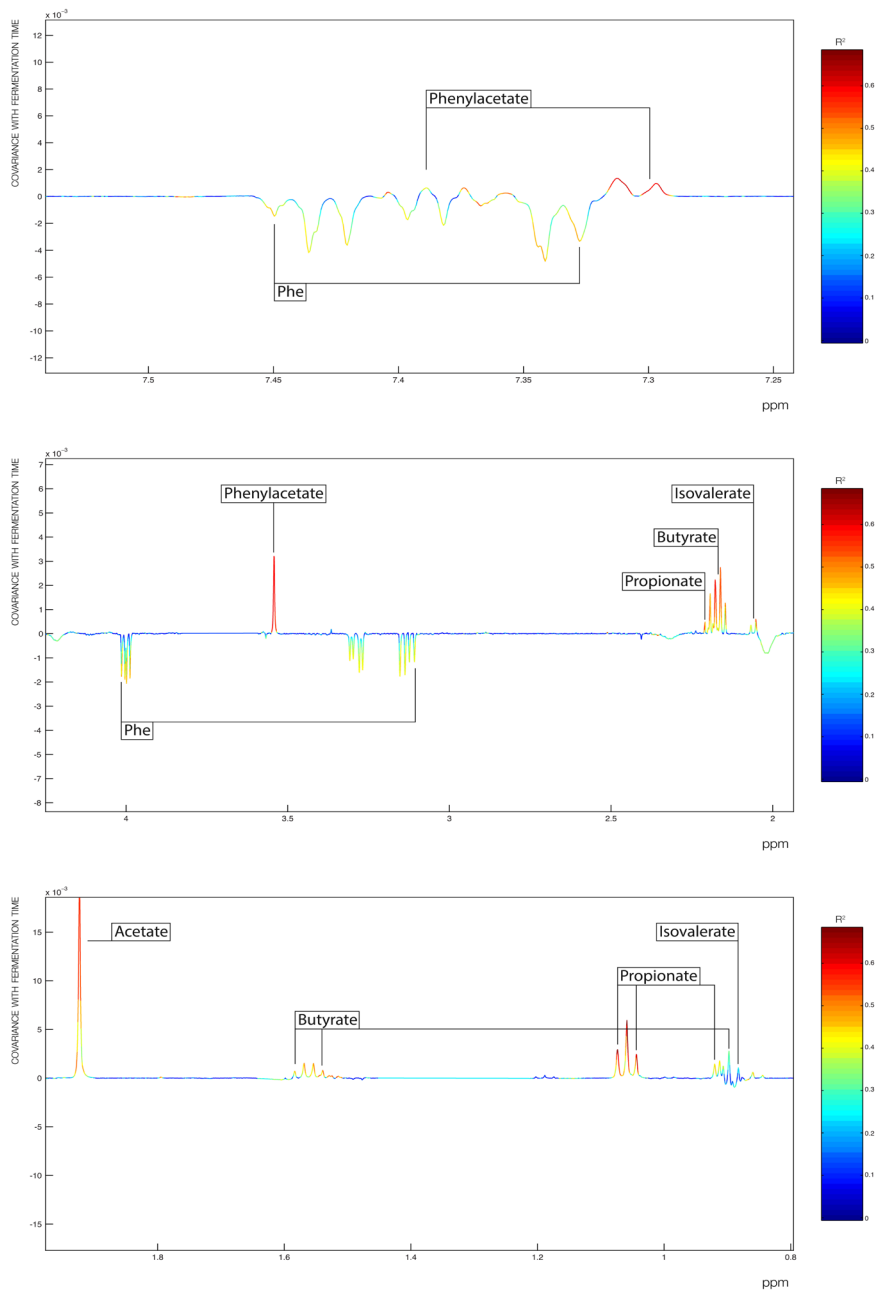
Fig. 1b OPLS coefficient-coded loading plot derived from the ¹H-NMR spectra of PHE *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).
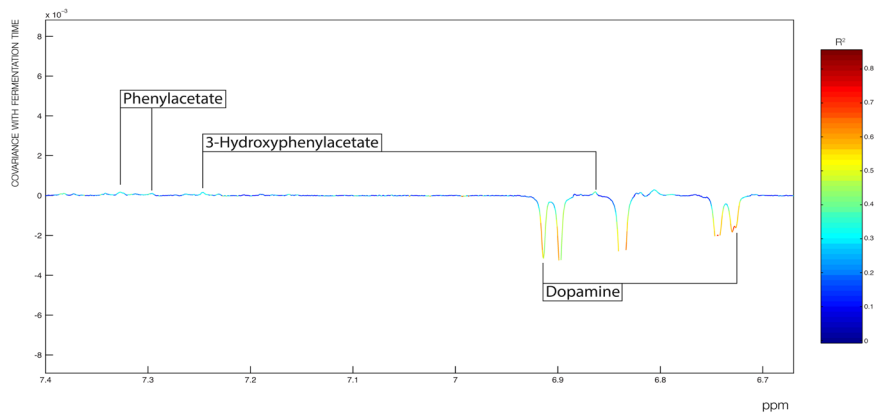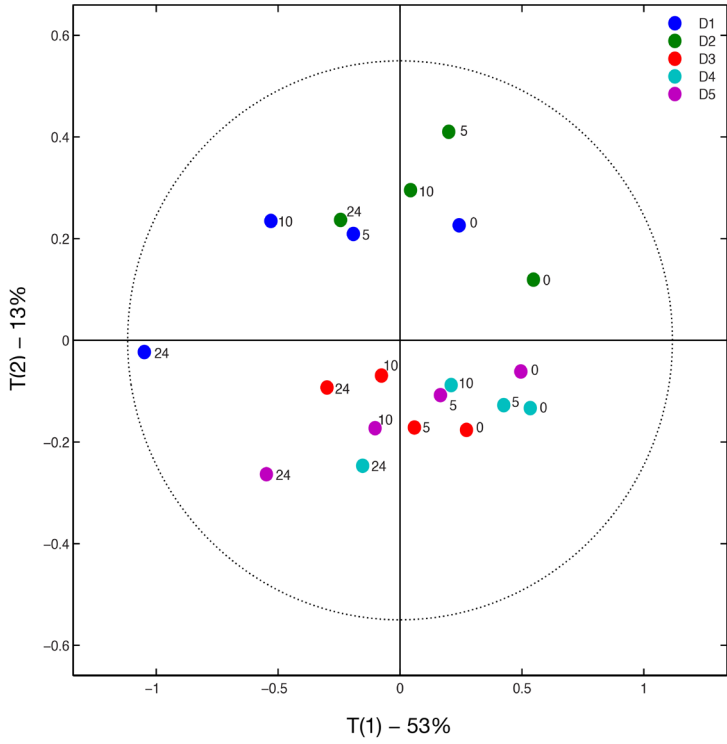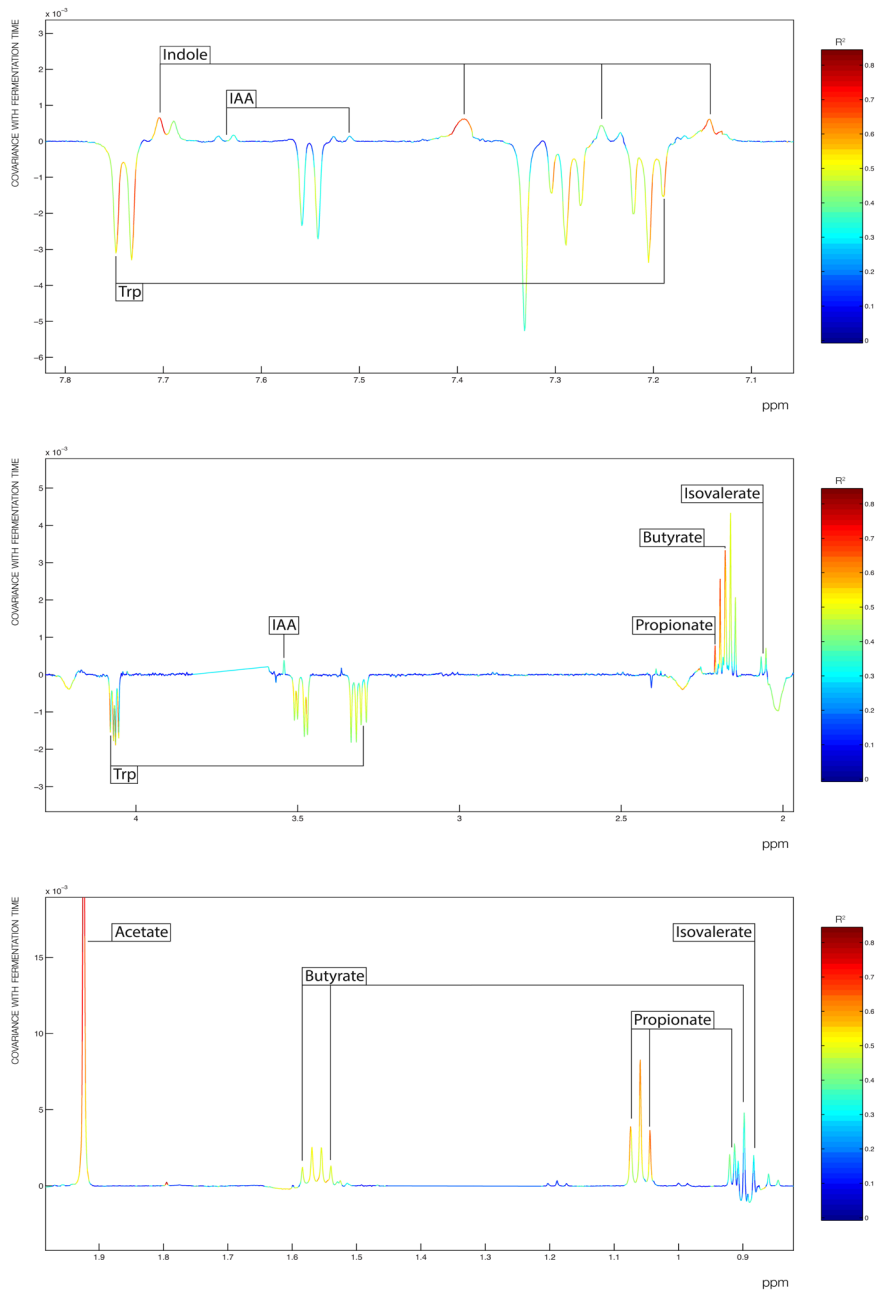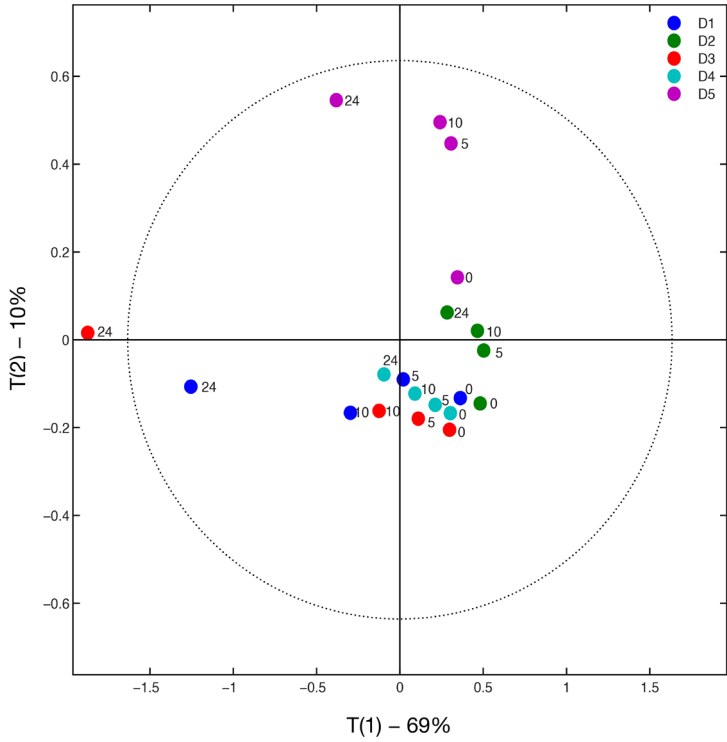
Fig. 2a Principal component analysis (PCA) score plot of TYR *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
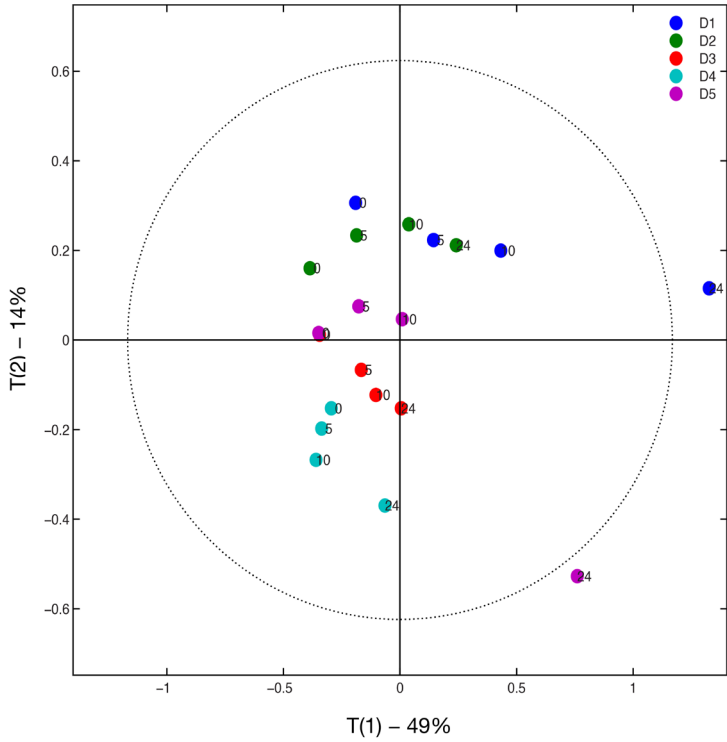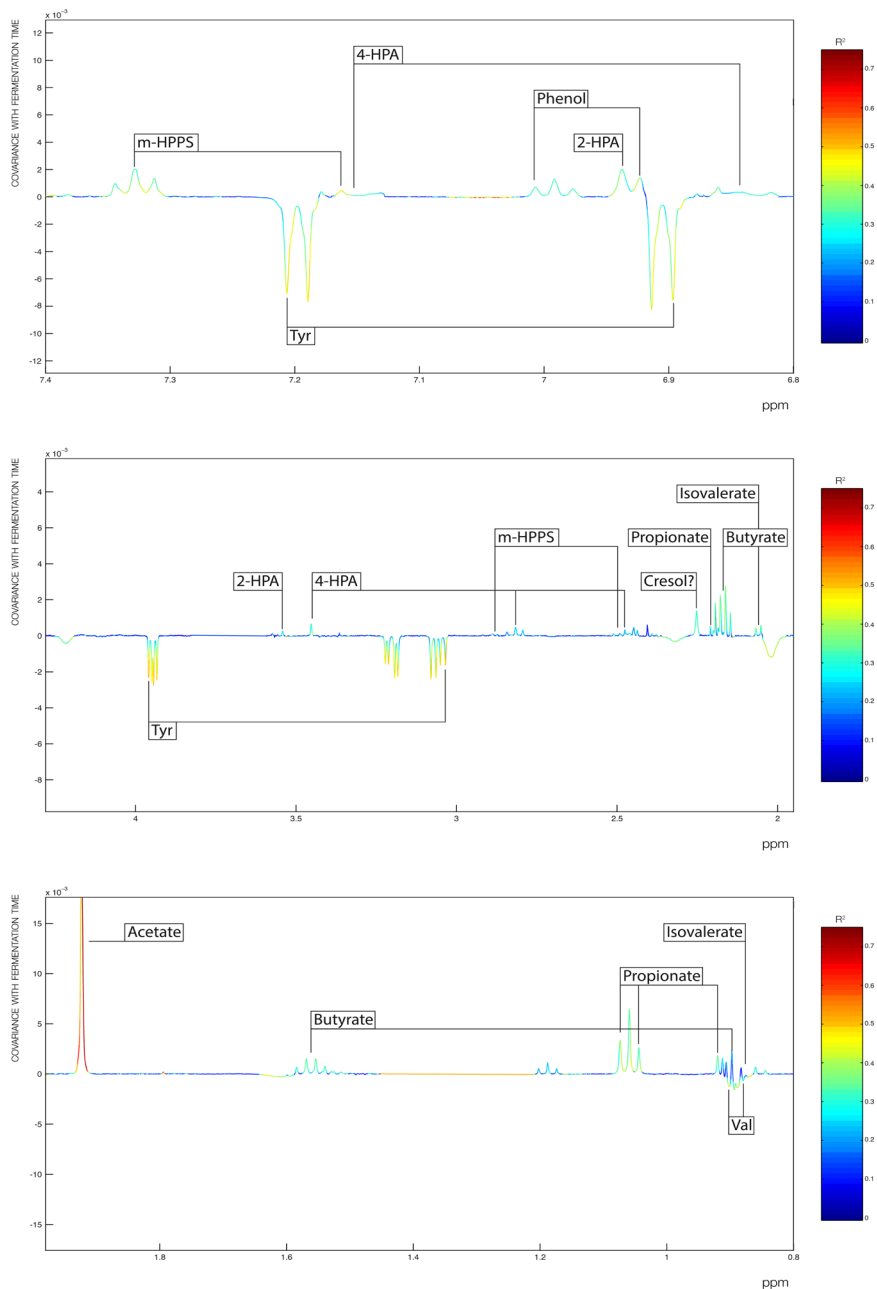
Fig. 2b OPLS coefficient-coded loading plot derived from the ^{1}H-NMR spectra of TYR *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

Fig. 3a Principal component analysis (PCA) score plot of DA *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
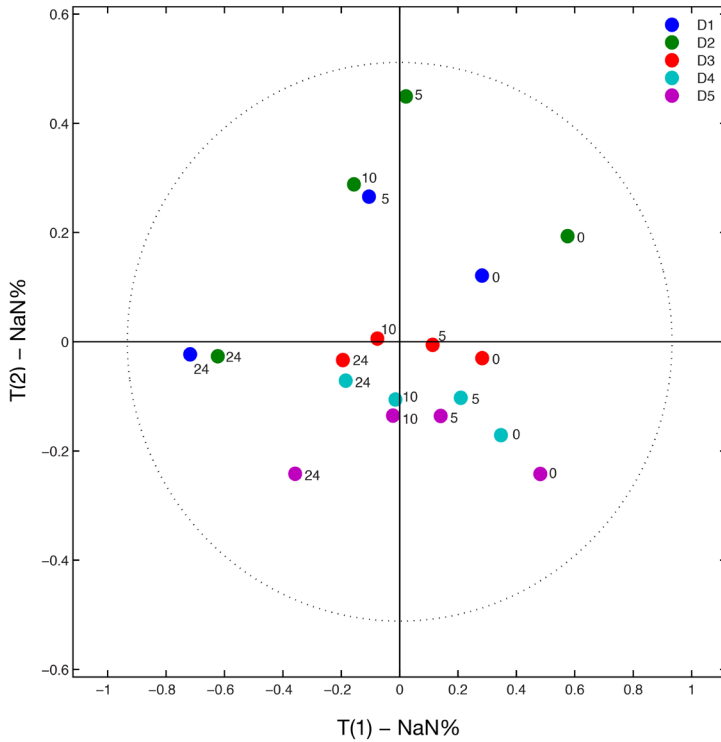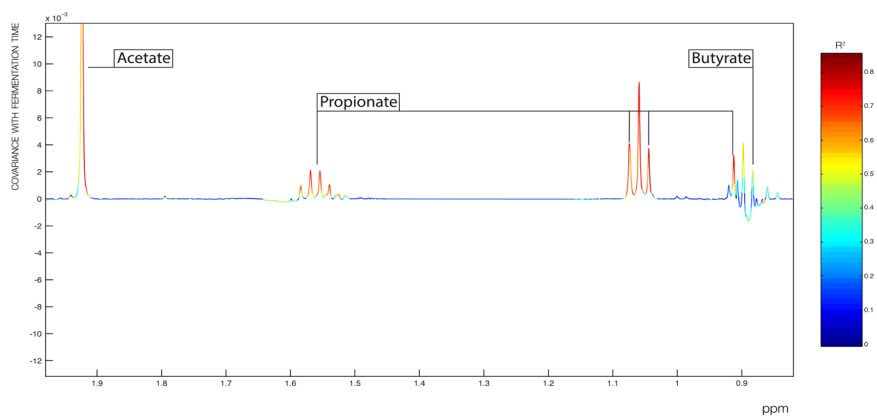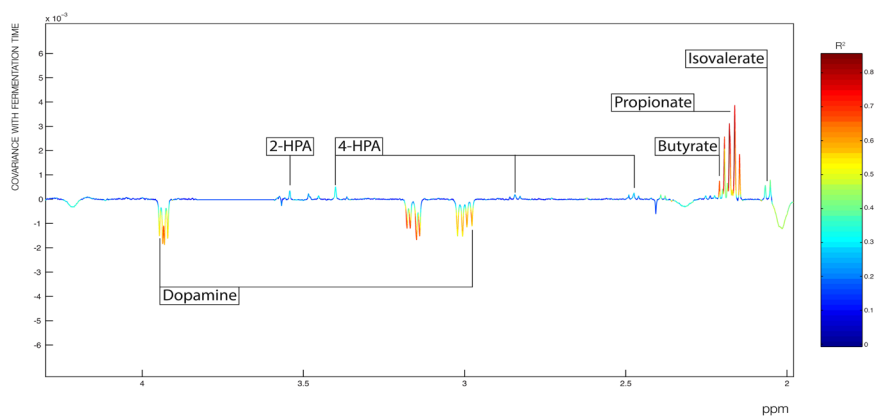
Fig. 3b OPLS coefficient-coded loading plot derived from the ^{1}H-NMR spectra of DA *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

Fig. 4a Principal component analysis (PCA) score plot of TRP *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).

Fig. 4b OPLS coefficient-coded loading plot derived from the ¹H-NMR spectra of TRP *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

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Fig. 5a Principal component analysis (PCA) score plot of GLU *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
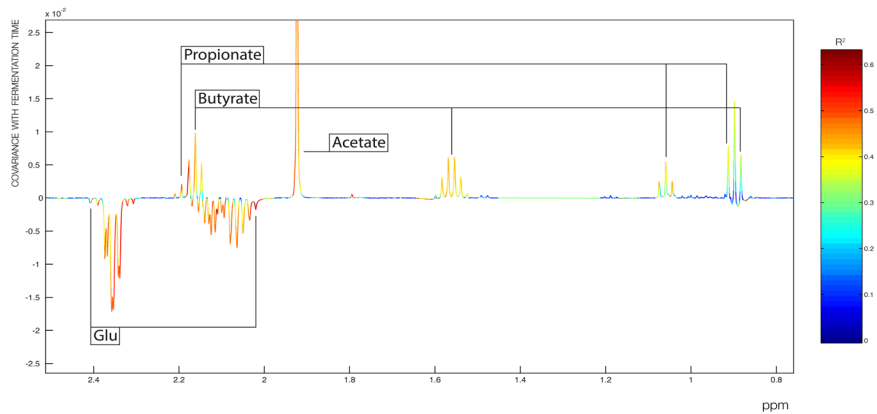
Fig. 5b OPLS coefficient-coded loading plot derived from the ^{1}H-NMR spectra of GLU *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

Fig. 6a Principal component analysis (PCA) score plot of MET *in vitro* fermentation over the time (0 - 24 h) and for 4 human healthy faecal donors (D1 - D4).
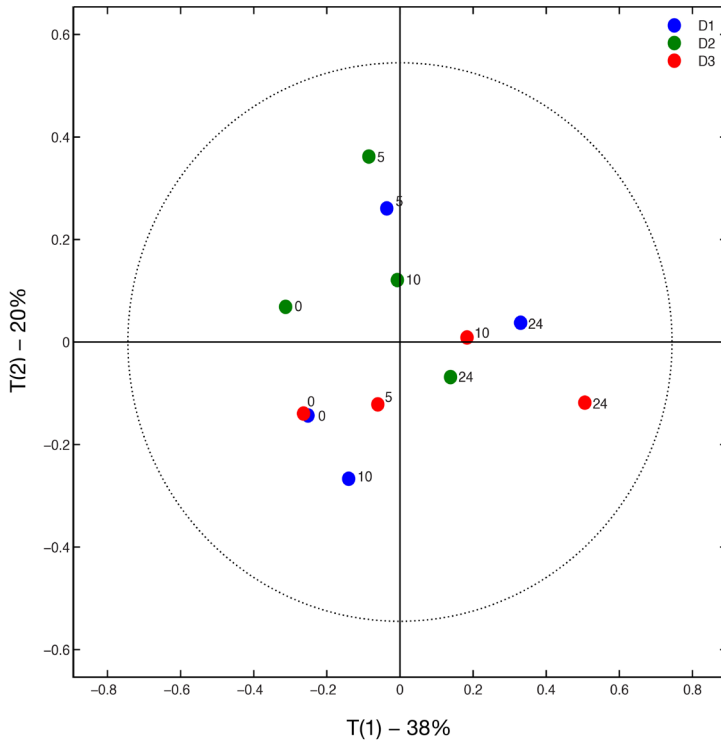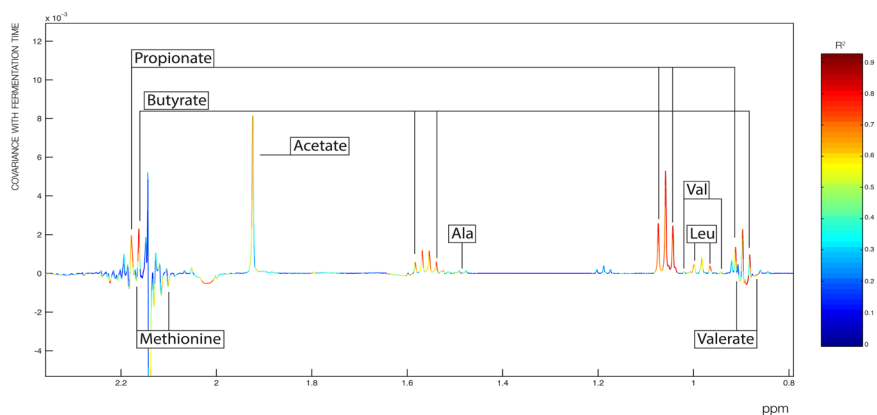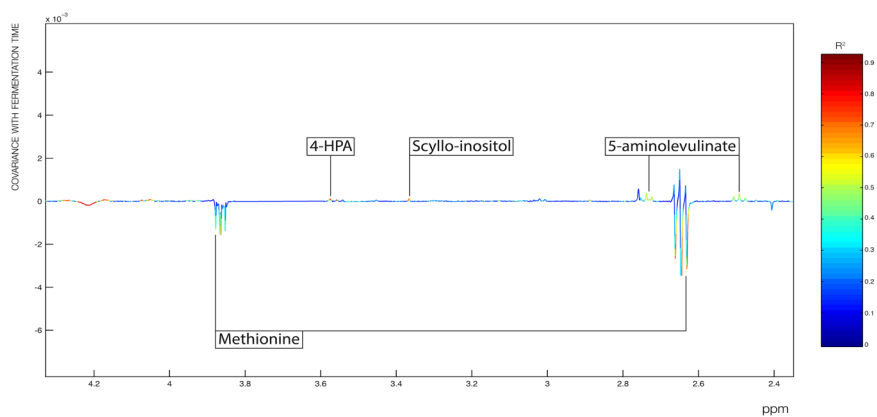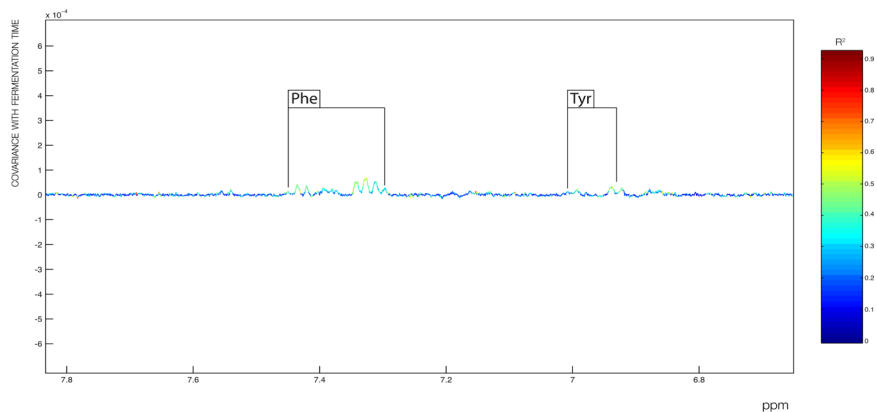
Fig. 6b OPLS coefficient-coded loading plot derived from the ¹H-NMR spectra of MET *in vitro* fermentation by five human faecal donor, over the time (0 - 24 h).

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METABOLITE	CHEMICAL SHIFTS (δ)
Dopamine	2.88(t), 3.23(t), 6.76(d), 6.87(s), 6.91(d)
Tyrosine	3.06(dd), 3.20(dd), 3.94(dd), 6.89(d), 7.18(d)
Tryptophan	3.30(dd), 3.48(dd), 4.05(dd), 7.19(t), 7.27(t), 7.31(s), 7.53(d), 7.72(d)
Phenylalanine	3.13(dd), 3.28(dd), 4.0(dd), 7.34(d), 7.39(m), 7.44(m)
Glutamate	2.08(m), 2.34(m), 3.75(t)
Methionine	2.14(s), 2.16(m), 2.65(t), 3.86(t)
Phenylacetate	3.54(s), 7.31(t), 7.39(t)
3-Hydroxyphenylacetate (3-HPA)	3.48(s), 6.79(d), 6.81(s), 6.86(d), 7.26(t) = 3.48(s), 6.79(m), 6.81(m), 6.86(dd), 7.26(t)
o-Hydroxyphenylacetate (2-HPA)	3.54(s), 6.91(d), 6.93(d), 7.18(dd), 7.21(td) = 3.54(s), 6.93(m), 7.21(m)
p-Hydroxyphenylacetate (4-HPA)	3.45(s), 6.87(d), 7.17(d)
Propionate	1.06(t), 2.19(q)
Isovalerate	0.92(d), 1.94(m), 2.05(d)
Acetate	1.92(s)
Butyrate	0.90(t), 1.56(m), 2.16(t)
m-hydroxyphenylpropionyl-sulfate (m-HPPS)	7.38 (t), 7.21 (m), 2.91 (t), 2.51 (t), 7.16 (dd)
Phenol	7.3 (t/m), 6.93 (t/m), 6.84 (d/m), 5.34 (s)
Ethanol	1.19(t), 3.66(q)
Valerate	0.89(t), 1.30(m), 1.55(m), 2.19(t)
Indole	6.61(s), 7.18(t), 7.27(t), 7.42(s), 7.56(d), 7.72(d)
Indole-3-acetate	3.66(s), 7.17(t), 7.24(t), 7.5(d), 7.63(d)
Indoleacetate (IAA)	7.62 (d), 7.50 (d), 7.24 (m), 7.15 (dd), 3.65 (s)
Phenylacetate	3.54(s), 7.31(t), 7.39(t)

Tab. 1 Proton NMR chemical shifts of main compounds found during *in vitro* colonic fermentation of neuroactive compounds.

METABOLITE	CHEMICAL SHIFTS (δ)
Hydroxypropyl propionate (HPP)	4.5 (d), 3.8 (m), 2.28 (q), 1.2 (d), 1.05 (t)
3-phenyllactate	7.33 (m), 4.26 (dd), 3.09 (dd), 2.87 (dd)
scyllo-inositol	3.36(s)
5-aminolevulinate	4.06 (s), 2.8 (t), 2.5 (t)
Alanine	1.48(d), 3.79(q)
Leucine	0.95(t), 1.71(m), 3.73(t) or 0.94(d), 0.96(d), 1.71(m), 3.73(t)
Valine	0.99(d), 1.04(d), 2.28(m), 3.62(d)

	DOPAMINE	TYROSINE	TRYPTOPHAN
R^2X	0,41	0,14	0,1
Q^2Y	0,41	0,45	0,45
p value	0,004	0,01	0,001
metabolites	acetate	acetate	acetate
	butyrate	butyrate	butyrate
	3-HPA	cresol	Indole
	2-HPA	ethanol	indole-3-acetate
	4-HPA	m-HPPS	indoleacetate
	isovalerate	2-HPA	propionate
	phenylacetate	4-HPA	
	propionate	isovalerate	
		phenol	
		propionate	

Tab. 2 Summary of the parameter values (R^2X, Q^2Y and p-value) describing the OPLS model and significantly representative metabolites found during fermentation of each treatment.

PHENYLALANINE	GLUTAMATE	METHIONINE
0,1	0,1	0,17
0,48	0,16	0,51
0,001	0,15	0,006
acetate	acetate	acetate
butyrate	butyrate	alanine
hydroxypropyl pyruvate	propionate	5-aminolevulinate
isovalerate		butyrate
phenylacetate		4-HPA
propionate		leucine
valerate		phenylalanine
		propionate
		propionate
		scyllo-inositol
		tyrosine
		valine
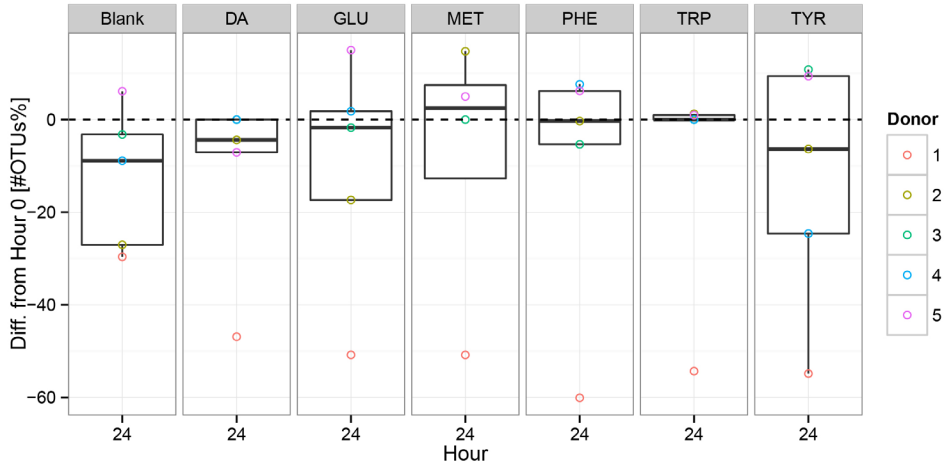
Fig. 7 Alpha-diversity, ranges from time 0 of sampling time (24 h). Repeated measure for five different human faecal donors (1 - 5), are marked with colored dots.
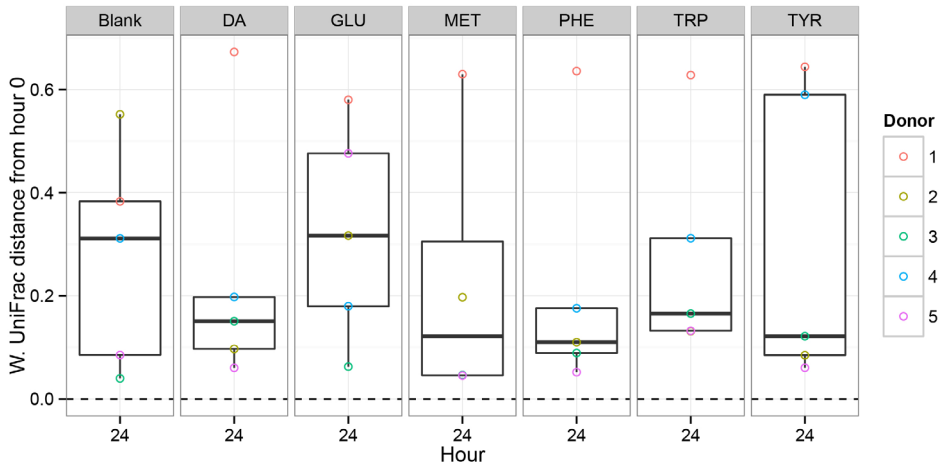
Fig. 8 Weighed UniFrac distances from hour 0 of samoling time (24 h). Only intra-donor distances were computed.
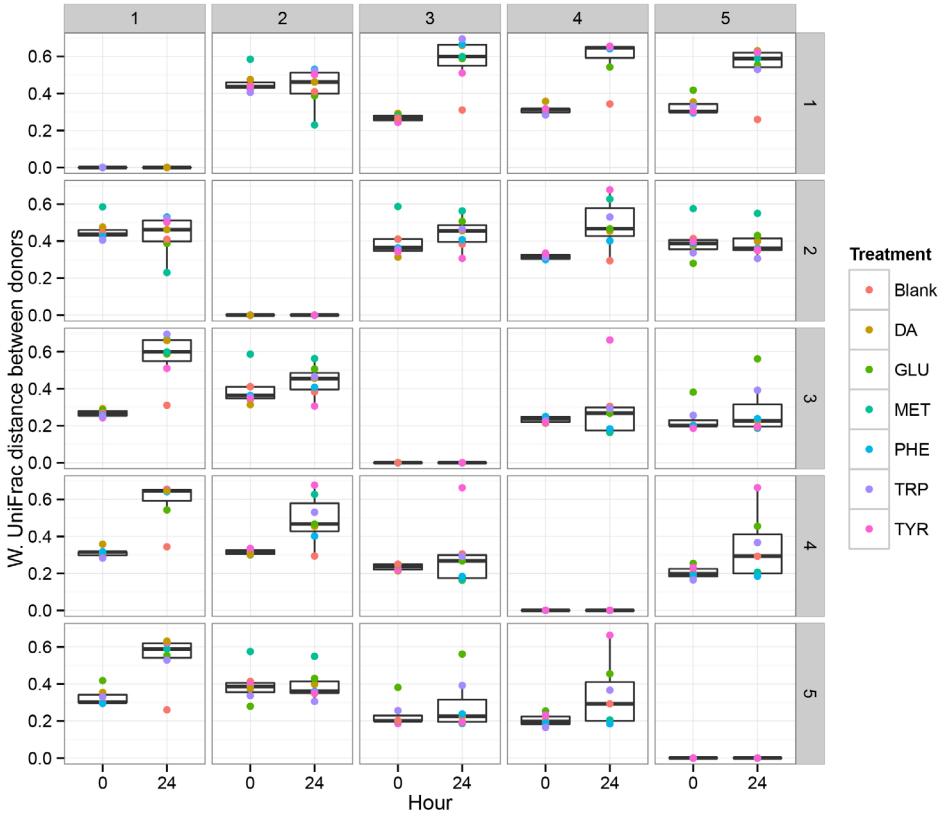
Fig. 9 Weighted unifrac distances between donors (1 - 5), at time 0 and 24 h. The donors are shown to the side of the frame, while treatments are indicated with a color – code dots.
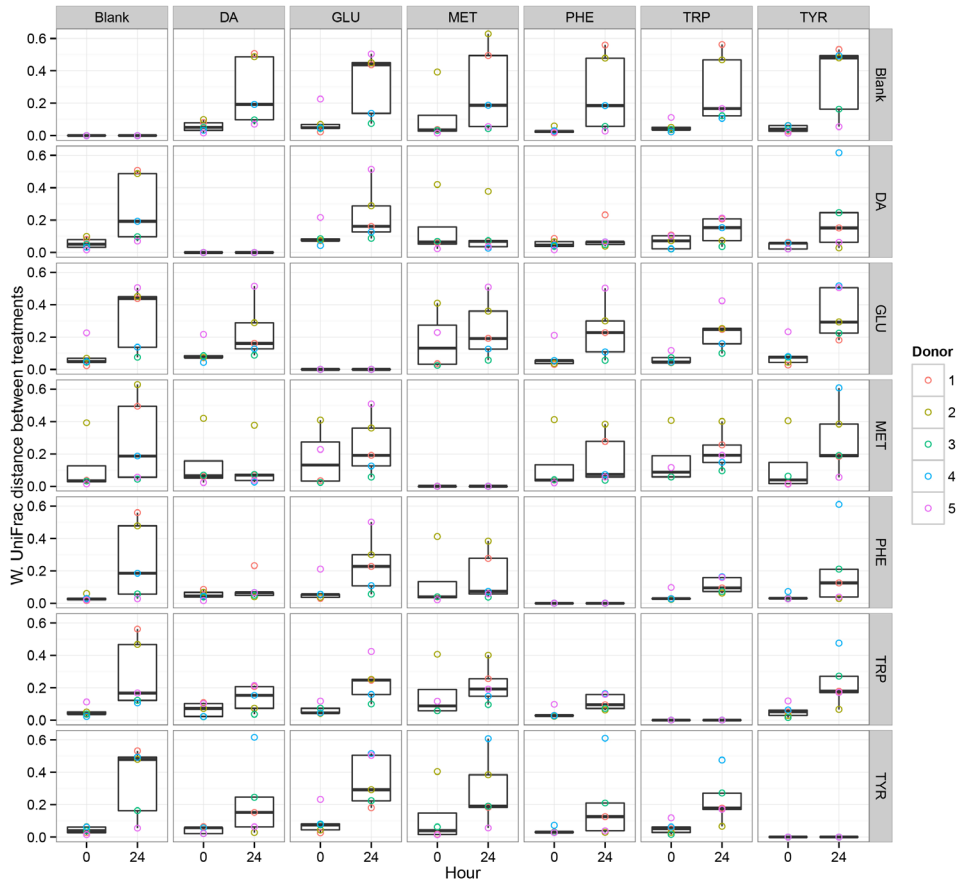
Fig. 10 Weighted unifrac distances between treatments (n = 6), at time 0 and 24 h. The treatments are shown to the side of the frame, while donors are marked with a color – code dots.

	Group A	Group B	St.dev A	St.dev B	p value
Hyphomicrobiaceae	1,57	3,58	1,12	1,09	1,68E-05
Desulfovibrionaceae	0,53	0,04	0,28	0,06	1,86E-05
Oxalobacteraceae	0,03	0,00	0,03	0,00	9,00E-03
Comamonadaceae	0,01	0,00	0,01	0,00	3,46E-02
Pasteurellaceae	0,01	0,07	0,01	0,09	5,60E-03
Enterobacteriaceae	0,06	0,44	0,08	0,28	8,56E-06
Verrucomicrobiaceae	2,97	3,59	0,02	0,04	9,05E-03
Victivallaceae	0,07	0,00	0,07	0,00	2,59E-03
Lachnospiraceae	16,53	22,76	1,83	7,22	1,24E-03
Peptococcaceae 1	0,02	0,00	0,02	0,00	1,01E-02
Acidaminococcaceae	0,77	0,14	0,31	0,19	9,88E-07
Enterococcaceae	0,06	0,00	0,07	0,00	8,36E-03
Synergistaceae	0,57	0,00	0,66	0,00	6,08E-03
Bifidobacteriaceae	1,85	5,76	0,40	3,59	1,04E-04
Actinomycetaceae	0,02	0,00	0,01	0,00	5,28E-04
Prevotellaceae	14,72	0,00	15,08	0,00	2,92E-03
Bacteroidaceae	9,09	37,35	3,25	6,83	6,01E-16
Porphyromonadaceae	1,75	0,18	0,99	0,26	4,52E-05

Tab. 3 Differences in relative abundance at family taxonomic level between Group A (D1 and D2) and Group B (D3, D4 and D5). One-way ANOSIM, Bray-Curtis distance measure, 9999 permutations, and pair-wise comparisons with Bonferroni correction P<0.01.
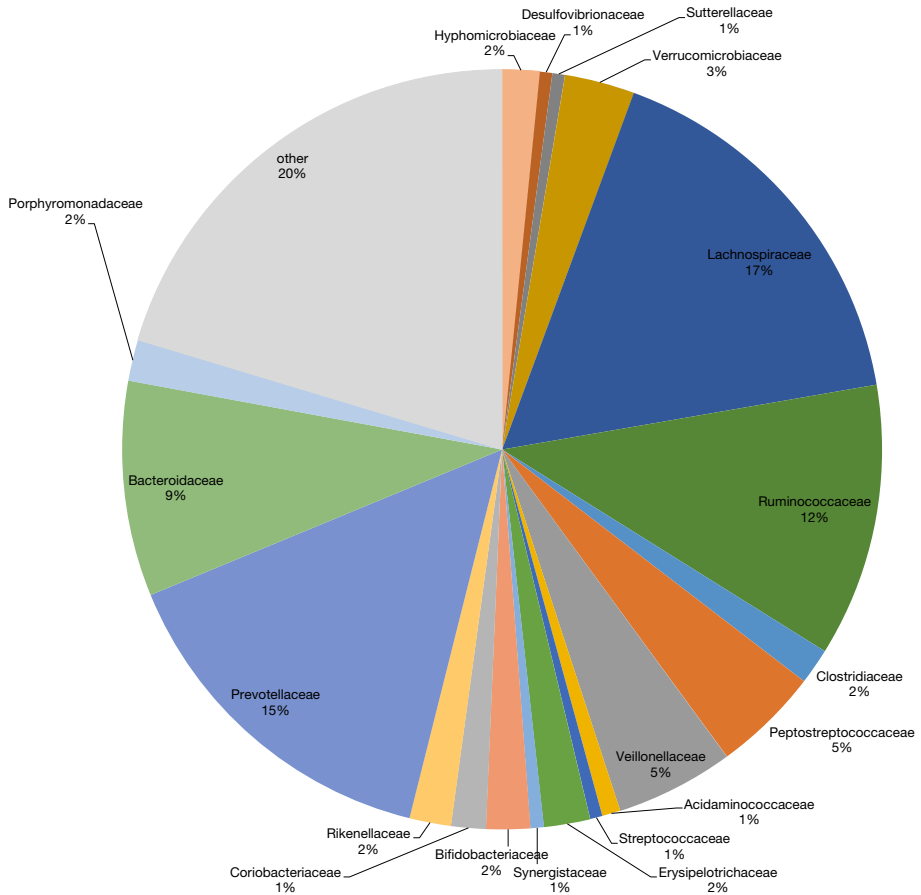
Fig. 11a Relative abundance, calculated using OTU table and relative bacterial species composition (%) based on DNA extracted from slurry human faecal of Group A (D 1 and D2) at time 0, before the beginning of fermentation.
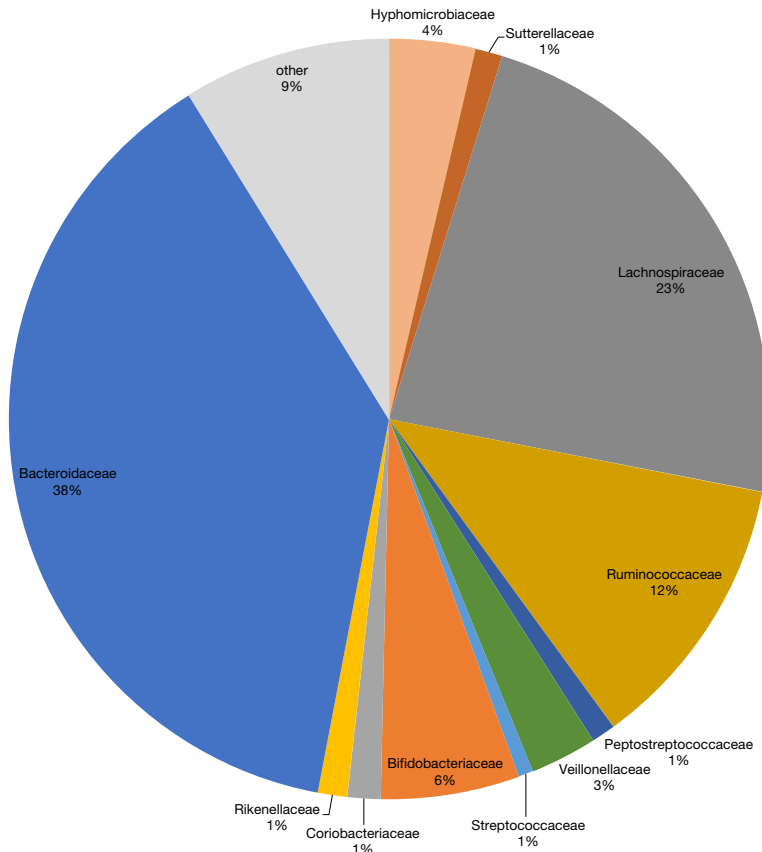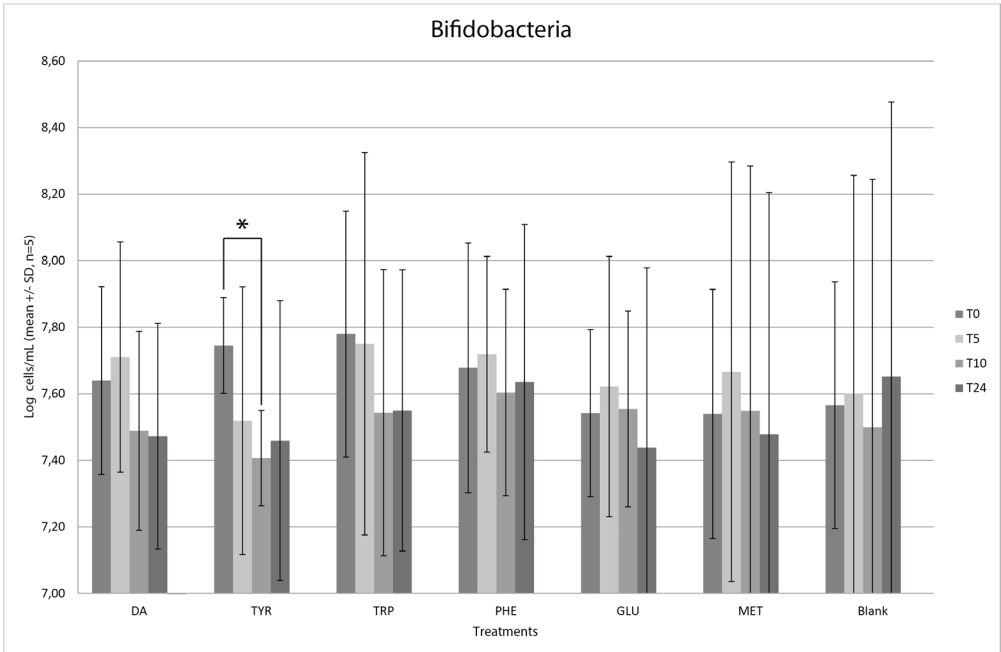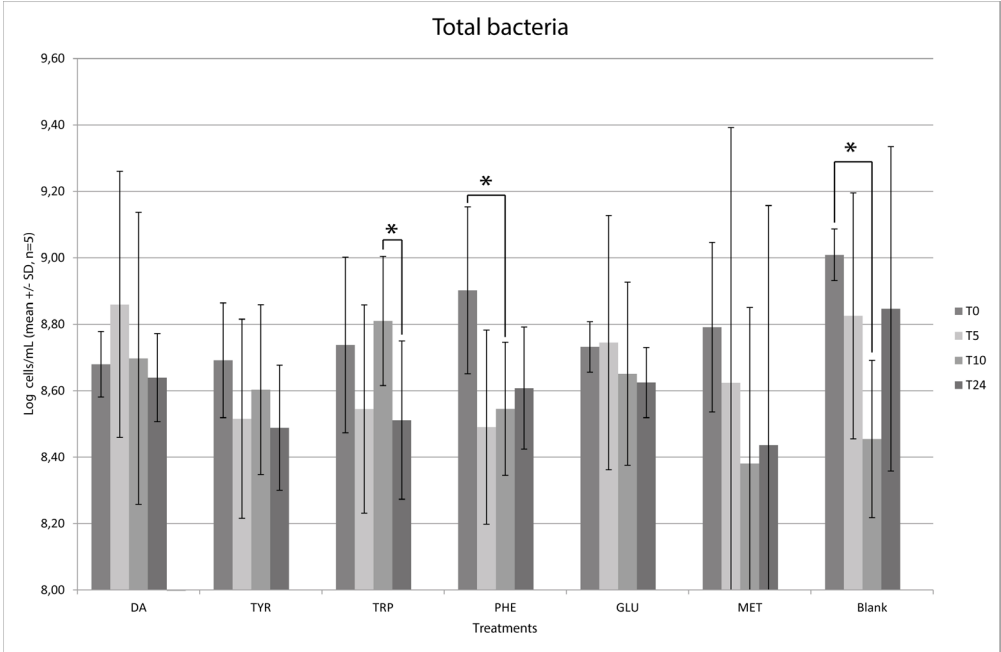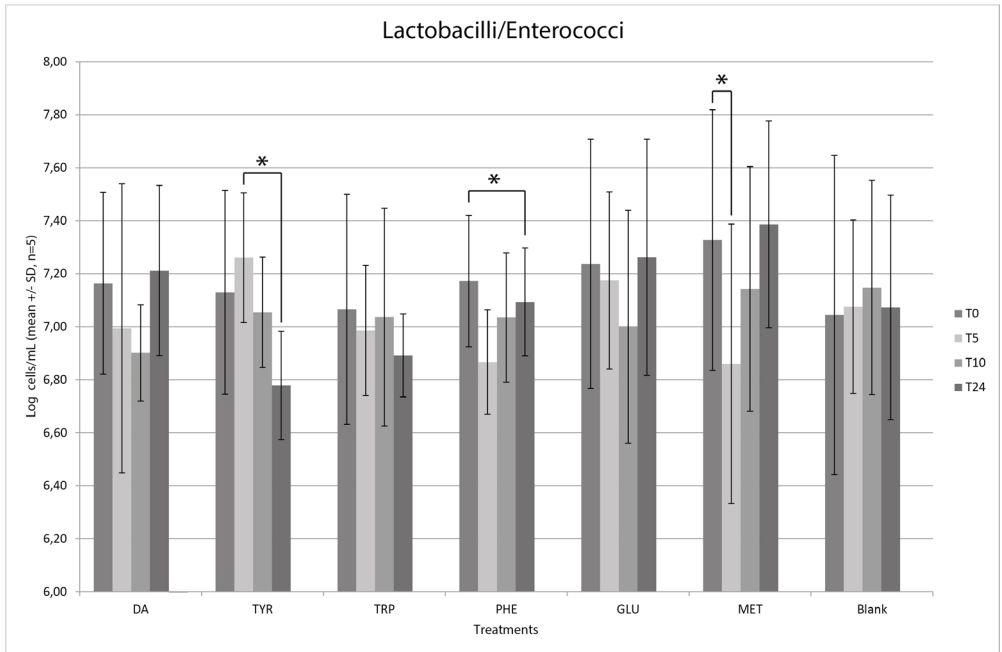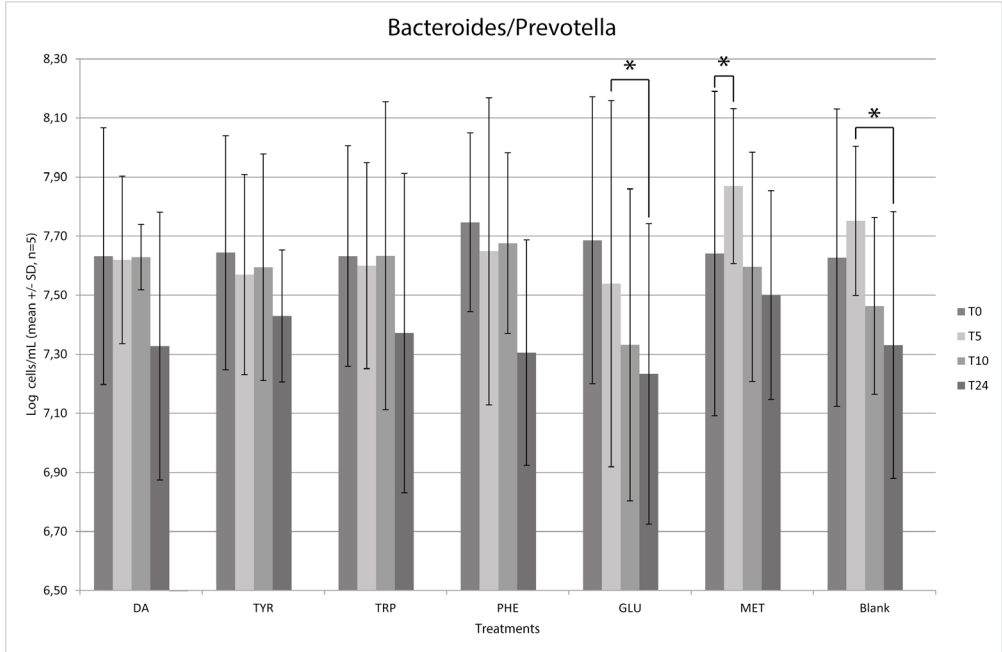
Fig. 11b Relative abundance, calculated using OTU table and relative bacterial species composition (%) based on DNA extracted from slurry human faecal of Group B (D 3, D4 and D5) at time 0, before the beginning of fermentation.
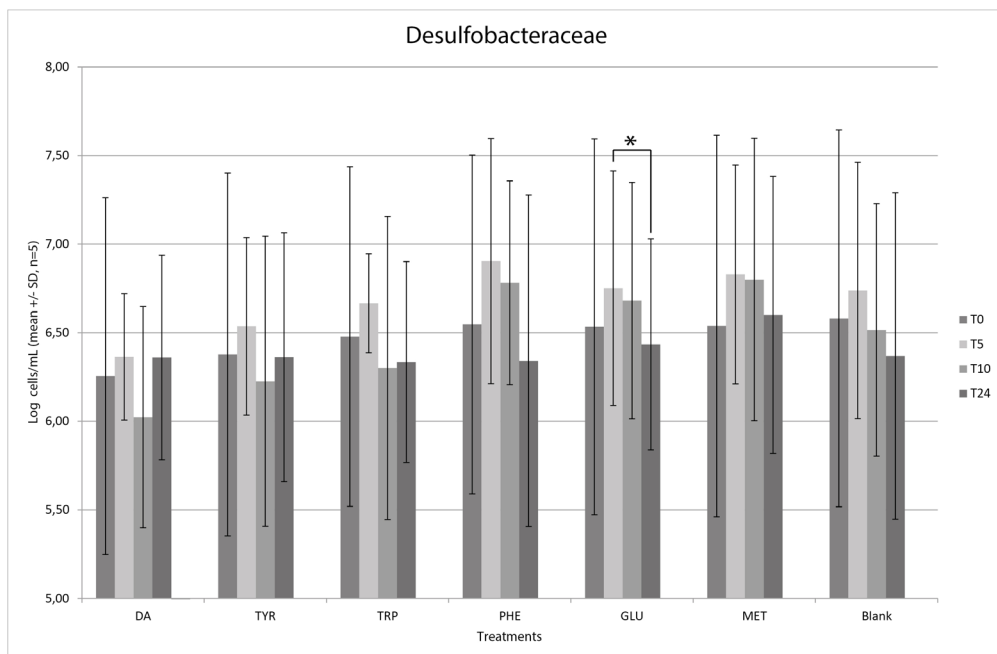
Total bacteria

Bifidobacteria

Bacteroides/Prevotella

Lactobacilli/Enterococci

Fig. 12 Total bacteria, bifidobacteria, *Bacteroides*/*Prevotella*, *Lactobacilli*/*Enterococci* and *Desulfobacteraceae* enumerated by FISH coupled with microscopy. Bacterial populations (log10 cells m^{l-1}) in pH-controlled batch cultures at 0, 5, 10 and 24 h of fermentation with basal medium (Blank), DA, TYR, TRP, PHE (5 mmol/l), and GLU, MET (10 mmol/l). Values are the average of five replicate batch cultures. Total bacterial and bacterial groups count was quantified using the following FISH probes: EUB 338 (I,II,III), Bif 164, Bac 303, Lab 158 and DSV 687.

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Growth, gut microbiota and fillet quality of rainbow trout

6. Growth, gut microbiota and fillet quality of rainbow trout

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Abstract

Developing fish farming to meet the demands of food security and sustainability in the 21st century will require new farming systems and improved feeds. Many physical and environmental variables can affect rainbow trout tolerance to the intensive aquaculture, and determine animal health and production yield. The recent recognition of the key role played by the gut microbiota in regulating host physiology and response to diet in particular, identifies diet:microbe interactions in the gut, as an important modifiable variable with potential to make a significant impact on future fish farming diets and production systems. Here, we monitored the gut microbiota of farmed rainbow trout (*Oncorhynchus mykiss*) using 16S rRNA profiling, over 75 weeks during standard rearing conditions and dietary regimes and during dietary supplementation of feed modified with antioxidant rich essential oils. Rainbow trout, from an initial body weight of 10.2 ± 2.5 g, were reared. From 24 weeks of age (T0), intestinal contents were collected after 4 (T1), 8 (T2), 18 (T3), 22 (T4), 27 (T5), 32 (T6) and 51 (T7) weeks. The gut microbiota 16S rRNA profiling indicated that, the fish gut was dominated by *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. Although, the dietary supplementation with plant derived essential oil had little impact on either the composition or architecture of the gut microbiota,

significant changes in alpha and beta diversity and relative abundance of important groups of gut bacteria were evident during different growth stages and standard feed, especially upon prolonged growth on finishing feed. Our results show that dietary supplementation with essential oil can have subtle effects on final fish fillet quality. We have also observed, significant differences within the gut microbiota of juvenile and adult rainbow trout under the same rearing conditions, highlighting the need for further studies examining how dietary microbiome modulation, at different life stages, can influence animal health, production yields and final product quality.

Since 1871 rainbow trout constitute one of the main species, used for fish farming (Halverson, 2010) and trout, is one of the most efficient species of fish, at converting macronutrients into biomass. However, it requires high-quality feed ingredients. Under standard production systems, the high proportion of animal protein (predominantly fishmeal), commonly used to support growth and health in the juvenile stage, is progressively reduced during the grow-out stage (from 12 to 24 months) (Naylor et al., 2009). Due to shortages and indeed collapse of wild fish stocks, the cost of fishmeal as a feed ingredient has risen sharply, promoting innovation in feed development towards use of cheaper plant-based protein sources (Gaylord and Barrows, 2009). Plant proteins derived from soybean, canola and peas, are used as ingredients of various aquaculture diets (Gatlin et al., 2007; Mundheim et al., 2004; Thiessen et al., 2003). However, plant protein ingredients contain also significant anti-nutrient factors, such as saponins, glucosinolates, phytate and trypsin inhibitors, which at high doses, have been associated with intestinal disorders, in farmed fish, including meal–induced enteritis (Francis et al., 2001; Refstie et al., 2000). Much current work focuses on optimal fish diet compositional development, to provide optimal dietary protein, from economically viable protein sources (e.g. vegetable food waste streams, poultry waste streams and alternative protein sources from farmed insects), but which also support animal health, especially gastrointestinal health and disease resistance.

In aquaculture, as well as in other animal husbandry, the misuse and over reliance on antibiotics, has lead to increased risk of resistant pathogenic or pest species and the spread of antibiotic resistance determinants (Guardabassi et al., 2008). Current use of antimicrobials, as growth promoters, is now banned in the EU and strict regulations control to use of pesticides and medicinal antibiotic are implemented in fish farming (Park et al., 2012; Giannenas et al., 2012). Essential oils, have recognized antimicrobial potential and also may have flavour traits, of potential interest from an organoleptic perspective, which has raised interest in their use as an innovative functional ingredient in fish feed. They also have recognized antioxidant activities and are used successfully, in other animal production systems, as alternatives to antibiotic growth promoters and to improve final food quality. Some of these bioactive effects, appear to be mediated through interactions within the gut, especially with the gut microbiota (Yang et al., 2015). Essential oils were found to exert antioxidant and antibacterial activities and to show beneficial effects on immune system and intestinal microbiota of fish (Giannenas et al., 2012). Other rese-

arch has demonstrated that essential oils, when added at the feed as preservatives, improves shelf life of fish fillets, by inhibiting lipid oxidation and bacterial spoilage (Burt, 2004; Kykkidou et al., 2009; Tironi et al., 2010; Erkan et al., 2011).

An influence of the intestinal microbiota on animal nutrition and health status of rainbow trout, was recently been suggested (Ingerslev et al., 2014). The gut microbiota plays an important role in animal host physiology, including digestion and absorption of nutrients, synthesis of vitamins, as well as development of intestinal mucosal and systemic immune system and assisting detoxification processes. Changes in the fish gut resident community, directly affect the growth and health of the animals, by influencing the nutritional status and the capability to build an efficacious immune defense, from pathogens (Kotzamanis et al., 2007; Abid et al., 2013). The close interaction between the animal host and gut symbiotic bacterial community, suggests that the host metabolism is the result of metabolic processes, involving the systemic organism in intimate collaboration, with its gut microbiota. Many exogenous and endogenous factors, may alter the composition of the gut microbiota of fish, including the environment (e.g. temperature, pH, salinity), the developmental age, fish species and individual genetics, use of antibiotics, during farming and diet (Pérez et al., 2010). In aquaculture, several dietary supplements for fish, including probiotic yeasts and bacteria, as well as a wide range of microalgae, have been shown to positively impact on the gut microbiota and, indirectly, to improve fish health, growth, survival and protection from disease (Hernández et al., 2016; Kiron, 2015; Navarrete and Tovar-Ramrez, 2014). In this study, we tested the hypothesis that dietary supplementation with essential oils, would induce a positive modulation of the fish gut microbiota and thus beneficially affect fish quality. To this aim, we used 16S rRNA profiling by pyrosequencing, to characterize the composition and structure of the gut microbiota over time and, in response to different diets, standard/control rearing diets and diets supplemented with essential oil. We also evaluated physical and chemical quality characteristics, of trout fillets.
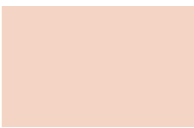
Fish, diets and culture conditions

A total number of 1296 selected parr (6 months old) of rainbow trout with an average weight of 10.2 ± 2.5 g were randomly distributed into 2 squared fiberglass tanks (0.7 m³) and assigned to 2 different treatments (Control and MixOil added diet) for a 51 weeks feeding trial (T0 or week 0). The fish were acclimatized for six days prior to the start of the trial. During the trial fish were fed manually, 5 days a week, and water temperature and dissolved O_2 (in the water outlet) were measured weekly. Feed was given and mortality recorded daily for each tank. The total biomass of each tank was assessed at different times during the trial at three stages of growth, Stage 1–8 weeks after start of test feed (end of 1st stage), end of stage 2–25 weeks after start of test feed and end of stage 3–51 weeks after start of the test feed. Water flow and size tanks, were increased accordingly with the growing fish biomass. Two different diets (Control–C and Treated–MO) were formulated to evaluate the effect of GrowNat-MixOil Aqua (an essential oils blend), at 200 ppm concentration, on growth performances of rainbow trout. The coating method, was used to combine GrowNat-MixOil Aqua (MixOil), with a commercial feed. To ease the coating process, the oils blend was diluted in fish oil (for control diet, C, pellets were coated with 1% fish oil (w/w), for MixOil diet, MO, pellets were coated with 0.99% fish oil (w/w) plus 0.1% MixOil (w/w)). The diets were iso-energetic and iso-lipidic. Pellet sizes and proximate composition of the commercial feed, are given in table 1, at each of the three stages of growth. Pellet size and feeding ratio were adjusted, according to manufacturer recommendations, as the fish grew. To insure growth performance, according to the manufacturer's specifications, the use of the feeds, were evaluated at the end of each stage, using the following parameters calculated from the data collected:

Specific Growth Rates (SGR, %/day) = [(Ln Wf – Ln Wi)/days] *100
Feed Conversion Ratio (FCR) = Total feed consumption / Weight gain.

Gut sampling and DNA extraction

The metagenomic analysis, was performed on samples obtained throughout the life stages of the fish, i.e. at time 0 (T0) and after 4 (T1), 8 (T2), 18 (T3), 22 (T4), 27 (T5), 32 (T6), 51 (T7) weeks of rearing. A total of 6 fish at each time was sampled, from the fiberglass tanks containing the fish fed control diet (C, n=3) and fish fed the same diet supplemented, with 200 ppm of Grownat-MixOil Aqua (MO, n = 3).

For the last time point (T7), the double of samples, was collected (C, n = 6 and MO, n = 6). Sampling, was performed at each time point, in order to obtain fish from two groups, with similar body weight, to avoid the influence of this last parameter on the metagenomics analysis results. The genomic DNA extraction from intestinal content, was carried out using FastDNA™ SPIN Kit for Feces (MP Biomedicals, USA), following the manufacturers' instructions. Total intestinal content (foregut, midgut and hindgut, excluding the stomach), was collected from each fish, immediately after sacrificing the animal, then stored in RNAlater® (Ambion, Life Technologies) at -80 °C. Genomic DNA, was extracted from 500 mg aliquots of intestinal content sample, after thawing and removal of RNAlater. DNA quality and quantity were assessed, by gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher). Only samples with Aλ260/Aλ280 within the range 1.8-2.0 were processed for analysis. Extracted DNA was stored at -20 °C.

PCR amplification of the V1-V3 region of bacterial 16S rRNA genes

For each sample, the 16S rRNA gene was amplified, by using special fusion primer set specific for V1-V3 hypervariable regions (27-Forward: 5'-AGAGTTTGATCMTG-GCTCAG-3' (Lane et al. 1991) and 533-Reverse: 5'-TTACCGCGGCTGCTGGCAC-3' (Muyzer et al. 1993)). The forward primer contained: the "LIB-L" primer A sequence specific for the "Lib-L" chemistry and the "One-Way Reads" sequencing methods (Roche, Branford, CT, USA), the key sequence TCAG, the barcode (Multiple IDentifier) sequence (specific for any sample) and the 8-forward sequence. The reverse primer contained the "Lib-L" primer B sequence, the key sequence TCAG and the 533-reverse sequence. For each sample, a PCR mix of 25 µl was prepared containing 1X PCR buffer, 1.25 U of FastStart High Fidelity polymerase blend (Roche) and dNTPs from the FastStart High Fidelity PCR system (Roche), 0,4 µM of each primer (PRIMM, Milano, Italy) and 10 ng of gDNA. Thermal cycling consisted of initial denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 1 minute, with a final extension of 8 minutes at 72 °C.

Library construction and pyrosequencing

The PCR products, were analysed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, Brea, CA, USA), following the manufactu-

rer's instructions. The products of the 54 samples, were quantitated via quantitative PCR, using the Library quantification kit – Roche 454 titanium (KAPA Biosystems, Boston, MA, USA) and pooled in equimolar way, in a final amplicon library. The 454 pyrosequencing, was carried out on the GS FLX+ system, using the XL+ chemistry, following the manufacturer's recommendations.

Data analysis
The pyrosequencing produced a total of 919,936 high quality reads of 16S rDNA reads. The sequences were assigned to samples, according to sample-specific barcodes. This allowed to collect FASTQ formatted files containing an average of 18,398 sequences per sample. Raw 454 files were demultiplexed using the Roche's sff file software. Raw data were submitted to the http://www.ebi.ac.uk/ena/submit/sequence-submission. Reads were processed using the MICCA pipeline (version 1.0, http://micca.org/) (Albanese et al., 2015). Forward and reverse primers trimming, were performed using micca trim (parameters -w AGAGTTTGATCMTG-GCTCAG -r TTACCGCGGCTGCTGGCAC -W -c). Quality filtering, were performed using micca filter (parameters -e 0.5 -m 400 -t), truncating reads shorter than 400 nt and discarding reads, with an expected error (EE) rate > 0.5%. De-novo sequence clustering, chimera filtering and taxonomy assignment, were performed by micca otu (parameters -d 0.97 -c): operational taxonomic units (OTU) were assigned by clustering the sequences, with a threshold of 97% pairwise identity, and their representative sequences were classified, using micca classify with the RDP classifier version 2.8 (Wang et al., 2007). Template-guided multiple sequence alignment (MSA), was performed using micca msa (parameters -m nast), against the multiple alignment of the Greengenes (DeSantis et al., 2006) database (release 13_05) clustered at 97% similarity. Finally, phylogenetic tree was inferred, using micca tree (parameters -m fasttree), using FastTree (Price et al., 2010). Sampling heterogeneity, was reduced by rarefaction (3,972 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates, were computed using the phyloseq R package (McMurdie & Holmes, 2013).

Experimental set-up for fillet quality characteristics
To test of the effect of the essential MixOil added in the diet, on quality characteristics of fillets, a total of 15 fish were sampled, from the fiberglass tanks containing, the fish C and the fish MO. The sampling was performed, in order to obtain fish

from two groups, with similar body weight, to avoid the influence of this last para-meter, on the selected quality indicators.

All 30 fish were filleted immediately, after slaughter and sent to DISPAA laboratories in Florence, where the fillets were stored in a refrigerated room, at 1 °C on ice until analyses. A number of 5 fillets per diet (i.e. n. 10 fillets in total), were analyzed at the 1st, 4th and 7th day of storage. The physical-chemical analyses, were carried out on the left fillets, while the microbiological ones on the right fillets. All analyses sche-duled for the samples at each time of storage, were performed at the same time. The physical-chemical determinations, on the left fillets, were the followings:

Drip loss (DL). Drip loss was determined for each fillet. The initial weight of the left fillets was recorded (Wi), in the day of slaughtering. Fillets were re-weighed at each storage time (1st, 4th and 7th; n = 10 at each storage time), to register the final weight (Wf). Drip loss, was then calculated as follows: DL (%) = [(Wi–Wf) /Wi] × 100.

Water Holding Capacity (WHC). It was determined, by a low speed centrifugation at 210 g for 5 minutes (Eide et al., 1982 modified by Hultmann and Rustad, 2002), on a sample withdrawn from the epaxial site of the fillet (Figure 1). The moisture of the sample, was determined by drying 2 g of flesh in an oven at 105 °C for 24 h.

Colour. The colorimetric attributes lightness (L*), redness index (a*), yellowness index (b*), saturation (Chroma) and hue (Hue), according to the method of the Com-mission Internationale de l'Éclairage (CIE, 1976), were registered by the Spectro-co-lor® colorimeter (software: Spectral qc 3.6). The measurements were done on three sites for each fillet (epaxial, ventral and caudal; Figure 1), with three replicates for each site.

Texture. Textural parameters were obtained by instrumental analysis, using the Zwick Roell® texturometer (software: Text Expert II) . A cyclic compression test was performed twice on the epaxial region (Figure 1). The parameters of the test were the followings: Probe: cylindric (length: 12 cm, diameter: 1 cm); Load cell: 1 kN; Number of cycles: 2; Constant speed: 100 mm/min; Deformation: 50% of the original length.

Total lipids. Total lipids were determined, by the Bligh and Dyer (1959 mod.) method. Five grams of muscle, were weighed and homogenized by adding chlo-roform, methanol and distilled water, to a final ratio of 2:2:1.8. Each homogenized sample, was centrifuged at 5000 rpm per 10 minutes, to allow the two phases

separation. One millilitre of the lower phase, was collected and transferred to a crucible. Solvents were left to evaporate and the net weight of the crucible was registered. Total lipids were calculated, according the formula:

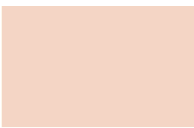g of lipid/100 of muscle = [net weight (g) x chloroform (ml)] / [g of muscle / evaporated chloroform (ml)]
of which:
Chloroform (ml) = 20 ml
Evaporated chloroform (ml) = 1 ml

Malondialdheyde (MDA). MDA was determined as reactive substances to 2-thiobarbituric acid (TBARs) according to the method of Siu and Draper (1978), modified by Luciano et al. (2013). A quantity of 2.5 g of muscle was homogenized in 5% (w/v) TCA (trichloroacetic acid) and then centrifuged at 5000 rpm for 30 minutes. The supernatant was filtered and 2 ml of the extract was collected to react with 2 ml of 40 mM TBA (2-thiobarbituric acid). Samples were incubated at 90 °C for 20 minutes. Absorbance was read at 532 nm by the spectrophotometer. MDA content was calculated by using a calibration curve of TEP standard solutions (TEP concentrations from 0.8 to 8 μM).

Vitamin E. Tocopherols concentration in rainbow trout muscle, previously submitted to the saponification and extraction phases, was determined by reverse phase chromatography and quantified by FID detection. The saponification procedure was performed according to Liu et al. (1996). Saponification solution was prepared fresh each day and comprised 11% KOH (wt/vol), 55% EtOH (vol/vol), and 45% deionized distilled water (vol/vol). One gram of frozen sample was weighed into a 20- x 150-mm test tube, in duplicate for each muscle sample. Ascorbic acid (0.25 g) and saponification solution (7.3 ml) were added to each tube. Tubes were briefly vortexed until the ascorbic acid was dissolved. Tubes were then placed into a test tube rack and incubated in a 80 °C shaking water bath for 15 min at 200 rpm. After the incubation, test tubes were cooled in ice and 4 ml of isooctane was delivered to each tube. Tubes were vortexed for 2 min and allowed to stand briefly to allow separation of aqueous and isooctane phases. The concentrations and volumes of the saponification solution and isooctane were designed to provide the final proportions of H_2O - 4 ml, EtOH - 4 ml, isooctane - 4 ml, the muscle sample was assumed to contribute 0.7 ml of H_2O.
The chromatographic separation of tocopherols was performed by HPLC, on a 150

mm x 4,6 mm i.d., 5 µm Cosmosil π NAP column (Nacalai-Tesque, Kyoto, Japan). The mobile phase used was methanol-water with 0.2% phosphoric acid (90:10 v/v), prepared fresh daily. The flow rate was 1.0 ml/min, and analyses were made at room temperature. The injection volume was 20 µl. The total run time was 25 minutes. The separated tocopherols were quantified with FID detector (Agilent mod. HP 050) at emission wavelenght λ_{em} = 295 nm and excitation wavelenght λ_{exc}= 330 nm. An external calibration curve was calculated by using α-tocopherols (DL-alpha-tocopherol, Sigma Aldrich) at different concentrations. Results were expressed as mg of α- and γ- tocopherol per kg of muscle.

On the right fillets the followings determinations were performed:

Activity of water (Aw) and pH. Aw and pH (pHmeter Mettler Toledo, Milan, Italy) were performed by using the official methods ISO (2004a) and MFHPB-03 (2003), respectively.

Microbial counts. Fillets were examined for total mesophilic count (CMT), Enterobacteriaceae and Pseudomonadaceae, to whichthe Specific Spoilage Organisms (SSOs) belong, deriving from gut, water, ice and skin and responsible for fish quality deterioration (Gram and Dalgaard, 2002). Strain typing of Enterobacteriaceae and Pseudomonadaceae was performed. CMT, Enterobacteriaceae and Pseudomonadaceae counts were performed by using the official methods ISO (2003a, 2004b and 2009). Fillets were also examined for the pathogens *Yersinia enterocolitica* and *Salmonella spp.*, according to the methods ISO (2003b and 2004c), and *Listeria monocytogenes* by using the qualitative and quantitative methods according to UNI EN ISO (2005a and 2005b). *Staphylococcus coagulase* positive bacteria were determined according to ISO (1999) and the lactic bacteria according to ISO (1998).

Statistical analysis

Data related to fillet quality characterization were analysed using the General Linear Model procedures of the statistical analysis software SAS (2004) for Windows.
A two-ways ANOVA tested the diet (D, with two levels: Control and MixOil diets) and the day of storage (S, with three levels: 1st, 4th and 7th day of storage) as fixed effects. The interaction diet x day of storage (D x S) was also tested.
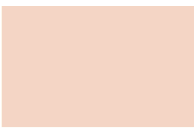
Growth performance

During the whole trial, the rearing conditions remained within a range optimal, for the fish growth. The mean dissolved oxygen (DO), measured in the tank water outlet, was 8.10 mg/l ± 1.03 and the water temperature fluctuated close to 12.58 °C ± 0.78. The maximum rearing density in the experimental tanks, was limited to: 21.4 kg/m^3 during the first stage, 41.4 kg/m^3 in the second one, and 37.4 kg/m^3 in the third growth stage. Fish live weight, increased from 9 g to 1000 g, within the 51 weeks period on the test diets. Similarly, the total biomass increased a hundredfold. Survival at the end of the trial, was high and no relevant differences were noted amongst the diets, in mortality rates. Limited differences in terms of total biomass are observed, between the control tank and the MO treated tank one, as show in the Figure 2.

The overall Feed Conversion Ratio (FCR) and Specific Growth Rates (SGR), were very similar but the MO group, showed a faster growth ratio and a better feed conversion, in the whole trial period. In the first stage the fish, 6–8 months old, show similar performances and the gap between control and treated group, become more evident in the second stage, when the fish are 30–150 g and 8–12 months old. On the other hand, the control group partially makes up, for the gap in the third stage.

Gut microbiota

The characterization, of the gut microbiota 16S rRNA gene community profiling (using the V1-V3 hypervariable regions), was carried out using Roche 454 GS-FLX Titanium and produced 186.791 high-quality sequences. The primer set employed in this study, prevented overestimation of species richness, which was observed previously by Youssef et al. (2009), by using primers targeting the V1-V2 regions of 16S rRNA. Average sequence lengths, were 501 nt (length SD 56 nt). Over the course of the intervention period and especially between growth stage 3 and the earlier growth stages, microbiota species richness and diversity, increased significantly over time (observed OUT's, Chao 1 and Shannon Index), with P value < 0.05 (Figure 3). The only exceptions was at T6, two weeks after the initiation of the growth stage 3, or finishing diet. Over the following 24 weeks, alpha-diversity increased dramatically in both groups of fish, irrespective of the presence of essential oil. A similar shift in beta-diversity, Unweighted UniFrac measure, of phylogenetic

distances between the gut microbiota of individual trout from, diets C and MO, over time-points T3 to T7, clear separation of samples from T7, upon principle components analysis (PCoA), with PC 1 explaining 41.6% of the variation, within the dataset and clearly separating T7, from the other time points (Figure 4). The second and third components of the PCoA, show a separation of microbiota phylogenetic distances, roughly according to time, from T3 (week 18) to T6 (week 32) and T7 (week 51). Examining changes in relative abundance, at different phylogenetic levels, according to diet and time, no significant differences were observed, between the control diet and MO diet. However, the distribution of predominant bacteria at the Class level, with respect to sampling time, revealed significant changes in dominant members of the Actinobacteria, Bacilli and Clostridia within the Firmicutes, and the α- and γ-Proteobacteria into gut of over the course of the experiment (Figure 5). After the 51st week (T7), the microbiota appeared to reach its maximum diversity, in terms of species richness, as observed by the number of OTU. Up until T6, or week 32 after the initiation of the dietary intervention, the gut microbiota of all fish, was dominated by the Corynebacteria, a member of the Actinobacteria Phylum. The fish were about 21 months of age and weighted about 350g at this stage. They had undergone shifts in feed at week 25. Two weeks later, all animals were placed on a diet lower in protein, with three times the dietary fiber. By week 51, the microbiota had experienced radical alterations both in α- and β-diversity. Major shifts in relative abundance, were also observed and manifest as statistically significant changes in *Actinobacteria*. Genus *Corynebacteria* decreased dramatically, becoming a sub-dominant genus, concomitantly with a small, but significant increase in genus *Propionibacterium* (Figure 6). Within the Firmicutes, genus *Bacillus*, *Lactobacillus*, *Carnobacterium*, *Pediococcus* and especially *Oenococus* all increased significantly in relative abundance. *Oenococcus* became a dominant member of the microbiota, representing about 15% of the microbiota. Changes occurred in both the *α*- and *γ-Proteobacteria*. *Enterobacteria* and *Pseudomonas* increased in relative abundance to 6.2% and 4.5%, respectively, and a small, but significant increase in *Serratia* relative abundance, was also observed within the *γ-Proteobacteria*. *α-Proteobacteria* became the dominant class of bacteria in the fish gut, with several predominant genera: *Acetobacter* (1.1%), *Gluconacetobacter* (30.1%), *Sphingomonas* (19.1%) and *Glucobacter* (11.3%) and is in start contrast to the Actinobacteria dominated gut microbiota of the juvenile animal.
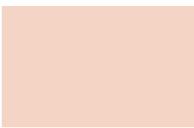
Fillet quality characteristics

Dietary supplementation with Mixed Essential oils (MO diet) did not significantly affect the fillet yields (Fig. 7). The storage time but not the test diets significantly increased drip loss ($p < 0.01$) (Fig. 8). The two groups of fish had a different behaviour during the storage, since differences were detected only over storage time in relation with diet (Fig. 8): fish fed control diet showed higher water losses from the 1st to the 4th storage day, while fish fed MixOil diet released a greater amount of water at the 7th day.

The muscle colour measurements did not show significant differences between fish fed two diets, except in the case of values registered at caudal region (Tab. 2;). In this site, fillets from MixOil had higher L* and Hue and lower redness index (a*) values, presumably due to the influence of the diet in pigmentation and indicating a tendency towards a slightly more yellowish shade. In all the sites where the measurements were carried out, the lightness was systematically higher in MixOil fillets. The storage time markedly influenced the behaviour of the colour parameters, that highlighted at the 4th day of storage L* values significantly ($p < 0.001$) higher, while a* and b* values, chroma and hue were lower than in the other storage days.

Concerning textural parameters (Tab. 3), no differences were found between diet groups except for hardness. Fillets from MixOil were softer than those from fish fed control diet. No differences were detected over the storage time but by the significant interaction resulted for cohesiveness and adhesiveness showed a different trend during storage in relation with diet, in the case of these textural parameters (Fig. 9).

No significant differences, were found in lipid content, between the diet groups (5.32 vs 5.57%), while there were significant differences in MDA content, between control and MixOil (1.230 vs 0.917 mg/kg of sample) fish. The lower MDA values, registered in the MixOil group, could suggest that the essential oils, included in feed, exerted an antioxidant effect, preventing the MDA formation. However, no significant differences were found, over storage time, in MDA content. As shown in Tab. 4, the tocopherol isomers α- and γ- were detected in fillet samples and α-tocopherols, generally, the most widespread in marine lipids (Parazo et al., 1998; Ortiz et al., 2013), were the most abundant in the muscle. The amount of tocopherols was similar to what was found by other authors on rainbow trout (Baron et al., 2009; Ortiz et al., 2013; Timm-Heinrich et al., 2013).
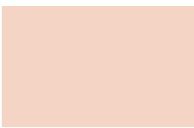
Differences between diets, were found in α-tocopherol, and fish fed MixOil showed

a significant lower amount of α-tocopherols. The amount of α- and γ- tocopherol significantly differed in samples analysed at different storage time.

No differences were found in pH values between the groups differently fed (Table 4) while this parameter significantly (p < 0.01) increased from the 1st to the 4th storage day and decreased from the 4th to the 7th storage day. In this study, the antibacterial effect attributed to the essential oils supplemented to the farmed animals diet (Govaris et al., 2007; Nieto et al., 2010; Álvarez et al., 2012) was not evidenced, since no significant differences were detected in microbial growth between the two groups of trouts (Tab. 5). Consequently the storage of fillets on ice, the CMT growth was significant for both groups of fish and yielded values of approximately 2.0-4.5 log cfu g^{-1}, while the variations of the Enterobacteriaceae and Pseudomonadaceae counts with the storage time were not significant (Tab. 5). By the microbiological characterization, resulted that in fish fed control diet, the type of Enterobacteriaceae detected at the 4th day of storage, were *Pantoea spp.*, while in fish fed MixOil were *Pantoea spp.* and *Hafnia alvei*. At the 7th day of storage the type of Enterobacteriaceae was *Pantoea spp.* and the type of Pseudomonadaceae was *Pseudomonas fluorescens*. No pathogens and lactic acid bacteria were detected in the fillet samples.
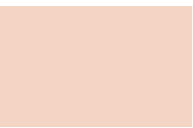
6.4 Conclusions

In this study, we examined the impact of a novel feed for rainbow trout, containing 200 ppm GrowNat-MixOil Aqua (an essential oils blend), compared to standard commercial feed alone as control, on fish production yield over 51 weeks, fillet quality and the composition of the intestinal microbiota. There is a clear and present need for improved aquaculture practices and feed innovation, to meet the food security and sustainability demands of the 21st century. Essential oils, have a long history as functional ingredients, in animal production, providing useful alternatives to antibiotic growth promoters, now banned in the EU, improving production animal yield and health, and reducing infectious disease, through pathogen inhibition. They have also been shown to impact on meat quality, including physiochemical characteristics of fish fillet texture, colour and taste, often through their antioxidative activities. In this study, although supplementation with essential oil mix, did not mediate any obvious change in fish yield (neither biomass nor calculated mean weight), it did improve trout fillet quality. The MixOil diet had a modest, but statistically significant influence, on certain qualitative parameters of fillets. FY, pH, WHC, colorimetric attributes in dorsal and ventral region, cohesiveness, resilience, gumminess and adhesiveness, lipid content and spoilage microorganisms, were not influenced by diet added with antioxidants. Fillets from fish fed MixOil, resulted in improved oxidative stability, since the MDA content was lower than the fish fed Control. Oxidative stability is an important physiochemical chatacteristic for fish storage, especially fish fillets, as it impacts significanty on organoleptic properties. They also had a smaller tendency to loose more water from the 4th to the 7th storage day at 1 °C, that can be considered a positive aspect for marketability. MixOil fillets, also showed higher tenderness and cohesiveness and lower adhesiveness, by increasing storage time, again important product characteristics for fillet quality. The intestinal microbiota is emerging, as an important metabolic and immune regulator, in all animals with an organized intestine. In trout, little work has been done, but it is recognized that the gut microbiota, plays an important role in immune function and education, contributing to resistance to pathogenic challenge (Balcázar et al., 2007; Araújo et al., 2015). Similarly, interactions between the animal diet or feed and the gut microbiota in early life, can have important implications on animal metabolism, especially insulin resistance (Geurden et al., 2014). A number of recent studies have identified the potential of using probiotic microorganisms in feed, to impact on trout production yields, protection from infection, antioxidant status and overall health (Ramos et al., 2013; Schubiger et al., 2015; Ghosh et al.,

2016). Essential oils are recognized for their anti-microbial activities and for their ability to impact both on metabolic and immune health in different production animal species. Recent studies have identified essential oils as potential bioactive feed ingredients and alternatives to antibiotic growth promoters, in aquaculture. Certain essential oils, have been shown to inhibit a number of important trout pathogens in vitro (Menanteau-Ledouble et al., 2015). However, few studies have examined their impact on trout during production. In the present study, we used 454-pyrosequencing of amplified V1-V3 regions of the 16S rRNA gene, present within the intestinal microbiota of farmed trout, over a 51 week dietary intervention, with either MO (mixed essential oil) supplemented feed or a control feed (standard feed without MO addition). The diets were iso-energetic and iso-lipidic, with fish oil replacing MO in the control diet. Although, no statistically significant effect of the dietary treatments, on the trout intestinal microbiota, was observed, the gut microbiota changed dramatically from juvenile stage (< 500 g body weight) to mature adult stage (> 500 g body weight). The microbiota of the juvenile fish, was characterized by relatively lower species richness and evenness, and was dominated by the Actinobacteria phylum, particularly the *Corynebacterium* genus. This bacterial genus comprised more than 50% of the resident intestinal microbiota in fish, up to the size of about 350 g of weight. However, in adult fish (about 1 Kg of weight) *Corynebacterium* and, more generally, the Actinobacteria phylum, comprised a sub-dominant minority of the intestinal microbiota, thus confirming recent work by other authors, who monitored the intestinal microbiota of rainbow trout using next generation sequencing (Wong et al., 2013). We showed that the adult fish microbiota was dominated by *α*- and *γ-Proteobacteria*, in good agreement with previous studies (Wong et al. 2013; Ingerslev et al. 2014).

In our study, the addition of essential oil to the standard rainbow trout feed, did not impact on the composition of the gut microbiota, or the relative abundances of the dominant genera either in juvenile, nor adult fish. However, we did not examine the impact of gross dietary change, e.g. changes in the relative proportions of protein, fat or carbohydrate in the animal feed or the type and quality of protein (e.g. animal/fish meal v vegetable protein). Gross dietary composition, has recently been shown to impact significantly, on the microbial ecology of the adult rainbow trout gut, and also impact on its contribution to host immune or anti-pathogen defences (Ingerslev et al. 2014). Similarly, rearing conditions, such as water quality and density of rearing, have also been shown to impact on animal health, physiology and gut

microbiota in farmed fish species (Giatsis et al., 2015).

In summary, although addition of essential oil (GrowNat-MixOil Aqua), at 200 ppm to commercial rainbow trout feed, did not impact significantly on production yield or on the intestinal microbiota of either juvenile or adult fish, it did have subtle effects on trout fillet physiochemical characteristics, including resistance to oxidative damage and reduced weight loss during storage, two important parameters, which determine product shelf-life and susceptibility to spoilage. High resolution 16S rRNA profiling, of the rainbow trout gut microbiota, identified significant differences in composition between, juvenile and adult fish, but further studies are required to delineate the relative influence of age, gross dietary composition and rearing conditions, on the successional development of the gut microbiota in rainbow trout and how it impacts on animal health and final product quality.
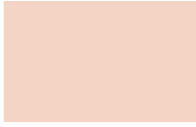
6.5 Figure & Table

	T3	T4	T5	T6
T4	0.0406	-	-	-
T5	0.0174	0.5291	-	-
T6	0.9307	0.0406	0.0054	-
T7	0.0054	0.0085	0.0043	0.0043

Table 1. Observed OTU: pairwise comparisons using Wilcoxon rank sum test. P value adjustment method: fdr.

	DIET (D)		DAYS OF STORAGE (S)		
	Control	MixOil	1	4	7
Nr of fish	15	15	10	10	10
Dorsal site					
L*	26.85	30.23	18.99 A	44.75B	21.87A
a*	17.95	16.49	22.17B	7.23A	22.25B
b*	16.37	19.13	20.95B	7.51A	24.78B
Chroma	24.70	25.45	31.12B	10.46A	33.64B
Hue	42.59A	48.97B	43.29	45.75	48.29
Ventral site					
L*	30.05	32.65	20.03A	44.7C	29.32B
a*	20.94	18.29	27.53C	9.39A	21.93B
b*	23.59	21.03	28.47B	8.59A	29.86B
Chroma	31.90	28.00	39.99B	12.74A	37.11B
Hue	46.33	47.83	45.86A	42.47A	52.90B
Caudal site					
L*	24.35A	28.53B	18.90A	41.17B	19.25A
a*	22.38B	19.44A	25.91B	10.31A	26.51B
b*	20.21	22.92	24.47B	9.25A	30.98B
Chroma	30.50	30.23	36.15B	13.86A	41.08C
Hue	41.66A	47.87B	43.21A	41.9A	49.19B

Table 2. Colour parameters measured at three sites of fillets from Control e MixOil fish at three times of refrigerated storage. [1] Residual Standard Deviation. Different superscripts in the same line, within criterion, indicate significant differences. ns: not significant.

P VALUE			RSD[1]
D	S	D x S	
Dorsal site			
ns	<0.001	ns	5.08
ns	<0.001	ns	3.82
ns	<0.001	ns	5.16
ns	<0.001	ns	5.20
<0.05	ns	ns	7.04
Ventral site			
ns	<0.001	ns	4.08
ns	<0.001	ns	3.80
ns	<0.001	ns	5.96
ns	<0.001	ns	6.22
ns	<0.001	ns	5.28
Caudal site			
<0.05	<0.001	ns	4.21
<0.05	<0.001	ns	3.86
ns	<0.001	ns	4.88
ns	<0.001	ns	4.72
<0.05	<0.05	ns	6.21

	Diet (D)		Days of storage (S)		
	Control	MixOil	1	4	7
Nr of fish	15	15	10	10	10
Hardness (N)	7.00B	5.98A	6.33	6.76	6.39
Cohesiveness	0.29	0.31	0.30	0.29	0.30
Resilience	0.06	0.07	0.06	0.07	0.07
Gumminess (N)	2.05	1.88	1.95	2.01	1.93
Adhesiveness (Nmm)	0.77	0.94	1.10	0.74	0.72

Table 3. Texture parameters of the fillets from Control e MixOil fish at three times of refrigerated storage. [1] Residual Standard Deviation. Different superscripts in the same line, within criterion, indicate significant differences. ns: not significant.

	Diet (D)		Days of storage (S)		
	Control	MixOil	1	4	7
Nr of fish	15	15	10	10	10
MDA	1.230B	0.917A	1.009	1.121	1.090
α-tocopherol (1)	22.87B	20.46A	25.29C	18.78A	20.93B
γ-tocopherol (1)	5.23	5.46	5.51B	4.77A	5.77B

Table 4. MDA (mg/kg of muscle) and tocopherols content (mg/kg of muscle) of the fillets from Control e MixOil fish at three times of refrigerated storage. [1] Residual Standard Deviation. Different superscripts in the same line, within criterion, indicate significant differences. ns: not significant.

	Diet (D)		Days of storage (S)		
	Control	MixOil	1	4	7
Nr of fish	15	15	10	10	10
pH	6.31	6.22	6.160A	6.420B	6.230A
CMT*	2.059	2.306	0A	2.092B	4.456C
Enterobacteriaceae*	0.372	0.656	0	0.580	0.962
Pseudomonadaceae*	0	0.325	0	0	0.488

Table 5. pH and microbiological characteristics of the fillets from Control e MixOil fish at three times of refrigerated storage. [1] Residual Standard Deviation. Different superscripts in the same line, within criterion, indicate significant differences. ns: not significant.

P VALUE			RSD[1]
D	S	D x S	
<0.05	ns	ns	1.14
ns	ns	<0.05	0.03
ns	ns	ns	0.01
ns	ns	ns	0.43
ns	ns	<0.05	0.70

P VALUE			RSD[1]
D	S	D x S	
<0.01	ns	ns	0.285
<0.05	<0.001	ns	3.189
ns	<0.001	ns	0.905

P VALUE			RSD[1]
D	S	D x S	
ns	<0.01	ns	0.148
ns	<0.001	ns	0.582
ns	ns	ns	1.062
ns	ns	ns	0.547
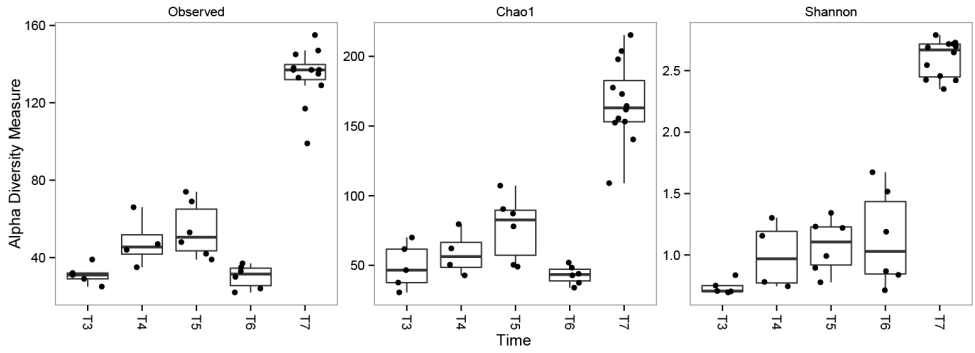
Figure 1. Comparison of alpha diversity of fish samples from T3 to T7, regardless of diet (observed OTUs, Chao 1 and Shannon Index).
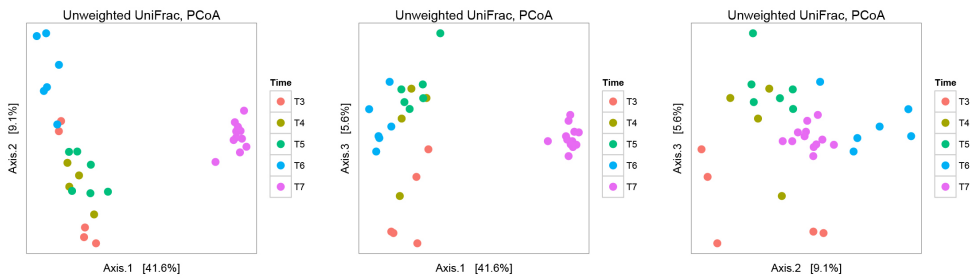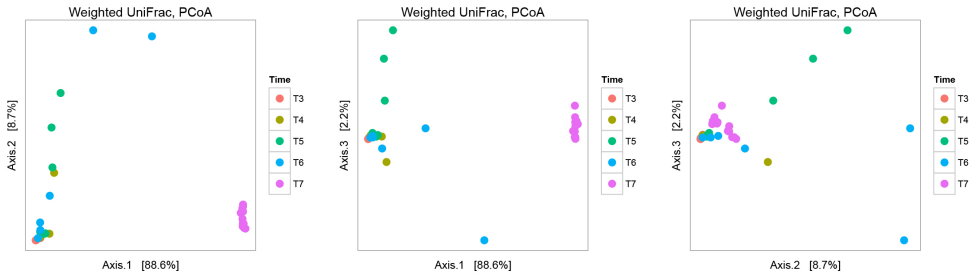

Figure 2. Beta diversity estimates of the rainbow trout gut microbiota. (A to C) Use of UniFrac to measure phylogenetic distances between the gut microbiotas of individual trout from all treatment groups. Unweighted UniFrac PCoA plotted against the PC1 versus PC2 axes (A), the PC1 versus PC3 axes (B) and the PC2 versus PC3 axes (C).
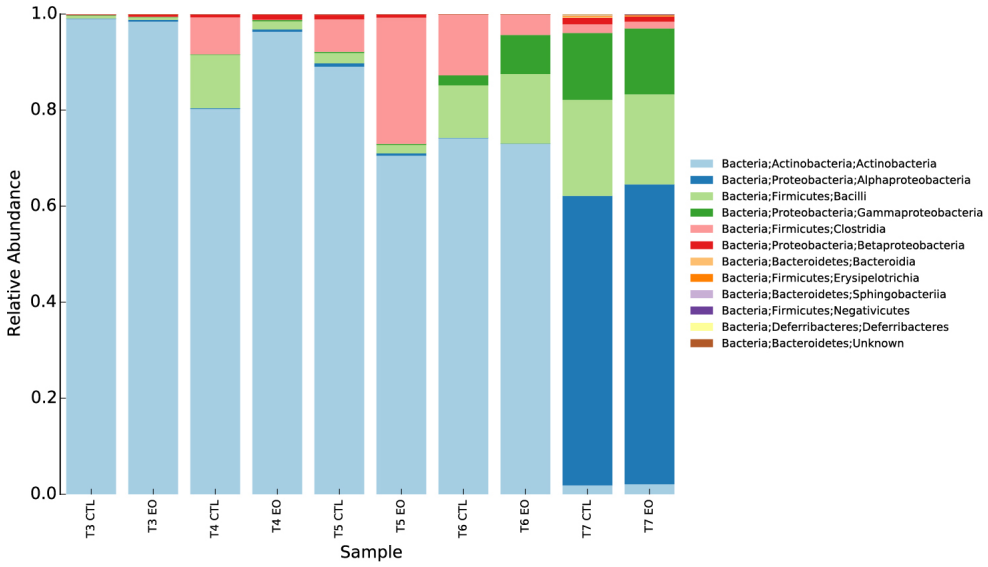
Figure 3. Relative abundance of classes identified in the intestinal tract of rainbow trout. The labels under each column indicate the sample names corresponding to fish tank with C and MO diets at different time (T3, T4, T5, T6 and T7). The legend includes only the 12 most abundant taxa.
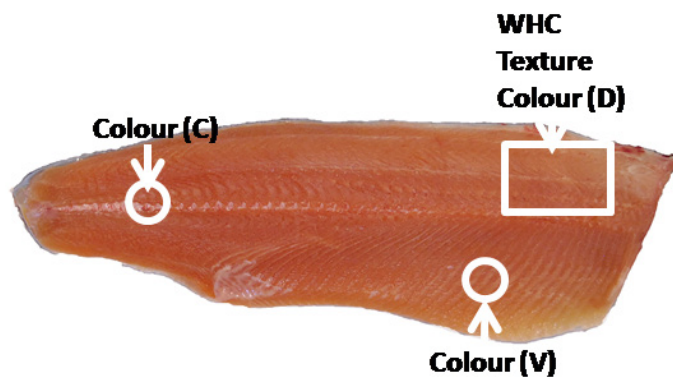
Figure 4. Sites where colour measurements were performed and where samples for the WHC and the texture analyses were taken.
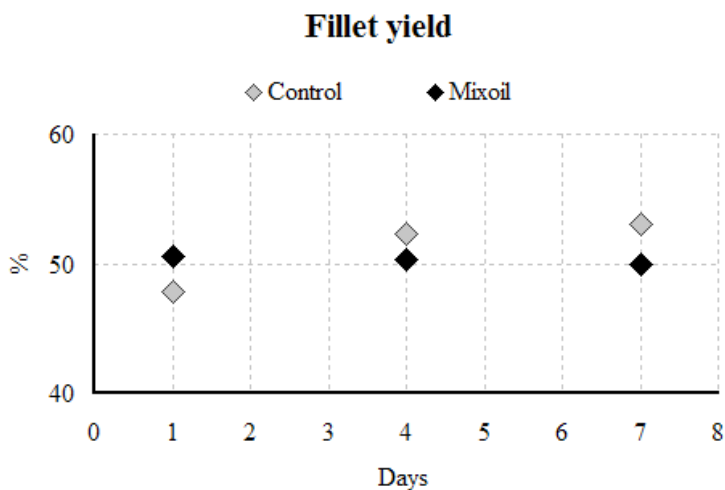
Figure 5. The fillet yield of fish fed two diets, analysed at different time during the storage.
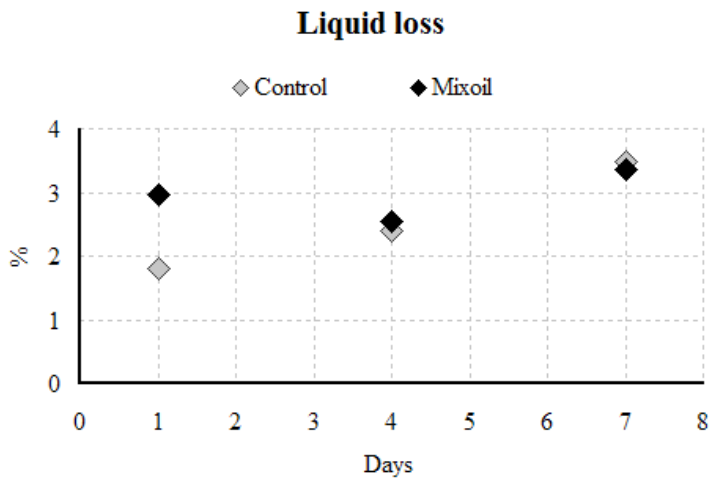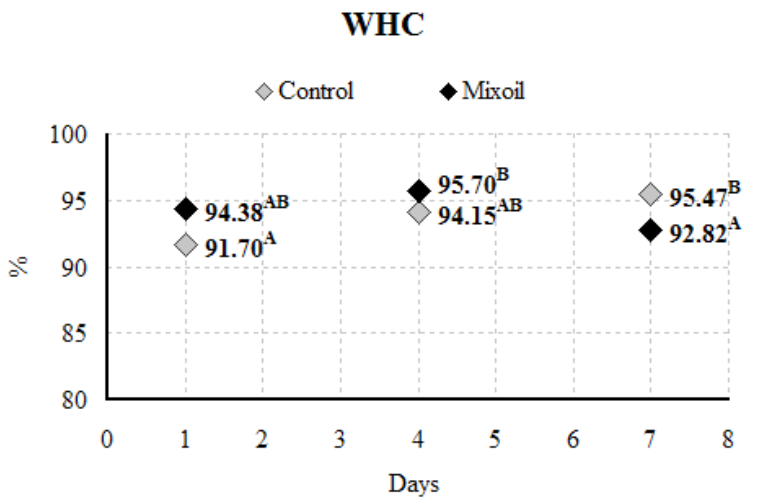
Figure 6. The drip loss and the Water Holding Capacity (WHC) of fillets from fish fed two diets, analysed at different time during the storage. Different superscripts indicate significant differences.
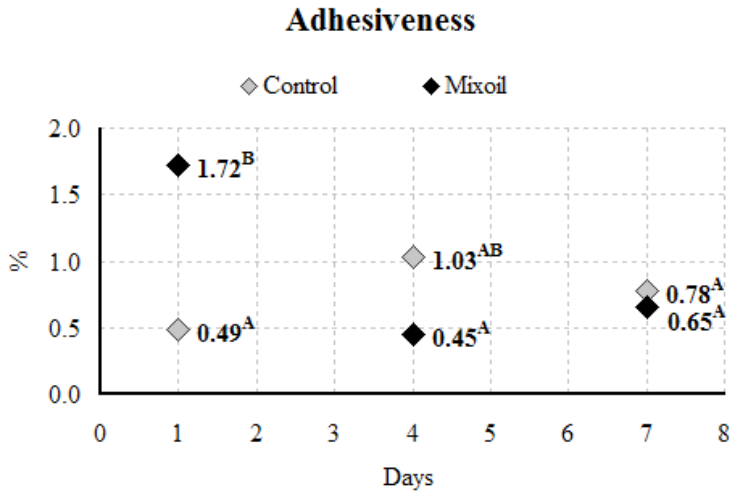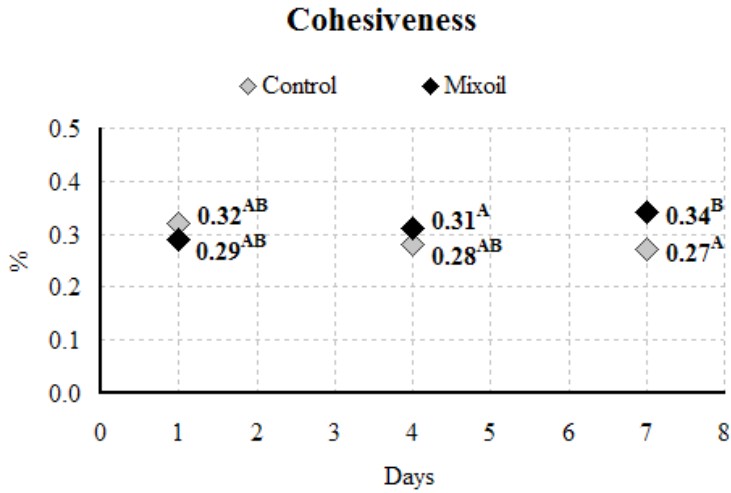
Figure 7. The cohesiveness and the adhesiveness of fillets from fish fed two diets, analysed at different time during the storage. Different superscripts indicate significant differences.

6.6 Supplementary Material

Figure 1. Beta diversity estimates of the rainbow trout gut microbiota. (D to I) Use of UniFrac to measure phylogenetic distances between the gut microbiotas of individual trout from all treatment groups. (D, E and F) Weighted UniFrac PCoA plotted against the PC1 versus PC2 axes (D), the PC1 versus PC3 axes (E) and the PC2 versus PC3 axes (F). Along axis 1, accounting for 88.6 % distance show a separation between T7 (purple, n = 12) and others time (T3, T4, T5, T6, n = 21).
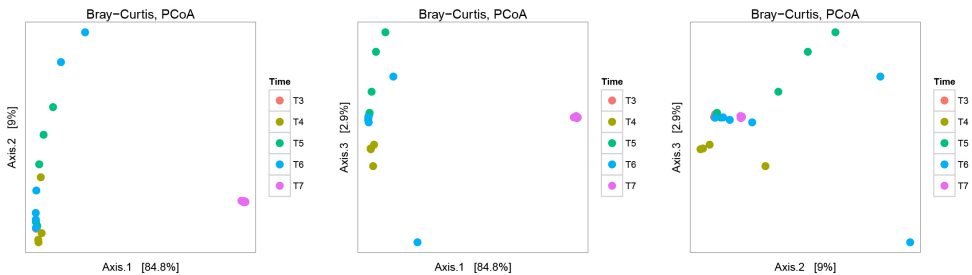
Figure 2. PCoA of rainbow trout microbiota, at different time points (T3-T7), based upon Bray-Curtis dissimilarity. Along axis 1, accounting for 84.8 % dissimilarity show a separetion between T7 (purple, n = 12) and others time (T3, T4, T5, T6, n = 21).
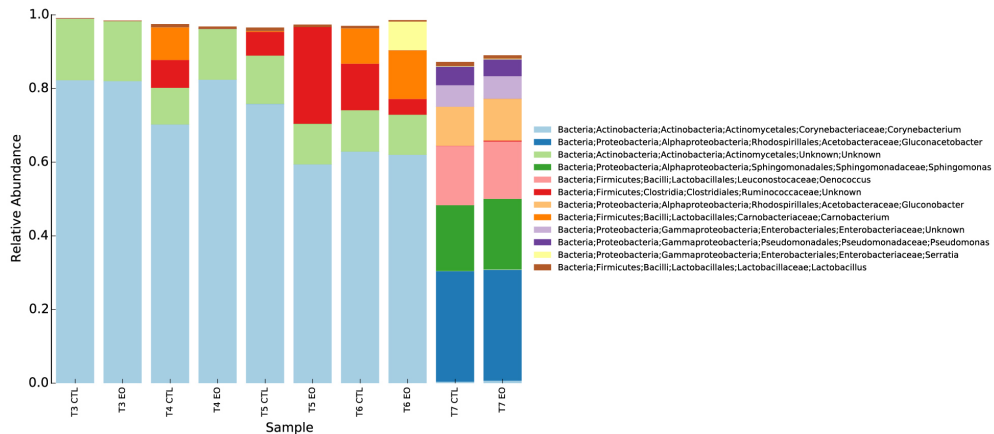
Figure 3. 16S rRNA gene sequences, at genus level, reveal similarities between intestinal microbiota of rainbow trout raised under different diet. Shown are relative abundances of bacterial genus in each sample. The labels under each column are sample names corresponding to fish tank with control and MixOil diets at different time (T3, T4, T5, T6 and T7). The legend includes only 12 taxa most abundant.
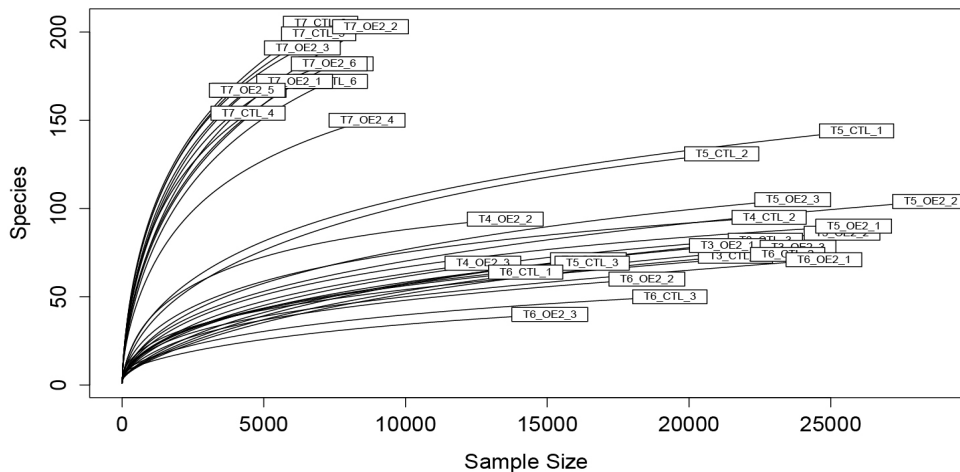
Fig. 4 Rarefaction curves of OTUs clustered at 97% phylotype similarity level. The samples were labeled respect to sampling time (T3, T4, T5, T6, T7), and treatments (C – control and MO – MixOil).

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Methods for study the human gut microbiota

7. Methods for study the human gut microbiota

Intestinal microorganisms constitute a rich ecosystem, with about 17 families, 50 genera and more than 1,000 species of bacteria. Their composition, which depends largely on environmental conditions, is also variable between individuals. A hidden ocean of bacterial diversity that had never been seen by cultivation, began to appear from microscopic observations, where the number of cells far outweighed colonies grown, on nutrient media agar plates. Many of these cells were shown to be metabolically active, even though they could not replicate on laboratory media. Additional evidence for the presence of bacterial taxa that cannot be grown in the laboratory, came from molecular tools, especially DNA based molecular tools, as resumed below (Tab. 4).

Technique	Description	Advantages	Disadvantages
Culture	Isolation of bacteria on selective media	Cheap, semi-quantitative	Labour intensive, <30% of gut microbiota have been cultured to date
qPCR	Amplification and quantification of 16S rRNA. Reaction mixure contains a compound that fluorescens when it binds to double-stranded DNA	Phylogenetic identification, quantitative, fast	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences -unable to identify unknow species
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize complementary nucleotide sequences. Fluorescence detected with a laser	Phylogenetic identification, semi-quantitative, fast	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons	Phylogenetic identification, quantitative, fast, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome	Phylogenetic identification, quantitative	Expensive, analysis of data is computationally intense

Tab. 4 Culture independent molecular techniques used to characterize the gut microbiota

7.1 Culture independent molecular biology techniques

Most molecular methods use the phylogenetic information present in the 16S rRNA gene, universally present across the kingdoms Bacteria and Archaea, which includes DNA regions conserved across different phylogenetic levels and hyper-variable DNA sequences regions, allowing species level classification. Typical molecular methods used to identify microorganisms present in an environmental sample or to enumerate microorganisms in a culture independent manner, are very briefly discussed below:

Fluorescent in situ hybridization (FISH)

Used for visualising phylogenetically known microorganisms in various environmental samples such as stool, clinical specimens and animal tissues. One of the advantages of this method is that it allows the analysis of intact microbial cells under the microscope or with the aid of flow cytometry. Another main advantage of FISH is that it is a quantitative method allowing accurate enumeration of microbial cells directly in samples. Based on the chosen probe, this technique can be used to detect and enumerate microorganisms at different phylogenetic levels down to species level in some cases. FISH allows the study of differential microbial abundance, it is rapid, quantitative and accurate but unknown species can not be enumerated or identified and in the case of 16S rRNA probes it can not be used to enumerate bacteria to the strain level (Amann et al., 1995). Other disadvantages of FISH are that hybridization efficacy depends on the permeability of target cells, the specificity of probes, the activity and abundance of ribosomes and 16S rRNA within the target cell (Swann et al., 2011) and the manual scoring and signal integration is difficult, time consuming and prone to operator error. Combining FISH with flow cytometry can overcome some of these problems.

Flow cytometry (FCM)

Represents a powerful tool to analyze intestinal microbiota especially when combined with FISH (Rigottier-Gois et al., 2003). It was first developed for counting and studying mammalian cells (Festin et al., 1987), but now it is commonly used to study the intestinal microbiota (Collado et al., 2008). The principle is that the cells are streamed through a capillary, during which a laser beam is used to detect the cells. The advantages of this technique are that it allows for the analysis of microbiota quantitatively and qualitatively, analyzing different parameters, such as the cell size, the metabolic state, and the density; it is time efficient and can proces smore than

10 000 cells/s, it does not require DNA extraction or amplification and it has high accuracy. The disadvantages of this technique are that it relies on liquid samples, since the size of bacteria cells is smaller than the mammalians this may affect the accuracy of the results, and so requires a complex data analysis (Bunthof, et al., 2002).

Quantitative polymerase chain reaction (q-PCR)

Based on the amplification of defined DNA sequences, quantitative PCR (qPCR) targeting specific groups of gut bacteria uses primers targeting phylogenetically informative regions of the 16S rRNA gene are commonly employed. The advantages of this technique are that it has a low detection limit (lower to 102 cell/g of faeces) and it allows large scale studies thanks to robotic manipulation of 96 well plates (Swann et al., 2011). However, the accuracy of the method is dependent upon the efficacy/reproducibility of DNA extraction and the presence/absence of PCR inhibitory compounds and PCR clean-up (Rastogi and Sani, 2011). It is also difficult to relate intensity signals to the real number of bacteria present in a given sample, since different bacteria possess different copy numbers of the target 16S rRNA gene. Combined, these limitations make qPCR generally less accurate than FISH for enumeration of bacterial taxonomic groups in intestinal/faecal samples.

Phylogenetic microarray

The comprehensive analysis of the diversity and dynamics of the gut microbiota composition is possible by microarrays targeting ribosomal RNA gene sequences (Kovatcheva-Datchary et al., 2009). The advantage of the phylogenetic microarray analysis is that constitutes high resolution of profiling of complex intestinal ecosystems with tens of thousands of individual phylogenetic probes targeted different species and groups of bacteria. This is a considerable advantage over q-PCR or FISH, which are labour intensive. The Human Gut Chip (HuGChip) is an example microarray designed and developed to analyze and characterise the human gut microbiota (Rajilić-Stojanović et al., 2009). Explorative probe design strategies coupled to the microarrays are well suited to survey complete microbial communities, including microorganisms with uncharacterized sequences (Dugat-Bony et al., 2012). The usefulness of standard microarray formats is often limited by hard to interpret signal patterns caused by an accumulated number of false-positive signals (Peplies et al., 2003).

Denaturing/temperature gradient gel electrophoresis (DGGE)

D/TGGE is a microbial fingerprinting technique that separates amplicons - of roughly the same size based on double stranded sequence melting properties across a gradient of denaturing chemicals (e.g. urea) or temperature. The main advantage is that it allows the seperation and visualization of DNA amplicons of different sequence but of the same length, allowing seperation of DNA species from a single PCR from mixtures of different microorganisms. It provides a quick way of looking at biodiversity in a microbial sample and does not require bacterial culture. Furthermore it does not require any specific sequence information before probe design, as primers targeting phylogenetically conserved regions of the 16S rRNA gene can be used to amplify even uncultured or un-classified microorganisms within phylogenetically related groupings. The disadvantages are that this is a qualitative assessment of biodiversity, technically challanging, often prone to low reproducibility and one must sequence DNA amplicons or bands seperated by D/TGGE to obtain any phylogenetic information, which for complex microbial consortia can be labour intensive. Another problem is the depth of coverage, as only a limited number of different amplicons can be seperated on any one gel, limiting analysis to the top few percent of microorganisms present within a given sample (Douterelo et al., 2014;.Rettedal et al., 2010).

Next Generation Sequencing (NGS)

The increasing use of NGS to analyze complex microbial communities, is due to lower costs of sequencing and to an ever greater development of bioinformatic tools essential for their interpretation and provide a better understanding than traditional culture based methods. Several technical variables can significantly impact results, such as DNA extraction protocol, amplification and purification, along with sequencing depth, which can limit the ability to accurately quantify proportions of microorganisms present in a given sample or to compare results between different laboratories. Bacterial 16S ribosomal RNA (rRNA) genes contain nine "hyper-variable regions" (V1 – V9), that allows the discrimination and then identification/classification of specific microorganisms in ecological samples (eg. organic material, soil). The length of the 16S rRNA fragment used to assign taxonomic identity increases the confidence of phylogenetic classification. However, none of the currently available technologies (e.g. Roche 454 GS-FLX, Illumina MiSeq, HiSeq

and Ion PGM), offer full-length (about 1600 bp) gene sequencing at sufficient depth to allow for multiplexing large numbers of samples on the same run to species level identification.

Bioinformatics Methods and Biological Interpretation for NGS Data

High throughput sequencing technologies have made it essential the development of bioinformatic tools capable of analyzing and processing efficiently huge data sets and enable biological interpretation of the results obtained. NGS pipelines were built to performs in a single workflow, with little to no impact on standard computing performance. Here are reported only two of these, QIIME, which is currently the most widespread and MICCA that was developed in Fondazione Edmund Mach (S. Michele all'Adige, Italy).

QIIME

Quantitative Insights Into Microbial Ecology (QIIME) is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. The software package is designed for microbial community analysis based on DNA sequence data, and provides a single analysis framework for analysis of raw sequence data through publication-quality statistical analyses and interactive visualizations. QIIME was originally developed to analysis of Roche 454 amplicon sequence data. In the latest versions workflows have been added to analyze data from different sequencing platforms, such as Illumina, and different types of data, such as shotgun data. The package contains many tools that enable the user to analyse and compare microbial communities, such as OTU picking, taxonomic assignment, and construction of phylogenetic trees from representative sequences of OTUs, and through downstream statistical analysis, allows to display and produce publication-quality graphics. It provides three high-level protocols for OTU picking. Described as de novo, closed-reference, and open-reference OTU picking (Navas-Molina et al., 2013).

MICCA

Microbial community analysis (MICCA), is a open-source pipeline that efficiently combines quality filtering, clustering of Operational Taxonomic Units (OTUs), taxonomy assignment and phylogenetic tree inference, providing accurate results

and reaching a good compromise among modularity and usability (Albanese et al., 2015). It can be used with any kind of reads obtained by different sequences as Roche 454 or Illumina. MICCA is composed by several executable Python scripts and wraps lots of external applications. Main features are: supports gapped alignment and IUPAC codes for primer trimming; quality filtering with sliding windows; denovo and reference-based clustering (Greengenes, Silva and UNITE QIIME-formatted databases are supported); denovo chimera filtering; taxonomy assignment with RDP classifier and BLAST+ (Greengenes, Silva and UNITE QIIME-formatted databases are supported); denovo and template multiple sequence alignment (MSA); phylogenetic tree reconstruction. Despite this new platform slightly underestimates the number OTU compared to QIIME, it is able to precisely reconstruct OTU composition of the samples analyzed and covers a similar variety of biological diversity. Moreover it showsa greater consistency in the results obtained, compared to QIIME (Albanese et al., 2015).

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7.2 Metabolomics

The molecular foundations of the gut microbial–mammalian cross talk is raising interest in the research world at both physiological and biochemical levels (Martin et al., 2012). Metabolites are the byproducts of cellular metabolism, (Villas-Bas et al., 2007), whose formation critically depends on enzymes responsible for the pathways in which they are involved. As intermediates of biochemical reactions, metabolites have an important role in connecting many different pathways in and across organisms. The metabolome is the complete set of metabolites associated with an organism, which is divided into endo- and exo metabolomics, covering intra- and extra-cellular metabolites, respectively; changes in the metabolome typically reflect gene and protein expression (Hollywood et al., 2006). An unspecified analysis of intracellular metabolites is called "metabolic fingerprinting" (Liang et al., 2015). The metabolomics is a research field which aims at comprehensive analysis of a metabolome. It provides a key understanding of cell and organism communication mechanisms, which play a crucial role in the symbiotic relationships between gastrointestinal tract (GIT) microbiota and the host (Putignani et al., 2014). Recently, some studies have highlighted the potential to combine culture independent microbiological techniques with biofluid metabolite profiling, linking bacteria within the gut microbiota with metabolic activities within the host animal at the systems level, through the creation of data matrices (Swann et al., 2011). Below a summary table of the main analytical techniques used (Tab. 5).

Method	Description	Advantages	Disadvantages
NMR spectrometry			
¹H - NMR	Analyses high energy and phospholipid metabolim to quantify amino acids, carbohydrates, fatty acids, lipids and phospholipids. Gives detailed structural information, particularly using 2-D-NMR of isolated metabolites, provides technical insights for characterizing the interactions of hosts and symbionts	Non-invasive solution, non-destructive, no-derivatization request, high precision, high temporal resolution, fast, sensitivity can be improved by magic angle spinning, low per-experiment cost, minimal sample preparation required, average analytical time of ten minutes, non-destructive, quantitative	Less sensitive than mass spectrometry, small chemical bias, low sensitivity, convoluted spectra, more than one peak per component, libraries of limited use to complex matrix, time - from few min to h depends on the strength of the magnet
³¹P - NMR	Quantifies high energy phosphates (e.g. ATP, phosphocreatine), phospholipid precursors and sugar phosphates		
¹³C - NMR	Quantifies metabolic fluxes of 13C-labelled precursors (e.g. 13C-labelled glucose fluxes through glycolysis), TCA cycle, pentose-phosphate cycle and de novo fatty acid synthesis		
HR - MAS - ¹H - NMR	In vitro tissue applications		
Mass spectrometry (MS)			
LC - MS	Full spectrum analysis of solutions with multiple metabolites	High sensitivity and specificity, full spectrum analysis, current gold standard for analyzing solutions	Derivatization/chemical modification needed, destructive to sample, not fully quantitative without appropriate standards
GC - MS			

Tab. 5 Common analytical techniques used in metabolomics

GC-MS

Mass spectrometry (MS) has played an important role in the development of methods for profiling of metabolites due to its selectivity and sensitivity. Gas chromatography coupled with mass spectrometry (GC-MS) is commonly used for total and targeted profiling and despite the high sensitivity, requires a more comprehensive sample preparation. In the study of intestinal microbiota, GC-MS Solid-Phase Microextraction (SPME), has been used to compare human faecal profiles during aging (Mayor A, 2014), to identify volatile markers of GI diseases (Probert et al., 2009), to evaluate the in vitro effect of prebiotics and probiotics (Vitali et al., 2012) or effects, after drugs administration, on the gut microbiota (Maccaferri et al., 2010). Its wide use is due to its rapid qualitative and quantitative analysis of metabolites, especially short and volatile molecules, present in biological samples and with the possibility to further improving the separation peak capacity through the addition of a second and orthogonal dimension of separation (Metz et al., 2007).

LC-MS

Liquid chromatography-mass spectrometry is used for both targeted metabolomics (allowing accurate quantitation of up to a few hundred specified metabolites) or untargeted metabolomics or metabolite profiling (which has the potential to qualitatively profile thousands of metabolites in a given sample). The untargeted metabolic profiling allows the detection and quantitation of candidate markers that can be used to describe an altered physiological state, growth condition, or intake of particular foods for example (Metz et al., 2007). High-performance liquid chromatography (HPLC) is the most widely used analytical separation technique for the qualitative and quantitative determination of compounds in solution. Although with less separation efficiency than capillary gas chromatography, the dominance of HPLC as a premier analytical technique is no accident. The applicability to diverse analyte types, from small organic molecules and ions to large biomolecules and polymers is one of the most prominent advantage. The coupling of HPLC to MS gave it an excellent separation capability with high sensitivity and specificity, as well as precision and robustness. Orbitrap mass spectrometer., which bring the specificity and confidence of high-resolution, accurate-mass (HRAM) data with liquid chromatography (LC), has begun to be widely used to investigate putative biomarkers of dietary intake in human studies (Ulaszewska et al., 2016). While Hydrophilic interaction chromatography (HILIC) provides an alternative approach to effectively separate

highly polar and ionic compounds and therefore suitable for samples that contain predominantly polar metabolites such as urine (Lei et al., 2011). Mass spectrometry is becoming a universal detection method for ionic or ionizable compounds with high speed, sensitivity, and selectivity. The developments of triple-quadrupole MS-MS, high-resolution MS (e.g. time-of-flight (TOF) and orbital trap), and hybrid MS (e.g. Q-TOF or ion trap–orbital trap) in combination with UHPLC and 2D-LC allowed the development and application for bioanalytical assays, multiresidue analysis of complex samples, and life science research (Fekete et al., 2014; Dillen et al., 2012).

NMR

Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive analytical tool that can be used to study biological fluids without intensive sample processing. Despite this, the low resolution and sensitivity which enables the annotation and quantification of only a limited number of low molecular weight molecules, in addition to being an expensive technique, limits its use (Jansson et al., 2009). 1H-NMR is especially attractive since protons, with high isotopic natural abundance (99.985%), are ubiquitous presence in organic compounds and its sensitivity is the highest among all the NMR observable nuclei (Hu et al., 2008). Recently, NMR-based metabolic profiling of biological fluids has emerged as a valuable tool to investigate the profiling of at least part of the mammalian metabonome and can monitor how it changes under different perturbations, such as nutritional studies (Savorani et al., 2013), human disease diagnosis (Emwas et al., 2013) and detection of altered metabolic activity of the gut microbiota (Le Gall et al., 2011). The physical connection of HPLC (or LC) and MS (LC–MS) to NMR (LC–NMR) increases the capability of solving structural problems of mixtures of unknown compounds (Silva Elipe, 2003).

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7.3 In vitro / in vivo models of the gut microbiota

In vitro models

Since the healthy intestine is not easily accessible for most research purposes, attempts have been undertaken to simulate the intestinal microbial ecosystem in vitro. Thus far, several authors have described successfully a continuous multi-stage culture system for simulating the microbial community in the large intestine (Miller and Wolin 1981; Veilleux and Rowland 1981; Gibson et al. 1988; Macfarlane et al. 1989). Currently available models of the large intestine range from simple batch fermentation systems to more or less sophisticated, well-controlled, single- and multi-vessel continuous bioreactor systems.

Batch cultures have the advantage of providing results within a relatively short time period of about 24–48 h (Gibson and Fuller 2000), but do not reflect the near stable in vivo conditions, even for such short incubation times (Rumney and Rowland 1992).

Continuous culture systems are, therefore, preferred, formed either of one or of more vessels, depending on complexity and/or purpose. A single stage chemostat simulating the human colon, was developed more then 20 years ago, mimicking the nutrient availability and environmental conditions extrapolated from our knowledge of the in vivo conditions (Allison et al. 1989). Several dynamic fermentation models have been developed in recent years, with the purpose to establish in vitro a relatively stable microbial ecosystem under physiologically relevant colon conditions. Continuous or semi-continuous flow systems, allow to replicate the ecological conditions of the human gut, thanks to studies performed by Macfarlane in the 90's. Continuous growth media flow, elimination of waste materials (mimicking the fecal expulsion), allows the maintenance of a high-like large bowel, microbial biodiversity. These in vitro model have been used to study a huge (pato)physiolical conditions, as dietary interventions, DNA transfer between microbial members of intestinal tract and pre-probiotic activities (Steer et al., 2000).

In vivo models

In human populations many factors are difficult or impossible to control, such as genotype variation, large interindividual variation of the gut bacterial species, and current and past environmental exposures. Animal models provide the ability to control most of these parameters. The dominant bacterial phyla of the gut microbiome found across a wide range of mammals are conserved. Despite this many species of bacteria found in mice are not present in humans (Ley et al., 2005), limiting the ability to directly extrapolate human data. One solution to this problem is to colonize a germ-free animal with human faeces. The species found in each animal after transplantation resemble the donor human microbiota, but the relative abundance of each taxonomic group IS altered to more closely resemble the typical microbiota of the recipient animal (Rawls et al., 2006). "Humanized animals" have been established by several groups in an attempt to study the human microbiome under controlled conditions (Gootenberg and Turnbaugh, 2011). Another animal model is zebrafish, which provides advantages in the study of the human gut microbiome, such as transparent body, allowing real-time visualization of fluorescently labeled microorganisms throughout the gut (Rawls et al., 2007), the possibility to perform chemical screens and genetic tests (Patton and Zon, 2001) for investigating host genes or signaling pathways that are regulated by members of the gut microbiota. Pigs have been proposed as model for studying the human gut microbiome since their diet is omnivorous and the digestive tract and physiological traits are similar to humans and with the possibility to humanize them as well (Pang et al., 2007). To discover the molecular underpinnings of host–commensal interactions ì insect models such as drosophila have sometimes been employed. Their rapid growth and high reproductive capacity allow to be cultivated on a large scale and are particularly suited to studies focused on ageing. Furthermore the microbiota combined with the vast collections of mutant flies can produce a model of symbiosis with enormous potential to reveal new insights into host–symbiont interactions at single gene the level (Douglas et al. 2011; Kostic et al., 2013).

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Conclusion

There are many potential signaling mechanisms by which gut microbiota could influence human (patho)physiology, including through microbial metabolism and production of several bioactive-compounds (e.g. SCFA, 3-indoxyl sulphate, TMA, serotonin). The study shown here has sought to shed light, through a multidisciplinary approach, on the interconnected dynamics governing the fermentative activity of the intestinal microbiota in the distal colon and to identify the metabolic profiles generated in order to understand the possible impact on the body human.

Inulin modulates microbial metabolism of digested red meat
In support of early in vitro studies from the 1980's, the prebiotic inulin improved protein metabolism by the faecal microbiota under simulated distal colon environmental conditions, by increasing the consumption of amino acids, such as tryptophan and phenylalanine, but at the same time reducing the production of potentially toxic metabolites such as TMA, 5-aminopentanoic acid (cadaverine pathway) and phenol. Shifting relative abundance in favour of *Butyricicoccus*, *Butyricimonas*, *Coprococcus* and *Dorea* in detriment of other genera, such as *Blautia* and *Lachnospiraceae incertae sedis* and *Clostridium cluster XVIII*, have been observed. However, there was considerable inter-individual variation in response to either digested meat or inulin between faecal donors, limiting statistical strength of observations. Moreover, inulin did not appear to increase the relative abundance of bifidobacteria, suggesting a greater effect of pH than substrate availability in determining the relative abundance of different bacteria within the distal colon. Several factors are involved in the onset of a predominant macronutrient-utilizing organisms, such as compounds related to partial degradation products, fermentation products and growth or inhibitory factors, which arise not only by the presence of specific compounds, but also competitive or synergistic interactions between two or more bacterial populations. Many microbial communities show significant division in metabolic work. End products of some organisms are metabolites for others; working together they turn raw resources into fully metabolized nutrients. The enteric microbiota play also a major role (largely through enzymatic deconjugation) in re-absorbing many compounds excreted in bile and eliminate them via the urine or recycle back into the bile. Such analysis of diet microbial metabolism would allow researchers to piece together a metabolic network that goes beyond genus boundaries. Therefore, combined analysis of the metagenome and metabolome enable estimates of how metabolites are transferred and transformed through a community. Fermentable carbohydrates (such as Inulin) may decrease the concentration

of putrefactive compounds that are generated during proteolytic fermentation by providing the microbiota an alternative source of energy. Although in vitro batch culture provide a technically simple way of studying complex biological systems, they give us the opportunity to correlate ecological niches and metabolic activities with particular phylogenetic groups among the microbiota of the human bowel.

Measuring the impact of the prebiotic inulin on gut microbiota tryptophan metabolism

The role of tryptophan metabolites in intestinal and systemic (patho)physiology is still poorly understood. In the TRP fermentation with inulin supplementation, the amount of BCAA's such as valine, leucine, and isoleucine, were reduced compared to TRP alone, while there was an increase of lactate, acetate and ethanol. BCAA may have been consumed by a more metabolically active microbiota in the presence of the fermentable substrate inulin or alternatively, less BCFA may have been produced due to an energetic preference for carbohydrate fermentation over amino acid fermentation. Both TRP and TRP with inulin added fermentations, modulated the microbial diversity within the fermentations over time and, at the genus level *Alistipes*, *Barnesiella*, *Clostridium IV*, *Desulfovibrio*, *Odoribacter*, *Oscillibacter*, *Phascolarctobacterium* had significantly lower relative abundance ($p < 0.0011$) in the presence of inulin, compared to TRP alone. This in vitro study confirms that the presence of a fermentable carbohydrate reduces microbial metabolism of TRP into indole metabolites redirecting it towards microbial biomass formation. Diet:microbe interactions in the distal colon may therefore play an important role in the bioavailability of TRP and its indole derivatives possibly impacting on TRP pathways linked to host health in the gut, periphery and along the gut:brain axis. TRP bioavalability and conversion into indole metabolites in particular appears to have important consequences for human health, impacting on the pathophysiology of immune and metabolic diseases, including obesity and on brain function. Inulin had a dramatic impact not only on the production of indole TRP catabolites, but also apparently on the bioavailability of TRP itself. Both were reduced in the presence of the fermentable carbohydrate, indicating that diet:microbiota interactions might play an important role in TRP availability and associated biological activities in vivo. Similarly, this study also supports the recent findings that pH as well as the type and quantity of growth substrate can have a dramatic impact on both the composition and metabolic activity or growth of bacteria and in this case illustrating that the bifidogenic effect of inulin may not be as obvious in the distal colon as it is in the proximal or

transverse colon.

Measuring gut microbiota metabolism of neuroactive compounds

Metabolites produced by the faecal microbiota under simulated distal colon conditions were measured for selected neuroactive compound (dopamine, tyrosine, tryptophan, phenylalanine, glutamate and methionine). Considerable inter-individual variation in response to different fermentation substrates was observed, with the faecal microbiota of different donors showing different metabolite production profiles. This may account for the lack of statistical significance of changes within microbial relative abundance and alpha – or beta – diversity within and amongst the fermented substrates. However, some differences were noted in terms of absolute abundances using FISH. Indeed, for the aromatic amino acids and dopamine, changes in metabolite profiles appeared to cluster into two distinct groups suggesting the existence of metabotypes or signature metabolic responses dependent on microbiome structure. Intriguingly, comparative analysis of 16S rRNA community structures between these two "metabotypes" revealed statistically significant differences between important groups of gut bacteria at the family level, including organisms already suspected of forming alternative community structures within the human microbiome, specifically the *Prevotellaceae* and *Bacteroidaceae*. Nutrition has an important impact on the gut:brain axis. Some neurotransmitters, essential molecules for the brain functioning, are derived from essential amino acids, which in turn are freed by breakdown of dietary protein. Gut bacteria modulate various host metabolic reactions and have the capacity, through digestion and fermentation of food, to generate many neurotransmitters and neuromodulators. These metabolites released through gut lumen may induce epithelial cells and modulate neural signaling acting directly on primary afferent axons or indirectly within the enteric nervous system. Physiologic factors that influence brain pools of these amino acids, notably diet, influence their rates of conversion to neurotransmitter products with functional consequences. Using NMR based metabolomics with multivariate data analysis, this study aimed to confirm the importance amino acid fermentation in the distal colon to production of neuroactive compounds and also the highlight the utility of using metabolomics in examining questions related colon fermentation by gut microbiota and metabolome level changes. Although this is only an in vitro study, with its natural limitations, it offered food for thought on how gut microbiota could be involved in the metabolism of these compounds, and how it could affect human physiology. We believe this to be the first step for screening small molecules

that can provide effective modulation of metabolic pathways and maybe neuronal activities. Through future metatranscriptomics analysis we could identify microbial expression profiles related with a specific catabolism and further understand amino acid breakdown by the gut microbiota.

Growth, gut microbiota and fillet quality of rainbow trout

In this experiment we evaluated the effects of diet on the gut microbiota of farmed trout over the course of production and in response to a dietary intervention with essential oils. During production, animal diet changes according to life stage, with a reduction of 10% of protein from the young (<3 months) to adult fish, with a shift from fish protein flour to soybean and vital wheat gluten vegetal proteins, while carbohydrates increased by 7 % and fats by 3%. Previous metagenome-based fingerprinting and high-throughput sequencing on bacterial 16S rRNA genes indicated that fish gut was dominated by *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes* and *Firmicutes*. Strong impact of diet on the gut microbiota of an important human food production species, suggests that gut microbial ecology may be an important factor to consider when designing new and improved fish production systems or diets. Importantly, this study showed clear differences in the gut microbiota composition and diversity between juvenile trout and adult trout, with the later having signficiantly higher alpha- and beta-diversity and a microbiota dominated by *Proteobacteria*, while the microbiota of young animals was dominated by *Actinobacteria*, mainly *Corynebacterium*. The high inter-individual variability reduces our ability to evaluate the effect of mixoil on the microbiota and develop microbial species to be used as biomarkers (in every time point-condition we have an outlier). This high variability suggests to preselect the animals for further analyses based on a proven zootechnical effect of mixoil, on growth performance parameters or on markers of inflammation and intestinal mucosa integrity. The juvenile gut microbiota observed was lower in species diversity with respect to the other published studies on the effects of plant diet, yet biodiversity increases in some animals at later timepoints (T6). We can speculate that further investigation should focus on mature animals exposed to a more complex diet. Finally our results show the potential of this animal model to study the host–microbiota interactions, using a metagenomics approach to evaluate the interaction of environment, genetics, and microbiota in health and disease.

Only certain nutrients are absorbed by the gut, which is a selective system able through this control, to exchange chemical signals and information with the human

body. The gut acts as a signal transducer, a neuroendocrine sensor, and an immunological recognition and presentation system. The gut microbiota play a vital role in converting the foods we eat into nutrients and also bioactive compounds with activities both within the gut and systemically. By leveraging the knowledge gained through these studies, we may understand the interactions which bind diet and microbe and improve human health thanks to improved or in-formed dietary advice and/or new functional foods. Furthermore by levering the gut:brain axis through dietary modulation of the gut microbiota it may be possible to increase mental health, regulate brain development and improve cognitive function. Future studies need to focus on discovery and understanding at the functional level of specific microbial pathways and products that contribute to our physiology and that may contribute to disease processes. The exploration of gut microbiota contributions to human host physiology and diseases represents an incredibly exciting and potentially fertile territory in biomedical research. These findings have important consequences for our understanding of nutritional specialisation amongst human colonic bacteria and for predicting how dietary composition, including the addition of prebiotics, can be used to manipulate microbiota composition and metabolic output.

Acknowledgements

"E quando penso che sia finita,

è proprio allora che comincia la salita.

Che fantastica storia è la vita."