Investigating the eukaryotic microbial microbiome in the gastrointestinal tract of individuals residing in Thailand

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Contents

Abbreviations

AIDS- Acquired immunodeficiency syndrome

BMI- Body Mass Index

DNA- Deoxyribonucleic Acid

DNP- Dynamic nuclear polarisation

DSS- Dextran Sulfate Sodium salt

EU- European Union

IBD- Irritable Bowel Disease

IBS- Irritable Bowel Syndrome

IPTG- Isopropyl β-D-1-thiogalactopyranoside

LB- Lysogeny Broth

MS- Mass Spectroscopy

NMR- Nuclear Mass Spectroscopy

PCR- Polymerase Chain Reaction

RNA- Ribonucleic Acid

Abstract

The microbiome of humans is a complex network of microorganisms that interact with each other and their human hosts. Its role in disease and pathogenicity is well known, this is particularly apparent in the role of the bacteria in the gastrointestinal tract. However, what is currently under researched is the part that the eukaryotic microbiome plays in the gastrointestinal tract. To better understand this using NMR, metabolites were extracted from faecal samples from humans in Thailand. This is one of the first thorough investigations of microbial alterations in populations in Thailand and possibly the whole South East Asia region. Recently the diet in Thailand has been changing to a more westernised diet, as diet is known to play a factor in the composition of gut microbiome, this is an important factor to consider. The results have been compared to human faecal samples from the UK. These samples were taken from a range of locations and varying BMI levels. The DNA from these samples was screened for various microbial parasites to better understand the eukaryotic microbiome. These results have also been compared to recently established data on metabolomic bacterial population to identify links between the bacterial and eukaryotic microbiome of the gut. Metabolites that were identified in both normal weight and overweight showed no significant difference between BMI. However, some metabolites that were present in only overweight individuals in this study such as acetoacetate and allantoin have been linked to obesity in other studies. These metabolites could be potential markers for obesity. The faecal DNA from these individuals were sequence negative, this could be due to the lack of suitable primers to target eukaryotic parasites in this region. Future work would involve expanding the scope of this study with a larger sample size and a focus on key metabolites associated with obesity.

1.0 Introduction

1.1 Microbiome

1.1.1 Defining the Microbiome

Microorganisms are found in a large variety of environments ranging from extremes in temperature to other parameters, one of these environments is the human body, microorganisms that reside here are known as the microbiome.

The human microbiome is made up of microorganisms that reside in or on a body in a symbiotic, commensal and pathogenic relationship (Turnbaugh P. et al, 2007). This microbiome largely consists of bacterial microorganisms that reside in the gut, which currently is the key focus of research into the microbiome. The microbiome is a variety of microorganisms, which include bacteria, archaea, viruses, unicellular eukaryotes and fungal organisms (D'Argenio V. et al, 2015).

These microorganisms can be found on the skin, nasal passages, oral cavity, gastrointestinal tracts and genital tracts of humans, outnumbering the number of human cells. There are approximately 10 times more bacterial cells than human cells present within the gut microbiome (Eckburg B, *et al* 2005), this only provides a snapshot of the total number of microorganisms that make up the human microbiome. Across the different parts of the body the microbiome can differ greatly; looking at healthy individuals it has been found that oral and gastrointestinal microbiome population were multifaceted in comparison to the vaginal population which was limited (Human Microbiome Project, 2012).

Bacteria are the microorganisms that have been the most widely researched component of the microbiome, however still only a small fraction of the species that inhabit the human microbiome have been cultured successfully (Lau, J.T, 2016). This presents an opportunity for further research into the human microbiome.

1.1.2 The Human Gut Microbiome in Health and Disease

The human gastrointestinal microbiome has been linked to a number of different diseases and health complications. This connection highlights the need for further study in this area. It is well known that the bacterial microbiome plays a pivotal role in disease; it has been linked to inflammatory bowel disease (IBD), Crohn's disease, colorectal cancer and allergies among others (Bäckhed, F et al, 2012).

There has been an increased prevalence of these diseases in developed countries, with very little sign of these diseases in developing countries (Haahtela, T., et al, 2013) (Karvonen, M et al, 2000). This finding could be due to better healthcare in developed countries. Thailand, the focus of this study is becoming more urbanised which has led to the increase of these diseases being seen among the population in this region. This rise in the incidence of diseases linked to the microbiome has been linked to changes in diet to a more westernised diet as well as increased use of antibiotics. The reduction of intestinal parasites that were very common in developing countries can be potentially linked to the rise in allergies as well (Caraballo, L., et al, 2011).

In addition the gut microbiome has been linked with a number of different cancers, which include colorectal cancer. This has been related to the rising use of antibiotics, which are known to alter the microbiome significantly (Yang, Y.-X.,et al, 2014).

Additionally, those with Crohn's disease, a subset disease of the group Inflammatory Bowel Disease (IBD) have been linked to a high concentration of Bacteriodetes and Proteobacteria not seen in individuals without this affliction (Gophna et al, 2006). As established in literature, this does not indicate that these organisms are directly responsible for the disease but it does establish links between the microbiome and disease.

The majority of studies focus on how the bacterial microbiome of the gut affects health and disease. The eukaryotic microbiome, however, has often been overlooked in this area. In the past eukaryotes such as protists and helminths of the gut have been classed as being pathogenic and of being a detriment to human health (Bogitsh et al, 2012).

Additionally there are some notable intestinal microbial eukaryotes that are pathogenic and do cause damage to their human hosts. *Crytosporidium* spp. is one example, an intracellular parasite that can cause gastrointestinal problems in immunocomromised individuals, with symptoms such as diarrhea (Rossle, N.F, et al, 2013). *Giardia lamblia* is another microbial eukaryote that is noted for its pathogenicity, it is particularly prevalent in developing countries and once again is particularly damaging to those who are immunocompromised (Muhsen, et al, 2012).

There are however microbial eukaryotes that have questionable pathogenicity; the protist *Blastocystis* is one of these organisms, and it has even been considered commensal. This classification has been brought about by it being

very commonly found in the gastrointestinal microbiome of humans (Scanlan PD, et al, 2014). It has been found in 10% to 100% of individuals depending on the population that was looked at. It has been found both in those who are immunocompromised and those who are healthy with no significant preference in either conditions (Parfrey, L.W., 2014).

1.1.3 The Microbiome and Obesity

Obesity is a non-communicable disease, it is defined as having a body mass index (BMI) of 30 or more, and it has been linked to a number of diseases including diabetes, cancer and cardiovascular disease. Since 1975 the mean BMI of adults, both males and females has increased, with 39% of men and women being classified as overweight or obese (World Health Organization, 2018).

This therefore highlights the obesity pandemic that is now worldwide, normally a problem in developed countries, where obesity is now becoming a problem for those in developing countries. This rise in obesity in developing countries also shows a rise in the obesity linked diseases described above among others, including hypertension, stroke, sleep apnea, respiratory problems and gynaecological problems (Centers for Disease Control and Prevention, 2018).

The reasons for the increased amount of those individuals who are overweight or obese have been due to an increased availability of food in these areas and decreased levels of physical activity (Hoffman, D.J, 2004). These factors have dramatically increased the risk of obesity and numerous linked diseases in these countries. Obesity is caused by an imbalance of intake and output in energy; this has caused a dramatic rise in obesity in both developed and developing countries. Current research is looking at how the gut microbiome plays a part in the development of obesity.

One of the studies that have looked at the role of the microbiome in obesity involves the use of rodents, they are used as models of the human gastrointestinal tracts. In one study they observed that when mice that had been raised in an environment without microorganisms and were then subsequently introduced to gut microbiota, their body fat increased even though their total food intake had decreased (Bäckhed, F., et al, 2004). This shows at least a tenuous link between the gut microbiome and obesity. Another study found that mice that had no gut microbiota and were put on a high fat, high sugar diet did not become overweight like their counterparts. These mice had an increased fatty acid metabolism, which prevented them from becoming overweight (Bäckhed, F, et al, 2007).

With the link of microbiome and obesity, certain metabolites can also be associated with BMI, with specific metabolites either increasing or decreasing in concentration as the BMI of an individual increases (Table 1).

Metabolite	Direction of change in concentration with increase in BMI
Urate	Up
Glutamate	Up
1-(1-enyl-palmitoyl)-2-oleoyl-GPC	Down
1-stearoyl-2-dihomo-linolenoyl-GPC	Up
1-eicosenoyl-GPC	Down
N2,N2-dimethylguanosine	Up
1-arachidoyl-GPC	Down
1-(1-enyl-stearoyl)-2-oleoyl-GPC	Down
N-acetylglycine	Down
5-methylthioadenosine (MTA)	Up
Valine	Up
Propionylcarnitine	Up
Succinylcarnitine	Up
1-nonadecanoyl-GPC	Down
1-linoleoyl-GPC	Down
Aspartate	Up
Mannose	Up
N-acetylvaline	Up
Kynurenate	Up
Sphingomyelin	Up

Table 1: Metabolites and their direction of change in concentration with increase in BMI. With up showing that the metabolite increases in concentration with an in increase in BMI. With down showing that the concentration of the metabolite decreases when BMI increases, Adapted from "Profound Perturbation of the Metabolome in Obesity is Associated with Health Risk" (Cirulli, *et al,* 2019).

These are just a few examples of what has been seen in mice models, however this does not show whether the microbiome of the gut plays a role in obesity in humans.

1.2 The Gastrointestinal Eukaryotic Microbiome

Eukaryotes that reside in the gastrointestinal tract can range from purely commensals to pathogenic. The focus of this study was to identify the microbial eukaryotes present within the gastrointestinal tract whether commensal or pathogenic. However metazoan parasites can be included within the eukaryotic microbiome (Bogitish B, 2005) as eukaryotes found in the gastrointestinal tract are spread across the eukaryotic phylogenic tree (Figure 1). The eukaryotic microbiome covers a number of distinct categories of metazoan parasites, which are comprised of organisms such as nematodes, helminthes etc., fungi and protozoans (Lukeš, J. et al, 2015).

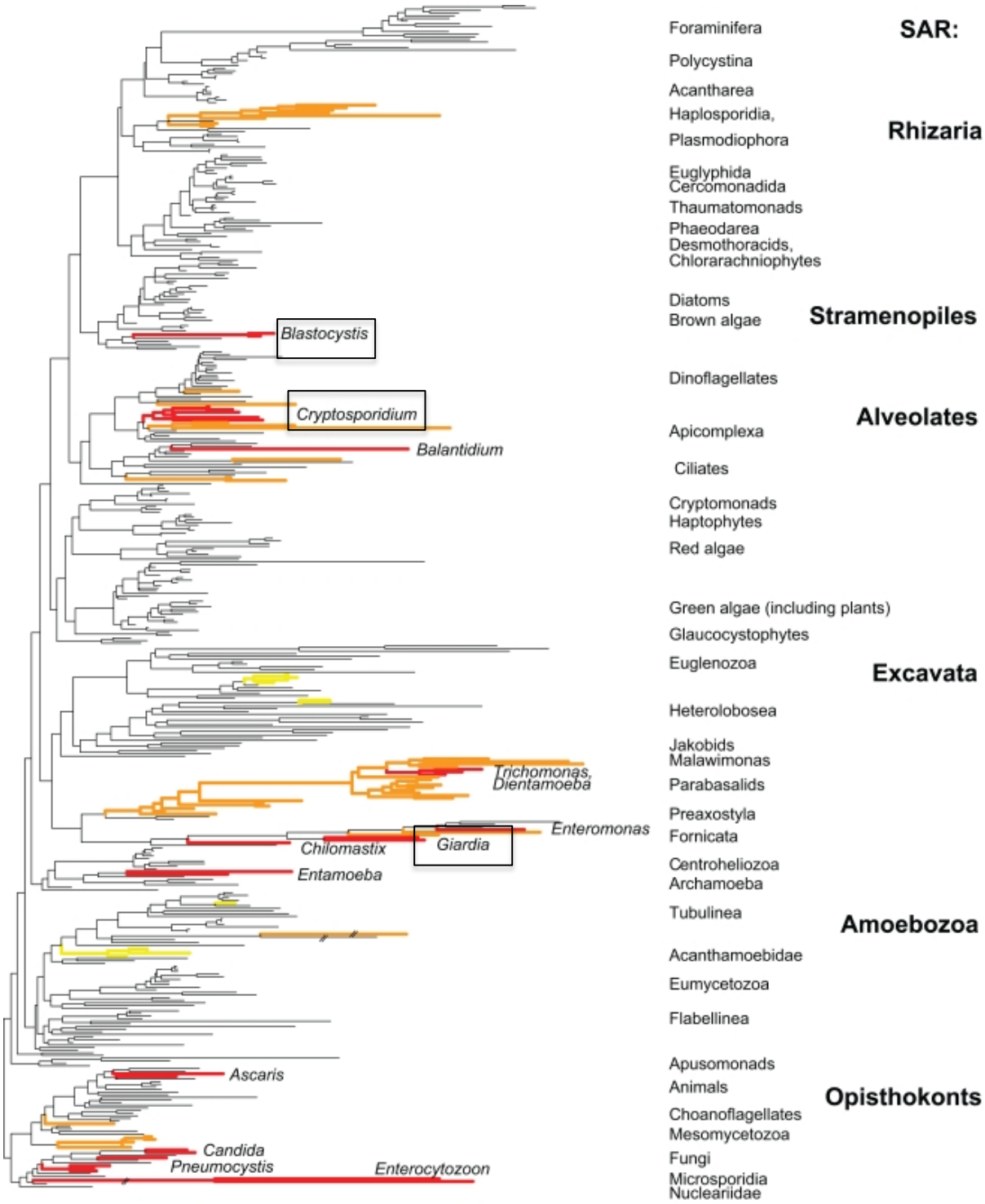

Figure 1- Distribution of Eukaryotes that Reside in a Host Microbiome. Those eukaryotes outlined will be focused on in this study. (*Blastocystis*, *Cryptosporidium* and *Giarda*). Red are those, which reside in vertebrates and orange lineages are those that reside in invertebrates, yellow are those that are non-gut parasites of humans. Boxes have been placed around those that were investigated in this study. Adapted from Parfrey, 2011.

As an overview, the gastrointestinal tract of humans is host to a number of eukaryotes with the main focus of research being from a parasitological standpoint. However there is a lack of research done into the role eukaryotes play in the gut microbiome. Additionally, investigations into the eukaryotic microbiome in non-Western communities are missing from the literature with the focus being primarily on western populations (Scanlan, P.D et al, 2008). Current research into the eukaryotes that are a part of the gastrointestinal microbiome is severely lacking in comparison to what is presently known about the bacteria that reside in the gastrointestinal tract (Hmpdacc.org, 2019).

The majority of eukaryotes that reside within the gastrointestinal tract of humans are commensal in nature, with a lack of pathogenicity being observed (Lukeš, J. *et al*, 2014). For example there is also the controversial pathogenicity of the protist *Blastocystis spp.* This organism has been linked to gastrointestinal distress but is also found to be asymptomatic with it being found in a high number of individuals (Scanlan, P.D *et al*, 2014). There are, however, some gastrointestinal eukaryotes that are purely opportunistic organisms, for example *Cryptosporidium spp*. and *Giardia intestinalis.*

Overall there is a range of relationships that eukaryotes residing in the gut can have with a human host. Furthermore, this relationship can change, eukaryotes that may not be pathogenic can become so and vice versa if there are any alterations within the microbiome. These changes can arise due to a number of factors, stress and diet being key examples. Immunocompromised individuals are also more likely to be affected by the more pathogenic organisms.

1.2.1. *Giardia intestinalis*

Giardia intestinalis is a eukaryotic microorganism that causes giardiasis, a gastrointestinal disease that affects human populations worldwide. *Giardia* infections have been found at 2-7 % prevalence in high-income countries and up to 30% in low-income countries (Fketcher S, *et al,* 2012). Although more common in the developing parts of the world, giardiasis can also be seen in the developed world with cases being seen in Europe, in 2014 23 countries of the EU reported 17278 incidences of giardiasis (Annual epidemiological report, ECDC, 2014). This highlights the tenacity of this microorganism, where even high water filtration techniques present in these European countries cannot prevent infection.

Giardiasis can be asymptomatic, although this is rare in comparison to those who are symptomatic (Nikillic A, *et al,* 2011). Human infection by *Giardia* only requires a low number of cysts, with only 10 cysts needed for infection to occur (Smithe, H. *et al,* 2006). Those who have immune deficiency problems such as those with acquired immune deficiency syndrome (AIDS) are more likely to suffer from complications of giardiasis, developing anorexia and dehydration (Angarano, G, *et al* 1997). There has also been a link between IBS (Irritable Bowel Syndrome) and having had giardiasis with the organism not needing to be present to manifest symptoms of IBS (Haliez M, *et al,* 2013).

Within the *Giardia* genus there are six species currently identified, with *Giardia intestinalis* being hosted by humans and other mammals. Other species, including *G. agilis* and *G. muris*, are host specific unlike *G.intestinalis*, which can infect a number of mammals (Caccio F, *et al,* 2005). Within the *G. intestinalis* species there are currently eight assemblages A-H (Lasek-Nesslquist, *et al,* 2010).

The microorganism itself has two forms; a trophozoite form and a cyst form, the cyst form is the way in which the infection is spread. These cysts are ingested which is then followed by the trophozoite form that emerges from the cyst and multiplies attaching themselves to the upper part of the small intestine of the host (Figure 2). Its flagellated form aids in its attachment to the wall of the small intestine. This form is the cause of the typical symptoms of giardiasis, with it being responsible for inflammation and diarrhoea (Buret AG. *et al,* 2008) (Cotton JA. *et al,* 2011).

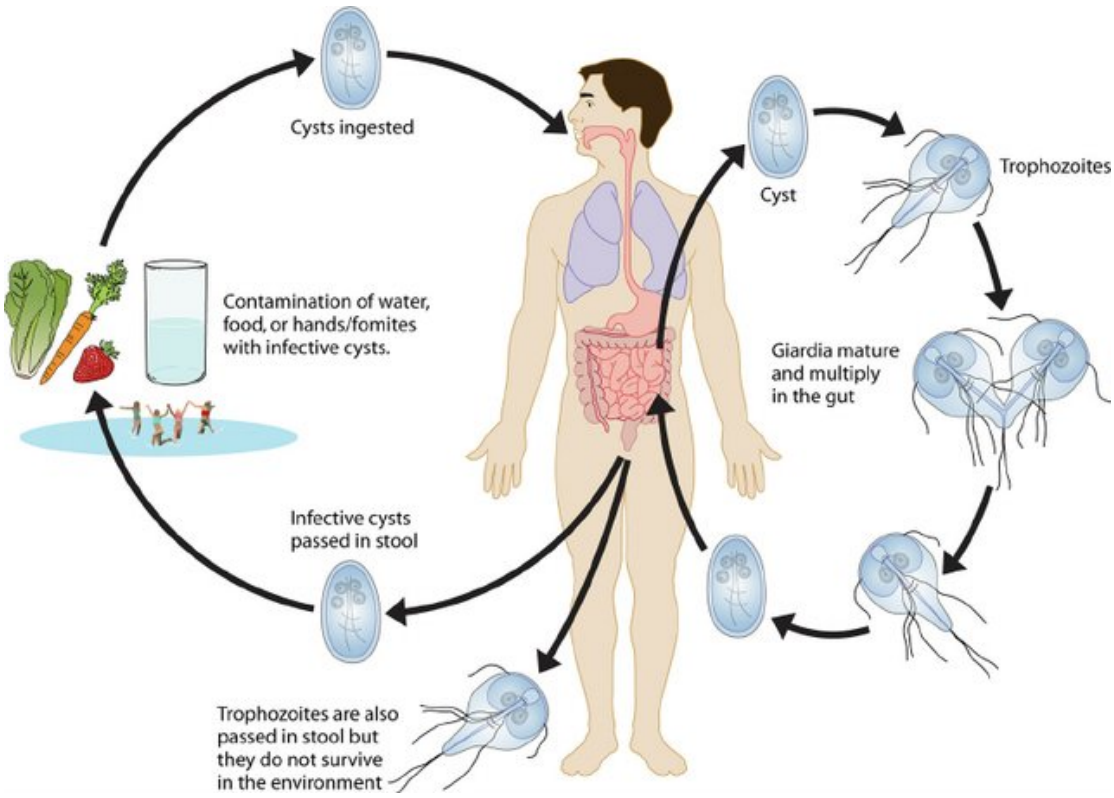

Figure 2- Life cycle of *Giardia spp*. showing the two forms the infectious cyst for and the trophozoite form. Adapted from Esch K. 2013.

The identification and diagnosis of *Giardia intestinalis* is typically done through microscopic analysis of faecal samples in a medical setting, this is due to the impracticality of PCR-based screening in areas where the prevalence of giardiasis is high (Behr M, *et al,* 1997). Once identified giardiasis can be treated with nitrimidazole drugs such as metronidazole, tinidaxole and ornidazole (Gardner, T. *et al,* 2001).

In Thailand the prevalence of *Giardia intestinalis* is variable across the country, and the highest number of cases is seen in western regions with 13.6% to 23.3%. The northern region showed low prevalence with 1.25% to 2.21% (Hassan H. 2008). The study of *Giardia* in Southeast Asia is limited in its scope, with only a small number of studies having been carried out.

G.intestinalis is one of the organisms screened for in samples for this investigation acquired from Thailand due to its high presence in the country.

1.2.2. *Cryptosporidium spp.*

Cryptosporidium is a genus of protozoan parasites and the cause of the disease cryptosporodiosis, which affects the gastrointestinal tract of humans and other animals. To date there are 26 species of *Cryptosporidium,* with human infections being typically caused by *Cryptosporidium hominis* and *Cryptosporidium parvum,* which is seen throughout the world (Chalmers, M, 2013).

Cryptosporidium can be asymptomatic, however, for the vast majority of those infected it causes severe gastrointestinal distress. Common symptoms include diarrhoea, vomiting and nausea; these symptoms can vary depending on the species present. Children and the elderly are the most severely affected by cryptosporidiosis (Bushen O, *et al* 2007). Those who are immunocompromised such as those with AIDS suffer even more severely with malnutrition, which can even lead to death without sufficient treatment (Asseda B, *et al,* 2009).

Infection by *Cryptosporidium* can take place by faecal contamination, as it is a zoonotic infection, this can be done via infected animals (Cama, V, *et al,* 2008). The infective oocysts can withstand some extremes of temperatures with their ideal environment being cool (Moriaty E, *et al,* 2005).

The entire life cycle of *Cryptosporidium* takes place in the gastrointestinal tract of one host, with it having asexual and sexual stages. Once the host has ingested the infected oocysts and the right conditions have been detected they begin excystation with four sporozoites being formed. These undergo a number of different steps shown in Figure 3. This culminates in the formation of oocysts, which then exit the host, thus continuing the life cycle (Bouzid, M. 2013).

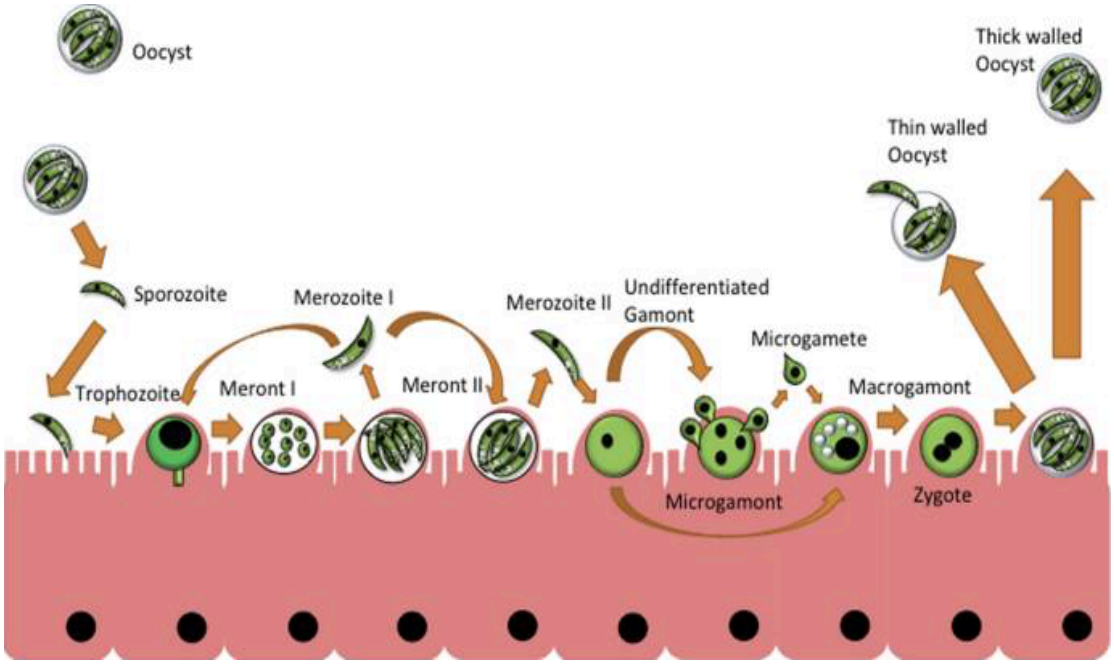

Figure 3-The life cycle of *Cryptosporidium parvum.* A detailed overview of the life cycle, showing the different stages that is undergone within the human host. Taken from (Bones A. *et al*., 2019).

The identification and thus diagnosis of *Cryptosporidium* is typically done via microscopy and ELISA, which allows for rapid detection of the parasite. However there are issues with these methods as they lack sensitivity and specificity, ELISA has greater sensitivity than microscopy and microscopy has greater specificity. To overcome this PCR can be used to identify the specific infective species (Mittal, S, *et al,* 2014).

A study in 2012 showed that up to 11% of the river and ocean water sampled from various locations in Thailand had *Cryptosporidium* present (Koompapong K, *et al,* 2012). It has also been seen that there is a high prevalence of *Cryptosporidium parvum* water buffalo in northeast Thailand (Inpankaew T, *et al,* 2014). As a zoonotic species this presents a risk to humans in the area via faecal contamination. This is why this orgaism was chosen to be dcreened for in this study.

1.2.3 Blastocystis spp.

Blastocystis is an anaerobic parasite that resides in the intestinal tracts of animals including humans (Tan C, *et al,* 2008). It can be found in individuals that are healthy and present no symptoms to those who are symptomatic which has led to it having a controversial pathogenicity (Moosavi A. *et al*, 2013).

This controversial pathogenicity is possibly derived from a lack of research into the genetic subsets of *Blastocystis hominis* that infects humans. This can be seen in research, where different genotypes could be responsible for its pathogenesis and other genotypes being responsible for the dismissal of this pathogenesis (Graham C. *et al*, 1997).

To date there are 17 subtypes of *Blastocytis* classified which is in contrast to its lack of morphological diversity (Cifre S. *et al*, 2018). These subtypes are based on the sequencing of partial small subunit (18s) ribosomal RNA, with ST1-9 having been found in humans (Adiyaman K., *et al*, 2015).

In humans *Blastocystis* has been linked to irritable bowel syndrome with those afflicted with this condition being found more frequently infected with *Blastocystis*, in comparison to those without IBS (Da C, *et al*, 2016). Subtypes one, three and four of *Blastocystis* have been linked to those with IBS. This suggests that the subtype may play a role in whether *Blastocystis* is pathogenic (Lepczyńska L. *et al*., 2016).

Unlike *Cryptosporidium* and *Giardia* mentioned above whose own life cycles have been observed, the life cycle of *Blastocystis* is ambiguous. It is thought that is has both asexual and sexual life stages with the asexual stage being binary fission and sexual reproduction forming a cyst; this form is the infectious form of *Blastocystis* (Basak D, *et al,* 2013).

A recent study conducted in Northern Thailand investigated the prevalence of *Blastocystis* in the population. It was found that 23% were identified as *Blastocystis* positive, these individuals also showed no signs of being infected (Yowang, A., et al, 2018). This shows a high infection rate amongst the Thai population.

The above reasons outline why this organism was chosen to be screened for in this study.

1.3. Approaches to Researching Gut associated Microbial Eukaryotes

Until recently the study of the human gut microbiome has been limited due to its complicated and diverse nature. The study of the bacterial gut microbiome has grown in research interest at an exceptional rate. This has been due to the push into high-throughput sequencing yielding a greater understanding of the interactions the bacterial community has with the gut (Mcfall N, *et al,* 2013) (Figure 4). In contrast knowledge about the eukaryotic microbiome is lacking due to the focus on the bacterial component of the human gut microbiome.

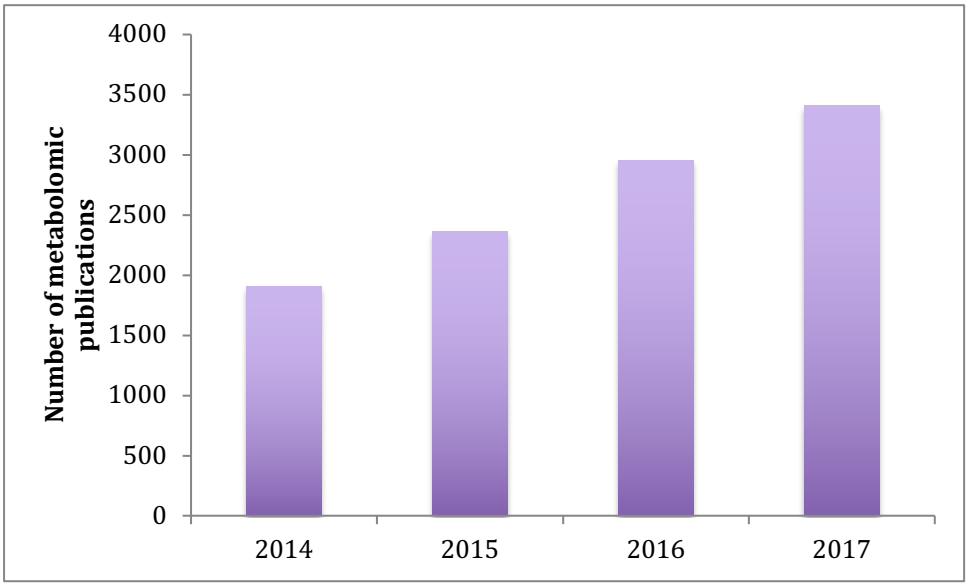

Figure 4. Number of metabolomic publications, using key word search (metabolomics) in Web of Knowledge (Web of Knowledge, 2020)

The primary methods for the study of microbial eukaryotes in the gut have been with microscopy and culturing of eukaryotic organisms (Bogitsh et al, 2005). These methods are obviously limited with the same restrictions being seen in the study of bacterial communities in the gut microbiome. Restrictions include that only those organisms that can be easily grown in a lab setting will be identified. This ultimately reduces the successful identification of the plethora of microorganisms that are present, but are unable to be cultured. This portion consists of the vast majority of the microorganisms that exist inside the human gut microbiome (Ericsson. A, *et al*, 2015). There is also the difficulty of a large amount of the species present in the gut microbiome are anaerobic, suitable for proliferation in the gut but a detriment to culturing (Zoetendal et al, 2004).

However there are some advantages to identifying organisms of the gut microbiome via culturing. One advantage includes the mass growth of organisms that can be studied for their pathological properties; they can also be tested under different conditions (Suchodolski, 2011). The limitations of culturing however far outnumber the advantageous aspects, culturing the microbiome of the gut would not reflect the relationships between the communities within the microbiome. It is also a complicated, time-consuming

process, which is expensive. These are all factors against using culturing as a method to study the gut microbiome.

Using methods that are not drawn upon culturing techniques can circumvent the problems with culturing microorganisms of the gut microbiome. These methods can identify the relationships between different subsets of microorganisms within the gut and difficult to culture or impossible to culture; microorganisms can subsequently be identified and studied. This can create a view of the diversity of the gut microbiome to a fuller extent.

There are predictably limitations to these other possible techniques as well as advantages. Recently the focus has been on molecular based approaches to studying the human gut microbiome, which has been particularly successful with the bacterial microbiome of the gastrointestinal tract (Stensvold, C. R, et al, 2011). Methods such as PCR have been used extensively to observe the bacterial communities and have also been applied to eukaryotic microorganisms (Caron D, A et al, 2009). This however this has been limited when looking at the eukaryotes of the gut microbiome. Limitations include the design of experimental protocol, extraction, bioinformatics and database discrepancies among others. This will be expanded upon later in this introduction.

Currently the focus of microbiome research uses gene sequence analysis, which looks at targeted genes. There is also metagenomics that provides an overview of the complete genomic picture present in a cohort of microorganisms and metatranscriptomics, which expands on how these genes are being expressed, and their functional capacity.

The characteristic approach to studying the human gut microbiome begins with the assembling of faecal samples from a population of interest, this can be done over a period of time to track any changes. DNA from the samples can then be extracted and assayed to identify the organisms present (Franzosa, E, *et al,* 2015). This analysis is done using bioinformatics tools to determine how these communities may interact.

Faecal samples are the easiest way to observe the human gut microbiome, which provides a look at the microorganisms present in the colon (Yasuda, K. et al 2015). Faecal samples are preserved for further analysis, typically by freezing (Song, S.J. et al, 2016). This method prevents any changes in the microbial makeup which would affect any analytical results.

Non-culturable methods are well established for the bacterial microbiome of the gut. The 16s rRNA sequences commonly used as a target gene for the identification of bacterial organisms with the human gut microbiome, the 16s rRNA is also used when identifying archaea (Rong, X etal, 2014). The targeting of this sequence is a quick and convenient marker for sequencing; it is accurate and is commonly employed by researchers.

With eukaryotes there is not an effective target for identification and comparison, as there is with bacteria across all eukaryotic microorganisms.

This prevents standard protocol being formed, which in turn can hinder bioinformatical systems being created. A solution to this could be found in targeting the 18s ribosomal RNA of eukaryotes, from which a database can be created to create an overview of eukaryotes present in the gut microbiome. Currently the most utilized method is to identify microbial eukaryotes present via the targeting of specific genes, which have been identified to be present in certain eukaryotic organisms.

Metabolomics is another tool used for the profiling of the human gut microbiome, using both mass spectrometry (MS) and/or nuclear magnetic resonance (NMR). It can be used to analyse the metabolites present in the human gut, this will be expanded on below.

1.4. Metabolomics

Metabolites are small organic compounds; these compounds are the product of the various stages of the many pathways in metabolism. They can be the starting products of pathways, intermediaries or end products. They are typically 1.5 KDa in size, and examples include sugars, nucleotides and lipids (Breitling C, et al., 2006).

Metabolomics is a study of these metabolites for their identification and quantification. One of the more recent additions to the "-omics" field, which includes genomics (genome identification), transcriptomics (study of gene expression) and proteomics (protein identification) (Figure 5). Metabolomics can be seen as the best characterisation of the phenotype of an organism.

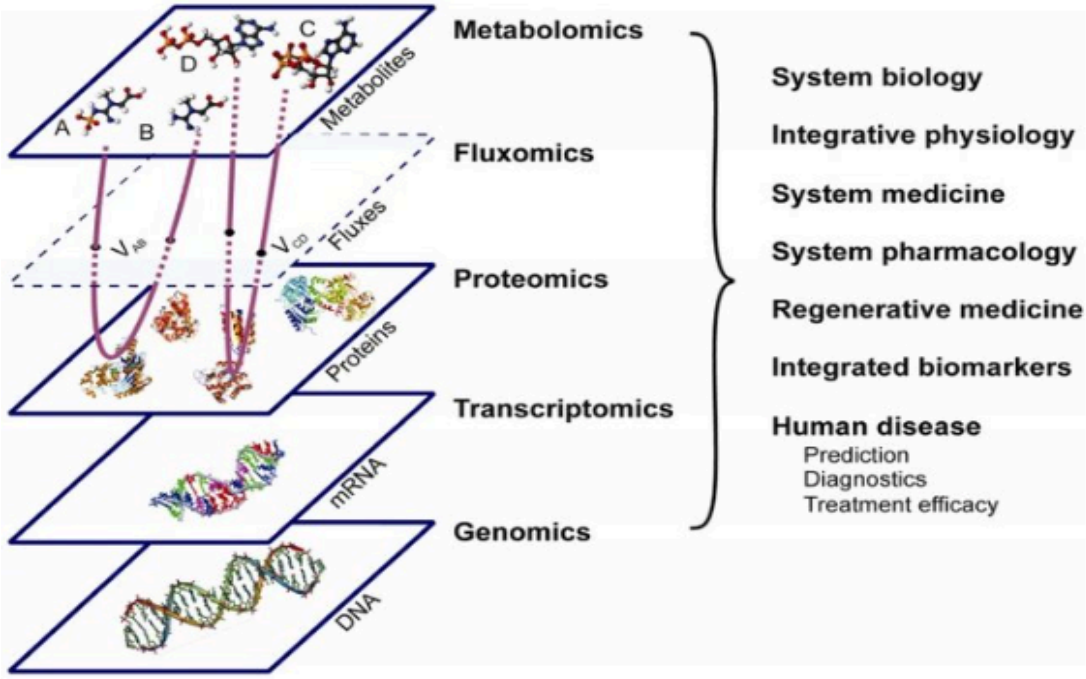

Figure 5 - Representation of the current –omics fields and their applications in health and medicine. Taken from (Nemutlu G, *et al*, 2012).

The metabolome however is the total number and identification of all metabolites that may be distinguished in any given biological sample. There are two different types of metabolites, primary and secondary, each with their own functions. This study will look at both of these types of metabolites.

Primary metabolites are made during the growth phase of cells; these metabolites are important and vital for the growth of these cells. They ultimately become a key component for compounds such as amino acids and other essential compounds. They can also be used for the creation of energy in the cell, another essential part of growth. Examples of primary metabolites include lactic acid and ethanol (Sanchez, S. 2008).

Secondary metabolites on the other hand are not essential for the survival and growth of the organism (Agostini-Costa T. *et al,* 2012). They are however useful in a variety of ways such as an advantage over other organisms (antibiotics) and as a defense mechanism (plant toxins). These are just some examples of the roles that secondary metabolites play (Breitling, R. *et al*, 2013).

Metabolomics is used in a number of ways in different areas of research. This includes bioengineering, drug development and metabolomic pathway analysis. This highlights its importance in research.

Unlike genomics and transriptomics, the compounds that are considered a part of the metabolome are diverse in number; the current number of metabolites identified by the Human Metabolome Database is 114,100 (Wishart, D, *et al,* 2017). This means that there is currently no method of identifying every one of the metabolites known. This is further compounded by the range in concentration that the metabolites can be present in. High sensitivity is needed to identify those at very low concentrations.

1.5 Nuclear Magnetic Resonance versus Mass Spectroscopy

Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) are techniques that are routinely used in the study of metabolites. Like other methods they both have their advantages over one another as well as their own limitations.

These are just two of the ways that metabolites can be identified and quantified, other methods include chromatography and UV-based methods. For this study the focus will be on the above-mentioned methods as they are the most common tools used to study metabolomics (Emwas A-HMS, 2013). Both NMR and MS can be used to qualitatively identify the metabolites in a given sample as well as quantitatively measure the amount of the metabolite present.

NMR is commonly used to look at the whole cohort of metabolites present in a sample, and quantifying the concentration of each of the metabolites. This is in contrast to targeted metabolomic analysis whereby specific metabolites are looked at and their role in a pathway. Mass spectroscopy is a technique used for this targeting of metabolites.

NMR is a tool used for metabolomics that is not damaging to the sample, an important point if there are to be further experiments on the sample. It uses a magnetic field to dislocate electrons from the nuclei of specific atoms in the sample from a low energy level to a high energy level. The radiation that is emitted when the electrons return to their original position is measured. For metabolite investigations the 1H nuclei is preferred. Although less sensitive than MS when using large amounts of sample it is adequate enough to detect metabolites. NMR can be used on a wide range of sample types; it highlights the overarching benefit of using NMR to study metabolites. It can be used on gas, liquid, solid and tissue samples (Bouhrara M, *et al,* 2013).

One of the main disadvantages of NMR includes its relative lack of sensitivity in comparison to MS. There have been various methods implemented to reduce this effect of low sensitivity. This includes dynamic nuclear polarization (DNP) that increases the polarization effectively increasing the sensitivity of NMR (Ardenkjær-Larsen JH, *et al,* 2003).

1.6 Hypothesis and Objectives

33 faecal samples were collected from two different regions in Thailand, Mae Fa Luang and Mae Chan. These locations are districts located in the Northern regions of Thailand (Figure 6).

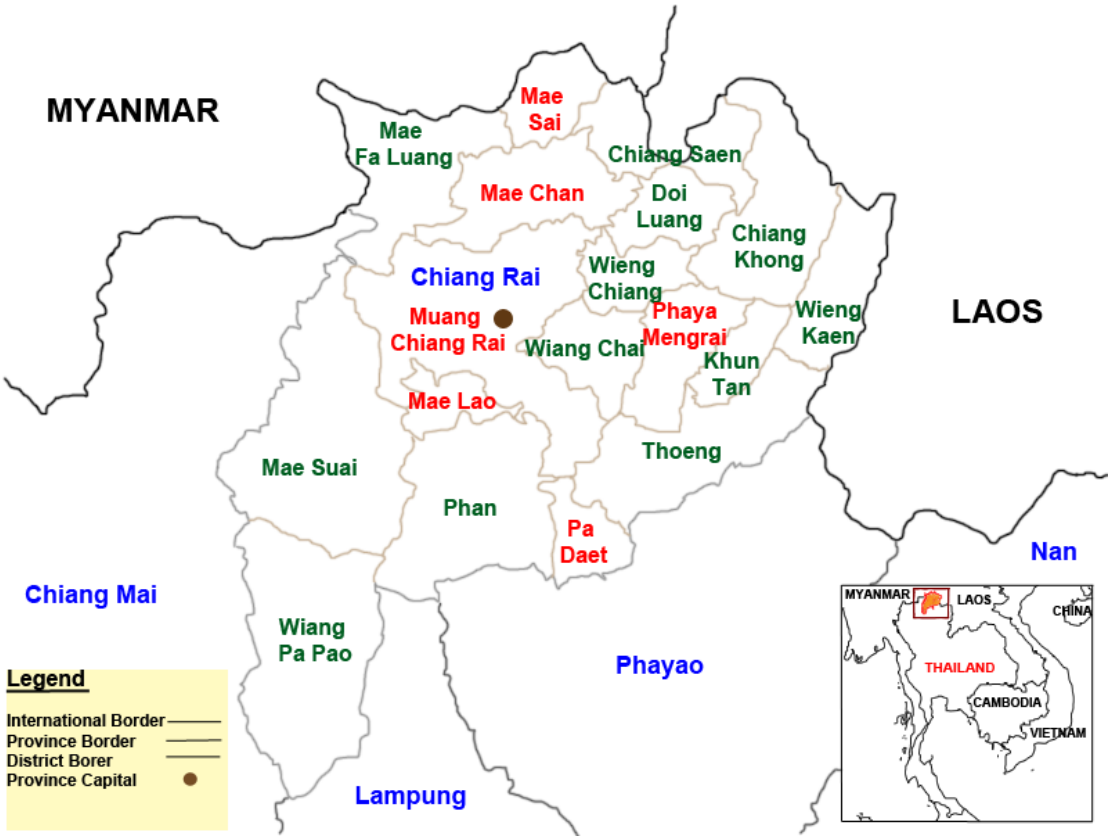

Figure 6- Map of Thailand and Chiang Rai Province districts. Blue-name of province. Green-name of locality. Red- areas that were sampled for this study and joint study. Taken from (Yowang A., et al, 2018).

NMR analysis of the metabolites present in these feacal samples was chosen as the preferred method over MS. This was due to the minimal processing requirements and the ease of identification of metabolites present in favour of the specificity offered by MS.

As discussed earlier there is evidence that the metabolite profile can be altered by the BMI of an individual. This study aimed to identify the metabolites in the gastrointestinal microbiome of Thai individuals, and then determine whether these metabolites are altered depending on the individual BMI.

The different locations of sample selection also allowed a comparison between the different districts and whether this has an effect on the metabolites present.

The samples were also screened using PCR for the identification of the three above-mentioned eukaryotes. This was done to provide an overview of the eukaryotic prevalence of known pathogenic organisms in the Thai population.

2.0 Materials and Methods

The method used in this study was a combination of NMR and PCR screening; the samples in this study were collected from the regions described in the introduction and underwent several processes outlined in these materials and methods (Figure 7).

Figure 7- Schematic showing the outline of the methods used in this study.

2.1 Exploring the presence of *Giardia, Cryptosporidium* and *Blastocystis* in Faecal Samples

Stock Solution	Reagents			
50X Tris-Acetate Ethylenediaminetetraacetic acid (Tris-Acetate-EDTA)	Tris Base (242 g) (Fisher)	Glacial acetic acid (57.1 mL) (Fisher)	0.4 M EDTA (pH 8.0) (100 mL) (Fisher)	Distilled water (made to 1 L)
1x Tris-Acetate-EDTA	50 X TAE (40 mL)	Distilled water (made to 2 L)	-	-
0.1 M Isopropyl β-d-1-thiogalactopyranoside	IPTG (0.238 g) (Melford)	Distilled Water (10 mL)	-	-
X-Gal 20mg/mL	X-Gal (200 mg) (Melford)	DMSO (10 mL)	-	-
Ampicillin 100 mg/mL	Ampicillin (1 g) (Melford)	Distilled water (Made to 10 mL)	-	-

Table 2 - Stock solutions and the reagents used to make them

2.1.1 Media

Lysogeny Broth

- Tryptone (10 g) (Oxoid)
- Sodium Chloride (10 g) (Fisher)
- Yeast Extract (5 g) (Oxoid)
- Distilled water (up to 1 L)

The above were mixed and sterilised via an autoclave at 121°C and then stored at 4°C.

Lysogeny Ampicillin Broth

Using the same procedure as described above with ampicillin at 100 mg/mL added to a concentration of 100 µg/mL.

2.1.2 X-Gal and IPTG Agar Plates

15 g of Agar (BD) was mixed in to 1 L of LB broth, autoclaved and cooled to a touchable temperature. 1 mL of X-Gal (20 mg/mL) (Melford) was aseptically

added for a concentration of 1 mL/L and 1 mL of IPTG (Melford) for a final concentration of 1 mM. Ampicillin (100 mg/mL) (Melford) was added to a concentration of 100 µg/mL.

The plates were then poured and cooled until they had set. The plates were then covered to protect them from the light and stored at 4 °C.

2.2 Sample Collection

2.2.1 Ethics Statement

Mae Fah Luang University's human ethics committee approved the sampling of adult human subjects from Thai individuals. (License approval number REH-60104). The individuals participating in the study gave consent.

2.2.2 Sample Collection from Individuals

All of the individuals taking part were over 18 and resided in the Chiang Rai Province when samples were collected. 23 individual volunteers took part in the study; there was no known incidence of gastro-intestinal symptoms in the individuals medical history and a lack of diarrheal issues within a month. All volunteers had also not received any antibiotic treatment in the last two months. The samples were collected from Mae Fa Luang (n=13) and Mae Chan (n=20) districts in the Chiang Rai Province (Figure 6). Faecal samples were taken from the individuals and stored at – 80 °C in sealed, sterilised containers. The DNA was extracted from the samples via the QIAamp DNA Stool Mini Kit (Qiagen, Thailand) following the protocol included. The DNA was then stored at -20 °C.

This protocol was adapted from (Yowlang A., 2018).

2.3 PCR Screening

2.3.1 PCR Mastermix

For all PCR reactions that were performed, a positive (using DNA extracted from axenic *Giardia intestinalis* culture) and negative control was used.

A nested PCR refers to the use of two sets of primers with successive PCRs being done to improve sensitivity and specificity. The result of the first PCR is used as a template for the second PCR.

2.3.2 *Cryptosporidium spp.*

A nested PCR was used to screen the faecal samples for *Cryprosporidium* using primers that targeted the *Cryptosporidium* oocyst wall protein (*COWP*) gene.

For the final mastermix for both the initial and nested PCRs 1 µL of DNA from each diluted sample was added to 25 µL of premixed mastermix (PCR Biosystems) with 2 µL each of the primers (Table 3).

2.3.3 *Giardia spp.*

A semi nested PCR was used to screen for the presence of *Giardia* in the samples. The primers were used to target the glutamate dehydrogenase (*gdh*) gene of *Giardia duodenalis.* The same mastermix used above for *Cryptosporidium* was used for this protocol with the corresponding primers being used.

2.3.4 *Blastocystis spp.*

For the screening of Blastocystis a nested PCR was used using primers that targeted the barcoding region of *Blastocystis spp .*

2µL of each of the extracted DNA from 1/10 diluted samples were added to 25 µL of premixed mastermix (PCR Biosystems) with 2 µL of both the reverse and forward primers.

A second nested PCR reaction used 2 µL of the initial PCR reaction with 25 µL of premixed mastermix (PCR Biosystems) with 2 µL of the nested primers.

2.3.5 Primers

Table 3 - Initial and nested primers used for primers with their sequences included. Primers are from Eurofins Genomics. The primers were based on [1] Coklin et al, 2011. [2] Feng, Y., 2011 [3] Betts, E., 2018.

	Initial		**Nested**	
	Forward	**Reverse**	**Forward**	**Reverse**
Cryptospridium spp.[1]	***COWP****:* GGAAGAGAT TGTGTTGC	***COWP****:*GCAGGAGC TACATATAG	***SSU****:* GTGTT CAATC AGACA CAGC	***SSU****:* CTGTATATCCT GGTGGGCAGA C
Giardia spp[2].	***GDHeF****:* TCAACGTYAA YCGYGGYTT CCGT	***GDHiF****:* CAGTACAACTCYG CTCTCGG	***GDHeF****:* TCAAC GTYAAY CGYGG YTTCC GT	***GDHiR***: GTTRTCTTGCA CATCTCC
Blastocystis spp. [3]	***RD3****:* GGGATCCTG ATCCTTCCGC AGGTTCACCT AC	***RD5****:* GGAAGCTTATCTG GTTGATCCTGCCA GTA	***RD5F****:* ATCTG GTTGA TCCTG CCAGT	**BhRDr**: GCTTTTTAACT GCAACAACG

2.3.6 Protocols

Table 4 - Protocols for the PCR reactions of Cryptosporidium spp., Giardia spp., and Blastocystis spp.. Protocols adapted from [1] Coklin et al, 2011. [2] Feng, Y., 2011 [3] Betts, E., 2018.

	Initial		**Nested**
	Step	**Time/ Temperature**	**Time/ Temperature**
Cryptospridium spp. [1]	Denaturing	2 minutes/ 94 °C	2 minutes/ 94 °C
	Annaeling	50 seconds/ 45 °C/ x45	50 seconds/ 45 °C/ x45
	Extending	50 seconds/ 55 °C	50 seconds/ 55 °C
	Final	1 minute/ 72 °C	1 minute/ 72 °C
Giardia spp. [2]	Denaturing	2 minutes 20 seconds/ 96°C	2 minutes 20 seconds/ 96°C
	Annaeling	10 seconds/ 45 °C/ x40	10 seconds/ 45 °C/ x40
	Extending	5 seconds/ 53 °C	5 seconds/ 53 °C
	Final	4 minutes/ 60 °C	4 minutes/ 60 °C
Blastocystis spp. [3]	Denaturing	5 minutes/ 95 °C	5 minutes/ 95 °C
	Annaeling	30 seconds/ 45 °C/ x30	30 seconds/ 45 °C/ x35
	Extending	1 minute 40 seconds/ 72 °C	1 minute 40 seconds/ 72 °C
	Final	5 minutes/ 72 °C	5 minutes/ 72 °C

2.4 Agarose Gel Electrophoresis

1 g of agarose (Melford) was dissolved in 100 mL using a microwave to make a 1 % agarose solution. 2 µL of ethidium bromide was mixed into this solution, which then set into a gel dock to aid in the visualization of the gel.

Each sample was loaded into the wells with 1 µL of 6X loading buffer (Qiagen). 5 µL of the relevant ladder was also used either 100 bp or 1 kbp (Promega).

The bands were extracted under UV light and purified following the GeneJet Gel Extraction Kit (Thermo Scientific) protocol.

2.5 Ligation of Positive PCR Samples

The products of the PCR were ligated into a pGEM T easy Vector (Promega) following the protocol set out by the company. A negative and a background control were also used.

2.6 Transformation of ligated plasmid into competent cells and selection of successful colonies

2.6.1 Preparation of Competent Cells

Using a current cell line of *E. coli* dh5 alpha cells 10 mL of LB medium was inoculated and grown overnight at 37 °C. Of this 1:100 was placed in fresh 100 mL LB media until the O.D reached 0.4-0.5 at an absorbance of 600 nm.
This was then incubated at 4 °C for 30 minutes followed by centrifugation at 5000 rpm for 5 minutes at 4 °C. The supernatant was then discarded with 25 mL of ice cold 0.1 M $MgCl_2$ being added then incubated on ice for 15 minutes. The cells were then centrifuged again (5000 rpm, 5 minutes) and the supernatant discarded. $CaCl_2$ (25 mL, 0.1 M) was then used to resuspend the cells and incubated for 15 minutes. Followed by the exact same centrifugation and discarding of the supernatant steps described above. Finally the cells were resuspended in ice cold 0.1 M $CaCl_2$ 20% Glycerol (1 mL) and separated into 100 µL aliquots where they were then stored at -70 °C until use. A 50 µL aliquot of DH5 alpha competent cells was defrosted on ice per a PCR positive sample. 3 µL of the ligation mixture was mixed with the competent cells and left on ice for 30 minutes. This was then placed into a 42 °C water bath for 45 seconds and then placed back onto ice for 1 minute. 250 µL of LB was added to the aliquots and incubated at 37 °C for 1 hour. 50 µL of this was then plated onto an ampicillin/ X-gal/ IPTG agar plate in sterile conditions and incubated at 37 °C overnight.

2.6.2 Selection of colonies for overnight growth

White colonies were selected from the agar plates as described above in sterile conditions and grown in 5 mL LB with 200 µL ampicillin (200 mg/mL). This was then left overnight at 37 °C.

2.6.3 Miniprep of overnight cultures

The miniprep was carried out by following the protocol for the GeneJet Plasmid Miniprep Kit (Thermo Scientific).

The samples were then digested using EcoR1 restriction enzyme and positive samples were sent for sequencing at Eurofins Genomics.

2.7 Sequencing of Positive PCR Samples

The sequencing results that were obtained from Eurofins Genomics were trimmed using Snap Gene software. The tripped sequences were then put into the NCBI Blast tool where they were compared with existing sequenced DNA.

2.8 NMR Sample Preparation

The faecal samples obtained from the human individuals were washed with 6 mL of 75 % ethanol at 80 °C with the wash through being collected. 2 mL of 2 mm glass beads were added to the samples and vortexed for 30 seconds before incubating for three minutes at 80 °C. The samples were then vortexed for a further 30 seconds until completely liquefied. The media was then drained and 6 mL of 75 % ethanol was added and then decanted.

The samples were subsequently placed in the centrifuge at 16,000 xg for 10 minutes and the supernatant was collected.

The samples were then dried via a rotor vac at 40 °C and suspended in 330 µL of double distilled water and centrifuged at 2,500xg for ten minutes, The supernatant was then collected and frozen at -20 °C until the day before use.
24 hours before use random samples were selected and freeze dried until desiccated. The samples were then resuspended in 650 µL of D_2O (Melford) with 60 mM of D6 DSS (Sigma-Aldrich) was added to each sample as both a concentration and chemical shift reference.

2.9 NMR Experimental Design and Analysis

Spectra were measured at 298K on a Bruker Avance III spectrometer at 600MHz (Topspin 3.5pl7) equipped with a QCI cryogenically cooled probe. All spectra were measured using a Noesy presaturation sequence using a mixing time of 100 ms with presauration of the on resonance water signal throughout the 3s relaxation delay used. Spectra were measured over a sweep width of 9590Hz using 16k data points, signals were averaged over 1024 scans to improve signal to noise.

Data were processed and analysed using the chenomx v 8.2 using standard processing parameters Spectra were automatically processed in batches with chenomx which was used for line broaden the spectra, transforming them, applying automatic and manual phasing, deleting the residual water peak and correcting baseline errors and shims. Spectra were then globally fitted to chenomx's internal library of compounds to analyse metabolites present and their concentrations.

Statistical analysis of the identified metabolites was done using Microsoft Excel, and pathway predictions were produced via the Metaboanlalyst 3.0 online tool using the *Homo sapiens* database.

3.0 Results

3.1 Metabolite Identification of Faecal Samples from Thailand

Thirteen individuals were chosen from the Mae Fa Luang district in the Chiang Rai Province for gut metabolite screening. From these twelve individuals eight were identified as being of normal weight according to the WHO standard and four individuals were observed as overweight with one individual being obese.

In the normal weight samples a total of 135 metabolites were identified via NMR in at least one of the samples (Table 5). In those who were overweight 43 metabolites were identified in at least one sample (Table 5). In the obese samples 24 metabolites were identified.

The metabolites that were seen in all of the samples screened both normal weight and overweight were 18 in total. The average concentration of these metabolites was calculated and their P value in comparison to each other to test for a significant difference between normal weight and overweight.

The metabolites were identified via NMR; the spectrums of example samples from each BMI category can be seen in figure 8.

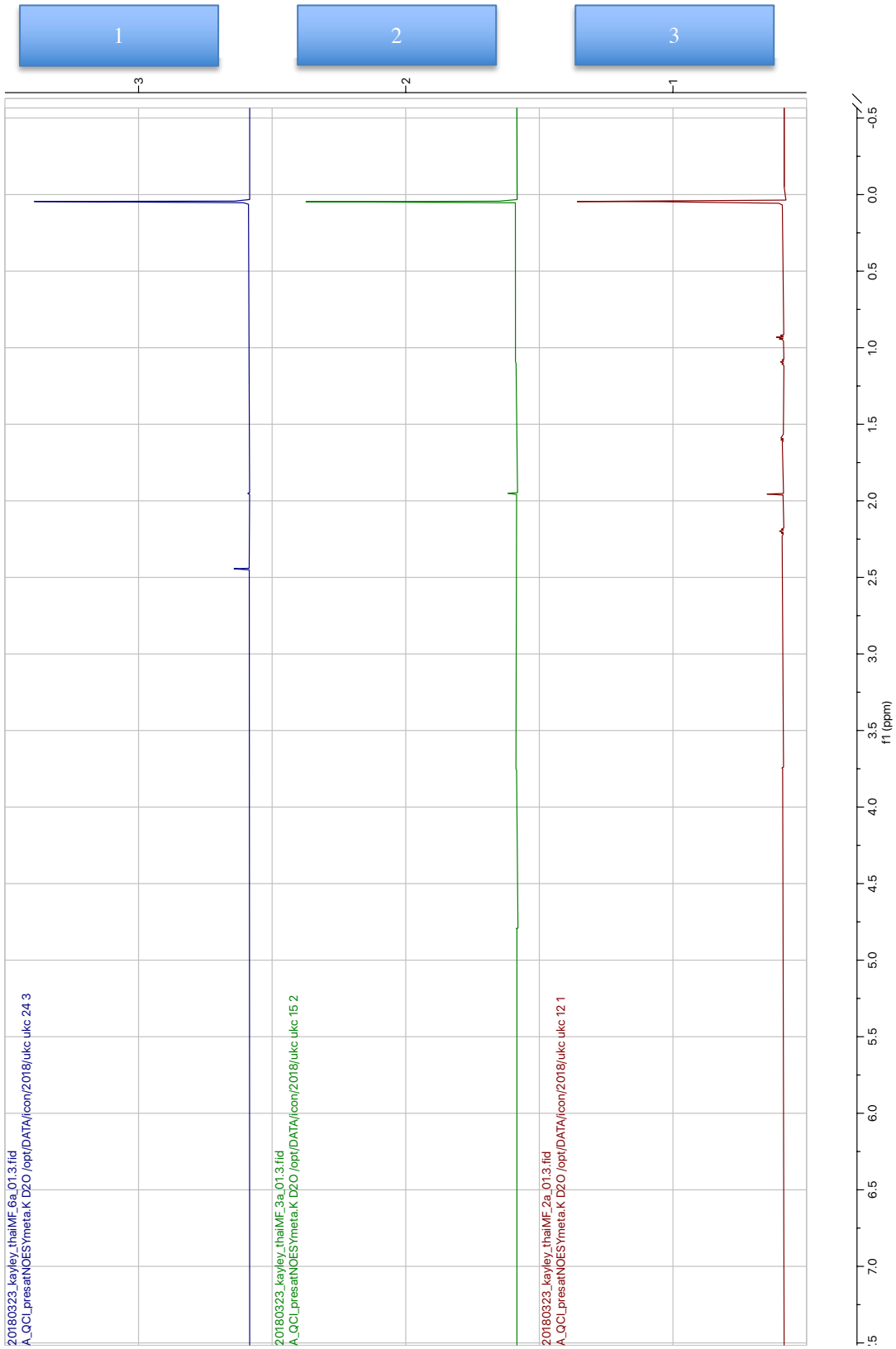

Figure 8 - Comparative diagram of normal weight (1), overweight (2) and obese (3) NMR spectra.

Table 5- Table comparing the metabolites identified in at least one of the samples in both normal weight and overweight sampled. Red- not present in any samples. Green- present in at least one sample.

Compound Name	Normal Weight	Overweight
1,3-Dimethylurate		
1,6-Anhydro-β-D-glucose		
1,7-Dimethylxanthine		
2-Aminobutyrate		
2-Hydroxyisobutyrate		
2-Hydroxyisocaproate		
2-Hydroxyphenylacetate		
2-Hydroxyvalerate		
2-Oxocaproate		
2'-Deoxyuridine		
3-Hydroxy-3-methylglutarate		
3-Hydroxyisobutyrate		
3-Hydroxyisovalerate		
3-Hydroxymandelate		
3-Hydroxyphenylacetate		
3-Methylxanthine		
3-Phenyllactate		
3-Phenylpropionate		
3,4-Dihydroxybenzeneacetate		
3,5-Dibromotyrosine		
4-Aminobutyrate		
4-Guanidinobutanoate		
4-Hydroxy-3-methoxymandelate		
4-Hydroxyphenylacetate		
4-Pyridoxate		
5-Aminopentanoate		
5-Hydroxyindole-3-acetate		

Compound Name	Normal Weight	Overweight
5,6-Dihydrothymine		
Acetamide		
Acetaminophen		
Acetate		
Acetoacetate		
Acetoin		
Acetone		
Adenine		
Alanine		
Allantoin		
Anserine		
Arabinose		
Aspartate		
Azelate		
Benzoate		
Butanone		
Butyrate		
Cadaverine		
Caprate		
Carnosine		
Cellobiose		
Chlorogenate		
Cholate		
Choline		
cis-Aconitate		
Citraconate		
Citrulline		
Creatine		
Creatine phosphate		
Creatinine		
Cytidine		
Desaminotyrosine		
Dimethyl sulfone		

Compound Name	Normal Weight	Overweight
Dimethylamine		
DSS (Chemical Shape Indicator)		
DSS-d6 (Chemical Shape Indicator)		
Ethanol		
Ethylene glycol		
Formate		
Fumarate		
Galactose		
Glucitol		
Glucose		
Glucose-6-phosphate		
Glutamate		
Glutarate		
Glycerol		
Glycine		
Glycolate		
Guanosine		
Histamine		
Histidine		
Homocystine		
Homovanillate		
Hydroxyacetone		
Hypoxanthine		
Imidazole		
Indole-3-acetate		
Isobutyrate		
Isocitrate		
Isoleucine		
Isovalerate		
Kynurenate		
Kynurenine		
Lactate		
Lactulose		

Compound Name	Normal Weight	Overweight
Levulinate		
Maleate		
Malonate		
Methanol		
Methionine		
Methylamine		
N-Acetylaspartate		
N-Acetylglycine		
N-Acetyltyrosine		
N-Methylhydantoin		
N-Phenylacetylglycine		
N,N-Dimethylformamide		
N,N-Dimethylglycine		
N6-Acetyllysine		
Nicotinate		
o-Cresol		
O-Phosphocholine		
Oxypurinol		
p-Cresol		
Pantothenate		
Phenylacetate		
Phenylalanine		
Pimelate		
Propionate		
Protocatechuate		
Pyruvate		
Riboflavin		
Sarcosine		
Suberate		
Succinate		
Succinylacetone		
Tartrate		
Taurine		

Compound Name	Normal Weight	Overweight
Theophylline		
Threonine		
Trimethylamine		
Trimethylamine N-oxide		
Tryptophan		
Tyramine		
Tyrosine		
UDP-N-Acetylglucosamine		
Uracil		
Uridine		
Valerate		
Valine		
Valproate		
Vanillate		
Xanthine		
Xanthurenate		
Xylitol		
β-Alanine		
π-Methylhistidine		
τ-Methylhistidine		

3.1.1 Mae Fa Luang Faecal Metabolite Screening

The figure below (figure 9) shows a comparison of the metabolites that were present in both normal weight and overweight samples. As seen in figure 9 there is no significant difference between normal weight and overweight samples and their respective concentrations of the metabolites present.

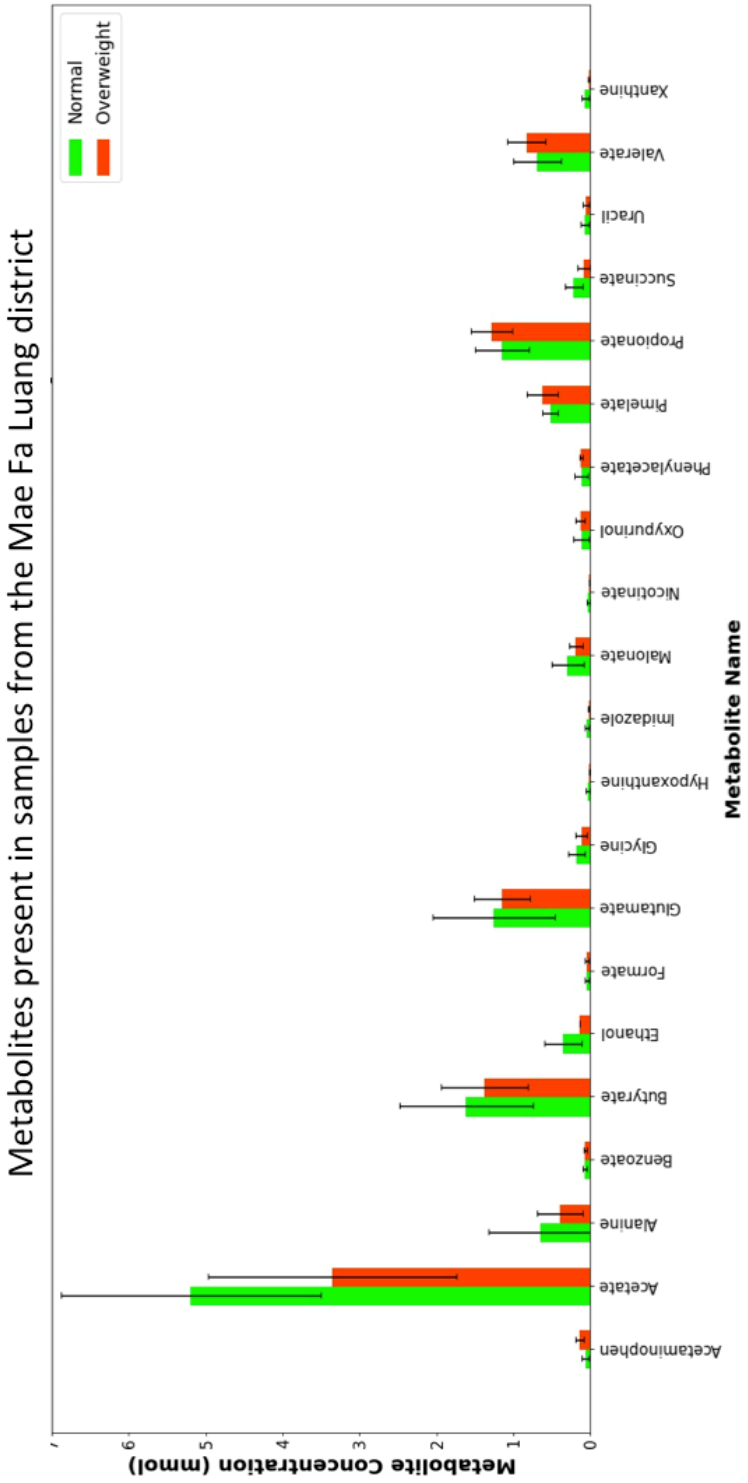

Figure 9- Metabolites present in samples from the Mae Fae Luang District. The metabolites that were identified via NMR are shown with their respective concentrations. Green- Normal weight. Red-Overweight. Error bars can also be seen.

All metabolites identified were statistically analysed with a P value of greater than 0.05 for each comparison between normal weight and overweight. This demonstrated that there was no statistical difference between the concentrations of normal weight metabolites and overweight metabolites.

Following this there were some metabolites actetate (figure 10), glycine (figure 11), hypoxanthine (figure 12) and xanthine (figure 13), which had P values close to being of statistically significantly different.

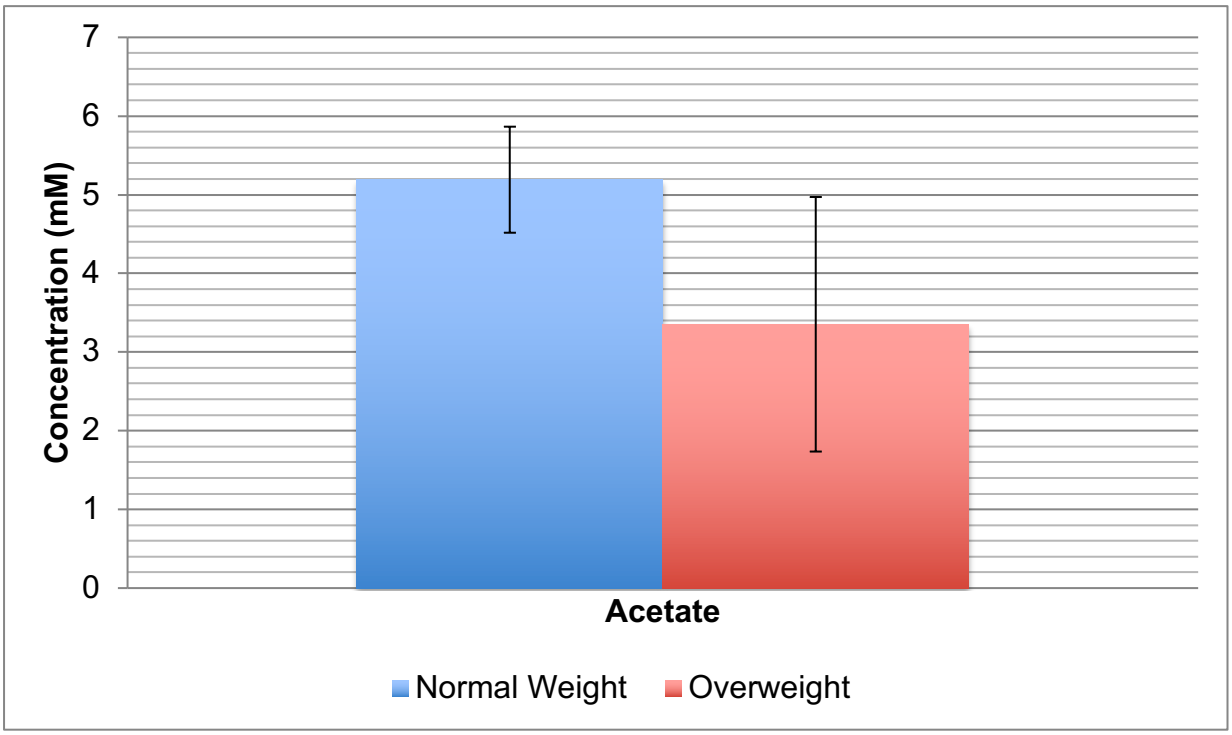

Figure 10 - Bar chart displaying the average concentration of Acetate in normal weight and overweight individuals. Error bars have been included. P value of 0.12, showing no statistical difference.

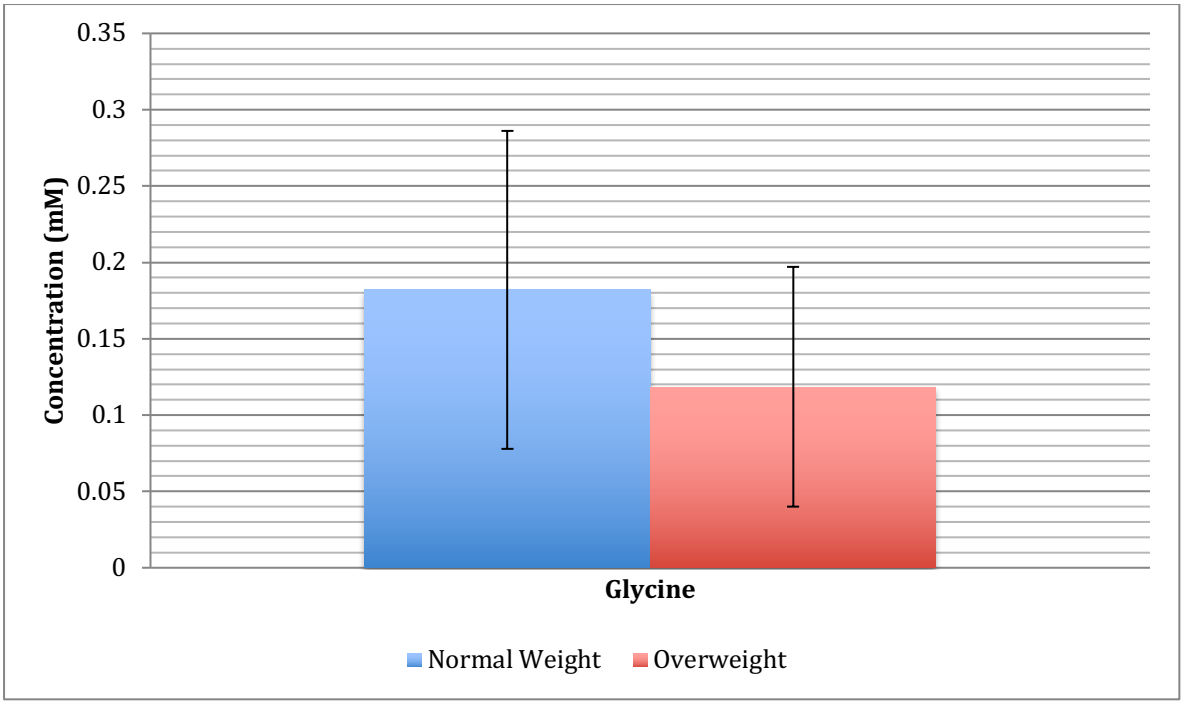

Figure 11 - Bar chart displaying the average concentration of Glycine in normal weight and overweight individuals. Error bars have been included. P value of 0.12, showing no statistical difference.

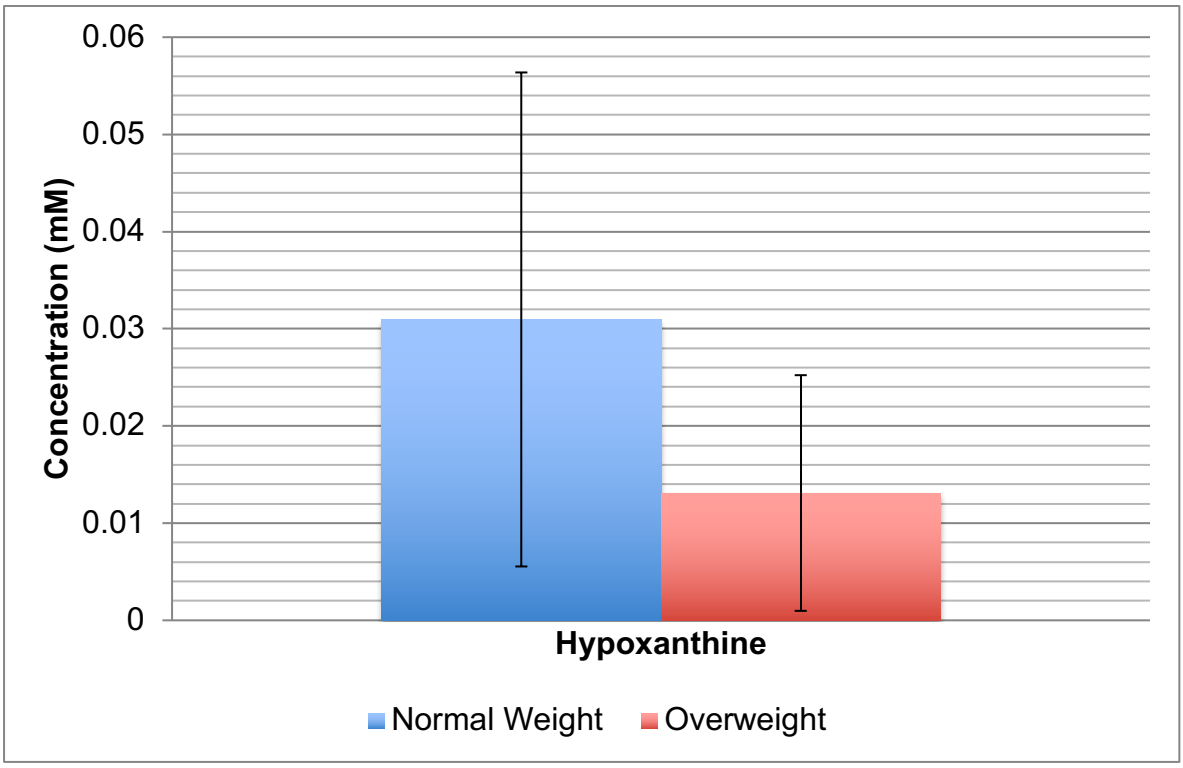

Figure 12 - Bar chart displaying the average concentration of Hypoxanthine in normal weight and overweight individuals. Error bars have been included. P value of 0.12, showing no statistical difference.

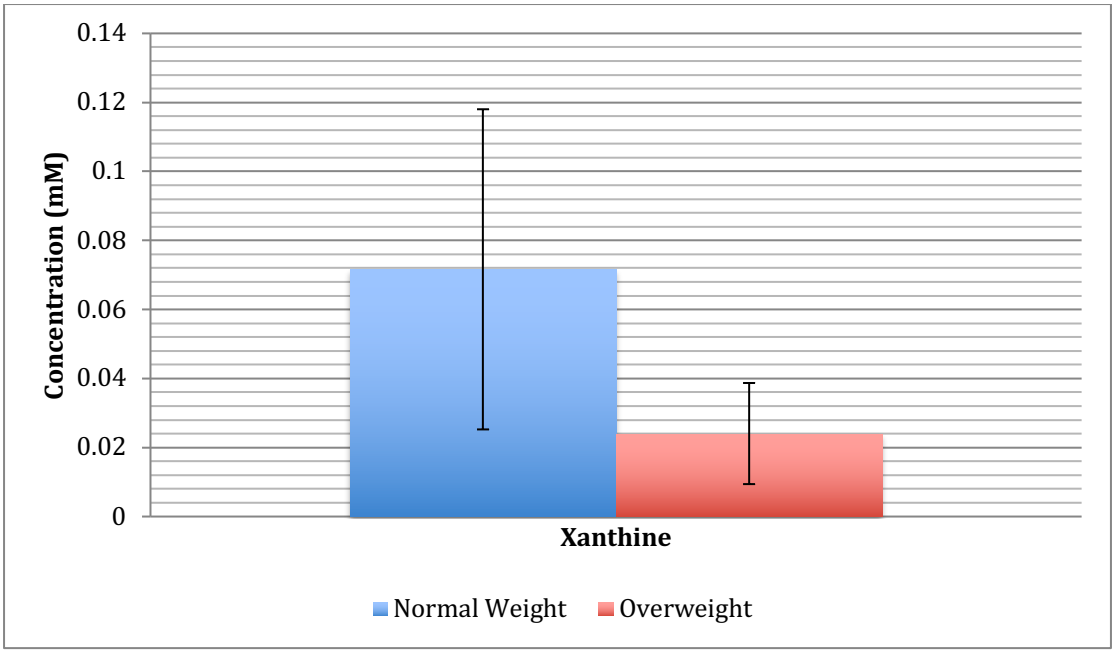

Figure 13 - Bar chart displaying the average concentration of Xanthine in normal weight and overweight individuals. Error bars have been included. P value of 0.06, showing no statistical difference.

3.1.2 Mae Chan Faecal Metabolite Screening

Seventeen individuals were chosen from the Mae Chan district in the Chiang Rai Province, with seven being of normal weight as described by the parameters above and ten being overweight.

58 metabolites were collectively identified in at least one of the samples that were from individuals of normal weight. 70 metabolites were positively detected in those who were overweight. Of these metabolites 26 were seen in the both normal weight and overweight individuals. They were then compared and statistical analysis was carried out.

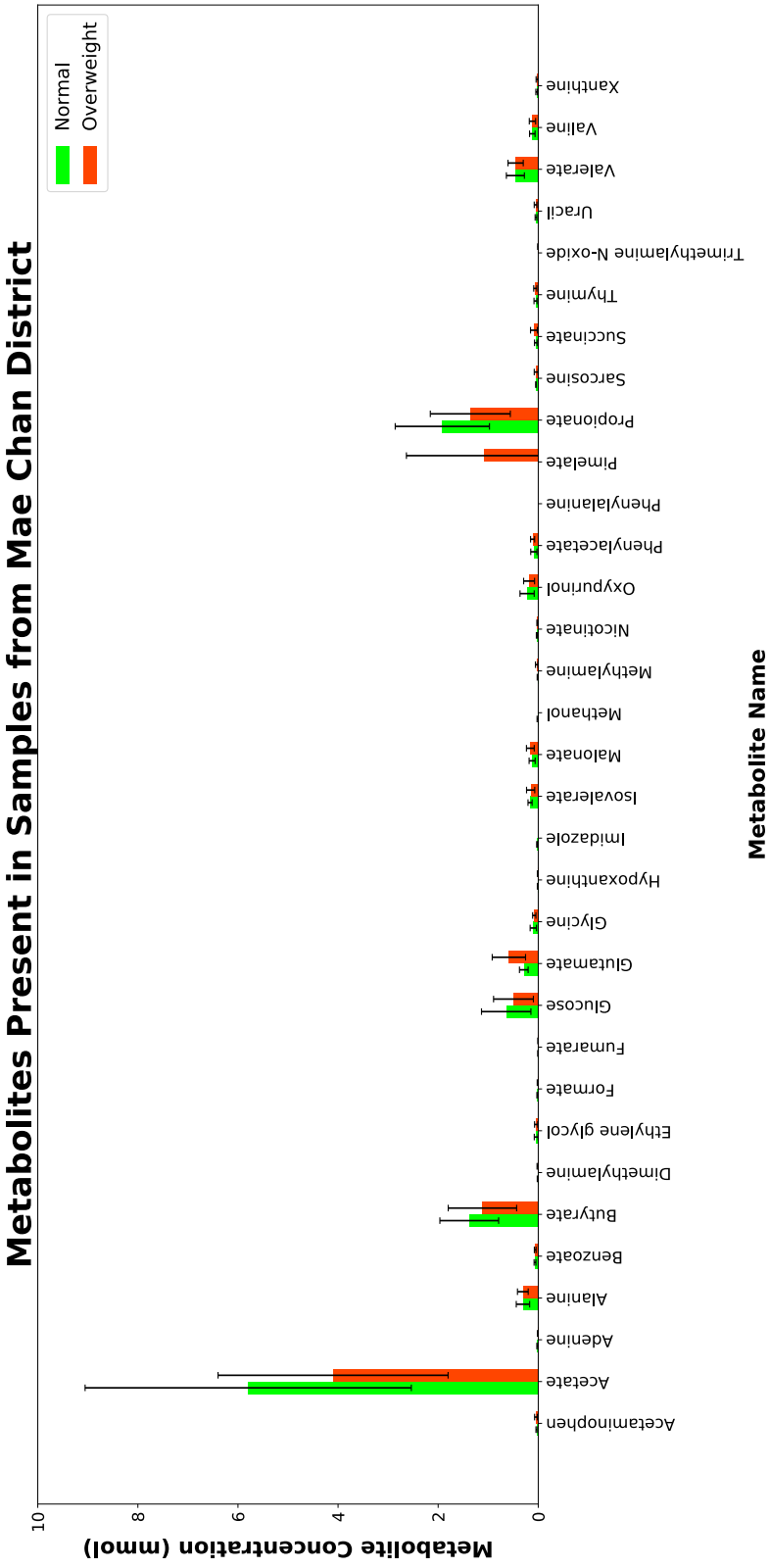

Figure 13 Metabolites present in samples from the Mae Chan District. The metabolites that were identified via NMR are shown with their respective concentrations. Green- Normal weight. Red-Overweight. Error bars can also be seen.

None of the metabolites identified in the individuals sampled from the Mae Chan district has statistically significant P values less than 0.05. Only one metabolite glutamate (figure 14) shows a P value (0.08) close to being significant.

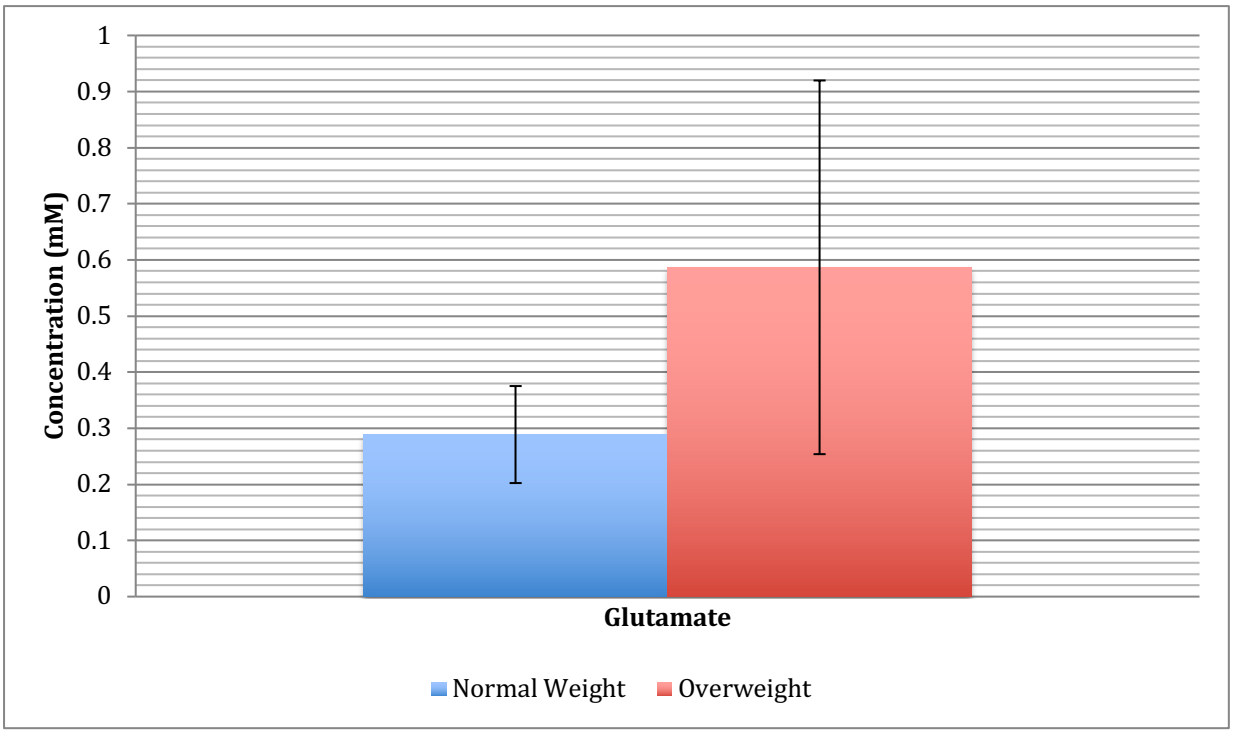

Figure 15 - Bar chart displaying the average concentration of Glutamate in normal weight and overweight individuals. Error bars have been included. P value of 0.08, showing no statistical difference

3.1.3 Pathway analysis of metabolites identified in Mae Fa Luang and Mae Fae samples

Metaboanalyst was used to identify the key pathways that the metabolites are involved in and their impact. The metabolites are involved in thirteen key pathways (Table 6).

A pathway analysis overview was also created to determine the impact of each pathway in comparison to its P value (Figure 16).

Table 6 - The hits (metabolites) identified in the individual pathways. Table showing various different metabolomic pathways and the total number of metabolites involved in these pathways. The hits are the number of metabolites that were identified in this investigation that are involved in these metabolomic pathways.

	Total	Hits
Methane metabolism	34	6
Alanine, aspartate and glutamate metabolism	24	4
Phenylalanine metabolism	45	5
Nitrogen metabolism	39	4
Butanoate metabolism	40	4
Aminoacyl-tRNA biosynthesis	75	5
Propanoate metabolism	35	3
Nicotinate and nicotinamide metabolism	44	3
Citrate cycle (TCA cycle)	20	2
Taurine and hypotaurine metabolism	20	2
Purine metabolism	92	4
Selenoamino acid metabolism	22	2
Pyrimidine metabolism	60	3

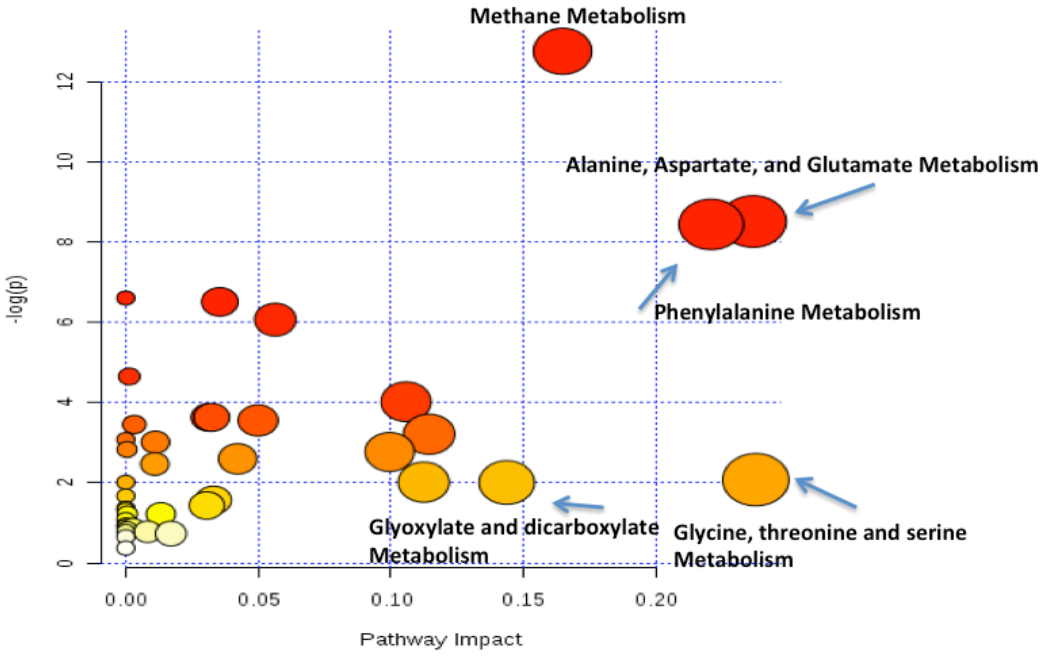

Figure 16- Overview of Pathway Analysis. Matched pathways according to the P values and pathway impact values. Yellow coloured circles have higher P values. Red coloured circles have lower P values. The size of the circle shows the impact score, the larger the circle the greater the impact of the metabolite hits.

The above figure 16 identifies the relevant metabolome pathways in this study in comparison with the metabolomic profile of a human.

3.2 Screening of Thai samples for gastrointestinal eukaryotes

Tables 7 and 8 below show an outline of the results of screening the DNA extracted from the faecal samples screened via PCR.

Table 7- Ma Fa Luang samples and their respective BMI's with regards to their results with PCR screening. Green- Normal weight. Orange- Overweight. Red-obese.

Sample Number	Origin	BMI	*Cryptosporidium*	*Blastocystis*	*Giardia*
1	Mae Fa Luang	Normal	Positive	Negative	Negative
2	Mae Fa Luang	Normal	Positive	Negative	Negative
3	Mae Fa Luang	Overweight	Positive	Negative	Negative
4	Mae Fa Luang	Normal	Positive	Negative	Negative
5	Mae Fa Luang	Overweight	Positive	Negative	Negative
6	Mae Fa Luang	Obese	Positive	Negative	Negative
7	Mae Fa Luang	Overweight	Positive	Negative	Negative
8	Mae Fa Luang	Normal	Positive	Negative	Negative
9	Mae Fa Luang	Overweight	Positive	Negative	Negative
10	Mae Fa Luang	Normal	Positive	Negative	Negative
11	Mae Fa Luang	Normal	Positive	Negative	Negative
12	Mae Fa Luang	Normal	Positive	Negative	Negative
13	Mae Fa Luang	Normal	Positive	Negative	Negative

Table 8- Ma Fae samples and their respective BMI's with regards to their results with PCR screening . Green- Normal weight. **Orange-** Overweight**. Red-**obese.

Sample Number	Origin	BMI	*Cryptosporidium*	*Blastocystis*	*Giardia*
1	Mae Fae	Normal	Negative	Negative	Negative
2	Mae Fae	Overweight	Negative	Negative	Negative
3	Mae Fae	Normal	Negative	Negative	Negative
4	Mae Fae	Overweight	Positive	Negative	Negative
5	Mae Fae	Obese	Positive	Negative	Negative
6	Mae Fae	Normal	Negative	Negative	Negative
7	Mae Fae	Overweight	Negative	Negative	Negative
8	Mae Fae	Normal	Positive	Negative	Negative
9	Mae Fae	Normal	Positive	Negative	Negative
10	Mae Fae	Obese	Positive	Negative	Negative
11	Mae Fae	Overweight	Positive	Negative	Negative
12	Mae Fae	Overweight	Positive	Negative	Negative
13	Mae Fae	Normal	Positive	Negative	Negative
14	Mae Fae	Overweight	Positive	Negative	Negative
15	Mae Fae	Overweight	Positive	Negative	Negative
16	Mae Fae	Overweight	Positive	Negative	Negative
17	Mae Fae	Normal	Positive	Negative	Negative
18	Mae Fae	Normal	Positive	Negative	Negative
19	Mae Fae	Overweight	Positive	Negative	Negative
20	Mae Fae	Overweight	Positive	Negative	Negative

The positive results shown in the tables above are these samples having a band present in the agorase gel. They were only determined as positive when the band was present at the correct size for the respective primers.

The results of these tables are expanded on in the following sections.

3.2.1 Screening of Mae Fa Luang and Mae Chan samples for *Cryptosporidium spp.*

Thirteen samples from Mae Fa Luang were screened for *Cryptosporidium* using COWP primers in a nested PCR. They were a mix of normal weight, overweight and obese individuals (Table 9).

Table 9 - Each Mae Fa Luang sample and their respective BMI's

BMI	Sample Number
Normal Weight	1,2,4,8,10,11,12,
Overweight	3,5,7,9
Obese	6

Of the 12 samples all were PCR positive with a band being seen at the expected 312 bp (Figure 17). The band below this is excess primers. These bands were extracted and sent for sequencing. The results of this sequencing showed no *Cryptosporidium* spp. being present in the faecal samples. Various flora (*Cymbogon citrtus* and *Alpinia galangal*) from the Thai region were instead identified this could be due to the diet of the individuals.

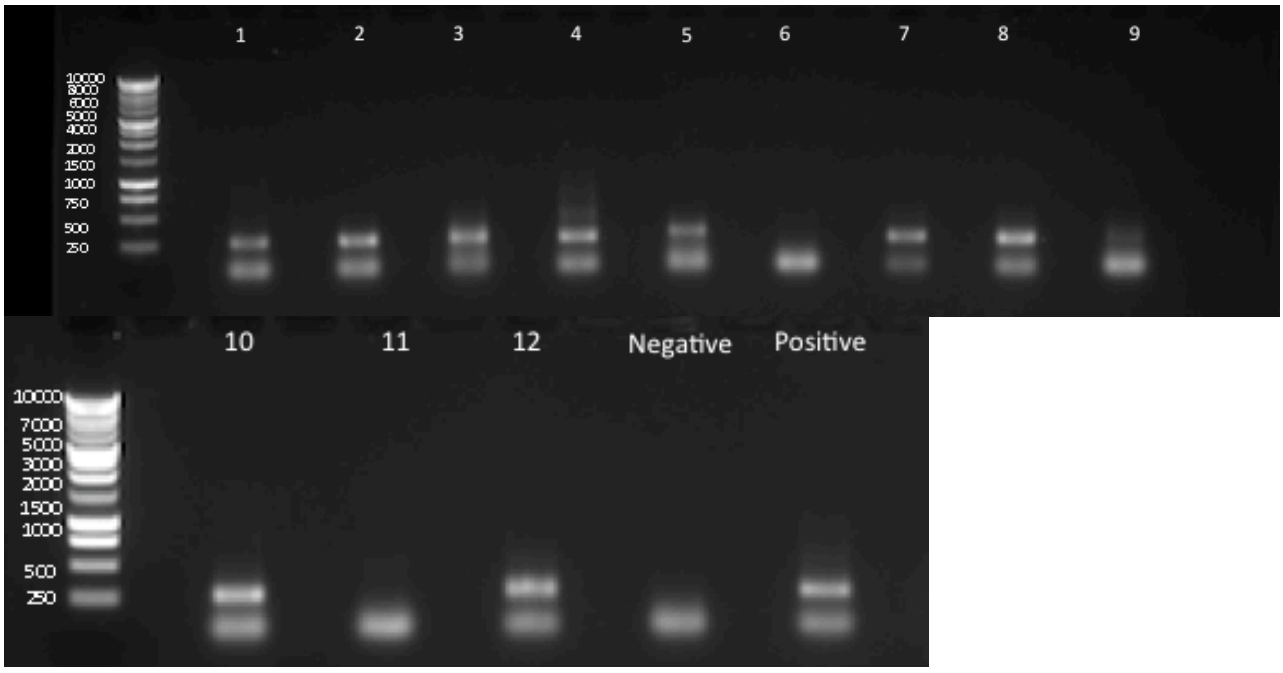

Figure 17- Agarose gel of the 12 samples from Mae Fa Luang, a band can be seen at 312 bp in relation to the ladder (10 kB).

The Mae Fae samples also underwent PCR screening via the above parameters however unlike Mae Fa Luang none of the samples were PCR positive therefore no DNA extracts were sent for sequencing.

3.2.2 Screening of Mae Fa Luang and Mae Fae samples for *Giardia spp.*

Twenty samples that were taken from individuals that resided in the Mae Fae district were screened for *Giardia spp.* using PCR. These samples like the Mae Fa Luang samples also were varied by their BMI (Table 10).

Table 10- Mae Fae samples and their corresponding BMI's

BMI	**Sample Number**
Normal Weight	1,3,6,8,9,13,17,18
Overweight	2,4,7,11,12,14,15,16,19,20
Obese	5,10

Fifteen samples were seen with a band at the approximately 500 bp mark which is close to the expected band size of 432 for the *gdh* primers used as described in the materials and methods (Figure 18) These bands were subsequently extracted and sent for sequencing where *Giardia spp.* was not seen but only as with the screening for *Cryptosporidium spp.* fauna and bacteria.

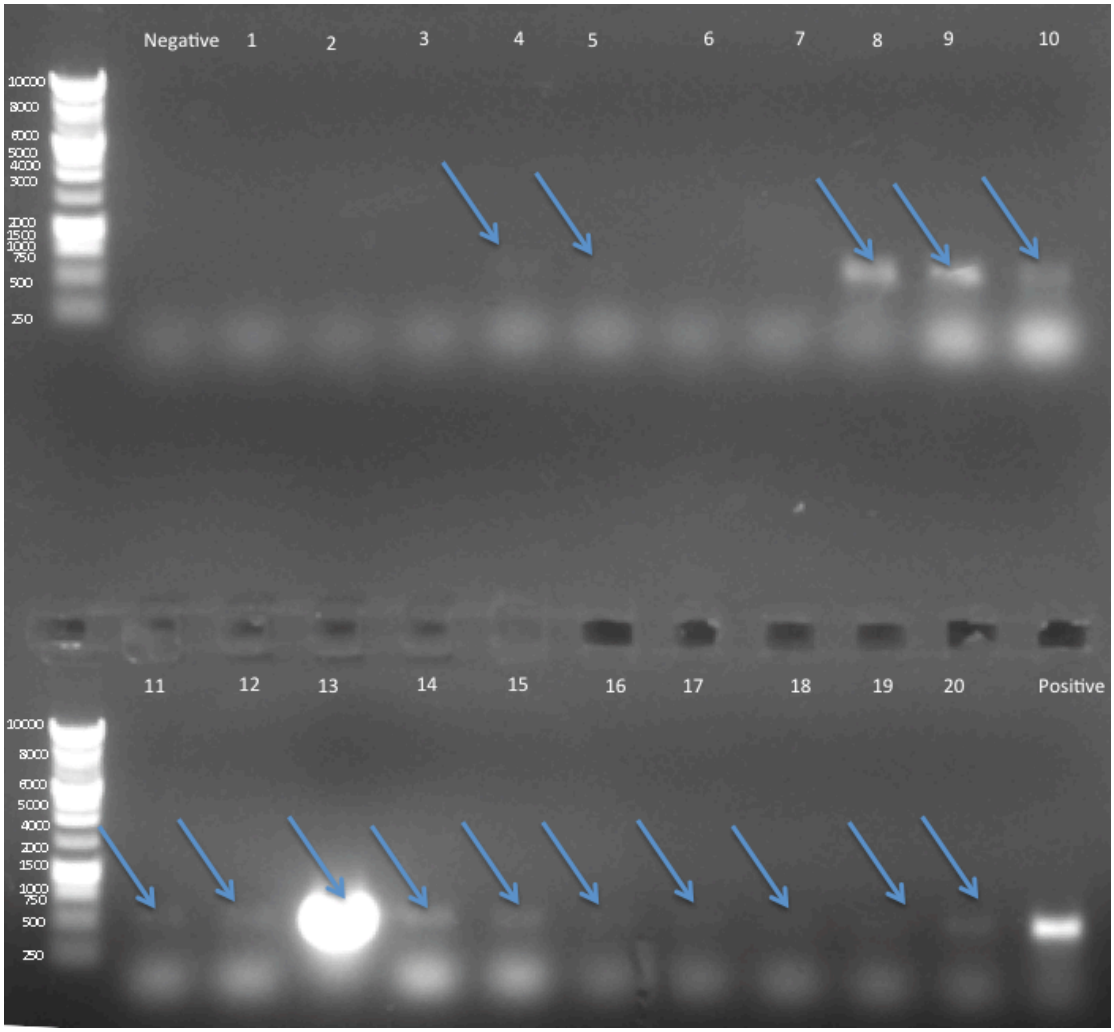

Figure 18- Gel electrophoresis of twenty faecal samples taken from individuals in the Mae Fae district. 1kb ladder. Arrows indicate the bands at approximately 500 bp

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With the Mae Fa luang samples with thirteen samples being screened for Giardia spp. the entire faecal sample DNA did not show any PCR positive results.

3.2.3 Screening of Mae Fa Luang and Mae Fae samples for *Blastocystis spp.*

All twelve of the Mae Fa Luang faecal DNA samples and twenty of the Mae Fae samples were screened for *Blastocystis spp.* using PCR methods. Like with some of the previous screening there were no indications of PCR positive results for these samples.

4.0 Discussion

This project had two aims; the first goal was to investigate whether BMI affected the gastrointestinal metabolite profile of people in Thailand. This comparison was performed between individuals from two different BMI categories, normal weight and overweight. Other studies have shown that there is a difference between the concentration of metabolites and their association with BMI (Moore, C, 2014). There is some evidence from the results of this study that the metabolomic profile is altered by BMI.

The other aim of the study was to determine the eukaryotic pathogens present in the gastrointestinal tract of the same individuals in Thailand. This study showed that even though there were some PCR positive results for certain organisms subsequent sequencing showed no affirmative confirmation of the presence of either *Crytosporidium spp,, Giardia spp.,* and *Blastocystis spp.*

4.1 Comparison between the metabolomic profiles of normal weight and overweight Thai individuals

4.1.1 Notable metabolites present in either overweight of normal weight individuals

A number of metabolites were found in this study to be seen only in obese or normal weight faecal samples. These metabolites could be viewed a biomarkers for obesity, with their presence or absence being a determinate for BMI.

In this study acetoacetate was identified in individuals with an obese BMI, it was not found in those with a normal weight BMI (Table 5). This metabolite has been shown in other studies to show a positive correlation with BMI (Zhao et al., 2016) (Palomino-Schätzlein et al., 2019). The liver releases Acetoacetate during periods of fasting as a source of energy, however it is also used as a source of for those with diabetes (Carmant, 2008). However there are other studies where no positive correlation could be identified between acetoacetate and BMI (Valcárcel et al., 2014). This irregularity in findings could present problems when using this metabolite as an indicator of obesity.

The metabolite allantoin is an intermediate metabolite that has been seen to activate the imidazoline I_I receptor that is linked to the secretion of insulin (Kinsky et al., 2016). In this study it was not seen in those with an overweight BMI (Table 5), this could suggest that these individuals may be prone to type 2 diabetes mellitus where a risk factor includes obesity.

Anserine decreases in concentration when mice are on a high fat diet in comparison to mice fed with a low fat diet (Fujisaka et al., 2018). This comparison can also be seen in this study where those who have the higher BMI of overweight do not have anserine present in faecal samples whereas those with a lower BMI do (Table 5). From this a conclusion could be made that those who have a higher BMI have a higher fat diet than those of normal weight BMI that would explain the lack of anserine in the overweight individuals.

Carnosine is a dipeptide found primarily in the brain and heart, it is seen to be present at lower levels in those with obesity and type 2 diabetes. Studies have found that increasing carsosine in humans reduced insulin resistance, thereby reducing the risk of developing type 2 diabetes (de Courten et al., 2016). This result is also reflected in this study where the metabolite carsosine was not observed in any overweight individuals (Table 5).

The metabolite chlorogenate has been seen to alter lipid and glucose metabolism for the better, preventing the onset of obesity (Cho et al., 2010) (Lin et al., 2017). In other findings it has also been found to increase the abundance of gut microbiota associated with a low fat diet such as *Bacteroides* (Wang et al., 2019). This is supported by the findings in this study with the lack of chlorgenate in those who have a obesity BMI profile (Table 5). The results from this study could explain the results of another study which used faecal samples from the same individuals in this study comparing the gut microbiota of normal weight and overweight individuals. In this study *Bacterioides* were abundant in those with a normal weight BMI profile (Jinatham et al., 2018).

The metabolite creatinine was not present in the metabolomic screening of overweight individuals (Table 5), this can be also be seen in another study where the levels of creatinine decreases in obese non-human primates (Chaudhari, U, et al, 2018).

The presence of the metabolite histidine, an important amino acid used in the synthesis of protein (Bhagavan, 2002) is not present in overweight samples (Table 5). Like the metabolite allantoin it lowers insulin resistance (Feng et al., 2013) and can reduce food intake by supressing the appetite (DiNicolantonio B *et al* 2018). This would correlate with the results seen in this study as those with higher histamine levels would ultimately have a lower appetite and therefore be more likely in the normal weight BMI category.

Indole 3-acetate was also found to be reduced in those who are overweight (Table 5), in other studies indole 3-acetate was reduced in mice who were fed a high fat diet. Another metabolite that reduced linearly with increasing BMI was N-acetylglycine (Fujisaka et al., 2018). This was also reflected in the results of this study.

The metabolite lactate has also been linked to increasing the level of resistance to insulin in obese individuals; its metabolomic pathway is changed in obese individuals (Lovejoy et al., 2002). This trend can also be seen in the presence of lactate in overweight individuals (Table 5).

The metabolite lactulose has been used as an inhibitor of galectin-1, which is involved in adipose metabolism. When lactulose was administered to obese rats on a high fat diet observations included a reduction in weight (Mukherjee and Yun, 2016). The absence of lactulose of in overweight individuals in this study and their presence in normal weight individuals could account for their BMI (Table 5).

The above metabolites have been seen in literature and in this study to be involved in BMI in some aspect. These could then be a potential avenue as biomarkers for a metabolomic profile with regards to BMI.

There were also a number of metabolites identified that currently have no literature supporting their involvement with BMI. Those metabolites associated with decreasing BMI can be seen in table 11 and those associated with decreasing BMI can be seen in table 12. These metabolites could be investigated in future studies to identify whether they have a link with BMI.

Table 11- Metabolites identified by NMR present only in normal weight individuals.

1,6-Anhydro-β-D-glucose	2-Aminobutyrate	2-Hydroxyisocaproate	2-Hydroxyvalerate
2-Oxocaproate	2'-Deoxyuridine	3-Hydroxyisobutyrate	3,5-Dibromotyrosine
4-Guanidinobutanoate	4-Hydroxyphenylacetate	5-Aminopentanoate	5,6-Dihydrothymine
Acetamide	Acetoin	Arabinose	Azelate
Cellobiose	Cholate	Choline	cis-Aconitate
Citraconate	Citrulline	Creatine	Cytidine
Glucitol	Glucose-6-phosphate	Guanosine	Homovanillate
Hydroxyacetone	Isobutyrate	Isocitrate	Isoleucine
Kynurenine	Levulinate	N-Acetylaspartate	N-Acetylglycine
N-Acetyltyrosine	N,N-Dimethylformamide	N,N-Dimethylglycine	N6-Acetyllysine
O-Phosphocholine	Pantothenate	Phenylalanine	Protocatechuate
Riboflavin	Theophylline	Threonine	Tryptophan
Tyramine	Tyrosine	UDP-N-Acetylglucosamine	Uridine
Vanillate	π-Methylhistidine		

Table 12- Metabolites identified by NMR present only in overweight individuals.

3,4-Dihydroxybenzeneacetate	Caprate	Galactose	Succinylacetone
4-Aminobutyrate	Desaminotyrosine	Glutarate	Valproate
Cadaverine	DSS (Chemical Shape Indicator)	Kynurenate	Xylitol

4.1.2 Metabolites present in both normal weight and overweight individuals

The metabolites identified in the Thai samples from both the Mae Fa Luang and Mae Chan provinces are involved in a number of processes in the body, from methane metabolism to purine metabolism (Table 6). This highlights the wide variety of metabolites that are present in the gastrointestinal tracts of those in Thailand.

The identified metabolites are to some extent expected to be found in the gastrointestinal tract. Acetiminophen was found in both the Mae Fa Luang samples and the Mae Chan samples (Table 5). Acetiminophen is more commonly known as paracetomol it is not metabolised by the gut microbiota which explains its presence in the faecal samples (Wilson,I et al, 2017).

A number of short-chain fatty acids (SCFA) were also identified in both the Mae Fa Luang and Mae Chan faecal samples.These short chain fatty acids includes acetate, butyrate, formate, isovalerate, propionate and valerate. These metabolites are produced during the fermentation of carbohydrates that are not digested in the small intestine (Louis P., et al, 2016).

These metabolites can give indications of the type of diet that this selection of individuals have, it has been seen in other studies that those with low fibre and high fat western diets see a reduction in Butyrate producing organisms such as *Roseburia intestinalis* and *Clostridium symbiosum* (Morrison D., et al, 2016).

The results of this project however show that there is no significant difference in Butyrate concentrations between those who are overweight and normal weight (Table 5). It has been seen that those who are overweight have a lower fibre intake and a higher fat intake in comparison to those of normal weight (Hadrevi, J et al, 2017) so it should be expected so see a reduction in Butyrate concentration in those people who are overweight. There is a slight reduction when looking at the data but nothing that is statistically significant; this could show a potential link that needs future work (Table 5).

Alanine was another metabolite seen in both sets of samples (Table 5), an amino acid produced by protein catabolism it can be an indicator of IBS when present in elevated levels (Ponnusamy, K, 2011). IBS is present in Thailand with a 4.3 % prevalence (Yan-Me Tan, 2003), in comparison to western countries where the prevalence ranges from 10-15% (Chang, Lu and Chen, 2010). The presence of alanine in these faecal samples could show a rise in the incidence of IBS in this region of the world.

The data collected from the metabolite profiling from both cohorts from Thailand showed no statistically significant difference between those who were normal weight and those who were overweight (P value greater than 0.05). This provides evidence that there is no discernible difference in concentration of the metabolites when BMI is taken into account.

This is contradicted by the most recent research where certain metabolites are associated with BMI. This has been seen when metabolites such as methyl succinate, asparagine, urate, glycine, glutamic acid and serine are associated with BMI (Zhao, H., 2016). Although not human, in mice models obese mice have higher levels of both acetate and butyrate (Davis, C., 2016). These elevated levels were not seen in this study to that extent, which suggests that perhaps BMI doesn't have as much effect on the metabolome of the gastrointestinal tract.

The only metabolites that were close to being statistically significant was Xanthine in the Mae Fa Luang samples (Figure 15) with a P value of 0.06. It showed that the level of Xanthine was reduced in those who are overweight. This is seen in other studies where the levels of Xanthine are reduced in those of higher BMIs (Zhao, H).

4.2 Screening of Thai samples for gastrointestinal eukaryotic parasites

The screening of the Thai samples by PCR showed some potential, as outlined in the intro the parasites mentioned have been seen in Thailand with *Giardia lamblia* being the most common protozoan gastrointestinal parasite (Nuchprayoon, S., 2002). However this study has surprisingly shown that there was no *Blastocystis spp., Cryptospordium spp. or Giardia spp*. (Figure 17 and Figure 18) in these individuals. The prevalence of *Blastocystis spp.,* in Thailand can be as high as 37% (Pipatsatitpong, D*, et al 2012)*, which makes the results of this study contradictory to known research*.*

Blastocystis spp. identification has in past research been done in most studies by microscopy (Sagnuankiat et al., 2014). The molecular methods used in this study are a relatively new approach to identifying *Blastocystis spp.* which causes it to be limited in approach (Boondit et al., 2014). This causes a large variation in the prevalence detected in these studies (Pintong H, et al. 2014), which could be responsible for the lack of *Blastocystis spp.* seen in this study. Other reasons for this include the variation in the different regions of Thailand and the incidence of *Blastocystis spp.* in these regions (Pintong et al., 2014).

Mae Chan and Mae Fa Luang are predominantly urban regions of Thailand; this could explain the lack of *Crytosporidium spp.* seen in those observed in this project. Typically *Cryptosporidium spp.* is found at high prevalence in rural regions due to large amounts of domesticated animals (Bamaiyi, P. and Redhuan, N., 2016).

One other reason why there was such a lack of positive sequencing data for the above parasites could be the primers used. The primers are developed to target these parasites in western countries; the difference in species prevalence/ subtype in Thailand could mean that these primers could not effectively target the specific subtypes in Thailand.

4.3 Future Work

The difference in the concentrations of metabolites identified in the Thai samples could be more effectively seen in normal weight and obese individuals. This is a more extreme difference in BMI, which has the potential to show a more significant difference. The samples provided for this project did include some individuals that were obese, however due to inconsistencies in the metabolites collected statistical analysis could not be carried out. Comparing the metabolites of those who are normal weight and obese has been done in previous studies. These studies have seen a more profound difference in the metabolites of those who are normal weight and obese in comparison to those who are overweight (Ottosson, F, *et al,* 2018)

This more significant difference can be seen as a consequence of the wider range between the BMI's of normal weight and obese, with the obese individuals have a greater effect on their metabolites than those who are overweight. Repeating this project but including those who are obese will provide a better insight into how the BMI can affect the metabolomic profile of an individual.

Using current research a more focused and targeted approach could be used in the future. As discussed earlier several metabolites have been identified as being strongly associated with BMI, these metabolites could then be targeted not only just using NMR but also MS to provide higher specificity. This could provide a clearer picture in the role these metabolites play in BMI.

The primers used in this study were clearly not specific enough to identify the gastrointestinal parasites that may be present in the Thai people. In future work more specific primers could be designed based on the subtypes/ species that are present in this part of the world. This could increase the positive identification of these microorganisms, which are likely to be present based on current findings in the area.

Doing this study with a greater number of samples would provide a greater insight in not only the metabolomic profile of the Thai people in these regions but also what gastrointestinal parasites are present. This would increase the scope and impact of the findings. The limited number of samples also influence the statistical validity of this study, whereby statistical analysis could not take place. This is in combination with limited controls being used.

Another approach could be looking at the metabolites identified in this study that were only found in either normal weight individuals or overweight individuals. Applying this could be screening a greater variation in BMI with a larger sample size to see if these metabolites showed the same correlation as they did in this study. This could create a greater link between an individuals gut metabolome and obesity.

4.4 Conclusion

This project demonstrated that there was an unexpected lack of difference in metabolites that were in both individuals of normal weight and overweight in the Mae Chan and Mae Fa Luang regions. This suggests that BMI may play a smaller role in the metabolomic profile that what has been previously seen. However a wider ranging sample size and better controls will help to determine whether this is a potential avenue.

However there were a number of metabolites identified that could be potential biomarkers of obesity, with their presence in only one BMI category, this is supported by current literature. This study also identified metabolites that could be investigated in future work.

There was a wide range of metabolites in this study that were identified with them playing a vast range of roles in the human body. This shows the great influence that the metabolome can play.

Although *Cryptosporium spp., Giardia spp., and Blastocystis spp*. were not positively identified by sequencing in this study it does present the interesting idea that there may be unknown subtypes present in this region that have yet to be identified by current primers.

This project has provided the potential for future work into the gut microbiome and how it can be affected by the BMI. This can then also be linked to what roles the eukaryotic gut microbiome may play, an underdeveloped research area, which this project has now contributed to.

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