Exploring methylmercury demethylation phenotypes in the human gut microbiome

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Abstract:

Methylmercury, a potent neurotoxicant with global prevalence, poses significant risks to human and ecosystem health. The metabolism of methylmercury still holds many uncertainties, such as the high variability in methylmercury half-lives across individuals. Gut microbiota have been suspected to play a role.

This thesis investigated the impact of the human gut microbiome on the demethylation rate of methylmercury. I conducted batch incubations with fecal samples collected from 10 healthy individuals and measured the demethylation rate under different conditions. Their microbiomes were characterized by metagenomic analysis.

Results indicate high variability among individual's methylmercury demethylation rate, correlating with pyruvate reduction. Some individuals carried bacterial genes for mercury detoxification, but none of these genes correlated with mercury demethylation.

These findings underscore the interplay between diet, gut microbiome, and neurotoxin transformation. This work highlights the link between human microbiome activity and methylmercury metabolism, providing a fresh perspective on neurotoxin exposure modulation.

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CHAPTER 1. INTRODUCTION:

1.1 Methylmercury Impact and Historical Context

As of 2015, the global mercury emissions into the atmosphere totaled approximately 2220 tonnes per year, with anthropogenic mercury emissions rising 20% between 2010 and 2015 (UN Environment, 2019). The projected global economic loss attributed to mercury pollution, including developmental IQ loss and increased heart attack deaths, is estimated to be $19 trillion USD by 2050 under current climate policies (Zhang et al., 2021). Primary anthropogenic sources of mercury pollution are industrial processes like mining and coal burning, which release elemental mercury (Hg(0) into the atmosphere. This volatile form converts to inorganic divalent mercury (Hg(II)) in the atmosphere. Hg(II) exhibits decreased volatility, shows high water solubility and accumulates in small particles. This solubility facilitates the transport of mercury from the atmosphere to the ground through rain (wet deposition) or gravity (dry deposition). In anoxic environments such as lakes, rivers and rice paddies, microorganisms methylate mercury, forming methylmercury (Poulain & Barkay, 2013).

Methylmercury (MeHg), a potent neurotoxicant, is the predominant form that effectively bioaccumulates in individuals and biomagnifies across food webs (Ruus et al., 2015). Exposure to MeHg among general human populations primarily occurs through ingestion of contaminated fish and grain (Hong et al., 2012). The most well-known example of acute MeHg exposure was seen in Minamata, Japan and became officially recognized as Minamata Disease in 1956 (Ekino et al., 2007). During the 1950's and 1960's, the chemical company Chisso discharged substantial quantities of MeHg into the Yatsushiro Sea near the city of Minamata, resulting in its

accumulation in fish and shellfish (Hachiya, 2006). Residents of Minamata became exposed to the toxic metalloid through daily consumption of the highly contaminated seafood (Ekino et al., 2007).

The severe effects of Minamata disease, including sensory disturbances, ataxia, dysarthria, visual and auditory disturbances, tremors and clonic seizures were extensively studied (Bernhoft, 2012; Harada, 1995). Infants born to exposed mothers faced even more severe consequences (Sakamoto et al., 2018). Although many were exposed to extremely high levels of MeHg, even more were chronically exposed to lower levels, the effects of which are still being studied today (Ekino et al., 2007). Chronic exposure to low doses of MeHg in adults can result in distal paresthesias, increased touch threshold, sensory ataxia and pseudoathetoid movement with damage to the somatosensory cortex of the brain (Hurley et al., 2008).

Despite often being associated with Minamata, MeHg is a significant issue in Canada. MeHg is widely distributed throughout lakes and rivers, posing risk for those consuming fish from these bodies of water (Government of Canada, 2013). Grassy Narrows in Ontario shares similarities with Minamata (Mosa & Duffin, 2017). Grassy Narrows is part of the Asubpeechoseewagong/Grassy Narrows First Nation Reserve. A paper mill released mercury-containing effluent into the Wabigoon River, upstream of the Grassy Narrows Reserve, leading to high MeHg concentrations in the local diet (Philibert et al., 2020). While less is known about Grassy Narrows than Minamata, studies on Grassy Narrows indicate a decrease in life expectancy as hair mercury levels increase and emphasize the enduring impact of MeHg exposure (Philibert et al., 2020).

1.2 Methylmercury in the environment

1.2.1 Mercury release and cycling

MeHg is a product of natural environmental processes driven by mercury cycling (Barkay & Poulain, 2007; Poulain & Barkay, 2013). Although MeHg is formed naturally, the predominant source of its precursor (mercury) is anthropogenic activities such as coal burning, gold mining and chlor-alkyl paper mills (Hylander & Goodsite, 2006). Non-anthropogenic sources, like volcanoes and forest fires can also contribute to mercury release (Rakociński et al., 2020). This release of vaporous elemental mercury (Hg(0)) is of particular concern to Canadians due to the longevity and capacity of mercury vapour to travel long ranges in the atmosphere, propelled by prevailing atmospheric wind currents to the apex of the northern hemisphere (Loewen et al., 2007; Travnikov, 2005). Over time, released Hg(0) undergoes oxidation in the atmosphere to divalent inorganic Hg(II), which condenses and falls to the ground through both wet and dry deposition (Li et al., 2020). In anoxic environments, such as lakes, rivers and rice paddies, microorganisms methylate Hg(II), producing MeHg (Poulain & Barkay, 2013). This is the form that can most effectively bioaccumulate and biomagnify across food webs (Ruus et al., 2015).

1.2.2 Mercury speciation

Mercury exhibits various species and complexes in the environment, ranging from extremely dangerous to benign. Within this range, there are three main species of mercury: elemental mercury Hg(0), inorganic (mercuric) mercury Hg(II), and organic mercury, which includes MeHg (O'Connor et al., 2019). Despite the methyl group (CH_3^-) rendering

methylmercury (MeHg or H_3CHg) only slightly more lipophilic than inorganic mercury, it remains less lipophilic than elemental mercury (the log p values of Hg(0), MeHg, and Hg(II) are 0.62, 0.41 and -0.22 respectively) (Scarmoutzos & Boyd, 2003). While the methyl group enhances lipophilicity, it marginally increases the size and keeps MeHg in small molecule range at 215.63 Da (Li & Kang, 2020).

Since Hg(0) is vaporous and lipophilic, it can easily penetrate the blood brain barrier (BBB) when inhaled, making it extremely potent to exposed individuals (Aschner & Aschner, 1990). However, size or lipophilicity are only part of what allows MeHg to be so easily distributed in the body. MeHg's ability to bioaccumulate and biomagnify arises from its ability to form complexes with thiols and chemical groups that contain selenium (Ruus et al., 2015). Most notable of these is L-Cysteine, a non-essential amino acid that contains a thiol (Sameem et al., 2019). When bound to L-Cysteine, this complex forms a strong resemblance in size and shape to L-Methionine, an essential amino acid (Marston & Wright, 1984). The similarity between these two compounds allows MeHg to access bodily systems that are inaccessible to ingested inorganic mercury (Aschner & Aschner, 1990; Bridges & Zalups, 2005; Kerper et al., 1992; Lohren et al., 2016; Simmons-Willis et al., 2002).

1.2.3 Methylmercury uptake, bioaccumulation and biomagnification

In the environment, MeHg is taken up by both aquatic plants (such as rice) and phytoplankton (Liu et al., 2019), increasing concentration in phytoplankton relative to their aquatic environment habitat (Lee & Fisher, 2016). Animals that ingest these plants and phytoplankton absorb this MeHg. As trophic levels increase, MeHg concentrations also increase. This is referred to as *biomagnification* (Baeyens et al., 2003). Higher trophic levels with more

predators exhibit higher MeHg concentrations. Predatory fish and other animals at the top of the chain accumulate much higher concentrations than the initial water concentration, posing a more significant risk through food consumption than water ingestion (Ruus et al., 2015). This poses a particular threat to individuals and communities, such as Canadian First Nations, that rely heavily on predatory fish and mammals as a food source (Wheatley & Paradis, 1995). MeHg has a prolonged half-life in animals, and ingestion can often outpace elimination. Consequently, the concentration of MeHg within an individual will increase over time – referred to as *bioaccumulation* (Li et al., 2020).

1.3 Methylmercury and climate change

Climate change is expected to have a significant impact on the presence of MeHg in the environment. Warming of lakes and rivers, coupled with permafrost thawing, is anticipated to expand anoxic environments, accelerating the conversion of inorganic mercury to MeHg (Varty et al., 2021; Yang et al., 2016). Compounding this increase, overfishing is also projected to increase the concentration of MeHg found in fish and other marine predators (Schartup et al., 2019). Given the persistent nature of mercury and its ability to be transported over distances, individuals residing in countries without significant mercury production, such as Canada or Norway, are likely to face continued negative from MeHg (Ruus et al., 2015). While mercury production is declining in Canada, the level of MeHg is still expected to rise due to these and other factors, such as dam construction (Calder et al., 2016; Environment Canada, 2010).

1.4 Exposure and Exposure Limits

1.4.1 Seafood consumption

As previously highlighted, the primary pathway for human exposure to MeHg is through the consumption of fish and seafood contaminated with MeHg (Basu et al., 2023). This is exemplified by Grassy Narrows and Minamata, where the effects of MeHg are still being felt over 50 years after mercury was introduced to the environment (Mosa & Duffin, 2017). Each had populations that relied heavily on fish/seafood as a staple for their diet. Although fish and seafood are the primary source of exposure for MeHg, there are other exposure pathways.

1.4.2 Grain and rice

Grain and rice have served as significant vectors for MeHg exposure, particularly in regions with mercury-rich environments, like the mercury mines of Guizhou Province, China (Philibert et al., 2020). Rice paddy fields contribute to MeHg production by microorganisms, and the resulting MeHg is taken up by the rice (Liu et al., 2019). Ingestion of rice from these areas is a substantial source of chronic MeHg exposure (Philibert et al., 2020).

A more extreme case of exposure occurred in Iraq during the early 1970s, where rural communities inadvertently ingested home-made bread from grain that was treated with a fungicide containing MeHg (Bakir et al., 1980). This incident resulted in significant hospitalizations (6350) and death (459), mirroring the severity and acute exposure seen in Minamata (Bakir et al., 1973; Basu et al., 2023).

1.4.3 Small scale artisanal gold mining

Communities who engage in in small scale gold mining operations face an uncommon, but significant source of MeHg exposure (Basu et al., 2023). Burning of gold ore with mercury in these operations without personal or environmental protective measures leads to mercury deposition in the surrounding environment (Esdaile & Chalker, 2018). When this mercury is deposited into the aquatic environments that fish are being consumed from, significant MeHg exposure combined with exposure to mercury vapour can occur (Calao-Ramos et al., 2021; Riaz et al., 2018).

1.4.4 Acute vs chronic exposure

All mercury compounds can be fatal in acute exposure scenarios at high concentrations (Basu et al., 2023). Unlike other forms of mercury, MeHg's primary concern arises from bioaccumulation of small doses over extended periods of time due to repeated consumption. While the risk of acute exposure is evident in extreme cases like the Iraq grain incident or Minamata, chronic exposure remains a more prevalent concern (Bakir et al., 1973; Harada, 1995). In Iraq and Minamata, such large quantities of mercury were dumped or ingested that acute MeHg exposure was of clinical concern (Bakir et al., 1973; Harada, 1995). However, without any remediation in such scenarios, chronic exposure will continue to be and has continued to be a concern for a long period of time, affecting far more individuals than those acutely exposed (Ekino et al., 2007; Hachiya, 2006). Chronic exposure is associated with adverse neurodevelopmental and cardiovascular outcomes (Basu et al., 2023). Other chronic exposure outcomes continue to be investigated, including low levels of adult and prenatal MeHg exposure in Seychelles (Davidson et al., 1998, 2008), the United States (Sun et al., 2021), New Zealand (Gearhart et al., 1995), and Canada (Philibert et al., 2020; Wheatley & Paradis, 1995).

1.4.5 Prenatal Exposure

MeHg has a significant impact on fetal development due to active transportation of MeHg across the placental barrier, leading to increased concentrations of MeHg in fetal blood relative to their mother's blood (Ha et al., 2017). Thus, prenatal MeHg exposure occurs through maternal MeHg consumption entering the developing fetus through the umbilical cord, with umbilical cord blood MeHg concentration serving as a proxy for prenatal MeHg exposure (Ha et al., 2017; Murcia et al., 2016).

1.4.6 Exposure limits

In Canada, the established guideline for fish to be considered a dietary exposure concern is 0.5 parts per million (ppm) of Hg, with maternal hair having an accepted threshold of 2 ppm (Health Canada, 2007, 2020; Legrand et al., 2010). These exposure limits underscore the extreme nature of cases like Minamata and Grassy Narrows. In Minamata, seafood from Minamata Bay had mercury content ranging from 5.61 to 35.7 ppm (Hachiya, 2006; Yorifuji et al., 2009), and in Iraq, hair MeHg ranged from 50 to 100 ppm (Yorifuji et al., 2009). Even in Grassy narrows, reported fish levels reached as high as 24 ppm Hg, with 13.1% of First Nations women aged 15-45 having hair Hg above 10 ppm from 1972-1992 (Wheatley & Paradis, 1995). However, it is important to note that clinical symptoms are estimated to set in when hair levels exceed 50 ppm (Clarkson & Magos, 2006).

1.5 Toxicological Effects

1.5.1 Neurotoxic Effects

For other species of mercury (Hg(II) or (0)), toxicity primarily arises from large acute exposures or inhalation, resulting mainly in kidney damage, and also central nervous system (CNS) and lung damage when Hg(0) is inhaled (Clarkson & Magos, 2006). Chronic poisoning from ingested inorganic mercury is uncommon due to poor absorption, low bioavailability and high elimination rate (Park & Zheng, 2012). In contrast, concerns with MeHg exposure are primarily due to its neurotoxic effects. MeHg's ability to penetrate the BBB and accumulate in the CNS distinguishes it from other forms of ingested mercury (Clarkson & Magos, 2006). Major signs and symptoms of MeHg poisoning, referred to as Minamata disease, includes paresthesia (numbness in extremities), ataxia (uncoordinated movement), dysarthria (problems speaking), and loss of vision (Ekino et al., 2007; Hachiya, 2006; Harada, 1995). Symptoms can exhibit significant latency periods between exposure and onset of symptoms. In cases such as the Iraq grain incident, this caused individuals to continue to consume the contaminated bread for several weeks (Weiss et al., 2002). Potential latent onset effects include neurodegenerative diseases such as Alzheimer's and Parkinson's, however, more research is needed on the link between MeHg exposure and these diseases (Cariccio et al., 2019; Clarkson & Magos, 2006; Pamphlett & Kum Jew, 2018).

1.5.2 Fetal Development

Neurodevelopmental effects are a paramount concern when it comes to MeHg exposure, evident in Minamata and Iraq, where children born to mothers showing limited or no symptoms

displayed severe brain damage (Bakir et al., 1980; Clarkson & Magos, 2006). Developing fetuses prove to be much more sensitive to MeHg exposure than adults, as observed in autopsies of severely affected infants, revealing extensive brain damage compared to localized lesions in adults (Choi et al., 1978).

Some indications suggest that total MeHg exposure is more critical for developmental effects than total mercury and inorganic Hg(II) (Papadopoulou et al., 2021; Pugach & Clarkson, 2009). MeHg's ability to cross the BBB and placental barrier, unlike Hg(II) contributes to this importance in developmental effects (Kajiwara et al., 1996). Variances in the effects on exposed children in Iraq and Minamata underscore the complexity of MeHg's impact. Site specific effects included intellectual disability, impaired speech, motor and autonomic function, blindness, deafness, akinetic mutism, decreased coordination, trouble swallowing, increased muscle tone, cerebral palsy, and severe CNS neuronal degeneration were observed in severe cases at one or both locations (Bakir et al., 1980; Sakamoto et al., 2018). In Minamata, there also was an observed change in the male:female sex ratio among babies being born (0.39 vs 0.515 in a control city) (Sakamoto et al., 2018). Prenatal MeHg exposure has also been linked to decreased birth weight (Murcia et al., 2016; Vigeh et al., 2018). In animal models, low doses of MeHg have been shown to change neuronal differentiation and cause autism like behaviour in rodents (Loan et al., 2023; Yuan et al., 2018). Low doses have also been shown to adversely impact human embryonic stem cells (Li et al., 2021) and embryonic stem cell differentiation (Li et al., 2023; Loan et al., 2023).

1.5.3 Cardiovascular Effects

MeHg exposure has been correlated with several adverse cardiovascular outcomes including increased risk of heart disease, cardiovascular disease, and acute coronary events (Virtanen et al., 2005), increasing the risk of myocardial infarction (Hong et al., 2012). MeHg exposure has also been shown to increase blood pressure in a dose-dependent manor, which could cause or add to these effects (Hu et al., 2018).

Contrary to the neurotoxic effects, cardiovascular effects of MeHg exposure are more pronounced in adults than children (Hong et al., 2012). However, MeHg exposure to pregnant women also shows developmental impacts on their children's cardiovascular health. Like adults, blood pressure was also increased in children whose mothers were exposed to low levels of MeHg during pregnancy (Kalish et al., 2014). Prenatal MeHg exposure is also associated with decreased cardiac autonomic function in children (Chan et al., 2021).

1.5.4 Reproductive toxicity

Sex-differences in MeHg toxicity has prompted exploration of the effects of MeHg on reproductive toxicity in low doses (Kimiko et al., 1987). Notably, there have been studies that show MeHg may accumulate in the testes (Friedmann et al., 1998), with reduced serum testosterone levels (McVey et al., 2008), and decreased sperm motility (Fossato da Silva et al., 2011; Friedmann et al., 1998) at low doses. However, these findings are primarily from rat studies, and human studies primarily focus on developmental defects in fetuses exposed to MeHg (Burbacher et al., 1987). Research examining MeHg levels and reproductive success in wild animals extends the impact of environmental MeHg on toxicology beyond human exposure (Fuchsman et al., 2017; Hu et al., 2021).

1.6 Mechanism of MeHg Toxicity

It is widely acknowledged that the primary mechanism of MeHg toxicity centers around oxidative stress induced by the generation of reactive oxygen species (ROS) (dos Santos et al., 2018; Farina et al., 2011; Reardon & Bhat, 2010). This is substantiated by the upregulation of various cellular responses to oxidative stress in the presence of MeHg (Ni et al., 2010; Wang et al., 2009). The generation and stress from ROS occur in two distinct pathways.

First, MeHg amplifies ROS production in neuronal mitochondria. Production of hydrogen peroxide, superoxide radicals and nitric oxide in neuronal mitochondria are all increased in the presence of MeHg (Fretham et al., 2012; Lee et al., 2020).

Secondly, MeHg diminishes a cell's ability to respond to ROS by binding thiol groups on glutathione, a key antioxidant responsible for reducing and detoxifying ROS. Consequently, MeHg inhibits the efficacy of glutathione in reducing ROS, thereby elevating ROS levels within the cell (Fretham et al., 2012). The outcome of this increased ROS availability includes lipid peroxidation, protein oxidation, DNA oxidation, impaired mitochondrial function and ultimately cell death (Fretham et al., 2012).

Another potential mechanism contributing to neuronal cell death is MeHg's interference with cellular calcium homeostasis. MeHg enhances Ca^{2+} uptake by neurons, leading to decreased mitochondrial function, increased ROS species generation and Ca^{2+} mediated cell death (Roos et al., 2012).

MeHg also shows strong affinity with selenium and effectively induces selenium deficiency by depleting selenium stores in the body (Raymond & Ralston, 2020; Spiller, 2018). This interferes with selenium's role in intracellular redox control and can permanently inhibit the function of selenoproteins (including glutathione) (Spiller, 2018). Inhibition of these selenium containing proteins could lead to suppression of their function in metabolism, cell signalling and oxidative stress (Arteel & Sies, 2001).

1.7 Toxicokinetics

1.7.1 Absorption

Given that ingestion is the predominant exposure route for MeHg, research has focused on ingestion, with limited data available on absorption through dermal and inhalation exposure (Clarkson & Magos, 2006). Approximately 95% of ingested MeHg is absorbed in the small intestine (CTEM, 2000). This is remarkably high compared to the 15% of inorganic Hg(II) that is absorbed and approximately 0.01% of elemental Hg(0) absorption through ingestion (National Research Council, 2000). The intestinal absorption of MeHg involves both passive transcellular diffusion and amino acid transporters by MeHg forming bonds with L-cysteine, cysteinylglycine, and glutathione to utilize the amino acid transporters (Tsutomu et al., 1990; Vázquez et al., 2014).

1.7.2 Distribution

1.7.2.1 Whole Body

Upon absorption in the small intestine, MeHg readily permeates red blood cells, forming bonds with available amino acid residues containing sulfhydryl groups, such as albumin containing cysteine residues in plasma (Komatsu et al., 1975; White & Rothstein, 1973; Wu, 1995; Yasutake et al., 1990). The concentration in red blood cells is higher than in plasma (Kajiwara et al., 1996; Kershaw et al., 1980). This absorption into red blood cells allows MeHg to be distributed throughout the body, reaching metabolic sites such as the liver and kidneys, and facilitates the deposition of mercury in hair, which is often used as a biomarker for MeHg exposure over time (Berglund et al., 2005; Kippler et al., 2021).

1.7.2.2 Central Nervous System (CNS)

MeHg's potent neurotoxicity comes from the ability of MeHg to traverse the BBB. The MeHg-L- cysteine complex is biosimilar to L-methionine, which allows the MeHg-L-Cys complex to cross the BBB using the L-type large neutral amino acid transporter (LAT1) by mimicking L-methionine, a substrate for the transporter (Simmons-Willis et al., 2002; Yin et al., 2008). Once in the brain, MeHg is demethylated to inorganic Hg(II), which cannot cross the BBB (Korbas et al., 2010). This entry into the CNS, coupled with slow metabolism and elimination from the CNS results in a significant accumulation of Hg in CNS tissue (Berlin et al., 1975). This is a significant mechanism for chronic low doses of MeHg exhibiting neurotoxic effects after long periods of low exposure. There are observed differences for acute and chronic exposure in brain distribution. Acute high-dose exposures of MeHg show Hg associating with sulfur containing compounds and chronic low doses show more Hg bound to selenium containing compounds that are less bioavailable (James et al., 2022).

Within the CNS, distribution was found to be significantly uneven in both mice, guinea pigs and monkeys. In mice, oral doses were evenly distributed amongst the posterior cerebral cortex, subcortex, brain stem and cerebellum. However, the anterior cerebral cortex had significantly higher MeHg than the rest of the brain (Vandewater et al., 1983). In the guinea pigs, distribution to the cerebrum was higher than the cerebellum, which was higher than the spinal cord (Iverson et al., 1973). In monkeys, the distribution to the brain was differential among individuals who showed clinical signs of intoxication following oral/stomach tube dosage, and the concentration in the occipital cortex was 50% higher than the rest of the brain. It was also observed that 4 days after a single oral dose, the concentration in the brain and the blood reached an equilibrium (Berlin et al., 1975). Monkeys were also shown to have significant changes in protein expression in both the occipital lobe and cerebellum after oral dosages with MeHg (Shao et al., 2015; Yamamoto et al., 2012).

1.7.2.3 Placenta

Similar to the BBB, MeHg can cross the placental barrier via the LAT1 transporter, mimicking L-methionine when combined with L-cysteine (Kajiwara et al., 1996). This poses a significant hazard, subjecting developing fetuses to MeHg induced neurotoxic effects (Ask et al., 2002; Lewandowski et al., 2002). Notably, the demethylated inorganic Hg(II) crosses the placental barrier very poorly compared to MeHg (Kajiwara et al., 1996).

1.7.3 Metabolism

A pivotal process in MeHg metabolism, facilitating both elimination and inducing toxic effects, involves its demethylation to inorganic Hg(II). At the cellular level, Hg(II) manifests

toxic effects and serves as the primary species through which ingested MeHg is excreted. The primary focus of this thesis lies in unraveling the intricacies of microbial MeHg demethylation occurring in the human gut.

1.7.3.1 Microbiome

Studies on mouse and rat models indicate that the microbiome modifies mercury metabolism and elimination. Disrupted microbial communities in mice, caused by mercury poisoning, showed increased elimination of MeHg after restoration of the microbiome (Rowland et al., 1984). Additionally, rats treated with antibiotics to eliminate microbial communities exhibited reduced mercury elimination (Rowland et al., 1980). Rats with depleted microbiomes from mercury poisoning alternatively showed enhanced mercury elimination upon microbiome restoration combined with selenium treatment (Liu et al., 2019).

Microbial demethylation of MeHg to inorganic Hg(II) in the intestinal lumen is estimated to account for 73% of MeHg metabolism in adults and 61% in children (Pope & Rand, 2021). The exact mechanism of MeHg demethylation, bacteria involved and variability in this demethylation among humans remains unknown. Direct demethylation has only been observed in two *in vitro* human samples (Edwards & McBride, 1975; Guo et al., 2018). However, it was observed that two individuals who underwent a course of antibiotics during a MeHg metabolism study had significantly reduced rates of MeHg elimination following exposure (Rand & Caito, 2019).

There are instances of mercury interaction with bacteria and archaea in nature. Microbes both produce and degrade MeHg, reduce Hg(II), oxidizing Hg(0) in addition to sequestering

these and other Hg species (Grégoire & Poulain, 2018). A significant step in Hg detoxification is the volatilization (by reduction) of mercury in aquatic environments to Hg(0), so the gaseous Hg(0) is effectively removed from the bacterial environment (Barkay et al., 2003; De et al., 2008; Joshi et al., 2022). This often involves the *mer operon,* a sequence of genes that contribute to bacterial MeHg demethylation and volatilization (Barkay et al., 2003; Boyd & Barkay, 2012). The focal point of the *mer operon* centers around 2 genes, an organomercury lyase (MerB) that splits carbon-Hg bonds (like MeHg), and a mercuric ion reductase (MerA) that reduces Hg(II) to Hg(0) (Barkay et al., 2003). The *mer operon* also involves a Hg(II) scavenging protein (MerP), membrane transport proteins (Mer T, MerC, MerE, MerF, MerG) and transcriptional regulators (MerR and MerD) (Boyd & Barkay, 2012). These genes have been recovered from primate fecal samples (Liebert et al., 1997) and human fecal samples (Rothenberg et al., 2016), but none of these genes were observed in human fecal samples when previously investigated in our lab (Guo et al., 2018).

1.7.3.2 Liver

The liver partially metabolizes MeHg found in blood cells, excreting it into the bile (Ballatori & Truong, 2009; Dutczak & Ballatori, 1994; Stein et al., 1988). This metabolism involves conjugation of MeHg with glutathione (a tripeptide containing a glycine, a cysteine and a glutamic acid residue) and subsequent release into the bile (Ballatori et al., 1998; Ballatori & Clarkson, 1983). This conjugated form is hydrolyzed to the MeHg-L-Cysteine complex and ultimately eliminated as Hg(II) combined with the demethylated MeHg from the microbiome in the feces (Ballatori et al., 1998; Dutczak, 1994; Pope & Rand, 2021). Another factor increasing neonatal toxicity of MeHg is due to the neonatal liver's limited secretion of glutathione therefore

increasing half-life, though this was only observed in rats below 4 weeks of age (Ballatori & Clarkson, 1982).

Enterohepatic circulation enables the re-absorption of MeHg metabolized by the liver. This occurs through MeHg-glutathione transport across the intestine (Tsutomu et al., 1990) as well as metabolism of the MeHg-glutathione complex by intestinal lumen enzymes, resulting in MeHg L-cysteine being being reabsorbed (Ballatori et al., 1998; Cikrt & Tichý, 1974; Clarkson & Magos, 2006; Magos et al., 1978).

1.7.3.3 Kidneys

Although only 3% of a single oral dose of MeHg is eliminated through the urine within the first 49 days (Aberg et al., 1969), there is still some limited metabolism of MeHg that occurs in the kidneys (Carrier et al., 2001; P. Li et al., 2020; Pope & Rand, 2021). The kidneys eliminate the MeHg bound to the albumin and other plasma proteins, which hold a much smaller fraction of MeHg compared to other tissues (Kajiwara et al., 1996; Kershaw et al., 1980). In the kidneys, MeHg from the albumin is conjugated with glutathione, which is then hydrolyzed to the MeHg-L-cysteine complex and ultimately excreted in urine as inorganic Hg(II) (Yasutake et al., 1989; Yasutake & Hirayama, 1994).

1.7.3.4 Brain

Ingested MeHg is commonly found in the brain as both MeHg and inorganic mercury indicating demethylation that occurs within the brain (James et al., 2022; Korbas et al., 2010; Vandewater et al., 1983). Mouse studies suggest that this demethylation rate is extremely slow and contributes to an exceedingly long half-life of MeHg in the brain (Friberg & Mottet, 1989).

Monkeys exposed to MeHg orally had significantly higher concentrations of inorganic Hg(II) in their brains compared to monkeys exposed intravenously to inorganic Hg(II), which suggests that the demethylation is taking place in the brain (Vahter et al., 1995). In rats, it was shown that both species of mercury are equally toxic in the brain (Gallagher & Lee, 1980). However, in monkeys MeHg half-life was 37 days in the brain, and the resulting inorganic Hg(II) half-life was 230-540 days in the brain (Vahter et al., 1995) showing that even when demethylated, the remaining Hg in the brain is persistent.

1.7.4 Elimination

Over 90% of ingested MeHg is excreted in the feces as Hg(II) (Berglund et al., 2005; CTEM, 2000). Approximately 1% of the human MeHg body burden is excreted daily (Clarkson et al., 1988). Additional excretion pathways include urine, hair deposition, sweat and fingernails (to a lesser extent) (Pope & Rand, 2021). As hair deposition is dependent on the blood concentration of MeHg, hair is often used as a biomarker for MeHg levels in the blood over time (Kippler et al., 2021; Rand & Caito, 2019).

Modelling MeHg proves extremely challenging due to substantial variation in elimination/metabolism among individuals (Jo et al., 2015). Reported half-lives of MeHg in the blood range widely from less than 30 to over 120 days (Rand & Caito, 2019). Attempts to predict MeHg concentration in hair among high-frequency fish consumers using variables such as age, obesity, smoking, could only account for 32% of variation between actual and expected results (Li et al., 2016). One offered explanation is the difference in microbiomes between individuals contributing to increased or decreased elimination rates (Caito et al., 2018; Rand & Caito, 2019; Rand et al., 2016). This is because microbiome composition is plastic and highly variable

between individuals (Koontz et al., 2019; National Academies of Sciences et al., 2017). However, this aspect has not been fully explored and needs to be investigated further.

1.8 The human gut microbiome

1.8.1 What is the Microbiome?

The human microbiome comprises all microbiota, with bacteria being the predominant component, alongside protozoa, archaea, eukaryotes and viruses, which symbiotically or commensally inhabit the human body and the collective genomes that they possess (Kho & Lal, 2018; Peterson et al., 2009). The human microbiome is divided into distinct communities. This includes the gut microbiome, oral microbiome, skin microbiome, respiratory microbiome and vaginal microbiome (Hou et al., 2022). The bacterial-to-human cell ratio within the human body is approximately 1.3:1 in men and 2.2:1 in women (Sender et al., 2016). Among these communities, the gut microbiome is the most densely populated, with the highest concentration (10^{11} cells/mL) inhabiting the colon (Sender et al., 2016). The jejunum, where a majority of MeHg absorption occurs, has a lower density (10^4 cells/mL) compared to the colon and other high-density areas like skin, saliva, the ileum, and dental plaque (Sender et al., 2016).

Each microbial community serves distinct symbiotic functions, including the breakdown of nutrients, fermentation of food, prevention of infection by pathogenic microbiota, vitamin production, immune system development and regulation, and the maintenance of homeostasis (Hillman et al., 2017; Hou et al., 2022; Ottman et al., 2012). In turn, humans provide the

microbiota with the appropriate habitat and nutrients for growth and function (Ottman et al., 2012; Zhou et al., 2013).

1.8.2 How is the Gut Microbiome Acquired and How does it Change?

During development, the fetus lacks a microbiome, which is acquired at birth when the newborn passes through the birth canal and interacts with their mother during the first moments of life (Donovan, 2020). Subsequently, the microbiome continues to develop throughout childhood until the age of 12, closely resembling the fully developed adult microbiome at that point (Derrien et al., 2019).

As previously highlighted, the adult gut microbiome is plastic and exhibits high variability between individuals (Koontz et al., 2019; National Academies of Sciences et al., 2017). This variation is measured in alpha and beta species diversity, species richness, evenness, total abundance, and composition (Falony et al., 2016; Vandeputte et al., 2021; Yatsunenko et al., 2012). Variation can also be measured as functional diversity, encompassing variables such as vitamin biosynthesis, metabolic profile, and disease presence (Jackson et al., 2018; Yatsunenko et al., 2012). Differences between individuals start at birth and are influenced by genetic and environmental factors such as birth method and breastfeeding during infancy (Donovan, 2020). In adulthood, the gut microbiome varies inter-individually based on factors such as age (Mueller et al., 2006; Zhernakova et al., 2016), diet (Falony et al., 2016; Zhernakova et al., 2016), medication (Falony et al., 2016; Jackson et al., 2018; Zhernakova et al., 2016), disease (Falony et al., 2016; Jackson et al., 2018; Zhernakova et al., 2016), gender (Falony et al., 2016; Mueller et al., 2006; Zhernakova et al., 2016) and geography (Mueller et al., 2006;

Yatsunenko et al., 2012). Additionally, intra-individual variations occur over time (Vandeputte et al., 2021), season (Davenport et al., 2014), and location in the GI tract (Flint et al., 2012).

1.8.3 Healthy Microbiomes

Despite significant variation in composition throughout life, two microbiomes with distinct compositions can still be considered "healthy". A presumed core set of microbiota that is common to most healthy adults is predominantly composed of *Firmicutes* and *Bacteroidota*, but also includes *Actinobacteria*, *Proteobacteria* and other bacterial phyla (Flint et al., 2012; Gill et al., 2006; Sweeney & Morton, 2013). The concept of a core microbiome and many subsequent attempts to define a healthy microbiome are often controversial (Shanahan et al., 2021). One explanation for functional similarity despite wide variation in composition is through redundancy – even without certain microbiota, gene overlap covers the missing functionality (Blakeley-Ruiz et al., 2019; Eng & Borenstein, 2018; Shanahan et al., 2021). Rather than relying on single time-point measures like microbiome diversity, richness or abundance, definitions of health should be contextual, considering the broader overall health picture of the individual (Ghosh et al., 2022).

1.8.4 The Gut-Brain Axis

The gut microbiome has far-reaching implications for both health and disease beyond gastrointestinal (GI) health (Hou et al., 2022). A major factor in these implications is the Gut-Brain Axis (GBA), which represents the bidirectional communication between the CNS and enteric nervous system, with the gut microbiome significantly influencing these interactions (Carabotti et al., 2015). The GBA is implicated in many GI disorders with psychological symptoms such as IBS, with intestinal microbiota playing a pivotal role in signalling and

communication between the GI tract and the brain (Margolis et al., 2021). The absorption and potential modification of MeHg introduces an additional layer into this intricate relationship, reshaping our evolving understanding of this system.

1.9 Rationale for Thesis

Despite the extensive research conducted since the Minamata Disaster, MeHg presents unique challenges in health monitoring and risk assessment. Upon exposure, MeHg absorbed into the blood is deposited into the growing hair follicles accumulating over time (Hong et al., 2012; Singh et al., 2023). This hair mercury concentration is proportional to blood mercury concentration (Hong et al., 2012), with the ratio of blood Hg concentration to hair Hg concentration being population-specific (Singh et al., 2023).

However, in attempts to predict MeHg concentration in hair among high-frequency fish consumers, researchers can account for little variation between actual and expected results (Li et al., 2016). Compounding this variation, the half-life of MeHg in individual bloodstreams is measured to have a wide range depending on the individual (Rand & Caito, 2019). This massive variation in elimination/metabolism between individuals makes modelling MeHg extremely difficult (Jo et al., 2015).

One proposed explanation for this variability lies in the differences in gut microbiome, contributing to individual elimination rate fluctuations (Caito et al., 2018; Rand et al., 2016). It has been suggested that the microbiome should be studied as an organ able to metabolize ingested xenobiotics and toxicants (Dheer et al., 2015). Microbiome composition's dynamic and

diverse nature could allow for individual variation in this xenobiotic metabolism (Koontz et al., 2019; National Academies of Sciences et al., 2017). Considering that MeHg exposure primarily occurs through ingestion, and the significant microbial impact on Hg cycling, there is a plausible interface between the microbiome and MeHg metabolism. Given the anticipated rise of MeHg levels within Canada and other arctic countries, there is a pressing need to identify solutions and mitigate risk for populations commonly exposed to MeHg. I aim to explore the relationship between the microbiome and MeHg metabolism, contribute to understanding the associated health risks of MeHg exposure, and inform targeted risk management strategies at an individual level for those vulnerable to MeHg and its impacts.

1.10 Objectives:

The overarching objectives of my thesis are to define interindividual variations in microbial metabolism of MeHg, elucidate the underlying biochemical mechanism used by microbiota to demethylate MeHg, and identify factors contributing to varying abilities among individuals in metabolizing MeHg. Addressing these knowledge gaps is pivotal for refining existing MeHg models to incorporate variations in MeHg demethylation by the microbiome.

The specific aims of this research are:

1. To investigate and define the extent of interindividual variability in microbial MeHg metabolism (Chapter 2).
2. To identify pathways, enzymes, and metabolites that play a role in the microbial metabolism of MeHg (Chapters 2 and 3).

3. To explore the factors that contribute to varying abilities among individuals in metabolizing MeHg. This includes investigating the influence of genetic, environmental, and lifestyle factors on the efficiency of MeHg metabolism by the microbiome. Identifying these factors will provide valuable insights into the determinants of MeHg resistance at the individual level (Chapter 3).

The ultimate goal is to enhance our understanding of individualized MeHg metabolism, enabling for the refinement of risk assessment models. This knowledge will allow the development of personalized risk profiles, allowing individuals to make informed decisions based on their specific microbiome-mediated MeHg demethylation capacity. Individuals with compromised microbiomes or inherently lower MeHg metabolism could take proactive measures to minimize MeHg exposure. Determining the underlying mechanisms of bacterial MeHg metabolism in the gut is a first step toward enhancing an individual's inherent microbial resistance to MeHg. This would allow for better protection of vulnerable individuals who may have depleted microbiomes residing in communities impacted by environmental MeHg exposure and limited viable food alternatives.

CHAPTER 2: IN-VITRO VARIATION IN MICROBIOME DEMETHYLATION RATES, MERCURY SPECIATION, AND METABOLISM INHIBITION

2.1 Introduction

Previously, our lab has demonstrated *in-vitro* demethylation of MeHg in human fecal samples (used to simulate the intestinal microbiome), under conditions mimicking the intestinal environment (Guo et al., 2018). Notably, *in-vitro* demethylation rates varied significantly between two individuals, reflecting the variability seen from *in-vivo* studies on human MeHg elimination rates. Over a 48-hour period, one individual was able to demethylate 52.5% of the MeHg in the media, while the other was able to demethylate 27.3%. When samples were exposed to media that was rich in peptone (simulating a high protein diet), these numbers changed to 90.6% and 21.3% respectively. Considering that demethylated MeHg is more readily eliminated in the Hg(II) and Hg(0) form, the observed increase in demethylation rate suggests a potential implication for an increased *in-vivo* elimination rate of MeHg and hence a decrease in bioavailability and toxicity.

Building upon this foundation, this study aims to broaden our understanding of MeHg demethylation by expanding the cohort to a larger number of individuals. By incorporating a larger sample size, I seek to capture the spectrum of demethylation abilities within the human gut microbiome. By incorporating better representation from a more diverse range of microbial communities, I will attempt to approximate the prevalence of this higher MeHg demethylation phenotype. This approach enables us to discern patterns, identify factors contributing to

demethylation variability, and explore potential links between demethylation rates and individual characteristics.

An intriguing observation is that the demethylation rate was increased for the initial 2 individuals when protein amendments were added to the media to simulate a high-protein diet (Guo et al., 2018). Adding glucose to simulate a carbohydrate rich diet decreased the demethylation rate of the higher demethylating microbiome. I speculate that the gut microbes use protein fermentation as an energy source (Gilbert et al., 2018), leading to increased demethylation. Alternatively, when the energy source for the microbes is glycolysis (Palmnäs-Bedard et al., 2022), this leads to decreased demethylation. Given the established relationships between diet, MeHg exposure, microbiome composition and host health, it is feasible that this interplay is also impacting the metabolism of MeHg in the gut.

A critical concern arises from the potential association of MeHg demethylation with protein fermentation, as protein fermentation leads to the formation of more toxic metabolites and is associated with microbiome dysbiosis, colorectal cancer, and inflammation (Diether & Willing, 2019; Gilbert et al., 2018; Windey et al., 2012; Yao et al., 2015). Many of these studies focus on the balance between protein fermentation and starch fermentation from starches. Starch fermentation to generate energy is a healthier metabolic state for the microbiome (Bird et al., 2000; DeMartino & Cockburn, 2020). Although we have explored how glucose influences MeHg demethylation, the impact of other starches on MeHg demethylation remains unexplored.

Starches can be broken down into 3 categories: rapidly digestible starches (RDS), slowly digestible starches (SDS) and resistant starches (RS) (Raigond et al., 2019). The study by Guo et al. has already investigated the impact of RDS and SDS, as RDS is converted to glucose rapidly

by salivary enzymes in the mouth and upper GI tract, and SDS is also converted to glucose in the small intestine (Raigond et al., 2019). I will investigate RS, as well as two non-starch polysaccharides: highly fermentable (1-3)-β-D-glucan (BDG) and cellulose (Choct, 1997). BDG is derived from oats, is widely available as an over-the-counter supplement, and has been investigated for its ability to increase the fermentation of resistant starches (De Vries et al., 2016; Zyła et al., 2019). Cellulose is a main component of plant cell walls and is poorly digested by humans (Fujimori, 2021).

This investigation assesses the rate, prevalence and metabolic drivers of MeHg demethylation. Furthermore, it delves into the fate of the demethylated mercury species to predict the exact mechanism of demethylation. There are 3 major pathways of MeHg demethylation used as detoxifying mechanisms by Hg-resistant organisms, along with 2 forms of abiotic chemical demethylation. They are: i) reductive demethylation involving the *mer operon*, producing gaseous Hg(0) and CH_4; ii) oxidative demethylation producing Hg (II), CO_2, and some CH_4; and iii) methanotrophic demethylation producing Hg(II), CO_2 and some other unknown products (this process is less well understood than the others). Abiotic demethylation consists of i) photochemical lysis that can produce Hg(0), Hg(II) or HgS, and ii) "dark" demethylation that involves chemical breakdown of MeHg by oxidants or sulfides into Hg(II), HgS, dimethyl mercury (DMeHg) and CH_4 (Barkay & Gu, 2021). A mass balance of the demethylated MeHg will aid to identify potential pathways involved in the demethylation of MeHg, providing insights for future investigations into the MeHg demethylating mechanism and guiding researchers in exploring responsible detoxification mechanisms using similar benchtop experiments and appropriate inhibitors.

2.2 Hypothesis and predictions

Building on prior research, and the available literature, I propose that the human microbiome is playing a critical role in the variation observed in MeHg metabolism. My central hypothesis is that the human microbiome actively contributes to MeHg demethylation, leading to the formation of more easily eliminated species of elemental mercury (Hg^0) or inorganic Hg(II). Since pronounced microbiome diversity occurs even within similar populations, I contend that this variability directly influences MeHg metabolism, contributing to observed differences in MeHg metabolism among populations with comparable mercury exposure.

From our previous research, which appears to show two distinct in-vitro phenotypes, I anticipate observing the same two phenotypes when expanding to a larger population. I predict the existence of a high demethylation phenotype in some individuals, and a low demethylation phenotype in others, less favourable for MeHg detoxification. Those with an increased ability to detoxify MeHg in their gut lumen should show increased elimination and reduced MeHg accumulation compared to those with the low demethylation phenotype.

Additionally, I propose that this demethylation is driven by fermentation, providing the demethylating bacteria with energy for growth and metabolism. I predict that the capacity for MeHg demethylation varies between individuals based on their ability to ferment. Inhibiting fermentation is expected to significantly hinder demethylation, while inhibiting cell respiration is not anticipated to reduce demethylation. I also predict that when treated with fermentable fibre, the demethylation rate in balanced media will match the protein-only demethylation rate. In

contrast, resistant starch is expected to resemble results previously observed with glucose, either showing no change or a decreased demethylation rate

Given the absence of the *mer operon* in the two previously studied individuals (Guo et al., 2018), I also predict that the resulting mercury species will be divalent Hg(II). This aligns with the previously reported absence of methane production in the demethylation reaction (Edwards & McBride, 1975). These hypotheses and predictions aim to explore the impact of the human microbiome on MeHg metabolism, contributing a deeper understanding of the complexity within the gut-brain axis.

2.3 Methodology:

2.3.1 Individual demethylation ability batch incubations:

To test the proposed hypotheses, participants voluntarily provided stool samples and completed a questionnaire assessing relevant factors such as age, seafood consumption habits, dental amalgams existing GI disorders, and recent antibiotic courses (Appendix). This information was to assess potential mercury exposure and the health of the participant's microbiomes. An ethics application was made for the University of Ottawa eReviews system (H-03-20-4702) and was granted on 05/06/2022 (See appendix for ethics certificate).

10 volunteers, aged 21 to 53, were recruited. There were 7 male and 3 female participants. Participants were asked to physically attend the lab to provide the fecal samples. The participants were given a fecal sample collection kit, and the kits were promptly placed in the anaerobic chamber following completion to minimize the death of strict anaerobes.

Collection of the fecal samples and suspension in the anaerobic chamber was completed immediately due to the anaerobic nature of the gut microbiome.

Following placement in the chamber, samples were suspended in Phosphate-buffered saline (PBS) (1 g/10 mL w/v ratio) to control pH and protect cells from lysing. Since protein amendments are known to stimulate the high demethylation phenotype (Guo et al., 2018), the samples were divided and used to inoculate both control (balance) and protein-enriched anaerobic bacterial growth media. For each individual, 5 mL of the fecal/PBS suspension was added to 45 mL of the media. Protein-enriched media contained higher levels of peptone to match the level of protein digestion seen in the jejunum (see appendix for media recipe). The samples were spiked with MeHg [45 nM]. Controls with no fecal sample but spiked with mercury were prepared for each media type to account for background sources of demethylation, including UV photodegradation (Tai et al., 2014). Controls were treated with the same volume of PBS as the experimental bottles (5 mL of PBS in 45 mL of media). Samples were sealed to maintain the anaerobic conditions and incubated in darkness at 37 °C with continuous shaking to replicate human intestinal conditions. Each treatment and control were prepared in triplicate to minimize sampling error. Aliquots of 5mL were taken at 0, 24 and 48 hours, promptly frozen with liquid nitrogen to halt microbial metabolic activity and stored at -20 °C until subsequent mercury extraction and speciation analyses could be performed.

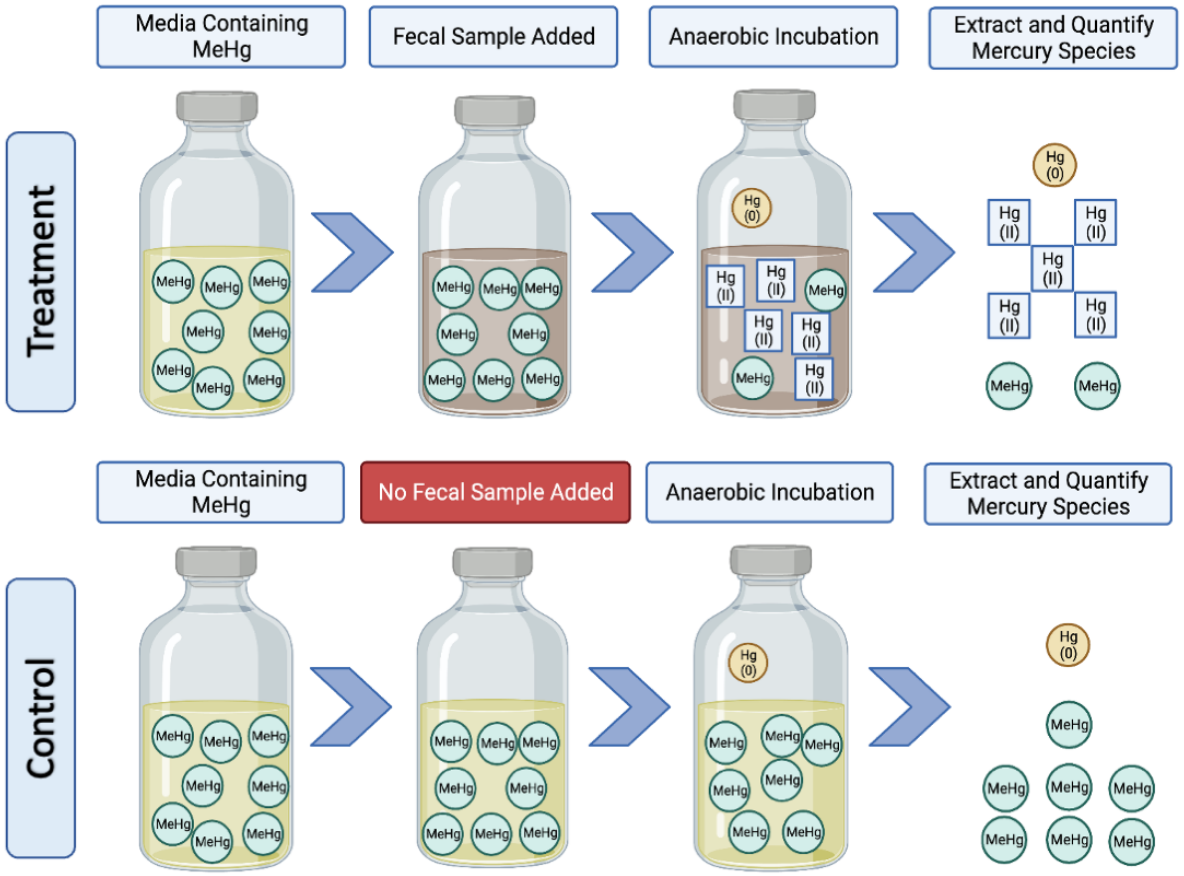

Figure 1:Batch Incubation Visualization. *Experiments were run using media simulating a balance diet, and a protein rich diet (containing increased peptone). Controls were run using the same media (balanced and high protein). Created with BioRender.com*

2.3.2 Mercury Extraction and Speciation

To evaluate demethylation rates, MeHg extraction was performed to isolate the remaining MeHg at each time point. Additionally, the isolation of Hg(0) and Hg(II) during extraction enabled a mass balance to determine the final products of MeHg demethylation.

The 5 mL aliquots from the sample are initially bubbled for 1 min each to remove any gaseous Hg(0) from the aliquots. Following bubbling, 1 mL of the aliquot is added to 3 mL of 4

M trace metal grade HNO_3 and incubated at 55 °C for 16 hours. Subsequently, the aliquots were cooled and 2 g of dichloromethane (DCM) were added. Aliquots were shaken for 2 hours at 330RPM, followed by centrifugation for 10 minutes at 3500 RPM. After centrifugation, 2 mL of the top inorganic layer, containing HNO_3 and Hg(II), was removed and set aside at -20 °C for later analysis. The organic layer containing DCM and MeHg was isolated, weighed and placed in 1mL of 0.01 M L-Cysteine prepared with ultrapure Milli-Q water (Sigma-Aldrich, Germany). This was shaken for 30 minutes at 330 RPM and centrifuged for 10 minutes at 3500 RPM. 200 uL was withdrawn and analyzed for MeHg content using an MA-3000 Mercury Analyzer (Nippon Instrument Corporation, Japan). Actual MeHg concentration was determined using the formula:

$$C_{Hg} = \frac{M_{Hg} \times \left({}^{DCM_{add}}/_{DCM_{rec}} \right)}{V_s}$$

Figure 2: Concentration calculation for MeHg batch incubations. *The formula used to adjust for DCM evaporation and calculate the concentration of MeHg at each timepoint where CHg is the actual concentration of MeHg, DCMadd is the added weight of DCM, DCMrec is the recovered weight of DCM, and Vs is the volume of L-Cysteine added to the sample.*

Two-tailed heteroscedastic student's t-tests were conducted on each individual's samples. Specifically, these tests compared the protein-rich samples with the protein-rich control, the balanced media samples with the balanced control, and the protein-rich sample with the balanced sample. The purpose of these tests was to determine the significance of each individual's demethylation rate compared to the control conditions demethylation rate and to determine whether the protein media significantly elevated the demethylation rate in comparison to the balanced media.

The inorganic layer was thawed and analyzed using a modified version of EPA Method 1631 (United States Environmental Protection Agency, 2002). I did not add BrCl to the sample, to prevent any remaining MeHg from reacting with $SnCl_2$. This approach facilitated the quantification of Hg(II) produced from MeHg demethylation. A mass balance was performed to identify the MeHg demethylation pathway (reductive or oxidative). Absence of Hg(II) and low recovery would suggest Hg(0) as the main product. Conversely, quantifiable Hg(II) and satisfactory Hg recovery would indicate Hg(II) and not Hg(0) as the final product (Gu et al., 2011; United States Environmental Protection Agency, 2002).

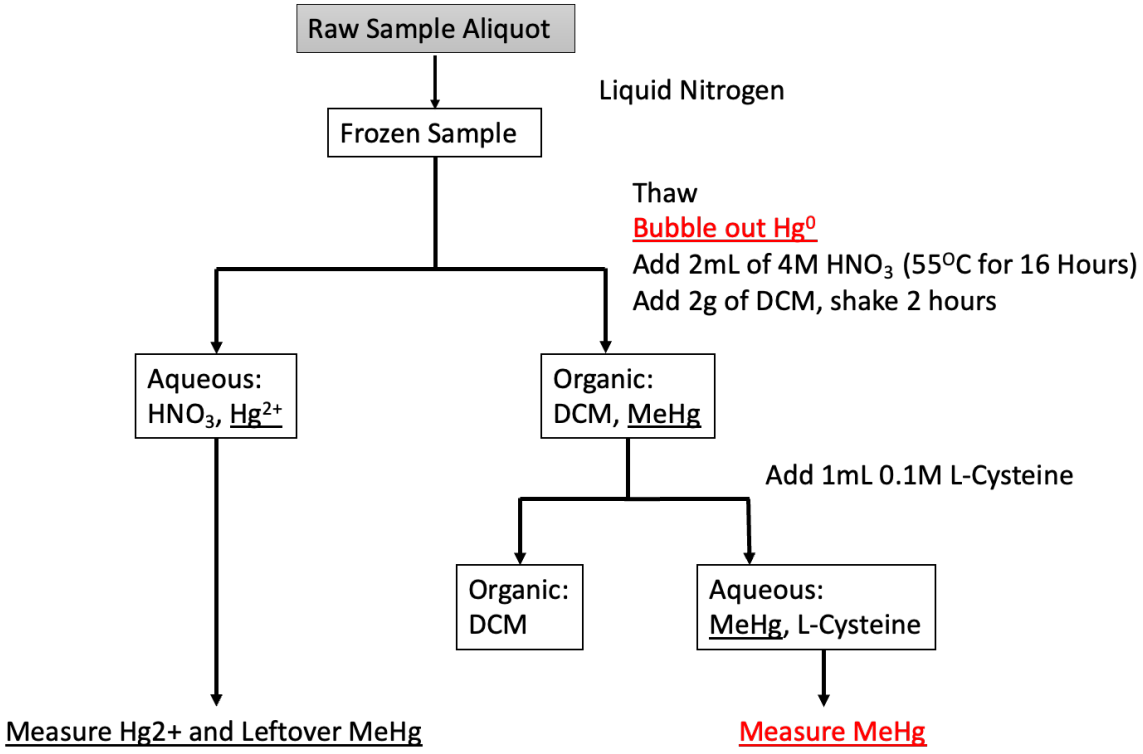

Figure 3: Mercury Extraction Protocol Overview.

2.3.3 Inhibition of Cell Metabolism and Fermentation Incubations

To test if protein fermentation was related to MeHg demethylation, I repeated incubations using the same methodology as previous batch incubations. In these repeated experiments using one individual (Individual A), I added 2 inhibitors at different concentrations. The first inhibitor of pyruvate oxidation is nitazoxanide (NTZ), an inhibitor of pyruvate ferredoxin oxidoreductase (PFOR). PFOR catalyzes the conversion of pyruvate into acetyl-CoA during anaerobic fermentation (Wang et al., 2022). NTZ was added in 3 concentrations, 10 uM, 50 uM and 100 uM (Lü et al., 2021). Dimethyl sulfoxide (DMSO) is a carrier for NTZ, so another set of controls was prepared to control for any increase or reduction in demethylation due to the presence of DMSO. Extraction methodology followed the same protocol as before, but mercury speciation was not performed. MeHg was extracted at T0 and T48 (Figure 7).

These experiments were repeated with a protonophore, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) using another sample from Individual A. CCCP was added to the incubations at concentrations of 0.1 uM, 1.0uM and 10uM (Lim et al., 2001). MeHg concentration was quantified at T0 and T48 (Figure 6).

To test for the impact of starch/fibre fermentation, the batch incubations were performed a final time with samples from Individual A. These were performed using 5% soluble starch, 10% cellulose and 100 uM of (1-3)-β-D-glucan (BDG) (Figure 8) (Zhang et al., 2018).

To compare significance between treatments, one-way analysis of variance (ANOVA) was performed with Tukey multiple comparisons using RStudio (version 1.4.1106) (R Core Team, 2023) and the base functions aov/TukeyHSD.

2.4 Results

2.4.1 Individual Demethylation Rates

All 10 invited volunteers agreed to participate with 100% participation rate. The MeHg in the fecal samples collected from 8 of 10 individuals exhibited significant ($p < 0.05$) demethylation above fecal-negative controls over 48 hours (Figure 4). However, the demethylation amount and rate varied significantly among participants. Some achieved complete demethylation of MeHg approaching the limit of detection (LOD) for the MA-3000, while others exhibited only slight demethylation that is not statistically significant ($p > 0.05$). Notably, all individuals demonstrated significantly ($p < 0.05$) enhanced *in-vitro* demethylation when subjected to the simulated high protein diet. These high protein demethylation rates were significant ($p < 0.05$) compared to both the fecal negative control and the balanced media test (Figure 4).

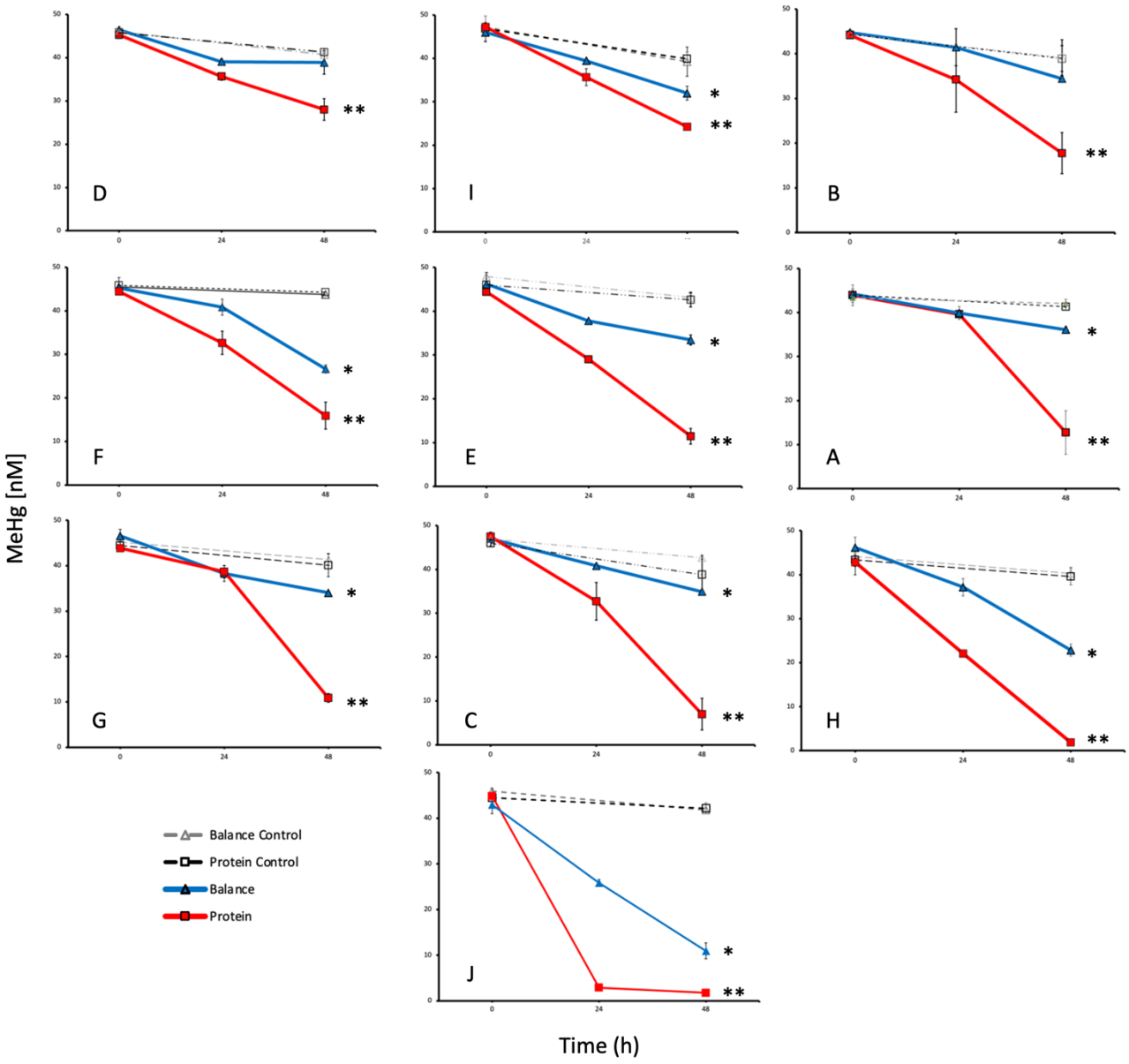

Figure 4: Demethylation rate of recruited participants microbiomes. Concentration of MeHg shown over time (n=10). *Concentrations are calculated according to the formula shown in figure 2. Individuals correspond to the letter in each corner and are ordered according to demethylation rate (left to right, top to bottom)..* indicates significant difference (p <0.05) between balance sample and balance control. ** indicates significant difference (p <0.05) between the protein treatment and both the balance treatment and protein control.*

Investigating Hg(II), revealed an evident inverse relationship over time between the concentration of MeHg and Hg(II). Figure 5 shows one high (Individual H) and one low demethylator (Individual D), measuring the MeHg concentration and Hg(II) concentration over time. As MeHg concentration decreases, Hg(II) increases proportionally for both individuals. For the mass balance, these experiments yielded a recovery rate between 75-95% for most participants (Table 1) with the exception of one individual (Individual F). Please note that Individual B was not included in the mass balance as their sample was not stored in a manner that would allow for total mercury analysis.

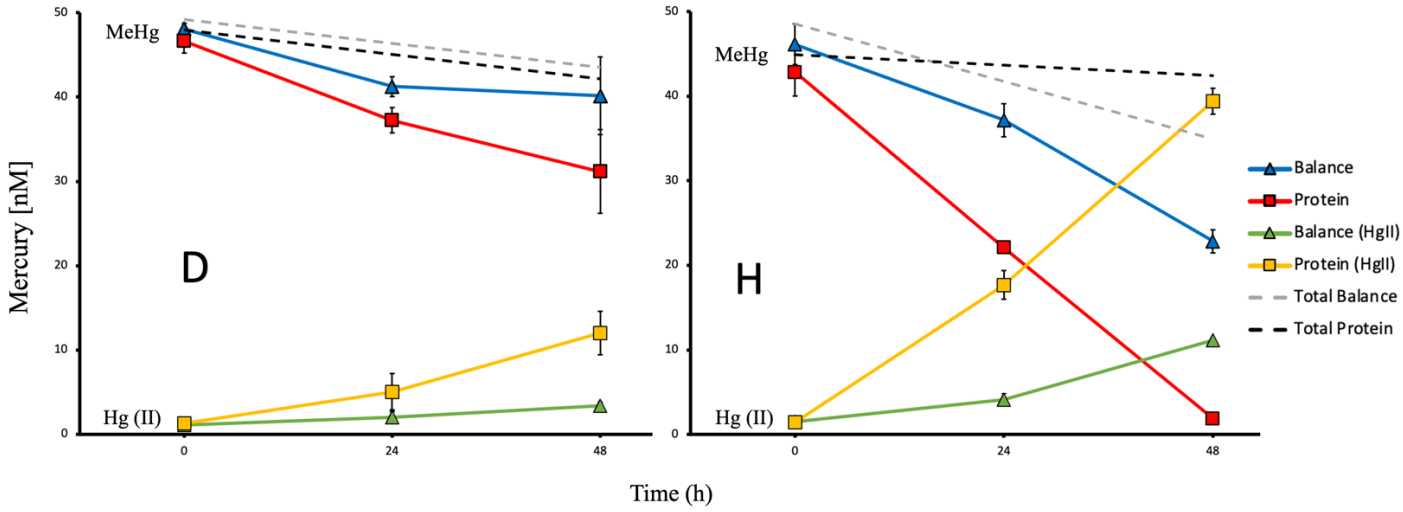

Figure 5: Concentration of MeHg (top) and Hg(II) (bottom) over time for a low (D) and high (H) demethylating microbiome. *Both balanced and high protein media are shown with total Hg (dashed lines).*

Individual	Initial MeHg [nmol]	Final MeHg [nmol]	▲MeHg	Hg(II) [nmol]	% Recovery
A (Balance)	41.63	29.89	11.75	8.85	89.26
A (Protein)	46.66	9.67	36.99	36.12	95.30
C (Balance)	49.65	36.95	12.70	3.60	81.68
C (Protein)	49.49	7.45	42.04	32.80	81.62
D (Balance)	48.07	40.15	7.92	3.39	88.50
D (Protein)	46.63	30.12	16.52	12.00	87.89
E (Balance)	46.84	33.95	12.89	6.53	83.49
E (Protein)	44.83	11.47	33.36	30.87	91.10
F (Balance)	46.63	28.08	18.55	7.59	74.25
F (Protein)	47.36	17.39	29.97	14.37	65.00
G (Balance)	47.52	34.89	12.63	3.38	78.39
G (Protein)	44.64	11.94	32.70	29.40	89.69
H (Balance)	47.00	23.79	23.21	11.16	72.08
H (Protein)	43.42	3.04	40.38	39.42	94.82
I (Balance)	47.32	32.97	14.34	4.54	77.24
I (Protein)	48.53	26.02	22.51	14.59	81.47
J (Balance)	43.55	12.87	30.68	26.62	87.67
J (Protein)	45.61	9.23	36.44	25.80	74.64

Table 1: Mass balance for MeHg and Hg(II) for 9 of the 10 participants. *ΔMeHg represents the change in MeHg from T0 to T48.*

2.4.3 Protein/Starch Fermentation and Cellular Respiration

Due to difficulties in recruiting individuals and the requirement of fresh fecal samples for batch incubations, fermentation and cellular respiration inhibition experiments were performed using only Individual A to test the efficacy of each treatment. Inhibiting cell respiration demonstrated no change in demethylation at 48 hours across concentrations in the balanced media ($p = 0.225$). In high protein media, the highest concentration [10μM] of CCCP increased demethylation in high protein media compared to both the control ($p = 0.495$) and medium [1μM] CCCP concentration ($p = 0.007$) (Figure 6).

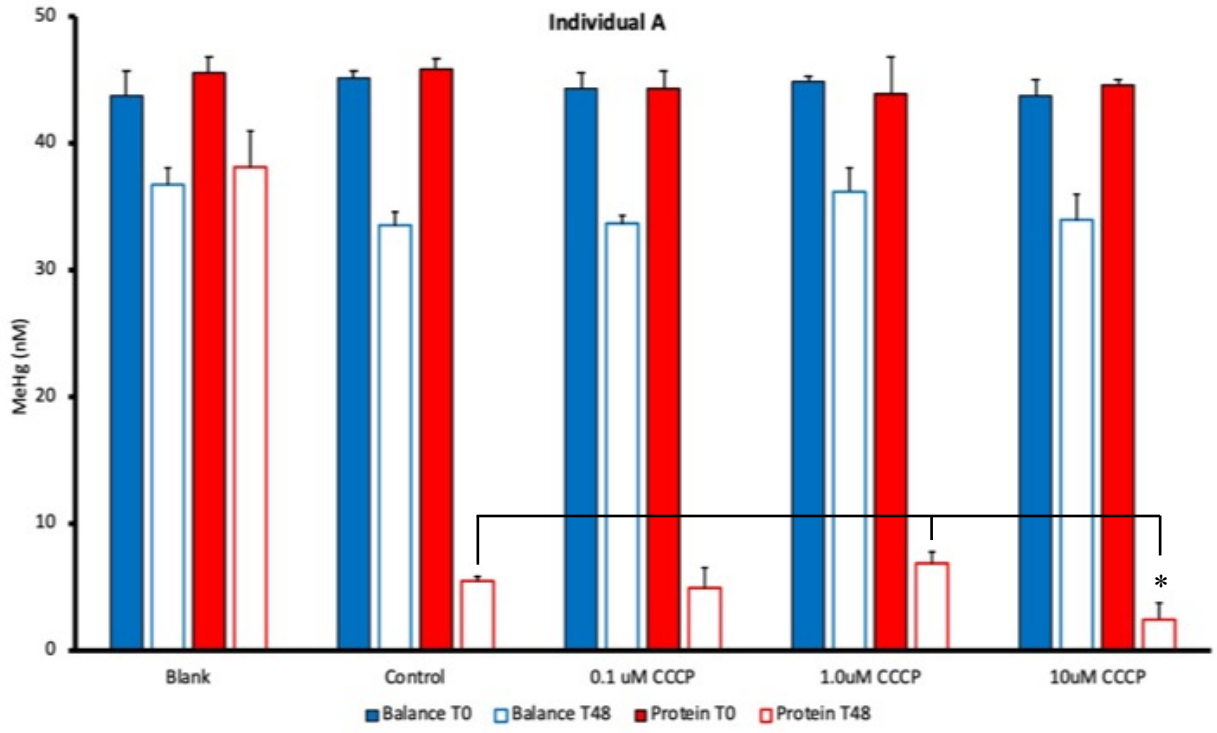

Figure 6: Use of a protonophore (CCCP) to inhibit cellular respiration. *Blank contains no fecal sample, Control contains fecal sample with no inhibitor and the others contain the stated concentration with fecal sample added. Blue is balance and red is high protein. MeHg is measured at 0 and 48 hours with standard deviation shown. * indicates a significant difference (p = <0.05) between treatments.*

NTZ treatment, using the DMSO control as a baseline, exhibited no difference among the balance treatments (p = 0.0817) (Figure 7). There was a significant decrease in demethylation as NTZ concentration increased (p = 0.011) in high protein media. At the highest and middle concentration tested ([100μM] and [50μM]), demethylation rate showed insignificant variation from the germ-free blank media ((p = 0.783) and (p = 0.265)).

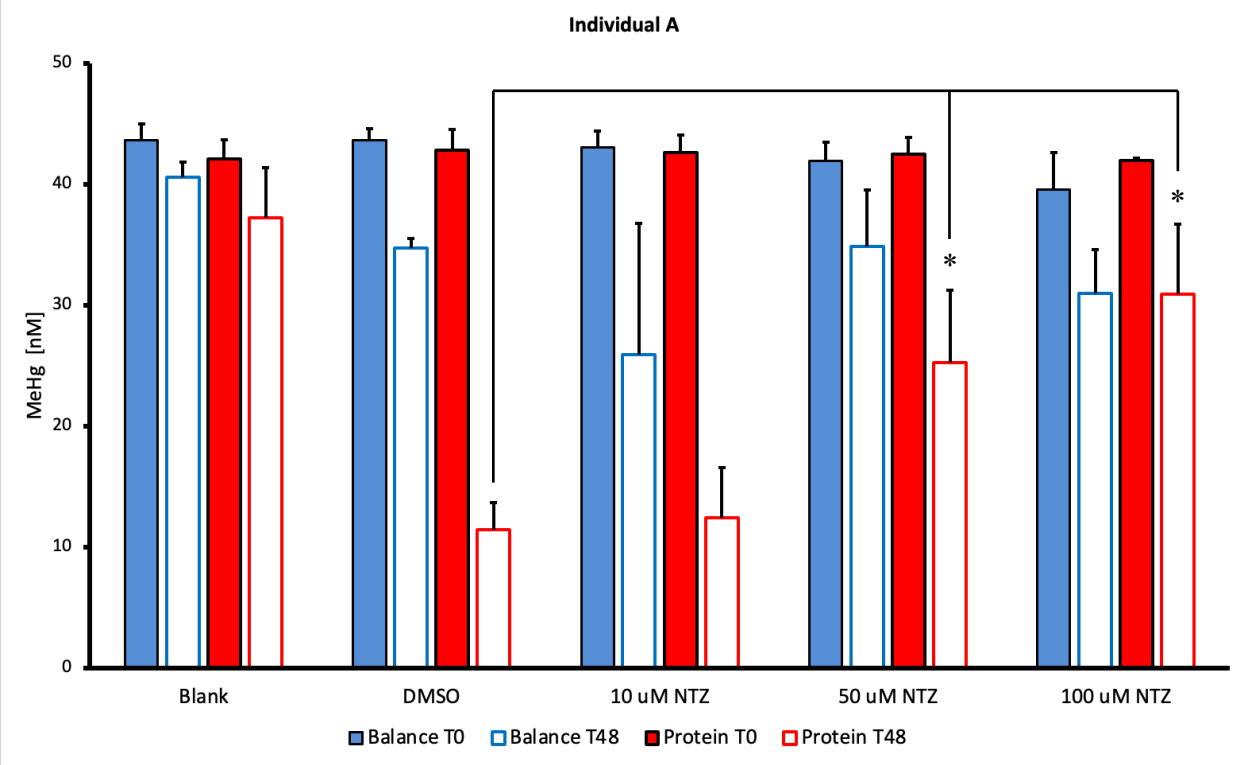

Figure 7: Inhibition of protein fermentation using nitazoxanide (NTZ). *Blank contains no fecal sample, DMSO contains fecal sample with no inhibitor (DMSO to control for the effects of the carrier) and the others contain the stated concentration with fecal sample added. Blue is balance and red is high protein. MeHg is measured at 0 and 48 hours with standard deviation shown. * indicates a significant difference (p = <0.05) between treatments.*

Exploring different fermentable substrates (Figure 8), RS showed insignificant change in both balanced (p = 0.783) and high protein media (p = 0.942) compared to the DMSO control. Cellulose and BDG increase demethylation in the high protein media ((p = 0.010) and (p = 0.016)), but not in balanced media ((p = 0.997) and (p = 0.504)) when compared to the DMSO control. Notably, cellulose, due to insolubility, hindered MeHg extraction evident in lower concentrations at time 0 for both media types.

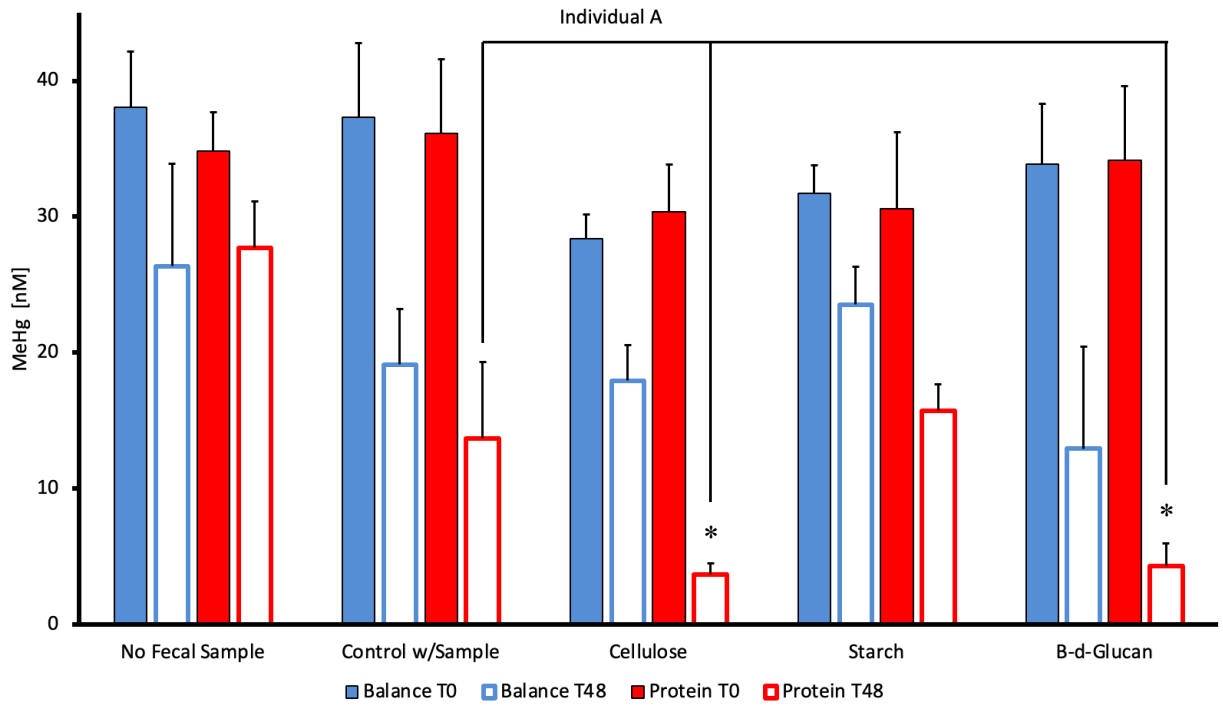

Figure 8: Impacts of starches and fibres on protein fermentation. *Blank contains no fecal sample; Control contains fecal sample with no added fibres/starches; and the others contain the stated fibre/starch with fecal sample added. Blue is balance and red is high protein. MeHg is measured at 0 and 48 hours. Standard deviation is shown. * indicates a significant difference (p = <0.05) between treatments.*

2.5 Discussion

2.5.1 Individual Demethylation Rates and Protein Treatment

In examining individual demethylation rates, our initial anticipation of a distinct high and low demethylation phenotype evolved into a spectrum across the 10 tested individuals. Rather than a clear separation between high and low (Figure 4), the observed variation aligns with the complex variation observed in MeHg elimination *in-vivo* (Li et al., 2016; Rand & Caito, 2019), emphasizing the need for in-depth investigation to compare these *in-vitro* rates to *in-vivo* elimination. This could be achieved through controlled-dose studies correlating in-vitro

demethylation rates with hair mercury content using experimental design similar to recent studies (Caito et al., 2018). This study design also allows us to test the effectiveness of *in-vitro* interventions like supplementation with BDG. Knowledge of these individual demethylation rates is a potential replacement for current models that either assume constant demethylation rates by the microbiome (Pope & Rand, 2021), or calculate individualized rates based on fecal Hg content (Rand et al., 2016).

Increasing the level of peptone in the media heightened the level of demethylation across all individuals (Figure 4), confirming prior laboratory results (Guo et al., 2018), and providing a starting point for further inquiry described in Chapter 3. The consistent impact of peptone on demethylation rate suggests a plausible connection to protein fermentation by the microbiome. Individual variations in fermentation potential may underpin the diversity in demethylation rates, raising mechanistic possibilities such as specific fermentative bacteria, MeHg mimicking substrates, or by-products of fermentation influencing demethylation.

2.5.2 Mercury Speciation and Demethylation Pathway

The recovery of spiked MeHg as Hg(II) aligns with oxidative or methanotrophic demethylation, eliminating reductive demethylation as a predominant pathway. The absence of the *mer operon* in our previous research further supports this conclusion. Exclusion of UV radiation during incubation further suggests oxidative, methanotrophic, or "dark" abiotic demethylation as potential remaining mechanisms.

While oxidative demethylation is strongly supported, it has been reported that oxidative demethylation of MeHg by anaerobic bacteria such as sulfate reducing bacteria (SRB) is falsely

reported and does not account for the interaction between MeHg and high concentrations of H_2S in the environment (Kanzler et al., 2018). Protein fermentations does produce higher levels of H_2S (Yao et al., 2018), and this could explain why demethylation is always higher in protein rich media. However, this is reported for MeHg degradation analysis via direct ethylation. Our method of MeHg and total mercury (THg) analysis (liquid-liquid extraction and thermal decomposition-gold amalgamation) would not falsely report these end products as demethylated MeHg, but as MeHg that was never demethylated. Therefore, any abiotic demethylation from decomposition with H_2S would theoretically not be reported instead of oxidative demethylation but would be occurring in addition to oxidative demethylation.

There is still evidence to support the "dark" demethylation hypothesis. The insoluble intermediate of this reaction [$(CH_3Hg)_2S$] also sticks to glassware during extraction. This has been reported to decrease recovery of Hg in mass balance experiments and could further explain why recovery is less than 100% in our analysis (Kanzler et al., 2018; Zhang et al., 2020). This effect would be predicted to be more prominent in high protein media, since H_2S would be expected to be in higher concentration. From our results, we see that the recovery is similar for high and low protein media. This implies that oxidative or methanogenic demethylation is still the dominant pathway, and abiotic demethylation via H_2S is occurring in negligible or un-accounted for. Although evidence strongly supports the oxidative rather than "dark" demethylation, this pathway should be explored in the future. A suggested experiment to confirm this and quantify the level of demethylation from H_2S in these experiments is to measure H_2S production in the media, recreate the H_2S concentration in the germ-free control bottles and detect MeHg concentration over time. If the level of demethylation matches the individual demethylation level, there would be significant proof of this pathway.

The direct relationship observed between MeHg and Hg(II) concentrations supports oxidative or methanotrophic demethylation in the human gut microbiome. Further metagenomic analysis in Chapter 3 will explore the influence of methanotrophic bacteria on demethylation. For the outlier individual (F), metagenomic analysis in Chapter 2 will be used to determine if there is presence of the *mer operon*, potentially explaining the low recovery due to increased Hg(0) production.

2.5.3 Protein/Starch Fermentation and Cellular Respiration

As predicted, inhibiting protein fermentation reduces demethylation while inhibiting cellular respiration has no effect. The decline in demethylation with increasing NTZ concentration supports the hypothesis of protein fermentation driving the demethylation reaction. Fermentation has been implicated in Hg detoxification before, being identified as a key pathway in Hg reduction in anoxic environments (Grégoire et al., 2018). Although driven by fermentation, I show that demethylation of MeHg is oxidative in nature (producing Hg(II)). This aligns with previous research that shows an absence of CH_4 generated in gut MeHg demethylation (Edwards & McBride, 1975).

The logical next step would be to test if increasing protein fermentative conditions in the gut leads to increased demethylation and increased MeHg elimination for hosts. However, the potential health risks associated with protein fermentation by-products, such as H_2S, ammonia, and *p*-Cresol, warrant careful consideration (Diether & Willing, 2019; Windey et al., 2012).

Interestingly, our results deviate from the expected decrease in protein fermentation with increased starch fermentation (Diether & Willing, 2019), as fermentable starches increased

demethylation in high protein media. Of particular interest is the ability of BDG to increase demethylation in high protein media. BDG, is a widely available prebiotic (Vetvicka et al., 2019) that is also used in clinical diagnostic testing of fungal infection (Theel & Doern, 2013). BDG presents a potential means to modulate MeHg degradation in the gut, offering avenues for risk reduction. However, further exploration into the intricate balance between protein and carbohydrate fermentation in MeHg metabolism is imperative.

To refine our understanding, future studies may employ metabolomics to accurately measure protein and carbohydrate fermentation levels in the media. The ratio of branched chain fatty acids (BCFAs) to short chain fatty acids (SCFAs) could serve as an indicator (Rios-Covian et al., 2020; Silva et al., 2020; Trefflich et al., 2021). BCFAs are produced by bacterial catabolism of branched amino acids while SCFAs are produced by bacterial catabolism of carbohydrates and other amino acids in the gut (Rios-Covian et al., 2020). Higher BCFA/SCFA ratios could potentially correlate with superior demethylators if protein fermentation is the primary driver of MeHg demethylation.

2.6 Limitations

An evident constraint in our study stems from the *in-vitro* nature of the experiments. Despite replication of small intestine conditions in the media (matching pH, temperature, oxygen concentration, and nutrient composition), some conditions cannot be matched *in-vitro*. Fecal samples, serving as proxies for an individual's colonic microbiome, introduce limitations as the colonic microbiome differs in microbial composition across distinct segments of the

gastrointestinal (GI) tract (Sundin et al., 2017). The fixed system utilized in batch incubations overlooks the nuanced dynamics of MeHg absorption, potential pre-demethylation absorption, and the continuous influence of MeHg/Hg(II) from successive meals and entero-hepatic cycling. Comprehensive in-vitro modeling would require a more intricate representation of the dynamic GI environment using tools such as a simulator of the human intestinal microbial ecosystem (SHIME) (van de Wiele et al., 2015).

A notable limitation also arises from the modest increase in participant numbers from n = 2 to n = 10, highlighting the challenge of producing a significant sample size. While this expansion revealed substantial variation in demethylation rates, caution is warranted in generalizing these findings to the broader population. Ideally, a comprehensive clinical trial would involve a more extensive and diverse participant pool, encompassing various genders, age groups, races, and geographical locations, ensuring a full understanding of MeHg demethylation dynamics. The wide variability observed in our small sample emphasizes the need for further studies with larger and more diverse populations to truly capture the spectrum of demethylation abilities. Additionally, repeated study of the same individuals MeHg demethylation rates over time would allow for the capture of intra-individual variability.

The translation of inhibitor (NTZ, CCCP) and nutrient concentrations (cellulose, resistant starch, BDG) from pure bacterial or cellular cultures to complex fecal microbiome samples poses a methodological challenge. Fecal microbiomes complicate the direct application of concentrations derived from the literature. Bridging the gap from organismal-level studies, such as mouse feed containing 5% cellulose, to microbiome studies requires careful consideration of nutrient bioavailability to the microorganisms engaged in MeHg demethylation. The complex

fecal microbial milieu demands nuanced interpretation and application of established concentrations.

Considering nutrient dynamics, an important limitation emerges concerning protein fermentation in the small intestine—the primary site of MeHg absorption. Protein fermentation is more pronounced in the distal colon and increases when host enzyme digestion is limited (Rist et al., 2013). Given the restricted protein fermentation in the small intestine, the potential interface between fermentative bacteria and MeHg might be constrained at the absorption site. This limitation further emphasizes the need for exploration of nutrient dynamics specific to MeHg and the small intestine's microbial landscape.

CHAPTER 3: METAGENOMIC ANALYSIS OF INDIVIDUAL MICROBIOMES

3.1 Introduction

The human microbiome, an emerging frontier in health research, has garnered increased attention as advances in genomic sequencing technologies make it more accessible for exploration (Gao et al., 2023; Proctor, 2019). Traditionally, research focused predominantly on disease-causing microbes, neglecting the intricate interplay between environmental toxicants and the microbiome (Peterson et al., 2009). With the cost of genomic sequencing decreasing drastically in the last decade (NIH & Wetterstrand, 2021; Peterson et al., 2009), these technologies have been more accessible to investigate the interactions between the microbiome and the ingested environmental chemicals.

The microbiome has a large impact in an individual's overall health, particularly through the gut-brain axis (GBA) (Appleton, 2018; Benakis et al., 2020; Margolis et al., 2021). Given MeHg's toxicity to both bacteria and the CNS, MeHg could impact on this very complex system (Pinto et al., 2020). Existing evidence suggests acute and sub-chronic MeHg exposure can perturb the gut microbiome, shift intestinal communities and alter GBA metabolites in rats (Lin et al., 2020; Zhao et al., 2020). It has also been shown in mice that disruption of the gut microbiota caused by MeHg exposure increases the accumulation of MeHg in organs since there is less microbiota available to demethylate MeHg (Seki et al., 2021). It is worth noting that these disruptions have not been observed in humans. They are occurring at high concentrations and in mice/rats, which are not good models for the extremely complex human microbiome

communities (Fritz et al., 2013). In pregnant women, microbiome composition was found to be associated with MeHg concentration in the blood. However, this only correlated with early gestation and was not associated in late gestation (Rothenberg et al., 2019). Far more human research and epidemiological data are necessary.

In alignment with the broader GBA, the reciprocal impact of MeHg and the microbiome on each other and the CNS underscores the importance of understanding the underlying bacterial processes responsible for MeHg demethylation. This knowledge will form the foundation for manipulating this relationship to enhance overall health by manipulating the microbiome to mitigate MeHg burden on the CNS. Importantly, every individual in both Chapter 2 and this chapter possesses functional, healthy microbiomes with no reported GI disorders (Rinninella et al., 2019).

Drawing from Chapter 2 results and prior lab research (Guo et al., 2018), our findings emphasize the influence of protein fermentation on MeHg demethylation (Figure 2) (Guo et al., 2018). This revelation, coupled with the inhibitory effect on demethylation when protein fermentation is hindered, underscores the significant implications of diet on MeHg demethylation. Given that food and nutrient levels directly impact microbiome structure and function (Choct, 1997; Rios-Covian et al., 2020; Rist et al., 2013; Trefflich et al., 2021; Walker et al., 2010), the investigation into this relationship has major implications for diet and its impact on the microbiome regarding MeHg demethylation.

To investigate this relationship, I employed a shotgun metagenomic analysis. This was chosen over amplicon sequencing (16S rRNA) due to the gut microbiome's complexity and the pivotal role of functional potential. Unlike taxonomic analyses of health, functional microbiome

health remains stable due to gene redundancy (Blakeley-Ruiz et al., 2019; Eng & Borenstein, 2018; Shanahan et al., 2021; Vandeputte et al., 2021) and high instances of horizontal gene transfer (Groussin et al., 2021; Huddleston, 2014; Lerner et al., 2017). This makes shotgun metagenomics more adept at investigating the microbiome's functionality, while amplicon sequencing would only allow us to infer metabolic activity based on the presence or absence of microbes (Pérez-Cobas et al., 2020). Additionally, there is evidence that shotgun metagenomics is better suited for taxonomic characterization of gut microbiota, making it the more effective approach in either case (Durazzi et al., 2021). With the goal of understanding the relationship between different metabolic pathways and MeHg demethylation, I focused on fermentation as a starting point, expanding our exploration to pathways involving acetyl-CoA (Since PFOR catalyzes the reaction of pyruvate to acetyl-CoA in anaerobic bacteria (Ballard et al., 2011)), sulfur metabolism and methanotrophy based on Chapter 2 findings.

Lastly, this investigation extends to the presence or absence of genes related to the *mer operon* (Boyd & Barkay, 2012). Given the observed association between Hg detoxification/resistance and antibiotic resistance (Barkay et al., 2003), I broadened the scope to encompass genes related to vancomycin and beta-lactam resistance, aiming for a comprehensive understanding of Hg resistance. This multifaceted approach sets the stage for unraveling the intricate interplay between MeHg, the microbiome, and the human host.

3.2 Hypothesis and predictions

In this exploratory chapter, the primary objective is to unveil genes or metabolic pathways exhibiting greater prevalence among higher demethylators compared to individuals with lower demethylating microbiomes. The overarching aim is to unravel metabolic pathways correlated with heightened MeHg demethylation, laying the groundwork for the generation of testable hypotheses for specific pathways.

I first explored the role of fermentation on MeHg demethylation, building upon insights from Chapter 2's findings. I posit that certain metabolic pathways, potentially harboring our demethylating gene of interest, will exhibit higher abundance in individuals with superior demethylation capabilities. I anticipated that diets favoring these pathways would correspondingly increase the MeHg demethylation rate. The purpose of this research is to identify and understand the metabolic landscape that contributes significantly to MeHg demethylation.

Based on the prior research, I hypothesize that protein fermentation, specifically amino acid degradation, represents the most favorable metabolic pathway for MeHg demethylation. To test this hypothesis, I isolated genes from various metabolic pathways and scrutinized their correlation with variations in demethylation rates.

Additionally, I revisited the hypothesis from Chapter 2, challenging the notion that reductive demethylation, facilitated by the *mer operon*, serves as the primary mechanism for MeHg demethylation. Analysis was conducted on samples from all participants to evaluate the presence of the *mer operon*. Given our preliminary findings, I do not anticipate detecting any genomic components of the *mer operon* (Guo et al., 2018), further substantiating the notion that an alternative mechanism is responsible for MeHg demethylation in the studied microbiomes.

3.3 Methodology

3.3.1 DNA Extraction

During incubations conducted in Chapter 2.3.1, 5mL aliquots were reserved for DNA extraction. These included samples from both the balance and protein media at 0 and 48 hours. These were promptly frozen in liquid nitrogen and stored at -20°C until DNA extraction could be performed. Once ready for extraction, samples were thawed at room temperature. In Chapter 2.3.1, batch incubations were performed in triplicate. DNA from these triplicate samples was pooled (combining 3 balance T0 aliquots into 1 balance T0 sample for extraction). 1mL from each aliquot was removed and transferred into a sterile 10mL centrifuge tube. To concentrate the DNA, tubes were centrifuged for 5 min at 3000 RPM, followed by resuspension in 500uL of ultrapure Milli-Q water (Sigma-Aldrich, Germany). After suspension, DNA was extracted using a QIAamp PowerFecal Pro DNA Kit (Qiagen, Germany). If needed, DNA was diluted following Genome Quebec's protocol. Samples were shipped overnight on dry ice for 150bp shotgun sequencing (Genome Quebec, Montreal).

3.3.2 Metagenomic Pipeline and Analysis

A total of 4 sequences were downloaded to the Aliance Canada Server for every individual (n=10) (Balance T0, Protein T0, Balance T48, Protein T48). Additionally, data from the preceding 2 individuals (Guo et al., 2018) was also incorporated, resulting in a total of n=12. These individuals each contributed 3 DNA samples (Balance T0, Balance T48 and Protein T48).

The sequencing pipeline was adapted from a methodology used in the analysis of mercury methylation and demethylation in rice paddy soils (Zhang et al., 2023). Sequences were analyzed for quality, length and repeated sequences using FastQC (Andrews, 2010). Subsequently, adapters were trimmed from sequences using fastp (Chen et al., 2018). Summarization of FastQC reports was summarised using MultiQC (Appendix) (Ewels et al., 2016).

Trimmed reads were co-assembled using MEGAHIT (D. Li et al., 2015) to generate contigs, and metaQuast (Mikheenko et al., 2016) provided preliminary statistics on these contigs, including the quantity, length and GC content (available as supplemental data). Contigs with lengths below 1000 bp were removed using anvi'o (Eren et al., 2021). Eukaryotic sequences were removed using EukRep (West et al., 2018) to filter out any potential host DNA. Contig databases were generated, and contigs were mapped accordingly. Anvi'o was used to create a profile database for each individual and generate taxonomy based on the most abundant single copy gene (SCG) using "anvi-estimate-scg-taxonomy". This identified and used the ribosomal S2 gene (rS2) as the most abundant SCG. Hidden Markov Models (HMMs) were employed to functionally annotate genes from the Kyoto Encyclopedia of Genes and Genomes (KEGG) kofams database (Aramaki et al., 2020; West et al., 2018). Gene coverage for each individual and treatment was calculated and normalized by adding the coverage for the gene on in each contig and dividing by the total coverage for the highest SCG (rS2) (Zhang et al., 2023).

Genes for fermentation (identified from the MetaCyc database (Caspi et al., 2020)), glycolysis, the TCA cycle, gluconeogenesis, methanogenesis, nitrate fixation/reduction, sulfate reduction, cell respiration, sulfur metabolism, mercury and antibiotic resistance were all

extracted from the KEGG MODULE database (Jin et al., 2023). Correlation matrices were performed in RStudio (version 1.4.1106) (R Core Team, 2023) using the base function cor to calculate Pearson Correlation, comparing metadata collected from the individuals studied and demethylation rates to gene coverage for each gene identified from KEGG. Demethylation rates were calculated for both the protein and balance media using the slope of the lines from T0 to T48 (Figure 4). Metatata was collected from the questionnaire provided to participants when samples were taken (Appendix). Correlation matrices were plotted using pheatmap, where the scale ranges from -1 (strong negative relationship) to 1 (strong positive relationship), with a value of 0 indicating no relation. (https://CRAN.R-project.org/package=pheatmap). Specific values are interpreted against Evans' classification of magnitude where < 0.2 is very weak, 0.2-0.39 is weak, 0.4-0.59 is moderate, 0.6-0.79 is strong and > 0.8 is very strong (Evans, 1996; Papageorgiou, 2022).

Sequences of Mer A hits identified by scanning HMMs were translated using "anvi-get-sequences-for-HMM-hits" and aligned using MAFFT (Katoh et al., 2018). Amino acid sequences were aligned to a reference MerA and true positives were identified using key amino acid residues from the reference (Christakis et al., 2021). These residues include a pair of cysteine (C) residues at the carboxy terminus (position 628 and 629), a cysteine pair at 207 and 212, a tyrosine (Y) at 264, and an addition tyrosine at 605 (in the archaeal MerA, this tyrosine is replaced with phenylalanine (F)).

Alpha and beta diversity were both calculated in RStudio (R Core Team, 2023) using the Vegan package for community ecology (https://CRAN.R-project.org/package=vegan) (Oksanen et al., 2022). Diversity figures were plotted in RStudio using ggplot2 (Wickham, 2006).

3.4 Results

3.4.1 Taxonomic Diversity

The most notable variation among the 12 individuals arises from the initial composition of the additional 2 individuals (K and L) collected previously in the lab. These two participants had significantly higher representation of bacteroidota at the phylum level. Despite these differences, there is no apparent pattern relating to demethylation ability based on phylum-level-taxonomy.

There are unexpected differences when comparing high protein and balanced media treatments. While there is a significant shift in the composition between time 0 and time 48 for every individual this change is more pronounced in the balanced media than in the high protein media (Figure 9). Examination of diversity measures at time 0 reveal that the balanced and high protein media are equally diverse, tending to cluster together except for outliers belonging to individuals K and L.

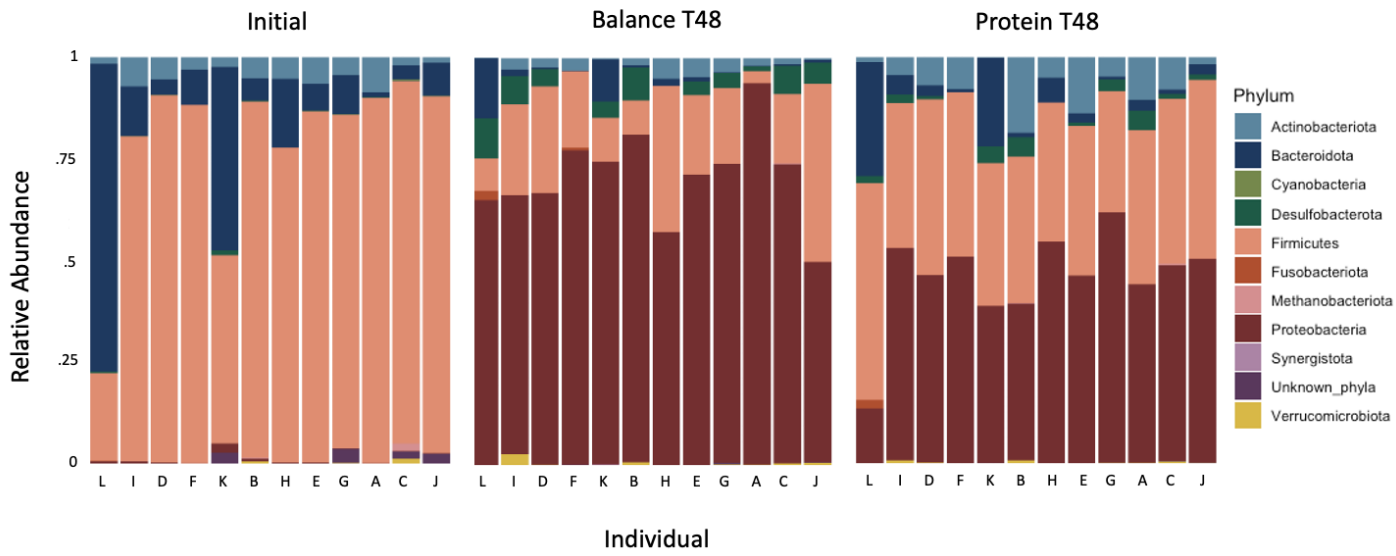

Figure 9: Taxonomy of individual microbiomes at the phylum level before and after batch incubation. *Individuals are ordered based on the difference between their protein and balanced media demethylation rate (protein rate – balance rate). For the initial compositions, the individuals average between balanced and high protein media are used to calculate relative abundances.*

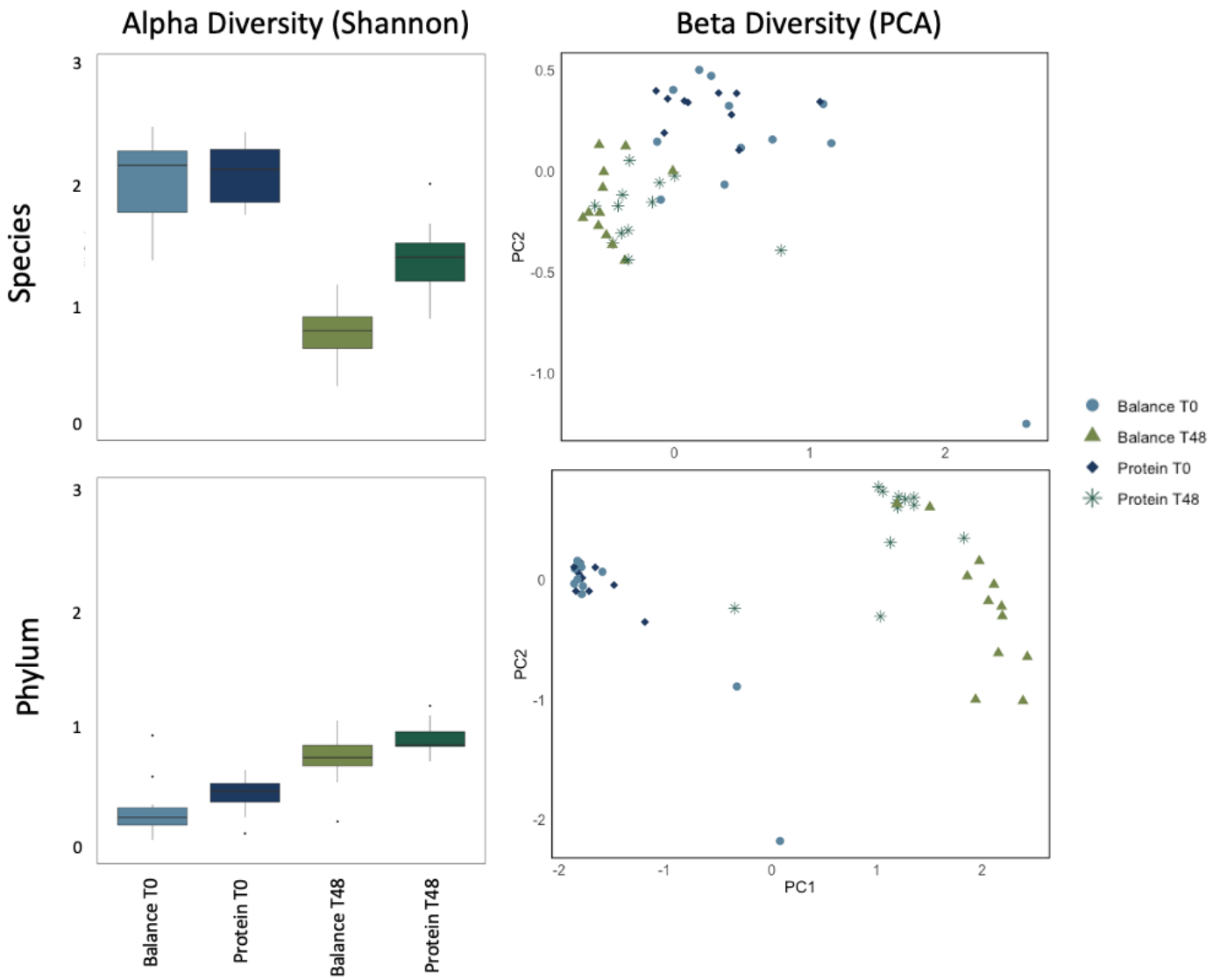

Figure 10: Measures of diversity from the 12 participants at each timepoint and growth media*. For alpha diversity, Shannon diversity was calculated at each timepoint. The average for each timepoint and growth media is plotted at both the species (top) and phylum (bottom) level. Ordination was performed using Principal Component Analysis again at the phylum and species level.*

3.4.2 Mer Operon

As described in chapter 2, prior investigation found no evidence of the *mer operon* in the 2 individuals studied. However, among the 12 individuals in this sudy, evidence of the *mer*

operon was detected in 7 participants using HMMs. From these suspected individuals, 6 of the 7 were confirmed as true positives, while 1 is likely a true positive but truncated (Figure 11). Although 6 individuals had true hits for merA (which catalyzes the reduction of Hg(II) to Hg^0), there were no positive HMM hits for merB, the gene responsible for demethylating MeHg.

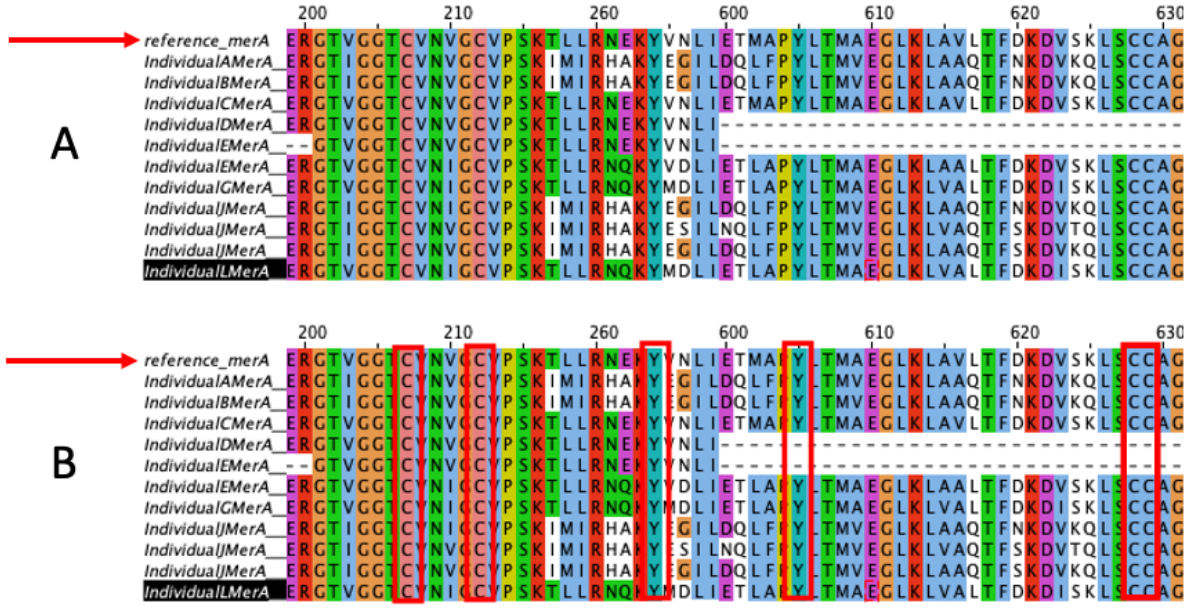

Figure 11: MerA hits using anvi-run-hmms with key amino acid residues highlighted. *Contigs identified using anvi-run-hmms were translated and compared to the reference merA in panel A and shown again with key amino acids are highlighted in panel B. Gaps between key residues were removed.*

3.4.3 Fermentative and Other Gene Pathways

For fermentative genes, correlations between individual metadata (x-axis) are depicted against coverage for genes belonging to each metabolic pathway (y-axis), ranging from -1 (no relation) to 1 (strong relationship) (Figure 12). Clustering of metadata along the x-axis is based on correlation with each other. At T0, focusing on genes related to fermentation reveals a moderate relationship between balance (correlation coefficient > 0.4) and protein demethylation

rates (correlation coefficient ≥ 0.5), and genes (mainly pyruvate ferredoxin oxidoreductase (PFOR)) involved in pyruvate oxidation (Figure 12).

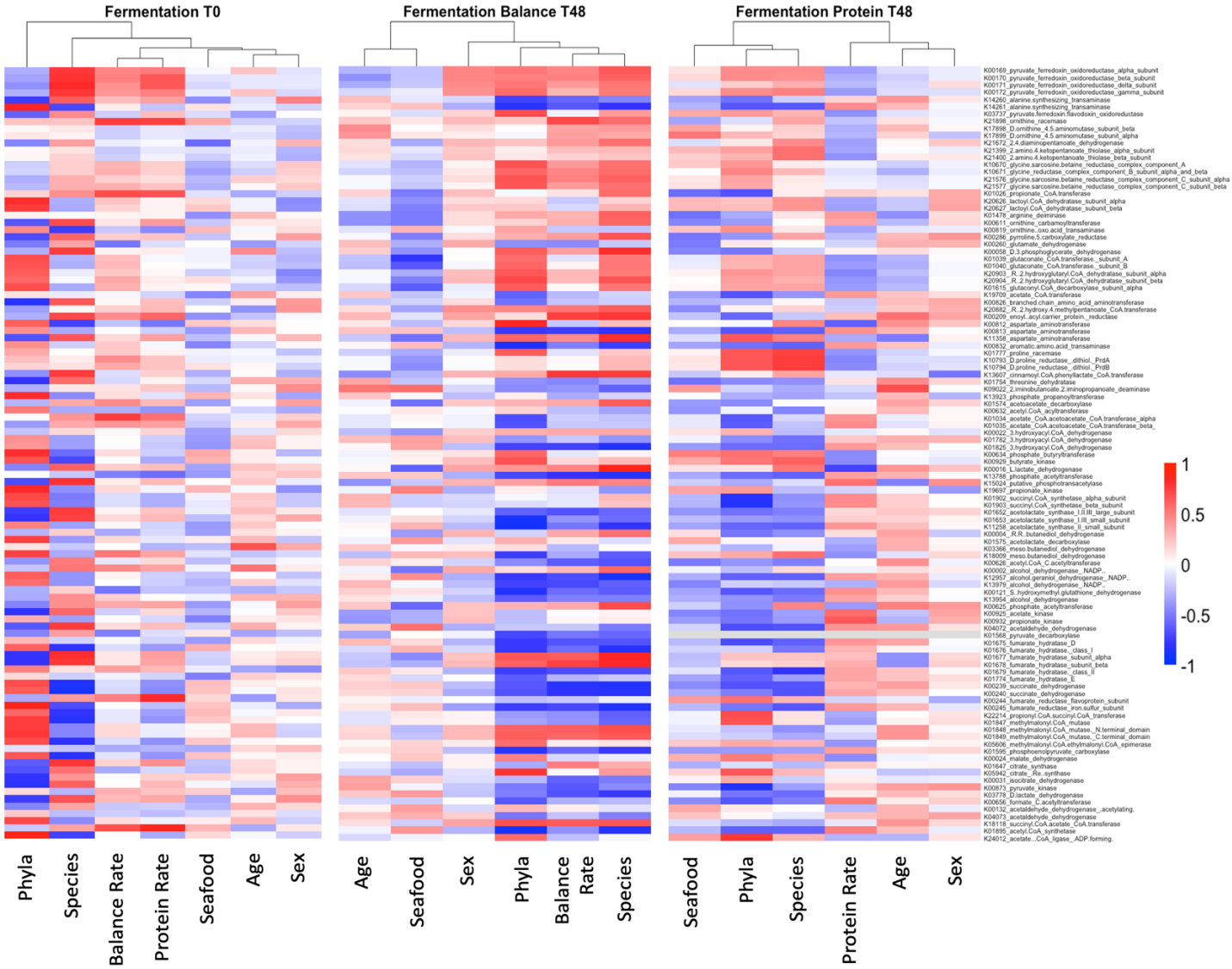

Figure 12: Fermentative gene correlation with individual metadata and demethylation rate. *Metadata was identified from the questionnaires filled out by volunteers including the number of seafood meals consumed monthly (Seafood). Alpha diversity (Shannon) was calculated for individuals (Figure 10) at both the phylum level (Phyla) and species level (Species). The balance demethylation rate (Balance) and protein demethylation rate (Protein) were calculated by taking the slope from the demethylation incubations in Chapter 2. Pearson's correlation coefficient was calculated using the cor function in R.*

Exploring various metabolic pathways other than protein fermentation shows positive relationship values between demethylation and methanogenesis (> 0.3 in balance media and > 0.5 in high protein media), cellular respiration (> 0.1 in balance media and > 0.2 in high protein media) and the Wood-Ljungdahl pathway (> 0.3 in balance and ≥ 0.5 in high protein media) (Figure 13). Notably, these relationships do not appear consistent at the 48-hour timepoints for both media.

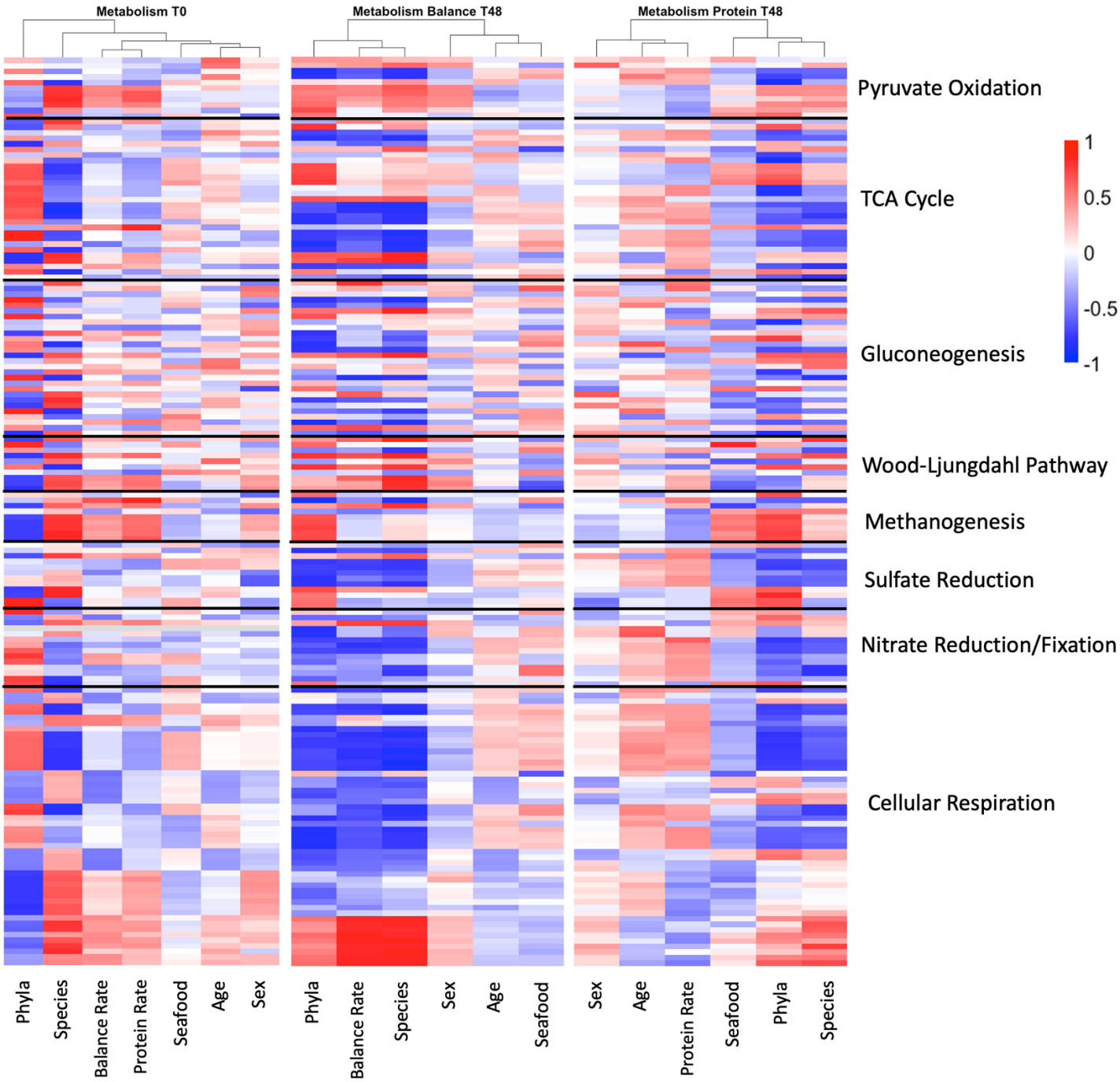

Figure 13: Metabolism type correlation with individual metadata and demethylation rate. *Metadata was identified from the questionnaires filled out by volunteers including the number of seafood meals consumed monthly (Seafood). Alpha diversity (Shannon) was calculated for individuals (Figure 10) at both the phylum level (Phyla) and species level (Species). The balance demethylation rate (Balance) and protein demethylation rate (Protein) were calculated by taking the slope from the demethylation incubations in Chapter 2. Pearson's correlation coefficient was calculated using the cor function in R.*

Further investigation into sulfur-related metabolism, at time 0 demonstrates moderate relationships between DMSO reductase (both aerobic and anaerobic > 0.4), and sulfhydrogenase (≥ 0.4) with the balance and protein demethylation rates (Figure 14). However, again these rates do not correlate to these genes at the T48 timepoints.

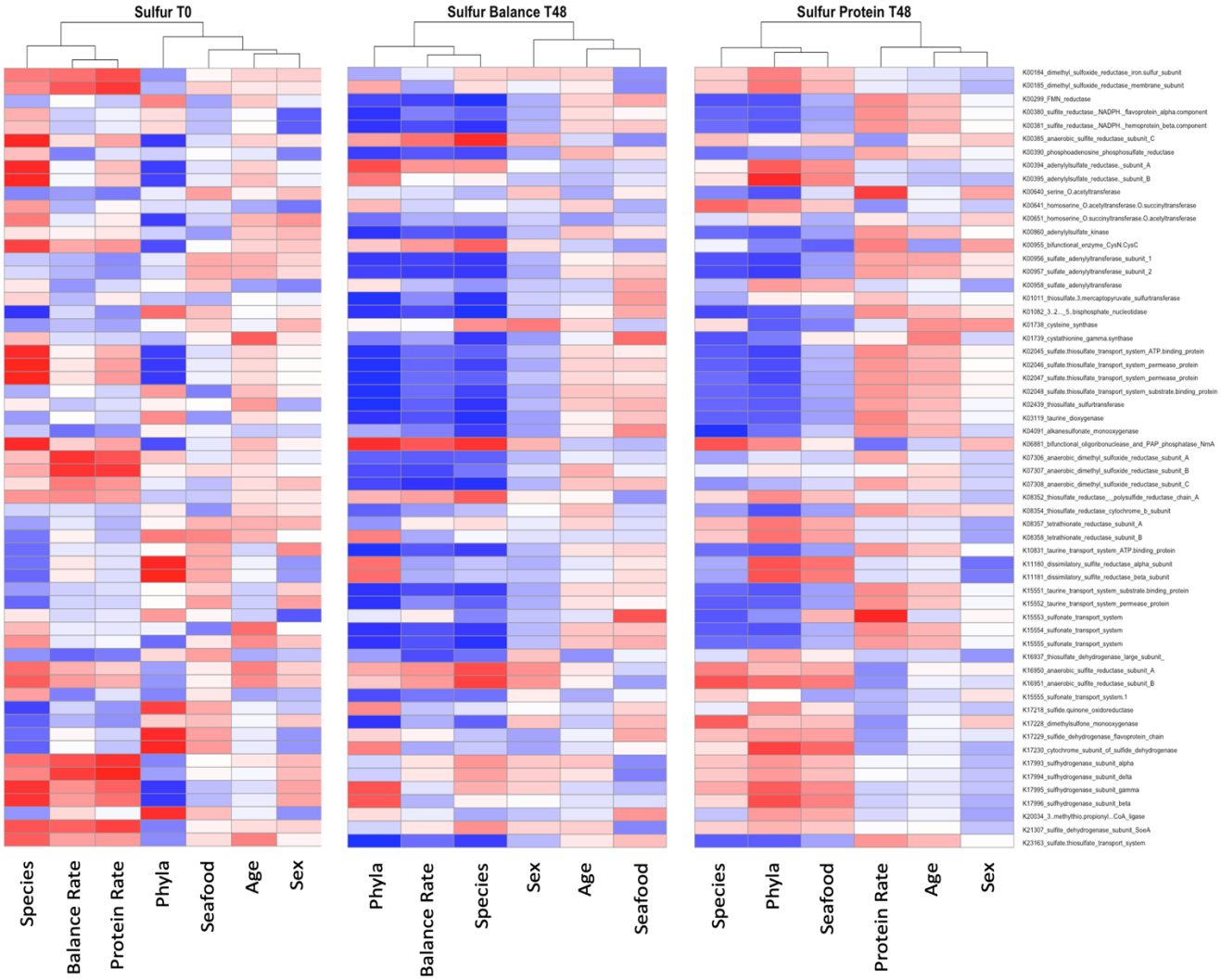

Figure 14: Sulfur correlation with individual metadata and demethylation rate*. Metadata was identified from the questionnaires filled out by volunteers including the number of seafood meals consumed monthly (Seafood). Alpha diversity (Shannon) was calculated for individuals (Figure 10) at both the phylum level (Phyla) and species level (Species). The balance demethylation rate (Balance) and protein demethylation rate (Protein) were calculated by taking the slope from the demethylation incubations in Chapter 2. Pearson's correlation coefficient was calculated using the cor function in R.*

Examining mercury and antibiotic resistance in relation to MeHg demethylation rate, clearly indicates no relationship between MeHg demethylation rate and the *mer operon* (relationship values < 0). Vancomycin resistance appears to strongly correlate with MeHg demethylation in the balanced media (relationship values > 0.6), but this relation does not hold for the high protein media (relationship values < 0).

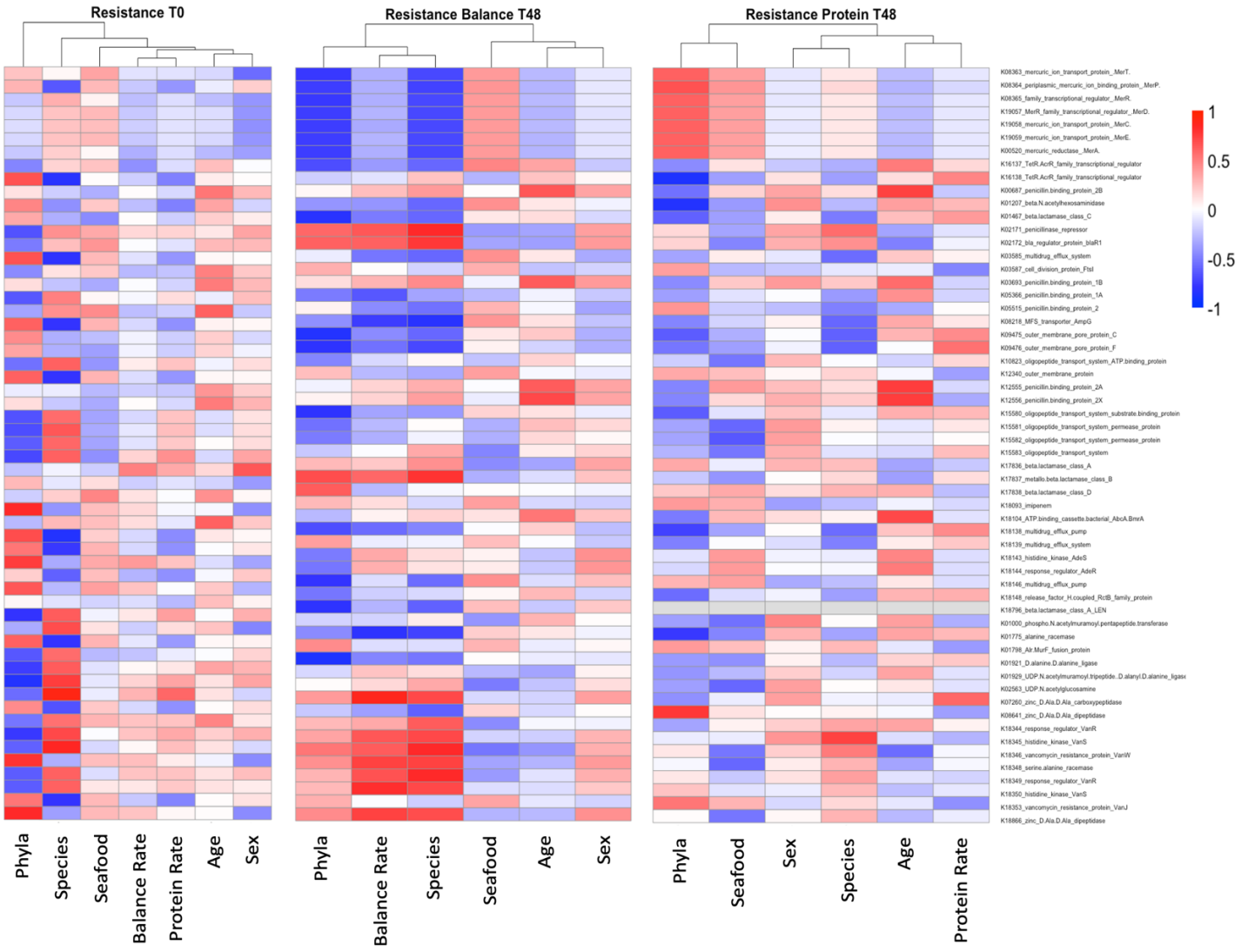

Figure 15: Mercury and antibiotic resistance correlation with individual metadata and demethylation rate *Metadata was identified from the questionnaires filled out by volunteers including the number of seafood meals consumed monthly (Seafood). Alpha diversity (Shannon) was calculated for individuals (Figure 10) at both the phyum level (Phyla) and species level (Species). The balance demethylation rate*

(Balance) and protein demethylation rate (Protein) were calculated by taking the slope from the demethylation incubations in Chapter 2. Pearson's correlation coefficient was calculated using the cor function in R.

3.5 Discussion

As previously emphasized, diet is a large factor in both microbiome health and composition, as well as the primary route of MeHg exposure. In this section, I investigate the impact of diet on microbiome composition and explore the reciprocal influence of microbiome composition on MeHg metabolism. Examining taxonomic data reveals comparable species-level diversity between high protein and balanced media, suggesting that the media does not significantly impact the initial measurements. Consequently, for subsequent analysis at time 0, I used the average coverage of the balanced and protein media for each gene.

The marked difference between the compositions at T0 and T48 is an intriguing finding in the taxonomic data. As discussed in Chapter 2.6, the substantial shift is expected due to these *in-vitro* experiments exposing anaerobic bacteria to oxygen before returning to anaerobic conditions. Additionally, the media was designed to replicate conditions in the small intestine. Compositions observed at T48 more closely represent jejunal microbiomes than the initial colonic microbiomes at T0 (Sundin et al., 2017).

Among the metabolic pathways and genes investigated, presence of the *mer operon* stands out as surprising. Contrary to the initial prediction that the *mer operon* would not be present in any individual, evidence of the *mer operon* was found in 7 out of 12 participants. Although it is present, it shows unexpectedly poor correlation with MeHg demethylation, despite

a positive association with seafood consumption (Figure 15). The hypothesis that the *mer operon* could be responsible for the low recovery in Individual F appears to be incorrect, as Individual F shows no MerA presence (Figure 11). While the *mer operon* is in fact present in some of the human microbiomes, it does not seem to play an important role in MeHg demethylation. This finding prompts further exploration into the *mer operon*'s potential involvement in ingested Hg(II) detoxification within the human gut. possibly preventing methylation by commensal gut microbes.

Considering the impact of protein fermentation on MeHg demethylation, the proportion of proteobacteria in the balanced vs protein media at T48 represents an unexpected pattern. Proteobacteria are more represented in the balanced media for every individual (Figure 9), despite proteobacteria possessing the broadest gene coverage of amino-acid reactions and expected to contribution to protein fermentation (Diether & Willing, 2019). As mentioned in Chapter 1.8.2, the microbiome does exhibit taxonomic variation temporally (Vandeputte et al., 2021). Functionally however, individual's microbiomes show metabolic stability over time (Blakeley-Ruiz et al., 2019). I have shown that protein fermentation is important to demethylation of MeHg, despite the taxa that is most capable of protein fermentation being represented less in the high protein, high demethylation media. This discrepancy emphasizes the importance of functional stability over taxonomic variation.

Examining correlation data reveals intriguing patterns, notably the hierarchal clustering of the demethylation rates with metadata along the x-axis. At T0, both balance and protein demethylation associate together, they also associate more with seafood consumption, age and

sex rather than alpha diversity. As mentioned, measures such as diversity are not reliable predictors of gut health or metabolic state due to the redundancy and high incidence of HGT.

Interestingly, MeHg demethylation rate in the balanced media associates with alpha diversity, while in high protein media, demethylation rate tends to associate with sex, and age. In no treatment does MeHg demethylation rate cluster with seafood consumption at T48. The absence of clustering with seafood consumption at T48 suggests that MeHg exposure may not be a sufficiently selective pressure to enhance MeHg demethylation in this participant group. This observation might be altered when studying individuals with higher MeHg exposure, such as those in coastal communities that rely more on predatory fish for sustenance.

Exploring the correlation in the metabolic profile reveals key observations, particularly the association between demethylation rates and PFOR gene coverage at T0. As PFOR coverage increases, demethylation rate increases, supporting the hypothesis that protein fermentation plays a crucial metabolic role in MeHg demethylation. Higher PFOR gene coverage may serve as a potential predictor or biomarker for MeHg demethylation ability, and the burden faced when exposed to ingested MeHg in the diet. However much further research would be needed to confirm the role in the diet of increasing PFOR coverage and the predictive ability of this set of genes. A notable relation between diet and gene coverage is the association between fish consumption and the same set of PFOR genes. Furthermore, the negative correlation between fish consumption and PFOR, along with other genes positively correlated with MeHg demethylation rate, again raises intriguing questions about the interplay between diet and gene coverage.

Another noteworthy correlation exists between MeHg demethylation rate and genes related to the metabolism of sulfur. As mentioned in chapter 2, H_2S is a potentially toxic product of protein fermentation that is also capable of abiotic demethylation of MeHg. Strong positive relations (correlation coefficients > 0.6) are observed for sulfhydrogenase, aerobic and anaerobic dimethylsulfoxide (DMSO) reductase in both balanced and high protein media. This suggests a potential role of pyruvate fermenting bacteria in producing sulfidic compounds that abiotically demethylate MeHg. There is evidence of pyruvate fermenting bacteria that form dimethylsulfide (DMS) from H_2S as a product of a demethylation reaction (Bak et al., 1992). These bacteria are however found in freshwater, demethylate aromatic methyl groups, and were not found in thetaxonomic analysis for any individual (Bak et al., 1992). Based on the results, it is possible that a pyruvate fermenting bacteria is producing sulfidic compounds that are abiotically demethylating MeHg.

Sulfate reducing bacteria (SRB) are key methylators of MeHg in marine sediments and would possess the correct genes to form H_2S in the gut (King et al., 2000). They are often found in the human gut (Gibson et al., 1993; Singh et al., 2023), and have been suggested to be an indicator of IBD occurrence (Kushkevych et al., 2019). Isolates of SRB have also been shown to demethylate MeHg *in-vitro* (Baldi et al., 1993; Bridou et al., 2011). Despite this, we see in Figure 13, that there are weak to negative correlations between sulfate reduction and demethylation rate other than for the high protein media at T48. I have also found no species of SRB (Bridou et al., 2011; Lu et al., 2023) in our 12 individuals studied. Although this evidence points away from SRB and sulfate reduction being implicated in intestinal MeHg demethylation, based on the correlation data with H_2S and DMS sulfur metabolism, it is worth exploring in the future this relationship between pyruvate/protein fermentation, and H_2S/ DMS production.

The correlation between methanogenesis and MeHg demethylation at T0 raises intriguing possibilities, given the potential involvement of methanotrophs in oxidative demethylation. We see in the data (Figure 13), that there are moderate to very strong correlations (0.4 – 0.85) between methanogenesis and MeHg demethylation rate at T0. One of the potential MeHg demethylation mechanisms involves oxidative demethylation by methanotrophs (Barkay & Gu, 2021). While we observe this using the fecal microbiome, this is not feasible where MeHg absorption is observed, since methanogenesis takes place in the colon, and is inhibited by the presence of bile acid in the small intestine (Florin et al., 1995). Future analyses, possibly utilizing difluoromethane (DFM) to inhibit methanotrophy, could shed light on this mechanism. (Miller et al., 1998).

A crucial connection between sulfur metabolism, methanogenesis, and pyruvate fermentation is the generation of acetyl-CoA (Shi & Tu, 2015). I have shown through *in-vitro* inhibition and validated with metagenomics that protein fermentation to acetyl-CoA is an important process in the microbial demethylation of MeHg in the human gut.

In addition, the Wood-Ljunghal pathway (WLP) is responsible for carbon fixation producing acetyl-CoA (Jiao et al., 2021), and displays consistent correlation with MeHg demethylation at both T48 and T0. These findings strongly suggest that the generation of acetyl-CoA, facilitated through either carbon fixation or protein fermentation, represents a critical step in microbial demethylation of MeHg. Investigating and inhibiting pathways subsequent to acetyl-CoA formation are the next step to identifying the exact mechanism and potential bacteria responsible for this demethylation process.

3.6 Limitations

While metagenomics provides a valuable snapshot of the microbiome at specific point in time, it does not fully capture the active genes, only providing insight to into their presence. Incorporation of a multi-omics approach, further including metabolomics and proteomics would allow for a more comprehensive understanding of the microbiota and their metabolic state (Baysoy et al., 2023; Ottman et al., 2012).

Additional limitations arise from the incubations and observations. Absence from the current analysis doesn't necessarily imply the absence of specific bacteria. Notably, *Desulfovibrio* isn't present in this analysis, but there still may be undetected SRB present in the microbiome. Future experiments could benefit from enrichment strategies to enhance representation of microbes or genes that might be under-represented or filtered out of the sequencing process (Lu et al., 2023).

CHAPTER 4: THESIS CONCLUSION

4.1 MeHg Risk

A major issue surrounding MeHg risk is the communication around diet, health and mercury exposure, particularly in expecting mothers (Kuntz et al., 2010). Several studies have focused on how the diet impacts MeHg exposure, absorption, metabolism, and toxicity (Chapman & Chan, 2000; Landry et al., 1979). Many studies look at the protective relationship between selenium ingestion and MeHg toxicity due to selenium's ability to bind MeHg (Berry & Ralston, 2008; Ganther, 1978; Ralston & Raymond, 2010). Some studies also focus on dietary cysteine intake and MeHg exposure, once again due to how cysteine binds MeHg (Mok et al., 2014). The best predictor of MeHg intoxication is still dietary intake of contaminated seafood regardless of what it is co-ingested with (Chapman & Chan, 2000). Although, it is the biggest source of MeHg exposure, dietary fish consumption benefit often far outweighs the risk unless the circumstances are extreme. In some cases, when residents were warned about the risk of MeHg in the fish, there was no alternative and cessation of the fish in the diet caused a far greater health epidemic than the MeHg would have (Wheatley & Paradis, 1995).

I have hypothesized and confirmed that protein fermentation increases MeHg demethylation. Naturally, I would assume that increased fish consumption would increase the rate of MeHg demethylation as well as genes associated with protein fermentation. However, the genes identified in Chapter 3 associated with protein fermentation and MeHg demethylation were often not closely correlated to fish consumption. This highlights the importance of

understanding the factors surrounding MeHg demethylation and helping to increase demethylation ability or reducing risk for those who have no alternative is of utmost importance.

4.2 Potential Contributions to the Field

This research delves into the intricate microbial processes governing MeHg demethylation in the human gut microbiome. The study utilizes metagenomic analyses and in-vitro inhibition to elucidate key pathways and mechanisms involved. I have shown in a limited number of participants a wide-ranging variation in the ability to demethylate MeHg using fecal sample *in-vitro*. I have also shown that the major pathway for this demethylation is oxidative and driven by protein fermentation, although the exact underlying mechanism of demethylation remains unknown. I have also shown that this fermentative demethylation can be increased in-vitro with additional fermentation from non-starch polysaccharides like cellulose and of (1-3)-β-D-glucan. This has significant implications for the role of diet in MeHg metabolism and determination of individual MeHg risk. Additionally, (1-3)-β-D-glucan serves as a potential candidate for decreasing MeHg burden in individuals who live in high-risk communities.

Furthermore, the metagenomic work investigating differences in individual microbiomes highlights and supports my hypotheses, implicating acetyl-CoA formation either through pyruvate reduction or the Wood-Ljungdhal Pathway. This implication of PFOR activity with oxidative MeHg demethylation suggests a novel demethylation pathway, but further investigation is warranted. Relationships with methanogenesis, methanotrophy and sulfur metabolism warrant further analysis, but circumstantial evidence downplays the ability for these microbes to impact MeHg demethylation in the small intestine, and the taxonomic data does not

implicate any single microbe in this process. Finally, I have shown finally that there is *mer operon* presence in the gut, but these genes are not related to MeHg demethylation.

While it has long been hypothesized that the microbiome is contributing to human MeHg metabolism, there has been no direct evidence that the variation in MeHg demethylation by the microbiome contributes to variance in MeHg metabolism. Recent PBPK models do acknowledge microbiome contributions to whole body MeHg metabolism, but this assumes a constant rate of demethylation across all individuals. To get to this demethylation rate, researchers work backwards based on average half-life to calculate the constant. As previously mentioned, the half-life of MeHg in individuals varies widely, making it hard to predict the burden of MeHg exposure at an individual level. Using personalized demethylation rates to calculate an individual's gut-lumen MeHg half-life would provide a more personalized approach to MeHg risk based on factors that impact an individual's microbiome health. Additionally, the characterization of individual microbiomes allows us to understand the metabolic state and allows us to shift the individual microbiome to increase acetyl-CoA production, therefore theoretically increasing MeHg demethylation. While I have not been able to directly identify the microbial demethylation process, this knowledge narrows the focus on the quest to gain better understanding of this phenomenon.

4.3 Future Directions

Further exploration of the correlation between in-vitro MeHg demethylation rate and in-vivo MeHg elimination is required to robustly confirm the main hypothesis. Once this relationship is substantiated and quantified, updating PBPK models will be critical for accurately predicting MeHg risk at an individual level. Additionally, investigation of acetyl-CoA pathways

using a comprehensive multi-omics approach will help identify the mechanistic underpinnings of MeHg demethylation.

I have already mentioned the potential therapeutic applications of this research, with supplementation with BDG needing to be explored for future use in increasing MeHg demethylation. However, interventions are not limited to supplementation with probiotics or prebiotics. Alterations of the microbiome are already widely used therapeutic interventions in the form of fecal microbiome transplants (FMT). Changing microbiome composition by FMT, diet (Flint et al., 2012) or lifestyle is an established form of therapy with larger implications for host health. Enacting similar changes may be explored to increase individual resistance to MeHg where necessary in extreme cases. Applications such as these could be critical in assisting communities where the risk of MeHg ingestion is high.

5 APPENDIX

5.1 Bibliography

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5.2 Supplemental Figures

Sample Name	% Duplicates	% GC	Read Length	Sequence length (millions)
A_Balance_T0_fastp.R1	17.20%	45%	150 bp	45.5
A_Balance_T0_fastp.R2	16.40%	46%	150 bp	45.5
A_Balance_T48_fastp.R1	71.30%	50%	150 bp	56
A_Balance_T48_fastp.R2	69.80%	50%	150 bp	56
A_Protein_T0_fastp.R1	19.70%	45%	150 bp	44.7
A_Protein_T0_fastp.R2	19.30%	45%	150 bp	44.7
A_Protein_T48_fastp.R1	46.00%	49%	150 bp	53.3
A_Protein_T48_fastp.R2	45.00%	50%	150 bp	53.3
B_Balance_T0_fastp.R1	18.80%	44%	149 bp	44.9
B_Balance_T0_fastp.R2	18.50%	44%	149 bp	44.9
B_Balance_T48_fastp.R1	58.70%	50%	148 bp	43.5

B_Balance_T48_fastp.R2	58.60%	50%	148 bp	43.5
B_Protein_T0_fastp.R1	19.50%	45%	149 bp	54
B_Protein_T0_fastp.R2	19.40%	45%	149 bp	54
B_Protein_T48_fastp.R1	39.50%	49%	150 bp	46.6
B_Protein_T48_fastp.R2	38.80%	50%	150 bp	46.6
C_Balance_T0_fastp.R1	17.20%	44%	149 bp	53.7
C_Balance_T0_fastp.R2	17.00%	44%	149 bp	53.7
C_Balance_T48_fastp.R1	62.30%	49%	150 bp	56.1
C_Balance_T48_fastp.R2	61.60%	49%	150 bp	56.1
C_Protein_T0_fastp.R1	15.00%	44%	150 bp	48.4
C_Protein_T0_fastp.R2	14.80%	45%	150 bp	48.4
C_Protein_T48_fastp.R1	43.10%	49%	149 bp	49.8
C_Protein_T48_fastp.R2	42.40%	49%	149 bp	49.8
D_Balance_T0_fastp.R1	22.60%	45%	150 bp	53.9
D_Balance_T0_fastp.R2	21.90%	45%	150 bp	53.9
D_Balance_T48_fastp.R1	58.50%	49%	150 bp	56.1
D_Balance_T48_fastp.R2	57.10%	49%	150 bp	56.1
D_Protein_T0_fastp.R1	23.20%	45%	150 bp	54.5
D_Protein_T0_fastp.R2	22.40%	45%	150 bp	54.5
D_Protein_T48_fastp.R1	43.90%	49%	150 bp	54.8
D_Protein_T48_fastp.R2	42.40%	49%	150 bp	54.8
E_Balance_T0_fastp.R1	18.00%	46%	150 bp	53.1
E_Balance_T0_fastp.R2	17.40%	46%	150 bp	53.1
E_Balance_T48_fastp.R1	59.50%	50%	150 bp	55.1
E_Balance_T48_fastp.R2	58.00%	50%	150 bp	55.1
E_Protein_T0_fastp.R1	15.90%	46%	149 bp	44.8
E_Protein_T0_fastp.R2	15.50%	46%	149 bp	44.8
E_Protein_T48_fastp.R1	44.20%	50%	150 bp	57.1
E_Protein_T48_fastp.R2	42.70%	50%	150 bp	57.1
F_Balance_T0_fastp.R1	19.40%	44%	149 bp	45.4
F_Balance_T0_fastp.R2	19.30%	44%	149 bp	45.4
F_Balance_T48_fastp.R1	61.70%	49%	150 bp	50
F_Balance_T48_fastp.R2	60.20%	49%	150 bp	50
F_Protein_T0_fastp.R1	21.30%	43%	149 bp	55.9
F_Protein_T0_fastp.R2	21.10%	43%	149 bp	55.9

F_Protein_T48_fastp.R1	48.10%	48%	150 bp	51.8
F_Protein_T48_fastp.R2	46.50%	48%	150 bp	51.8
G_Balance_T0_fastp.R1	20.60%	46%	150 bp	51.2
G_Balance_T0_fastp.R2	20.00%	46%	150 bp	51.2
G_Balance_T48_fastp.R1	58.10%	50%	150 bp	60
G_Balance_T48_fastp.R2	56.80%	50%	150 bp	60
G_Protein_T0_fastp.R1	22.30%	46%	150 bp	53.6
G_Protein_T0_fastp.R2	21.80%	46%	150 bp	53.6
G_Protein_T48_fastp.R1	48.90%	49%	150 bp	48.1
G_Protein_T48_fastp.R2	47.30%	49%	150 bp	48.1
H_Balance_T0_fastp.R1	21.20%	42%	150 bp	50.4
H_Balance_T0_fastp.R2	20.70%	42%	150 bp	50.4
H_Balance_T48_fastp.R1	44.60%	49%	150 bp	52.5
H_Balance_T48_fastp.R2	43.40%	49%	150 bp	52.5
H_Protein_T0_fastp.R1	20.80%	42%	150 bp	45.3
H_Protein_T0_fastp.R2	20.20%	42%	150 bp	45.3
H_Protein_T48_fastp.R1	50.80%	49%	149 bp	59.2
H_Protein_T48_fastp.R2	49.60%	49%	149 bp	59.2
I_Balance_T0_fastp.R1	17.60%	44%	151 bp	52.4
I_Balance_T0_fastp.R2	17.00%	44%	151 bp	52.4
I_Balance_T48_fastp.R1	52.70%	50%	150 bp	60.2
I_Balance_T48_fastp.R2	50.90%	50%	150 bp	60.2
I_Protein_T0_fastp.R1	15.20%	44%	150 bp	43
I_Protein_T0_fastp.R2	14.90%	44%	150 bp	43
I_Protein_T48_fastp.R1	44.10%	48%	150 bp	55.3
I_Protein_T48_fastp.R2	42.50%	49%	150 bp	55.3
J_Balanace_T48_fastp.R1	40.30%	50%	150 bp	59.9
J_Balanace_T48_fastp.R2	39.00%	50%	150 bp	59.9
J_Balance_T0_fastp.R1	14.80%	45%	150 bp	48.8
J_Balance_T0_fastp.R2	14.10%	45%	150 bp	48.8
J_Protein_T0_fastp.R1	16.50%	45%	150 bp	53.3
J_Protein_T0_fastp.R2	16.20%	45%	150 bp	53.3
J_Protein_T48_fastp.R1	50.00%	49%	150 bp	57.7
J_Protein_T48_fastp.R2	48.80%	50%	150 bp	57.7
K_Balance_T0_fastp.R1	21.90%	46%	100 bp	54.3

K_Balance_T0_fastp.R2	16.10%	46%	100 bp	54.3
K_Balance_T48_fastp.R1	28.30%	53%	100 bp	48.6
K_Balance_T48_fastp.R2	21.40%	53%	100 bp	48.6
K_Protein_T48_fastp.R1	24.10%	50%	100 bp	48.4
K_Protein_T48_fastp.R2	18.50%	50%	100 bp	48.4
L_Balance_T0_fastp.R1	49.50%	43%	100 bp	62.7
L_Balance_T0_fastp.R2	38.30%	43%	100 bp	62.7
L_Balance_T48_fastp.R1	22.90%	52%	100 bp	46.4
L_Balance_T48_fastp.R2	17.70%	51%	100 bp	46.4
L_Protein_T48_fastp.R1	21.20%	47%	100 bp	63.5
L_Protein_T48_fastp.R2	15.80%	47%	100 bp	63.5

Table S 1: MultiQC report for sequences analyzed.

Degradation of methylmercury by human gut microbiota: prevalence, cause, and determination of genotype

Name: _____

Individual: _____

Age: _____

Sex: _____

Have you taken antibiotics for any reason in the past 60 days? Y/N _____

Do you have any known gastrointestinal disorder (e.g. Crohn's, IBD, Celiac) Y/N? _____

Name of Disorder: _____

Do you currently have any dental amalgams fillings? Y/N _____

How often do you consume seafood (please circle one):

Daily

4-6 Meals per week

1-3 Meals per week

A few times a month

Rarely

Never

Figure S 1: Questionnaire provided to participants.

SampleID	Age	Demethylation rate	Sex	Shannon Diversity Phyla (T0)	Shannon Diversity Species (T0)	GI Disorder	Amalgams
A Balance	25	0.168969574	1	0.3606444	1.979994	no	no
A Protein	25	0.650444435	1	0.3820751	1.9385532	no	no
B Balance	28	0.215106977	0	0.3791438	2.2440911	no	no
B Protein	28	0.549444155	0	0.6125544	2.3429405	no	no
C Balance	28	0.251195489	0	0.4533414	2.5279208	no	no
C Protein	28	0.842766405	0	0.6030167	2.4849906	no	no
D Balance	28	0.157336616	1	0.335175	2.3212256	no	no
D Protein	28	0.35911113	1	0.4429099	2.307252	no	no
E Balance	25	0.265400292	1	0.4239315	1.8001485	no	no
E Protein	25	0.686271206	1	0.6071396	1.7791403	no	no
F Balance	53	0.38818293	1	0.3359059	2.4855262	no	no
F Protein	53	0.593196347	1	0.4996838	2.1426462	no	no
G Balance	47	0.260609958	0	0.4675727	2.1735171	no	no
G Protein	47	0.688387929	0	0.7727269	2.1879945	no	no
H Balance	21	0.485221298	1	0.4418942	2.2289612	no	no
H Protein	21	0.853702851	1	0.7986505	1.8561565	no	no
I Balance	47	0.292422398	1	0.5756986	1.7924478	no	no
I Protein	47	0.478545322	1	0.7483103	1.8649145	no	no
J Balance	23	0.70689207	1	0.379372	2.3267107	no	no
J Protein	23	1.748514903	1	0.5601559	2.3791903	no	no
K Balance	31	0.389376618	0	1.0402642	1.5986272	no	no
K Protein	31	0.653230891	0	1.0402642	1.5986272	no	no
L Balance	31	0.170064064	1	0.6734473	1.3895621	no	no
L Protein	31	0.153190288	1	0.6734473	1.3895621	no	no

Table S 2: Individual Metadata from questionnaire

Metabolic Process	KEGG Orthology	Gene
Pyruvate Oxidation	K00161	pyruvate_dehydrogenase_E1_component_alpha_subunit_[EC:1.2.4.1]
	K00162	pyruvate_dehydrogenase_E1_component_beta_subunit_[EC:1.2.4.1]
	K00163	pyruvate_dehydrogenase_E1_component_[EC:1.2.4.1]
	K00627	pyruvate_dehydrogenase_E2_component_(dihydrolipoamide_acetyltransferase)_[EC:2.3.1.12]
	K00382	dihydrolipoamide_dehydrogenase_[EC:1.8.1.4]
	K00169	pyruvate_ferredoxin_oxidoreductase_alpha_subunit_[EC:1.2.7.1]
	K00170	pyruvate_ferredoxin_oxidoreductase_beta_subunit_[EC:1.2.7.1]

	K00171	pyruvate_ferredoxin_oxidoreductase_delta_subunit_[EC:1.2.7.1]
	K00172	pyruvate_ferredoxin_oxidoreductase_gamma_subunit_[EC:1.2.7.1]
	K03737	pyruvate-ferredoxin/flavodoxin_oxidoreductase_[EC:1.2.7.1_1.2.7.-]
	K01960	pyruvate_carboxylase_subunit_B_[EC:6.4.1.1]
TCA Cycle	K01647	citrate_synthase_[EC:2.3.3.1]
	K05942	citrate_(Re)-synthase_[EC:2.3.3.3]
	K01681	aconitate_hydratase_[EC:4.2.1.3]
	K01682	aconitate_hydratase_2_/_2-methylisocitrate_dehydratase_[EC:4.2.1.3_4.2.1.99]
	K00031	isocitrate_dehydrogenase_[EC:1.1.1.42]
	K00030	isocitrate_dehydrogenase_(NAD+)_[EC:1.1.1.41]
	K00164	2-oxoglutarate_dehydrogenase_E1_component_[EC:1.2.4.2]
	K00658	2-oxoglutarate_dehydrogenase_E2_component_(dihydrolipoamide_succinyltransferase)_[EC:2.3.1.61]
	K00174	2-oxoglutarate/2-oxoacid_ferredoxin_oxidoreductase_subunit_alpha_[EC:1.2.7.3_1.2.7.11]
	K00175	2-oxoglutarate/2-oxoacid_ferredoxin_oxidoreductase_subunit_beta_[EC:1.2.7.3_1.2.7.11]
	K00177	2-oxoglutarate_ferredoxin_oxidoreductase_subunit_gamma_[EC:1.2.7.3]
	K00176	2-oxoglutarate_ferredoxin_oxidoreductase_subunit_delta_[EC:1.2.7.3]
	K01902	succinyl-CoA_synthetase_alpha_subunit_[EC:6.2.1.5]
	K01903	succinyl-CoA_synthetase_beta_subunit_[EC:6.2.1.5]
	K18118	succinyl-CoA:acetate_CoA-transferase_[EC:2.8.3.18]
	K00239	succinate_dehydrogenase_/_fumarate_reductase,_flavoprotein_subunit_[EC:1.3.5.1_1.3.5.4]
	K00240	succinate_dehydrogenase_/_fumarate_reductase,_iron-sulfur_subunit_[EC:1.3.5.1_1.3.5.4]
	K00241	succinate_dehydrogenase_/_fumarate_reductase,_cytochrome_b_subunit
	K00242	succinate_dehydrogenase_/_fumarate_reductase,_membrane_anchor_subunit
	K00244	fumarate_reductase_flavoprotein_subunit_[EC:1.3.5.4]
	K00245	fumarate_reductase_iron-sulfur_subunit_[EC:1.3.5.4]
	K00246	fumarate_reductase_subunit_C
	K00247	fumarate_reductase_subunit_D
	K01676	fumarate_hydratase,_class_I_[EC:4.2.1.2]
	K01677	fumarate_hydratase_subunit_alpha_[EC:4.2.1.2]
	K01678	fumarate_hydratase_subunit_beta_[EC:4.2.1.2]
	K01679	fumarate_hydratase,_class_II_[EC:4.2.1.2]
	K00024	malate_dehydrogenase_[EC:1.1.1.37]
	K00116	malate_dehydrogenase_(quinone)_[EC:1.1.5.4]
Gluconeogenesis/ Glycolysis	K01596	phosphoenolpyruvate_carboxykinase_(GTP)_[EC:4.1.1.32]
	K01610	phosphoenolpyruvate_carboxykinase_(ATP)_[EC:4.1.1.49]
	K01689	enolase_[EC:4.2.1.11]

	K03841	fructose-1,6-bisphosphatase_I_[EC:3.1.3.11]
	K02446	fructose-1,6-bisphosphatase_II_[EC:3.1.3.11]
	K04041	fructose-1,6-bisphosphatase_III_[EC:3.1.3.11]
	K00844	hexokinase_[EC:2.7.1.1]
	K00845	glucokinase_[EC:2.7.1.2]
	K00886	polyphosphate_glucokinase_[EC:2.7.1.63]
	K01810	glucose-6-phosphate_isomerase_[EC:5.3.1.9]
	K06859	glucose-6-phosphate_isomerase,_archaeal_[EC:5.3.1.9]
	K00850	6-phosphofructokinase_1_[EC:2.7.1.11]
	K16370	6-phosphofructokinase_2_[EC:2.7.1.11]
	K21071	ATP-dependent_phosphofructokinase_/_diphosphate-dependent_phosphofructokinase_[EC:2.7.1.11_2.7.1.90]
	K00918	ADP-dependent_phosphofructokinase/glucokinase_[EC:2.7.1.146_2.7.1.147]
	K01623	fructose-bisphosphate_aldolase,_class_I_[EC:4.1.2.13]
	K01624	fructose-bisphosphate_aldolase,_class_II_[EC:4.1.2.13]
	K11645	fructose-bisphosphate_aldolase,_class_I_[EC:4.1.2.13]
	K16305	fructose-bisphosphate_aldolase_/_6-deoxy-5-ketofructose_1-phosphate_synthase_[EC:4.1.2.13_2.2.1.11]
	K16306	fructose-bisphosphate_aldolase_/_2-amino-3,7-dideoxy-D-threo-hept-6-ulosonate_synthase_[EC:4.1.2.13_2.2.1.10]
	K01803	triosephosphate_isomerase_(TIM)_[EC:5.3.1.1]
	K00134	glyceraldehyde_3-phosphate_dehydrogenase_[EC:1.2.1.12]
	K00927	phosphoglycerate_kinase_[EC:2.7.2.3]
	K01834	2,3-bisphosphoglycerate-dependent_phosphoglycerate_mutase_[EC:5.4.2.11]
	K15633	2,3-bisphosphoglycerate-independent_phosphoglycerate_mutase_[EC:5.4.2.12]
	K15634	2,3-bisphosphoglycerate-dependent_phosphoglycerate_mutase_[EC:5.4.2.11]
	K15635	2,3-bisphosphoglycerate-independent_phosphoglycerate_mutase_[EC:5.4.2.12]
	K00873	pyruvate_kinase_[EC:2.7.1.40]
Wood-Ljungdahl Pathway	K00198	anaerobic_carbon-monoxide_dehydrogenase_catalytic_subunit_[EC:1.2.7.4]
	K15022	formate_dehydrogenase_(NADP+)_beta_subunit_[EC:1.17.1.10]
	K22015	formate_dehydrogenase_(acceptor)_[EC:1.17.99.7]
	K01938	formate--tetrahydrofolate_ligase_[EC:6.3.4.3]
	K01491	methylenetetrahydrofolate_dehydrogenase_(NADP+)_/_methenyltetrahydrofolate_cyclohydrolase_[EC:1.5.1.5_3.5.4.9]
	K01500	methenyltetrahydrofolate_cyclohydrolase_[EC:3.5.4.9]
	K00297	methylenetetrahydrofolate_reductase_(NADPH)_[EC:1.5.1.20]
	K15023	5-methyltetrahydrofolate_corrinoid/iron_sulfur_protein_methyltransferase_[EC:2.1.1.258]
	K14138	acetyl-CoA_synthase_[EC:2.3.1.169]
	K00197	acetyl-CoA_decarbonylase/synthase,_CODH/ACS_complex_subunit_gamma_[EC:2.1.1.245]

Methanogenesis	K11261	formylmethanofuran_dehydrogenase_subunit_E_[EC:1.2.7.12]
	K01895	acetyl-CoA_synthetase_[EC:6.2.1.1]
	K00194	acetyl-CoA_decarbonylase/synthase,_CODH/ACS_complex_subunit_delta_[EC:2.1.1.245]
	K08264	heterodisulfide_reductase_subunit_D_[EC:1.8.98.1]
	K03388	heterodisulfide_reductase_subunit_A2_[EC:1.8.7.3_1.8.98.4_1.8.98.5_1.8.98.6]
	K03389	heterodisulfide_reductase_subunit_B2_[EC:1.8.7.3_1.8.98.4_1.8.98.5_1.8.98.6]
	K03390	heterodisulfide_reductase_subunit_C2_[EC:1.8.7.3_1.8.98.4_1.8.98.5_1.8.98.6]
	K14127	F420-non-reducing_hydrogenase_iron-sulfur_subunit_[EC:1.12.99.-_1.8.98.5_1.8.98.6]
	K00125	formate_dehydrogenase_(coenzyme_F420)_beta_subunit_[EC:1.17.98.3_1.8.98.6]
Sulfate Reduction	K00958	sulfate_adenylyltransferase_[EC:2.7.7.4]
	K00860	adenylylsulfate_kinase_[EC:2.7.1.25]
	K00955	bifunctional_enzyme_CysN/CysC_[EC:2.7.7.4_2.7.1.25]
	K00956	sulfate_adenylyltransferase_subunit_1_[EC:2.7.7.4]
	K00957	sulfate_adenylyltransferase_subunit_2_[EC:2.7.7.4]
	K00390	phosphoadenosine_phosphosulfate_reductase_[EC:1.8.4.8_1.8.4.10]
	K00380	sulfite_reductase_(NADPH)_flavoprotein_alpha-component_[EC:1.8.1.2]
	K00381	sulfite_reductase_(NADPH)_hemoprotein_beta-component_[EC:1.8.1.2]
	K00394	adenylylsulfate_reductase,_subunit_A_[EC:1.8.99.2]
	K00395	adenylylsulfate_reductase,_subunit_B_[EC:1.8.99.2]
	K11180	dissimilatory_sulfite_reductase_alpha_subunit_[EC:1.8.99.5]
	K11181	dissimilatory_sulfite_reductase_beta_subunit_[EC:1.8.99.5]
Nitrate Reduction/Fixation	K00370	nitrate_reductase_/_nitrite_oxidoreductase,_alpha_subunit_[EC:1.7.5.1_1.7.99.-]
	K00371	nitrate_reductase_/_nitrite_oxidoreductase,_beta_subunit_[EC:1.7.5.1_1.7.99.-]
	K00374	nitrate_reductase_gamma_subunit_[EC:1.7.5.1_1.7.99.-]
	K02567	nitrate_reductase_(cytochrome)_[EC:1.9.6.1]
	K02568	nitrate_reductase_(cytochrome),_electron_transfer_subunit
	K00362	nitrite_reductase_(NADH)_large_subunit_[EC:1.7.1.15]
	K00363	nitrite_reductase_(NADH)_small_subunit_[EC:1.7.1.15]
	K03385	nitrite_reductase_(cytochrome_c-552)_[EC:1.7.2.2]
	K15876	cytochrome_c_nitrite_reductase_small_subunit
	K00372	assimilatory_nitrate_reductase_catalytic_subunit_[EC:1.7.99.-]
	K00366	ferredoxin-nitrite_reductase_[EC:1.7.7.1]
	K02588	nitrogenase_iron_protein_NifH
	K02586	nitrogenase_molybdenum-iron_protein_alpha_chain_[EC:1.18.6.1]
	K02591	nitrogenase_molybdenum-iron_protein_beta_chain_[EC:1.18.6.1]
Cellular Respiration	K00330	NADH-quinone_oxidoreductase_subunit_A_[EC:7.1.1.2]

K00332	NADH-quinone_oxidoreductase_subunit_C_[EC:7.1.1.2]
K00333	NADH-quinone_oxidoreductase_subunit_D_[EC:7.1.1.2]
K00331	NADH-quinone_oxidoreductase_subunit_B_[EC:7.1.1.2]
K13378	NADH-quinone_oxidoreductase_subunit_C/D_[EC:7.1.1.2]
K00334	NADH-quinone_oxidoreductase_subunit_E_[EC:7.1.1.2]
K00335	NADH-quinone_oxidoreductase_subunit_F_[EC:7.1.1.2]
K00336	NADH-quinone_oxidoreductase_subunit_G_[EC:7.1.1.2]
K00337	NADH-quinone_oxidoreductase_subunit_H_[EC:7.1.1.2]
K00338	NADH-quinone_oxidoreductase_subunit_I_[EC:7.1.1.2]
K00339	NADH-quinone_oxidoreductase_subunit_J_[EC:7.1.1.2]
K00340	NADH-quinone_oxidoreductase_subunit_K_[EC:7.1.1.2]
K00341	NADH-quinone_oxidoreductase_subunit_L_[EC:7.1.1.2]
K00342	NADH-quinone_oxidoreductase_subunit_M_[EC:7.1.1.2]
K00343	NADH-quinone_oxidoreductase_subunit_N_[EC:7.1.1.2]
K03886	menaquinol-cytochrome_c_reductase_iron-sulfur_subunit_[EC:1.10.2.-]
K03887	menaquinol-cytochrome_c_reductase_cytochrome_b_subunit
K03888	menaquinol-cytochrome_c_reductase_cytochrome_b/c_subunit
K02275	cytochrome_c_oxidase_subunit_II_[EC:7.1.1.9]
K02274	cytochrome_c_oxidase_subunit_I_[EC:7.1.1.9]
K02277	cytochrome_c_oxidase_subunit_IV_[EC:7.1.1.9]
K00425	cytochrome_bd_ubiquinol_oxidase_subunit_I_[EC:7.1.1.7]
K00426	cytochrome_bd_ubiquinol_oxidase_subunit_II_[EC:7.1.1.7]
K00424	cytochrome_bd-I_ubiquinol_oxidase_subunit_X_[EC:7.1.1.7]
K22501	cytochrome_bd-II_ubiquinol_oxidase_subunit_AppX_[EC:7.1.1.7]
K02297	cytochrome_o_ubiquinol_oxidase_subunit_II_[EC:7.1.1.3]
K02298	cytochrome_o_ubiquinol_oxidase_subunit_I_[EC:7.1.1.3]
K02299	cytochrome_o_ubiquinol_oxidase_subunit_III
K02300	cytochrome_o_ubiquinol_oxidase_subunit_IV
K02827	cytochrome_aa3-600_menaquinol_oxidase_subunit_I_[EC:7.1.1.5]
K02826	cytochrome_aa3-600_menaquinol_oxidase_subunit_II_[EC:7.1.1.5]
K02828	cytochrome_aa3-600_menaquinol_oxidase_subunit_III_[EC:7.1.1.5]
K02829	cytochrome_aa3-600_menaquinol_oxidase_subunit_IV_[EC:7.1.1.5]
K02111	F-type_H+/Na+-transporting_ATPase_subunit_alpha_[EC:7.1.2.2_7.2.2.1]
K02112	F-type_H+/Na+-transporting_ATPase_subunit_beta_[EC:7.1.2.2_7.2.2.1]
K02113	F-type_H+-transporting_ATPase_subunit_delta
K02114	F-type_H+-transporting_ATPase_subunit_epsilon
K02115	F-type_H+-transporting_ATPase_subunit_gamma
K02108	F-type_H+-transporting_ATPase_subunit_a

K02109	F-type_H+-transporting_ATPase_subunit_b
K02110	F-type_H+-transporting_ATPase_subunit_c
K02117	V/A-type_H+/Na+-transporting_ATPase_subunit_A_[EC:7.1.2.2_7.2.2.1]
K02118	V/A-type_H+/Na+-transporting_ATPase_subunit_B
K02119	V/A-type_H+/Na+-transporting_ATPase_subunit_C
K02120	V/A-type_H+/Na+-transporting_ATPase_subunit_D
K02121	V/A-type_H+/Na+-transporting_ATPase_subunit_E
K02122	V/A-type_H+/Na+-transporting_ATPase_subunit_F
K02107	V/A-type_H+/Na+-transporting_ATPase_subunit_G/H
K02123	V/A-type_H+/Na+-transporting_ATPase_subunit_I
K02124	V/A-type_H+/Na+-transporting_ATPase_subunit_K

Table S 3: Metabolism Genes from Figure 13

5.3 Ethics Certificate

10/07/2023

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

CERTIFICAT D'APPROBATION ÉTHIQUE | CERTIFICATE OF ETHICS APPROVAL

Numéro du dossier / Ethics File Number	H-03-20-4702
Titre du projet / Project Title	Degradation of methylmercury by human gut microbiota: prevalence, cause, and determination of genotype
Type de projet / Project Type	Thèse de maîtrise / Master's thesis
Statut du projet / Project Status	Fermé / Closed
Date d'approbation (jj/mm/aaaa) / Approval Date (dd/mm/yyyy)	05/06/2020
Date d'expiration (jj/mm/aaaa) / Expiry Date (dd/mm/yyyy)	04/06/2023

Équipe de recherche / Research Team

Chercheur / Researcher	Affiliation	Role
Jacob DROUILLARD	Département de biologie / Department of Biology	Chercheur Principal / Principal Investigator
Laurie CHAN	Département de biologie / Department of Biology	Superviseur / Supervisor
Alexandre POULAIN		Co-superviseur / Co-supervisor
Galen GUO	Département de biologie / Department of Biology	Collaborateur / Collaborator

Conditions spéciales ou commentaires / Special conditions or comments

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Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

Le Comité d'éthique de la recherche (CÉR) de l'Université d'Ottawa, opérant conformément à l'*Énoncé de politique des Trois conseils* (2014) et toutes autres lois et tous règlements applicables, a examiné et approuvé la demande d'éthique du projet de recherche ci-nommé.

L'approbation est valide pour la durée indiquée plus haut et est sujette aux conditions énumérées dans la section intitulée "Conditions Spéciales ou Commentaires". Le formulaire « Renouvellement ou Fermeture de Projet » doit être complété quatre semaines avant la date d'échéance indiquée ci-haut afin de demander un renouvellement de cette approbation éthique ou afin de fermer le dossier.

Toutes modifications apportées au projet doivent être approuvées par le CÉR avant leur mise en place, sauf si le participant doit être retiré en raison d'un danger immédiat ou s'il s'agit d'un changement ayant trait à des éléments administratifs ou logistiques du projet. Les chercheurs doivent aviser le CÉR dans les plus brefs délais de tout changement pouvant augmenter le niveau de risque aux participants ou pouvant affecter considérablement le déroulement du projet, rapporter tout évènement imprévu ou indésirable et soumettre toute nouvelle information pouvant nuire à la conduite du projet ou à la sécurité des participants.

The University of Ottawa Research Ethics Board, which operates in accordance with the *Tri-Council Policy Statement* (2014) and other applicable laws and regulations, has examined and approved the ethics application for the above-named research project.

Ethics approval is valid for the period indicated above and is subject to the conditions listed in the section entitled "Special Conditions or Comments". The "Renewal/Project Closure" form must be completedfour weeks before the above-referenced expiry date to request a renewal of this ethics approval or closure of the file.

Any changes made to the project must be approved by the REB before being implemented, except when necessary to remove participants from immediate endangerment or when the modification(s) only pertain to administrative or logistical components of the project. Investigators must also promptly alert the REB of any changes that increase the risk to participant(s), any changes that considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project or the safety of the participant(s).

Coordonateur / COORDINATOR
Coordonnateur de l'éthique / Ethics Coordinator
Pour/For **Daniel LAGAREC** Président(e) du/ Chair of the **Comité d'éthique de la recherche en sciences de la santé et sciences / Health Sciences and Sciences Research Ethics Board**

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