Metabolomics analysis of bacterial interactions with the environment : perspective from human gut and heavy metal stress

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**METABOLOMICS ANALYSIS OF BACTERIAL
INTERACTIONS WITH THE ENVIRONMENT: PERSPECTIVE
FROM HUMAN GUT AND HEAVY METAL STRESS**

ABHISHEK JAIN

**INTERDISCIPLINARY GRADUATE SCHOOL
NANYANG ENVIRONMENT & WATER RESEARCH INSTITUTE**

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ABHISHEK JAIN

**Interdisciplinary Graduate School
NANYANG ENVIRONMENT & WATER RESEARCH INSTITUTE**

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SUMMARY

The number of research questions in microbiology is as many as the habitats the organisms come from. Microbes play a vital role in the number of areas from food, agricultural, environmental to medical and health technology. Therefore, it becomes important to understand how microorganisms interact with, survive in and influence their environment. Particularly, there are approximately 100 trillion microbes residing in the human gut and they are linked with health and disease. Thus, an understanding of what constitutes a health-promoting or disease-promoting microbial group becomes important.

In addition to their role in human health, over the years, due to rising levels of environmental pollution, microbes capable of degrading organic xenobiotics and additionally those able to survive and biotransform toxic heavy metals, have gained economic value. Humans also take up metals through food, drinking water and inhalation, in turn, the microbiota residing in the human gut are often exposed to heavy metals. Microbes are mostly studied in ideal laboratory conditions, but it becomes important to understand how microbes detect and cope with the environmental stress (e.g. heavy metals) in their surroundings.

In this thesis, we established an untargeted metabolomics platform to study the bacterial interactions with the environment such as human gut and heavy metal stress.

In our first project, we compared the fecal bacterial diversity and composition of healthy Indian and Chinese adults using next-generation sequencing. Our analysis revealed a unique community structure, dominant *Firmicutes*, *Actinobacteria* and underrepresented *Bacteroides*, of Indian and Chinese gut bacteria. Partial least squares discriminant analysis and non-metric multidimensional scaling plots showed dietary habit wise clustering of subjects. Thus, our finding confirmed an important role of diet in determining gut bacterial composition.

Next, we established an untargeted GC-MS and LC-MS metabolomics methods to study the association of urine and fecal metabolites with diet and gut microbiome composition. Our GC-MS method enabled the detection of 122 and 86 metabolites including amino acids, phenolics, indoles, carbohydrates, sugars and metabolites of microbial origin from fecal and urine samples respectively. 41 compounds were confirmed using external standards. Partial least squares discriminant analysis and hierarchical cluster heat map

showed dietary habit or ethnicity wise grouping of urine and fecal metabolite profiles of subjects. We have also showed a strong association of metabolites with gut bacterial profiles in the genus and species level.

In our second project, we applied GC-MS metabolomics to elucidate the mechanism that allows alpha proteobacterium *Caulobacter crescentus* to survive in Ni (II) stress condition. We identified an increased level of nine important metabolites including TCA cycle intermediates and amino acids in Ni (II) stressed *C. crescentus*. This indicates that changes in central metabolism are linked with the disruption of cell division process. We further characterized the 5 metabolites including malic acid, citric acid, alanine, proline, and glutamine to 0.015 mM that showed a protective effect on Ni(II) toxicity.

In summary, our results highlight the potential of using metabolomics to study the gut microbiome interaction with diet and human metabolome. Comparison between the metabolites profiles of Indian and Chinese adults demonstrates characteristic metabolites signature and their association with diet or ethnicity and gut microbiome. Metabolomics was further applied to investigate the NI(II) stress condition in *C. crescentus*, which revealed the metabolites that show a protective effect on Ni (II) toxicity. Metabolomics based platforms developed in this study can be applied to future research on how bacteria interact with, survive in and influence different types of environment such as human gut and heavy metal contaminated soil, water or air

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CHAPTER 1

INTRODUCTION

Metabolomics is the comprehensive, non-biased, high throughput technique of science, that allows the characterization of metabolites within a complex biological mixture such as cell, tissue or biofluid of an organism in response to external stress conditions such as disease, environmental pollutants exposure, or nutritional imbalances[1, 2]. As compared to genes and proteins, metabolites are the final products of cellular regulatory processes and reflect changes in phenotype and function[3, 4]. Our research is based on the application of metabolomics to simultaneously detect as many metabolites as possible in a given biological system and to compare the overall changes in metabolome in response to xenobiotic exposure of an organism. This thesis combines two major branches of science, metabolomics and microbiology, to investigate some of the complex questions of environmental microbiology and human gut microbiology.

The current research focus in microbiology is as diverse as the habitats the organisms come from. Microbes play a vital role in a number of areas from food, agricultural, environmental to medical and health technology[5]. Therefore, it becomes important to understand how microorganisms interact with, survive in and influence their environment. Particularly, scientists uncovered that microbiota residing in the gut contains 150 fold more genes than the entire human genome and they are linked with human health[6]. For example, microbes residing in the human gut have been linked to a plethora of disease, from diabetes, autism to anxiety and obesity[7, 8, 9]. The gut microbiome could also determine the response of an individual to a certain drug; recently, Alexander et al. (2017) reviewed how gut microbiota influence the response

of cancer patients to chemotherapy[10]. Even a possible link of gut microbiota with how well humans sleep has been suggested[11]. Therefore, an understanding of what constitutes a health-promoting or disease-promoting microbial group has turned into the area of huge research.

In addition to their role in human health, microbes also play a vital role in the variety of industrial applications. Over the years, due to rising levels of environmental pollution, microbes capable of degrading organic xenobiotics and additionally those able to survive and biotransform toxic heavy metals, have gained economic value. Therefore, scientists are keen on unveiling how the microbes are able to do such useful metabolic accomplishments. In the recent years, the focus of the microbiology field has been shifted towards the investigation of microbial responses under unknown and fluctuating environmental stress conditions such as heavy metals stress [12]. Another direction is the understanding of uncharacterized and obscure metabolic pathways involved in microbial stress responses[13]. Humans also take up metals through food, drinking water and inhalation, in turn, the microbiota residing in the human gut are often exposed to heavy metals. Microorganisms are mostly studied in ideal laboratory conditions but despite years of research, it is still not fully understood how microbes detect and cope with environmental stress in their surroundings. Furthermore, external environmental conditions (e.g. nickel stress) can influence the cellular physiology of microorganisms, but it is not entirely clear how the different external signals or cues impinge on fundamental biological processes in microorganisms

.

1.1 Aims and objectives

In this thesis we have established untargeted metabolomics platforms to understand how bacteria interact with, survive in and influence different kinds of environments such as the human gut and heavy metal contaminated soil, water or air.

In our first project our objective was to develop untargeted GC-MS and LC-MS metabolomics platform to identify the association of human metabolites with diet or ethnicity and gut microbiome of Asian adults. We aimed to (1) characterize the prevalent bacterial taxa to define the community structure in gut samples from healthy Indian and Chinese adults, (2) compare the bacterial diversity distribution within and between the two groups studied, and (3) discover possible relationship of gut bacteria with diet and identify gut bacterial biomarkers of diet which distinguish Indian and Chinese adults (4) establish the GC-MS and LC-MS based metabolomics platforms to determine fecal and urine metabolites composition (5) compare metabolites composition between two groups and to identify the association of metabolites with diet and gut microbiome composition.

In the second project, our objective was to apply untargeted metabolomics to gain a deep mechanistic understanding of how microbes respond to nickel (Ni (II)) stress condition in the environment, focusing on the alpha proteobacterium *Caulobacter crescentus*. It is a ubiquitous microorganism that has been found in many different environments, including wastewater, seawater, soil, deep subsurface goldmines and human gut. We aimed to (1) investigate how Ni (II) stress in the environment can affect the cell proliferation and cell cycle of microorganisms, such as *C. crescentus*, and (2) identify the metabolites that enhance or reduce *C. crescentus*'s ability to survive in Ni (II) stress condition.

Conclusively, our study showed that metabolomics is an effective technology to investigate the bacterial interaction with different kind of environments, focusing on human gut and heavy metal stress. Knowledge from this study could aid future research aim to modulate human gut microbiota, with probiotics or synbiotic dietary supplementations, which may provide new approaches to control the diet-microbiota-human health interactions. This thesis also strengthens our understanding towards links between metabolite signatures with specific bacterial genera or species and a possibility of foreseeing gut bacterial profile in light of less expensive and less tedious metabolomics analysis. Moreover, our results shed light on the mechanism of increased Ni(II) tolerance in *C. crescentus* which may be useful in bioremediation or other antimicrobial strategies, antibiotics development and synthetic biology applications such as the development of whole cell biosensor. It could also help us investigate the biotransformation of environmental pollutants by gut microbiomes and how the contaminants would affect the metabolism of microbiota

CHAPTER 2

LITERATURE REVIEW

Part I - Metabolomics as a monitoring tool

2.1 Metabolomics

Metabolomics is the comprehensive study of metabolites within a cell, tissue and biofluid involving in different chemical processes in organisms[14]. To be more specific, metabolomics is the analysis of small molecule metabolites that are the final products of the cellular processes. The levels of metabolites reflect the ultimate response of a biological system to genetic or environmental changes. Metabolomics is regarded as the end point of the “omics” cascade[15].

Fig. 2.1 A representation of the “Omics” cascade[15]

As compared to other omics approaches such as genomics and proteomics, metabolomics indicates changes in the phenotype and function of a particular tissue, or an organism (Fig. 2.1). Thus, it gives an instantaneous snapshot of the entire physiology

of an organism[16]. In other words, genomics, transcriptomics and proteomics show the probability of a process to occur, but metabolomics provides information about what is happening.

2.1.1 Targeted and untargeted metabolomics approaches

Metabolomics is broadly divided into two categories: Untargeted and targeted metabolomics approaches[17]. The targeted approach aims to detect a specific class of compounds; for example, amino acids, fatty acids or a specific group of compounds related to particular pathways and to quantify the metabolites biomarkers of disease or xenobiotics exposure[4]. Targeted metabolomics focuses on the quantification of the already known compounds by using external analytical standards[18]. As such, in terms of application, targeted metabolomics is not much different from conventional analytical chemistry detection methods. The main disadvantage of the targeted approach is that it is restricted to only already known or predicted analytes or biochemical reactions, thus it is unable to provide a holistic overview of changes occurred in a biological system[15]. Additionally, our present information of biological systems or chemical reaction is still extremely constrained, consequently there is still a huge number of unknown compounds present in the crude environmental extract, bacterial cultures and human biofluid i.e. they are completely novel or ambiguously identified.

The main objective of a metabolomics experiment is to simultaneously detect as many metabolites as possible in a given biological system and to compare the overall changes in metabolome in response to disease and xenobiotic exposure. Untargeted metabolomics approach is more useful and effective tool to identify the metabolites that play a key role in modulating the biochemical processes in response to xenobiotics exposure, disease or any other specific changes of interest, as it provides the information of both known and unknown compounds[19, 20]. Since, the untargeted approach can be

simultaneously applied to an extensive range of metabolites, regardless of whether they have already been identified or not, it is a true “omics” approach[21, 22]. Overall, untargeted metabolomics is more comprehensive and provide the holistic overview of metabolome changes in a given biological system.

2.2 Analytical tools in metabolomics

There are two major analytical platforms used in metabolomics studies: nuclear magnetic resonance (NMR) and mass spectrometry (MS)[23]. In this thesis we focused on mass spectrometry-based metabolomics techniques.

2.2.1 Gas chromatography -mass spectrometry (GC-MS) based metabolomics

GC-MS is the well-developed technology that allows simultaneous detection of hundreds of metabolites in a single run. GC-MS is a combination of two powerful techniques, in which thermally stable and volatile compounds are separated by GC and then eluting metabolites are detected by electron-impact (EI) mass spectrometers. GC-MS offers several advantages, including high efficiency, reproducibility and sensitivity[24, 25]. However, this technique has a drawback that it can be applied to only volatile compounds or derivatization is needed to make the compounds stable. Thus, sample preparation for GC-MS analysis is more complex and time-consuming process as compared to LC-MS[26]. Also, large size metabolites with high polarity are not detectable by GC[27].

GC-MS is particularly suitable for identification of volatile organic compounds (alcohols, esters, aldehydes, ketones, phenols, SCFAs,) and amino acids. Selection of solvent for metabolite extraction is the first important step of sample preparation. The suitability of solvent for a particular extraction depends on the application. Acetonitrile,

hexane, methanol, ethyl acetate, dichloromethane is some of the most commonly used solvents for metabolites extraction.

The next important step of sample preparation is derivatization, which introduces chemical groups to increase the volatility and thermal stability of analytes to be detected. It also improves compound ionisation. Alkylation, acylation and silylation are widely applied derivatization methods. Trimethylsilylation is a very effective method but, to date, very few literature about fecal water or urine metabolomics on such derivatization is available[28].

2.2.2 Liquid chromatography -mass spectrometry (LC-MS) based metabolomics

Liquid chromatography coupled with mass spectrometry (LC/ MS) has become a method of choice for many metabolomics analyses because of its ability to separate, ionize and detect an extensive variety of chemicals with little effort in few pre-analytical steps. LC-MS sample preparation is easier as there is no need of derivatization[29]. The combination of LC with MS permits the analysis of polar, non-polar and neutral compounds including organic acids, polyamines, nucleosides and nucleotide in a complex matrix. In addition, LC/MS analysis acquires chemically distinct information such as mass to charge (m/z), retention time and fragment ion spectra of the detected metabolites, which provides insight into chemical identity[30].

The metabolites are of diverse chemical nature (e.g. organic acids, sugars, lipids, amino acids) with a large dynamic range, therefore unlike other omics where a single instrument is enough to perform the entire analysis, metabolomics needs a variety of instrument depending on the type of sample and compounds to be detected. Thus, a combination of both GC-MS and LC-MS is more suitable for a comprehensive

metabolite profiling[15]. A summary of different classes of metabolites, based on their compatibility with GC-MS and LC-MS techniques, is shown in figure 2.2.

Fig. 2.2 Comparison of metabolites based on the compatibility with GC-MS and LC- MS

2.3 Applications of metabolomics

Metabolomics technology can be applied to a wide range of applications, from microbial biotechnology, food technology, pharmacology, to plant biotechnology and medical technology[31]. In this thesis we combine metabolomics with microbiology to understand how bacteria interact with, survive in and influence different kinds of environments such as the human gut and heavy metal. The following section describes the role of metabolomics in environmental and gut microbiology.

2.3.1 Environmental metabolomics

Environmental metabolomics is an emerging field focuses on the application of metabolomic tools to analyse the interactions of living organisms with their environment[1]. It is an effective technique to investigate the bacterial response to unknown and fluctuating environmental stress conditions, such as heavy metal stress. Another direction of environmental metabolomics is the investigation of uncharacterized and obscure metabolic pathways involved in organism's response to stress. A typical workflow of environmental metabolomics experiment design is depicted in figure 2.3. The first step is the selection of a model organism (e.g. plants, bacteria, humans) according to the hypothesis of the experiment, a type of

environmental stress (e.g. temperature or PH change, carbon starvation or heavy metals) and the method that will be used to expose the organism to the external stress. We need to decide the optimum time of exposure, amount of stressor and after that next step is the collection of biological samples. it may include organs or tissue extracts, microbial or human cell culture and biofluids such as urine, serum, blood. In addition, other steps of typical metabolomics workflow are used in an environmental metabolomic experiment such as sample preparation, data acquisition using GC-MS, LC-MS or NMR, data analysis, metabolites identification and biological interpretation of the data obtained [3, 32].

Fig. 2.3 A typical workflow of environmental metabolomics experiment

2.3.2 Metabolomics and gut microbiome

Over the past two decades, the development of high throughput omics technologies such as metagenomics and metabolomics provided us with effective tools to monitor and determine the human gut microbiome phenotype. Specifically, we will be using 16S

rRNA sequencing which enables us to obtain the taxonomic profile of gut microbial species in human subjects, without the need of any classical plate culture techniques[2]. On the other hand, metabolomics has been utilized to track metabolites generated from the gut microbes, and to investigate the interaction between the host and dietary components[33]. Metabolites mirror the health status of an individual by acquiring extensive insights into the functioning of a biological system. Metabolomics is a powerful technique that simultaneously detects hundreds of small molecules present in a given biological system such as fecal, urine or saliva samples, thus it can capture the host-microbial-co-metabolites, i.e. the metabolites that are shared between the host and microbes in the gut [4]. Fecal metabolites are the final product of both cellular and microbial metabolism undergoing inside the human intestinal tract. Some of the metabolites from the gut are absorbed into the circulation and eventually chemically modified (that is, co-metabolized) by the host, then finally excreted with the urine[34]. In the previous section we have discussed the presence of various microorganisms in human gastrointestinal tract which affect nutrient absorption, energy regulation, detoxification, or transformation of xenobiotics and the health of an individual[35, 36, 37]. It is known that gut microbiota composition is largely affected by diet, consequently metabolites produced by gut microbiota also determined by diet[38, 39]. The complex gut microbial community utilizes both diet and host derived energy sources for growth, predominantly through fermentative metabolism[40]. Analysis of fecal and urine metabolic compositions has received a lot of attention, as it does not just reflect the status of the gut microbiome yet additionally bridge the connections between symbiotic microbes and the host's health. Several previous studies have proposed the usefulness of fecal and urine metabolites in disease diagnosis, e.g. higher concentrations of amino acids, saturated fatty acids, and ursodeoxycholic acid were reported in fecal samples of

colorectal cancer patients [41]. In another study higher levels of choline, trimethylamine N-oxide (TMAO) and betaine produced from dietary phosphatidylcholine in the gut were found to be associated with cardiovascular disease risk and atherosclerosis[42]. Moreover, some metabolites such as short chain fatty acids, phenolics and vitamins decreases the risk of gastrointestinal disorder[43], cancer[44], diabetes[45] and cardiovascular diseases[46]. Thus, examining the fecal and urine metabolomes serve as a vigorous strategy for understanding the interactions between diet, human metabolism, and the gut microbiota composition in health and disease.

Part II Role of the gut microbiome in human nutrition and metabolism

2.4 Human gut microbiome

Microorganisms impact entire biosphere due to their ubiquitous nature. As a result of the quantitative magnitude of microbial life (approximately 5.0×10^{30} cells, which is estimated to be half of the biomass on the earth[47]), microorganisms play an essential role in directing biogeochemical systems in nearly all of our planet's surroundings, including some of the most extreme, from frozen environments and acidic lakes, to hydrothermal vents at the bottom of profound oceans, and some of the most well-known, for example the human gastrointestinal tract[48, 49]. In particular, approximately, 100 trillion microbes reside in the human gut (collectively termed “gut microbiota”) and together, the gut microbiome has approximately 150-fold more gene than the entire human genome[6]. Humans are considered to be a complex superorganism because of the magnitude of the symbiotic gut microbiome. On the whole, the data on gut microbiome reveals an astonishing fact that 90% of human body is occupied by microbes and only the rest of the 10% part is human[50].

Fig. 2.4 Humans are considered as a complex superorganism. Human body is composed of several species: Eukaryotic, bacterial and archaea. It is estimated that 90% of human body is composed of microbes and only the rest of the 10% part is human[50]

The term microbiome was coined by Joshua Lederberg to “signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease” [51]. The contribution of the human gut microbiome to health and disease is now emerging as an important aspect to be considered in parallel to the human genome sequence[52, 53]. Imbalance in normal gut microbiota has been associated with inflammatory and metabolic disorders including inflammatory bowel disease [54], irritable bowel syndrome [55], and obesity [56]. Therefore, an understanding of what constitutes a health-promoting or disease-promoting microbial group has become an important area of research. The role of key members of the gut microbiome in health and diseases is represented in figure 2.5.

Fig. 2.5 The functional roles of key members of human microbiota in health and disease[57]

The human gut microbiome is mainly represented by two major phyla *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria*. Other bacteria such as *Lactobacilli*, *streptococci* and *enterobacteria* are present in a small percentage (Fig. 2.6) [58]. However, the exact structure of gut microbiota is influenced by multiple intrinsic and extrinsic factors including microbes acquired from mother and from the environment, diet, host genetics and physiology, xenobiotics, drug intake and disease (Fig. 2.7) [59][60]. However, extensive research has shown diet as the most important factor in modulating the composition and function of gut microbes in humans and other mammals. Diet is easiest to modify and provides the simplest route for therapeutic intervention[61]; consequently, diet is now widely acknowledged to regulate human health at the molecular level. Accordingly, modern nutrition has turned its focus towards molecular biology and genetics with the objective of preventing disease and improving the health and well-being of an individual[62].

Fig. 2.6 Human gut microbiome composition

Fig. 2.7 Intrinsic and extrinsic factors that influence gut microbiome composition. Gut microbiota is important for homeostasis of the immune system[58, 63]

Recent studies have linked diet and microbiome with health [64, 65]. Changes in gut microbiota reported in experimental animals fed a high fat diet that induces[66]. Moreover, controlled diets consist of non-digestible carbohydrates gave to overweight

men actuate remarkable changes in certain dominant species, although the responses vary among subjects [67]. The effects of different dietary patterns on gut microbiome composition are discussed in the next section

2.5 Diet is the most influential factor in determining gut microbiome composition

This section presents a review of studies on the contribution of diet to gut microbiome composition and functions.

2.5.1 Long term vs short term dietary patterns

Human intervention studies from the past decade have revealed the extent to which short term and long term dietary patterns influence the different aspects of the gut microbiota. Rapid changes in gut microbiota has been reported in response to large changes in diet. It could be evidenced from subjects who switched between plant- and meat-based diets, who added extra specific dietary fibres to their diet or who consumed either a high-fibre–low fat diet or a low-fibre–high-fat diet for 10 days; The composition and function of microbiota shifted within 1-2 days in all the cases[61, 67, 68]. It could be conceived by the fact that microbes can double their population within an hour. Despite these rapid dynamics, long term dietary patterns are proved to be the dominant force in determining gut microbiota composition[69]. Short term dietary interventions might induce detectable changes in the microbiota, but it does not affect the major compositional features and the overall classification of each subject's microbiota. A few, yet not all, cross-sectional investigations revealed that long term dietary patterns are connected to main features of microbiota. Another important aspect to be considered is, that a particular change in diet is not necessarily to have same impact on microbiota but a highly variable effect on different people can be expected due to the individualized nature of their gut microbiota. For instance, a study on obese men reported the increase

of *Ruminococcus bromii*-related taxa in response to resistant-starch intervention in most of the participants, whereas the lack of response in the other members might show an absence of such taxa in those individuals[67].

2.5.2 Vegetarian diet and Western diet

Western diet contains higher animal fat and protein, lower fibre, higher refined carbohydrate, as compared to traditional rural or vegetarian diet. De Filippo et al. (2010) reported that rural African children consuming predominantly vegetarian diet rich in starch, fibre and complex carbohydrate showed higher abundance of *Bacteroidetes*, *Prevotella*, *Xylanibacter*, *Treponema* and lower abundance of *Firmicutes* when compared with Italian children consuming western style diet[70]. Similarly, the gut microbiome of the Bangladeshi and Egyptian population showed a higher abundance of *Prevotella* and lower abundance of *Bacteroides* as compared to US population [71, 72].

A review of the effect of different dietary patterns on gut microbiome composition is presented in table 2.1:

Table 2.1: A review of the effect of different dietary patterns on gut microbiome composition

Diet	Microbiota Changes	Metabolites	References
Agrarian diet- cereals, legumes, vegetables (Rural African) vs Western diet high in sugar, starch, animal protein and fat (Italian)	↑ <i>Bacteroidetes</i> , <i>Prevotella</i> , <i>Xylanibacter</i> , <i>Treponema</i> ↓ <i>Firmicutes</i> in Africans	↑ Propionic, butyric acid	[70]
Carbohydrate-rich- rice, bread, lentils with rarely any meat (Bangladeshi) vs Typical western diet (US)	↑ <i>Lactobacillus</i> , <i>Bifidobacterium</i> , <i>Prevotella</i> , <i>Catenibacterium</i> , <i>Mitsuokella</i> ↓ <i>Bacteroides</i> in Bangladeshi		[71]

<p>Mediterranean diet rich in plant foods (Egyptian)</p> <p>vs</p> <p>Western diet high in animal protein, fats, and highly processed carbohydrates (US)</p>	<p>↑ <i>Prevotella</i>, <i>Megasphaera</i>, <i>Eubacterium</i>, <i>Mitsuokella</i>, <i>Catenibacterium</i></p> <p>↓ <i>Bacteroides</i>, <i>Faecalibacterium</i>, <i>Akkermansia</i>, <i>Ruminococcus</i>, <i>Coprococcus</i>, and <i>Blautia</i></p> <p>in Egyptian</p>	<p>↑ Acetate, butyrate, and propionate</p> <p>↓ Ethanol, lactate, malate, pyruvate, and succinate, amino acids, taurine, choline</p> <p>In Egyptian</p>	[72]
Barley kernel based bread	<p>↑ <i>Bacteroidetes</i>, <i>Prevotella</i></p> <p>↓ <i>Bacteroides</i></p>		[73]
<p>Animal based diet</p> <p>vs</p> <p>Plant based diet</p>	<p>↑ <i>Bacteroides</i>, <i>Alistipes</i>, <i>Bifidobacterium</i></p> <p>↓ <i>Prevotella</i>, <i>Roseburia</i>, <i>Eubacterium rectale</i>, <i>Ruminococcus bromii</i></p>	<p>↓ SCFA</p> <p>↑ amino acids</p>	[74]
Whole grain wheat cereals or wheat bran	<p>↑ <i>Bifidobacteria</i> (whole grain)</p> <p><i>Lactobacilli/Enterococci</i> (both products)</p> <p><i>Clostridium</i> (wheat bran)</p>	No changes in acetic, propionic or caproic acid	[75]
Maize-based whole grain breakfast cereals	<p>↑ <i>Bifidobacterium</i> spp.</p> <p><i>Atobacterium</i> cluster spp.</p>	changes in lactic, acetic, propionic, butyric, valeric acid and branched-chain fatty acids	[76]
almonds and almond peel	<p>↑ <i>Lactobacillus</i> spp.</p> <p>↓ <i>Clostridium perfringens</i></p>		[77]
Apple	<p>↑ <i>Bifidobacteria</i></p> <p>↓ <i>Clostridia</i></p>	↑ Acetic acid	[78]
Chickpea or raffinose	<p>↓ <i>Clostridium</i> cluster XI, <i>Clostridium</i> cluster I/II</p>	No change in short chain and branched chain fatty acids	[79]

2.5.3 Food Constituents

Carbohydrates are one of the major classes of biologically essential organic molecules found in all living organisms. Dietary fibre is one of the most important complex, non-starch carbohydrates which is found in whole grain, legumes and beans. The complex carbohydrates such as dietary fibres are indigestible in small intestine and are metabolized by gut bacteria in the large intestine to produce short chain fatty acids, which acts as a primary energy source for colonic cells and also protects from digestive disorders and colon cancer[80]. Resistant starches, non-starch polysaccharides, and oligosaccharides, are the three major classes of complex carbohydrate.

In contrast to complex carbohydrate, refined carbohydrates, present in refined grains, pasta, and white rice, are immediately absorbed into the blood stream, resulting in higher blood sugar and insulin level.

Fats and proteins are another two essential macronutrients which have been extensively studied for their effect on human gut microbiome composition. Meat, poultry, nuts, milk and fish are some of the major sources of fat and proteins[81]. The effect of major dietary components on gut microbiome composition is summarized in figure 2.8.

Fig. 2.8 Influence of major dietary components on gut microbiome composition[81]

2.6 Metabolic functions associated with gut microbiome

The gut microbiota is solely in charge of several essential metabolic functions, including vitamin and short chain fatty acid production, amino acid synthesis, bile acid biotransformation, hydrolysis and fermentation of non-digestible substrates[82]. Microbes that reside in the human gut frequently produce various little molecules through primary and secondary metabolic pathways, huge numbers of which are dependent on the diet of the host. Microbial metabolism in the colon is impacted by the quantity and kind of dietary compounds that survive small intestinal digestion[83]. Albeit a portion of the metabolites produced by microbes is held inside the gut biological system and secreted with faeces, others will be absorbed into the circulation and eventually chemically modified (that is, co-metabolized) by the host, then finally excreted with the urine(Fig. 2.9) [84] .

Fig. 2.9 Metabolic route of non-digestible food in humans[84]

These metabolites can have various effects on the host immune system. A summary of metabolites produced from the interaction of microbiota with dietary components is presented in table 2.2.

Table 2.2: Metabolites associated with microbial metabolism or microbial host co-metabolism[83, 85, 86, 87]

Metabolite	Bacteria	Substrate
SCFAs: acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate	<i>Clostridial clusters IV and XIVa</i> <i>Lactobacillus</i> , <i>Eubacterium</i> , <i>Roseburia</i> , <i>Faecalibacterium</i> , <i>Coproccoccus</i> , <i>Bifidobacterium</i>	indigestible oligosaccharides, dietary plant polysaccharides or fibers, non-digested proteins and intestinal mucin
Organic acids: benzoate, hippurate, phenylacetate, phenylpropionate, hydroxybenzoate, hydroxyphenylacetate, hydroxyphenylpropionate 3,4-dihydroxyphenylpropionat and D-lactate	<i>Clostridium difficile</i> , <i>Faecalibacterium prausnitzii</i> , <i>Bifidobacterium</i> , <i>Subdoligranulum</i> , <i>Lactobacillus</i>	dietary polyphenols or unassimilated AAs or carbohydrates
Vitamins: vitamin B9, vitamin B2, vitamin B12, niacin, pyridoxine, vitamin K, vitamin B1, vitamin B5, vitamin B8	<i>Bifidobacterium bifidum</i> , <i>Bifidobacterium longum subsp. infantis</i> , <i>Bifidobacterium breve</i> , <i>Bifidobacterium adolescentis</i> , <i>commensal Lactobacilli</i> , <i>B. subtilis</i> <i>E. coli</i> and <i>anaerobes</i> , <i>Bacteroidetes</i> , <i>Fusobacteria</i> , <i>Proteobacteria</i> , <i>Actinobacteria</i>	
Bile salts: cholate, hyocholate, deoxycholate, chenodeoxycholate, α -muricholate, β -muricholate, taurocholate, glycocholate, taurochenoxycholate, glycochenodeoxycholate, taurocholate, lithocholate, ursodeoxycholate, hyodeoxycholate, glycodeoxylcholate, taurohyocholate, taurodeoxylcholate	<i>Bacteroides</i> , <i>Clostridium</i> , <i>Lactobacillus</i> , <i>Bifidobacterium</i> , <i>Enterobacter</i> , <i>Eubacterium</i> , <i>Escherichia</i>	
Polyphenol: Hydroxycinnamic acids and flavonoids	<i>Lactobacillus</i> , <i>Bifidobacterium</i>	apples, berries and kiwifruit, in vegetables such as potatoes and coffee
Amino Acids	Colonic bacteria, <i>Clostridium</i> , <i>Peptostreptococcus anaerobius</i>	

Part III Heavy metal toxicity and microbial life

2.7 Food as a source of toxic metals

Despite providing essential nutrients to the humans and animals, diet also carries toxic compounds such as heavy metals over a wide range of concentrations. The US Environmental Protection Agency has placed 13 heavy metals on its priority pollutants list of 126 compounds [88]. Food is found to be one of the most important route for accumulating most environmental pollutants, including heavy metals[89, 90]. The intake of heavy metals by humans through the food chain has been noticed in many countries and nowadays this problem is receiving a lot of attention from the public as well as governmental agencies[91]. Metal accumulation in vegetables may pose a direct threat to human health[92, 93]. It could be supported by the fact that nearly half of the mean ingestion of lead, cadmium and mercury through the food of plant origin[94].

The dietary contribution to toxic metal intake has been extensively studied. An estimated dietary intake of heavy metals from the total diet, in some selected countries, is presented in table 2.3.

Table 2.3: Dietary intake of heavy metals from the total diet, in some selected countries (μg per person per day) [95, 96, 97]

Country	Pb	Cd	Hg	Ni
China	86.3	13.8	10.3	
Japan	85.0	29.0	-	-
USA	9.80	13.0	3.2	300-600
UK	60.5	18.9	-	
Sweden	17.0	12.0	1.80	
Denmark	42.0	20.0	7.0	150-900
Canada				286
Global	153	25.0	-	

2.8 Other sources of heavy metals in the environment

Heavy metal toxicity is one of the most established environmental issues and remains a serious health concern today. Human activities, such as industrial production, agriculture, mining, and smelting operations significantly contribute to the elevated level of heavy metals in water, soil, and air[98]. A list of major heavy metal pollutants in the environment is presented in table 2.4:

Table 2.4: Major heavy metal pollutants in the environment

S. No.	Metal	Guideline Value ($\mu\text{g/l}$) Drinking Water	Occurrence
1.	Arsenic	10	Natural Water- 0.5-2 $\mu\text{g/l}$, Areas containing natural sources (upto 12 $\mu\text{g/l}$), Soil 1-50 mg/kg
4.	Chromium	50	Sea Water- 0.2-0.6 ppm Con. Of drinking Water- 2 $\mu\text{g/l}$ Soil Concentration- 500-6000ppm
5.	Selenium	40	Con. In drinking Water-less than 10 $\mu\text{g/l}$ Soil-0.1 to 3.5 ppm
6.	Uranium	30	Con. In drinking Water-less than 1 $\mu\text{g/l}$ Con. In soil- 300 $\mu\text{g/kg}$ Con. In Sea Water- 3.3 $\mu\text{g/l}$
7.	Cadmium	3	Con. In drinking Water-less than 1 $\mu\text{g/l}$ Soil 0.1-1 ppm 0.03-1mg/kg in marine environment
8.	Mercury	6	Sea water- 0.3-1 ppm Drinking water- 0.002 mg/l Ground water-2 $\mu\text{g/l}$
9.	Antimony	20	Ground water- >0.001 $\mu\text{g/l}$ Surface water- >0.2 $\mu\text{g/l}$ Drinking Water- > 5 $\mu\text{g/l}$
10.	Copper	2000	Con. In drinking Water- ≤ 0.005 ->30mg/l Con in soil- below 15ppm Con. In dump sites- 7-80 ppm
11.	Lead	10	Con. In drinking Water- below 5 $\mu\text{g/l}$ Con. In soil- 50-590 ppm
12.	Nickel	70	Con. In drinking Water-less than 0.02mg/l

			Sea Water- 0.5-2ppm Soil 4-80 ppm sometimes up to 5000ppm
14.	Fe	300	Natural fresh water- 0.5-50mg/l Sea water- 1-3 ppb River water- 0.5-1ppm Drinking water- less than 200ppb
15.	Mn	400	Surface water- 0.01-1.70 mg/l (CCME,1987) Drinking water- upto 0.05 mg/l (CCME,1987) Soil- upto 10mg/l (CCME,1987)
16.	Zn	3000	Con. In surface water- less than 0.01 mg/l Ground water- >0.05 mg/l Sea water- 0.6-5ppb
17	Tl	2	Sea Water- 11,000 ppm Rivers- 9ppm Drinking water- 50mg/l
18.	Silver	50-100	Surface & drinking water- < 5 µg/l Soil- upto 44ppm Sea water- 2-100ppt
19	Cobalt	100	Soil- 8ppm USA river- 1-36 µg/l

Source: WHO guidelines for drinking water quality, 2011 and US EPA [88, 99]

2.9 Microbial interactions with the heavy metals in the environment

humans take up metals through ingestion, inhalation and dermal absorption, in turn, the microbiota residing in the human gut are often exposed to heavy metals(Fig. 2.10) [100].For example the Ni content of in Danish diet ranges from 150 µg/d to 900 µg/d. while the average nickel content in the American diet was 300–600 µg Ni/d [101]. It is known that gut microbes alter the absorption, metabolism and excretion of environmental pollutants that might affect obesity and diabetes[102]. Gut microbes also protect human from the absorption of the ingested pollutants. In a study, Breton et al. (2013)showed that mice lacking intestinal microbiota were more susceptible to the accumulation of heavy metals in blood and target organs[103]. Similarly, certain members of gut microbiota, such as *Lactobacilli* resist metal toxicity through the active

expulsion of metals due to the presence of mer and ars operon which encode efflux transporter [104]. Various past examinations have explained that environmental chemicals alter gut microbiota composition which leads to disorders of energy metabolism, nutrient absorption and immune system functions but it is not clear that how gut microbiota cope with these stresses and what role these microbes play in interacting with the metals[105, 106].

Fig 2.10 Microbial interactions with the heavy metals in the environment, focusing on *C. crescentus*. Microbiota residing in the human gut are often exposed to heavy metals through contaminated food and water. The gut microbiota alters chemical structures of ingested compounds including heavy metals. In addition to the human gut, microbes living in other environments such as water, soil, air are routinely exposed to heavy metals

As discussed above, various Human activities significantly contribute to the elevated level of heavy metals in water, soil, and air. Thus, in addition to the human gut, microorganisms are routinely exposed to heavy metals in many other environments,

including wastewater, seawater, soil, and deep subsurface metal mines. one such microbe is the α -proteobacterium, *Caulobacter crescentus* (Fig. 2.10), which is equipped to survive in extreme environments or polluted sites that are hostile to humans. *C. crescentus* is a ubiquitous microorganism that has been found in many different environments, including wastewater, seawater, soil, deep subsurface goldmines and human gut [107, 108]. It is known that bacteria employ different molecular mechanisms to survive in often unknown and fluctuating environmental conditions (e.g.: heavy metals stress). However, despite years of research, it is still not fully understood how microbes can detect and cope with so many different stresses in their surroundings. Furthermore, external environmental conditions can influence the cellular physiology of microorganisms, but it is not entirely clear how the different external signals or cues impinge on fundamental biological processes, like the cell cycle, in bacteria. A deep understanding of bacterial stress responses is important for bioremediation or other antimicrobial strategies, antibiotics development and synthetic biology applications such as the development of whole cell biosensor. It could also help us understand the biotransformation of environmental pollutants by gut microbiomes and how the contaminants would affect the metabolism of microbiota. Therefore, we seek to investigate how heavy metals in the environment can affect the proliferation of microorganisms, such as *Caulobacter crescentus*.

2.10 *Caulobacter crescentus*: a model organism to study cell cycle

C. crescentus is a model organism for understanding the regulation of cell cycle, asymmetric cell division and cellular differentiation owing to the following unique characteristics [109, 110]:

- 1) Cell populations can be easily synchronized with a density gradient centrifugation that separates swarmer cell from the stalked cell population.

- 2) Cell cycle phases can be easily observed in the laboratory by monitoring the changes in morphology.
- 3) Unlike *E. coli*, DNA replication takes place once and only once in a complete cell cycle of *C. crescentus*.
- 4) Length of the cell cycle is not too fast. Chromosome replication in *C. crescentus* takes around 150 minutes as compared to *E. coli* in which chromosome is replicated in 20 minutes. The longer cell cycle time allows us to observe the sequential occurrence of different events taking place during the cell cycle.

2.11 Cell cycle of *Caulobacter crescentus*

C. crescentus utilizes changes in the form and function of a cell to create a life style that allows it to survive in hostile conditions. *C. crescentus* produces two daughter cells namely, swarmer cell and stalked cell, during its cell division. These two cells are morphologically and functionally distinct to each other. Motile swarmer cells contain a polar flagellum and multiple pili at one pole. At the later stage pili are retracted and polar flagellum of swarmer cell is replaced by a stalk during the differentiation of motile swarmer cell into a sessile stalked cell. Stalked cell adheres to the surface tightly using an organelle called “holdfast” [111].

The cell cycle of *C. crescentus* initiates with a motile swarmer cell which is incapable of starting DNA replication or cell division and remains in G1 Phase. Then this swarmer cell is differentiated into a stalked cell which can initiate DNA replication. The stalked cell is present in ‘S’ phase. DNA replication and movement of the chromosomes to opposite ends of the pre-divisional cell ensues. During the pre-divisional cell stage, a flagellum and a stalk are formed at swarmer pole and stalked pole respectively. Now the cell enters the G2 phase and becomes incompetent in DNA replication. Subsequently, the pre-divisional cell divides into two distinctive daughter cells namely, the motile

swarmer cell and the sessile stalked cell [109]. The stalked cell enters the ‘S’ phase and reinitiates the DNA replication, while the swarmer cell is not able to participate in DNA replication and remains in the G1 phase. A diagrammatic representation of a typical cell cycle of *C. crescentus* is shown in fig 2.11:

Fig. 2.11 Life cycle of *C. Crescentus* [109]

2.11.1 The core engine driving cell cycle progression in *C. crescentus*

The cell cycle of *C. crescentus* is regulated by five key master proteins, DnaA, GcrA, CtrA, SciP, and CcrM [112]. The chromosome replication in *C. crescentus* is tightly controlled by DnaA and CtrA which are opposing in roles to each other. The CtrA protein binds to five sites in the origin of replication and thus silences the chromosomal replication process [113]. Therefore, CtrA must be deactivated to initiate the process of replication in *C. crescentus*. When CtrA vacates origin of replication this position is occupied by DnaA, thus initiation of replication takes place [114].

The interaction amongst core cell cycle regulatory proteins is shown in figure 2.12:

Fig. 2.12 Core engine of cell cycle regulation in *C. crescentus* [115]. DnaA, GcrA, CtrA, SciP, and CcrM are master regulatory proteins

CtrA is also a key transcription factor in the cell and activates or represses the transcription of around 25 percent of cell cycle regulated genes. It is involved in important cell cycle events including initiation of DNA replication, DNA methylation and cell division [116]. the activity of this master regulator is controlled in multiple ways. CtrA is present in swarmer cell but disappears during swarmer cell to stalked cell transition to initiate replication. The overall accumulation level of CtrA during the cell cycle is shown in figure 2.13

Fig 2.13 Polar localization and proteolysis of CtrA during the *C. crescentus* cell cycle [117]. SW- swarmer cell, ST-stalk cell, PD- Pre-divisional cell

2.11.2 DnaA/GcrA/CtrA regulatory cascade is critical for cell cycle progression

DnaA/GcrA/CtrA regulatory cascade plays important role in driving the forward progression of cell cycle. GcrA promoter contains a CtrA binding site, a DnaA box and two putative methylation site [118]. CtrA negatively regulates the transcription of *gcrA* by binding at its site on *gcrA* promoter while DnaA binds to the DnaA box at *gcrA* and positively regulates the transcription of this gene. The accumulation level of GcrA during cell cycle is shown in figure 2.14 :

■ GcrA accumulation

Fig. 2.14 Accumulation level of GcrA during cell cycle progression [119]. SW- swarmer cell, ST-stalk cell, PD- Pre-divisional cell

Initially, in the swarmer progeny cell, CtrA represses the transcription of *gcrA*. CtrA is degraded and DnaA accumulates during swarmer cell to stalked cell transition. Then this accumulated DnaA promotes the transcription of *gcrA* that results in increasing level of GcrA during swarmer to stalked cell transition. Once cell division is initiated *gcrA* induces the transcription of *ctrA*. The increased level of CtrA and reduced level of DnaA together are responsible for turning off *gcrA* transcription in predivisional cell [57].

2.11.3 Mechanism of interaction between essential proteins, MipZ FtsZ and ParB, during cell division

There are many proteins involved in the cell division process of *C. crescentus*. The two most important proteins are FtsZ and MipZ. FtsZ is critical for initiation of cell division process in bacteria. It interacts with another important protein MipZ to facilitate chromosome segregation and cell division in *C. crescentus*. MipZ, a member of ParA superfamily of P loop ATPases, is an essential protein for chromosome segregation and cell division process in *C. crescentus*. MipZ has two properties that make it an important cell division protein, one its ability to interact with ParB and the other it inhibits the polymerization of FtsZ. MipZ pattern of localization was found to be parallel to the origin of replication during the cell cycle [75, 76].

The DNA partitioning protein, ParB, binds to a conserved site ParS located very near to the origin of replication (Cori). It was observed that ParB and origin of replication occupy the same position in the cell and share a similar pattern of localization over the cell cycle [77, 78]. The MipZ protein interacts with ParB, thus resulting in the formation of a MipZ-ParB- ParS complex. MipZ localization pattern is mediated by interaction with ParB.

FtsZ localization is restricted to subcellular locations with the lowest concentration of MipZ. Origin of replication and MipZ-ParB complex is present at the flagellated pole of swarmer cell and FtsZ positioned to the pole opposite to it. After commencement of chromosome replication and duplication of the origin, the newly formed sister duplexes are quickly reoccupied by ParB and MipZ, and one of the MipZ ParB origin complexes immediately moves towards the opposite cell pole. Once MipZ-ParB-origin complex reaches the opposite pole, it destabilizes FtsZ and displaces FtsZ towards the midcell, where MipZ concentration is lowest.

Later in the predivisional cell, FtsK, FtsA, and FtsQ also localize at the midcell, thus forming a 'Z' ring by combining with FtsZ [67]. The Model for FtsZ localization by the origin-bound MipZ-ParB complex is shown in figure 2.15:

Fig. 2.15 Model for the FtsZ localization by the origin bound MipZ-ParB complex [117]

CHAPTER 3
SIMILARITIES AND DIFFERENCES IN GUT MICROBIOME
COMPOSITION CORRELATE WITH DIETARY PATTERNS OF INDIAN
AND CHINESE ADULTS

Abstract

The interaction between diet and gut microbiota, and ultimately their link to health, has turned into the concentration of huge research. However, this relationship still needs to be fully characterized, particularly in the case of the Asian population. We compared the fecal bacterial diversity and composition of healthy Indian and Chinese adults, ages 22 to 35 years, using next-generation sequencing analysis on IlluminaHiSeq2500 platform. Our analysis revealed a unique community structure, dominant *Firmicutes*, *Actinobacteria* and underrepresented *Bacteroides*, of Indian and Chinese gut bacteria. This community structure closely matched with the gut bacterial composition of the Russian population. Therefore, we hypothesized that enrichment of these bacterial clades is supported by high consumption of starch-rich diet such as rice, potato, refined grains. The dominance of genus *Bifidobacterium* due to carbohydrate-rich diet is another notable feature of this study. Moreover, Indian gut bacteria are significantly represented by *Bacteroidetes* ($p=0.001$) and *Prevotella* ($p=0.002$) in contrast to Chinese, which could be associated with whole grains and plant-based vegetarian diet of Indians. The gut bacterial population of Indian adults were as diverse as Chinese adults ($p>0.1$), but significant difference was noticed in gut bacterial composition and relative abundance between two populations ($R = 0.625$, $p<0.005$). Partial least squares discriminant analysis and non-metric multidimensional scaling plots showed dietary habit wise clustering of subjects. Thus, the present work confirms an important role of diet in determining gut bacterial composition. LEfse analysis revealed genera *Prevotella*, *Megasphaera*, *Catenibacterium*, *Lactobacillus*, *Ruminococcus* and species *Prevotella copri*, *Lactobacillus ruminis* as the potential biomarkers of diet.

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3.1 Introduction

The human gut is a host of trillions of bacteria. The entire gut microbiota is estimated to contain 150-fold more genes than our host genome [6]. Tremendous progress has been made in linking human gut microbiota with health and disease [52, 53, 120]. Imbalance in normal gut microbiota has been associated with inflammatory and metabolic disorders including inflammatory bowel disease [54], irritable bowel syndrome [55, 56], and obesity [56]. Therefore, an understanding of what constitutes a health-promoting or disease-promoting microbial group has turned into the concentration of huge research. Gut microbiota composition varies among individuals within and between communities [121]. Several factors such as diet, geography, host genetics and physiology, and drug usage, influence gut microbial composition [59, 60, 122] but diet has been considered as the most prominent factor amongst all [38, 123, 124, 125, 126]. Moreover, diet is simplest to modulate and provides the easiest route for therapeutic intervention [61]. Recent studies have linked diet and microbiome with health [65, 127, 128]. Changes in gut microbiota reported in experimental animals fed a high fat diet that induces obesity [66, 129, 130]. Moreover, controlled diets consist of non-digestible carbohydrates gave to overweight men actuate remarkable changes in certain dominant species, although the responses vary among subjects [67].

Over the last two decades, microbiome analysis of fecal samples using culture-independent methods, such as high-throughput DNA sequencing has emerged as a non-invasive tool to study nutrition and health [131]. The development of tools has enabled researchers to explore the interaction between diet and gut microbiota, but this relationship still needs to be fully characterized, especially in the case of the Asian population. Research in this area is still at preliminary stages and most of the studies have been performed on European and American population. The diets of Asians vary

markedly within the continent and differ substantially from those of other continents. In particular, two most populated countries India and China have a unique diet profile. Moreover, India and China together accounted for 15% of world's total obese population and placed immediately after the US with 13% obese population [132] . Therefore, it becomes important to characterize the gut microbiota of people from these two countries to further comprehend the correlation between dietary components and the profile of gut bacteria and, eventually, their link to health and disease such as obesity.

In this study, we aimed to (1) characterize the prevalent bacterial taxa to define the community structure in gut samples from healthy Indian and Chinese adults, (2) compare the bacterial diversity distribution within and between the two groups studied, and (3) discover possible relationship of gut bacteria with diet and identify gut bacterial biomarkers of diet which distinguish Indian and Chinese adults. The gut bacterial profiling of 16 healthy Asian adults, including 11 Indians and 5 Chinese, obtained using next-generation sequencing. We further explored the influence of dietary habits on the composition of gut microbiota.

3.2 Materials and Methods

3.2.1 Recruitment of volunteers

A total of 16 healthy adults, including 11 Indians and 5 Chinese, were recruited for the current study (Table 3.1). All the volunteers were university students, ages 22 to 35, studying in Singapore for past 1-3 years

Table 3.1: Sample information on age, gender and ethnicity

Sample ID	Age	Gender	Ethnicity
IN1	31	Male	Indian
IN2	30	Female	Indian
IN3	30	Male	Indian
IN4	30	Female	Indian
IN5	23	Female	Indian
IN6	26	Female	Indian
IN7	27	Male	Indian
IN8	26	Female	Indian
IN9	30	Male	Indian
IN10	27	Female	Indian
IN11	23	Female	Indian
CH1	26	Male	Chinese
CH2	23	Female	Chinese
CH3	22	Female	Chinese
CH4	35	Male	Chinese
CH5	23	Female	Chinese

Healthy individuals without any gastrointestinal disorder and who did not use any antibiotics, laxatives or other drugs known to influence gastrointestinal function in the 3 months before the study, were selected. The written informed consent forms and a standard questionnaire were taken from the volunteers. Food Frequency Questionnaire (FFQ) was used to recall food diary. Ethical approval was granted by Nanyang Technological University- Institutional Review Board, Singapore

3.2.2 Sample collection

All participants were asked to refrain from smoking, eating, drinking for at least 1-2 hour prior to samples collection. Study participants were provided with two different containers: a sterile pot and a 50 mL sterile centrifuge tube. The volunteers were asked to transfer fresh feces from the sterile pot to the tube immediately after defecation. The samples were anonymized in the same order with the questionnaire (e.g. IN1, IN2...IN11 for Indians and CH1, CH2...CH5 for Chinese). Samples were homogenized, 10 g of feces were taken in 50 ml falcon tube and centrifuged (50,000 x g at 10°C for 2 h). The fecal water was removed and samples were stored at -80 °C freezer prior to DNA extraction.

3.2.3 DNA extraction

0.25 g of frozen fecal sample was used for total genome DNA extraction. DNA was extracted using Mo Bio Powersoil DNA isolation kit (Qiagen). The concentration of DNA sample was measured using NanoDrop and purity was monitored on 1% agarose gel. The concentration of DNA sample was diluted to 1 ng/μL in sterile water.

For each sample, 16S rRNA genes of V4 region were amplified. The primer set corresponding to primers 515F -806R with the barcode was used for amplification. All PCR reactions were performed with Phusion high fidelity master mix (New England Biolabs). The same volume of loading buffer containing SYBR green was mixed with PCR products. Gel electrophoresis with 2% agarose was performed for detection. Samples with the bright main strip between 400-450 bp were selected for facilitating experiments. PCR products were mixed in equidensity ratios and afterward purified with Qiagen gel extraction kit (Qiagen, Germany)

3.2.4 Library preparation and sequencing

Sequencing and analysis were performed by NovogeneAIT (Singapore). Sequencing libraries were generated using TrueSeq DNA PCR-free sample preparation kit (Illumina, USA) according to manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Then, sequencing was performed on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated.

3.2.5 Data analysis

Samples were assigned paired-end reads and reads were merged using FLASH[133] . Quality filtering of raw tags was performed according to QIIME quality-controlled process. UCHIME [134] algorithm was used to detect and remove chimera sequences, finally, effective tags were obtained. The Uparse software was used to analyse sequences. Same OTUs were assigned to sequences with $\geq 97\%$ similarity. The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Greengenes database was used based on RDP classifier algorithm to annotate taxonomic information. PyNAST software (Version 1.2)[135] was used to execute multiple sequence alignment against the “Core Set” dataset in the Greengenes database. Thus, phylogenetic relationship of different OTUs and the distinction of the prevailing species in various samples(groups), were contemplated. Normalization of OTUs abundance information was conducted using a standard of sequence number corresponding to the sample with the least sequences.

Alpha diversity was analysed using, Observed-species, Chao1, ACE, Goods-coverage, Shannon, and Simpson diversity indices. Beta diversity analysis, based on both weighted and unweighted unifracs distances were performed to evaluate compositional

heterogeneity among bacterial communities. Unweighted Pair-group Method with Arithmetic Means (UPGMA) hierarchical clustering was performed to interpret the distance matrix using average linkage. All these analyses were conducted by QIIME software (Version 1.7.0) and R software (Version 2.15.3). Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity was applied with Analysis of similarities (ANOSIM) and Multi-response permutation procedure (MRPP) to analyse the difference in microbial composition among subjects and test the significance of the difference [136].

3.2.6 Statistical analysis

Metastats analysis [137], two tailed t-test, Wilcoxon two-sample test and Pearson correlation [138] were performed. Metastats identifies the differentially abundant feature in metagenomics dataset via nonparametric t-test, Fisher's exact test and False discovery rate (FDR). LEfSe (linear discriminant analysis (LDA) effect size) [139] was applied to gut bacterial profiling data to identify bacterial biomarkers differentiating Indians and Chinese based on their dietary habits. The $LDA > 4.0$ was set as the threshold for selection of features. MetaboAnalyst 3.0 [138] was used to perform Partial least square discriminant analysis (PLS-DA) to see the separation between gut bacterial profiles of subjects based on their dietary habits.

3.3 Results

To compare the bacterial composition of GI tracts of healthy Asian adults, we collected fresh fecal samples from 16 adults aged 22 to 35 years old together with food frequency questionnaire. The diet of Indian cohorts mainly consists of whole wheat, rice, lentils, legumes, green vegetables, fruits and dairy products. Apart from this other whole grains, ghee, white flour, and fast food are also substantially included in the Indian diet. Their Chinese counterpart consumed a diet high in animal fat and protein in addition to carbohydrate and vegetables. Chinese food includes rice, noodles, beans, refined grains, white flour, peanut oil, sea food, fish, a lot of variety of meat and animal fat such as lard. Amplicon sequencing on IlluminaHiSeq2500 platform covering the V4 region of

Table 3.2: The summarized information of amplicon sequencing of 16 fecal DNA samples

Sample	Raw PE	Combined	Qualified	Nochime	Base(nt)	AvgLen(nt)	Q20	Q30	GC%	Effective%
CH1	108,477	101,735	93,372	87,097	35,588,171	409	98.64	97.27	53.97	80.29
CH2	102,446	95,911	87,368	80,974	33,210,664	410	98.62	97.2	55.24	79.04
CH3	104,371	98,527	89,207	84,417	34,629,671	410	98.58	97.11	55.84	80.88
CH4	107,509	99,405	89,302	81,821	33,995,999	415	98.54	97.07	53.78	76.11
CH5	99,340	92,944	84,778	77,949	31,856,476	409	98.52	97.06	54.01	78.47
IN10	96,681	89,660	80,787	76,119	31,716,233	417	98.65	97.27	53.04	78.73
IN11	102,112	94,360	84,342	79,439	33,026,096	416	98.49	96.94	54.67	77.8
IN1	94,186	86,888	77,787	72,601	30,215,865	416	98.58	97.12	53.48	77.08
IN2	102,296	95,745	86,504	80,986	33,589,734	415	98.62	97.15	55.25	79.17
IN3	95,251	89,115	81,270	74,796	30,685,000	410	98.64	97.28	54.05	78.53
IN4	101,549	94,857	86,562	80,032	32,857,121	411	98.65	97.26	54.66	78.81
IN5	99,728	93,306	84,954	79,340	32,578,937	411	98.63	97.23	54.38	79.56
IN6	93,086	86,553	78,110	73,226	30,313,544	414	98.52	97.02	53.99	78.66
IN7	106,418	98,665	88,173	83,996	35,148,149	418	98.6	97.17	53.93	78.93
IN8	109,214	102,082	92,543	88,088	36,321,085	412	98.59	97.13	55.44	80.66
IN9	104,681	96,815	86,976	81,110	33,839,805	417	98.65	97.27	53.56	77.48

bacterial 16S rRNA of 16 fecal DNA samples representing 11 Indian and 5 Chinese produced on an average of 85,752 high quality reads (average read length 413.12 nt) in each sample (Table 3.2). These reads were clustered at 97% similarity threshold into 7223 unique OTUs (388-521 per subject). The classification tree of particularly concerned gut bacteria (Top 10 genera for each sample) from both Indian and Chinese samples is shown in Fig. 3.1.

Fig. 3.1 Taxon composition profiles of Indian and Chinese gut bacteria. The numbers after the taxonomic ranks are the relative abundances of the corresponding taxon in gut bacteria.

The four major bacterial phyla detected were *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*, in agreement with previous studies reporting these phyla contributed to the majority of human gut bacteria[140, 141] . The dominant taxa at all the taxonomic levels were found to be the same in both the groups. The preponderant bacteria were belonging to *Firmicutes* (phylum), *Clostridia* (class), *Clostridiales*(order), *Lachnospiraceae*(family), *Bifidobacterium*(genus) at different taxonomic levels.

The 10 major bacterial clades from the gut bacterial profiles of both the groups at various taxon levels including phylum, class, order, family, genus, and species, are represented in Fig. 3.2.

Fig. 3.2 Relative abundance distribution of top 10 bacterial clades from the gut bacterial profiles of Indian and Chinese adults at various taxon levels including (a) phyla, (b) classes, (c) orders, (d) families, (e) genera

Metastats analysis and t-test were performed to determine species with significant variation between groups ($p < 0.05$) . *Bacteroidetes* ($p=0.001$) and *Cyanobacteria* ($p=$

0.003) were significantly higher in the Indian group at the phylum level. Particularly, the abundance of *Bacteroidetes* in Indians (16.39%) was almost 4 times higher as compared to *Bacteroidetes* in Chinese (4.27%). The abundance of *Firmicutes* (68.08%), *Actinobacteria* (25.48%), in Chinese were not significantly different and slightly higher, as compared to *Firmicutes* (60.5%), *Actinobacteria* (20.57%) in Indians. It is noticeable that *Firmicutes* has outnumbered *Bacteroidetes* in both the populations (Fig. 3.2a).

The bacterial classes that showed a significant difference ($p < 0.05$) between the two groups were *Bacteroidia*, *Bacilli*, *Gammaproteobacteria*, and *Chloroplast*. The bacterial orders ($p < 0.05$) accounting for significant variation between Indians and Chinese were found to be *Bacteroidales*, *Lactobacillales*, *Bacillales*, *Streptophyta*. The bacterial families ($p < 0.05$) *Prevotellaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Carnobacteriaceae* showed significantly higher abundance in Indians whereas *Porphyromonadaceae* was higher in Chinese.

The bacterial genera ($p < 0.05$) *Prevotella*, *Megasphaera*, *Catenibacterium*, *Lactobacillus*, *Mitsuokella*, *Carnobacterium*, *Lachnospira* were significantly higher in Indians as compared to Chinese. The genera, *Bifidobacterium* (15.2%) and *Prevotella* (13.07%) were almost equally dominant in Indian population (Fig. 3.2e). *Prevotella* was dominant in 5 Indian subjects whereas the rest of the 6 subjects were dominated by *Bifidobacterium*. As shown in Fig. 3.2e, the two most abundant genera in Chinese were *Bifidobacterium* (20.90%) and *Blautia* (13.19%). Previous studies on different populations have reported that human gut microbiome could be classified based on one of the four distinct communities, namely *Prevotella*, *Bacteroides*, *Clostridiales*, and *Bifidobacterium* [74]. In our study, both the populations were dominated by *Bifidobacterium* and *Clostridiales* but *Prevotella* in Indians (13.07%) is highly abundant

as opposed to very low percentage of *Prevotella* in Chinese (0.58%) (Fig. 3.2e). Thus, we can conclude that *Prevotella* is a potential biomarker which distinguishes Indian cohorts from Chinese.

To better understand if Chinese and Indians can be separated based on their gut bacterial profiles, hierarchical cluster analysis and partial least squares discriminant analysis (PLS-DA) were performed at all taxa levels (Fig. 3.3 a and b)

Fig. 3.3 Subjects are clustered based on their dietary habits (a)Partial least square discriminant analysis of gut bacterial profiles of Indian and Chinese adults **(b)** Relative abundance heat map of top 35 genera.

PLS-DA plot showed clustering of individuals based on dietary habits. Leave-one-out-cross-validation of PLS-DA plot gave $R^2 = 96.56$ and $Q^2 = 68.39$ which represent variance and predictive capability respectively. We could see differences at all taxonomic levels but the gut bacterial composition at the genus level showed the marked difference between the two groups. The abundance distribution of dominant 35 genera among all samples was displayed in the heatmap (Fig. 3.3a).

All the Indians clustered together and showed a clear separation from Chinese samples, thus dietary habits played an important role in clustering of the individuals based on the gut bacterial profiles.

3.3.1 Microbiota from Indian adults is as diverse as microbiota from Chinese adults

Alpha diversity is widely used for the analysis of microbial community diversity. It reflects the richness and diversity of the microbial community by using a series of statistical indices, species accumulation curve, and species richness curve.

Fig. 3.4 Rarefaction curves and rank abundance curves of alpha diversity: (Left) In Rarefaction curves plot, X-axis is number sequencing reads randomly chosen from a certain sample to obtain OTUs. Y-axis is corresponding OTUs. (Right) In Rank abundance curves plot, X-axis is the abundance rank. Y-axis is the relative abundance. Curves for different samples are represented by different colours.

As can be seen from Fig. 3.4, individual rarefaction and rank abundance curves revealed that the Indian adults exhibited similar diversity, including evenness, as the Chinese adults. Alpha diversity between the two groups was further compared using observed species, goods coverage, Chao1, ACE, Shannon and Simpson diversity indices. The box

plots and tabulated description of statistical indices of alpha diversity are shown in Fig. 3.5 and Table 3.3.

Fig. 3.5 Between group variation of alpha diversity indices (a) ACE (b) Simpson (c) Shannon (d) Chao1 (e) observed Species (f) goods Coverage

Overall, the gut bacterial profiles of Indians and Chinese had the similar value of these statistical indices, as calculated using two-tailed t-test with unequal variance and Wilcoxon two-sample test, the $p > 0.1$ for all the indices.

3.3.2 Gut microbiota composition and relative abundance differ between healthy Indian and Chinese adults

The alpha diversity indices were found to be similar in both the groups, now to estimate the extent of variation between gut bacterial profiles of Indians and Chinese, we proceeded to analyse beta diversity using unweighted and weighted unifracs distances.

Table 3.3: Alpha diversity indices

Sample	observed_species	shannon	simpson	chao1	ACE	good_coverage
IN1	417	5.241	0.936	516.878	502.915	0.999
IN2	392	5.353	0.949	435.4	439.532	0.999
IN3	476	5.843	0.961	517.6	519.106	0.999
IN4	478	5.992	0.96	488	496.259	0.999
IN5	426	4.889	0.893	463.82	464.431	0.999
IN6	400	5.24	0.931	433.108	430.346	0.999
IN7	349	4.706	0.915	385.273	390.915	0.999
IN8	422	5.275	0.93	461.783	460.514	0.999
IN9	395	5.306	0.949	431.667	431.41	0.999
IN10	429	4.964	0.899	450.738	459.778	0.999
IN11	399	5.079	0.937	438.722	449.411	0.999
CH1	392	5.23	0.947	417.554	427.136	0.999
CH2	466	5.554	0.953	489.459	496.189	0.999
CH3	453	4.518	0.832	508.222	505.235	0.999
CH4	388	4.916	0.928	402.786	415.561	0.999
CH5	411	5.335	0.943	435.5	440.901	0.999

The unweighted unifracs distance is calculated according to phylogenetic relationships of OTUs and weighted unifracs distance is based on the OTU's abundance information. The beta diversity indices were significantly different ($p < 0.02$) between Indian and Chinese groups, as shown by two-tailed t-test and Wilcoxon two-sample test. It reveals that two groups can be distinguished based on both community composition and relative abundance of gut bacteria. The beta diversity indices box plots which describe differences in species diversity between groups are shown in Fig. 3.6b. Box plots also directly reflect the mean value, the degree of dispersion, the maximum and minimum value which describes intragroup species diversity. Furthermore, the unweighted pair-

group method with arithmetic means (UPGMA), using unweighted and weighted unifracs distances, was applied to study the similarities among the subjects. UPGMA hierarchical clustering produced distinct clusters which distinguished Indian adults and Chinese adults (Fig. 3.6c)

Fig. 3.6 Gut microbiota composition and relative abundance differ between healthy Indian and Chinese adults (a)NMDS plot of the gut bacterial profiles. Each data point represents a subject. The stress factor value <0.2 shows the reliability of results. (b) Beta diversity box plots of unweighted and weighted unifracs distances (c)UPGMA hierarchical clustering based on unweighted and weighted unifracs distances produced distinct clusters which distinguished Indian adults and Chinese adults

Non-metric multi-dimensional scaling (NMDS) plot of the gut bacterial profiling data indicates the tendency of dietary habit wise grouping of subjects (Fig. 3.6a). The stress factor value is <0.2 , which confirms the reliability of NMDS analysis results. As shown in Fig. 3.6a, most of the Indian samples were grouped closely and showed clear separation from Chinese samples, which were more dispersed. The significance of differences was confirmed by the test of Analysis of similarity (ANOSIM) ($R=0.6205$, $p=0.001$), and multi-response permutation procedures (MRPP) ($p=0.003$). $R >0.5$ implies that separation between groups is good and intergroup variation is significantly greater than intragroup variations.

3.3.3 LEfse analysis identified gut bacterial biomarkers of diet

Our analysis has revealed a noteworthy contrast in gut bacterial profiles of Indian and Chinese adults. It suggests that different dietary habits could be the major drivers towards distinction in gut microbiota. This led us to examine gut bacterial biomarkers of diet. LEfse (linear discriminant analysis (LDA) effect size) analysis, which couples statistical significance with biological consistency and effect size estimation, detected 15 bacterial clades (LDA score >4) showing marked differences between Indian and Chinese adults (Fig. 3.7a). The histogram with LDA scores and cladogram are shown in Fig. 3.7 a and b. In the histogram, green colour represents the taxa found to be more abundant in Indian adults and red colour represents the taxa more abundant in Chinese adults.

The discriminating features at higher taxonomic levels are unlikely to be an effective biomarker, as they incorporate a diversity of genera and species. In this regard, genera *Prevotella*, *Megasphaera*, *Catenibacterium*, *Lactobacillus*, *Ruminococcus* and species *Prevotella copri*, *Lactobacillus ruminis* can potentially serve as biomarkers of diet to distinguish Indian and Chinese adults.

Fig. 3.7 LEfse analysis identified gut bacterial biomarkers of diet (a) The histogram of the LDA scores presents species(biomarker) whose abundance showed significant differences between Indian and Chinese adults. The length of each bin, namely, the LDA score, represents the effect size i.e. the extent to which a biomarker can explain the differentiating phenotypes between groups (b) In Cladogram, circles radiating from inner side to outer side represents taxonomic level from phylum to genus(species). Each circle's diameter is proportional to the taxon's relative abundance. Red nodes refer to the bacteria contributed a lot in Chinese adults, Green nodes refer to the bacteria dominant in Indians

The colours in the cladogram represent the branch of the phylogenetic tree more significantly represents a certain group. In this case, Indians are significantly represented by *Bacteroidetes*.

3.4 Discussion

Long-term diet has been noted as one of the significant factors linked to gut bacterial composition in US and European subjects[61, 70]. In our study, the unique community structure, dominated by *Firmicutes* and *Actinobacteria* and underrepresented *Bacteroides*, of Indian and Chinese gut bacteria closely matches with the gut bacterial composition of eastern Russia and rural regions consuming starch-rich diet[142]. It is known that a large portion of bacteria belongs to *Firmicutes* and *Actinobacteria*, are

nutritionally specialized towards starch [143]. Presumably, the unique bacterial community is due to high consumption of starch-rich food such as rice, oatmeal or other breakfast cereals, potatoes, refined grains, white flour, noodles, tortillas, beans, in Indian and Chinese adults.

The *Firmicutes* to *Bacteroidetes*(F/B) ratio was 14:1 in Chinese adults and the ratio was 3.7:1 in the Indian adults (Fig. 3.8).

Fig. 3.8 *Firmicutes* to *Bacteroidetes* (F/B) ratio in Indian and Chinese adults

Previous studies have reported that high (F/B) ratio and higher abundance of *Actinobacteria* are associated with obesity[124, 144, 145]. The high abundance of *Firmicutes* and *Actinobacteria* (Fig. 3.9) in Indians (81.07%) and Chinese (93.56%) justifies the study carried out by Ng et al. (2014) [132] in which China and India are placed at second and third position respectively after the US in the world's most obese population list. Indian and Chinese together accounted for 15% of world's obese people with 46 million and 30 million obese population respectively.

Fig. 3.9 Relative abundance of *Firmicutes* and *Actinobacteria*

Another notable feature of our study was the dominance of genus *Bifidobacterium* in gut bacteria of both the communities. *Bifidobacteria* have been reported to confer protection against pathogens and maintain immune system and exertion of nutritional effects to the intestinal cells and the host [146, 147]. The high abundance of *Bifidobacterium* has previously been noted as a particular feature in Asian children, rural Indian tribes, and rural Russian population[142, 148, 149] . It could be conceived that carbohydrate-based Asian diet drives the colonization of *Bifidobacteria*.

3.4.1 Dominance of *Bacteroidetes*, *Prevotella*, and *Lactobacillus* in gut bacteria of Indians is consistent with their dietary habits

We found that *Bacteroidetes* and *Prevotella* are significantly dominant in Indians as compared to that of Chinese. These bacterial clades have previously been reported to be more prevalent in the populations consuming vegan or vegetarian diet[70, 150]. *Prevotella* is also known to be associated with indigestible carbohydrate-rich diet,

particularly grains such as whole wheat, barley[61, 151]. US American and Europe population consuming high animal fat and protein diet had shown a significantly lower abundance of *Bacteroidetes* and *Prevotella*, as compared to Egyptian and rural African population which consumed the vegetarian diet rich in carbohydrate and dietary fibers[70, 72]. *Prevotella* are known degraders of xylan and other fibrous polysaccharides [152] which justifies their presence in Indian group where a large fraction of diet is comprised of vegetables and whole grains. It could be inferred that differential abundance of *Bacteroidetes* and *Prevotella* is consistent with the difference between Chinese and Indian diet. *Prevotella* is very well known as an important biomarker of diet[74].

In our study, *Lactobacillus* was identified as another important biomarker of diet. Past reports have suggested that dairy associated microbes such as *Lactobacillus* can survive the transit through the digestive system[68]. It implies that the significantly higher abundance of *Lactobacillus* in Indians could be due to their high intake of milk products (including cheese or paneer, curd, buttermilk and other dairy products) and fermented products containing *Lactobacillus*.

Catenibacterium, *Megasphaera*, and *Mitsuokella* were also found to be enriched in Indian gut. Higher abundance of these three genera was noted in Egyptian children when compared to US children[72]. Similarly, *Catenibacterium* and *Mitsuokella* were found only in Bangladeshi children but were absent in the gut of US children [71] and they have been previously linked to the abundances of *Prevotella* [72, 153]. In the present study also, *Prevotella* was found to be in positive correlation with *Catenibacterium*, *Megasphaera*, *Mitsuokella* ($r=0.562-0.90$). In comparison to Indians, starch-degrading genera, namely, *Ruminococcus*, and *Blautia*, were more abundant in the stools of Chinese, feasibly due to the high preponderance of starch as a dietary polysaccharide in

their diet. It concurs with De Filippis et al. (2016) [150] who previously reported a lower abundance of *Prevotella* and higher abundance of *Ruminococcus* in Omnivores. In addition to this, mucin degrading genus *Akkermansia* which has previously been associated with non-vegetarian[154] was detected in all Chinese samples whereas only 3 out of 11 Indian samples have shown its presence. *Prevotella* has shown a negative correlation with *Ruminococcus*, *Blautia*, *Dorea*, *Megamonas* *Bacteroides* $-(0.587-0.797)$), while a positive correlation with *Faecalibacterium* *Dialister*, *Lactobacillus* $(0.641-0.775)$. The correlation of *Prevotella* with these bacteria is in agreement with previously reported results[148, 149] .

3.5 Conclusions

Overall this study deepens our understanding of gut microbiota composition of healthy adults from two largest Asian countries, India and China. In spite of the fact that, the prevalent gut bacteria were similar to those found in other populaces, we discovered some exceptional community structure also. This information expands our knowledge of healthy human microbiome ecology and serves as a reference point for future epidemiological investigations and translational applications. Moreover, we examined the correlations among the gut bacterial community. We further suggested the possible link between diet and gut bacterial composition. We identified bacterial biomarkers of diet, which are very well known for their interaction with diet, distinguishing Indian and Chinese adults. However other hidden factors such as host physiology and genetics may also interact with their microbiota. Thus, Knowledge from this study could aid future research aim to modulate human gut microbiota, with probiotics or synbiotic dietary supplementations, which may provide new approaches to control the diet-microbiota-human health interactions.

CHAPTER 4

ASSOCIATION OF FECAL AND URINE METABOLITES WITH DIET OR ETHNICITY AND GUT MICROBIOME OF INDIAN AND CHINESE ADULTS: AN UNTARGETED METABOLOMICS ANALYSIS

Abstract

Human fecal and urine metabolites are involved in multiple host metabolic pathways. The metabolites composition is used in disease diagnosis and it is largely affected by diet or ethnicity and gut microbiota. We established an untargeted GC-MS metabolomics method based on two solvent extraction steps, first acetonitrile: methanol for protein precipitation, second methanol: water, followed by trimethylsilylation derivatization. This method enabled the detection of 120 and 86 metabolites including amino acids, phenolics, indoles, carbohydrates, sugars and metabolites of microbial origin from fecal and urine samples respectively. 41 compounds were confirmed using external standards. Next, we compared urine and fecal metabolites composition of healthy Indian and Chinese adults using untargeted GC-MS and LC-MS metabolomics approaches. Partial least squares discriminant and hierarchical cluster heat map showed dietary habit or ethnicity wise grouping of urine and fecal metabolite profiles of subjects. Our analysis revealed 53 differentiating metabolites including a higher abundance of amino acids and phenolics in Chinese and higher abundance of fatty acids, glycocholic acid, metabolites related to tryptophan metabolism in Indian adults. Correlation analysis showed a strong association of metabolites with gut bacterial profiles of the same subjects in the genus and species level. Our study showed that diet or ethnicity and gut microbiota play an important role in determining urine and fecal metabolites composition. We also confirmed a link between metabolites and gut bacteria, thus it could be possible to predict gut bacterial profile with the help of less expensive and less tedious metabolomics technology.

4.1 Introduction

Metabolites mirror the health status of an individual by acquiring extensive insights into the functioning of a biological system. Metabolomics is a powerful technique that simultaneously detects hundreds of small molecules present in a given biological system such as fecal, urine or saliva samples[4]. Fecal metabolites are the final product of both cellular and microbial metabolism undergoing inside the human intestinal tract. Some of the metabolites from the gut are absorbed into the circulation and eventually chemically modified (that is, co-metabolized) by the host, then finally excreted with the urine[34]. The development of culture-free techniques, for example high-throughput DNA sequencing, suggests the presence of various microorganisms in the human gastrointestinal tract which affects nutrient absorption, energy regulation, detoxification, or transformation of xenobiotics and the health of an individual[35, 36, 37]. It is known that gut microbiota composition is largely affected by diet, consequently metabolites produced by gut microbiota also determined by diet[38, 39]. The complex gut microbial community utilizes both diet and host derived energy sources for growth, predominantly through fermentative metabolism[40]. Analysis of fecal and urine metabolic compositions has received a lot of attention, as it does not just reflect the status of the gut microbiome yet additionally bridge the connections between symbiotic microbes and the host's health. Several previous studies have proposed the usefulness of fecal and urine metabolites in disease diagnosis, e.g. higher concentrations of amino acids, saturated fatty acids, and ursodeoxycholic acid were reported in fecal samples of colorectal cancer patients [41]. In another study higher levels of choline, trimethylamine N-oxide (TMAO) and betaine produced from dietary phosphatidylcholine in the gut were found to be associated with cardiovascular disease risk and atherosclerosis[42]. Moreover, some metabolites such as short chain fatty acids, phenolics and vitamins

decrease the risk of gastrointestinal disorder[43], cancer[44], diabetes[45] and cardiovascular diseases[46]. Thus, examining the fecal and urine metabolomes serve as a vigorous strategy for understanding the interactions between diet, human metabolism, and the gut microbiota composition in health and disease.

In this regard, there is a growing need for developing a high-throughput and large-scale sample analysis method which is pivotal to the results of metabolomics in such a field. Several mass spectrometry-based techniques (MS) and nuclear magnetic resonances spectroscopy (NMR) have been employed to analyze metabolites levels in biological samples but GC-MS is the most robust method due to higher sensitivity, resolution, reproducibility and better reliability as compared to LC-MS and NMR. However, the choice of extraction solvents and derivatization method largely affect the simultaneous detection of the total number and different classes of metabolites within a single GC-MS analysis, thus it makes the sample preparation a tedious process[26]. In the last few years, the use of LC-MS in nutritional metabolomics has also been increasing. LC-MS is more suitable for labile compounds and in addition to those that are difficult to derivatize[29]. An untargeted global investigation of urine or fecal samples is useful to identify metabolite biomarkers of diet or disease.

The two most populated Asian countries, India and China, have a unique diet profile. In our previous study, we determined the gut microbiota composition of healthy Indian and Chinese adults. In this study, an untargeted GC-MS metabolomics was established for fecal and urine samples. Untargeted GC-MS and LC-MS metabolite profiling were performed on 16 fecal and urine samples obtained from 11 Indian and 5 Chinese adults. Dietary habits or ethnicity wise grouping of subjects were observed based on their

metabolite profiles. Next, we performed a correlation analysis between metabolites and gut bacteria.

4.2 Material and methods

4.2.1 Chemicals

All chemicals were of analytical grade. Amino acid standard AAS18 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used for the identification of amino acids. Lactic acid, benzoic acid, succinic acid, 4-hydroxybenzaldehyde, 3- phenylpropionic acid, malic Acid, 4-methoxyphenylacetic acid, Gamma-Aminobutyric acid, trans-Cinnamic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, adonitol, hydrocinnamic acid, 3,4-dihydroxyphenylacetic acid, citric acid, D-Fructose, d-Galactose, d-Glucose, 3,4-dihydroxyhydrocinnamic acid, 3-(4-hydroxyphenyl)propionic acid, 1H-indole-3-acetic Acid, D-mannitol, dextrose, sucrose, indole, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used for identification purposes. Stock solutions of all the analytical standards were prepared by dissolving the compounds in MilliQ water.

4.2.2 Recruitment of volunteers

A total of 16 healthy adults, including 11 Indians and 5 Chinese, were recruited for the current study (Table 3.1). All the volunteers were university students, ages 22 to 35, studying in Singapore for past 1-3 years. Healthy individuals without any gastrointestinal disorder and who did not use any antibiotics, laxatives or other drugs known to influence gastrointestinal function in the 3 months before the study, were selected[155]. The written informed consent forms were taken from the volunteers. Ethical approval was granted by Nanyang Technological University- Institutional Review Board, Singapore.

4.2.3 Sample collection

All participants were asked to refrain from smoking, eating, drinking for at least 1-2 hour prior to samples collection. Study participants were provided with two different containers: a sterile pot and a 50 mL sterile centrifuge tube. The volunteers were asked to transfer fresh feces from the sterile pot to the tube immediately after defecation and urine samples were collected directly in 50 mL sterile centrifuge tube. The samples were anonymized as, IN1, IN2...IN11 for Indians and CHI, CH2...CH5 for Chinese [155]. Samples were homogenized, 10 g of feces were taken in 50 ml falcon tube and centrifuged (50,000 x g at 10°C for 2 h), the supernatant is collected[156]. The urine samples in 50 ml falcon tubes were centrifuged to remove any debris (50,000 x g at 10°C for 15 mins). The fecal water or urine samples were transferred to 1.5 ml Eppendorf tubes and immediately stored at -80 °C freezer prior to metabolite extraction.

4.3 GC-MS sample preparation and metabolite extraction

Fecal water or urine samples were thawed and 100 µl of samples were taken in fresh Eppendorf tubes. Five microliters of 4 mg/mL ribitol dissolved in MilliQ water was added to every sample as an internal standard to correct for any loss of metabolite during the extraction process. For protein precipitation, 450 µl of acetonitrile/methanol (3:1) was added, vortexed the mixture for 2 minutes and kept at room temperature for 10 minutes. The samples were then centrifuged at 12000 rpm at 4°C for 20 minutes and the supernatant was transferred to a fresh Eppendorf tube. A second extraction was conducted by adding 200 µl of methanol/water (8:1) to the remaining residue, vortexed for 2 minutes, kept at room temperature for 10 minutes, then centrifuged the mixture at 12000 rpm at 4°C for 20 minutes. Now the previous supernatant was added to the tube and whole mixture was centrifuged for 5 minutes, transferred the whole supernatant to

a fresh Eppendorf tube. The samples were air dried using a heat block at 30°C for 24 hours. Samples were derivatized by adding 50 µl of 2% methoxyamine HCL in pyridine (ThermoFisher Scientific) and incubated for 1 h at 37 °C. Next, 100 µL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich) was added to all samples and incubated at 70 °C for 30 min. Samples were centrifuged for 1 h at room temperature and then transferred to GC-MS glass vials[157]. 15 µl of amino acids standard and all other analytical standards (1 mg/ml) were air dried and derivatized same as the samples, and transferred to GC-MS vials.

4.3.1 GC-MS analysis and metabolites detection

The analysis of samples and standards was done using Agilent Technologies 5973N GC/MS. Metabolites were isolated through a HP-5MS capillary 54 column (30 m× 0.250 mm i.d.; 0.25-µm film thickness; Agilent J&W Scientific). Autosampler injected 1 µl of each sample and the separation was performed using the column in splitless mode. The carrier gas was Helium with a flow rate of 1.1 mL/min. Temperatures for inlets and MS source were taken as 250°C and 230°C, respectively. The temperature of the oven was kept at 75 °C for 4 min and increased to 280 °C with a rate of 4°C/min then held for 1.56 min. Mass spectrum was recorded from 40 to 600 m/z with a scan time of 0.2 s.

Data processing and metabolite identification were performed according to the previous study[158]. Briefly, data extraction was performed by GC-MS solution software (GC/MSD Chemstation Data Analysis, Agilent). The total ion chromatogram was obtained, and then mass spectra were identified. The detected metabolites peaks were identified using the NIST 08 mass spectral library (National Institute of Standards and Technology) and the extracted compounds were aligned, normalized according to

internal standard ribitol. Peaks with a similarity index more than 80% were used for feature identification.

4.4 LC-MS metabolomics analysis and metabolites identification

100 µl of fecal water or urine samples were thawed and filtered using 0.22 µm pore size membrane (Jiménez-Girón et al. 2015). Metabolomic analysis of filtered solution was performed using Agilent 6550 iFunnel Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA), operated in both positive and negative ion mode. 2 µl of samples were injected into an Agilent ZORBAX Rapid Resolution HD SB C18 (2.1 × 100 mm, 1.8 µm) maintained at 45 °C. The flow rate was set at a constant 0.4 ml/min and the pressure was 600 bar. The gradient mobile phase was composed of phase A (water containing 0.1% formic acid) and phase B (acetonitrile containing 0.1% formic acid). The gradient started with 95% A from 0 to 1 min and decreased to 5% from 1 min to 13 min, holding at 5% A till 16 min then turned to 95% in next 10 minutes and holding at 95% A for 4 minutes.

The parameters were the following: capillary voltage 3500 V, nozzle voltage 1000 V, skimmer voltage 65 V, drying gas temperature 200, sheath gas temperature 350, fragmentor voltage 175 V, drying gas flow rate 14 l/min, sheath Gas flow rate 11 l/min, nebulizer pressure 35 psi. MS data were recorded across the range of 50– 1700 m/z at 1.5 spectra/s.

All raw data extracted and processed using Agilent MassHunter Qualitative Analysis B.07.00 software. A list of peak areas, retention time and mass to charge (m/z) were obtained and metabolites were identified by comparing the data to selected databases, namely, KEGG, HMDB, and METLIN

4.5 Statistical analysis

Multivariate statistical analysis was performed using MetaboAnalyst 3.0[138]. Partial least squares discriminant analysis was performed to see the difference between metabolite profiles of Indian and Chinese adults. The $VIP > 1$ was taken to identify the features significantly differentiating between Indian and Chinese adults, then fold change ratio was obtained for each feature. Hierarchical cluster analysis heat map was obtained using ward clustering algorithm and Euclidean distance calculation to further confirm the results of PLS-DA and to show the distribution of metabolites among all individuals. Pathways analysis was performed and correlations between microbiome and metabolites were obtained using Pearson r distance measure.

4.6 Results and discussion

Metabolome analysis of fecal and urine samples are emerging as a non-invasive tool to study the relation of diet and gut microbiota with human health. Our aim was to establish an untargeted GC-MS metabolomics method which allows the simultaneous detection of as many important metabolites as possible in the fecal samples. We applied two solvent extraction steps, first acetonitrile: methanol followed by methanol: water, with trimethylsilylation derivatization. Although methanol alone has been proved to be a suitable solvent for metabolite extraction from human biofluids but most of these methods did not consider the importance of protein precipitation step in the fecal GC-MS analysis[159]. Precipitation of protein from fecal samples can be achieved by adding salts or acids but the addition of water miscible solvents prior to GC-MS could be a better approach. It decreases the electrolyte which improves MS sensitivity and avoids instrument capillary blockage[160]. The solubility of phospholipids in methanol is high, hence, in the event that methanol is utilized as an extraction solvent, lipids (including triacylglycerides and phospholipids) are extracted in sizable quantity which are involatile in GC-MS under trimethylsilylating conditions and would, consequently increase the carry-over background fatty acid signals detected in the chromatograms[161, 162, 163]. We chose acetonitrile as it is more effective in removing the phospholipids due to poor solubility of phospholipids in acetonitrile[28, 164]. The second step with just methanol was likely to get a more complete extraction and as proteins already denatured and precipitated in the first step, it cannot get into solution. Moreover, the nonpolar nature of methanol as a solvent can help maximize the metabolome coverage. Also, methanol is an effective desalting agent

To study the metabolic activity in the gut ecosystem of healthy humans and understand the relationship between diet, gut microbiome and fecal or urine metabolites, we applied

our GC-MS method on the fecal samples of 16 healthy Asian adults. We extracted 122 metabolites including amino acids, phenolics, indoles, dicarboxylic acids and other metabolites of microbial origin (Table 4.1). The same method was applied to urine samples and it enabled the detection of 86 metabolites as listed in the table 4.1.

Table 4.1: List of metabolites detected in fecal and urine samples of Indian and Chinese adults using GC-MS metabolomics .41 metabolites confirmed using analytical standards and metabolites of microbial origin are shown

RT	Fecal Metabolites	Urine metabolites	Origin
7.9	Lactic acid	confirmed	Microbial
8.26	Acetic acid		Microbial
8.58	2-propenoic acid		
9.07	L-alanine	confirmed	
10.22	Propanedioic Acid		
10.84	3-hydroxybutyric acid		
11.89	Cyclohexanecarboxylic acid		
12.67	L-valine	confirmed	
13.4	Benzoic acid	confirmed	Microbial
14.58	L-leucine	confirmed	
14.93	3-pyridinecarboxylic acid		
15.14	Benzeneacetic acid		Microbial
15.27	L-isoleucine	confirmed	
15.6	Glycine	Glycine	confirmed
15.88	Succinic acid	Succinic acid	confirmed
16.26	Methylsuccinic acid	Methylsuccinic acid	Microbial
16.47	n-valeric acid	n-valeric acid	Microbial
16.68	Pyrimidine	Pyrimidine	
16.89	2-butenedioic acid		
17.17	4-hydroxybenzaldehyde	4-hydroxybenzaldehyde	confirmed
17.25	5-hydroxyhexanoic acid		
17.33	Pipecolic acid	Pipecolic acid	Microbial
17.5		2,3-Dihydroxybutanoic acid	
17.61	Serine	Serine	confirmed
17.94	benzene		
18.47	L-threonine	L-threonine	confirmed
18.75	Pentanedioic acid		Microbial
18.83	3- phenylpropionic acid		confirmed
19.5	Beta-alanine	Beta-alanine	

19.6	Indole		confirmed	Microbial
19.9	3,4-dihydroxybutanoic acid	3,4-dihydroxybutanoic acid		
20.05	Propylene glycol			
20.35	L-homoserine	L-homoserine		Microbial
20.99	Pyruvic acid			
21.63	Malic Acid		confirmed	
21.75	4-pentenoic acid			
21.8	2-pyrrolidone-5-carboxylic acid			
21.85	Hexanedioic acid			
21.98	2-aminocaprylic acid			
22.08	pyroglutamic acid			
22.25		Butane		
22.31	L-methionine	L-methionine	confirmed	
22.36	L-proline		confirmed	
22.5	L-aspartic acid	L-aspartic acid	confirmed	
22.53		Pyrogallol		
22.76	4-methoxyphenylacetic acid	4-methoxyphenylacetic acid	confirmed	
22.78	Gamma-Aminobutyric acid		confirmed	
22.8	trans-Cinnamic acid		confirmed	Microbial
22.83		2-Furancarboxylic acid		
22.93		Creatinine		
23.35	L-cysteine		confirmed	
23.48		2,3,4-trihydroxybutyric acid		
23.51	Dodecanol			
24.07	L-threonic acid	L-threonic acid		
24.11	Linolenic acid	Linolenic acid		
24.72	3-hydroxybenzoic acid	3-hydroxybenzoic acid	confirmed	Microbial
24.9	Cyclohexylacetate			
25.14	L-ornithine			
25.18	4-hydroxybenzoic acid	4-hydroxybenzoic acid	confirmed	Microbial
25.26	Phenylalanine		confirmed	
25.34	L-glutamic acid		confirmed	
25.37		Mannonic acid		
25.45	Acetamide			
25.48		2,3-dimethyl-3-hydroxyglutaric acid		
25.57	3,5-dihydroxybenzoic acid	3,5-dihydroxybenzoic acid		
25.76	4-hydroxybenzeneacetic acid	4-hydroxybenzeneacetic acid	confirmed	Microbial
26.26		Tartaric Acid		
26.31	Arachidonic acid			

26.57	Phenol	Phenol		
26.7	3,4,5-trihydroxy pentanoic acid	3,4,5-trihydroxy pentanoic acid		
26.89	2-propenoic acid	2-propenoic acid		
26.96	D-arabinonic acid	D-arabinonic acid		
27.02	d-xylose	d-xylose		
27.45	5-hydroxyindole	5-hydroxyindole		
27.63		Arabinitol		
27.69	Threitol			
27.72	Glycyl-1-glutamic acid			
27.8	1,6-anhydro-.beta.-d-glucose	1,6-anhydro-.beta.-d-glucose		
27.83		L-Arabinose		
27.93	3-(3-hydroxyphenyl)propanoic acid	3-(3-hydroxyphenyl)propanoic acid		Microbial
28.2	3-hydroxyhex-2-enedioic acid			
28.23	1,4-butanediamine	1,4-butanediamine		Microbial
		acetamide		
28.35				
28.43	Tricarballic acid			Microbial
		d-(+)-Arabitol		
28.53				
28.65	Adonitol	Adonitol	confirmed	
28.85	Hydrocinnamic acid	Hydrocinnamic acid	confirmed	Microbial
29	trans-Aconitic acid	trans-Aconitic acid		
29.25	Phenylacetic acid	Phenylacetic acid		Microbial
29.6	Phosphoric Acid	Phosphoric Acid		
29.81	Azelaic Acid	Azelaic Acid		
30.11	Ribonic Acid	Ribonic Acid		
30.64		L-sobopyronase		
30.88	Cadaverine			Microbial
30.93	D-Arabinose			Microbial
31.05		1,2,3-propanetricarboxylic acid		
31.06	3,4 -dihydroxyphenylacetic acid		confirmed	Microbial
31.13		(4-hydroxy-3-methoxyphenyl) ethylene glycol		
31.22	N-alfa-acetyl-L-lysine			
31.33	1H-Indole-3-ethanamine			
31.38		Pinitol		
31.45	3-(3-hydroxyphenyl)-3-hydroxypropionic acid			
31.55	Citric acid	Citric acid	confirmed	
31.62	Ethylmalonic acid	Ethylmalonic acid		

32.01	Hydrobenzoin			
32.14	Arabinitol	Arabinitol		
32.23		Gluconolactone		
32.3	3-hydroxy-3-phenylpropanoic acid	3-hydroxy-3-phenylpropanoic acid		
32.48	L-(-)-Sorbitol	L-(-)-Sorbitol		
32.64	Galactonic acid	Galactonic acid		
32.85	D-Fructose	D-Fructose	confirmed	
32.91	d-Galactose	d-Galactose	confirmed	
33.14	d-Glucose	d-Glucose	confirmed	
33.2	L-Lysine		confirmed	
33.35	Tyrosine		confirmed	
33.57	d-Mannose	d-Mannose		
33.64	3,4-dihydroxyhydrocinnamic acid	3,4-dihydroxyhydrocinnamic acid	confirmed	Microbial
33.88	3-(4-hydroxyphenyl)propionic acid	3-(4-hydroxyphenyl)propionic acid	confirmed	
33.92	1H-indole-3-Acetic Acid		confirmed	Microbial
33.99	D-mannitol	D-mannitol	confirmed	Microbial
34.15	Dulcitol	Dulcitol		
34.4	MyoInositol	MyoInositol		
34.53	Aniline			
34.93	Pantothenic acid	Pantothenic acid		
35.2	Dextrose		confirmed	
35.56		D-Gluconic acid		
35.72	Hexadecanoic acid	Hexadecanoic acid		
35.78	(3,4-dihydroxy phenyl)pentanoic acid			
36.15	Scyllo-Inositol	Scyllo-Inositol		
36.41	cis-5,8,11-Eicosatrienoic acid	cis-5,8,11-Eicosatrienoic acid		
36.57	3-Indolepropionic acid			Microbial
37.4	Inositol	Inositol		
37.88	Heptadecanoic acid	Heptadecanoic acid		
38.14	Sedoheptulose	Sedoheptulose		
38.63	D-Arabinopyranose	D-Arabinopyranose		
38.73	D-Glucitol	D-Glucitol		
38.95	5-hydroxyindolepropionic acid			
40.06	Octadecanoic acid	Octadecanoic acid		
46.96	Hexacosane	Hexacosane		
49.15	Sucrose	Sucrose	confirmed	
49.94		Cellobiose		
50.51	maltose	Maltose		

Two technical replicates were run for each sample and only the metabolites detected in both the cases were listed, which shows the reproducibility of the GC-MS method. Out of all the metabolites detected, the presence of 41 compounds was further confirmed

Fig. 4.1 A representative GC-MS chromatogram of the fecal extract

using commercial external standards. The representative GC-MS chromatogram of fecal extract is shown in figure 4.1. In order to further understand the usefulness of the metabolites detected with the GC-MS method, we performed pathway analysis to relate the metabolites with their corresponding pathways.

A network map, a bar graph and a table with the probable functional role of metabolites are generated (Table 4.2, Figs. 4.2 a and b).

Table 4.2: Functional roles of fecal and urine metabolites detected using GC-MS

Pathway	Total	Hits
Glutathione Metabolism	21	6
Glucose-Alanine Cycle	13	4
Galactose Metabolism	38	8
Glutamate Metabolism	49	9
Malate-Aspartate Shuttle	10	3
Urea Cycle	29	6
Alanine Metabolism	17	4
Ammonia Recycling	32	6
Glycine and Serine Metabolism	59	9
Homocysteine Degradation	9	2
Lactose Degradation	9	2
Spermidine and Spermine Biosynthesis	18	3
Cysteine Metabolism	26	4
Methionine Metabolism	43	6
Aspartate Metabolism	35	5
Gluconeogenesis	35	5
Arginine and Proline Metabolism	53	7
Carnitine Synthesis	22	3
Transfer of Acetyl Groups into Mitochondria	22	3
Warburg Effect	58	7
Fructose and Mannose Degradation	32	4
Amino Sugar Metabolism	33	4
Beta-Alanine Metabolism	34	4
Glycolysis	25	3
Fatty Acid Biosynthesis	35	4
Biotin Metabolism	8	1
Phenylalanine and Tyrosine Metabolism	28	3

Selenoamino Acid Metabolism	28	3
Pyruvate Metabolism	48	5
Alpha Linolenic Acid and Linoleic Acid Metabolism	19	2
Phenylacetate Metabolism	9	1
Lactose Synthesis	20	2
Starch and Sucrose Metabolism	31	3
Pyruvaldehyde Degradation	10	1
Pantothenate and CoA Biosynthesis	21	2
Propanoate Metabolism	42	4
Citric Acid Cycle	32	3
Glycerol Phosphate Shuttle	11	1
Phosphatidylethanolamine Biosynthesis	12	1
Taurine and Hypotaurine Metabolism	12	1
Ketone Body Metabolism	13	1
Thyroid hormone synthesis	13	1
Nicotinate and Nicotinamide Metabolism	37	3
Inositol Phosphate Metabolism	26	2
Valine, Leucine and Isoleucine Degradation	60	5
Sphingolipid Metabolism	40	3
Lysine Degradation	30	2
Phosphatidylinositol Phosphate Metabolism	17	1
Butyrate Metabolism	19	1
Ethanol Degradation	19	1
Mitochondrial Electron Transport Chain	19	1
Inositol Metabolism	33	2
Catecholamine Biosynthesis	20	1
Nucleotide Sugars Metabolism	20	1
Threonine and 2-Oxobutanoate Degradation	20	1
Ubiquinone Biosynthesis	20	1

Vitamin B6 Metabolism	20	1
Betaine Metabolism	21	1
Tryptophan Metabolism	60	4
Tyrosine Metabolism	72	5
Sulfate/Sulfite Metabolism	22	1
Glycerolipid Metabolism	25	1
Oxidation of Branched Chain Fatty Acids	26	1
Phytanic Acid Peroxisomal Oxidation	26	1
Plasmalogen Synthesis	26	1
Histidine Metabolism	43	2
Mitochondrial Beta-Oxidation of Long Chain Saturated Fatty Acids	28	1
Folate Metabolism	29	1
Pentose Phosphate Pathway	29	1
Purine Metabolism	74	4
Fatty Acid Elongation In Mitochondria	35	1
Porphyrin Metabolism	40	1
Pyrimidine Metabolism	59	2
Fatty acid Metabolism	43	1
Bile Acid Biosynthesis	65	2
Steroid Biosynthesis	48	1
Arachidonic Acid Metabolism	69	2

The functional analysis of urine and fecal metabolites of Indian and Chinese adults showed that most of the metabolites were involved in more than one pathway. For example, the metabolites of glutamate metabolism (Gamma-Aminobutyric acid, glycine, L-glutamic acid, L-alanine, L-aspartic acid, pyruvic acid, succinic acid, L-cysteine, phosphoric acid) represented most in the study.

Fig. 4.2 (a) A network map and (b) a bar graph with the functional role of metabolites detected using GC-MS metabolomics in fecal and urine samples of Indian and Chinese adults

The metabolites related to galactose metabolism were galactitol, D-Glucose, D-Galactose, D-mannose, myo-Inositol, sorbitol, sucrose, D-Fructose. The metabolites of glutathione metabolism detected were glycine, L-glutamic acid, L-alanine, pyroglutamic acid, L-cysteine, phosphoric acid. Glycine, L-glutamic acid, L-proline, L-aspartic acid, ornithine, succinic acid, phosphoric acid were found to be involved in arginine and proline Metabolism. The metabolites of methionine metabolism detected were glycine, L-serine, L-cysteine, L-methionine, L-homoserine, putrescine.

Metabolites associated with ammonia recycling were glycine, L-glutamic acid, L-serine, L-aspartic acid, pyruvic acid. Ornithine, putrescine, L-methionine were associated with spermidine and spermine biosynthesis.

4.6.1 Fecal and urine metabolomics revealed differences between Indian and Chinese adults

The Indian adults recruited for this study were consuming food which closely matches the Mediterranean style diet. The main components of their diet were whole wheat or other whole grains, nuts, rice, lentils, legumes, green vegetables, fruits, dairy products, sweets, ghee, refined flour and fast food. On the other hand, Chinese adults consumed a diet including seafood, fish, chicken, pork, beef, a lot of variety of other meat, which was high in animal fat and protein. In addition, rice, noodles, beans, peanut oil, green vegetables, white flour, refined grains were a substantial part of the Chinese diet. In our previous study, we have reported the differences in gut microbiome composition between Indian and Chinese adults[155]. To assess whether the differences in gut microbiome composition and dietary habits between Indian and Chinese adults can alter the luminal environment, GC-MS and LC-MS metabolomics were performed on 16 fecal and urine samples, including 11 Indian and 5 Chinese. Overall 69 and 123 metabolites were extracted from LC-MS metabolomics of fecal and urine samples, respectively. LC-MS is more suitable for labile compounds, polar compounds and in addition to those that are difficult to derivatize[29]. Some specific classes of metabolites such as organic acids, organic amines, nucleosides, ionic species, nucleotides, and polyamines are more compatible with LC-MS analysis. Thus, a combination of both GC-MS and LC-MS will be more suitable for a comprehensive metabolomics analysis[165].

In order to further strengthen our results, the multivariate statistical analysis was applied on urine metabolites profiles also. PLS-DA plot with $R^2=80.81$, $Q^2=63.18$ and heat map of urine metabolites data also showed a clear distinction between Indian and Chinese based on their urine metabolite profiles. The heatmap representing the distribution of urine metabolites among all the individuals and PLS-DA plots are shown in figure 4.4a and b.

Fig. 4.4 Urine metabolites profiles are influenced by dietary habits or ethnicity of subjects (a) Partial least square discriminant analysis of urine metabolites profiles of Indian and Chinese adults (b) Heat map of the distribution of urine metabolites among all individuals

Overall dietary habits or ethnicity were found to play an important role in clustering of individuals based on urine and fecal metabolite profile. These results justify our previous findings where diet or ethnicity was found to be important in determining gut microbiome composition[155]

4.6.2 Metabolites that differentiate between Indian and Chinese adults could be associated with their diet

It has been reported that variable importance in the projections (VIP) values greater than 1 could be considered as the most relevant metabolites for explaining the differences[166]. Based on the criteria of $VIP > 1$, 53 compounds distinguishing Indian and Chinese adults were identified. The metabolites with VIP values and fold change ratio between Indian and Chinese are presented in table 4.3.

Table 4.3: Metabolites that differentiate between Indian and Chinese adults. All the metabolites are presented with variable importance in projection (VIP) values and fold change ratio (Chinese/Indian)

Metabolite	VIP	Fold (Chinese/Indian)
L-alanine	1.71	1.72
L-leucine	1.87	3
L-isoleucine	1.73	2.06
Glycine	1.62.	1.871
L-proline	1.67	2.2
Serine	1.75	2.62
L-glutamic acid	1.69	9.84
L-threonine	1.22	1.7
Gamma aminobutyric acid	1.71	2.65
L-homoserine	1.81	2.18
Benzeneacetic acid	2.34	3.96
3-(4-hydroxyphenyl) propionic acid	1.67	3.94
3-hydroxy-3-phenylpropanoic acid	1.93	4.66
3-(3-hydroxyphenyl)-3-hydroxypropionic acid	2.09	37.09
3-hydroxybenzoic acid	1.43	2.18
Malic acid	2.03	4.26
Citric acid	1.36	3.03

Sedoheptulose	1.76	2.56
5-hydroxyindole	1.3	1.76
2-hydroxydecanedioic acid	2.39	3.7
L-urobilin	1.59	7.92
3-hydroxyisovalerylcarnitine	1.82	6.98
Pentanedioic acid	1.97	3.72
Tricarballic acid	1.46	29.72
3-hydroxyphenylglycine	1.69	3
Myoinositol	1.74	4.09
2-piperidinecarboxylic Acid	1.25	4.13
5,6-dihydroxyindole	0.98	3.26
4-sulfobenzyl alcohol	2.49	0.229
5-sulfosalicylic acid	1.26	4.12
L-sobopyronase	1.32	0.7
D-gluconic acid	1.97	0.54
Dulcitol	1.11	0.085
Tartaric Acid	1.30	0.008
L-isoleucyl-L-proline	1.54	0.082
Pantothenic acid	1.51	0.47
Palmitaldehyde	1.54	0.43
Diisopropyl adipate	1.41	0.166
Cyclohexanecarboxylic acid	1.541	0.106
S-ribosyl-L-homocysteine	1.86	*
Syringin	2.73	*
4-pyridoxic acid	1.22	0.38
Indole-3-ethanol	1.73	0.45
2-aminomuconic acid semialdehyde	1.65	0.11
3-hydroxy-sebacic acid	1.95	0.10
Glycocholic Acid	1.28	0.51

D-glucitol	1.55	2
Pinitol	2.07	0.21
Gluconolactone	1.78	0.08
Benzoic acid	1.49	0.39
3-phenylpropionic acid	1.32	3.89
Creatinine	1	1.31
Creatine	2.31	1.2

*detected only in Indian samples

The levels of 7 amino acids were higher in Chinese adults, consistent with high protein consumption in subjects consuming Chinese diet. Our results are in agreement with the study carried out by Shankar et al.(2017) where US children consuming the western diet with high protein showed a higher level of amino acids as compared to Egyptian consuming Mediterranean diet[72]. Microbial degradation of dietary proteins results in the production of amino acids. However, the bioavailability of amino acids in the host is controlled by the gut microbiota composition. It has been reported previously that distribution of free amino acids in the gastrointestinal tract of germ free and conventionalized mice can be altered by the gut bacteria as the amino acids could be utilized by the bacteria in SCFA synthesis [73, 167]. Pathway analysis on differentiating metabolites showed that the seven amino acid metabolites(glycine, L-serine, L-isoleucine, L-proline, L-glutamic acid, L-alanine, L-threonine) were found to be involved in aminoacyl-tRNA biosynthesis pathway. The etiology of diseases including cancer, neuronal pathologies, autoimmune disorders and abnormal metabolic conditions is found to be associated with aminoacyl-tRNA synthetases[168]. The metabolites L-glutamic acid, Gamma-Aminobutyric acid, L-alanine were associated with alanine, aspartate, and glutamate metabolism. Glycine, L-glutamic acid, L-alanine were related to glutathione metabolism and alanine metabolism. Metabolites of glutamate

metabolism (Gamma-Aminobutyric acid, glycine, L-glutamic acid, L-alanine) were higher in Chinese. Another group of five metabolites (L-threonine, L-serine, glycine, creatine, L-homoserine) was associated with glycine, serine and threonine metabolism

We have also noticed a marked increase in benzeneacetic acid in Chinese samples which has previously been associated to high protein diet[169, 170]. Interestingly, most of the phenolics (3-(4-hydroxyphenyl) propionic acid, 3-hydroxy-3-phenylpropanoic acid, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, 3-hydroxybenzoic acid, 3-phenylpropionic acid) were higher in Chinese except benzoic acid which was more abundant in Indian subjects. The high amount of phenolics could be associated with high consumption of soy products, eggplants, mushrooms, blueberry, cranberry and leafy green vegetables such as broccoli, cabbage, cauliflower, spinach[85]. It is evidenced that phenolics are derived from the plant-based diet but these compounds can also be produced from microbial fermentation of protein-rich diet[169, 170]. Phenolics displayed many important functions including inhibition of pathogens, prevention of various chronic diseases such as cancer, diabetes, and cardiovascular diseases, antioxidant[64, 171]. Levels of central metabolism intermediates (malate, citrate, sedoheptulose, myoinositol, and D-Glucitol) were also higher in Chinese samples, possibly indicating incomplete fermentation of complex polysaccharides in the guts of these adults. Furthermore, higher abundance of creatine and creatinine in Chinese adults concurs with Stella et al. (2006), which showed consumption of meat is associated with an increase of these metabolites[172]. Creatinine is a breakdown product of creatine and high levels of creatinine could be an indicator of kidney diseases[173].

The higher amount of fatty acids and conjugates (palmitaldehyde, 3-hydroxy-sebacic acid, diisopropyl adipate) were observed in Indian samples. The higher amount of fatty acids could be associated with higher secretion of glycocholic acid in Indians. It is a

secondary bile acid produced by the bacterial actions in the colon. Bile acids act as a fatty acid emulsifier to facilitate the digestion of fats and oil. The levels of metabolites related to tryptophan metabolism (2-aminomuconic acid semialdehyde, Indole-3-ethanol) were also higher in Indians. Higher abundance of tryptophan related metabolites has been associated with the Mediterranean diet[174]. The bioactive compound, a phenylpropanoid, syringin was detected only in Indian subjects which could be linked with plant based diet.

Fig. 4.5 Metabolites that differentiates Indian and Chinese adults are mapped onto metabolic pathways. Red color represents higher abundance in Chinese, green color represents higher abundance in Indian, blue color shows the metabolites that do not differ between the two groups and black represents the undetected metabolites

Syringin is known for its pharmacological properties including scavenging of free radicals ,anti-diabetic effect , anti-allergic effect, anti-inflammatory potential[175]. The alteration of metabolites in response to different dietary components has been studied but linking the metabolite changes to specific pathways still remains a challenge. The effect of diet on the human body and health or diseased status are directly correlated with presence or absence of specific combinations of metabolites. Generally, it is the combination of metabolites rather than the individual compounds, which is of great biological relevance[62]. Therefore, the alteration in the metabolites involved in the central metabolism and in the linking metabolites to amino acid synthesis w summarized on a simplified metabolic map (Fig. 4.5)

4.6.3 Gut microbiome is correlated with fecal and urine metabolites

We investigated the interactive features between metabolites differentiating Indian and Chinese adults, metabolites of microbial origin and gut microbiome. The metabolites showed a comprehensive correlation with available 16S rRNA sequencing data on the gut bacterial profiles of the same subjects in the genus and species level[155]. The heat map showing correlations between metabolites and gut microbial abundance in genus, species levels is presented in the figure 4.6

Three genera, *Ruminococcus*, *Dorea*, and *Blautia* which are member of one of the most abundant families, *Lachnospiraceae*, are positively correlated with L-alanine, L-leucine, L-isoleucine, glycine, serine, and L-proline. Consistent with our results, in a recent study of Swedish adults, all these genera were associated with increased levels of amino acids except a negative correlation was observed with serine[176]. Similarly, *Clostridium* which is known to be involved in the amino acid production [177]was found to be associated with L-alanine, L-norleucine, L-isoleucine, glycine, serine, L-proline

4-hydroxybenzoic acid concurs with the previous studies[181]. *Bacteroides ovatus* is correlated with n-valeric acid. *Collinsella aerofaciens* showed a positive correlation with sedoheptulose, citric acid, tricarballic acid. *E.coli* was positively correlated with pipercolic acid. Genus *Faecalibacterium* showed a negative correlation with L-leucine and serine and species *Faecalibacterium prausnitzii* showed a negative correlation with serine, L-leucine, and malic acid. *Lactobacillus ruminis* showed a negative correlation with glycine. Genera *Dialister*, *Catenibacterium*, *Turicibacter* showed a positive correlation with tartaric acid and species *Ruminococcus bromii*, *Parabacteroides distasonis*, *Bacteroides caccae* were negatively correlated with tartaric acid. Genera *Akkermansia*, species *Coprococcus catus* were positively correlated with creatine and genera *Mitsuokella*, *Weissella*, *Lactobacillus*, species *Mitsuokella multacida*, *Lactobacillus ruminis* were negatively correlated with creatine. Genera *Faecalibacterium*, *Succinivibrio*, *Macroccoccus* are negatively correlated with creatinine. Genera *Bilophila*, *Enterococcus*, *Dorea*, *Clostridium*, *Phascolarctobacter* and species *Faecalibacterium prausnitzii*, *Photobacterium angustum* are positively correlated with creatinine. Genera *Escherichia*, *Paraprevotella*, *Akkermansia* have a positive correlation with glycocholic acid. Genus *Carnobacterium* and species *Ruminococcus flavefaciens*, *Butyricicoccus pullicaecorum*, *Mitsuokella multacida*, *Bacteroides fragilis* have a negative correlation with glycocholic acid. Genera *Enterococcus*, *Dermacoccus*, *Kocuria*, *Roseburia* and species *Eggerthella lenta*, *Bacteroides fragilis* showed a negative correlation with 3-phenylpropionic acid. Genus *Oscillospira* is positively correlated with citric acid, concurs with maria et al [182]. Genera *Acinetobacter*, *Microbacterium*, *Dysgonomonas*, *Bulleidia*, *Oscillospira* and species *Bacteroides caccae*, *Kocuria rhizophila*, *Pseudomonas fragi*, *Ruminococcus bromii* were positively correlated with 4-pyridoxic acid.

4.7 Conclusions

In summary, we have established a trimethylsilylation based GC-MS metabolomics method which enabled the detection of various important fecal and urine metabolites such as amino acids, phenolics, indoles, sugars in health and nutritional studies. This study reported the fecal and urine metabolites of Indian and Chinese adults. Individuals with the same dietary habit or ethnic group had more similar profiles of gut microbiome and metabolites. A higher level of amino acids and phenolics were observed in Chinese population whereas Indian cohort showed more abundance of fatty acids and glycocholic acid. Overall, 53 metabolites differentiating Indian and Chinese adults were identified based on PLS-DA and fold change ratio. Further analysis on the gut microbial and metabolite profiles of the Indian and Chinese population should be carried out for more details. Various metabolites such as amino acids, phenolics, glycocholic acid were found to be correlated with bacterial genera or species. Although it was a preliminary analysis with small sample size, this study strengthens our understanding towards links between metabolite signatures with specific bacterial genera or species and a possibility of foreseeing gut bacterial profile in light of less expensive and less tedious metabolomics analysis. However, a study of a much larger population with different groups across Asia and the rest of the world will give a better picture of these con

CHAPTER 5

INVOLVEMENT OF ORGANIC ACIDS AND AMINO ACIDS IN AMELIORATING NI(II) TOXICITY INDUCED CELL CYCLE DYSREGULATION IN *CAULOBACTER CRESCENTUS*: A METABOLOMICS ANALYSIS

Abstract

Nickel (Ni(II)) toxicity is addressed by many different bacteria but bacterial responses to nickel stress are still unclear. Therefore, we studied the effect of Ni(II) toxicity on cell proliferation of α -proteobacterium *Caulobacter crescentus*. Next, we showed the mechanism that allows *C. crescentus* to survive in Ni(II) stress condition. Our results revealed that the growth of *C. crescentus* is severely affected when the bacterium was exposed to different Ni(II) concentrations, 0.003 mM slightly affected the growth, 0.008 mM reduced the growth by 50% and growth was completely inhibited at 0.015 mM. It was further shown that Ni(II) toxicity induced mislocalization of major regulatory proteins such as MipZ, FtsZ, ParB, and MreB, resulting in dysregulation of the cell cycle. GC-MS metabolomics analysis of Ni(II) stressed *C. crescentus* showed an increased level of nine important metabolites including TCA cycle intermediates and amino acids. This indicates that changes in central carbon metabolism and nitrogen metabolism are linked with the disruption of cell division process. Addition of malic acid, citric acid, alanine, proline and glutamine to 0.015 mM Ni(II) treated *C. crescentus* restored its growth. Thus, the present work shows a protective effect of these organic acids and amino acids on Ni(II) toxicity. Metabolic stimulation through the PutA/GlnA pathway, accelerated degradation of CtrA, and Ni-chelation by organic acids or amino

acids are some of the possible mechanisms suggested to be involved in enhancing *C. crescentus*'s tolerance.

Our results shed light on the mechanism of increased Ni(II) tolerance in *C. crescentus* which may be useful in bioremediation strategies and synthetic biology applications such as the development of whole cell biosensor.

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5.1 Introduction

Bacteria are often exposed to heavy metal ions in their environment. Human activities such as industrial production, agriculture, mining, and smelting operations, significantly contribute to the elevated level of heavy metals in the environment[98, 183, 184] . The U.S. Environmental Protection Agency has placed 13 heavy metals, including nickel (Ni), on its priority pollutants list of 126 compounds[88]. Nickel toxicity in plants, animals, and humans has been widely studied[185, 186, 187] . Nickel toxicity has received a lot of attention due to its role as a human carcinogen. Conversely, the importance of nickel toxicosis in microorganisms is less well studied[188] . However, the nickel defense system (RcnA) in *E.coli* and detection of putative homologues in archaea and throughout bacteria suggests that excessive nickel is a routine physiological concern[189] . Therefore, it becomes important to understand the effect of nickel exposure to bacteria. Nickel may support bacterial growth when present in trace amounts but at higher concentrations, this metal causes toxic effects to bacteria by inhibiting iron metalloenzymes like taurine/ α KG dioxygenase (TauD) in *E.coli* [190], zinc metalloenzymes like *Trypanosoma cruzi* metallocarboxypeptidase 1 (TcMCP-1) [191] or some other metalloenzymes such as nitrous oxide reductase from *Rhodobacter sphaeroides* [192] , Cobaltochelataase from *Pseudomonas denitrificans* [193]. Nickel is also known to inhibit non-metal enzymes, for example, Uricase (urate oxidase) from *Bacillus fastidiosus* [194] and N-carbamoyl D-amino acid amidohydrolase from *Agrobacterium* lose activity in the presence of nickel[195] . Hence it is essential for cells to maintain homeostasis by tightly regulating the cellular concentration of nickel. When the metal concentration is insufficient inside the cell, bacteria express metal import systems, such as, NikABCDE which is a part of the ABC transporter subfamily found in eubacteria and archaea [196, 197] . Conversely, if the cellular concentration of metal

is more than the required limit, bacteria use several types of metal transport system, such as the RcnA efflux pump in *E.coli* [189], the RND transport system in *C. metallidurans*, *A. xylosoxidans* and *H. pylori* to remove the excess metal ions [188, 198] .

It is known that bacteria employ different molecular mechanisms to survive in often unknown and fluctuating nickel stressed environmental conditions. However, despite years of research, it is still not fully understood how microbes detect and cope with nickel stress in their surroundings. Furthermore, external environmental conditions (e.g. nickel stress) can influence the cellular physiology of microorganisms, but it is not entirely clear how the different external signals or cues impinge on fundamental biological processes, like the cell cycle, in bacteria. Cell cycle regulation has been studied in several model bacteria. One prominent example is the asymmetrically dividing alpha proteobacterium *Caulobacter crescentus*. *C. crescentus* has been found in many different environments, including wastewater, seawater, soil, and deep subsurface goldmines [107].

It is particularly noted for its ability to survive in low nutrient conditions or in habitats that contain harmful levels of metal contamination. In particular, scientists have found that *C. crescentus* can tolerate at least up to 2-3 times more uranium stress than *E.coli*. *C. crescentus* could survive at 1 mM of uranium while *E.coli* was completely non-viable at 0.5 mM [199]. In addition, *C. crescentus* is a model organism for understanding the cell cycle regulation, asymmetric cell division and cellular differentiation [109, 110] . The cell cycle mechanism of *C. crescentus* has been studied mostly in ideal laboratory conditions but it is still not understood how natural environmental stresses (e.g. heavy metal stress) affect the complex regulatory cascade of the cell cycle. A deep understanding of bacterial stress responses is important for the development of antibiotics or other antimicrobial strategies as well as for synthetic biology applications

such as the development of whole cell biosensors. Previous studies have also shown that *C. crescentus* has applications in uranium biosensor and cadmium bioremediation [199, 200] .

In this chapter, we seek to (1) investigate how Ni(II) stress in the environment can affect the proliferation of microorganisms, such as *C. crescentus* and (2) identify the metabolites that enhance or reduce *C. crescentus* 's ability to survive in Ni(II) stress condition. This study began with the systematic analysis of the effect of Ni(II) stress on *C. crescentus* growth and survival. Our analysis revealed that certain Ni(II) concentration severely affect both cell morphology and cell cycle of *C. crescentus*. The diameter of Ni(II) stressed *C. crescentus* cells was increased due to mislocalization of important cytoskeleton protein MreB. We also found that mislocalization of the cell division proteins such as FtsZ, MipZ, and ParB was responsible for dysregulation in chromosome replication and cell division process. Next, GC-MS metabolomics analysis revealed nine important metabolites which were increased in Ni(II) stressed *C. crescentus*. The restoration of growth of 0.015 mM Ni(II) treated cells upon supplementation of malic acid, citric acid, glutamine, alanine, and proline confirmed their role in ameliorating Ni(II) toxicity in *C. crescentus*.

5.2 Materials and methods

5.2.1 Bacterial strains, media, and growth

The *Caulobacter crescentus* strain CB15N (ATCC 19089) and its derivatives cultures were maintained on PYE (peptone-yeast extract) agar plates containing 0.2% (wt/vol) bacto peptone (Difco), 0.1% (wt/ vol) yeast extract (Difco), 1 mM MgSO₄, 0.5 mM CaCl₂, and 3% (wt/vol) agar (Difco) supplemented with appropriate combinations of antibiotics. Broth cultures used for growth curve, qPCR and microscopic studies were

PYE and M2G Minimal media contains per litre [50 ml of 20X M2 salts (34.8 g Na_2HPO_4 , 21.2 g KH_2PO_4 , 10 g NH_4Cl per litre), 0.5 mM MgSO_4 , 10 ml of 20% Glucose, 1 ml 10mM FeSO_4 and 10mM EDTA (Sigma #F-0518), 0.5 mM CaCl_2]. The overnight culture was inoculated with colonies from PYE plates, grown at 30°C, and shaken continuously at 225 rpm [201].

5.2.2 Toxic metals effect on growth and survival

1M, 0.1 M and 10 mM stock solutions of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, K_2CrO_4 , CdSO_4 , Na_2SeO_3 , CuSO_4 (Sigma-Aldrich) were prepared by dissolving the metal compounds in MilliQ water. All metal stocks were sterilized by filtration through a 0.2 μm membrane. Similarly, 0.5 M and 0.25 M stock solutions of glutamine, proline, alanine, valine, glutamic acid, malic acid and citric acid (Sigma-Aldrich) were prepared.

5 ml culture of *C. crescentus* were grown in PYE overnight. The PYE culture is diluted 1:50 in minimal media and grew until bacteria reached mid log phase. The mid log phase culture then diluted again to OD 0.03 - 0.04 in fresh minimal media and 5 ml of culture were aliquot into different 14 ml snap cap tubes already containing heavy metals. A control was also prepared with no heavy metal. The OD was measured at 660 nm using the spectrophotometer. For spot dilution test, OD values were normalized for all the cultures after 24 h and 10-fold serial dilutions were prepared for each. 4 μl of each dilution was spotted on PYE agar plates. Plates were kept in 30°C incubator for 40-48 hrs, then images were taken using Gel doc system (Bio-Rad).

To test the effect of organic acids and amino acids on growth of 0.015 mM Ni(II) treated cells, 1 mM or 10 mM of each amino acid or organic acid exogenously added to *C. crescentus* culture in M2G minimal media containing 0.015 mM Ni(II).

5.2.3 RNA Extraction

The overnight *C. crescentus* culture in PYE was diluted in minimal media and grew until bacteria reached mid log phase. The mid log phase culture in minimal media was diluted again to OD 0.03 - 0.04 in fresh minimal media. When the bacteria reached exponential phase (OD 0.25 -0.30), 10 ml of culture were aliquot into different 50 ml tubes already containing Ni(II) concentrations and incubated for 30 mins. A control was also prepared with no heavy metal. After 30 mins of heavy metal shock, samples were centrifuged at $10,000 \times g$ for 5 min and the supernatant was removed. The cell pellets were stored at -80°C . RNA was first isolated by QiAzol-chloroform extraction. Then RNeasy mini kit from Qiagen including on-column DNase digestion was used for further extraction steps. RNA concentration was measured using Nanodrop.

5.3 Quantitative PCR

Quantitative PCR (qPCR) was performed in 96 well plates in a CFX96 Touch Real-Time PCR Detection System(Bio-Rad). qScript cDNA SuperMix (Quantabio) was used for reverse transcription. For quantitation of transcript levels, PerfeCTa SYBR Green FastMix (Quantabio) was used and thermocycling conditions were as follows: 3 min at 95°C , and 39 cycles (30 s at 95°C , 45 s at 55°C , 30 s at 60°C) for amplification. Primers used for qPCR are listed in table 5.1

Table 5.1 List of primers used for qPCR

Name	Sequence
CC_2677 (for)	TCTACTATCGCGGCTATCCC
CC_2677 (rev)	GTACATGCGCGAGGTGTC
ctrA (for)	CCGACGACTACATGACCAAG
ctrA (rev)	AGCATCTGGTACTCCTTGCC
sciP (for)	TGCGATCGTTACAAGCTGA
sciP (rev)	ACCCACCTTAACGGTACTGC
gcrA (for)	AGACGGGTAATGAGCTGGAC
gcrA (rev)	CAATTGCTTGGCGATCTG
dnaA (for)	CTGTCGGCGGGTCTGGTCTG
dnaA (rev)	AACGGTCGGCGAGGAAGTGC
ccrM (for)	CTGGATCCTCAACGACATC
ccrM(Rev)	TCGTAGTTGAAGGTGTAGC
ftsZ (for)	ATCGCTAACCCGCTGCTGGAC
ftsZ (rev)	CGCGCCGAAGATGATGTTGG
mipZ (for)	GCGCGTGGCTGGACAACAAGA
mipZ (rev)	TTCGAAACCGGCCACCTGCTC
CC_0464 (for)	ACTATGCCCCGTGAGGTCGTTGAG
CC_0464 (rev)	TGCCCCGTTTCCGCTTCTTTA

5.4 Fluorescence Microscopy

The overnight culture in PYE was diluted in minimal media and grew until *C. crescentus* strains reached mid log phase. This mid log phase culture in minimal media was diluted again to OD 0.03 - 0.04 in fresh minimal media and 5 ml of cultures were aliquot into different 14 ml snap cap tubes already containing heavy metals concentrations to be tested. A control was also prepared with no heavy metal. All the strains were exposed to heavy metal for 12 hours. Expression of xylose-inducible genes or vanillate-inducible genes was induced by adding 0.3% xylose(Sigma-Aldrich) or 0.5 mM vanillate (Sigma-Aldrich) for 2-3 hours[202] . All samples were imaged on freshly prepared 1% agarose

pad onto microscopy slides. Microscopy was performed on a DM6000B(Leica) upright microscope with Leica Application Suite X (LAS X) software. An external light source Leica EL6000 was used for enhanced fluorescence imaging. Cell dimensions were measured using ImageJ [203] Strains used in this study are listed in table 5.2.

Table 5.2: List of *C. crescentus* strains

Strain	Description	Reference
77	pMT464-CC0903; Pvan-ftsZ-mCherry (gentR)	[204]
1643	CB15N pMT335-CC0903; Pxyl-GFP-MreB (kanR)	[205]
MT15	CB15N ori:(lacO) _n ;xylX::pHPVV472	[206]
MT 97	CB15N mipZ::mipZ-yfp	[204]
1534	CB15N pVCHYN2-parB [207](vanA::mcherry-parB)	[207]

0.003 mM, 0.008 mM and 0.015 mM of Ni(II) concentrations. A control was also prepared with no heavy metal. Three separate sets of samples were collected after 30 mins, 2h and 4h of heavy metal shock respectively. Samples were centrifuged at 10,000 × g for 5 min and the supernatant was removed. The cell pellets were stored at -80°C. The cell pellets were stored at -80°C. Metabolites were extracted after collecting all the samples. 900 µL of 2:1 methanol: chloroform solution was added to frozen cell pellets for extraction.

5.5 Collection of samples and extraction of metabolites

The overnight *C. crescentus* culture in PYE was diluted in minimal media and grew until bacteria reached mid log phase. This mid log phase culture in minimal media was diluted again to OD 0.03 - 0.04 in fresh minimal media. When the bacteria reached mid log phase (OD 0.25 -0.30), 5 ml of culture were aliquot into different 14 ml tubes already containing Samples were then lysed using glass beads and a bead grinder. 5 µL of 4

mg/mL ribitol dissolved in MilliQ water was added to each sample as an internal standard to correct for any loss of metabolite during the extraction process. After this 200 μ L of water and chloroform solution was added to each sample. Samples were centrifuged for 10 min at 14,000 RPM and supernatant was transferred to a fresh Eppendorf tube (Booth et al. 2015). The supernatant was air dried using a heat block set at 37°C for 24 h. 50 μ L of 2% methoxyamine. HCL in pyridine (ThermoFisher Scientific) was added to all the samples and incubated for 1h at 37°C. Next, 100 μ L of N,O Bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1% TMCS, Sigma Aldrich) was added to all samples and incubated for 30 min at 70°C. Samples were centrifuged for 1 h at room temperature and then transferred to GC-MS glass vial [157].

5.6 GC-MS analysis

The samples were analyzed using an Agilent Technologies 7890B GC and 5977A GC-MSD system. Metabolites were isolated through a HP-5MS capillary 54 column (30 m $\times$ 0.250 mm i.d.; 0.25 μ m film thickness; Agilent J&W Scientific). 1 μ L of each sample was injected into the system by autosampler and separated using the column in splitless mode. Helium was used as carrier gas with a flow rate of 1.1 ml/min. Temperatures for inlets and MS source were set at 250°C and 230°C, respectively. The oven temperature was kept at 75 °C for 4 minutes and increased to 280 °C with a rate of 4 °C/min then held for 1.56 minutes. Mass spectrum was recorded from 40 to 600 m/z with a scan time of 0.2 second.

5.6.1 Metabolites detection and quantification

Acquisition of the total ion chromatogram and identification of mass spectra were done by GC-MS solution software (GC/MSD Chemstation Data Analysis, Agilent). NIST14

mass spectral library (National Institute of Standards and Technology) was used to identify the detected metabolite peaks. Peak area of Compounds was compared to internal control for normalization. Peaks with a similarity index of more than 80% were extracted to name the candidate compounds. Partial least square discriminant analysis using MetaboAnalyst 3.0 [208] was performed to see the difference between metabolite profiles of untreated *Caulobacter* and *Caulobacter* cells treated with different concentrations of Ni(II).

5.7 Results

5.7.1 Ni(II) toxicity inhibits cell growth and proliferation of *C. crescentus*

The effect of Ni(II) toxicity on cell growth was compared with five other heavy metals (Co(II), Cr(VI), Cu(II), Se(IV), Cd(II)) which are listed as priority pollutants [88]. In order to understand the effect of heavy metal toxicity on microbial life, we first tested the effect of heavy metals on the growth of some of the commonly found microbial strains such as *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. cerevisiae*, *S. oneidensis*, in natural environment or gut environment and compared them with *C. crescentus*. IC₅₀ values of all the heavy metals are presented in table 5.3. IC₅₀ value refers to the concentration of heavy metal responsible for 50% reduction in the growth of bacteria during mid log phase.

Table 5.3: Growth of different microbial strains in presence of heavy metals [IC₅₀ (mM)]

Metals	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. cerevisiae</i>	<i>S. oneidensis</i>	<i>C. crescentus</i>
Ni(II)	0.08	0.02	0.03	0.7	0.05	0.008
Co(II)	0.05	0.01	0.03	1.2	0.04	0.03
Cr(VI)	0.03	0.03	0.8	0.2-0.3	0.06	0.03-0.04
Cd(II)	0.001	0.005	0.001	0.03	0.05	0.004
Cu(II)	0.03	0.01	0.1	0.6-0.7	0.3	0.02
Se(IV)	0.4	1.5	0.005	*	0.05	0.8
Zn(II)	-	-	0.3	*		-

*Small effect (upto 1.5 mM)

From the analysis of results, it was found that out of all the six microbial strains studied, *S. cerevisiae* has shown the maximum tolerance against all the heavy metals tested except in case of Cr (VI). Our results for *S. cerevisiae* are in good agreement with those reported by Hosiner et al. (2014) [209] and Hyeong-Cheol et al. (2003) [210]. *B.*

subtilis showed maximum tolerance against Cr(VI) amongst all the strains. The results of effect of heavy metals on growth of *B. subtilis* are in good agreement with those reported in some previous studies [211, 212, 213]. *P. aeruginosa* growth results in minimal media were also in good agreement with the studies carried out by A. Hassen et al. (1998) [214], Teitzel et al. (2003) [215]. The effect of heavy metals on growth of *E. coli* were similar to the studies performed by Ackerley et al. (2006) [216], Nies et al. (1999) [217]. Growth of *Shewanella* is substantially affected in 0.06 mM Cr (VI), which is slightly different from those reported in studies carried out by Parker et al. (2011) [218] and Karuna et al. (2006) [219].

Our results of effect of heavy metals on the growth of model organism *C. crescentus* were consistent with those reported by Hu et al.(2005) [201]. It was observed that growth of *C. crescentus* was severely affected in presence of 0.006 mM Cd(II), 0.04 mM Cr(VI) but slightly affected in presence of 0.3 mM Se(IV) [201]. Therefore, we can conclude that these results are comparable with our findings in which IC₅₀ values of Cd(II) and Cr(VI) are 0.004 mM and 0.04 mM respectively, whereas growth of bacteria is slightly affected in presence of 0.3 mM Se(VI). The results obtained show that relatively lower concentrations of NiCl₂ and CdCl₂ were sufficient to inhibit the growth of *C. crescentus* (Table 5.3, Fig. 5.1a). We found firstly that 0.003 mM slightly affected the cell growth, secondly, that 0.008 mM reduced the cell growth by 50% and thirdly that 0.015 mM of Ni(II) completely inhibited the growth. Next, spot dilution test results confirmed that the cells did not only stop proliferating

but were also killed by 0.015 mM of Ni(II) (Fig. 5.1b). This is in contrast to the effect seen using 0.008 mM concentration of Ni(II), whereby cells only stopped proliferation but were still viable (Fig. 5.1b). On the other hand, growth reduced by half at 0.004 mM Cd(II) and the cells stopped proliferating when treated with 0.1 mM Cd(II) . However, spot dilution test results showed that cells were viable even after exposure to 0.2 mM Cd(II) concentration . Thus, our results showed that out of all six metals tested, Ni(II) produced the most toxic effect on cell growth and survival.

Fig. 5.1 Ni(II) stress severely affected growth and survival of *C. crescentus* CB15N (a) *C. crescentus* was grown in M2G minimal media in presence of 0.005 mM to 0.03 mM Ni(II) and the effect on growth with respect to control (without metal) is measured in terms of cell optical density at 660 nm. (b) Spot dilution test confirms the detrimental effect of Ni(II) on the growth of *C. crescentus* . The results are shown in presence of 0.008 mM to 0.02 mM Ni(II) .All the results are representative of at least three experiments. *C – Control without metal

5.7.2 Mislocalization of MreB leads to a severe cell shape defect in Ni(II) treated *C. crescentus*

The morphology of Ni(II) treated *C. crescentus* cells was severely affected. It has been shown that different stress conditions affect the *C. crescentus* morphology in different ways. The cell size of *C. crescentus* was largely maintained during carbon starvation, growth in stationary phase and acute heat shock [220, 221, 222] . In another study, *C. crescentus* cells grew into long filaments after treatment with 100 mM NaCl or 4%

ethanol [202] . However, we observed a different cell morphology when *C. crescentus* treated with Ni(II). The diameter of 0.015 mM Ni(II) stressed cells was significantly increased at the mid cell as compared to untreated or 0.003 mM Ni(II) treated cells (Fig. 5.2a).

Fig. 5.2 Mislocalization of MreB affected cell morphology in Ni(II) treated *C. crescentus* (a) Differential interference contrast(DIC) microscopy images of *C. crescentus* cells treated with different Ni(II) concentrations (b) Representative DIC and fluorescence microscopy images from cell expressing MreB-GFP. Shown are untreated cells, cells treated with 0.003 mM or 0.008 mM or 0.015 mM Ni(II)

The cells showed different morphologies when treated with 0.008 mM and 0.015 mM concentration of Ni(II), as shown in Fig. 5.2a. The expanded predivisional cells with constriction at mid cell were dominant in the presence of 0.008 mM Ni(II), whereas large lemon shaped cells were observed at 0.015 mM concentration of Ni(II) (Fig. 5.2a). The cells were also found to be increased in length in both the cases. A comparison of cell size under different Ni(II) stress conditions is presented in table 5.4.

Table 5.4: Cell size under different Ni(II) stress conditions

Ni(II) (mM)	Length (μm)	Width(μm)
No Ni(II)	2 - 2.2	0.45 - 0.49
0.003	2 - 2.3	0.45 - 0.49
0.008	3.7 - 4.3	1 - 1.2
0.015	2.8 - 3.3	0.85 - 1.1

The bacterial actin homologue MreB is required to maintain the rod shape of the cell in *C. crescentus*, *E. coli*, and *Bacillus* [205]. The lemon shape or expanded predivisional morphology of Ni(II) treated cell was similar to what observed in the *C. crescentus* cell with malfunctioning MreB protein [223] . This led us to examine the localization of MreB using fluorescence microscopy. We found that Ni(II) stress disrupted the localization pattern of MreB in *C. crescentus*. In the case of untreated or 0.003 mM Ni(II) treated cells, MreB bands were concentrated to the mid cell region whereas, at 0.008 mM and 0.015 mM concentrations of Ni(II), MreB was distributed throughout the cells or sometimes accumulated at the poles (Fig. 5.2b).

In the present study, we have also compared the effects of five other metals with Ni(II) stress on the morphology of *C. crescentus* (Fig. 5.3).

Fig. 5.3 Different heavy metals stresses affected cell morphology of *C. crescentus* in different ways

C. crescentus cells had shown different morphological patterns when exposed to various heavy metals. Cells were found to be slightly longer and more crescent shaped when exposed to 0.02 mM Co(II) and 0.05 mM Cr(VI), while 0.1 mM Cd(II) stress produced longer cells. An increase in width and length of the cells was observed in the presence of 0.02 mM Cu(II). On the other hand, Se(IV) did not affect the morphology of *C. crescentus* cells and were similar to the untreated cells. It can be concluded that the lemon shaped or expanded predivisional phenotype of cells under Ni(II) stress condition is not a common pattern under heavy metal stress condition but a unique effect specifically produced in Ni(II) treated cells.

5.7.3 Ni(II) stressed *C. crescentus* cells show altered expression of master cell cycle regulatory genes and abnormal localization of major cell division proteins

It was previously observed that the cell cycle master regulator CtrA was differentially expressed upon exposure of *C. crescentus* to ethanol and salt stress condition but FtsZ expression or localization pattern was found to be normal [202]. In another study carbon starvation in *C. crescentus* altered expression of important cell cycle proteins such as CtrA, DnaA, GcrA and cell division protein FtsZ [224]. Therefore, it was of interest to investigate if the expression of genes regulating the cell cycle is affected in Ni(II) stressed *C. crescentus* cells. qPCR results showed that the cell cycle regulatory genes *dnaA*, *gcrA*, and *ctrA* and gene responsible for cytokinesis, *ftsZ*, were upregulated in all three concentrations (0.003 mM, 0.008 mM, 0.015 mM) of Ni(II) treated *C. crescentus* cells (Fig. 5.4).

Fig. 5.4 Ni(II) stress altered expression of major cell cycle regulatory genes in *C. crescentus*: Relative expression levels of four key master regulatory genes (*dnaA*, *gcrA*, *ctrA*, *ccrM*) and cell division genes (*ftsZ*, *mipZ*), under different Ni(II) stress conditions. The error bars indicate the SD of three biological replicates.

The altered expression of *ctrA*, *dnaA*, *gcrA*, and *ftsZ* is consistent with the slowing down of the cell cycle in presence of Ni(II). The transcription of *ccrM* gene is dependent on CtrA protein but interestingly *ccrM* expression level were not found to be affected in Ni(II) stressed *C. crescentus* cells. CtrA regulates the expression of *ccrM* only in phosphorylated state and phosphorylation of CtrA depends on hybrid membrane kinase CckA and the histidine phosphotransferase ChpT [225]. It could be hypothesized that dysregulation in CtrA phosphorylation pathway resulting in unchanged expression of *ccrM*.

In our previous experiments, we have observed that Ni(II) affected cell growth and morphology. The difference in *C. crescentus* cell morphology in 0.008 mM and 0.015 mM Ni(II) stressed cells suggests that different concentrations of Ni(II) affected the cell cycle at different stages. At 0.008 mM Ni(II) treatment, most of the *C. crescentus* cells were at the predivisional stage which shows that the cells were capable of replicating initially but were arrested at the G2 phase later and unable to divide. On the other hand, 0.015 mM Ni(II) treated *C. crescentus* cell phenotype, lemon shaped cells without mid cell septa, suggested that both replication and cell division process were affected. This led us to hypothesize that the cell cycle is dysregulated. To better understand the underlying molecular mechanism, we proceeded to test the localization pattern of key proteins implicated in cell division like MipZ, FtsZ, and chromosome partitioning protein ParB in the presence of Ni(II). The localization of FtsZ- mCherry, MipZ-YFP and ParB-mCherry fusion proteins was examined in Ni(II) treated *C. crescentus* cells (Fig. 5.5). It was found that at Ni(II) concentration of 0.003 mM and 0.008 mM the localization pattern of these proteins was similar to untreated cells. But in the case of 0.015 mM Ni(II) treated cells multiple 3-4 abnormal MipZ, FtsZ and ParB foci were observed (Fig. 5.5).

Fig. 5.5 Ni(II) stress induced abnormal localization pattern of FtsZ, MipZ, and ParB: Representative DIC and fluorescence microscopy images from cell expressing ftsZ-mCherry, mCherry-ParB, MipZ-YFP. Shown are untreated cells, cells treated with 0.003 mM or 0.008 mM or 0.015 mM Ni(II)

Multiple MipZ and ParB foci suggest that there are multiple origins of replication. We confirmed by using a fluorescent repressor-operator system (FROS), which marks the origins of replication [206], that 0.015 mM Ni(II) treated lemon shaped cells contained multiple 3-4 well-segregated origins (Fig. 5.6).

Fig. 5.6 Localization pattern of origin of replication in 0.015 mM Ni(II) stressed *C. crescentus* cells as compared to untreated cells. $(lacO)_n$ /LacI-CFP- tagged ori were used for visualisation

The FtsZ-mCherry and MipZ-YFP localization patterns were also analyzed in the other five heavy metals stress. It was found that the FtsZ and MipZ had normal localization. However, mislocalization of MipZ was observed in 0.02 mM Cu(II)treated cells (data not shown). These results confirmed that different heavy metal stresses affected the cell cycle of *C. crescentus* at different stages. Additionally, 0.015 mM Ni(II) stress produced the most severe effect by disturbing the localization of important cell division protein such as FtsZ, MipZ, and ParB.

Fluorescence microscopy has shed light onto the mechanism of how Ni(II) toxicity affects cell morphology and cell cycle process in *C. crescentus*. Now as the second part of this paper we aspire to unveil the cellular metabolism involved in alleviating the Ni(II) toxicity in *C. crescentus*. Therefore, in our next section, we have described the GC-MS metabolomics performed on *C. crescentus* cells to identify the important metabolites which enhance or reduce its capability to survive in Ni(II) stress condition.

5.7.4 Comparison of metabolite profiles of *C. crescentus* treated with different Ni(II) concentrations

The role of amino acids and central metabolism in cell growth and division have been explored [226, 227]. The toxic effect on cell growth and division induced by Ni(II) stress

suggests that Ni(II) might affect the *C. crescentus* metabolome. To investigate this possibility, we performed untargeted GC-MS metabolomics to compare untreated *C. crescentus* with *C. crescentus* cells treated with three different Ni(II) concentrations. The changes in metabolome would help shed light on the mechanism for *C. crescentus*'s ability to cope with Ni(II) toxicity. In total 42 metabolites including amino acids, sugar and organic acids were identified (Table 5.5).

Fig. 5.7 The GC-MS spectra of untreated (black) and 0.015 mM Ni(II) treated(blue) *C. crescentus*.The observable differences are shown with red blocks or arrows

Table 5.5 GC-MS metabolites

	Metabolite	RT(min)
1	Alpha-Methylstyrene	6.03
2	Lactic Acid	8.21
3	alpha.-Hydroxyisobutyric acid	8.26
4	Glycolic Acid	8.63
5	Alanine	9.48
6	Hydroxylamine	9.748
7	3-Hydroxybutyric acid	11.34
8	Heptanoic acid	11.41
9	Methyl aminoacetate	11.9
10	Valine	13.19
11	L-Norleucine	15.09
12	Glycine	16.22
13	Butanedioic acid	16.42
14	Methyl Succinate	16.79
15	Glyceric Acid	17.23
16	2-Butenedioic Acid	17.46
17	M-Toluic Acid	17.73
18	Serine	18.16
19	Threonine	19.03
20	.beta.-Alanine	20.02
21	Erythrono- 1,4-lactone	20.14
22	Homoserine	20.93
23	Aminomelonic acid	21.52
24	Arabino-Hexos-2-ulose	21.7
25	Malic acid	22.2
26	Butylated hydroxytoluene	22.32
27	Triethylene Glycol	22.4
28	Proline	22.9
29	Cyanuric Acid	25.7
30	Glutamic acid	25.98
31	Benzene	26.23
32	3,4,5-Trihydroxypentanoic acid	27.3
33	Adonitol	29.38
34	Glutamine	30.1
35	Phosphoric Acid	30.16
36	Citric Acid	31.55
37	Myristic acid	31.6
38	Fructose	33.18
39	Galactose	33.55
40	Glucose	34.08
41	Talose	34.37
42	D-mannitol	34.58

The GC-MS spectra of untreated and 0.015 mM Ni(II) treated *C. crescentus* was overlaid, as illustrated in Fig. 5.7.

The visual inspection shows the differences in metabolite profiles between untreated and 0.015 mM Ni(II) treated cells. Multivariate analysis using Partial least square discriminant analysis was used to accurately describe the variations between GC-MS metabolite profiles.

Fig. 5.8 Ni(II) stress affected *C. crescentus* metabolome. Partial least square discriminant analysis of GC-MS metabolite profiles of untreated cells and cells treated with 0.003 mM(red) or 0.008 mM(green) or 0.015 mM(blue) Ni(II) for 4 h

As shown in Fig. 5.8, PLS-DA analysis clearly separated untreated *C. crescentus* cells from Ni(II) treated cells. 0.003 mM and 0.008 mM Ni(II) treated cells were grouped close to each other and clearly separated apart from 0.015 mM Ni(II) cells. Nine

important differential metabolites were identified and the changes in their levels are shown in Fig. 5.9.

Fig. 5.9 Relative abundance of metabolites in 0.003 mM, 0.008 mM and 0.015 mM Ni(II) treated *C. crescentus* cells as compared to untreated cells: changes in the TCA cycle metabolite malic acid, amino acid metabolites (alanine, proline, glutamic acid, valine, aminomalonic acid) and levels of 3,4,5 trihydroxypentanoic acid. The error bars indicate the SD of three biological replicates

Citric acid and glutamine could be detected only when cells were exposed to 0.015 mM of Ni(II) for 4h. The metabolites can be classified into amino acids (alanine, proline, glutamic acid, glutamine, valine, aminomalonic acid), TCA cycle intermediates (malic acid, citric acid) and 3,4,5 trihydroxypentanoic acid.

We mapped the metabolomic changes in relation to biochemical pathways (Fig 5.10).

Fig. 5.10 Metabolic changes in *C. crescentus* exposed to 0.003 mM, 0.008 mM and 0.015 mM Ni(II) for 4 h mapped onto metabolic pathways. Coloured symbols represent an increase or no change in the metabolite level at each Ni(II) concentration as compared to untreated *C. crescentus*. Open symbol represents metabolites below detectable level

This gives a view of metabolite changes in presence of different Ni(II) concentrations in comparison to untreated *C. crescentus* cells which help us understand (1) the connection of carbon and nitrogen metabolism with cell cycle dysregulation and (2) the cellular metabolism to ameliorate Ni(II) toxicity.

5.7.5 Exogenous addition of malic acid, citric acid, proline, glutamine, and alanine enhanced the Ni(II) tolerance of *C. crescentus*

GC-MS metabolomics revealed nine differential metabolites in Ni(II) stressed *C. crescentus* cells. Aminomalonic acid and 3,4,5-trihydroxypentanoic acid are generated as a product of stress damage. We have added 1 mM or 10 mM concentration of the remaining six metabolites (malic acid, citric acid, alanine, proline, glutamic acid, glutamine, valine) to 0.015 mM Ni(II) treated cells to test whether they have a direct role in Ni(II) tolerance in *C. crescentus*. Interestingly, we found that the growth of 0.015 mM Ni(II) stressed cells was significantly improved after the addition of 10 mM of malic acid or proline or alanine or glutamine or 1 mM of citric acid (fig. 5.11)

Fig. 5.11 Effect of exogenous addition of 1 mM or 10 mM of malic acid or citric acid or proline or alanine or glutamic acid or glutamine or valine on the growth of 0.015 mM Ni(II) stressed *C. crescentus* cells. Cell optical density was monitored to represent cell Ni(II) tolerance. The results shown are representative of at least three experiments.

The growth was slightly improved in presence of 1mM of malic acid or proline. There was no restoration of growth observed when glutamic acid or valine added to 0.015 mM

Ni(II) treated *C. crescentus* cells (Fig. 5.11). The observation from this experiment and GC-MS metabolomics combinedly confirmed the role of The growth was slightly improved in presence of 1mM of malic acid or proline. There was no restoration of growth observed when glutamic acid or valine added to 0.015 mM Ni(II) treated *C. crescentus* cells (Fig. 5.11). The observation from this experiment and GC-MS metabolomics combinedly confirmed the role of malic acid, citric acid, proline, glutamine and alanine in conferring the resistance against Ni(II) toxicity in *C. crescentus*.

5.8 Discussion

5.8.1 Comparison of the effect of different Ni(II) concentrations on *C. crescentus* cell cycle

MipZ, a member of ParA superfamily of P loop ATPases, is an essential protein for chromosome segregation and cell division in *C. crescentus*. The localization of MipZ is parallel to the origin of replication during the cell cycle [204] . The MipZ protein interacts with the DNA partitioning protein, ParB. The MipZ-ParB complex and the origin of replication occupy the same position in the cell and share a similar pattern of localization over the cell cycle [228, 229]. Another protein FtsZ is responsible for cytokinesis process in *C. crescentus*. FtsZ localization is MipZ dependent and restricted to subcellular locations with the lowest concentration of MipZ. The MipZ-ParB-origin complex displaces FtsZ towards the midcell, where MipZ concentration is lowest [204, 230] . Multiple mislocalized foci of MipZ, FtsZ, and ParB in 0.015 mM Ni(II) treated *C. crescentus* cells suggest that replication occurred multiple times. Cells did not increase in length and accumulated only 3-4 origin of replication in 15 hours. It could be inferred that cell growth and DNA replication proceeded for only 1-2 doubling times

without division then later cells got arrested at the G1 stage. These observations recommend that chromosome replication and segregations process has gone awry in 0.015 mM Ni(II) treated *C. crescentus*. Past investigations have also detailed that stress conditions such as carbon starvation, growth in stationary phase and acute heat shock caused DNA replication block and G1-arrest in *C. crescentus* [220, 221, 222]. All these cell cycle proteins showed the normal pattern of localization in 0.003 mM and 0.008 mM Ni(II) treated *C. crescentus* cells (Fig. 5.5). It implies that 0.008 mM of Ni(II) impaired cell proliferation, however, DNA replication process remain unaffected. The morphology of 0.008 mM Ni(II) treated cells also suggest that a large portion of the cells are captured at the predivisional stage (Fig. 5.2a) after completing replication process normally. The morphological manifestations of cell polarity are essential for the lifecycle of *C. crescentus* [231]. The bacterial actin homologue, MreB, is involved in generating cell polarity in *C. crescentus* and *E. coli* [205, 232]. We speculate that 0.008 mM Ni(II) treated *C. crescentus* cells were unable to establish correct polarity due to the abnormal localization of MreB. Because of mislocalization of MreB, cells were arrested at predivisional stage without further division. In contrast to Ni(II) stress, upon treatment with 100 mM NaCl or 4% ethanol cells grew into long filaments and accumulated 7-8 chromosomes within 8 hours. It demonstrated that cells continued to undergo DNA replication, chromosome segregation and cellular growth, however, cell division was ceased [202].

5.8.2 Role of organic acids and amino acids in Ni (II) stress response of *C. crescentus*

Metabolomic profiling can be useful to gain insight into the mechanism of cell cycle dysregulation in Ni(II) stressed *C. crescentus*. Aminomalonic [233] and 3,4,5-trihydroxypentanoic acid [234] have been reported as a product of stress damage. We could interpret that the increased level of aminomalonic acid is associated with errors in

protein synthesis and oxidative damage to amino acid residues in proteins whereas 3,4,5-trihydroxypentanoic acid production is related with DNA damage process in Ni(II) stressed *C. crescentus*.

Increased level of amino acids and organic acids have previously been associated with increased tolerance to various stress conditions [235, 236, 237, 238]. The higher accumulation of amino acids and organic acids may be a result of a major shift in metabolism and activation of defence mechanism in Ni(II) stressed *C. crescentus*. The restoration of growth upon exogenous addition of proline, glutamine, alanine, malic acid and citric acid in 0.015 mM Ni(II) stressed *C. crescentus* cells affirmed their role in enhancing Ni(II) tolerance (Fig. 5.11). While the role of these metabolites is very well documented in plant stress conditions, there is still a dearth of literature in the case of bacteria. Previous studies on Ni-accumulating plants have demonstrated nickel's tendency to form stable complexes with amino acids and organic acids as a mechanism of detoxification [237, 239, 240]. Elevated levels of organic and amino acids in *C. crescentus* may involve in Ni-chelation mechanism to protect against Ni toxicity related damages. Increased levels of alanine, citric acid, and malic acid have previously shown to contribute to nickel complexation to alleviate toxicity in *Stackhousia tryonii* [241]. Proline is very well known for its role as an osmoprotectant in bacteria and plants [242, 243, 244]. Furthermore, as compared to other amino acids, the proline accumulation under heavy metal stress is a more prevalent phenomenon [244, 245, 246]. Proline is involved in the synthesis of glutathione, an important antioxidant, which has been associated with presenting resistance against heavy metal stress including Ni(II) [247, 248, 249]. Elevated level of proline may help *C. crescentus* undergo osmotic adjustment and enhance anti-oxidant metabolism to secure against Ni(II) toxicity related damages. We also propose that 0.015 mM Ni(II) induced *putA* and *glnA* expression may be a

reason of improved growth in the presence of glutamine and proline. *C. crescentus* imported exogenous proline and PutA converted this proline into glutamate [250] . Glutamate is converted by glutamate dehydrogenase to alpha-ketoglutarate, an intermediate of TCA cycle, which can subsequently generate ATP via oxidative phosphorylation. GlnA (glutamine synthetase) catalyzes the reaction between ammonia and glutamate to produce glutamine which is a key nitrogen donor in nitrogen metabolism process [251] . It could be conceived that *C. crescentus* can take up exogenous proline and glutamine but not glutamic acid. In this manner, proline and glutamine encouraged metabolic stimulation through PutA/GlnA pathway may be an explanation behind improved resilience of *C. crescentus* against Ni(II).

Cheng et al. (2014) [252] reported another role of proline and glutamine in accelerating degradation of DNA-binding protein CtrA in alpha proteobacterium *E. chaffeensis*. Ni(II) treatment has significantly increased the expression of *ctrA* in *C. crescentus* (Fig. 5.4). The chromosome replication is tightly controlled by CtrA and it must be deactivated to start the process [113] . We speculate that like *E. chaffeensis*, increased level of glutamine and proline might be associated with the accelerated degradation of CtrA to facilitate replication process in 0.015 mM Ni(II) stressed *C. crescentus*.

The connection of carbon and nitrogen metabolism with cell growth and division has been established [226, 227, 253] . We perceive that changes in amino acids and TCA cycle intermediates are linked with the cell cycle dysregulation in Ni(II) stressed *C. crescentus*. Elevated level of malic acid and citric acid might affect FtsZ assembly. Our argument could be supported by a recently highlighted connection between central carbon metabolism and cell division in *B. subtilis* [227]. *B. subtilis* Z-ring assembly is regulated by pyruvate thus it was a key metabolite in the coordination of bacterial

growth with cell division. In another study glutamate dehydrogenase (GdhZ) was involved in the molecular mechanism that coordinates central metabolism with cell division in *C. crescentus* [253]. GdhZ also acts as a bridging molecule between nitrogen cycle and TCA cycle. GdhZ directly interferes with FtsZ polymerization and its catabolic activity is mainly controlled by glutamate. It is conceivable that change in glutamic acid concentration affect GdhZ activity which may lead to mislocalization of FtsZ in Ni(II) treated *C. crescentus*. Furthermore, Beaufay et al. (2015) [253] reported that alanine suppressed the growth and morphology defect in Δ gdhZ *C. crescentus* cells which concurs with the protective effect of alanine on Ni(II) toxicity.

5.9 Conclusions

In summary, Ni(II) toxicity severely affected growth, morphology and cell cycle of *C. crescentus*. The use of GC-MS metabolomics has enabled us to understand Ni(II) induced changes in metabolism and biochemical pathways of *C. crescentus*. The results suggest that changes in carbon and nitrogen metabolism are linked with the dysregulation of the cell cycle. Also, metabolite profiling helped us identify the role of amino acids and organic acids in ameliorating Ni(II) toxicity. However, more studies to uncover the exact mechanism of protective effect of amino acids and organic acid on Ni(II) toxicity is required. For example, gene manipulations or reporter gene assay to check the role of central metabolism and PutA/GlnA pathway, pulse chase experiment to calculate the half-life of CtrA. Knowledge from this study could aid future work to understand bacterial responses to environmental stress conditions

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVE

In the recent years, the focus of the microbiology field has been shifted towards understanding of uncharacterized and obscure metabolic pathways. Another direction is the investigation of microbes under unknown and fluctuating environmental stress conditions. Over the years, due to rising levels of environmental pollution, microbes capable of degrading organic xenobiotics and additionally those able to survive and biotransform toxic heavy metals have gained economic value. Therefore, we were keen on unveiling how the microbes are able to do such useful metabolic accomplishments. In addition to their large industrial applications, microbes also play a vital role in human health. For example, microbes residing in human gut has been linked to plethora of disease, from diabetes, autism to anxiety and obesity. The gut microbiome could also determine the response of an individual to a certain drug, recently Alexander et al. (2017) reviewed how gut microbiota influence the response of cancer patients to chemotherapy[10]. Even a possible link of gut microbiota with how well humans sleep has been suggested. Hence, we were interested to understand how external factors such as diet affect the gut microbiome composition in Asian adults

This thesis investigated how microorganisms interact with, survive in and influence different kind of environment such as human gut and heavy metal contaminated soil, water or air. We have combined metabolomics with microbiology to investigate bacterial interaction in different environment. Figure 6.1 is depicting how metabolomics is used as an effective monitoring tool to characterize the metabolites of bacteria in response to their interaction with external factors such as diet and heavy metals.

Fig. 6.1 Metabolomics is used as an effective monitoring tool to study bacterial responses in different environments such as the human gut and heavy metal stress. CA - Citric acid, MA - Malic acid

We compared the fecal bacterial diversity and composition of healthy Indian and Chinese adults, ages 22–35 years, using next-generation sequencing. In spite of the fact that, the prevalent gut bacteria were similar to those found in other populaces, we discovered some exceptional community structure also. The dominant *Firmicutes*, *Actinobacteria* and underrepresented *Bacteroides*, in Indian and Chinese gut community structure, closely matched with the gut bacterial composition of the Russian population. The dominance of genus *Bifidobacterium* due to carbohydrate-rich diet is another notable feature of this study. We further suggested the possible link between diet and gut bacterial composition. We identified bacterial biomarkers of diet, which are very well known for their interaction with diet, distinguishing Indian and Chinese adults. This information expands our knowledge of healthy human microbiome ecology and serves

as a reference point for future epidemiological investigations and translational applications

We further hypothesized that analysis of metabolites compositions could be useful to reflect the status of the gut microbiome and additionally bridge the connections between symbiotic microbes and the host's health. In the chapter 4, a pipeline workflow for the metabolomics study was developed. We optimized the metabolite extraction process and the sample preparation for the GC-MS analysis. Our untargeted GC-MS metabolomics method enabled the detection of 120 and 86 metabolites including amino acids, phenolics, indoles, carbohydrates, sugars and metabolites of microbial origin from fecal and urine samples respectively. 41 compounds were confirmed using external standards. In order to increase the metabolome coverage, we determined urine and fecal metabolites composition using a combination of both GC-MS and LC-MS metabolomics and compared them between Indian and Chinese adults. According to metabolomics analysis, urine and fecal metabolome compositions were influenced by diet or ethnicity of subjects. These results justify our previous findings in chapter 3, where diet or ethnicity was found to be important in determining gut microbiome composition. We identified 53 differentiating metabolites including higher abundance of amino acids and phenolics in Chinese and higher abundance of fatty acids, glycocholic acid in Indians. Correlation analysis further confirm the association of metabolites with gut bacterial profiles of the same subjects in the genus and species level. It shows the possibility of predicting gut bacterial profile with the help of less expensive and less tedious metabolomics technology.

The overall outline and major finding from chapter 3 and 4 is summarized in figure 6.2

Fig. 6.2 Schematic diagram summarizing the role of dietary habits on the gut microbiome and metabolite profiles of Indian and Chinese adults

In chapter 5, we investigated the Ni(II) stress condition in bacterium *C. crescentus*, a model organism to study cell cycle, which is found many different environments including wastewater, seawater, soil, deep subsurface goldmines and the human gut. Nickel toxicity in plants, animals, and humans has been widely studied. Conversely, the importance of nickel toxicosis in microorganisms is less well studied. Nickel stress is a routine physiological concern for bacteria, for example, human gut bacteria are exposed to 150 – 900 µg of nickel per day as a constituent of the diet. Therefore, it becomes important to understand the effect of nickel exposure to bacteria. The overall outline and major findings from chapter 5 are summarized in figure 6.3. Our analysis revealed that Ni(II) toxicity severely affected growth, morphology and cell cycle of *C. crescentus*.

The use of GC-MS metabolomics has enabled us to understand Ni(II) induced changes in metabolism and biochemical pathways of *C. crescentus*. The results suggest that changes in carbon and nitrogen metabolism are linked with the dysregulation of the cell cycle. Also, metabolite profiling helped us identify the role of amino acids and organic acids in ameliorating Ni(II) toxicity

Fig. 6.3 Schematic diagram summarizing the study of Ni (II) stress condition in bacterium *C. crescentus*

Conclusively, our study showed that metabolomics is an effective technology to investigate the bacterial interaction with different kind of environments, focusing on human gut and heavy metal stress. Knowledge from this study could aid future research aim to modulate human gut microbiota, with probiotics or synbiotic dietary supplementations, which may provide new approaches to control the diet-microbiota-human health interactions. This thesis also strengthens our understanding towards links between metabolite signatures with specific bacterial genera or species and a possibility of foreseeing gut bacterial profile in light of less expensive and less tedious

metabolomics analysis. Moreover, our results shed light on the mechanism of increased Ni(II) tolerance in *C. crescentus* which may be useful in bioremediation or other antimicrobial strategies, antibiotics development and synthetic biology applications such as the development of whole cell biosensor. It could also help us investigate the biotransformation of environmental pollutants by gut microbiomes and how the contaminants would affect the metabolism of microbiota

6.1 Future Perspective

In this study we compared the gut microbiome composition of healthy Indian and Chinese adults. We further suggested a link between diet and gut microbiome composition. However, other hidden factors such as host physiology and genetics, lifestyle, geography may also affect gut microbiota composition. The present study was based on the uncontrolled dietary habits of subjects. However, dietary intervention studies with a defined composition of Indian and Chinese diet will give us a better idea of changes in gut microbiota specifically in response to diet and it will certainly minimize the effect of other hidden factors. Another useful approach will be to develop in vitro culture conditions to determine the impact of diet on gut microbiota. Although, few studies attempted developing in vitro condition for maintaining gut bacteria, but it is still needed to set up better culture conditions to support the growth of as many as gut bacteria possible which can represent the human gastrointestinal tract in vitro.

GC-MS and LC-MS based untargeted metabolomics analysis of fecal and urine samples helped us identify the association of metabolites with diet or ethnicity and gut microbiome of Indian and Chinese adults. This study strengthens our understanding towards links between metabolite signatures with specific bacterial genera or species which could be useful to foresee gut bacterial profile in light of less expensive and less tedious metabolomics analysis. It was a preliminary analysis with small sample size,

However, a study of a much larger population with different groups across Asia and the rest of the world will give a better picture of the connection between gut microbiota, metabolites, and diet

In this thesis, we also studied the effect of Ni(II) toxicity on cell growth, morphology, and cell cycle of *C. crescentus*. The use of GC-MS metabolomics has enabled us to understand Ni (II)-induced changes in metabolism and biochemical pathways of *C. crescentus*. We have used only GC-MS for metabolites detection in *C. crescentus*. However, using an additional platform such as LC-MS in combination with GC-MS can enhance the metabolome coverage and will be able to provide additional information to the study. We have identified the role of amino acids and organic acids in ameliorating Ni(II) toxicity. However, more studies to uncover the exact mechanism of the protective effect of amino acids and organic acid on Ni (II) toxicity is required. Metabolic stimulation through the PutA/GlnA pathway, accelerated degradation of CtrA, and Ni-chelation by organic acids or amino acids are some of the mechanisms that could be involved in enhancing *C. crescentus*'s tolerance. We can use gene manipulations or reporter gene assay to check the role of central metabolism and PutA/GlnA pathway, and pulse chase experiment to calculate the half-life of CtrA.

Besides metabolomics analysis during heavy metal stresses, we plan to perform screens for genomic elements that can either enhance the ability of *C. crescentus* to survive in Ni(II) stress condition. We will perform transposon mutagenesis to identify genomic elements that can enhance the ability of *C. crescentus* to survive. We will grow the cells in a sub-lethal concentration of nickel (~0.008mM) that lowers proliferation rate and repeatedly mutagenize the cells, until we obtain a population of cells that can grow as quickly as wildtype untreated cells. Due to the relatively small size of the bacterial

genome (~4MB), we will sequence the whole genome of multiple mutant clones to identify changes in the DNA that have occurred.

In this thesis we have successfully set up the metabolomics platform to investigate bacterial interaction with different kind of environments. We hope this could aid future work, on the relation of diet and gut microbiota with human health and to investigate bacterial responses to environmental stress conditions.

LIST OF PUBLICATIONS

- 1) **Jain A**, Chen W (2018) Involvement of organic acids and amino acids in ameliorating Ni (II) toxicity induced cell cycle dysregulation in *Caulobacter crescentus*: a metabolomics analysis. *Applied Microbiology Biotechnology*, 102(10), 4563-4575
- 2) **Jain A**, Li XH, Chen W (2018) Similarities and differences in gut microbiome composition correlates with dietary differences between Indian and Chinese adults. *AMB Express*, 8(1):104
- 3) **Jain A**, Chen W (2018) Fecal and urine metabolites association with diet or ethnicity and gut microbiome of Indian and Chinese adults: an untargeted metabolomics analysis, *Submitted*

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REFERENCES

1. Booth S, Turner RJ, Weljie A. Metabolomics in Environmental Microbiology. *Emagres*. 2013;2(4):517-528. doi: 10.1002/9780470034590.emrstm1335. PubMed PMID: WOS:000218787200006.
2. Lamichhane S, Sen P, Dickens AM, et al. Gut metabolome meets microbiome: A methodological perspective to understand the relationship between host and microbe. *Methods*. 2018 2018/04/30/. doi: <https://doi.org/10.1016/j.ymeth.2018.04.029>.
3. García-Sevillano MÁ, García-Barrera T, Gómez-Ariza JL. Environmental metabolomics: Biological markers for metal toxicity. *ELECTROPHORESIS*. 2015 2015/09/01;36(18):2348-2365. doi: 10.1002/elps.201500052.
4. Fiehn O. Metabolomics - the link between genotypes and phenotypes. *Plant Molecular Biology*. 2002 Jan;48(1-2):155-171. doi: 10.1023/a:1013713905833. PubMed PMID: WOS:000173211000011.
5. Vitorino LC, Bessa LA. Technological Microbiology: Development and Applications. *Frontiers in Microbiology*. 2017 05/10;8:827. doi: 10.3389/fmicb.2017.00827. PubMed PMID: PMC5423913.
6. Vacharaksa A, Finlay BB. Gut Microbiota: Metagenomics to Study Complex Ecology. *Current Biology*. 2010 Jul;20(13):R569-R571. doi: 10.1016/j.cub.2010.05.020. PubMed PMID: WOS:000280024300018.
7. Upadhyaya S, Banerjee G. Type 2 diabetes and gut microbiome: at the intersection of known and unknown. *Gut Microbes*. 2015 2015/03/04;6(2):85-92. doi: 10.1080/19490976.2015.1024918.
8. Mulle JG, Sharp WG, Cubells JF. The Gut Microbiome: A New Frontier in Autism Research. *Current psychiatry reports*. 2013;15(2):337-337. doi: 10.1007/s11920-012-0337-0. PubMed PMID: PMC3564498.
9. Lach G, Schellekens H, Dinan TG, et al. Anxiety, Depression, and the Microbiome: A Role for Gut Peptides. *Neurotherapeutics*. 2018 2018/01/01;15(1):36-59. doi: 10.1007/s13311-017-0585-0.
10. Alexander JL, Wilson ID, Teare J, et al. Gut microbiota modulation of chemotherapy efficacy and toxicity. *Nature Reviews Gastroenterology & Hepatology*. 2017 Jun;14(6):356-365. doi: 10.1038/nrgastro.2017.20. PubMed PMID: WOS:000402116200008.
11. Thompson RS, Roller R, Mika A, et al. Dietary Prebiotics and Bioactive Milk Fractions Improve NREM Sleep, Enhance REM Sleep Rebound and Attenuate the Stress-Induced Decrease in Diurnal Temperature and Gut Microbial Alpha Diversity [10.3389/fnbeh.2016.00240]. *Frontiers in Behavioral Neuroscience*. 2017;10:240.
12. Rothschild LJ, Mancinelli RL. Life in extreme environments. *Nature*. 2001 02/22/online;409:1092. doi: 10.1038/35059215.
13. Lovley DR. Cleaning up with genomics: applying molecular biology to bioremediation [Review Article]. *Nature Reviews Microbiology*. 2003 10/01/online;1:35. doi: 10.1038/nrmicro731.

-
14. Putri SP, Nakayama Y, Matsuda F, et al. Current metabolomics: Practical applications. *Journal of Bioscience and Bioengineering*. 2013 Jun;115(6):579-589. doi: 10.1016/j.jbiosc.2012.12.007. PubMed PMID: WOS:000321026700001.
 15. Dettmer K, Aronov Pavel A, Hammock Bruce D. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*. 2006 2007/01/01;26(1):51-78. doi: 10.1002/mas.20108.
 16. German JB, Hammock BD, Watkins SM. Metabolomics: building on a century of biochemistry to guide human health. *Metabolomics*. 2005 2005/03/01;1(1):3-9. doi: 10.1007/s11306-005-1102-8.
 17. Dettmer K, Hammock BD. Metabolomics--a new exciting field within the "omics" sciences. *Environmental Health Perspectives*. 2004;112(7):A396-A397. PubMed PMID: PMC1241997.
 18. Roberts Lee D, Souza Amanda L, Gerszten Robert E, et al. Targeted Metabolomics. *Current Protocols in Molecular Biology*. 2012 2012/04/01;98(1):30.2.1-30.2.24. doi: 10.1002/0471142727.mb3002s98.
 19. De Vos RCH, Moco S, Lommen A, et al. Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nature Protocols*. 2007 04/05/online;2:778. doi: 10.1038/nprot.2007.95.
 20. Lommen A, van der Weg G, van Engelen MC, et al. An untargeted metabolomics approach to contaminant analysis: Pinpointing potential unknown compounds. *Analytica Chimica Acta*. 2007 2007/02/12;584(1):43-49. doi: <https://doi.org/10.1016/j.aca.2006.11.018>.
 21. Dunn WB, Erban A, Weber RJM, et al. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics*. 2013 2013/03/01;9(1):44-66. doi: 10.1007/s11306-012-0434-4.
 22. Alonso A, Marsal S, Julià A. Analytical Methods in Untargeted Metabolomics: State of the Art in 2015 [10.3389/fbioe.2015.00023]. *Frontiers in Bioengineering and Biotechnology*. 2015;3:23.
 23. Nicholson JK, Wilson ID. Understanding 'Global' Systems Biology: Metabonomics and the Continuum of Metabolism [Perspective]. *Nature Reviews Drug Discovery*. 2003 08/01/online;2:668. doi: 10.1038/nrd1157.
 24. Dias AP, Brown J, Bonello P, et al. Metabolite Profiling as a Functional Genomics Tool. In: Grotewold E, editor. *Plant Functional Genomics*. Totowa, NJ: Humana Press; 2003. p. 415-425.
 25. Jonsson P, Johansson AI, Gullberg J, et al. High-Throughput Data Analysis for Detecting and Identifying Differences between Samples in GC/MS-Based Metabolomic Analyses. *Analytical Chemistry*. 2005 2005/09/01;77(17):5635-5642. doi: 10.1021/ac050601e.
 26. Want Elizabeth J, Cravatt Benjamin F, Siuzdak G. The Expanding Role of Mass Spectrometry in Metabolite Profiling and Characterization. *ChemBioChem*. 2005 2005/11/04;6(11):1941-1951. doi: 10.1002/cbic.200500151.
 27. Schauer N, Steinhauser D, Strelkov S, et al. GC-MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Letters*. 2005 2005/02/28;579(6):1332-1337. doi: 10.1016/j.febslet.2005.01.029.

28. Gao X, Pujos-Guillot E, Sébédio J-L. Development of a Quantitative Metabolomic Approach to Study Clinical Human Fecal Water Metabolome Based on Trimethylsilylation Derivatization and GC/MS Analysis. *Analytical Chemistry*. 2010 2010/08/01;82(15):6447-6456. doi: 10.1021/ac1006552.
29. Gomez-Casati DF, Zanol MI, Busi MV. Metabolomics in Plants and Humans: Applications in the Prevention and Diagnosis of Diseases. *Biomed Research International*. 2013. doi: 10.1155/2013/792527. PubMed PMID: WOS:000323289100001.
30. Wang JH, Byun J, Pennathur S. Analytical Approaches to Metabolomics and Applications to Systems Biology. *Seminars in Nephrology*. 2010;30(5):500-511. doi: 10.1016/j.semnephrol.2010.07.007.
31. Goodacre R. Metabolomics – the way forward. *Metabolomics*. 2005 2005/03/01;1(1):1-2. doi: 10.1007/s11306-005-1111-7.
32. Lankadurai BP, Nagato EG, Simpson MJ. Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environmental Reviews*. 2013 2013/09/01;21(3):180-205. doi: 10.1139/er-2013-0011.
33. Cameron SJS, Takáts Z. Mass spectrometry approaches to metabolic profiling of microbial communities within the human gastrointestinal tract. *Methods*. 2018 2018/04/26/. doi: <https://doi.org/10.1016/j.ymeth.2018.04.027>.
34. Meyer TW, Hostetter TH. Uremic solutes from colon microbes [Review]. *Kidney International*. 2012 May;81(10):949-954. doi: 10.1038/ki.2011.504. PubMed PMID: WOS:000303543200005; English.
35. Li M, Wang B, Zhang M, et al. Symbiotic gut microbes modulate human metabolic phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*. 2008 Feb 12;105(6):2117-2122. doi: 10.1073/pnas.0712038105. PubMed PMID: WOS:000253261900062.
36. Goodacre R. Metabolomics of a superorganism. *Journal of Nutrition*. 2007 Jan;137(1):259S-266S. PubMed PMID: WOS:000243330800019.
37. Jia W, Li H, Zhao L, et al. Gut microbiota: a potential new territory for drug targeting. *Nature Reviews Drug Discovery*. 2008 Feb;7(2):123-129. doi: 10.1038/nrd2505. PubMed PMID: WOS:000252806500011.
38. Harakeh SM, Khan I, Kumosani T, et al. Gut Microbiota: A Contributing Factor to Obesity. *Frontiers in Cellular and Infection Microbiology*. 2016 Aug;6. doi: 10.3389/fcimb.2016.00095. PubMed PMID: WOS:000382282000003.
39. Flint HJ, Duncan SH, Scott KP, et al. Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*. 2015 Feb;74(1):13-22. doi: 10.1017/s0029665114001463. PubMed PMID: WOS:000352198500003.
40. Russell WR, Hoyles L, Flint HJ, et al. Colonic bacterial metabolites and human health. *Current Opinion in Microbiology*. 2013 Jun;16(3):246-254. doi: 10.1016/j.mib.2013.07.002. PubMed PMID: WOS:000323794800004.
41. Weir TL, Manter DK, Sheflin AM, et al. Stool Microbiome and Metabolome Differences between Colorectal Cancer Patients and Healthy Adults. *Plos One*. 2013 Aug 6;8(8). doi: 10.1371/journal.pone.0070803. PubMed PMID: WOS:000324401500051.

-
42. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011 Apr 7;472(7341):57-U82. doi: 10.1038/nature09922. PubMed PMID: WOS:000289199400036.
 43. Saito YA, Locke GR, Weaver AL, et al. Diet and functional gastrointestinal disorders: A population-based case-control study. *American Journal of Gastroenterology*. 2005 Dec;100(12):2743-2748. doi: 10.1111/j.1572-0241.2005.00288.x. PubMed PMID: WOS:000233627400023.
 44. Bruce WR, Giacca A, Medline A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiology Biomarkers & Prevention*. 2000 Dec;9(12):1271-1279. PubMed PMID: WOS:000166046900001.
 45. Weickert MO, Pfeiffer AFH. Metabolic effects of dietary and prevention of diabetes. *Journal of Nutrition*. 2008 Mar;138(3):439-442. PubMed PMID: WOS:000253345000001.
 46. Wong JMW, de Souza R, Kendall CWC, et al. Colonic health: Fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*. 2006 Mar;40(3):235-243. doi: 10.1097/00004836-200603000-00015. PubMed PMID: WOS:000236816600015.
 47. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95(12):6578-6583. PubMed PMID: PMC33863.
 48. Bowler C, Karl DM, Colwell RR. Microbial oceanography in a sea of opportunity. *Nature*. 2009 05/13/online;459:180. doi: 10.1038/nature08056.
 49. Konopka A. What is microbial community ecology? [Perspective]. *The Isme Journal*. 2009 08/06/online;3:1223. doi: 10.1038/ismej.2009.88.
 50. Cani PD, Delzenne NM. The gut microbiome as therapeutic target. *Pharmacology & Therapeutics*. 2011 2011/05/01/;130(2):202-212. doi: <https://doi.org/10.1016/j.pharmthera.2011.01.012>.
 51. Grice EA, Segre JA. The Human Microbiome: Our Second Genome. *Annual review of genomics and human genetics*. 2012 06/06;13:151-170. doi: 10.1146/annurev-genom-090711-163814. PubMed PMID: PMC3518434.
 52. Imhann F, Vila AV, Bonder MJ, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut*. 2018 Jan;67(1):108-119. doi: 10.1136/gutjnl-2016-312135. PubMed PMID: WOS:000417778600016.
 53. Qin JJ, Li YR, Cai ZM, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*. 2012 Oct;490(7418):55-60. doi: 10.1038/nature11450. PubMed PMID: WOS:000309446800031.
 54. Frank DN, Amand ALS, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America*. 2007 Aug;104(34):13780-13785. doi: 10.1073/pnas.0706625104. PubMed PMID: WOS:000249064700047.
 55. Jeffery IB, O'Toole PW, Ohman L, et al. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut*. 2012 Jul;61(7):997-1006. doi: 10.1136/gutjnl-2011-301501. PubMed PMID: WOS:000304443200009.

56. Claesson MJ, Jeffery IB, Conde S, et al. Gut microbiota composition correlates with diet and health in the elderly. *Nature*. 2012 Aug;488(7410):178-+. doi: 10.1038/nature11319. PubMed PMID: WOS:000307267000026.
57. Milani C, Ferrario C, Turrone F, et al. The human gut microbiota and its interactive connections to diet. *Journal of Human Nutrition and Dietetics*. 2016 2016/10/01;29(5):539-546. doi: 10.1111/jhn.12371.
58. Riaz Rajoka MS, Shi J, Mehwish HM, et al. Interaction between diet composition and gut microbiota and its impact on gastrointestinal tract health. *Food Science and Human Wellness*. 2017 2017/09/01;6(3):121-130. doi: <https://doi.org/10.1016/j.fshw.2017.07.003>.
59. Lozupone CA, Stombaugh JI, Gordon JI, et al. Diversity, stability and resilience of the human gut microbiota. *Nature*. 2012 Sep;489(7415):220-230. doi: 10.1038/nature11550. PubMed PMID: WOS:000308635900030.
60. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature*. 2012 Jun;486(7402):222-+. doi: 10.1038/nature11053. PubMed PMID: WOS:000305189000027.
61. Wu GD, Chen J, Hoffmann C, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science*. 2011 Oct;334(6052):105-108. doi: 10.1126/science.1208344. PubMed PMID: WOS:000295580300050.
62. Claus SP, Swann JR. Nutrimetabonomics: Applications for Nutritional Sciences, with Specific Reference to Gut Microbial Interactions. *Annual Review of Food Science and Technology*. 2013 2013/02/28;4(1):381-399. doi: 10.1146/annurev-food-030212-182612.
63. Schmidt TSB, Raes J, Bork P. The Human Gut Microbiome: From Association to Modulation. *Cell*. 2018 2018/03/08;172(6):1198-1215. doi: <https://doi.org/10.1016/j.cell.2018.02.044>.
64. Cardona F, Andres-Lacueva C, Tulipani S, et al. Benefits of polyphenols on gut microbiota and implications in human health. *Journal of Nutritional Biochemistry*. 2013 Aug;24(8):1415-1422. doi: 10.1016/j.jnutbio.2013.05.001. PubMed PMID: WOS:000322290100001.
65. Donovan SM. Introduction to the special focus issue on the impact of diet on gut microbiota composition and function and future opportunities for nutritional modulation of the gut microbiome to improve human health. *Gut Microbes*. 2017 2017/03/04;8(2):75-81. doi: 10.1080/19490976.2017.1299309.
66. Lecomte V, Kaakoush NO, Maloney CA, et al. Changes in Gut Microbiota in Rats Fed a High Fat Diet Correlate with Obesity-Associated Metabolic Parameters. *Plos One*. 2015 May;10(5). doi: 10.1371/journal.pone.0126931. PubMed PMID: WOS:000354917300067.
67. Walker AW, Ince J, Duncan SH, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *Isme Journal*. 2011 Feb;5(2):220-230. doi: 10.1038/ismej.2010.118. PubMed PMID: WOS:000290020000007.
68. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014 Jan;505(7484):559-+. doi: 10.1038/nature12820. PubMed PMID: WOS:000329995000042.

69. Muegge BD, Kuczynski J, Knights D, et al. Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans [10.1126/science.1198719]. *Science*. 2011;332(6032):970.
70. De Filippo C, Cavalieri D, Di Paola M, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America*. 2010 Aug;107(33):14691-14696. doi: 10.1073/pnas.1005963107. PubMed PMID: WOS:000281287600033.
71. Lin A, Bik EM, Costello EK, et al. Distinct Distal Gut Microbiome Diversity and Composition in Healthy Children from Bangladesh and the United States. *Plos One*. 2013 Jan;8(1). doi: 10.1371/journal.pone.0053838. PubMed PMID: WOS:000314019100034.
72. Shankar V, Gouda M, Moncivaiz J, et al. Differences in Gut Metabolites and Microbial Composition and Functions between Egyptian and U.S. Children Are Consistent with Their Diets. *Msystems*. 2017 Jan-Feb;2(1). doi: 10.1128/mSystems.00169-16. PubMed PMID: WOS:000408193500012.
73. Macfarlane GT, Allison C, Gibson SAW, et al. Contribution of the microflora to proteolysis in the human large intestine. *Journal of Applied Bacteriology*. 1988 1988/01/01;64(1):37-46. doi: 10.1111/j.1365-2672.1988.tb02427.x.
74. Gorvitovskaia A, Holmes SP, Huse SM. Interpreting Prevotella and Bacteroides as biomarkers of diet and lifestyle. *Microbiome*. 2016 Apr;4. doi: 10.1186/s40168-016-0160-7. PubMed PMID: WOS:000374464900001.
75. Costabile A, Klinder A, Fava F, et al. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. *British Journal of Nutrition*. 2008;99(1):110-120. doi: 10.1017/S0007114507793923.
76. Carvalho-Wells AL, Helmolz K, Nodet C, et al. Determination of the in vivo prebiotic potential of a maize-based whole grain breakfast cereal: a human feeding study. *British Journal of Nutrition*. 2010;104(9):1353-1356. doi: 10.1017/S0007114510002084.
77. Liu Z, Lin X, Huang G, et al. Prebiotic effects of almonds and almond skins on intestinal microbiota in healthy adult humans. *Anaerobe*. 2014 2014/04/01;26:1-6. doi: <https://doi.org/10.1016/j.anaerobe.2013.11.007>.
78. Shinohara K, Ohashi Y, Kawasumi K, et al. Effect of apple intake on fecal microbiota and metabolites in humans. *Anaerobe*. 2010 2010/10/01;16(5):510-515. doi: <https://doi.org/10.1016/j.anaerobe.2010.03.005>.
79. Fernando WMU, Hill JE, Zello GA, et al. Diets supplemented with chickpea or its main oligosaccharide component raffinose modify faecal microbial composition in healthy adults. *Beneficial Microbes*. 2010 Jun;1(2):197-207. doi: 10.3920/bm2009.0027. PubMed PMID: WOS:000208380800012.
80. Turner ND, Lupton JR. Dietary Fiber. *Advances in Nutrition*. 2011 03/10;2(2):151-152. doi: 10.3945/an.110.000281. PubMed PMID: PMC3065764.
81. Senghor B, Sokhna C, Ruimy R, et al. Gut microbiota diversity according to dietary habits and geographical provenance. *Human Microbiome Journal*. 2018 2018/04/01;7-8:1-9. doi: <https://doi.org/10.1016/j.humic.2018.01.001>.

82. Putignani L, Del Chierico F, Vernocchi P, et al. Gut Microbiota Dysbiosis as Risk and Premorbid Factors of IBD and IBS Along the Childhood–Adulthood Transition. *Inflammatory Bowel Diseases*. 2016;22(2):487-504. doi: 10.1097/MIB.0000000000000602.
83. Vernocchi P, Del Chierico F, Putignani L. Gut Microbiota Profiling: Metabolomics Based Approach to Unravel Compounds Affecting Human Health. *Frontiers in Microbiology*. 2016 07/26;7:1144. doi: 10.3389/fmicb.2016.01144. PubMed PMID: PMC4960240.
84. Doris MJ, Estelle G, John van Duynhoven and Elaine EV. Non-Digestible Food Ingredients, Colonic Microbiota and the Impact on Gut Health and Immunity: A Role for Metabolomics. *Current Drug Metabolism*. 2009;10(1):41-54. doi: <http://dx.doi.org/10.2174/138920009787048383>.
85. Aura A-M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews*. 2008 2008/10/01;7(3):407-429. doi: 10.1007/s11101-008-9095-3.
86. Dai Z, Wu Z, Hang S, et al. Amino acid metabolism in intestinal bacteria and its potential implications for mammalian reproduction. *MHR: Basic science of reproductive medicine*. 2015;21(5):389-409. doi: 10.1093/molehr/gav003.
87. den Besten G, van Eunen K, Groen AK, et al. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*. 2013 Sep;54(9):2325-2340. doi: 10.1194/jlr.R036012. PubMed PMID: WOS:000330533600005.
88. EPA U. Priority Pollutant List. 2014.
89. Calderón J, Ortiz-Pérez D, Yáñez L, et al. Human exposure to metals. Pathways of exposure, biomarkers of effect, and host factors. *Ecotoxicology and Environmental Safety*. 2003 2003/09/01;56(1):93-103. doi: [https://doi.org/10.1016/S0147-6513\(03\)00053-8](https://doi.org/10.1016/S0147-6513(03)00053-8).
90. Roychowdhury T, Tokunaga H, Ando M. Survey of arsenic and other heavy metals in food composites and drinking water and estimation of dietary intake by the villagers from an arsenic-affected area of West Bengal, India. *Science of The Total Environment*. 2003 2003/06/01;308(1):15-35. doi: [https://doi.org/10.1016/S0048-9697\(02\)00612-5](https://doi.org/10.1016/S0048-9697(02)00612-5).
91. Cui Y-J, Zhu Y-G, Zhai R-H, et al. Transfer of metals from soil to vegetables in an area near a smelter in Nanning, China. *Environment International*. 2004 2004/08/01;30(6):785-791. doi: <https://doi.org/10.1016/j.envint.2004.01.003>.
92. Li Y, Wang Y-b, Gou X, et al. Risk assessment of heavy metals in soils and vegetables around non-ferrous metals mining and smelting sites, Baiyin, China. *Journal of Environmental Sciences*. 2006 2006/11/01;18(6):1124-1134. doi: [https://doi.org/10.1016/S1001-0742\(06\)60050-8](https://doi.org/10.1016/S1001-0742(06)60050-8).
93. Li J, Xie ZM, Xu JM, et al. Risk assessment for safety of soils and vegetables around a lead/zinc mine. *Environmental Geochemistry and Health*. 2006 2006/02/01;28(1):37-44. doi: 10.1007/s10653-005-9009-x.
94. Islam Eu, Yang X-e, He Z-l, et al. Assessing potential dietary toxicity of heavy metals in selected vegetables and food crops. *Journal of Zhejiang University Science B*. 2007 12/18;8(1):1-13. doi: 10.1631/jzus.2007.B0001. PubMed PMID: PMC1764924.

95. Chen J, Gao J. The Chinese total diet study in 1990. Part I. Chemical contaminants. *Journal of AOAC International*. 1993 1993 Nov-Dec;76(6):1193-1205. PubMed PMID: 8286956; eng.
96. Tripathi RM, Raghunath R, Krishnamoorthy TM. Dietary intake of heavy metals in Bombay city, India. *Science of The Total Environment*. 1997 1997/12/22;208(3):149-159. doi: [https://doi.org/10.1016/S0048-9697\(97\)00290-8](https://doi.org/10.1016/S0048-9697(97)00290-8).
97. de W-SR, M.I. B, van DG, et al. Dietary intake of heavy metals (cadmium, lead and mercury) by the Dutch population. 2003. <https://www.rivm.nl/bibliotheek/rapporten/320103001.html>
98. Tchounwou PB, Yedjou CG, Patlolla AK, et al. Heavy Metals Toxicity and the Environment. *EXS*. 2012;101:133-164. doi: 10.1007/978-3-7643-8340-4_6. PubMed PMID: PMC4144270.
99. WHO. Guidelines of drinking water quality, fourth edition. 2011.
100. Breton J, Massart S, Vandamme P, et al. Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. *BMC Pharmacology and Toxicology*. 2013 2013/12/11;14(1):62. doi: 10.1186/2050-6511-14-62.
101. Barceloux DG, Barceloux D. Nickel. *Journal of Toxicology: Clinical Toxicology*. 1999 1999/01/01;37(2):239-258. doi: 10.1081/CLT-100102423.
102. Snedeker SM, Hay AG. Do Interactions Between Gut Ecology and Environmental Chemicals Contribute to Obesity and Diabetes? *Environmental Health Perspectives*. 2012 10/31;120(3):332-339. doi: 10.1289/ehp.1104204. PubMed PMID: PMC3295356.
103. Breton J, Daniel C, Dewulf J, et al. Gut microbiota limits heavy metals burden caused by chronic oral exposure. *Toxicology Letters*. 2013 2013/10/24;222(2):132-138. doi: <https://doi.org/10.1016/j.toxlet.2013.07.021>.
104. Monachese M, Burton JP, Reid G. Bioremediation and Tolerance of Humans to Heavy Metals through Microbial Processes: a Potential Role for Probiotics? *Applied and Environmental Microbiology*. 2012 Sep;78(18):6397-6404. doi: 10.1128/aem.01665-12. PubMed PMID: WOS:000308336400001.
105. Claus SP, Guillou H, Ellero-Simatos S. The gut microbiota: a major player in the toxicity of environmental pollutants? (vol 2, 16003, 2016). *Npj Biofilms and Microbiomes*. 2017 Jun;3. doi: 10.1038/npjbiofilms.2017.1. PubMed PMID: WOS:000412923200001.
106. Koppel N, Rekdal VM, Balskus EP. Chemical transformation of xenobiotics by the human gut microbiota. *Science*. 2017 Jun;356(6344). doi: 10.1126/science.aag2770. PubMed PMID: WOS:000403881700029.
107. Hughes V, Jiang C, Brun Y. *Caulobacter crescentus*. *Current Biology*. 2012 Jul;22(13):R507-R509. doi: 10.1016/j.cub.2012.05.036. PubMed PMID: WOS:000306379600003.
108. Bik EM, Eckburg PB, Gill SR, et al. Molecular analysis of the bacterial microbiota in the human stomach. *Proceedings of the National Academy of Sciences of the United States of America*. 2006 Jan;103(3):732-737. doi: 10.1073/pnas.0506655103. PubMed PMID: WOS:000234727800042.

109. Curtis PD, Brun YV. Getting in the Loop: Regulation of Development in *Caulobacter crescentus*. *Microbiology and Molecular Biology Reviews* : MMBR. 2010;74(1):13-41. doi: 10.1128/MMBR.00040-09. PubMed PMID: PMC2832345.
110. Jensen RB, Wang SC, Shapiro L. Dynamic localization of proteins and DNA during a bacterial cell cycle [Review Article]. *Nature Reviews Molecular Cell Biology*. 2002 03/01/online;3:167. doi: 10.1038/nrm758.
111. Jonas K. To divide or not to divide: control of the bacterial cell cycle by environmental cues. *Current Opinion in Microbiology*. 2014 2014/04/01/;18:54-60. doi: <https://doi.org/10.1016/j.mib.2014.02.006>.
112. Skerker JM, Laub MT. Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus* [Review Article]. *Nature Reviews Microbiology*. 2004 04/01/online;2:325. doi: 10.1038/nrmicro864.
113. Reisenauer A, Shapiro L. DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter* [10.1093/emboj/cdf490]. *The EMBO Journal*. 2002;21(18):4969.
114. Domian IJ, Quon KC, Shapiro L. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell*. 1997 Aug;90(3):415-424. doi: 10.1016/s0092-8674(00)80502-4. PubMed PMID: WOS:A1997XQ06300006.
115. Tan MH, Kozdon JB, Shen X, et al. An essential transcription factor, SciP, enhances robustness of *Caulobacter* cell cycle regulation [10.1073/pnas.1014395107]. *Proceedings of the National Academy of Sciences*. 2010;107(44):18985.
116. Quon KC, Marczyński GT, Shapiro L. Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell*. 1996 Jan;84(1):83-93. doi: 10.1016/s0092-8674(00)80995-2. PubMed PMID: WOS:A1996TQ17000011.
117. Ryan KR, Huntwork S, Shapiro L. Recruitment of a cytoplasmic response regulator to the cell pole is linked to its cell cycle-regulated proteolysis [10.1073/pnas.0402153101]. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(19):7415.
118. Collier J, Shapiro L. Spatial complexity and control of a bacterial cell cycle. *Current Opinion in Biotechnology*. 2007 Aug;18(4):333-340. doi: 10.1016/j.copbio.2007.07.007. PubMed PMID: WOS:000249980400008.
119. Collier J, Murray SR, Shapiro L. DnaA couples DNA replication and the expression of two cell cycle master regulators. *The EMBO Journal*. 2006 01/05;25(2):346-356. doi: 10.1038/sj.emboj.7600927. PubMed PMID: PMC1383511.
120. Zhao LP. The gut microbiota and obesity: from correlation to causality. *Nature Reviews Microbiology*. 2013 Sep;11(9):639-647. doi: 10.1038/nrmicro3089. PubMed PMID: WOS:000323205500012.
121. Conlon MA, Bird AR. The Impact of Diet and Lifestyle on Gut Microbiota and Human Health. *Nutrients*. 2015 Jan;7(1):17-44. doi: 10.3390/nu7010017. PubMed PMID: WOS:000348402900002.
122. Simon GL, Gorbach SL. INTESTINAL FLORA IN HEALTH AND DISEASE. *Gastroenterology*. 1984;86(1):174-193. PubMed PMID: WOS:A1984RV31600025.

-
123. Chakraborti CK. New-found link between microbiota and obesity. *World Journal of Gastrointestinal Pathophysiology*. 2015 11/15;6(4):110-119. doi: 10.4291/wjgp.v6.i4.110. PubMed PMID: PMC4644874.
 124. Clarke SF, Murphy EF, Nilaweera K, et al. The gut microbiota and its relationship to diet and obesity. *Gut Microbes*. 2012 2012/05/01;3(3):186-202. doi: 10.4161/gmic.20168.
 125. Lagier JC, Million M, Hugon P, et al. Human gut microbiota: repertoire and variations. *Frontiers in Cellular and Infection Microbiology*. 2012 Nov;2. doi: 10.3389/fcimb.2012.00136. PubMed PMID: WOS:000324638100005.
 126. Zhang JC, Guo Z, Lim AAQ, et al. Mongolians core gut microbiota and its correlation with seasonal dietary changes. *Scientific Reports*. 2014 May;4. doi: 10.1038/srep05001. PubMed PMID: WOS:000335949700001.
 127. Cardona F, Andrés-Lacueva C, Tulipani S, et al. Benefits of polyphenols on gut microbiota and implications in human health. *The Journal of Nutritional Biochemistry*. 2013 2013/08/01;24(8):1415-1422. doi: <https://doi.org/10.1016/j.jnutbio.2013.05.001>.
 128. Singh RK, Chang HW, Yan D, et al. Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*. 2017 Apr;15. doi: 10.1186/s12967-017-1175-y. PubMed PMID: WOS:000398612900001.
 129. Mullin GE. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. *Nutrition in Clinical Practice*. 2010 Jun;25(3):310-311. doi: 10.1177/0884533610368714. PubMed PMID: WOS:000278951700015.
 130. Murphy EF, Cotter PD, Healy S, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut*. 2010 Dec;59(12):1635-1642. doi: 10.1136/gut.2010.215665. PubMed PMID: WOS:000284109200009.
 131. Khanna S, Tosh PK. A Clinician's Primer on the Role of the Microbiome in Human Health and Disease. *Mayo Clinic Proceedings*. 2014 Jan;89(1):107-114. doi: 10.1016/j.mayocp.2013.10.011. PubMed PMID: WOS:000329163600018.
 132. Ng M, Fleming T, Robinson M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*. 384(9945):766-781. doi: 10.1016/S0140-6736(14)60460-8.
 133. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. 2011 ;27(21):2957-2963. doi: 10.1093/bioinformatics/btr507. PubMed PMID: PMC3198573.
 134. Edgar RC, Haas BJ, Clemente JC, et al. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. 2011;27(16):2194-2200. doi: 10.1093/bioinformatics/btr381.
 135. Caporaso JG, Bittinger K, Bushman FD, et al. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*. 2010;26(2):266-267. doi: 10.1093/bioinformatics/btp636.
 136. Ramette A. Multivariate analyses in microbial ecology. *Fems Microbiology Ecology*. 2007 ;62(2):142-160. doi: 10.1111/j.1574-6941.2007.00375.x. PubMed PMID: PMC2121141.

-
137. Paulson JN, Pop M, Bravo HC. Metastats: an improved statistical method for analysis of metagenomic data. *Genome Biology*. 2011 09/19;12(Suppl 1):P17-P17. doi: 10.1186/gb-2011-12-s1-p17. PubMed PMID: PMC3439073.
 138. Xia J, Sinelnikov IV, Han B, et al. MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research*. 2015;43(W1):W251-W257. doi: 10.1093/nar/gkv380.
 139. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biology*. 2011;12(6). doi: 10.1186/gb-2011-12-6-r60. PubMed PMID: WOS:000296646600008.
 140. Bäckhed F, Ley RE, Sonnenburg JL, et al. Host-Bacterial Mutualism in the Human Intestine [10.1126/science.1104816]. *Science*. 2005;307(5717):1915.
 141. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing [Article]. *Nature*. 2010 03/04/online;464:59. doi: 10.1038/nature08821
<https://www.nature.com/articles/nature08821#supplementary-information>.
 142. Tyakht AV, Kostyukova ES, Popenko AS, et al. Human gut microbiota community structures in urban and rural populations in Russia. *Nature Communications*. 2013 Sep;4. doi: 10.1038/ncomms3469. PubMed PMID: WOS:000325533900016.
 143. Martens EC, Lowe EC, Chiang H, et al. Recognition and Degradation of Plant Cell Wall Polysaccharides by Two Human Gut Symbionts. *Plos Biology*. 2011 Dec;9(12). doi: 10.1371/journal.pbio.1001221. PubMed PMID: WOS:000298673000011.
 144. Ley R, Turnbaugh P, Klein S, et al. Microbial Ecology: Human gut microbes associated with obesity. Vol. 444. 2007.
 145. Turnbaugh PJ, Hamady M, Yatsunenkov T, et al. A core gut microbiome in obese and lean twins. *Nature*. 2009 11/30;457(7228):480-484. doi: 10.1038/nature07540. PubMed PMID: PMC2677729.
 146. Turrone F, Ventura M, Butto LF, et al. Molecular dialogue between the human gut microbiota and the host: a Lactobacillus and Bifidobacterium perspective. *Cellular and Molecular Life Sciences*. 2014 Jan;71(2):183-203. doi: 10.1007/s00018-013-1318-0. PubMed PMID: WOS:000329223500001.
 147. Ventura M, Turrone F, Motherway MO, et al. Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends in Microbiology*. 2012 Oct;20(10):467-476. doi: 10.1016/j.tim.2012.07.002. PubMed PMID: WOS:000309946900003.
 148. Dehingia M, Devi KT, Talukdar NC, et al. Gut bacterial diversity of the tribes of India and comparison with the worldwide data. *Scientific Reports*. 2015 Dec;5. doi: 10.1038/srep18563. PubMed PMID: WOS:000367039000001.
 149. Nakayama J, Watanabe K, Jiang JH, et al. Diversity in gut bacterial community of school-age children in Asia. *Scientific Reports*. 2015 Feb;5. doi: 10.1038/srep08397. PubMed PMID: WOS:000349711900001.
 150. De Filippis F, Pellegrini N, Vannini L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut*. 2016 Nov;65(11):1812-1821. doi: 10.1136/gutjnl-2015-309957. PubMed PMID: WOS:000386471000008.

151. Kovatcheva-Datchary P, Nilsson A, Akrami R, et al. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of *Prevotella*. *Cell Metabolism*. 2015 Dec;22(6):971-982. doi: 10.1016/j.cmet.2015.10.001. PubMed PMID: WOS:000365993100006.
152. Dodd D, Mackie RI, Cann IKO. Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. *Molecular Microbiology*. 2011 Jan;79(2):292-304. doi: 10.1111/j.1365-2958.2010.07473.x. PubMed PMID: WOS:000286114200004.
153. Noguera-Julian M, Rocafort M, Guillen Y, et al. Gut Microbiota Linked to Sexual Preference and HIV Infection. *Ebiomedicine*. 2016 Mar;5:135-146. doi: 10.1016/j.ebiom.2016.01.032. PubMed PMID: WOS:000375078200027.
154. Ruengsomwong S, La-Ongkham O, Jiang JH, et al. Microbial Community of Healthy Thai Vegetarians and Non-Vegetarians, Their Core Gut Microbiota, and Pathogen Risk. *Journal of Microbiology and Biotechnology*. 2016 Oct;26(10):1723-1735. doi: 10.4014/jmb.1603.03057. PubMed PMID: WOS:000388662200009.
155. Jain A, Li XH, Chen WN. Similarities and differences in gut microbiome composition correlate with dietary patterns of Indian and Chinese adults. *AMB Express*. 2018 2018/06/23;8(1):104. doi: 10.1186/s13568-018-0632-1.
156. Gao X, Pujos-Guillot E, Martin J-F, et al. Metabolite analysis of human fecal water by gas chromatography/mass spectrometry with ethyl chloroformate derivatization. *Analytical Biochemistry*. 2009 2009/10/15;393(2):163-175. doi: <https://doi.org/10.1016/j.ab.2009.06.036>.
157. Wang M, Bai J, Chen WN, et al. Metabolomic Profiling of Cellular Responses to Carvedilol Enantiomers in Vascular Smooth Muscle Cells. *PLOS ONE*. 2010;5(11):e15441. doi: 10.1371/journal.pone.0015441.
158. Jain A, Chen WN. Involvement of organic acids and amino acids in ameliorating Ni(II) toxicity induced cell cycle dysregulation in *Caulobacter crescentus*: a metabolomics analysis. *Applied Microbiology and Biotechnology*. 2018 2018/05/01;102(10):4563-4575. doi: 10.1007/s00253-018-8938-0.
159. A J, Trygg J, Gullberg J, et al. Extraction and GC/MS Analysis of the Human Blood Plasma Metabolome. *Analytical Chemistry*. 2005 2005/12/01;77(24):8086-8094. doi: 10.1021/ac051211v.
160. Nováková L, Vlčková H. A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation. *Analytica Chimica Acta*. 2009 2009/12/10;656(1):8-35. doi: <https://doi.org/10.1016/j.aca.2009.10.004>.
161. Fiehn O, Wohlgemuth G, Scholz M, et al. Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant Journal*. 2008 Feb;53(4):691-704. doi: 10.1111/j.1365-313X.2007.03387.x. PubMed PMID: WOS:000252931800007.
162. Fiehn O. Metabolomics by Gas Chromatography–Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Current Protocols in Molecular Biology*. 2016 2016/04/01;114(1):30.4.1-30.4.32. doi: 10.1002/0471142727.mb3004s114.
163. Bylda C, Thiele R, Kobold U, et al. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *Analyst*. 2014;139(10):2265-2276. doi: 10.1039/c4an00094c. PubMed PMID: WOS:000334734200001.

-
164. Alzweiri M, Watson DG, Robertson C, et al. Comparison of different water-miscible solvents for the preparation of plasma and urine samples in metabolic profiling studies. *Talanta*. 2008 2008/01/15/;74(4):1060-1065. doi: <https://doi.org/10.1016/j.talanta.2007.07.037>.
165. Gowda GAN, Djukovic D. Overview of Mass Spectrometry-Based Metabolomics: Opportunities and Challenges. *Methods in molecular biology* (Clifton, NJ). 2014;1198:3-12. doi: 10.1007/978-1-4939-1258-2_1. PubMed PMID: PMC4336784.
166. Woo HM, Kim KM, Choi MH, et al. Mass spectrometry based metabolomic approaches in urinary biomarker study of women's cancers. *Clinica Chimica Acta*. 2009 2009/02/01/;400(1):63-69. doi: <https://doi.org/10.1016/j.cca.2008.10.014>.
167. Barker HA. Amino Acid Degradation by Anaerobic Bacteria. *Annual Review of Biochemistry*. 1981 1981/06/01;50(1):23-40. doi: 10.1146/annurev.bi.50.070181.000323.
168. Park SG, Schimmel P, Kim S. Aminoacyl tRNA synthetases and their connections to disease [10.1073/pnas.0802862105]. *Proceedings of the National Academy of Sciences*. 2008;105(32):11043.
169. Russell WR, Gratz SW, Duncan SH, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *The American Journal of Clinical Nutrition*. 2011;93(5):1062-1072. doi: 10.3945/ajcn.110.002188.
170. Russell Wendy R, Duncan Sylvia H, Scobbie L, et al. Major phenylpropanoid-derived metabolites in the human gut can arise from microbial fermentation of protein. *Molecular Nutrition & Food Research*. 2013 2013/03/01;57(3):523-535. doi: 10.1002/mnfr.201200594.
171. Graf BA, Milbury PE, Blumberg JB. Flavonols, flavones, flavanones, and human health: Epidemiological evidence. *Journal of Medicinal Food*. 2005 Fall;8(3):281-290. doi: 10.1089/jmf.2005.8.281. PubMed PMID: WOS:000232239100001.
172. Stella C, Beckwith-Hall B, Cloarec O, et al. Susceptibility of Human Metabolic Phenotypes to Dietary Modulation. *Journal of Proteome Research*. 2006 2006/10/01;5(10):2780-2788. doi: 10.1021/pr060265y.
173. Williamson L, New D. How the use of creatine supplements can elevate serum creatinine in the absence of underlying kidney pathology. *BMJ Case Reports*. 2014 :bcr2014204754. doi: 10.1136/bcr-2014-204754. PubMed PMID: PMC4170516.
174. Young VR, Pellett PL. Plant proteins in relation to human protein and amino acid nutrition. *The American Journal of Clinical Nutrition*. 1994;59(5):1203S-1212S. doi: 10.1093/ajcn/59.5.1203S.
175. Chu Y, Kwon T, Nam J. Enzymatic and metabolic engineering for efficient production of syringin, sinapyl alcohol 4-O-glucoside, in *Arabidopsis thaliana*. *Phytochemistry*. 2014 2014/06/01/;102:55-63. doi: <https://doi.org/10.1016/j.phytochem.2014.03.003>.
176. Ottosson F, Brunkwall L, Ericson U, et al. Connection Between BMI-Related Plasma Metabolite Profile and Gut Microbiota. *The Journal of Clinical Endocrinology & Metabolism*. 2018;103(4):1491-1501. doi: 10.1210/jc.2017-02114.

177. Dai ZL, Wu GY, Zhu WY. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Frontiers in Bioscience-Landmark*. 2011 Jan;16:1768-1786. doi: 10.2741/3820. PubMed PMID: WOS:000290094700013.
178. Marin L, Miguelez EM, Villar CJ, et al. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *Biomed Research International*. 2015. doi: 10.1155/2015/905215. PubMed PMID: WOS:000351112600001.
179. Raimondi S, Anighoro A, Quartieri A, et al. Role of bifidobacteria in the hydrolysis of chlorogenic acid. *MicrobiologyOpen*. 2014 2015/02/01;4(1):41-52. doi: 10.1002/mbo3.219.
180. Manach C, Scalbert A, Morand C, et al. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*. 2004;79(5):727-747. doi: 10.1093/ajcn/79.5.727.
181. Rowland I, Gibson G, Heinken A, et al. Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition*. 2018 2018/02/01;57(1):1-24. doi: 10.1007/s00394-017-1445-8.
182. Santoru ML, Piras C, Murgia A, et al. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Scientific Reports*. 2017 2017/08/25;7(1):9523. doi: 10.1038/s41598-017-10034-5.
183. He ZLL, Yang XE, Stoffella PJ. Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine and Biology*. 2005;19(2-3):125-140. doi: 10.1016/j.jtemb.2005.02.010. PubMed PMID: WOS:000234080900001.
184. Herawati N, Suzuki S, Hayashi K, et al. Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia, and China by soil type. *Bulletin of Environmental Contamination and Toxicology*. 2000 Jan;64(1):33-39. PubMed PMID: WOS:000084544300006.
185. Yusuf M, Fariduddin Q, Hayat S, et al. Nickel: An Overview of Uptake, Essentiality and Toxicity in Plants. *Bulletin of Environmental Contamination and Toxicology*. 2011 Jan;86(1):1-17. doi: 10.1007/s00128-010-0171-1. PubMed PMID: WOS:000285999900001.
186. Das KK, Das SN, Dhundasi SA. Nickel, its adverse health effects & oxidative. *Indian Journal of Medical Research*. 2008 Oct;128(4):412-425. PubMed PMID: WOS:000262164400006.
187. Denkhaus E, Salnikow K. Nickel essentiality, toxicity, and carcinogenicity. *Critical Reviews in Oncology Hematology*. 2002 Apr;42(1):35-56. doi: 10.1016/s1040-8428(01)00214-1. PubMed PMID: WOS:000175260200004.
188. Macomber L, Hausinger RP. Mechanisms of nickel toxicity in microorganisms. *Metallomics*. 2011;3(11):1153-1162. doi: 10.1039/c1mt00063b. PubMed PMID: WOS:000296382200009.
189. Rodrigue A, Effantin G, Mandrand-Berthelot MA. Identification of rcnA (yohM), a nickel and cobalt resistance gene in *Escherichia coli*. *Journal of Bacteriology*. 2005 Apr;187(8):2912-2916. doi: 10.1128/jb.187.8.2912-2916.2005. PubMed PMID: WOS:000228204700038.
190. Kalliri E, Grzyska PK, Hausinger RP. Kinetic and spectroscopic investigation of Coll, Nill, and N-oxalylglycine inhibition of the FeII/ α -ketoglutarate dioxygenase, TauD.

-
- Biochemical and Biophysical Research Communications. 2005
2005/12/09/;338(1):191-197. doi: <https://doi.org/10.1016/j.bbrc.2005.08.223>.
191. Niemirowicz G, Parussini F, Agüero F, et al. Two metallo-carboxypeptidases from the protozoan *Trypanosoma cruzi* belong to the M32 family, found so far only in prokaryotes. *Biochemical Journal*. 2007 Jan;401:399-410. doi: 10.1042/bj20060973. PubMed PMID: WOS:000243629800004.
 192. Sato K, Okubo A, Yamazaki S. Characterization of a multi-copper enzyme, nitrous oxide reductase, from *Rhodobacter sphaeroides* f. sp. *denitrificans*. *Journal of Biochemistry*. 1998 Jul;124(1):51-54. doi: 10.1093/oxfordjournals.jbchem.a022096. PubMed PMID: WOS:000074726400009.
 193. Debussche L, Couder M, Thibaut D, et al. ASSAY, PURIFICATION, AND CHARACTERIZATION OF COBALTOCHELATASE, A UNIQUE COMPLEX ENZYME CATALYZING COBALT INSERTION IN HYDROGENOBYRINIC ACID A,C-DIAMIDE DURING COENZYME B-12 BIOSYNTHESIS IN *PSEUDOMONAS-DENITRIFICANS*. *Journal of Bacteriology*. 1992 Nov;174(22):7445-7451. doi: 10.1128/jb.174.22.7445-7451.1992. PubMed PMID: WOS:A1992JY56000048.
 194. Bongaerts GPA, Uitzetter J, Brouns R, et al. URICASE OF *BACILLUS-FASTIDIOSUS* PROPERTIES AND REGULATION OF SYNTHESIS. *Biochimica Et Biophysica Acta*. 1978;527(2):348-358. doi: 10.1016/0005-2744(78)90349-2. PubMed PMID: WOS:A1978GB053000005.
 195. Louwrier A, Knowles CJ. The purification and characterization of a novel D(-)-specific carbamoylase enzyme from an *Agrobacterium* sp. *Enzyme and Microbial Technology*. 1996 1996/12/01/;19(8):562-571. doi: [https://doi.org/10.1016/0141-0229\(95\)00044-5](https://doi.org/10.1016/0141-0229(95)00044-5).
 196. Navarro C, Wu LF, Mandrandberthelot MA. THE NIK OPERON OF *ESCHERICHIA-COLI* ENCODES A PERIPLASMIC BINDING-PROTEIN-DEPENDENT TRANSPORT-SYSTEM FOR NICKEL. *Molecular Microbiology*. 1993 Sep;9(6):1181-1191. doi: 10.1111/j.1365-2958.1993.tb01247.x. PubMed PMID: WOS:A1993LX92900006.
 197. Mulrooney SB, Hausinger RP. Nickel uptake and utilization by microorganisms. *Fems Microbiology Reviews*. 2003 Jun;27(2-3):239-261. doi: 10.1016/s0168-6445(03)00042-1. PubMed PMID: WOS:000183744500008.
 198. Stahler FN, Odenbreit S, Haas R, et al. The novel *Helicobacter pylori* CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. *Infection and Immunity*. 2006 Jul;74(7):3845-3852. doi: 10.1128/iai.02025-05. PubMed PMID: WOS:000238591800015.
 199. Hillson NJ, Hu P, Andersen GL, et al. *Caulobacter crescentus* as a whole-cell uranium biosensor. *Applied and Environmental Microbiology*. 2007 Dec;73(23):7615-7621. doi: 10.1128/aem.01566-07. PubMed PMID: WOS:000251474400017.
 200. Patel J, Zhang Q, McKay RML, et al. Genetic Engineering of *Caulobacter crescentus* for Removal of Cadmium from Water. *Applied Biochemistry and Biotechnology*. 2010 2010/01/01;160(1):232-243. doi: 10.1007/s12010-009-8540-0.
 201. Hu P, Brodie EL, Suzuki Y, et al. Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. *Journal of Bacteriology*. 2005 Dec;187(24):8437-8449. doi: 10.1128/jb.187.24.8437-8449.2005. PubMed PMID: WOS:000233880000026.

-
202. Heinrich K, Sobetzko P, Jonas K. A Kinase-Phosphatase Switch Transduces Environmental Information into a Bacterial Cell Cycle Circuit. *Plos Genetics*. 2016 Dec;12(12). doi: 10.1371/journal.pgen.1006522. PubMed PMID: WOS:000392138700051.
 203. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis [Perspective]. *Nature Methods*. 2012 06/28/online;9:676. doi: 10.1038/nmeth.2019
<https://www.nature.com/articles/nmeth.2019#supplementary-information>.
 204. Thanbichler M, Shapiro L. MipZ, a spatial regulator coordinating chromosome segregation with cell division in *Caulobacter*. *Cell*. 2006 Jul;126(1):147-162. doi: 10.1016/j.cell.2006.05.038. PubMed PMID: WOS:000239224800023.
 205. Gitai Z, Dye N, Shapiro L. An actin-like gene can determine cell polarity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*. 2004 Jun;101(23):8643-8648. doi: 10.1073/pnas.0402638101. PubMed PMID: WOS:000222037000028.
 206. Viollier PH, Thanbichler M, McGrath PT, et al. Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proceedings of the National Academy of Sciences of the United States of America*. 2004 Jun;101(25):9257-9262. doi: 10.1073/pnas.0402606101. PubMed PMID: WOS:000222278600018.
 207. Ptacin JL, Lee SF, Garner EC, et al. A spindle-like apparatus guides bacterial chromosome segregation. *Nature Cell Biology*. 2010 07/25/online;12:791. doi: 10.1038/ncb2083
<https://www.nature.com/articles/ncb2083#supplementary-information>.
 208. Xia JG, Sinelnikov IV, Han B, et al. MetaboAnalyst 3.0-making metabolomics more meaningful. *Nucleic Acids Research*. 2015 Jul;43(W1):W251-W257. doi: 10.1093/nar/gkv380. PubMed PMID: WOS:000359772700039.
 209. Hosiner D, Gerber S, Lichtenberg-Fraté H, et al. Impact of Acute Metal Stress in *Saccharomyces cerevisiae*. *PLOS ONE*. 2014;9(1):e83330. doi: 10.1371/journal.pone.0083330.
 210. Yang H-C, Pon LA. Toxicity of Metal Ions Used in Dental Alloys: A Study in the Yeast *Saccharomyces cerevisiae*. *Drug and Chemical Toxicology*. 2003 2003/01/01;26(2):75-85. doi: 10.1081/DCT-120020403.
 211. Moore CM, Gaballa A, Hui M, et al. Genetic and physiological responses of *Bacillus subtilis* to metal ion stress. *Molecular Microbiology*. 2005 Jul;57(1):27-40. doi: 10.1111/j.1365-2958.2005.04642.x. PubMed PMID: WOS:000229581800004.
 212. Garbisu C, Alkorta I, Llama MJ, et al. Aerobic chromate reduction by *Bacillus subtilis*. *Biodegradation*. 1998 1998/03/01;9(2):133-141. doi: 10.1023/A:1008358816529.
 213. Chillappagari S, Seubert A, Trip H, et al. Copper Stress Affects Iron Homeostasis by Destabilizing Iron-Sulfur Cluster Formation in *Bacillus subtilis*. *Journal of Bacteriology*. 2010 May;192(10):2512-2524. doi: 10.1128/jb.00058-10. PubMed PMID: WOS:000277161400005.
 214. Hassen A, Saidi N, Cherif M, et al. Effects of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. *Bioresource Technology*. 1998 1998/07/01;65(1):73-82. doi: [https://doi.org/10.1016/S0960-8524\(98\)00011-X](https://doi.org/10.1016/S0960-8524(98)00011-X).

-
215. Teitzel GM, Parsek MR. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*. 2003 Apr;69(4):2313-2320. doi: 10.1128/aem.69.4.2313-2320.2003. PubMed PMID: WOS:000182151800057.
216. Ackerley DF, Barak Y, Lynch SV, et al. Effect of chromate stress on *Escherichia coli* K-12. *Journal of Bacteriology*. 2006 May;188(9):3371-3381. doi: 10.1128/jb.188.9.3371-3381.2006. PubMed PMID: WOS:000237171200026.
217. Nies DH. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology*. 1999 Jun;51(6):730-750. doi: 10.1007/s002530051457. PubMed PMID: WOS:000081207300003.
218. Parker DL, Borer P, Bernier-Latmani R. The response of *Shewanella oneidensis* MR-1 to Cr(III) toxicity differs from that to Cr(VI). *Frontiers in Microbiology*. 2011;2. doi: 10.3389/fmicb.2011.00223. PubMed PMID: WOS:000208863500231.
219. Chourey K, Thompson MR, Morrell-Falvey J, et al. Global molecular and morphological effects of 24-hour chromium(VI) exposure on *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology*. 2006 Sep;72(9):6331-6344. doi: 10.1128/aem.00813-06. PubMed PMID: WOS:000240474000079.
220. Jonas K, Liu J, Chien P, et al. Proteotoxic Stress Induces a Cell-Cycle Arrest by Stimulating Lon to Degrade the Replication Initiator DnaA. *Cell*. 2013 Aug;154(3):623-636. doi: 10.1016/j.cell.2013.06.034. PubMed PMID: WOS:000322629900015.
221. Boutte CC, Henry JT, Crosson S. ppGpp and Polyphosphate Modulate Cell Cycle Progression in *Caulobacter crescentus*. *Journal of Bacteriology*. 2012 Jan;194(1):28-35. doi: 10.1128/jb.05932-11. PubMed PMID: WOS:000298350800005.
222. Leslie DJ, Heinen C, Schramm FD, et al. Nutritional Control of DNA Replication Initiation through the Proteolysis and Regulated Translation of DnaA. *Plos Genetics*. 2015 Jul;11(7). doi: 10.1371/journal.pgen.1005342. PubMed PMID: WOS:000360622200019.
223. Figge RM, Divakaruni AV, Gober JW. MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. *Molecular Microbiology*. 2004 Mar;51(5):1321-1332. doi: 10.1046/j.1365-2958.2003.03936.x. PubMed PMID: WOS:000189142200008.
224. Britos L, Abeliuk E, Taverner T, et al. Regulatory Response to Carbon Starvation in *Caulobacter crescentus*. *Plos One*. 2011 Apr;6(4). doi: 10.1371/journal.pone.0018179. PubMed PMID: WOS:000289354100006.
225. Mohapatra SS, Fioravanti A, Biondi EG. DNA methylation in *Caulobacter* and other Alphaproteobacteria during cell cycle progression. *Trends in Microbiology*. 2014 2014/09/01;22(9):528-535. doi: <https://doi.org/10.1016/j.tim.2014.05.003>.
226. Ron EZ, Grossman N, Helmstetter CE. CONTROL OF CELL-DIVISION IN *ESCHERICHIA-COLI* - EFFECT OF AMINO-ACID STARVATION. *Journal of Bacteriology*. 1977;129(2):569-573. PubMed PMID: WOS:A1977CV77900001.
227. Monahan LG, Hajduk IV, Blaber SP, et al. Coordinating Bacterial Cell Division with Nutrient Availability: a Role for Glycolysis. *Mbio*. 2014 May-Jun;5(3). doi: 10.1128/mBio.00935-14. PubMed PMID: WOS:000338875900047.
228. Figge RM, Easter J, Gober JW. Productive interaction between the chromosome partitioning proteins, ParA and ParB, is required for the progression of the cell cycle

-
- in *Caulobacter crescentus*. *Molecular Microbiology*. 2003 Mar;47(5):1225-1237. doi: 10.1046/j.1365-2958.2003.03367.x. PubMed PMID: WOS:000181056400004.
229. Mohl DA, Easter J, Gober JW. The chromosome partitioning protein, ParB, is required for cytokinesis in *Caulobacter crescentus*. *Molecular Microbiology*. 2001 Nov;42(3):741-755. doi: 10.1046/j.1365-2958.2001.02643.x. PubMed PMID: WOS:000172341700013.
 230. Goley ED, Yeh YC, Hong SH, et al. Assembly of the *Caulobacter* cell division machine. *Molecular Microbiology*. 2011 Jun;80(6):1680-1698. doi: 10.1111/j.1365-2958.2011.07677.x. PubMed PMID: WOS:000292106100020.
 231. Lam H, Schofield WB, Jacobs-Wagner C. A landmark protein essential for establishing and perpetuating the polarity of a bacterial cell. *Cell*. 2006 Mar;124(5):1011-1023. doi: 10.1016/j.cell.2005.12.040. PubMed PMID: WOS:000237241200022.
 232. Carballido-López R. The Bacterial Actin-Like Cytoskeleton. *Microbiology and Molecular Biology Reviews*. 2006;70(4):888-909. doi: 10.1128/MMBR.00014-06. PubMed PMID: PMC1698507.
 233. Copley SD, Frank E, Kirsch WM, et al. DETECTION AND POSSIBLE ORIGINS OF AMINOMALONIC ACID IN PROTEIN HYDROLYSATES. *Analytical Biochemistry*. 1992 Feb;201(1):152-157. doi: 10.1016/0003-2697(92)90188-d. PubMed PMID: WOS:A1992HE33600022.
 234. Kroeger KM, Hashimoto M, Kow YW, et al. Cross-Linking of 2-Deoxyribonolactone and Its β -Elimination Product by Base Excision Repair Enzymes. *Biochemistry*. 2003 2003/03/01;42(8):2449-2455. doi: 10.1021/bi027168c.
 235. Jespersen D, Yu JJ, Huang BR. Metabolic Effects of Acibenzolar-S-Methyl for Improving Heat or Drought Stress in Creeping Bentgrass. *Frontiers in Plant Science*. 2017 Jul;8. doi: 10.3389/fpls.2017.01224. PubMed PMID: WOS:000405164400001.
 236. Singh S, Parihar P, Singh R, et al. Heavy Metal Tolerance in Plants: Role of Transcriptomics, Proteomics, Metabolomics, and Ionomics. *Frontiers in Plant Science*. 2016 Feb;6. doi: 10.3389/fpls.2015.01143. PubMed PMID: WOS:000369788400001.
 237. Sharma SS, Dietz KJ. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany*. 2006 Mar;57(4):711-726. doi: 10.1093/jxb/erj073. PubMed PMID: WOS:000235771700001.
 238. Lee J, Reeves RD, Brooks RR, et al. The relation between nickel and citric acid in some nickel-accumulating plants. *Phytochemistry*. 1978 1978/01/01;17(6):1033-1035. doi: [https://doi.org/10.1016/S0031-9422\(00\)94274-2](https://doi.org/10.1016/S0031-9422(00)94274-2).
 239. Kersten WJ, Brooks RR, Reeves RD, et al. NATURE OF NICKEL-COMPLEXES IN *PSYCHOTRIA-DOUARREI* AND OTHER NICKEL-ACCUMULATING PLANTS. *Phytochemistry*. 1980;19(9):1963-1965. doi: 10.1016/0031-9422(80)83013-5. PubMed PMID: WOS:A1980KK43100013.
 240. Montargès-Pelletier E, Chardot V, Echevarria G, et al. Identification of nickel chelators in three hyperaccumulating plants: An X-ray spectroscopic study. *Phytochemistry*. 2008 2008/05/01;69(8):1695-1709. doi: <https://doi.org/10.1016/j.phytochem.2008.02.009>.
 241. Bhatia NP, Walsh KB, Baker AJM. Detection and quantification of ligands involved in nickel detoxification in a herbaceous Ni hyperaccumulator *Stackhousia tryonii* Bailey.

-
- Journal of Experimental Botany. 2005 May;56(415):1343-1349. doi: 10.1093/jxb/eri135. PubMed PMID: WOS:000228636300009.
242. Kawahara Y, Ohsumi T, Yoshihara Y, et al. PROLINE IN THE OSMOREGULATION OF BREVIBACTERIUM-LACTOFERMENTUM. *Agricultural and Biological Chemistry*. 1989 Sep;53(9):2475-2479. doi: 10.1080/00021369.1989.10869668. PubMed PMID: WOS:A1989AT33300026.
243. Csonka LN. PHYSIOLOGICAL AND GENETIC RESPONSES OF BACTERIA TO OSMOTIC-STRESS. *Microbiological Reviews*. 1989 Mar;53(1):121-147. PubMed PMID: WOS:A1989T534200006.
244. Liang XW, Zhang L, Natarajan SK, et al. Proline Mechanisms of Stress Survival. *Antioxidants & Redox Signaling*. 2013 Sep;19(9):998-1011. doi: 10.1089/ars.2012.5074. PubMed PMID: WOS:000323886400009.
245. Talanova VV, Titov AF, Boeva NP. Effect of increasing concentrations of lead and cadmium on cucumber seedlings. *Biologia Plantarum*. 2000;43(3):441-444. doi: 10.1023/a:1026735603890. PubMed PMID: WOS:000089279700021.
246. Yusuf M, Fariduddin Q, Ahmad A. 24-Epibrassinolide modulates growth, nodulation, antioxidant system, and osmolyte in tolerant and sensitive varieties of *Vigna radiata* under different levels of nickel: A shotgun approach. *Plant Physiology and Biochemistry*. 2012 2012/08/01/;57:143-153. doi: <https://doi.org/10.1016/j.plaphy.2012.05.004>.
247. Siripornadulsil S, Traina S, Verma DPS, et al. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell*. 2002 Nov;14(11):2837-2847. doi: 10.1105/tpc.004853. PubMed PMID: WOS:000179264700015.
248. Freeman JL, Persans MW, Nieman K, et al. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *Plant Cell*. 2004 Aug;16(8):2176-2191. doi: 10.1105/tpc.104.023036. PubMed PMID: WOS:000223195000018.
249. Hoque MA, Banu MNA, Nakamura Y, et al. Proline and glycinebetaine enhance antioxidant defense and methylglyoxal detoxification systems and reduce NaCl-induced damage in cultured tobacco cells. *Journal of Plant Physiology*. 2008;165(8):813-824. doi: 10.1016/j.jplph.2007.07.013. PubMed PMID: WOS:000256527300004.
250. Zhou YZ, Larson JD, Bottoms CA, et al. Structural basis of the transcriptional regulation of the proline utilization regulon by multifunctional PutA. *Journal of Molecular Biology*. 2008 Aug;381(1):174-188. doi: 10.1016/j.jmb.2008.05.084. PubMed PMID: WOS:000258197500015.
251. Harth G, Maslesa-Galic S, Tullius MV, et al. All four *Mycobacterium tuberculosis* glnA genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis. *Molecular Microbiology*. 2005 Nov;58(4):1157-1172. doi: 10.1111/j.1365-2958.2005.04899.x. PubMed PMID: WOS:000232893800018.
252. Cheng ZH, Lin MQ, Rikihisa Y. *Ehrlichia chaffeensis* Proliferation Begins with NtrY/NtrX and PutA/GlnA Upregulation and CtrA Degradation Induced by Proline and Glutamine Uptake (vol 5, e02141, 2014). *Mbio*. 2015 Mar-Apr;6(2). doi: 10.1128/mBio.00242-15. PubMed PMID: WOS:000355312400017.

-
253. Beaufay F, Coppine J, Mayard A, et al. A NAD-dependent glutamate dehydrogenase coordinates metabolism with cell division in *Caulobacter crescentus*. *Embo Journal*. 2015 Jul;34(13):1786-1800. doi: 10.15252/embj.201490730. PubMed PMID: WOS:000357324100007.