Polyphenol-rich cocoa powder improves behavioural functionality and gut morphology in a zebrafish model of autism

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January, 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of

Master of Science

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Abstract

Valproic acid is an anticonvulsant that has been implicated as risk factor for autism spectrum disorder (ASD) when taken during pregnancy and has been used to induce ASD in animal models. Interventions for ASD predominantly aim at addressing specific comorbid conditions, encompassing therapeutic approaches for anxiety, behavioral issues, communication challenges, as well as occupational and nutritional needs. Although numerous treatments yield efficacy in mitigating the symptoms of ASD and enhancing quality of life, they invariably impose supplementary burdens in terms of financial expenditure and time commitment. This can be difficult to manage for busy families, which is why new treatments that are easy to administer and cheap to acquire are critical. Within this context, cocoa polyphenols have emerged as a compelling candidate for ASD therapy, backed by studies emphasizing their potential cognitive benefits in adults. Further supporting this proposition is a trial conducted with children diagnosed with ASD, which reported notable improvements in behavioral metrics. This thesis describes an evaluation of the efficacy of treating behavioural and physiological symptoms of autism spectrum disorder (ASD), using cocoa powder, in a zebrafish model. Firstly, a valproic acid (VPA) induced model of ASD was validated in zebrafish. VPA toxicology was performed to determine the optimal VPA dosage to induce the autistic phenotype in the zebrafish without causing severe physical damage such as pericardial edemas and swimming disability like ataxia which can obstruct behavioural assessments. Secondly, toxicological assessment of prebiotic cocoa powder, enriched in cocoa polyphenols, was conducted to determine the most favorable exposure concentrations, which would maximize survival rate and dosage, to ensure beneficial modulation of the VPA zebrafish. In terms of the above VPA and cocoa validation studies, the concentrations of compounds were 3 μM and 2.5 μM, respectively. The impact of the treatment in the validated zebrafish ASD model was assessed in terms of behaviour, gut morphology, metabolomics, and proteomics. The VPA-treated zebrafish demonstrated an ASD-like phenotype as they exhibited stressed behaviour with increased and more sporadic swimming patterns. The VPA-treated

zebrafish swam larger distances at greater velocities in both dark and light cycles when compared to the control, but also the cocoa and VPA + cocoa groups. Interestingly, the VPA-treated fish with cocoa remained calm and behaved similarly to controls in the dark cycle and remained calmer after transitioning into the light cycle of the assay. VPA treatment caused severe gastrointestinal shrinkage and deformations compared to controls whereas cocoa treatment in the VPA group was associated with gut morphology similar to the control group. VPA-treated zebrafish suffered from displaced and deformed livers compared to all the other groups. Metabolomics revealed significant differences in metabolites present in VPA-treated zebrafish versus the control group. The VPA treatment was associated with increased 5-methoxytryptophan, cystathionine, tryptophan, indoleacrylic acid, D-fructose 1,6-bisphosphate, L-valine, leucine, tyrosine, proline, and 2-octenoyl-carnitine. On the other hand, cocoa treatment of the VPA fish did not exhibit any metabolite differences from controls apart from increased caffeine and theobromine levels. Proteomics revealed significant differences in proteins present in VPA-treated zebrafish versus the control group. The VPA-treated group was associated with decreased synaptosomal-associated protein 25 (SNAP25). In contrast, cocoa treatment of the VPA fish did not exhibit any protein differences from controls apart from histone h3.2. Pathway analysis re-emphasized disrupted protein synthesis and amino acid metabolism such as tryptophan. This validated high throughput zebrafish VPA model of ASD lays the work for future research exploring the mechanistic pathways by which anti-epileptic drug exposure is associated with the development of ASD using large-scale omics. In conclusion, this study provides strong evidence on the efficacy of cocoa polyphenols in modulating behavioural symptoms of ASD in a chemically-induced zebrafish model, which could be mediated through their beneficial effects on the GI tract, metabolome, and proteome. Moreover, these results provide support towards further studies on the effectiveness of cocoa powder in treating ASD in other animal models, as well as in clinical studies in humans.

Abrégé

L'acide valproïque est un antiépileptique qui a été identifié comme un facteur de risque pour le trouble du spectre de l'autisme (TSA) lorsqu'il est pris pendant la grossesse. Il a aussi été utilisé pour induire le TSA dans des modèles animaux. Les interventions pour le TSA visent principalement à traiter des conditions comorbides spécifiques, incluant des approches thérapeutiques pour l'anxiété, les problèmes de comportement, les défis de communication, ainsi que les besoins occupationnels et nutritionnels. Bien que de nombreux traitements soient efficaces pour atténuer les symptômes du TSA et améliorer la qualité de vie, ils imposent invariablement des fardeaux supplémentaires en termes de dépenses financières et de temps d'administration. Cela peut être difficile à gérer pour les familles occupées, c'est pourquoi de nouveaux traitements faciles à administrer et pas chers à acquérir sont essentiels. Dans ce contexte, les polyphénols de cacao sont un candidat prometteur pour la thérapie du TSA, soutenus par des études qui soulignent leurs avantages cognitifs potentiels chez les adultes. Une étude menée auprès d'enfants diagnostiqués avec des TSA a rapporté des améliorations notables dans les analyses comportementales. De plus, en tant que complément nutritionnel, la poudre de cacao est également un prébiotique qui peut améliorer la santé gastro-intestinale, ce qui est important dans le TSA puisque la dysbiose intestinale et la dyspepsie sont des comorbidités courantes. Cette thèse décrit une évaluation de l'efficacité du traitement des symptômes comportementaux et physiologiques du trouble du spectre de l'autisme (TSA), en utilisant la poudre de cacao, dans un modèle de poisson-zèbre. D'abord, un modèle de TSA induit par l'acide valproïque (VPA) a été validé chez le poisson-zèbre. La toxicologie du VPA a été réalisée pour déterminer la dose optimale de VPA pour induire le phénotype autistique chez le poisson-zèbre sans causer de handicap physique sévère. Ensuite, une toxicologie de la poudre de cacao prébiotique a été menée pour déterminer les concentrations d'exposition les plus favorables pour assurer des effets bénéfiques sur le poisson-zèbre traité de VPA. En ce qui concerne les études sur le VPA et le cacao, les concentrations idéales des composés étaient quasiment similaires, respectivement de 3 μM et 2,5 μM. L'impact du traitement par le cacao dans le modèle de TSA de poisson-zèbre a été évalué en termes de comportement, de morphologie intestinale, de métabolomique et de

protéomique. Les poissons-zèbres traités au VPA ont démontré un phénotype semblable au TSA car ils ont présenté un comportement stressé avec des motifs de nage accrus et plus sporadiques. Les poissons-zèbres traités au VPA ont nagé de plus grandes distances à des vitesses plus élevées dans les cycles sombres et lumineux par rapport au contrôle, mais aussi par rapport aux groupes cacao et VPA + cacao. Les poissons traités au VPA avec du cacao sont restés calmes et se sont comportés de manière similaire aux contrôles dans le cycle sombre et sont restés plus calmes après la transition vers le cycle lumineux de l'expérience. Le traitement au VPA a causé une réduction sévère et des déformations du tractus gastro-intestinal par rapport aux contrôles alors que le traitement au cacao dans le groupe VPA a été associé à une morphologie intestinale similaire à celle du groupe de contrôle. Les poissons-zèbres traités au VPA ont souffert de déplacements et de déformations du foie par rapport à tous les autres groupes. La métabolomique a révélé des différences significatives dans les métabolites présents chez les poissons-zèbres traités au VPA par rapport au groupe de contrôle. Le traitement au VPA a été associé à une augmentation de 5-méthoxytryptophane, de cystathionine, de tryptophane, d'acide indoleacrylique, de D-fructose 1,6-bisphosphate, de L-valine, de leucine, de tyrosine, de proline et de 2-octénoyl-carnitine. D'autre part, le traitement au cacao des poissons VPA n'a montré aucune différence de métabolites par rapport aux contrôles à part des niveaux accrus de caféine et de théobromine. La protéomique a révélé des différences significatives dans les protéines présentes chez les poissons-zèbres traités au VPA par rapport au groupe de contrôle. Le groupe traité au VPA a été associé à une diminution de la protéine associée aux synaptosomes 25 (SNAP25) et à une augmentation du 5-méthoxytryptophane (5-MTP). En comparaison, le traitement au cacao des poissons VPA n'a montré aucune différence de protéines par rapport aux contrôles à part l'histone h3.2. L'analyse des voies de signalisation a réaffirmé la synthèse des protéines perturbée et le métabolisme des acides aminés comme le tryptophane. Ce modèle de VPA de poisson-zèbre de TSA validé pose les bases de recherches futures explorant les voies mécanistiques par lesquelles l'exposition aux médicaments antiépileptiques est associée au développement de TSA en utilisant des analyses omiques. En conclusion, cette étude fournit des preuves solides sur l'efficacité des polyphénols de cacao dans la modulation des symptômes comportementaux du TSA dans un modèle de poisson-zèbre induit chimiquement, grâce à leurs effets bénéfiques sur le tractus gastro-intestinal, le métabolome, et le protéome. De plus, ces résultats nous

permettront aussi de poursuivre des études supplémentaires sur l'efficacité de la poudre de cacao dans le traitement du TSA dans d'autres modèles animaux, ainsi que dans des études cliniques chez les humains.

Acknowledgements

First and foremost, I would like to extend my deepest gratitude to my supervisors, Dr. Stan Kubow and Dr. Kessen Patten. Their invaluable guidance and intellectual rigor have been pivotal to my academic journey. Their mentorship has undeniably shaped my growth as a researcher, and for this, I am profoundly grateful.

My sincere appreciation also extends to Dr. Ryan Mailloux, a member of my committee, whose expertise has been an asset throughout the progression of my research. I would be remiss not to acknowledge Dr. Michele Iskandar whose thoughtful feedback and detailed edits greatly contributed to shaping my proposal and final manuscript.

A special word of gratitude is due to Charlotte Zaouter. Her assistance in the planning and organization of my experiments, as well as in the development of my animal model, was truly indispensable. The success of this work can be attributed as much to her efforts as to mine.

I would also like to recognize the efforts of Dr. Lekha Sleno and Nathan Ghafari in helping me with the metabolomics and proteomics sample preparation and analysis. Your commitment to produce these results truly brought the whole project together.

My heartfelt thanks to all the lab members of Dr. Patten's group. Before all the jokes, I must seriously thank all of you for your support during the last two years from helping me with my experiments to giving me feedback on my thesis drafts and presentations. Without your support this thesis would have been many magnitudes more difficult to complete. First, I must thank Dr. Edouard Pan for his insights and seemingly endless bag of fun facts. Of course, I must express my deepest appreciation to Dr. Léa Lescourzières, Adeline Paimboeuf, and Fanny Nobileau for teaching me everything I know about zebrafish ... France, bread, cheese, and alcohol. I already miss our weekly Wednesday traditions. Finally, I could certainly not forget Jaskaran Singh, the brilliant PhD student who instills fear into presenters during the questions period. Thank you for sharing your unbelievably extensive research knowledge and delicious Indian food recommendations throughout the past year. I have no doubt you will become Hokage one day. Additionally, I'd like to extend a note of appreciation to the newcomers Sabrina Maher and Kelly Chan Chee. Their friendship made the final leg of this journey much more memorable.

To my wonderful girlfriend, Zhang Linhan, I owe an immense debt of gratitude. Her joy in our shared accomplishments has been a source of strength throughout this journey. This was certainly not an easy journey during which we shared many ups and downs (though it felt like there might have been more downs than ups), but we made it through together. I look forward to our many adventures ahead, with you by my side and our delightful Westie, Niunai, by our feet. Of course, I must dedicate a few words to our dog Niunai whose companionship brought much-needed moments of levity and comfort during the more stressful times.

致我的家人，你们一直以来是我最坚实的基础与支柱。感谢你们对我学业的支持与鼓励。你们在我的成长中授予了我知识与智慧，这篇论文仅能体现其中的一部分。其中，特别感谢我的父亲李景城，你对我的研究始终保持着浓厚的兴趣，会与我一起探究讨论并给予我很多反馈。我还要感谢我的母亲李燕，感谢你对我的信念从未动摇，感谢你不断培养我的意志，谢谢你成为我的灯塔。To my family, who are my foundation, I express my deepest gratitude. My father, Jingcheng Li, deserves special mention for his unwavering interest and efforts to understand my research and provide thoughtful feedback. This thesis is unfortunately only a small representation of the brilliance I gained from you and mom. My mother, Yan Li, whose faith in me never wavered and who continually fostered my aspirations, thank you for being my beacon. To my brother, Leon Li, who, while perhaps to this day does not know nor care about what I study, has always cared about me. Thank you, I love you all dearly.

This next one is for Rakeym Jean-Baptiste. You left us too soon, but your memory will live on. I dedicate all the hardships I went through in the past two years to you.

Thank you as well to all my friends, you know who you are, but you aren't each getting your own paragraphs. Nonetheless, I will acknowledge everyone's efforts in sending me memes and reels, pretending to be interested in cocoa powder and zebrafish, and having group therapy sessions. We will certainly play basketball and games (fight over Secret Hitler and scream over Stadium), travel, go fishing, and eat together again very soon.

Finally, to the hardworking employees at Monster Energy, your Ultra Paradise flavour is incredible. Thank you for fueling all those late nights and long days and giving me caffeine resistance.

This thesis is a testament to the collective effort and support from each one of you. I am eternally grateful.

Contribution of Authors

Jeffrey Xinyue Li (Candidate): participated in study design, was responsible for developing the protocols related to this thesis, performing in vivo data collection, and analyzing the data. The candidate wrote the thesis and generated figures and tables.

Dr. Michele Iskandar (Research Associate, School of Human Nutrition, McGill University): provided guidance for proposal development, statistical analyses and interpretation, and edited the thesis.

Nathan Ghafari (PhD Candidate, Sleno Lab, UQAM): performed the sample preparation and analysis of proteomic and metabolomic data.

Charlotte Zaouter (Research Associate, Patten Lab, INRS): provided guidance for the experiments conducted, aided in the development and husbandry of the animals and animal model, and helped collect data.

Dr. Lekha Sleno (Professor, UQAM): performed the sample preparation and analysis of proteomic and metabolomic data.

Dr. Kessen Patten (Co-supervisor of Candidate, Professor, INRS): provided guidance, research direction and feedback throughout this study and edited the thesis.

Dr. Stan Kubow (Co-supervisor of Candidate, Associate Professor, School of Human Nutrition, McGill University): provided guidance, research direction and feedback throughout this study and edited the thesis.

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5-HT	5-hydroxytryptamine.
5-HTT	Serotonin transporter.
5-MTP	5-methoxytryptophan.
AAAD	Aromatic L-amino acid decarboxylase.
ABA	Applied behaviour analysis.
ACTH	Adrenocorticotrophic hormone.
ADHD	Attention-Deficit/Hyperactivity Disorder.
AhR	Aryl hydrocarbon receptor.
ALS	Amyotrophic lateral sclerosis.
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.
ARID1	AT-rich interactive domain-containing protein 1.
ARS	Acute response stress.
ASD	Autism spectrum disorder.
ASMT	N-acetylserotonin O-methyltransferase.
ATEC	Autism Treatment Evaluation Checklist.
BBB	Blood brain barrier.
BDNF	Brain derived neurotrophic factor.
CDC	Centre for Disease Control.
CHARGE	Coloboma, heart disease, atresia of the choanae, retarded growth and mental development, genital anomalies, and ear malformations and hearing loss.
CHD7	Chromodomain helicase-binding protein 7.
CHD8	Chromodomain helicase-binding protein 8.
CNS	Central nervous system.
CNTNAP2	Contactin-Associated Protein-Like 2.
CRH	Corticotropin receptor hormone.
DAT	Dopamine transporter.
DPF	Days post-fertilization.

EEC	Enteroendocrine cell.
EMA	European Medicines Agency.
ENS	Enteric nervous system.
ESDM	Early Start Denver Model.
FC	Fold change.
FDA	Food and Drug Administration.
FDR	False discovery rate.
FMR1	Fragile X Messenger Ribonucleoprotein 1.
FMT	Fecal matter transplant.
FOS	Fructooligosaccharide.
FRAP	Ferric reducing antioxidant power assay.
FXS	Fragile X Syndrome.
GABA	Gamma-aminobutyric acid.
GBA	Gut-brain axis.
GF	Germ-free.
GI	Gastrointestinal.
GM	Gut microbiota.
GMBA	Gut-microbiota-brain axis.
GOS	Galactooligosaccharide.
GSH	Glutathione.
GSSG	Oxidized glutathione.
HDAC	Histone deacetylase.
HIOMT	Hydroxyindole O-methyltransferase.
HPA	Hypothalamic-pituitary-adrenal.
HPF	Hours post-fertilization.
HTS	High-throughput screen.
LC-MS	Liquid chromatography-mass spectrometry.
LDL	Low-density lipoprotein.
LPS	Lipopolysaccharide.
MECP2	Methyl-CpG binding protein 2.
MTC	Maximum tolerable concentration.
NAD	Nicotinamide adenine dinucleotide.

NADP	Nicotinamide adenine dinucleotide phosphate.
NADPH	Reduced NADP.
NLGN3	Neuroligin-3.
NMDAR	N-methyl-D-aspartate receptor.
NO	Nitric oxide.
NRF2	Nuclear factor erythroid 2-related factor 2.
NRXN1	Neurexin-1.
NT	Neurotransmitter.
PCA	Principle component analysis.
PEA	Phenylethylamine.
PET	Positron emission tomography.
PFP	Pentafluorophenyl.
PHTS	PTEN hamartoma tumor syndrome.
PI3	Phosphatidylinositol 3-phosphate kinase.
PNS	Parasympathetic nervous system.
PRE	Polyphenol-rich extract.
PTEN	Phosphatase and tensin.
PVA	Phenylvaleric acid.
PVL	Phenyl-δ-valerolactone.
REDOX	Reduction oxidation.
ROS	Reactive oxygen species.
RP	Reverse-phase.
SCFA	Short-chain fatty acid.
SERT	Serotonin transporter.
SFN	Sulforaphane.
SHANK3	SH3 and multiple ankyrin repeat domains 3.
sMRM	Scheduled multiple reaction monitoring.
SNAP25	Synaptosomal-associated protein 25.
SNP	Single-nucleotide polymorphism.
SPF	Specific pathogen free.
SREM	Structurally related (–)-epicatechin metabolites.
TAAR	Trace amine-associated receptor.

tBOOH	Tert-butyl hydroperoxide.
TLR	Toll-like receptor.
TOF-MS	Time-of-flight mass spectrometry.
TPH	Tryptophan hydroxylase.
UHPLC	Ultra-High-Performance Liquid Chromatography.
VPA	Valproic acid.
ZF	Zebrafish.

General Introduction

1.1 Introduction

Autism Spectrum Disorder (ASD) is an increasingly prevalent neurodevelopmental disorder affecting a considerable proportion of the global population. It is a condition of significant medical and societal impact, characterized by a wide spectrum of clinical presentations affecting social interaction, communication, and behavior. The Centers for Disease Control and Prevention (CDC) currently estimates that approximately 1 in 59 children worldwide are affected by ASD, highlighting its pervasive nature (for Disease Control and Prevention, 2009). Moreover, the rate of ASD diagnoses appears to be escalating, especially within developed nations, a trend which may be influenced by a combination of factors including heightened awareness and advances in diagnostic methodologies (Fombonne, 2018).

ASD is a complex condition with a multifaceted etiology that encompasses both genetic and environmental factors. Genetic influences have long been recognized in the development of ASD, with numerous genetic loci and associated genes being implicated, including mutations in genes such as SH3 and multiple ankyrin repeat domains 3 (SHANK3), phosphatase and tensin (PTEN), and others (Geschwind, 2011). Nevertheless, the genetic landscape of ASD is exceptionally heterogeneous and continues to be the subject of extensive research. High-throughput sequencing technologies, including whole-exome and whole-genome sequencing, are increasingly being employed to uncover novel ASD risk genes and genetic variants, shedding light on the intricate genetic

underpinnings of this disorder.

The interaction of these genetic susceptibilities with environmental influences adds an additional layer of complexity to ASD etiology. Prenatal and early postnatal environmental exposures, including certain medications, maternal health conditions, and possibly certain dietary factors, have been associated with an increased risk of ASD (C. Wang et al., 2017). Epigenetic modifications, induced by environmental exposures, may mediate some of these risks, marking an exciting frontier in ASD research (Schanen, 2006).

The clinical presentation of ASD is incredibly diverse, with a broad array of cognitive, behavioral, and emotional manifestations (Hodges et al., 2020). This heterogeneity is suggestive of a complex biological architecture, potentially encompassing multiple pathways and biological mechanisms. In this regard, studies investigating the neurobiology of ASD have reported alterations in synaptic function, neuronal connectivity, and neuroimmune regulation, among others (Hodges et al., 2020; Rylaarsdam and Guemez-Gamboa, 2019). Ongoing research is keenly focused on elucidating these mechanisms, with the hope of providing more targeted and effective therapeutic strategies.

Presently, treatments for ASD are primarily behavioral and aim to enhance social communication skills, reduce maladaptive behaviors, and improve overall functioning. Established interventions, such as applied behavior analysis (ABA) and the Early Start Denver Model (ESDM), have been demonstrated to improve outcomes in some children (Dawson et al., 2010). In terms of pharmacological management, certain medications can be employed to manage associated symptoms such as irritability, hyperactivity, and anxiety, although these do not address the core symptoms of ASD (Fung et al., 2016).

Despite the availability of these therapeutic options, ASD generally remains a lifelong disorder, leading to substantial disability and a considerable impact on the quality of life

for affected individuals and their families. Consequently, the need for novel, more effective therapeutic strategies is undeniable. Researchers are increasingly exploring potential therapeutic targets, such as modulating synaptic function or addressing neuroinflammation, as promising avenues for future ASD treatments (Cellot and Cherubini, 2014; Mossa et al., 2018; Baranova et al., 2021). Simultaneously, efforts are underway to develop reliable early markers for ASD, which would facilitate early intervention and potentially improve long-term outcomes (Gliga et al., 2014; Barbaro and Dissanayake, 2013).

In this regard, cocoa polyphenols have emerged as promising therapeutic candidates given their low cost to acquire and comparatively minimal time required to administer/consume. Indeed, studies have found that polyphenols in general have been found to be beneficial for ASD subjects' cognitive function. Polyphenols in general have been found to be important in maintaining and improving cognitive function (Vauzour, 2012). Additionally, intake of prebiotic polyphenol-rich cocoa extracts (PRE) has been associated with maintaining and improving cognitive function in various domains of cognition in adults (Mastroiacovo et al., 2015; Socci et al., 2017). Cocoa polyphenols have also been found to improve mood in healthy adults and children with ASD (Pase et al., 2013; Sadek et al., 2018). Nonetheless, despite promising results thus far, more research is needed on the efficacy of cocoa polyphenols in treating the symptoms of ASD. Indeed, a more mechanistic understanding of the impact of cocoa polyphenols in the pathophysiology of ASD is needed before establishing it as a reliable therapeutic for humans.

This thesis evaluated a novel VPA-induced zebrafish model of autism and examined the efficacy of a polyphenol-rich cocoa extract in treating the behavioural symptoms, gut morphology, and metabolomic and proteomic profiles of autistic zebrafish.

1.2 Hypotheses

1. The prebiotic cocoa treatment can counteract VPA-induced deficits in gut morphology and behaviour.

2. VPA-treated zebrafish will express dysregulated metabolism and protein expression assessed via metabolomics and proteomics.

3. Cocoa-treated VPA-treated zebrafish will express similar metabolomic and proteomic profiles to control zebrafish.

1.3 Objectives

1. Validate a chemically-induced model of autism in zebrafish using valproic acid (VPA) which will exhibit gut morphological and behavioural autistic-like deficits.

2. Evaluate the metabolome and proteome of VPA-treated zebrafish

3. Determine a non-toxic dose of polyphenol-rich cocoa powder co-treatment and its efficacy in preventing the behavioural and morphological symptoms of the VPA treatment in zebrafish.

4. Examine the impact of cocoa powder co-treatment on the metabolome and proteome of VPA-treated zebrafish.

1.4 Scope

This study aimed to validate the ASD zebrafish model using valproic acid. This objective was developed to provide a high-throughput screening approach of testing potential therapeutics as proof-of-concept towards testing in other animal models and possible future clinical applications. This ASD model was used to test the impact of polyphenol-rich cocoa powder co-treatment on outcomes related to behavioural symptoms and gut morphology as well as metabolomic and proteomic parameters. The study was directed towards furthering our understanding of how dietary interventions involving polyphenol-rich cocoa powder could influence ASD behavior with particular focus on the metabolome and proteome.

Literature Review

2.1 Autism Spectrum Disorder

In recent years, considerable strides have been achieved in the field of neurodevelopmental research, with a particular emphasis on Autism Spectrum Disorder (ASD). This complex, multifactorial condition is characterized by a range of symptoms, including social communication challenges, restrictive repetitive behaviors, and often, co-occurring conditions such as intellectual disability or epilepsy. The pathogenesis of ASD has traditionally been attributed to genetic factors, with numerous risk loci identified (Rylaarsdam and Guemez-Gamboa, 2019). However, recent research suggests that the development of ASD might also be influenced by environmental factors and the complex interplay between genes and environment (Hodges et al., 2020).

2.1.1 ASD as a developmental disorder

ASD is a neurodevelopmental disorder characterized by deficits in social interaction, verbal and non-verbal communication, and the presence of restricted and repetitive behaviors (Rylaarsdam and Guemez-Gamboa, 2019; Hodges et al., 2020). These symptoms typically manifest in early childhood and persist throughout the lifespan, impacting an individual's ability to function in social, academic, and occupational domains. While the exact etiology of ASD remains unknown, it is thought to result from a complex interplay of genetic and environmental factors (Rylaarsdam and Guemez-Gamboa, 2019; Hodges et al., 2020).

Genomic constituents are implicated in the predisposition to ASD. A heightened prevalence of ASD diagnosis is observed amongst biological siblings of ASD patients, presenting a deviation from population-based averages. Moreover, a substantially elevated yet not absolute concordance of ASD diagnosis is evident in monozygotic twin pairs (Sandin et al., 2014; Risch et al., 2014). Numerous genes and chromosomal regions have been implicated in the disorder, reflecting the considerable genetic heterogeneity (An and Claudianos, 2016; Bruining et al., 2010). Genome-wide association studies and whole exome sequencing methodologies have vastly expanded our knowledge of genes that contribute to Autism Spectrum Disorder (ASD) susceptibility. Further elucidation of these gene functions could illuminate possible biological mechanisms (Walsh et al., 2008). Examples of such ASD candidate genes encompass those that influence cerebral development or neurotransmitter activity, as well as genes that modulate neuronal excitability (McDougle, Erickson, et al., 2005; Rubenstein and Merzenich, 2003). A considerable number of genetic aberrations linked with ASD encode proteins pertinent to neuronal synapse functionality or proteins involved in activity-dependent modifications in neurons, inclusive of regulatory entities like transcription factors (H. Kim et al., 2019; Zoghbi, 2003). Hypothetical "networks" of converging ASD genetic risk may encompass pathways associated with neurotransmission and neuroinflammation (Voineagu et al., 2011). Transcriptional and splicing anomalies or modifications in epigenetic mechanisms, such as DNA methylation or histone acetylation and modification, may also be implicated (H. Kim et al., 2019; Voineagu et al., 2011). Overall, ASD continues to be one of the most genetically diverse neuropsychiatric disorders, with infrequent de novo and hereditary variants appearing in over 700 genes (Hodges et al., 2020). These genetic alterations often affect synaptic function, neural connectivity, and transcriptional regulation, which might

explain the broad spectrum of phenotypic presentations in ASD (Geschwind, 2011).

Although genetic factors undeniably contribute to the etiology of ASD, the expression of genetic susceptibility exhibits significant variability in ASD (Veenstra-VanderWeele et al., 2004). A recent meta-analysis highlighted several prenatal, perinatal, and postnatal risk factors leading to a raised relative risk of ASD in offspring (C. Wang et al., 2017), yet also exposed substantial heterogeneity, preventing definitive conclusions about the significance of these factors. Increased risk has also been observed with prenatal exposure to anti-epileptic drugs such as thalidomide and valproic acid (VPA) (Rasalam et al., 2005). Interestingly, Suren et al. (2013) found that prenatal supplements of folic acid may reduce the incidence of ASD by half (Surén et al., 2013). However, no research has evaluated whether the benefits of folic acid supplementation may mitigate the risk of ASD caused by prenatal exposure to VPA. Additionally, autoimmune conditions in the mother, such as diabetes, thyroid disease, or psoriasis, have been theorized as potential risk factors, although studies have yielded inconsistent results (Croen et al., 2005; Xiang et al., 2018). Another area of interest is maternal infection or immune activation during pregnancy, which recent studies suggest may constitute a potential risk factor (Croen et al., 2019; Malkova et al., 2012; G. B. Choi et al., 2016). Furthermore, premature birth has been linked with a heightened ASD risk, along with other neurodevelopmental disorders (Agrawal et al., 2018).

Overall, the study of ASD as a neurodevelopmental disorder is a rapidly evolving field that continues to unveil the intricate interplay of genetic, environmental, and possibly, microbiota factors in its etiology. More research is needed to elucidate these complex relationships, which would hopefully pave the way for novel preventive strategies and therapeutic interventions.

2.1.2 Neurochemistry of ASD

Over the last few years, there has been an exponential rise in interest in the neurochemistry of ASD. Unraveling the complex neurochemical mechanisms implicated in ASD can provide crucial insights into its etiology and could potentially lead to the development of targeted pharmacological interventions (Jiang et al., 2022).

ASD is typified by a highly heterogeneous neurochemical profile, with several neurotransmitter systems thought to play pivotal roles. Among these, the serotonergic, dopaminergic, and glutamatergic systems have been intensively studied (Marotta et al., 2020; Jiang et al., 2022).

Serotonin

Numerous research investigations have demonstrated the involvement of the serotonin system in early brain development and its potential link to the onset of autism (Yang et al., 2014). Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter in the monoamine family that influences several developmental processes, such as cellular division, cortical proliferation and differentiation, migration, cortical plasticity, and synaptogenesis (Celada et al., 2013; Gaspar et al., 2003). It also plays crucial roles in various cognitive functions like memory and learning, and acts as a modulator of sleep and mood (Jenkins et al., 2016). Elevated levels of serotonin or its transporter (SERT or 5-HTT) have been observed in both autistic children and animal models when compared to controls . Moreover, postmortem examinations have revealed a decrease in the binding of both 5-HT2A and 5-HT1A in the ASD brain (Rose'Meyer, 2013; Muller et al., 2016; Siemann et al., 2017; Abdulamir et al., 2018). Positron emission tomography (PET) studies have demonstrated

that typically developing children between two to five years exhibit elevated 5-HT synthesis that subsequently declines around puberty. However, this decline is not observed in children with autism. In fact, their ability to synthesize serotonin remained consistent over time, and the levels were notably lower in these children between two to five years when compared to controls, though there was a slight increase with age (B. J. Hwang et al., 2017; Chugani et al., 1999).

Investigations have identified hyperserotonemia, or high serotonin levels, in platelets of ASD subjects, with average increases ranging from 20% to 50% (Gabriele et al., 2014). This phenomenon appears to be specific to autism or ASD, as it has not been observed in cases of intellectual disability or other neuropsychiatric disorders (Mulder et al., 2004). Despite the mounting evidence confirming the role of serotonin in the pathophysiology of autism, the mechanism behind the elevation of serotonin levels remains unclear. Additionally, the connection between these elevated levels and the functioning of the central serotonergic system requires more in-depth investigation.

Dopamine

Dysfunction in the dopaminergic system has also been implicated in ASD. Dopamine is a neurotransmitter crucial to the regulation of reward and pleasure centers, mood, and motor function. Besides its crucial function in motor regulation, dopamine also plays a vital role in social cognition and behaviors, particularly through the mesocorticolimbic pathway.

An array of studies has proposed a potential association between ASD and dysfunctions in the dopaminergic system. These studies hypothesize that imbalances of dopamine in specific cerebral regions might contribute to the manifestation of autistic behaviors (Dichter et al., 2012). Genetic research has identified a link between autism and various

gene polymorphisms involved in dopaminergic pathways, including dopamine transporter (DAT) and dopamine receptors DR3 and DR4 (Gadow et al., 2010; Staal, 2015). A recent study on mouse models highlighted that DAT mutations could cause abnormal dopamine efflux and result in autism-like behavioral phenotypes (DiCarlo et al., 2020). Additionally, these dopaminergic gene polymorphisms are thought to influence emotion dysregulation and symptoms of ADHD in children with ASD (Gadow et al., 2014). Furthermore, the haploinsufficiency of *SHANK3*, a gene that has been strongly associated with ASD, has been associated with reduced neuronal dopaminergic activity in the ventral tegmental area, leading to behavioral anomalies, including social skills impairments (Bariselli et al., 2016). Finally dopamine receptor blockers (risperidone and aripiprazole) have been approved by the EMA/FDA for treating irritability, and these have also demonstrated efficacy in managing repetitive behaviors associated with ASD (McDougle, Scahill, et al., 2005; Marcus et al., 2009).

Glutamate

The glutamatergic system, the primary excitatory neurotransmitter system in the brain, is also believed to play a significant role in ASD. Glutamate is involved in synaptic plasticity, learning, and memory. Abnormalities in glutamatergic signaling could potentially disrupt the balance between neuronal excitation and inhibition, contributing to the cognitive and behavioral symptoms seen in ASD (Rubenstein and Merzenich, 2003).

Glutamate, the primary excitatory neurotransmitter in the mammalian cortex, interacts with three principal receptor classes: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), and metabotropic glutamate receptors. Evidence implicates both NMDARs and AMPARs in

ASD pathophysiology (M. Essa et al., 2013; Petroff, 2002). Autism rodent models induced by valproic acid demonstrated a selective upregulation of NMDA receptors subunits NR2A and NR2B. This overexpression induced enhanced NMDA receptor-mediated synaptic currents, leading to heightened postsynaptic plasticity in neocortical pyramidal neurons (Rinaldi et al., 2007).

Genetic studies highlighted a correlation between the development of autistic traits, glutamatergic dysregulation, and mutations in genes (*SHANK*, *NLGN3*, *NLGN4*, and *UBE3A*) implicated in synapse formation and maintenance, as well as protein targeting (Trobiani et al., 2018; Krishnan et al., 2017; Marro et al., 2019). However, a comprehensive analysis of glutamatergic and GABAergic gene sets in subjects with ASD and ADHD displayed a significant association only between the glutamate gene set and the severity of hyperactivity and impulsivity symptoms (Naaijen et al., 2017). No significant associations were found for autism symptoms in the glutamate and GABA gene sets, underscoring the necessity for additional research on the genetic aspects of excitatory/inhibitory imbalance in ASD.

Gamma-aminobutyric acid

Additionally, there is growing evidence for the involvement of the gamma-aminobutyric acid (GABA) system in ASD. GABA is the chief inhibitory neurotransmitter in the mammalian central nervous system, playing a central role in reducing neuronal excitability. Imbalances between excitatory glutamate and inhibitory GABA signaling have been hypothesized in ASD, although the exact mechanisms remain to be elucidated (Cellot and Cherubini, 2014).

GABA produced from glutamate via the activity of glutamate decarboxylase, serves to

balance neuronal excitability through a complex and homeostatic relationship. During brain development, GABA acts as the primary excitatory neurotransmitter, affecting cellular proliferation, migration, synapse maturation, differentiation, and apoptosis (Owens and Kriegstein, 2002). Evidence supporting the excitatory/inhibitory imbalance theory for social behavior deficits comes from studies where prolonged cell depolarization in mice medial prefrontal cortex elevated the excitatory/inhibitory balance, leading to significant impairment in information processing and social behavior dysfunction (Yizhar et al., 2011).

Melatonin

Children with ASD often suffer from ASD sleep disorder as comorbidities (Marotta et al., 2020). Melatonin, an integral regulator of the sleep-wake cycle, not only minimizes sleep latency but also acts as a potent antioxidant (Galano et al., 2011). Additionally, it plays a role in neurodevelopment, neural plasticity, placental homeostasis, and immune functions (Galano et al., 2011; Bubenik, 2002). Research involving autistic individuals indicates decreased plasma levels of melatonin or its metabolites and reduced urinary excretion rates of melatonin sulfate (Tordjman et al., 2013). Furthermore, a 2018 study demonstrated significantly lower levels of 6-sulfatoxymelatonin in mothers who have a child with ASD compared to control subjects (Braam et al., 2018). Indeed, melatonin is able to cross the placenta during pregnancy helping the fetus establish a normal sleep cycle essential to neurodevelopment (Jin et al., 2018). More recently, melatonin has been used a treatment for children with ASD suffering from sleep disorders and has also been found to be effective in improving daytime behaviours, anxiety, depression, and gastrointestinal dysfunctions (Malow et al., 2012; Cuomo et al., 2017; Gagnon and Godbout, 2018).

While significant progress has been made in understanding the neurochemistry of ASD,

the precise neurochemical changes and how they relate to the complex behavioral manifestations of ASD remain largely unknown. Considering the vast heterogeneity of ASD, it is likely that multiple neurochemical systems interact in a complex and individual-specific manner. As such, the pursuit of a singular neurochemical hypothesis for ASD may be overly reductive. Rather, an integrative approach considering the interaction of multiple neurochemical systems may hold the key to unraveling the complex neurochemistry of ASD.

2.1.3 Behavioural features of subjects with ASD

ASD is characterized by a complex array of behavioural features, which can vary considerably in their presentation and severity due to the spectrum nature of the condition. These features can be broadly classified into: social-communication difficulties and restricted interests, repetitive behaviors, and sensory deficits (Association et al., 2000; Samson et al., 2014).

Social communications difficulties are the most recognizable features of ASD and can manifest in various ways. Individuals with ASD may exhibit impaired social reciprocity where they may show less interest in social interaction, struggle to initiate and maintain conversations, or fail to understand social norms and conventions (Association et al., 2000). There may also be reduced sharing of enjoyment or showing items of interest to others, a behaviour that is commonly observed in early childhood (Association et al., 2013). Additionally, subjects with ASD may also face difficulties navigating non-verbal communication as they are unable to decrypt social cues or respond to physical expression such as body language, facial expression, or the use of gestures (Wimpory et al., 2002). Finally, patients with ASD often employ speech and language differently from typical

patterns. In fact, while some individuals may not speak much at all, others might have an extensive vocabulary but struggle heavily with pragmatic aspects of language such as conversation turn-taking, interpretation of idioms, or understanding the intent of others (Tager-Flusberg et al., 2005; Klin, Saulnier, et al., 2007).

Individuals with ASD often have intense fixations on specific topics or objects, and they may know a great deal about these subjects. However, these interests are typically narrow and may not include other related topics (Klin, Danovitch, et al., 2007). Similarly, many individuals with ASD also have a strong desire for routines and predictability. This can manifest as distress over minor changes in their environment, strict adherence to routines, rituals around eating (time of meals and components of the meal itself), or other daily activities (Richler et al., 2010).

Patients with ASD may also demonstrate repetitive motor movements which can include hand-flapping, rocking, spinning, or repetitive use of objects and mannerisms. These behaviours in many cases are self-soothing and can increase during times of stress or excitement (Leekam et al., 2011). Moreover, many individuals also exhibit unusual responses to sensory input. This can involve either hypo- or hyper-sensitivity to sensory stimuli, such as sounds, lights, textures, tastes leading to avoidance, stress, or seeking of specific sensory experiences (Ben-Sasson et al., 2009).

It is important to note that these features can manifest differently across individuals, can fluctuate over time, and are also influenced by other factors such as age, cognitive ability, and co-occurring conditions. As such, ASD is a highly heterogeneous condition with diverse behavioural presentations. It is crucial that healthcare professionals and caregivers adopt a flexible, individualized approach to understanding and supporting individuals with ASD. At the same time, research on ASD must also recognize the heterogeneity of the disorder in

order to better study and elucidate its causes and find potential treatments.

2.2 Role of the gut microbiome in ASD

Over the past decade, significant research has been dedicated to studying the dynamic intersection between microbiology and neuroscience. Specifically, many have investigated the interactions between hosts and their bacterial communities termed the microbiome (Kinross et al., 2011; Gould et al., 2018). The pathways are numerous, complex, and mechanistically involve chemical, neuronal, and even immunological signalling (Morais et al., 2021). The leaps made in characterizing and understanding the animal microbiome has changed the way we understand the pathology of disease. Recent research has highlighted how the microbiome can alter and influence the production of metabolic and neurochemical factors in the gut which can impact the nervous system (Rutsch et al., 2020; Cryan et al., 2019). These findings have led to research correlating gut health with many neuropsychiatric disorders such as depression, Alzheimer's Disease, and ASD (Carabotti et al., 2015; L. Liu et al., 2022).

The gut microbiome (GM) is a community of microorganisms that persist in the digestive systems of humans and animals (Morais et al., 2021; Pulikkan et al., 2019). Representing the greatest abundance of microoganisms in the body, the gut microbiota is also active in the healthy modulation of our immune system and metabolism (Cryan et al., 2019). In addition, the GM is also responsible for maintaining brain-gut communication, which can affect host neurological functions (Pulikkan et al., 2019; Rutsch et al., 2020). Overall, the composition of human microbiota can change in response to changing host factors such as age and genetics and environmental factors like diet and drug use (Ribeiro et al., 2022;

Góralczyk-Bińkowska et al., 2022). The genetic variance in the gut microbiome is extremely vast and estimated to be around 232 million genes, which significantly increases the gut's metabolic capabilities (Tierney et al., 2019). Therefore, the bacteria present in the human gut are not only a reflection of the environment, but also of the host's health and can even directly influence host health.

2.2.1 The gut-microbiota-brain axis and gut dysbiosis

The gut-microbiota-brain axis (GMBA) refers to a complex network of bidirectional pathways that connect the gut and brain (Carabotti et al., 2015; Cryan et al., 2019; Rutsch et al., 2020; Morais et al., 2021; L. Liu et al., 2022). The connections between the brain and gut are crucial in maintaining proper homeostasis of many physiological functions (Cryan et al., 2019; Carabotti et al., 2015). The communication through these channels is bi-directional and can be both direct and indirect through signalling via chemicals, immune factors, and neurotransmitters. Furthermore, the GMBA extends beyond just the digestive system and the brain. In fact, given this axis comprises the vagal, immune, and humoral pathways, it is therefore also connected to many other organs and biological systems as well (**Figure 2.1** below) (J.-G. Lee et al., 2021; Rutsch et al., 2020).

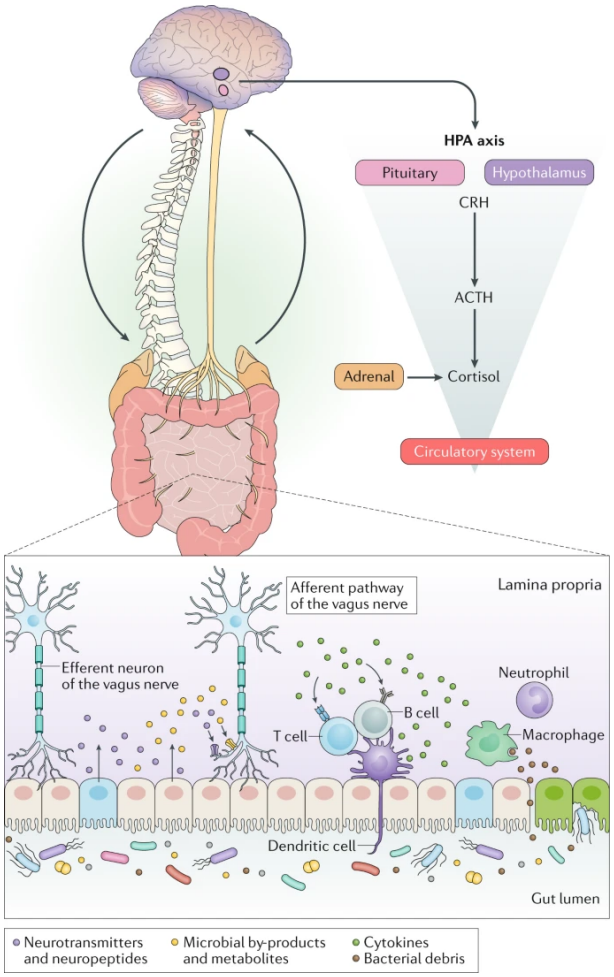

Figure 2.1: The gut microbiota-brain axis from Morais et al. (2021).

The gut microbiota and the central nervous system (CNS) engage in a two-way communication via various pathways within the gut-brain axis. The communication channels encompass components of the autonomic nervous system, such as the enteric nervous system (ENS) and the vagus nerve, the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and metabolic routes. Inside the gut, the microbiota generates neuroactive compounds and metabolites that can interact with the host immune system, influence metabolism, and impact local neuronal cells. The gut microbiome can also affect gut barrier integrity, which regulates the transmission of signaling molecules. Within the nervous system, stress can induce the HPA axis response, involving hypothalamic neurons that secrete hormones such as corticotropin receptor hormone (CRH) into the brain or the portal circulation, leading to the release of adrenocorticotrophic hormone (ACTH). This subsequently triggers the synthesis and release of cortisol, which modulates neuroimmune signaling responses that, in turn, impact intestinal barrier integrity. Stress hormones, immune mediators, and CNS neurotransmitters can stimulate neuronal cells of the ENS and afferent pathways of the vagus nerve, thereby altering the gut environment and the composition of the microbiota.

Gut dysbiosis refers to disturbances in the health of the gut microbiota caused by host and environmental factors leading to a change in microbial composition. Gut dysbiosis has been related to inflammatory bowel disease, autoimmune diseases, and cardiovascular diseases (Pulikkan et al., 2019) and has been shown to dysregulate brain-gut communication as well (Fröhlich et al., 2016; Dinan and Cryan, 2017).

Brain-gut crosstalk is further supported by the evidence that enteroendocrine cells (EECs) mediate the communication between the gut and brain via the vagus nerve using serotonin, a significant monoamine and neurotransmitter in brain development and functioning, which is primarily produced in the gut (Kulkarni et al., 2018; Li et al., 2000; Latorre et al., 2016; Mittal et al., 2017). On the other hand, EECs detect signals from the gut microbiota via toll-like receptors (TLRs) for bacterial products and metabolites like lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs) (Samuel et al., 2008; Abreu et al., 2005). Toll-like receptors are a family of transmembrane pattern recognition receptors and mediate the transduction of signalling upon recognition of damage and pathogen associated molecular patterns (Lin et al., 2019). TLRs have been identified to be involved in the pathogenesis of neurodegenerative diseases like Alzheimer's Disease and Parkinson's Disease (Lin et al., 2019; Holmqvist et al., 2014). The involvement of gut dysbiosis-related chronic inflammation and viral infection has been found to alter the permeability of the blood brain barrier resulting in behavioural and cognitive changes (Braniste et al., 2014; Larroya-García et al., 2019;Leclercq et al., 2017). Given the crosstalk of gut and brain in neurodegenerative diseases, there is evidence also pointing to similar effects of gut dysbiosis in neurodevelopmental diseases such as ASD.

2.2.2 Modulation of the gut microbiome

Modulation of the gut microbiome has become a highly researched solution to treating symptoms of ASD. In this framework, studies have evaluated probiotics which are living non-pathogenic microorganisms that can provide health benefits from their oral intake (Fattorusso et al., 2019). Probiotic treatments for ASD subjects have been reported to positively modulate the GM (Fattorusso et al., 2019). Specific combinations of probiotics detailed in the **Table 2.1** below in clinical trials have normalized gut microbial composition, which has been linked with improved disruptive social behaviors and decreased severity of ASD, evidenced by improved Autism Treatment Evaluation Checklist – ATEC scores (Fattorusso et al., 2019). Furthermore, Pärtty et al. (2015) describes an interesting study where a probiotic supplementation, *L. Rhamnosus* GG, was administered in a randomized control trial (RCT). They then examined the two groups, one control where the children received no treatment and one where the children got probiotics, and found that the incidence of ASD or ADHD in the non-treated group was 17% compared to 0% in the probiotic group. While these findings do not highlight the potential of probiotics in treating ASD, they do illustrate the importance of gut health in neurodevelopment, hence the gut-brain axis, after birth.

Probiotic Treatments in Humans			
Population	Intervention	Study type	Main Findings
Children with ASD n=33	*L. Acidophilus* *L. Rhamnosus* *L. Delbrueckii* *B. Longum* *B. Bifidum* *B. Casei*	Single-arm intervention	- Reduced ASD severity (West et al., 2013)
Children with ASD 4-10 years old n=22	*L. Acidophilus* Rosell-11	Cohort study	- Reported improvement in children concentration abilities (Kałużna-Czaplińska and Błaszczyk, 2012)
Children with ASD 5-9 years old n=30	*L. Acidophilus* *L. Rhamnosus* *B. Longum*	Single-arm intervention	- Improved GI symptoms - Reduced ASD severity (Shaaban et al., 2018)
Healthy infants n=75	*L. Rhamnosus* GG	RCT with 13-year follow-up	- 17% of control group diagnosed with ASD or ADHD - None in probiotic group (Pärtty et al., 2015)

Table 2.1: Effects of probiotic treatments in healthy human subjects and those with ASD.

Alongside, probiotics, prebiotics are defined as food products that when ingested result in specific changes in the composition and/or activity of the gut microbiota that may confer additional host health benefits (Gibson et al., 2017). Although prebiotics are ingested in the diet, they are considered to be nondigestible in the human digestive system. Instead, these products are selectively fermented by intestinal microflora which results in the stimulation and/or growth of the gut microbiota that is associated with human well-being (Gibson et al., 2017; Slavin, 2013).

2.2.3 Potential of polyphenol-rich prebiotics

Polyphenols are naturally occurring phytochemicals present in large quantities in fruits and vegetables (Scalbert et al., 2005). Their proposed benefits within the context of ASD are due to two factors. The first is their benefit for improved cognitive function (Vauzour, 2012). Indeed, polyphenols have been found to be beneficial for cognitive function in ASD subjects. For example, cocoa polyphenols have been found to improve mood in healthy adults and children with ASD (Pase et al., 2013; Sadek et al., 2018). The exact mechanism of these improvements is unknown. However, it has been postulated that cocoa polyphenols may induce nitric oxide (NO) mediated vasodilation leading to enhanced blood flow in the brain (Magrone et al., 2017; Marsh et al., 2017) in addition to activating anti-inflammatory and antioxidant pathways such as Nuclear factor erythroid 2-related factor 2 (NRF-2) (Sies et al., 2005). Moreover, cocoa flavonols were found to suppress lipid peroxidation in LDL induced by myeloperoxidase given sufficient levels of NO (Sies et al., 2005). In a meta-analysis, Barrera et al., 2020 found that polyphenol metabolites from cocoa such as structurally related (–)-epicatechin metabolites (SREM), phenyl-δ-valerolactones (PVLs), and phenylvaleric acids (PVAs) positively affected memory and executive function in ageing adults with mild cognitive impairment. Specifically, prebiotic polyphenol-rich cocoa extracts (PRE) have been associated with maintaining and improving cognitive function in various domains of cognition in adults (Socci et al., 2017; Mastroiacovo et al., 2015). These cognitive benefits are hypothesized to occur due to the microbial metabolites from polyphenols which can cross the blood brain barrier and localize to regions of the brain such as hippocampus, cerebral cortex, cerebellum, and striatum which are involved in learning and memory (Barrera-Reyes et al., 2020).

Mechanistically, a possible explanation for these effects is the role of polyphenol derived metabolites that inhibit NADPH oxidase, an enzyme responsible for the generation of superoxide which forms peroxynitrite radicals with excess nitric oxide (Tripathi et al., 2020, Radi, 2018) to induce glutamate release and cause excitotoxicity (Steffen et al., 2008). In addition, the formation of peroxynitrite, an important biomarker in autism (Goldani et al., 2014, Tripathi et al., 2020) also inhibits mitochondrial respiration and causes mitochondrial dysfunction (Boczkowski et al., 2001). The excess formation of glutamate and superoxide radicals align with the observed dysregulation of neurotransmitters and redox metabolism in ASD subjects (R. Frye et al., 2013, R. E. Frye, 2020, D. Rossignol and Frye, 2012, D. A. Rossignol and Frye, 2014, Siddiqui et al., 2016).

Secondly, polyphenols may also benefit ASD subjects due to their ability to mitigate gut dysbiosis and improve gut health due to their reported roles as prebiotics (Plamada and Vodnar, 2021; Martinez et al., 2017; Nazzaro et al., 2020). Indeed, prebiotics are commonly known components of food, which can induce the growth or activity of beneficial gut bacteria to positively modulate the GM (Grimaldi et al., n.d.). Polyphenols have been reported to improve gut health through the production of SCFAs that restore intestinal barrier integrity and tight junction function, which is critical for the proper selectivity of the BBB, leading to enhanced circulation of polyphenol metabolites (Medani et al., 2011; Braniste et al., 2014; Sorrenti et al., 2020; Zhang et al., 2021). Overall, subjects with ASD experience gut dysbiosis and abnormal behaviour and difficulty with emotional control. These effects could be due to the dysregulation of important neurochemicals such as glutamate which highlight the potential of prebiotics in treating ASD related symptoms.

2.3 Zebrafish as a model organism

The zebrafish (Danio rerio) has emerged as a preeminent model organism in biological research, contributing significantly to our understanding of developmental biology, genetics, and disease mechanisms (Briggs, 2002). The advantages of using zebrafish in research are multifaceted: they reproduce rapidly and in large numbers, their embryos are transparent allowing real-time visualization of organ development, and they share a substantial genetic similarity with humans (Briggs, 2002). Furthermore, their amenability to genetic manipulation makes them particularly valuable for creating disease models. From investigating fundamental processes of embryogenesis to modelling complex diseases like cancer and neurodevelopmental disorders, the zebrafish is undoubtedly a cornerstone of contemporary biological research.

2.3.1 High throughput model for early brain development and pharmacology

The zebrafish is a fresh water fish with several distinct stages such as embryonic pre-hatch (0 to 72 hours-post-fertilization (hpf)), embryonic post-hatch (72-120 hpf), larval (1-29 days-post-fertilization (dpf)), juvenile fish (30-89 dpf), adult zebrafish (90 dpf to 2 years), and ageing zebrafish (2 years onwards) (Kalueff et al., 2014). A major aspect of zebrafish which make it a high throughput model is its rapid embryonic development where major organs are formed after 1 dpf and the fish may start feeding from 3 dpf (Kimmel et al., 1995). The zebrafish has become a widely recognized and powerful model organism for studying early brain development and for pharmacological research, providing numerous advantages in a high-throughput setting (Kalueff et al., 2014). The optical transparency of

zebrafish embryos and larvae and their rapid development allow researchers to visualize the development of brain structures in real-time using non-invasive imaging techniques such as light sheet microscopy (Kalueff et al., 2014). The rapid external development of the zebrafish, with many major organs including the brain formed by 24-48 hours post-fertilization, allows for swift observations of neurodevelopmental processes (Kimmel et al., 1995).

A critical factor in establishing the zebrafish as a powerful animal model is that advanced genetic tools such as CRISPR-Cas9 can be employed to create targeted genetic modifications in zebrafish, facilitating the investigation of gene function during early brain development. By manipulating genes of interest, researchers can observe resultant changes in neurodevelopment, allowing for the identification of genes crucial for normal brain formation (W. Y. Hwang et al., 2013). Currently, the Patten lab employs several such zebrafish models which study neurodevelopmental and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Butti et al., 2021) and CHARGE syndrome (Jamadagni et al., 2021). Furthermore, despite the evolutionary distance between fish and mammals, many fundamental processes in early brain development are conserved. This includes neural induction, patterning of the neural tube, and the formation of major brain structures (Köster and Fraser, 2001). Also, zebrafish brain morphology is very similar to mammalian models including the macro-organization of the brain and its cellular morphology (Kalueff et al., 2014). In addition, zebrafish share extensive genetic homology to mammals and humans (Howe et al., 2013). Overall, zebrafish show conserved genetics and physiology of major brain processes and behavioural traits that are homologous in rodents (Howe et al., 2013). Thus, findings in zebrafish can often be extrapolated to higher vertebrates, including humans (Köster and Fraser, 2001; Kalueff et al., 2014).

The small size of zebrafish embryos and larvae, combined with their cost efficiency and

suitability for growth in multi-well plates, makes them an ideal system for high-throughput drug screening. By exposing zebrafish to various compounds, researchers can rapidly assess drug effects on neurodevelopment or neurobehaviour (MacRae and Peterson, 2015). Zebrafish are capable of absorbing small molecules from water, providing a simple and effective route for drug administration. Importantly, zebrafish have similar drug metabolism systems to humans, making them a relevant model for pharmacokinetic studies (Hill et al., 2005).

Zebrafish also display a wide range of quantifiable behaviors, making them useful for assessing the effects of drugs on the nervous system. Assays have been developed to test various aspects of behavior, from locomotor activity to complex behaviors like learning and memory, anxiety, and social behavior (Kalueff et al., 2014). Furthermore, many key neurophenotypic domains and associated disorders have also been studied and modeled in zebrafish providing solid foundations for studying neurodevelopmental disorders (Kalueff et al., 2014).

In conclusion, the zebrafish model combines the complexity of vertebrate biology with the experimental tractability of invertebrate systems, making it a uniquely versatile organism for studying early brain development and pharmacology. Its amenability to high-throughput studies will continue to accelerate the pace of discovery in these fields.

2.3.2 ASD models using zebrafish

ASD is a complex neurodevelopmental disorder with a strong genetic component (Muhle et al., 2004; Geschwind, 2011). Understanding the pathogenesis of ASD through human studies alone is challenging due to its heterogeneity relating to the interaction of several relevant genes alongside the impact that environment plays in the pathogenesis of ASD and

the inherent limitations of human research (Geschwind, 2011). As a result, animal models, including zebrafish, have been increasingly utilized to unravel the molecular underpinnings of ASD. Here, we discuss several zebrafish models used in ASD research, focusing on their scope, strengths, and limitations.

Chromodomain helicase DNA-binding protein 8 (*CHD8*) is one of the most commonly mutated genes in individuals with ASD and has been identified a likey root cause for a specific subtype of ASD (Bernier et al., 2014). Zebrafish with *CHD8* mutations show dysregulation of genes involved in neural development and brain volume, recapitulating some aspects of the human phenotype (Satterstrom et al., 2020; Bernier et al., 2014; Barnard et al., 2015; Sugathan et al., 2014). *SHANK3* is another high-confidence risk gene for ASD. *SHANK3* is a post synaptic scaffolding protein in glutamatergic synapses and its expression is disrupted in ASD and is completely lacking in Phelan McDermid Syndrome (C.-x. Liu et al., 2018). The zebrafish model using *SHANK3* displayed significant similarities to human ASD. *SHANK3* mutations in zebrafish, like in humans, result in abnormal social behavior and increased repetitive behavior, mirroring core symptoms of ASD (Kozol et al., 2015). The zebrafish *SHANK3* model also comes with limitations. The behavioural output of zebrafish does not map perfectly onto human behavior. Also, *SHANK3* mutations are associated with only a subset of ASD cases, limiting the generalizability of the findings (Moessner et al., 2007).

Environmental causes have also been found to contribute to the pathogenesis of ASD. In fact, exposure to the anti-epileptic drug valproic acid (VPA) during pregnancy is a known environmental risk factor for ASD (Dietert et al., 2011). The VPA-induced ASD model is well studied in mice showing high anatomical, pathological, and etiological similarities with human ASD (Schneider and Przewłocki, 2005). Further, the mice showed low pain sensitivity and high sensitivity to nonpainful stimuli illustrating dysregulated

sensory processes. The mice also exhibited locomotion and repetitive behaviour stereotypical of human ASD as well as decreased social behaviours (Schneider and Przewłocki, 2005). While the VPA-induced zebrafish model of autism is more recently developed, studies have shown that the affected fish also exhibited social deficits, anxiety and hyperactivity, upregulated metabotropic glutamate receptors - similar dysregulation with human ASD (Rea and Van Raay, 2020). In addition, VPA was also observed to interact with autism-related genes such as *SHANK3*, *NRXN1*, *NLGN3* (C. Liu et al., 2021; Dwivedi et al., 2019). Overall, zebrafish exposed to VPA exhibit abnormal social behavior, hyperactivity, and increased brain volume, consistent with some characteristics of ASD (Baronio et al., 2018). The VPA model provides a unique opportunity to study gene-environment interactions in ASD.

Overall, each of these zebrafish models brings unique advantages to ASD research. The *CHD8* and *SHANK3* models leverage the genetic conservation between zebrafish and humans, whereas the VPA model explores environmental influences on ASD. However, these models are limited by inherent differences between zebrafish and humans and the complex, multi-factorial nature of ASD. Despite these limitations, zebrafish models of ASD provide a valuable, high-throughput model for understanding ASD pathogenesis and for potential drug discovery.

2.3.3 Administration of natural food compounds and nutraceuticals in zebrafish

Zebrafish models have gained significant traction in nutritional studies over the past years. They are particularly useful for testing the effects of natural food compounds and nutraceuticals, due to their physiological similarities to humans, easy and rapid breeding,

and transparent embryos which allow for live imaging. Here, we explore the use of zebrafish in studying several natural food compounds and nutraceuticals, the scope of these studies, and their potential applications.

A compound that has garnered a lot of attention from the scientific community in recent years due to their purported beneficial health properties are polyphenols. Polyphenols are plant-derived compounds with antioxidant properties that are found in foods such as fruits, vegetables, tea, and wine (Scalbert et al., 2005). Zebrafish models have been used to study the neuroprotective effects of polyphenols like resveratrol, flavonoids, and phenolic acids on oxidative stress and neurodegeneration (N. Wang et al., 2019; Mhalhel et al., 2023; Szwajgier et al., 2017). Such studies have shed light on the potential benefits of polyphenols in managing conditions like Alzheimer's disease, Parkinson's disease, and autism-related symptoms (Richetti et al., 2011; Caruana et al., 2016; Ghidersa et al., 2021). **Table 2.2** below presents some recent evidence on the benefits of polyphenols and prebiotics in clinical studies. Using stool from healthy adults, Tzounis et al. (2011) reported that cocoa flavonols in a GI model resulted in significant increases in *Bifidobacteria* and *Lactobacilli* as well as reductions in triacylglyceral and c-reactive proteins which are associated with inflammation. Further, Wiese et al. (2019) found that lycopene, a carotenoid, and dark chocolate helped improve lipid metabolism in adults with $30 < \text{BMI} < 35$. Moreover, they also observed dose dependent changes in gut microbiota profile where greater consumption of the prebiotics resulted in healthier gut microbiota profile defined as greater proportions of healthy microbes versus harmful ones (Wiese et al., 2019). These two studies illustrate the benefits of prebiotics particularly cocoa flavonols and dark chocolate which are hypothesized to improve gut health through their antioxidant and anti-inflammatory properties and their ability to modulate the gut microbiome.

Within the context of ASD, high antioxidant cocoa powder was administered to children by Sadek et al. (2018) and found to reduce ASD severity and improve aberrant behaviour related with ASD. Using maltodextrin, a prebiotic produced from starch, to treat children with ASD, Grimaldi et al. (2018) found that the treatment was associated with positive metabolic shifts in urine spectra and fecal samples. While these studies do not necessarily provide an in-depth exploration of the mechanisms of action behind the prebiotics, overall they are able to emphasize their benefits in modulating gut health through reduced inflammatory factors and increased beneficial gut microbes. In children, prebiotics have even reduced ASD severity and reduced GI discomfort, a common comorbidity of ASD. Ultimately, it is evident that prebiotics and specifically cocoa polyphenols are promising candidates as treatments for ASD.

Prebiotic Treatments in Humans			
Population	Intervention	Study type	Main Findings
Adults with 30<BMI<35 n=30	Lycopene and Dark chocolate	RCT	- Dose dependent changes in gut microbiota profile - Improved lipid metabolism (Wiese et al., 2019)
Healthy adults n=22	Cocoa flavonols	RCT with GI model using fecal samples	- Significant increases in *Bifidobacteria* and *Lactobacilli* - Reductions in plasma triacylglyceral and c-reactive proteins (Tzounis et al., 2011)
Children with ASD n=30	Maltodextrin	RCT	- Metabolic shifts in urine spectra profile and fecal samples - Reduced GI discomfort (Grimaldi et al., 2018)
Children with ASD n=12	High antioxidant cocoa	Single-arm intervention	- Improved Aberrant Behaviour Checklist Score - Improved Autism Spectrum Rating Scale (Sadek et al., 2018)

Table 2.2: Potential of prebiotics and polyphenols in clinical studies.

It is important to note some limitations. The effects of cocoa and chocolate have largely been attributed to their flavonoid content, but these products contain other compounds that could also contribute to their effects. Moreover, the processing of cocoa into chocolate can significantly reduce flavonoid content, making it difficult to extrapolate findings with pure cocoa to chocolate products. Finally, while these studies demonstrated certain health benefits to prebiotics and polyphenols, they provided limited insight into the mechanisms through which these prebiotics function apart from gut microbiome modulation and certain plasma biomarkers (Tzounis et al., 2008; Wiese et al., 2019; Grimaldi et al., 2018).

2.4 Rationale and Objectives

In the past decades, research on ASD has picked up and we now have a much better understanding of the disorder. Despite understanding the disorder better, treating ASD remains a complex undertaking due to the wide-ranging symptoms and differing severity across patients. In addition, research has also identified various subtypes of ASD with different genetic and environmental causes. Relevant to this thesis is the understanding that prenatal exposure to VPA causes ASD in children. Though treatments for this exact subtype of ASD may not be perfectly transferable to other subtypes of ASD, understanding how a treatment can alleviate symptoms in a specific subtype/model of ASD may broaden our understanding on pathways and mechanisms of ASD in general. Ultimately, this would allow researchers to create more specialized treatments for a very complex disorder.

Furthermore, based on existing clinical and animal model studies, there is increasing evidence regarding benefits of polyphenols and prebiotics in supporting gut and brain health and possibly, in the case of high antioxidant cocoa, ASD. However, more research is needed to understand how polyphenol-rich cocoa could be utilized physiologically to potentially alleviate the symptoms of ASD. In that regard, investigations are needed into how cocoa polyphenols could exert their downstream effects involving metabolites identified via metabolomics and proteomics analyses.

I hypothesize that within this framework, the prebiotic cocoa treatment can counteract the VPA-induced deficits in gut morphology and behaviour. Also, the VPA-treated zebrafish will express dysregulated metabolism and protein expression whereas the cocoa-treated VPA-treated zebrafish will express similar metabolomic and proteomic profiles to control zebrafish.

In this study, I will aim to validate the chemically-induced model of autism in zebrafish

using valproic acid; evaluate the metabolome and proteome of VPA-treated zebrafish; determine a non-toxic dose of polyphenol-rich cocoa powder co-treatment and its efficacy in preventing behavioural and morphological symptoms of VPA treatment; and finally examine the impact of cocoa powder co-treatment on the metabolome and proteome of VPA-treated zebrafish.

Finally, many studies on ASD and nutraceuticals are conducted in humans and mice which are expensive and often conducted in low sample size and are statistically weak to considerable interindividual variability. Moreover, while these studies have provided insight into the potential benefits of prebiotics on health and in some cases ASD, few have examined the function of these treatments mechanistically. This is where zebrafish models can help fill these gaps. Zebrafish models offer substantial advantages in studying nutraceuticals, including their high genetic and physiological homology to humans, their fast reproductive rate, and their transparent embryos allowing for real-time visualization of physiological processes. It is important to note that the effects observed in zebrafish may not always translate directly to humans. Therefore, results from zebrafish studies must be validated in higher animal models and human clinical trials. Despite these caveats, the zebrafish model continues to be a robust platform for nutraceutical research and an important high-throughput first step in evaluating novel approaches to treating a complex disorder such as ASD. In this thesis, the effects of polyphenol-rich cocoa powder were examined on behavioural symptoms, gut morphology, and metabolic and proteomic profiles in a VPA-induced zebrafish model of autism. Overall, this project will provide a starting point for the development of a novel nutrition-based approach to treat ASD and will lay the foundation to identify new biological targets for future development of pharmaceutical interventions.

Materials and Methods

3.1 Zebrafish husbandry

Wild-type zebrafish (Danio rerio), specifically the AB/TL strain, were maintained at a temperature of 28°C, following a 12/12 hour light/dark cycle in accordance with "The Zebrafish Book" (Westerfield, 2000). The embryos were raised at 28.5°C, and collected and nurtured as previously described (Kimmel et al., 1995). All experiments were conducted in compliance with the guidelines of the Canadian Council on Animal Care and the local ethics committee of the INRS. For experiments that required additional compounds be added to the zebrafish rearing environment, the compounds were dissolved in the zebrafish rearing water E3 (“E3 medium (for zebrafish embryos)”, 2011) and before being introduced to the fish. All experiments were performed in line with the guidelines of the Canadian Council for Animal Care and the local ethics committee (1605-01 and 2005-01).

3.2 Experimental design

This experiment compares control zebrafish to cocoa treatment, VPA treatment, and VPA + cocoa treatments. The sample sizes are as follows: six different batches (N = 6), each batch containing 20 larvae (n = 20) for each treatment group. Three of the batches were dedicated to haematoxylin and eosin (H&E) staining and the other three were flash frozen and stored at $-80°C$ for metabolomics and proteomics. All the batches will undergo

the behavioural analysis in the Noldus (Leesburg, VA, USA) manufactured DanioVision machine, made for high-throughput tracking of zebrafish larvae and other small organisms, prior to sample preparation. Between group comparisons will be made for each treatment group to control as well as between treatment groups (i.e. cocoa versus VPA, cocoa versus VPA + cocoa, etc.).

3.3 Drug Treatment

Valproic acid (VPA) was purchased from Sigma-Aldrich (MilliporeSigma Canada Ltd., Etobicoke, Ontario), and a 50 mM stock solution was prepared in distilled water. The cocoa powder was purchased from CocoaVia (Mars Canada Ltd., Bolton, Ontario) and were prepared fresh daily for usage. Cocovia powder composition and supplement facts label are shown **Figure 3.1**. Embryos were treated from 6 hpf with VPA up to 4.5 dpf, and at 6 hpf with cocoa powder up to 6 dpf. The water was replaced every day with fresh water containing final concentration of the drug, until the activity measurement and/or imaging.

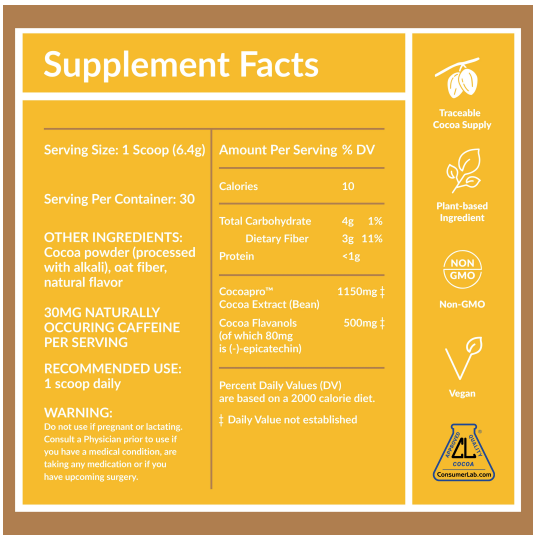

Figure 3.1: CocoaVia™ Cardio Health Supplement Facts Label

3.4 Prebiotic toxicology

The cocoa prebiotic toxicology was conducted similarly to Bugel et al. (2016). The fish were exposed to the cocoa powder dissolved in E3 medium from 6 hpf to 6 dpf. Concentration of polyphenol was determined by referencing the nutrition facts label provided by CocoaVia™ (Mars Inc., McLean, Virginia). The cocoa-E3 medium was prepared by dissolving the cocoa powder in E3 and then centrifuging the mixture at 10,000 x g for 5 min. Only the supernatant was kept for the zebrafish treatment. The medium was prepared freshly and changed daily. Concentrations for the prebiotic cocoa powder refer to the concentration of (-)-epicatechin present according to the Cocoavia™ nutrition facts label. Concentrations from 1-50 μM were tested and survival, morphology, and behaviour (DanioVision) were assessed as described below.

3.5 VPA model validation

Valproic acid zebrafish treatments in the literature ranged from 1 μM (Messina et al., 2020), 6.5 μM (Brotzmann et al., 2021), 12.5 μM (S. Lee et al., 2018), 48 μM (Robea et al., 2021), 75 μM (Dwivedi et al., 2019), to 500 and 1500 μM (J. Chen et al., 2018). Therefore, tests were prepared for the following concentrations: control (0 μM), 1 μM, 2.5 μM, 5 μM, 10 μM, 15 μM, 25 μM, 50 μM, 75 μM, 100 μM, 250 μM, 500 μM, and 1000 μM. The embryos were exposed in E3 to the VPA starting from 6 hpf until 4.5 dpf at which point they were switched to E3 only for the duration of the experiment (6 dpf) and their behaviour was analyzed using the DanioVision. The concentration of VPA was deemed in excess for modeling ASD when the larvae did not survive up to 6 dpf or were over-intoxicated and could not move. The concentration of VPA was deemed too weak to induce any ASD-like phenotypes when no morphological differences were observed compared to control and/or no behavioural differences were observed following the DanioVision assessment.

3.6 Behavioural analyses

Larvae (6 dpf) were separated into single wells of a 96-well plate containing 250 μL of E3 media and habituated in the Daniovision® recording chamber (Noldus) for 1 h before the start of the experiment. Larval locomotor activity was monitored over light-dark cycles using the Daniovision® apparatus. Analysis was performed using the EthoVision XT12 software (Noldus) to quantify the total swimming distance in given hours and the locomotor activity per second.

3.7 Gross morphology and survival assessment

Larvae (control, VPA, cocoa, VPA + cocoa) were assessed for their survival rate and morphological phenotypes. The sample sizes for the different treatment groups were as follows: three different batches (n = 3), each batch containing 20 larvae (n = 20) for each treatment group. Morphology was observed under a microscope noting any pericardial edemas, head malformations, and other deformations observed compared to control.

3.8 Haematoxylin and Eosin staining

For haematoxylin and eosin (H&E) staining, fish sections were fixed in paraffine. The sections were stained with Haematoxylin (StatLab) for 4 min, washed with alcohol-acid, and were rinsed with tap water. The sections were then soaked in saturated lithium carbonate solution for 10 sec and then rinsed with tap water. Finally, staining was performed with Eosin Y (StatLab) for 2 min, and mounted under a coverslip with Permount mounting media (Fisher Scientific, Saint-Laurent, Quebec).

3.9 Gut morphology assessment

Zebrafish gut morphology was assessed following H&E staining. The following cuts were used to examine the gut: sagittal, transverse, and coronal planes. GI structure was noted as well as the placement or displacement of organs surrounding the gut such as the liver.

3.10 Metabolomics and proteomics

Sample preparation involved homogenizing the zebrafish in methanol and separating the supernatant for untargeted metabolomics and the pellet for untargeted proteomics. First 500 μL of MeOH was added to each sample which underwent sonication with a probe 2x 10s. The samples were then centrifuged for 8 min at 14000 rpm at 4° C. Both the supernatant and protein pellet were extracted and dried. For untargeted metabolomics, the dried supernatant was reconstituted with 200 μL of 25% MeOH.

3.10.1 LC-MS/MS for Untargeted Metabolomics

Metabolomics analysis was performed using two columns: a reverse phase pentafluorophenyhl (PFP) column using water and methanol as mobile phases (with 0.1% formic acid) and the Scherzo SM-C18 column using the same gradient but with acetonitrile; this column has some cation exchange behaviour as well as C18 stationary phase. The Shimadzu Nexera UHPLC system was used with the following two columns and specifications. Scherzo SM-18 (mixed mode) had a flow of 300 μL/min using H_2O + 0.1% FA/ACN + 0.1% FA. PFP column had a flow of 250 μL/min using H_2O + 0.1% FA/MeOH. Overall injection volume was set to 20 μL. Source parameters was as follows: ESI +/-; ionization potential +5000V/-4500V; source temperature 450° C; curtain gas 35 psi; drying/nebulisation flow rate 50 psi. Data dependant acquisition using TOF-MS: m/z 80-800 (250 ms); TOF-MS/MS: m/z 40-800 (150 ms); CE = 30 +/- 10 V; MS/MS on top 8 ions (IDA). The data processing pipeline for untargeted metabolomics begins with feature generation (m/z + RT) using Markerview followed by identification with spectral library using Sciex OS-Q and library score $>$ 85 and ppm $<$ 10. Then there is integration

adjustment for quantitative assessment also in Sciex OS-Q. Finally statistical analyses are conducted in R, MetaboAnalyst 5.0 (heatmaps), and Markerview (t-testing, pairwise-comparisons).

3.10.2 Targeted Metabolomics

Targeted metabolomics was conducted using the Biocrates kit (Biocrates Inc, Aliso Viejo, California) (Baraniuk et al., 2021). Firstly, 50 μL of reconstituded sample from the untargeted workflow is taken and dried in speedvac. Then it is reconstitutded with 25 μL of EtOH 85% and 10 μL of this sample is added to the extraction plate from the Biocrates kit (MxP 500). The Biocrates protocol was followed for derivatization and extraction.

3.10.3 LC-MS/MS for Targeted Metabolomics

The Shimadzu Nexera UHPLC system was used with the a proprietary RP-column provided by Biocrates with the following specifications: flow rate 800 μL/min at 50° C; pressure up to 500 bar; injection volume 5 μL for LC1 (positive mode) and 15 μL for LC2 (negative mode); mobile phase A using H_2O + 0.2% FA and mobile phase B using ACN + 0.2% FA. SMRM was configured as follows: MRM detection window 90s; target scan time 0.1s; cycle time 0.1s; min dwell 3ms; max dwell 250ms; cycle 3300. The Sciex QTRAP was configured as follows for LC1 where ionisation ESI(+); voltage +5.5 kV; and TEM +500° C and as follows for LC2 where where ionisation ESI(-); voltage -4.5 kV; and TEM +650° C. The data processing for targeted metabolomics begins with peak integration, normalization and quantification using MetIDQ followed by statistical analysis in MetaboAnalyst 5.0.

3.10.4 Proteomics

Proteomics was conducted using the pellet from the metabolomics pipeline. First 50 μL of 7 M urea/2 M thiourea was added. The pellet was sonicated in bath for 15 min. Then 200 μL of ABC buffer (100 mM, pH 8,5) was added followed by sonication with probe (3 x 5s). The sample was then centrifuged for 2 min at 14000 rpm. Bradford protein quantification (5 μL sample + 45 μL H_2O for dilution) was performed. Then 50 μg of protein was extracted into a new tube and volume was completed to 400 μL with ABC buffer (100 mM, pH 8.5). The following additions were made: 8 μL of 100 mM DTT for 15 min at 37° C; 12 μL of 100 mM IAM for 30 min at 37° C; and 20 μL of 0.1 mg/mL trypsin for 100 μg of protein. This was followed by a 4h digest at 37° C and 600 rpm. Finally SPE was performed using HLB cartridge and dried in speedvac then reconstituted in 120 μL of 10% ACN + 0.2% FA.

3.10.5 LC-MS/MS for Proteomics

The Shimadzu Nexera UHPLC system was used with an Aeris PEPTIDE XB-C18 100 x 2.1 mm, 1.7 μm Gradient (47 min). The mobile phase consisted of H_2O + 0.1% FA - ACN + 0.1% FA at 300 μL/min and an injection volume of 20 μL. Data-dependent acquisition was conducted using TOF-MS: m/z 140-1250 (250 ms); TOF-MS/MS: m/z 300-1250 (50 ms); IDA (15 most intense ions); cycle time 1.1 s. Data-independent acquisition was conducted using TOF-MS: m/z 140-1250 (150 ms); MS/MS: m/z 80-1500 (25 ms), CE 30 V; 100 variable precursor windows; cycle time 2.7 s. Data processing for proteomics is as follows: ProteinPilot 5.0 software (OneOmics) was used to generate lists of peptides and proteins, identified in the raw data (peptide sequencing by MS/MS), and create the ion library. Parameters used on ProteinPilot search: Iodoacetamide (IAM) as

cysteine alkylation, trypsin for digestion, Zebrafish as a species and a protein threshold of 1% local false discovery rate (FDR). After the creation of an ion library using the proteins identified in the pool sample, all the samples were analyzed to quantify proteins. Up to 4 peptides per protein and 3 transitions per peptide were selected as criteria. Quantification of proteins in the sample was done using SWATH (OneOmics). Proteins were filtered based on p-value < 0.05 and fold change over 50%.

3.10.6 Pathway Analysis

Pathway analysis was conducted using MetaboAnalyst 5.0 with the kegg pathway library for zebrafish and integrated metabolites from both targeted and untargeted metabolomics and proteins in a joint-pathway analysis. Using only the metabolomics data, a metabolite-only pathway analysis was also conducted. Pathway impact is calculated as a ratio of identified metabolites and/or proteins to all the metabolites and/or proteins present in the pathway. In this analysis, the smaller the p-value, the more likely the association identified between the compound of interest and the pathway is not random.

3.11 Statistical Analysis

All zebrafish experiments were performed in at least three replicates (N) and each consisted of a sample size (n) of 15–30 fish. Data are presented as Mean ± SEM. Significance was generally set at $p < 0.05$ prior to multiple comparison corrections. Differences in mean group survival proportions were assessed using 95% confidence intervals (CIs) with Bonferroni correction. For behavioural assays, specifically the mean total distance moved for fish of each treatment, differences between groups were assessed

using one-way ANOVA with the post-hoc Tukey test. For differences in metabolites and protein, multiple t-tests were conducted with Bonferroni correction. Pathway analysis of metabolite and protein data consisting of pathway impact/enrichment ratio was calculated as a ratio of identified metabolites/proteins to all the metabolites/proteins present in the pathway. The p-values for this analysis are obtained from a Fisher's exact test based on hypergeometric distribution. The heatmaps from metabolomics and proteomics were obtained using non transformed/non normalized data with an euclidian distance measure. Clustering was based on the Ward method and the 50 features with the most significant p-values from ANOVA t-test are displayed. MetaboAnalyst 5.0 was used for t-testing, pairwise comparisons, and heatmaps for the metabolomics data. ProteinPilot 5.0 software (OneOmics) was used to generate lists of peptides and proteins, identified in the raw data from peptide sequencing using MS/MS, and create the ion library. Quantification of proteins in a sample were conducted using SWATCH (OneOmics) and proteins were filtered based on p-value < 0.05 and fold change over 50%. Data processing and visualization of volcano plots for metabolomics and proteomics was conducted in R (RStudio 2022.07.2+576). Dplyr (v.1.1.1) was used to clean and prepare the data while BiocManager (Bioconductor v.1.30.16) and EnhancedVolcano (v.1.12.0) packages were used to generate the volcano plots. All other analyses and graphs were completed in PRISM (v.10.0.0).

Results

4.1 Prebiotic toxicology

Following cocoa extract toxicology, there was increased mortality in all treatment groups above 5 μM in concentration of (-)-epicatechin. **Figure 4.1** illustrates the survival of the different treatment groups from 0 dpf to 6 dpf. Zebrafish treated above 2.5 μM (-)-epicatechin from the PRE cocoa powder had reduced survival proportion compared to control and 2.5 μM.

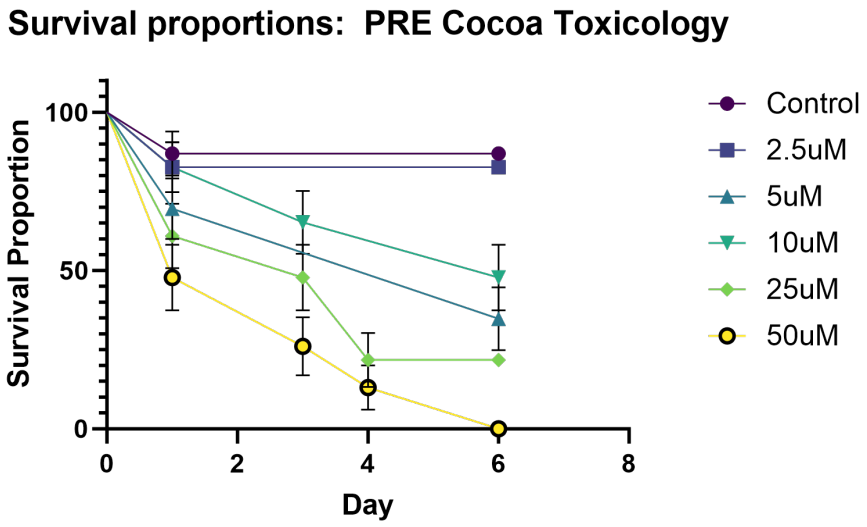

Figure 4.1: Zebrafish larvae (n = 23) survival proportions for PRE cocoa treatment from 0 dpf to 6 dpf

Figure 4.2 shows that treatment groups above 5 μM also experienced delayed hatching of the embryos whereas those under 5 μM and controls underwent normal hatching around 2-4 dpf with the majority of larvae hatching around the expected 3 dpf. Additionally, while control, 2.5 μM, and 5 μM saw a 100% hatch rate, the groups in higher concentrations saw much lower hatching success. Ultimately, no fish hatched in the 50 μM group which had all perished by 6 dpf as shown in **Figure 4.1**.

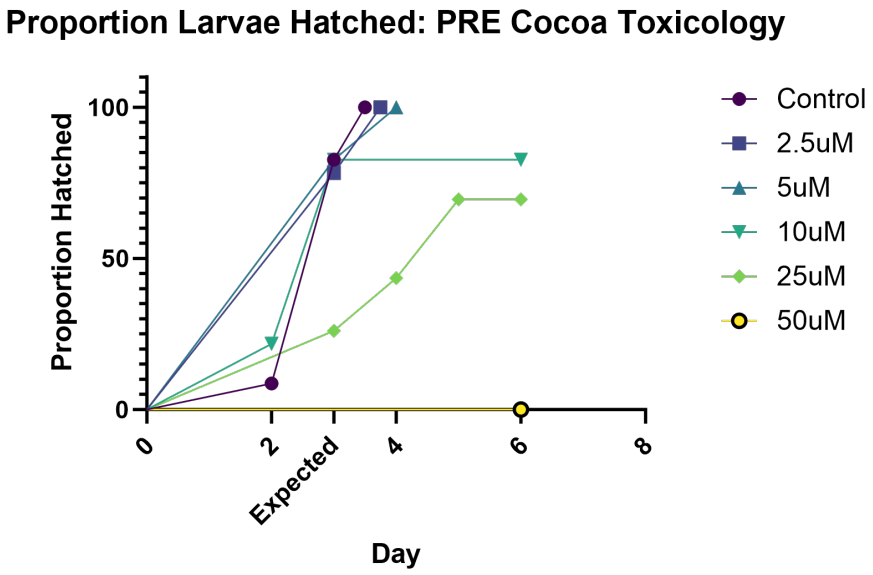

Figure 4.2: Zebrafish larvae (n = 23) hatching proportion for PRE cocoa treatment from 0 dpf to 6 dpf where normally larvae are expected to hatch around 3 dpf

Overall, 2.5 μM is the concentration of (-)-epicatechin from the PRE cocoa powder that was used to treat the zebrafish without adverse effects to their hatching and survival rates.

4.2 VPA model validation

To address the wide range of VPA concentrations used to induce ASD-like phenotype in zebrafish in the literature, an initial exploratory toxicology was conducted to test a large range of concentrations from 5 μM to 500 μM. **Figure 4.3** shows the results of the exploratory trial where it was determined that concentrations above 10 μM caused severe reduction in larvae survival rate and thus are not appropriate to use as an ASD model. Specifically, zebrafish in the 10-15 μM groups only survived up to days 3-4 post fertilization. Fish treated in concentrations higher than 25 μM survived less than 24 hours following the beginning of VPA exposure.

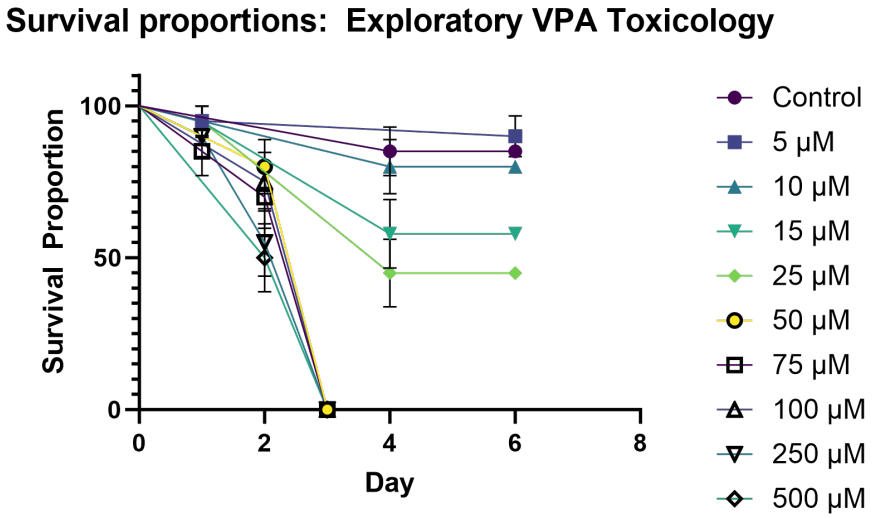

Figure 4.3: Exploratory VPA toxicology on zebrafish (n = 20) from 0 dpf to 6 dpf

Following the exploratory trials, a more precise range of concentrations was tested to determine the most appropriate ASD model. **Figure 4.4** illustrates the survival proportions for 1 μM, 3 μM, 5 μM, and 10 μM from 0 dpf to 6 dpf. In this category, both 5 μM and 10 μM had too low survival rates.

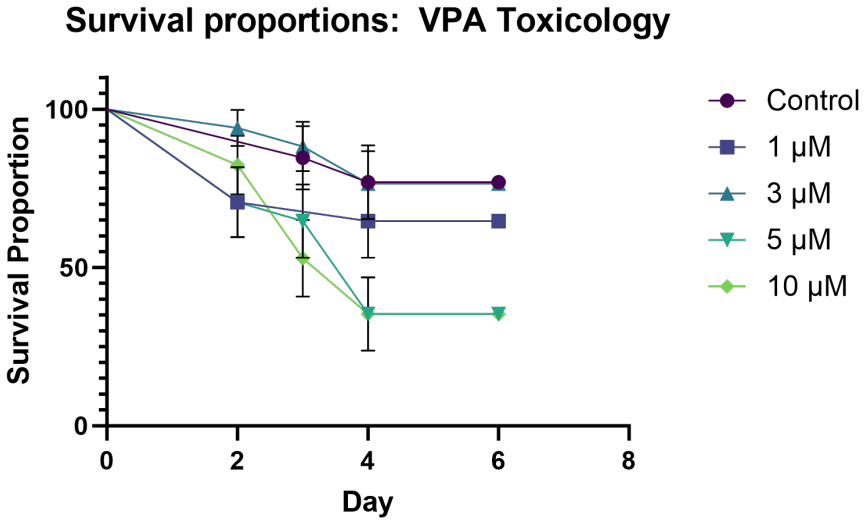

Figure 4.4: Zebrafish larvae (n = 20) survival proportions for VPA from 0 dpf to 6 dpf

While 1 μM was most similar to control, 3 μM remains a strong possibility given that it has previously been employed in the literature and it may express stronger ASD-like phenotype compared to 1 μM whose phenotype may be too mild. Therefore, assessments on hatching proportion and proportion of larvae with deformations was also assessed and results are shown in **Figure 4.5** and **Figure 4.6**.

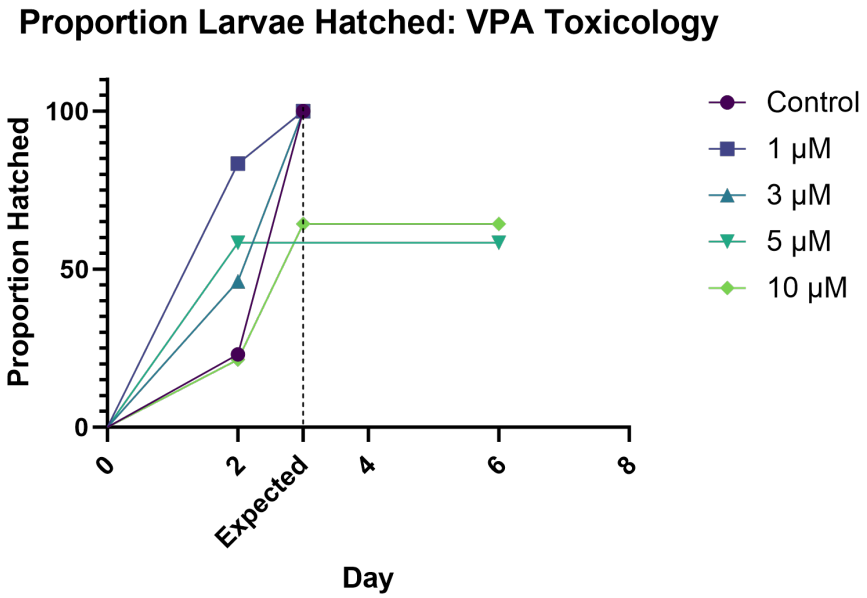

Figure 4.5: Zebrafish larvae (n = 20) hatching proportions for VPA treatments from 0 dpf to 6 dpf where normally larvae are expected to hatch around 3 dpf

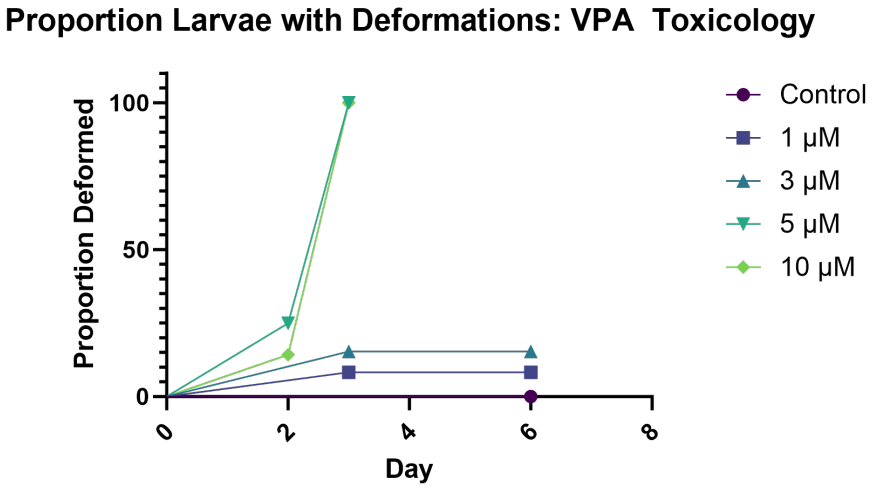

Figure 4.6: Zebrafish larvae (n = 20) deformity proportion for VPA treatments from 0 dpf to 6 dpf

The VPA toxicology revealed severe morphological deformations in zebrafish larvae treated with VPA at and above 5 μM. Zebrafish treated at 5 μM and 10 μM were severely deformed (i.e. pericardial edemas) but were nonetheless included in the behavioural analysis in the DanioVision to determine whether VPA-treated zebrafish exhibited autistic-like responses to stress.

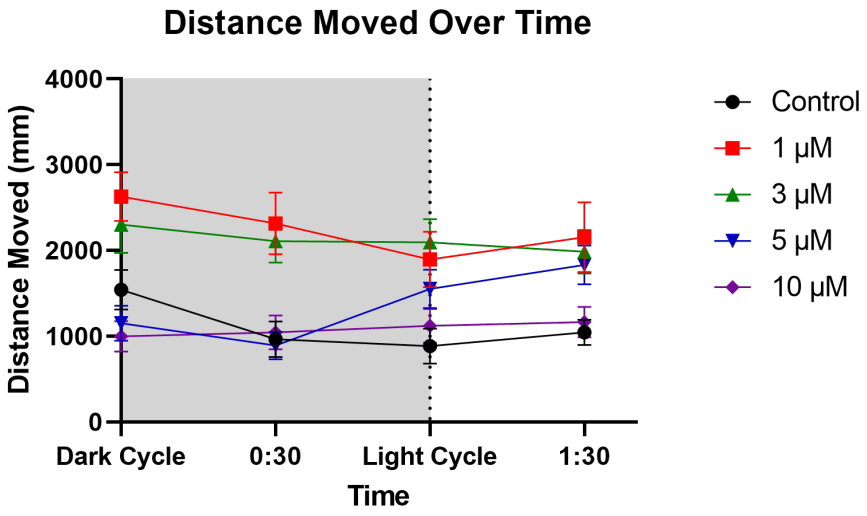

Figure 4.7: DanioVision zebrafish movement tracking illustrating VPA-treated fish response to a stress inducing change in environment (dark to light) revealing distance moved over time (n = 12)

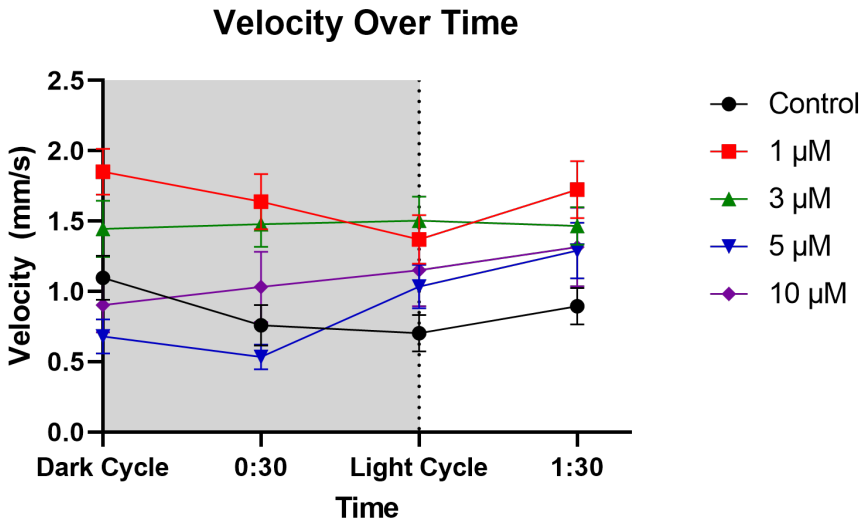

Figure 4.8: DanioVision zebrafish movement tracking illustrating VPA-treated fish response to a stress inducing change in environment (dark to light) revealing velocity over time (n = 12)

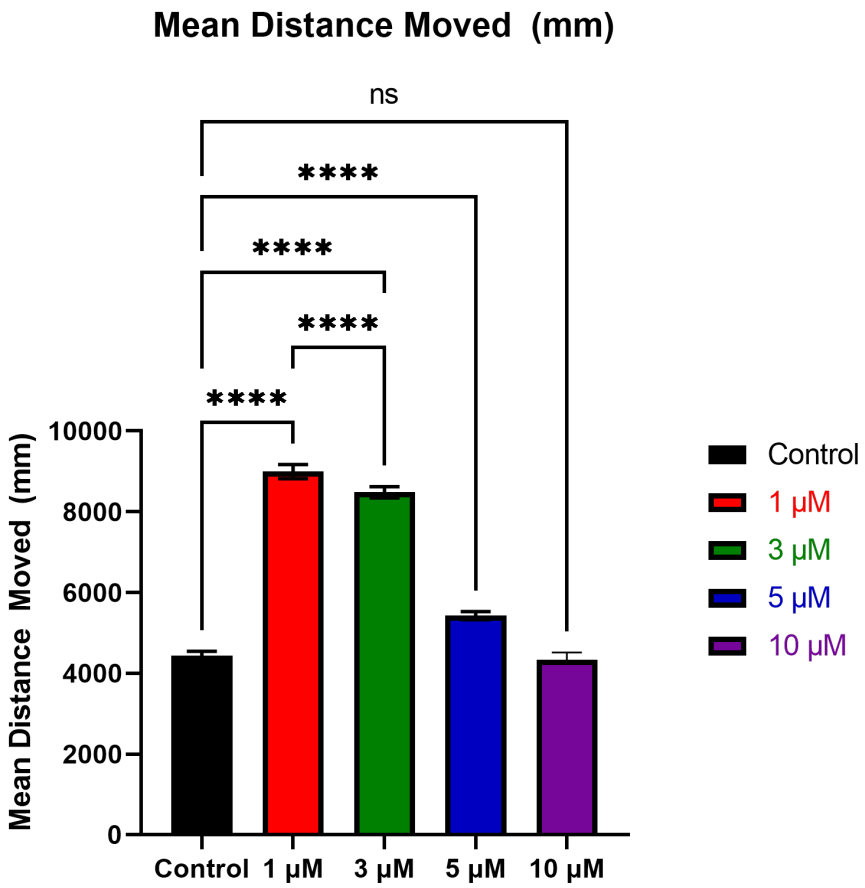

Figure 4.9: DanioVision zebrafish movement tracking illustrating mean total VPA-treated fish swim distance (n = 12)

The DanioVision results illustrate VPA-treated fish swimming distance over time (**Figure 4.7**); velocity over time (**Figure 4.8**); and total mean swim distance (**Figure 4.9**). Overall, while 5 μM and 10 μM performed decently in distance and velocity over time, 10 μM was similar to control in total distance ($p = 0.8495$). Though, 5 μM seemed acceptable, their survival rate was too low with too large a proportion of fish having deformations, leaving just 1 μM and 3 μM as options.

Ultimately, 3 μM was enough to induce anxiety-like behaviour in the zebrafish while

ensuring the fish survive to 6 dpf. In addition, 3 μM was chosen over 1 μM for consistency since there have been published papers where the concentration of 3 μM was used.

4.3 Effect of polyphenol-rich cocoa powder treatments

4.3.1 Survival

Figure 4.10 illustrates survival proportions of different groups of zebrafish up to 12 dpf. **Figure 4.11** illustrates the differences in zebrafish survival proportions using 95% CIs with Bonferroni correction. The control group had similar survival rates compared to cocoa only treatments, providing assurance that the cocoa powder did not negatively affect the zebrafish. However, VPA treated zebrafish had significantly reduced survival compared to control whereas the VPA + cocoa had slightly better survival rates compared to VPA only suggesting possible benefits of the PRE cocoa powder.

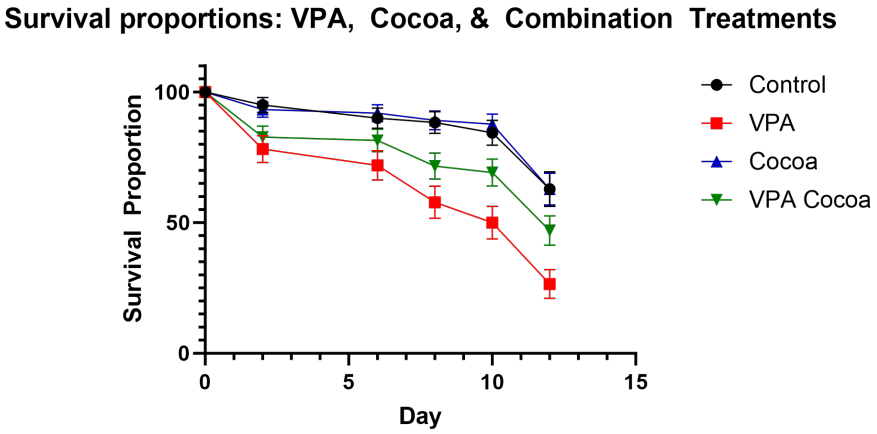

Figure 4.10: Zebrafish larvae (n = 12) survival proportions from 0 dpf to 12 dpf

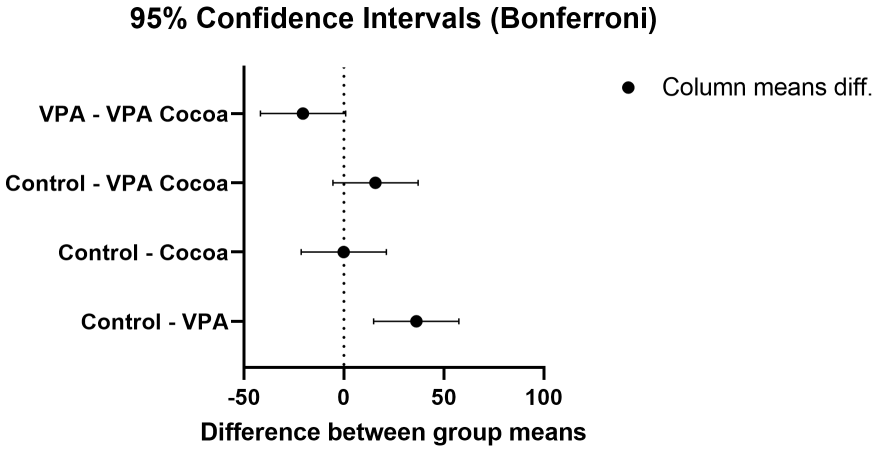

Figure 4.11: Differences in zebrafish survival proportion at 12 dpf as 95% CIs with Bonferroni correction

4.3.2 Behavioural functionality

DanioVision behavioural assays revealed that VPA-treated zebrafish swam at greater velocities and larger distances compared to all groups (control, cocoa, VPA + cocoa) during both light on and light off cycles (**Figure 4.12** & **Figure 4.13**). The VPA + cocoa group on the other hand swam similar distances and at similar mean velocity as the control and cocoa groups.

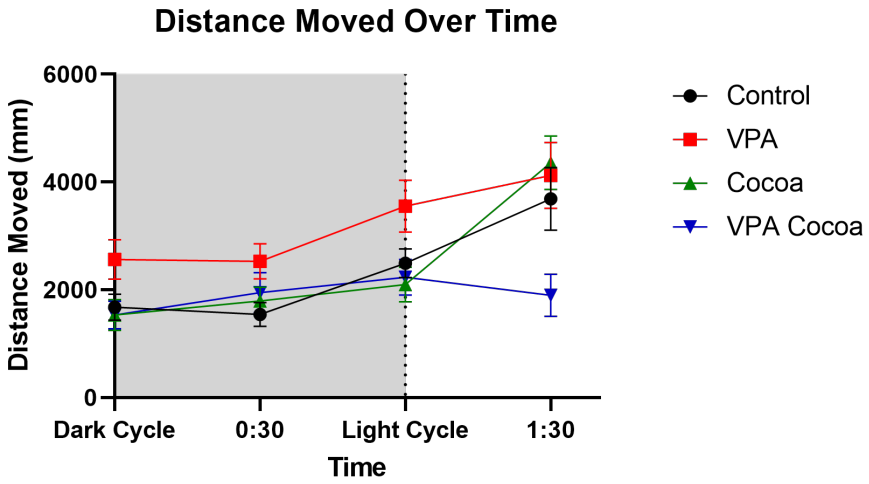

Figure 4.12: DanioVision zebrafish movement tracking illustrating fish response to a stress inducing change in environment (dark to light) revealing distance moved over time (n = 12)

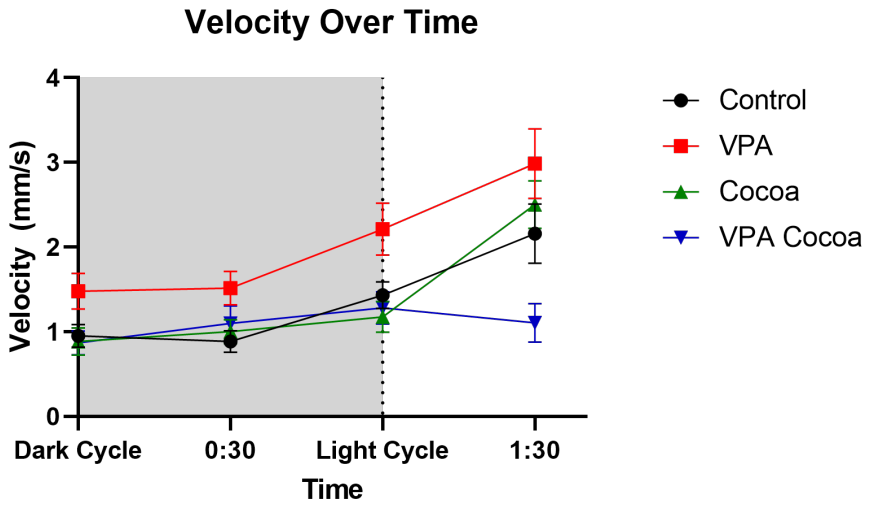

Figure 4.13: DanioVision zebrafish movement tracking illustrating fish response to a stress inducing change in environment (dark to light) revealing velocity over time (n = 12)

Figure 4.14 compares the mean total distances swam by fish in each group. Overall, VPA-treated fish exhibited anxious-like behaviour and swam larger distances compared to control whereas none of the other groups swam significantly more or less compared to control. VPA fish treated with cocoa powder behaved more like control- and cocoa-treated fish rather than VPA-treated fish with statistically significant lower mean total swim distance.

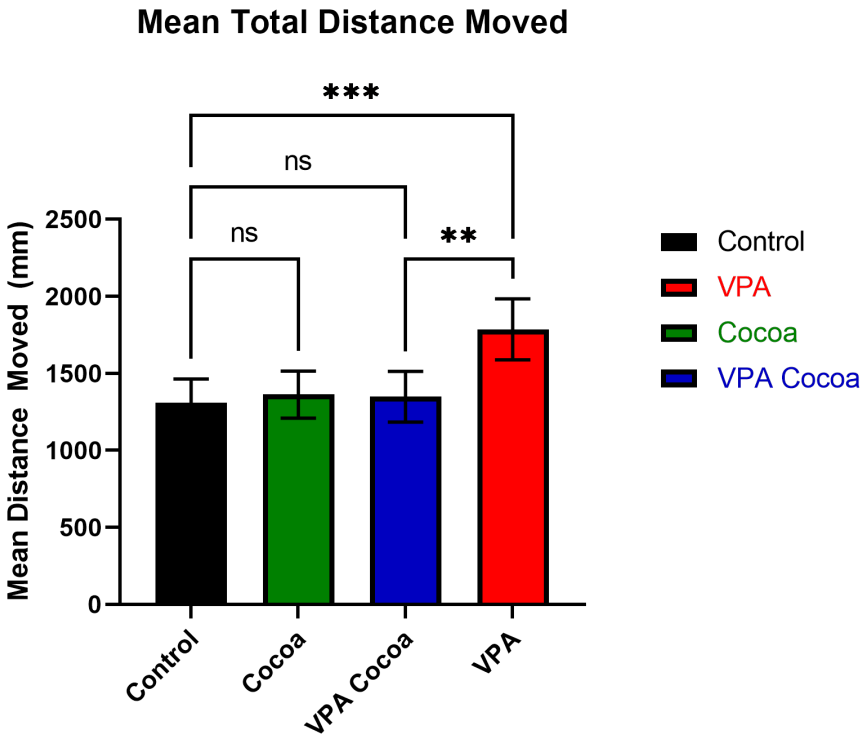

Figure 4.14: DanioVision zebrafish movement tracking illustrating mean total fish swim distance (n = 12). ** indicate p-value < 0.05. *** indicate p-value < 0.01.

4.3.3 Gut morphology

All zebrafish were processed with H&E staining. **Figure 4.15** shows the gut of a healthy control zebrafish at 6 dpf. The gut encircled in red is well-shaped and includes proper folds and structure.

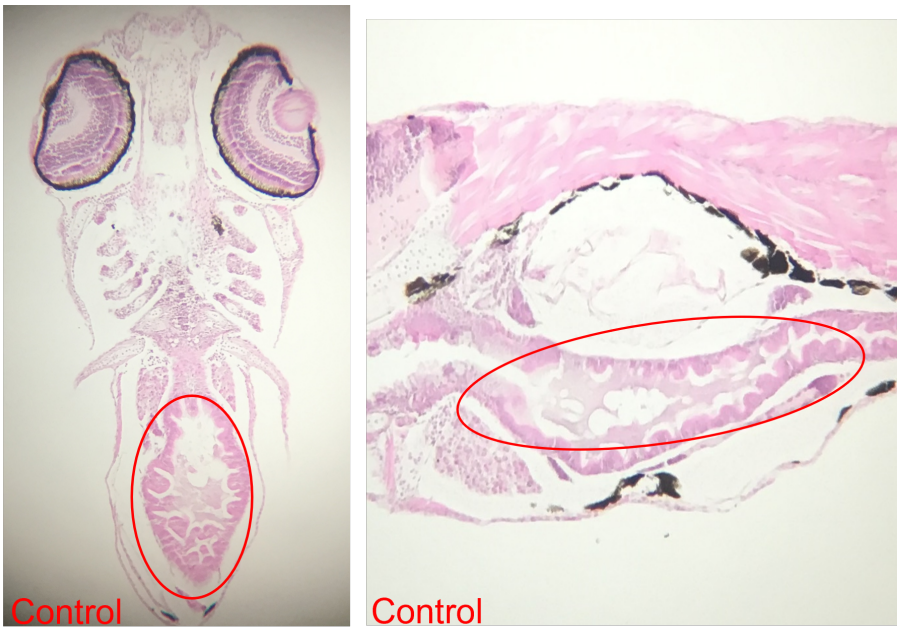

Figure 4.15: H&E staining of control zebrafish larvae at 6 days-post-fertilization showing coronal and sagittal cuts side-by-side with gut encircled

Figure 4.16 shows the same cuts on a VPA zebrafish. This gut, however, is different from the control gut as its structure is deformed and misplaced.

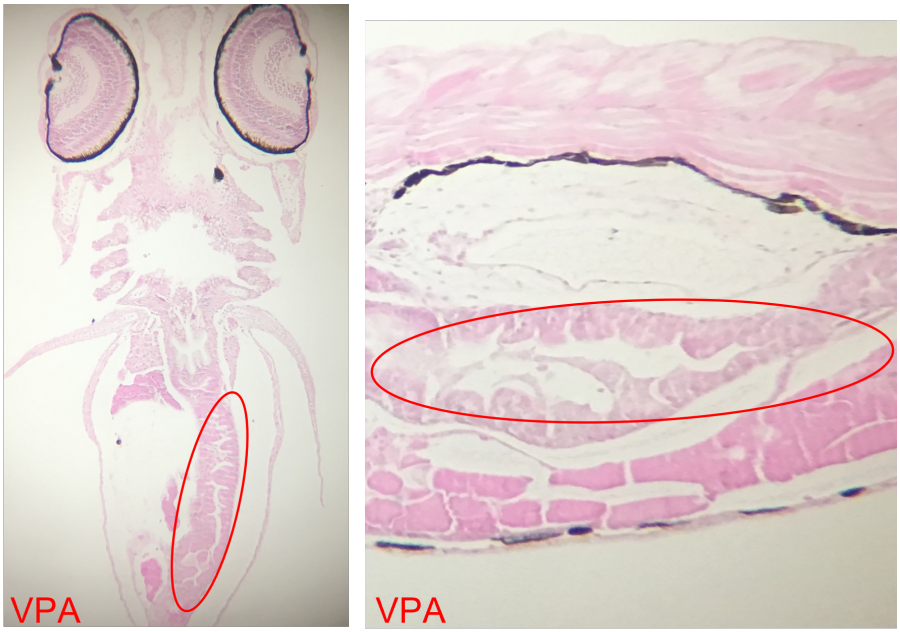

Figure 4.16: H&E staining of VPA treated zebrafish larvae at 6 days-post-fertilization showing coronal and sagittal cuts side-by-side with gut encircled

Figure 4.17 shows the cuts on VPA and cocoa treated zebrafish. This gut structure is recovered as it is similar to control. It is well-rounded and is neither deformed nor misplaced.

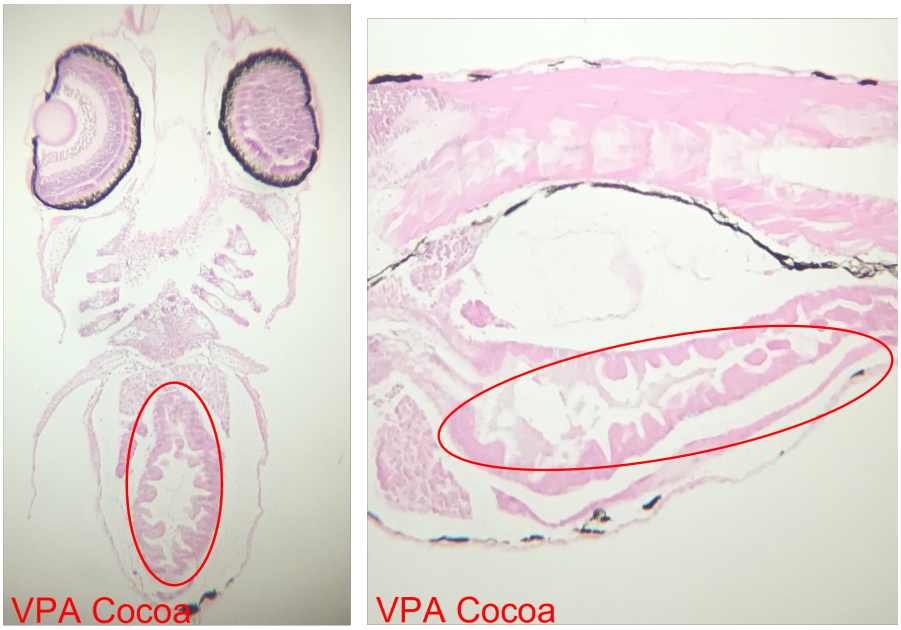

Figure 4.17: H&E staining of VPA and cocoa treated zebrafish larvae at 6 days-post-fertilization showing coronal and sagittal cuts side-by-side with gut encircled

Figure 4.18 shows all the zebrafish groups side-by-side where the differences between group gut structure are highlighted. While the VPA gut is severely deformed and misplaced, treatment with cocoa powder recovers overall gut morphology.

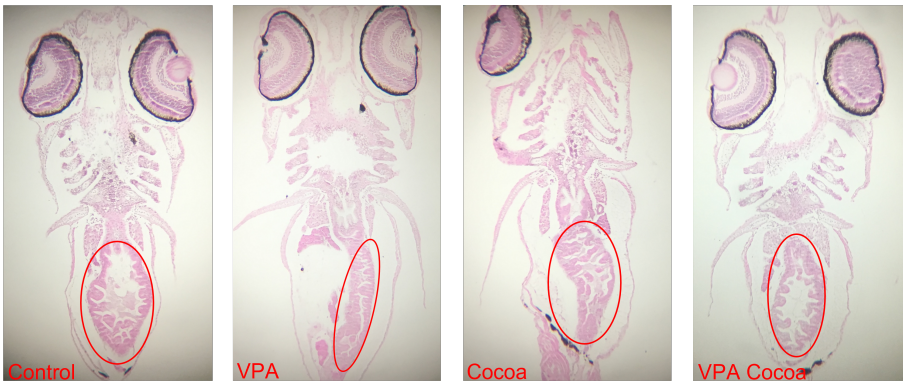

Figure 4.18: H&E staining of all zebrafish larvae side-by-side at 6 days-post-fertilization showing coronal cuts with the gut encircled

4.3.4 Metabolomics and Proteomics Data

Both metabolomics and proteomics data are presented in volcano plots and heatmaps. In the former, the x-axis is the $-\log_2$(fold change) which describes the ratio of change in expression levels of a protein/metabolite between two conditions. A positive value indicates upregulation under the condition being studied, and a negative value indicates downregulation. Significance in fold change (FC) is marked by the dotted dash line at 1.5 $<$ FC $<$ -1.5. The y-axis is a $-\log_{10} p\text{-}value$. Taking -log10 of the p-value adjusts the scale for easier viewing. Larger values on this scale indicate higher significance. By default, the horizontal dotted dash line represents significance at p-value $<$ 0.05 with Bonferroni correction.

Each point in the volcano plot represents a different metabolite/protein that was measured. If a point is positioned to the right of 0 on the X-axis, it means that the metabolite/protein is more prevalent in the first group versus the second group. If it is to the left of 0, it means it is less prevalent. The higher a point is on the Y-axis, the more statistically significant the difference in metabolite/protein prevalence between the first and second group. Points significant in p-value only are illustrated in blue whereas those significant in FC only are illustrated in green. Ultimately, significance in both FC and p-value is observed in the upper-right and upper-left sections, delimited by the dotted dash lines, illustrated by points in red. Overall, for omics each sample had n = 15 and was replicated three times.

4.3.5 Metabolomics

When comparing VPA samples versus control samples in **Figure 4.19**, metabolomics analyses revealed that VPA samples had increased oxitriptan (significant by FC only) and increased 5-methoxytryptophan (5-MTP) which was significant in both FC > 1.5 and p-value < 0.05.

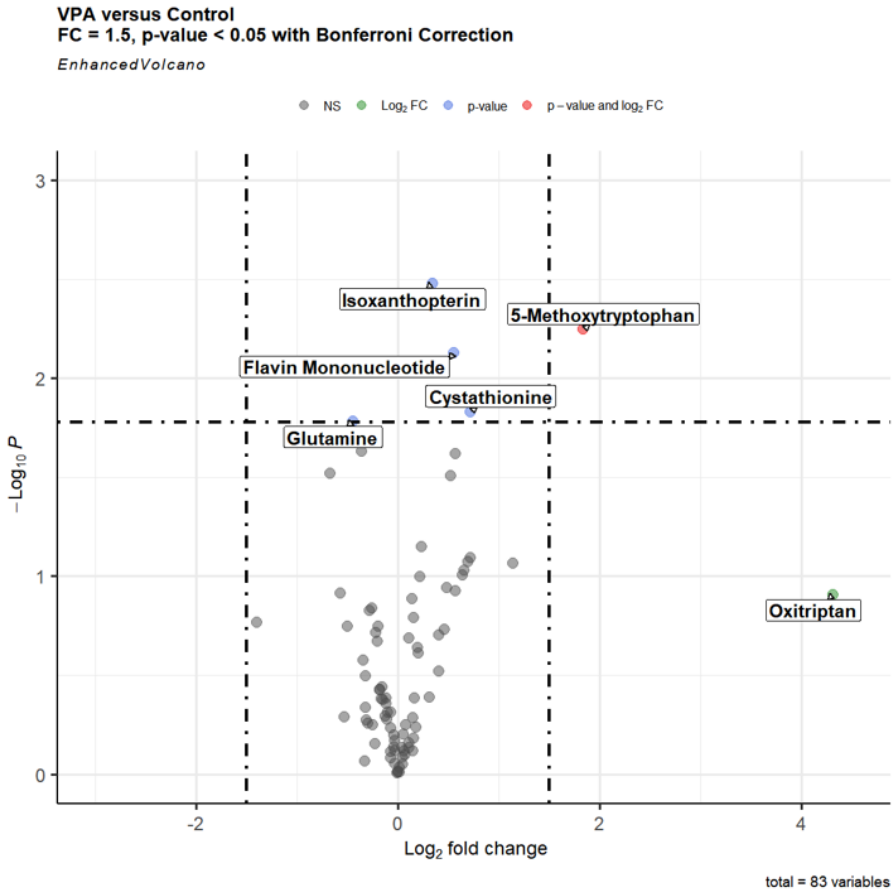

Figure 4.19: Volcano plot of the metabolite fold change between VPA and control zebrafish at significance p < 0.05 with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

On the other hand, when comparing cocoa versus control (**Figure 4.20**), there were no metabolites that were significant in both p-value and FC. However, there was a large FC increase of caffeine and theobromine in the cocoa group versus control.

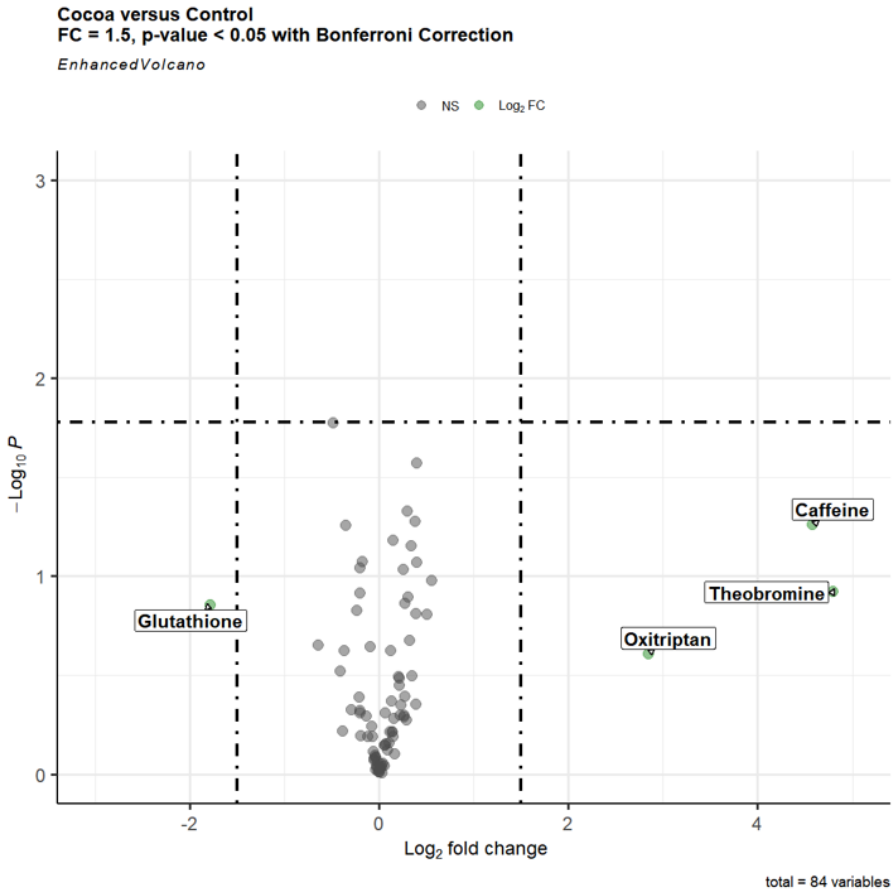

Figure 4.20: Volcano plot of the metabolite fold change between cocoa and control zebrafish at significance $p < 0.05$ with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

In the samples where VPA fish were also treated with cocoa versus control (**Figure 4.21**), only caffeine and theobromine were significant in FC only. No metabolites were identified to be statistically significantly different between VPA + cocoa and control samples.

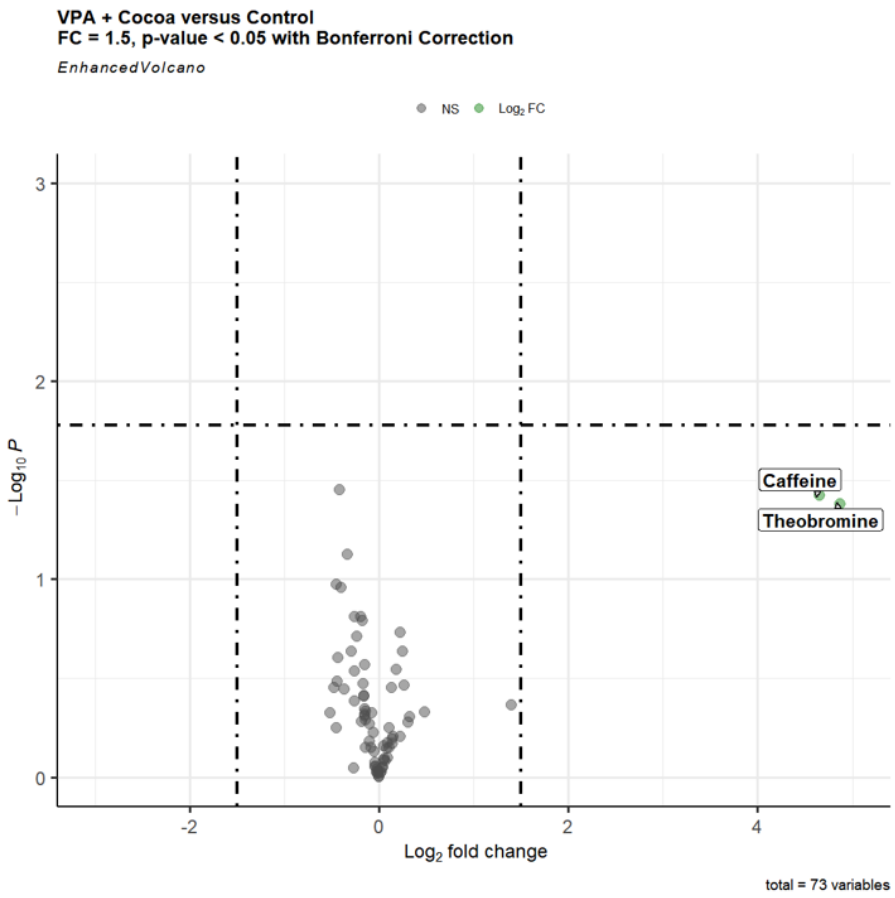

Figure 4.21: Volcano plot of the metabolite fold change between VPA & cocoa and control zebrafish at significance p < 0.05 with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

Figures 4.22; 4.23; and 4.24 illustrate metabolite heatmaps from the PFP column, Scherzo column, and targeted metabolomics respectively. The clustering in the heatmap was obtained using a Ward method with an Euclidian distance measure. A t-test was conducted on the different protein and the top 50 most significant by p-value were used for the heatmap. Untargeted metabolomics (**Figures 4.22 and 4.23**) show that VPA treated samples had significantly altered amino acid expression such as proline, leucine, adenine,

tyrosine, L-valine, tryptophan, L-tryptophan, and many metabolites of amino acid metabolism such as 5-methoxytryptophan.

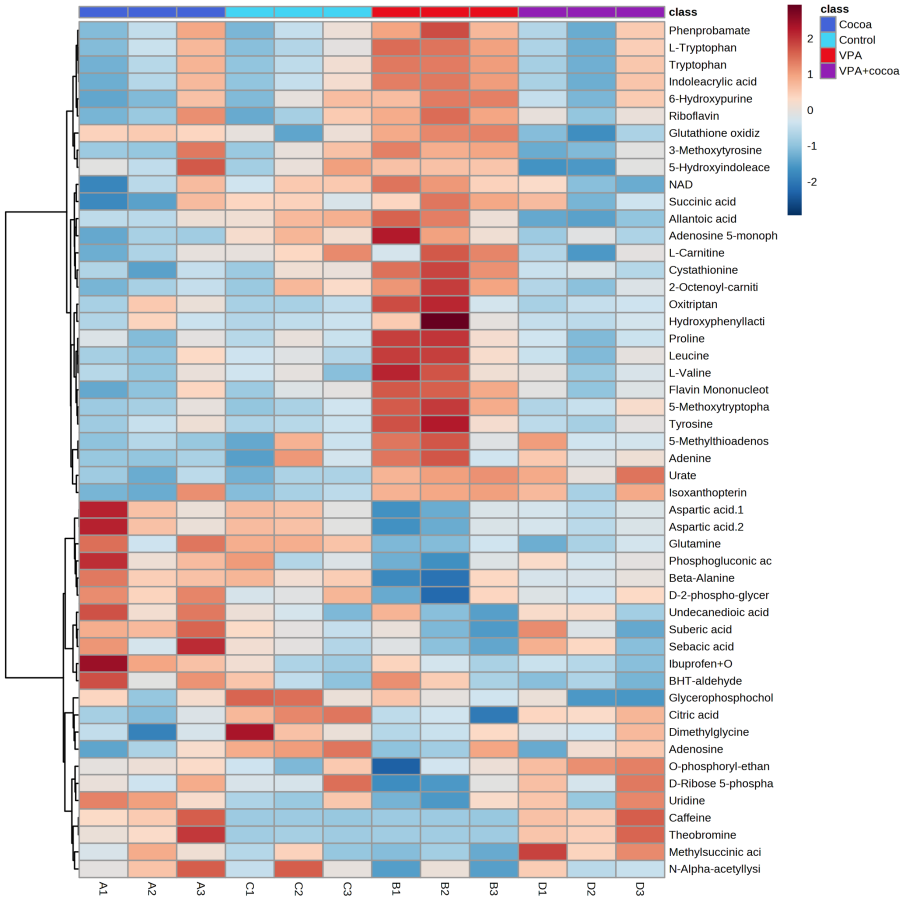

Figure 4.22: PFP column Metabolomics Heatmap where each row represents a distinct metabolite, while each column indicates a specific sample or condition. Color intensity denotes the relative concentration of each metabolite, with red indicating higher abundance and blue indicating lower abundance

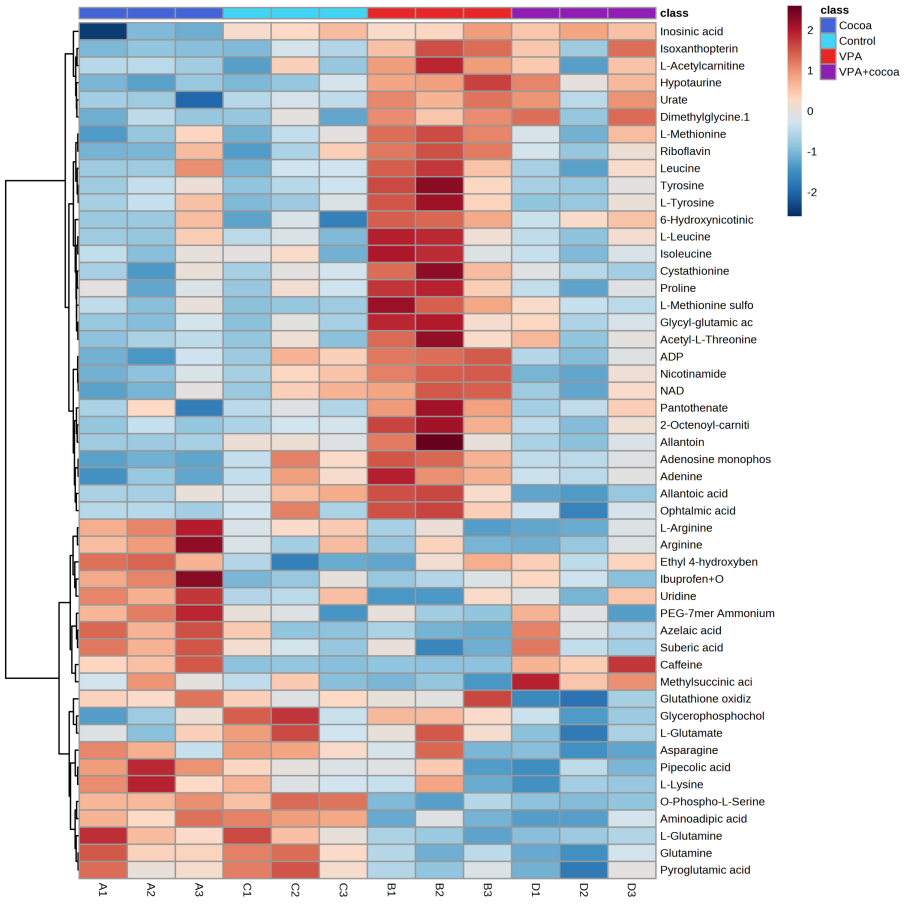

Figure 4.23: Scherzo column Metabolomics Heatmap where each row represents a distinct metabolite, while each column indicates a specific sample or condition. Color intensity denotes the relative concentration of each metabolite, with red indicating higher abundance and blue indicating lower abundance

Targeted metabolomics heatmap in **Figure 4.24** emphasize the significant differences in amino acid metabolism present in the VPA treated samples only while highlighting some further differences in metabolism in the cocoa only treated sample. The differences in amino acid metabolism observed in the VPA treated samples were not observed in the cocoa treated VPA samples.

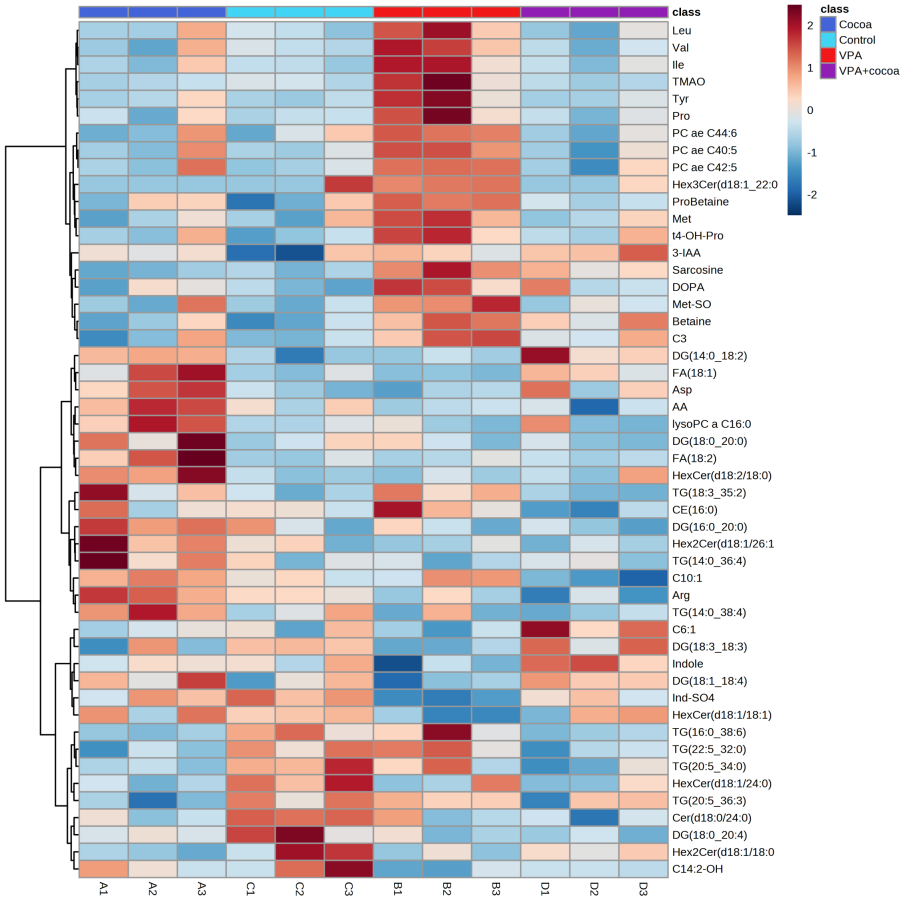

Figure 4.24: Targeted Metabolomics Heatmap where each row represents a distinct metabolite, while each column indicates a specific sample or condition. Color intensity denotes the relative concentration of each metabolite, with red indicating higher abundance and blue indicating lower abundance

4.3.6 Proteomics

When comparing VPA samples versus control samples in **Figure 4.25**, proteomics analyses revealed that VPA samples had increased cytoglobin-1, glyceraldehyde-3-phosphate dehydrogenase, desmoplakin A, epididymal secretory protein E1, betaine-homocysteine S-methoxytransferaase 1, fermitin family homolog 2, and hemoglobin subunit beta-1, which were significant in both FC > 1.5 and p-value < 0.05. On the other hand, VPA samples had decreased synaptosomal-associated protein 25 (SNAP25) which was also significant in both FC < -1.5 and p-value < 0.05.

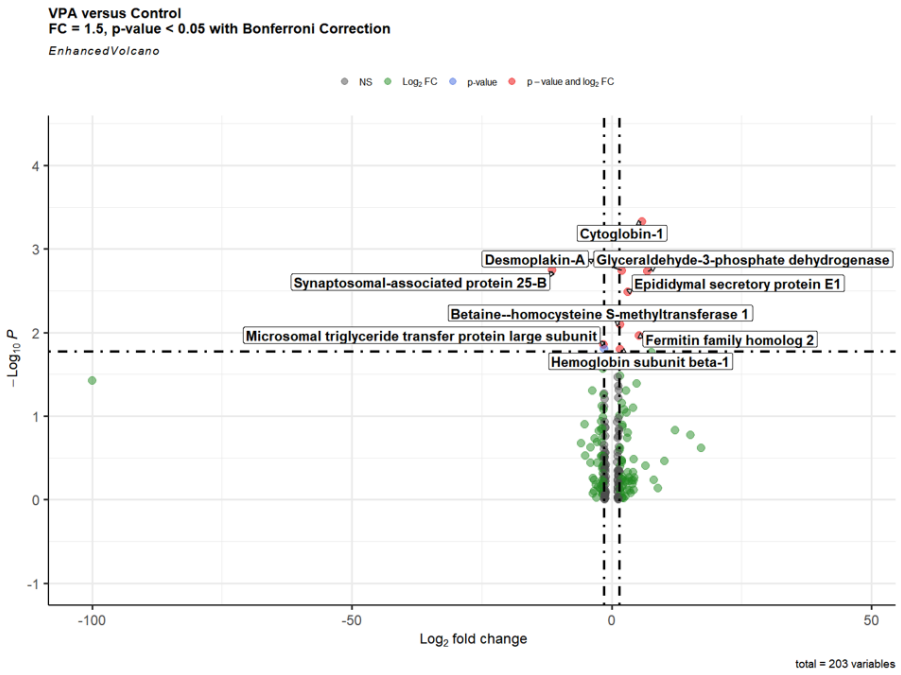

Figure 4.25: Volcano plot of the protein fold change between VPA and control zebrafish at significance p < 0.05 with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

Figure 4.26 shows that the only significant protein in both FC > 1.5 and p-value < 0.05 is parvalbumin-2 which was increased in cocoa-treated fish samples compared to control.

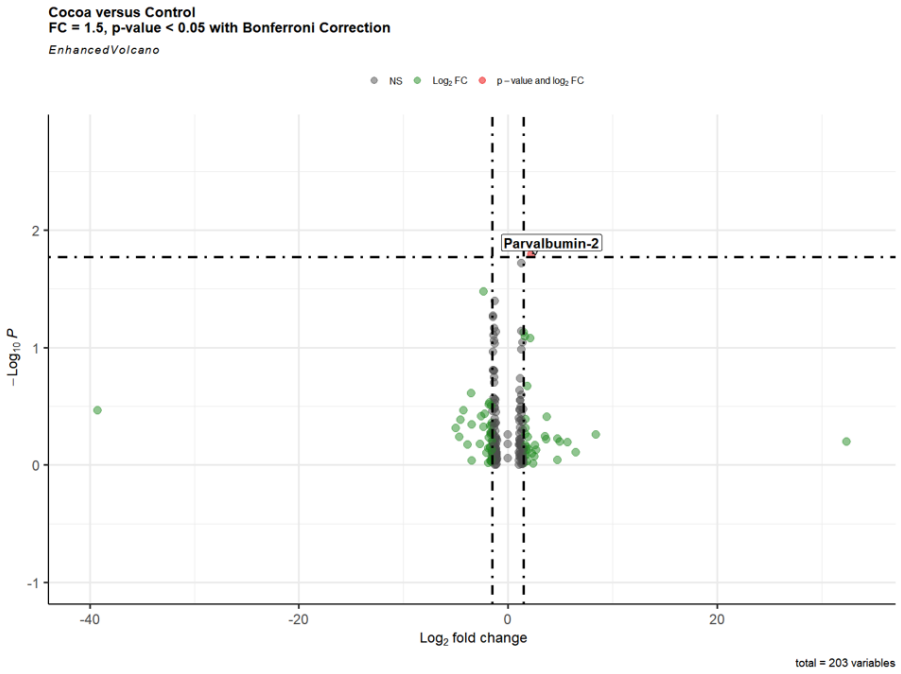

Figure 4.26: Volcano plot of the protein fold change between cocoa and control zebrafish at significance p < 0.05 with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

Figure 4.27 illustrates that VPA + cocoa fish samples only express significantly greater histone H3.2 when compared to control. The differences observed in the VPA versus control groups were no longer present in the VPA fish treated with PRE cocoa.

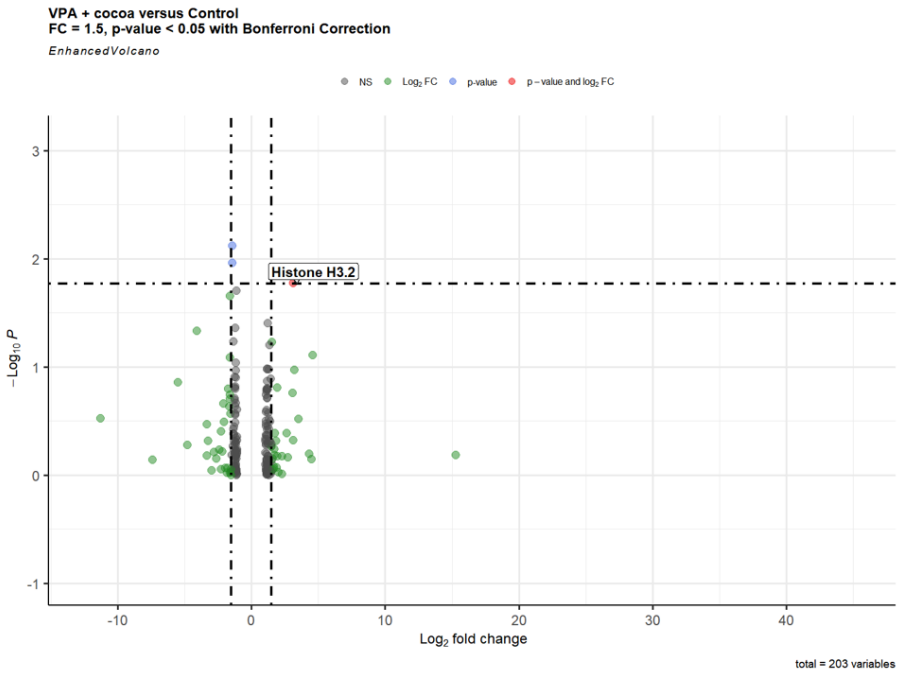

Figure 4.27: Volcano plot of the protein fold change between VPA & cocoa and control zebrafish at significance $p < 0.05$ with Bonferroni correction and a $-\log_{10} p\text{-}value$ transformation

Figure 4.28 illustrates the differences in protein expression between study samples highlighting that VPA treated zebrafish expressed both significantly decreased proteins (blue) and significantly increased proteins (red) compared to all other groups (control, cocoa, VPA + cocoa).

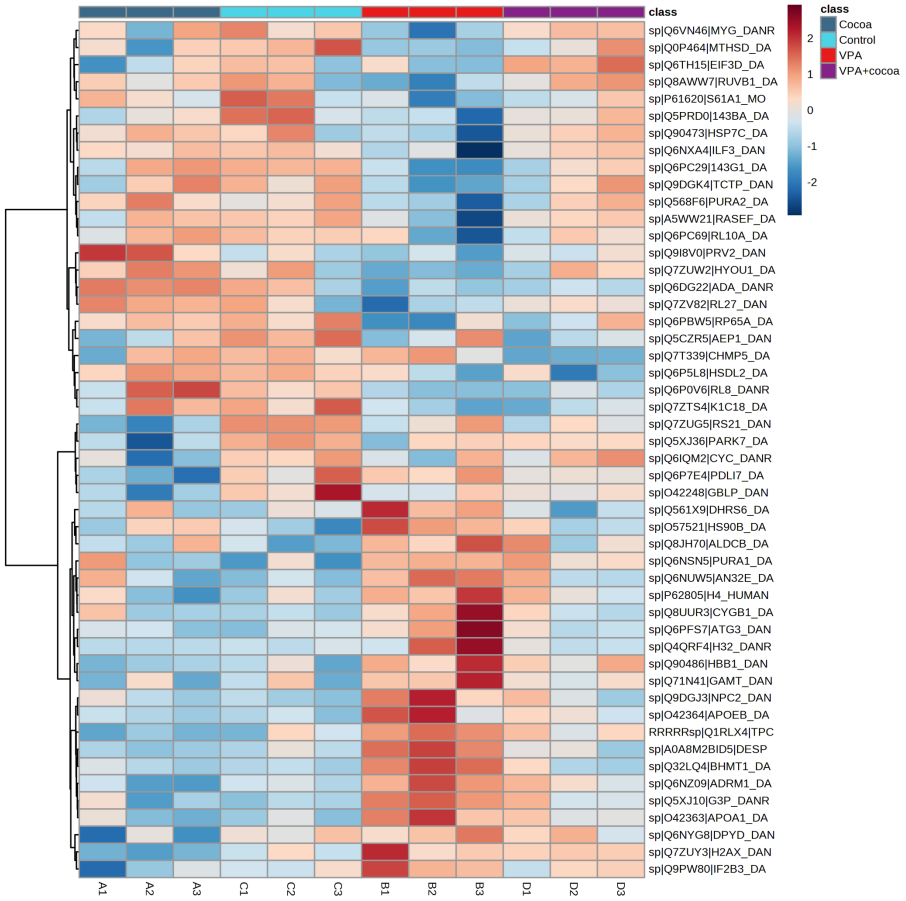

Figure 4.28: Proteomics Heatmap where each row represents a distinct protein, while each column indicates a specific sample or condition. Color intensity denotes the relative concentration of each protein, with red indicating higher abundance and blue indicating lower abundance

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Feature	Name	Cocoa vs Control		VPA vs Control		VPA + cocoa vs Control	
		FC	P-value	FC	P-value	FC	P-value
sp|Q6VN46|MYG_DANRE	Myoglobin	-1.1	0.8351	-1.6	0.0556	-1.1	0.9964
sp|Q0P464|MTHSD_DANRE	Methenyltetrahydrofolate synthase domain-containing protein	-1.3	0.3319	-1.7	0.0270	-1.2	0.4880
sp|Q6TH15|EIF3D_DANRE	Eukaryotic translation initiation factor 3 subunit D	-1.6	0.9261	-1.4	0.8930	-1.2	0.2766
sp|Q8AWW7|RUVB1_DANRE	RuvB-like 1	1.2	0.4794	-1.4	0.4158	1.2	0.4242
sp|P61620|S61A1_MOUSE	Protein transport protein Sec61 subunit alpha isoform 1	-1.2	0.7828	-1.6	0.3040	-2.2	0.6075
sp|Q5PRD0|143BA_DANRE	14-3-3 protein beta/alpha-A	-1.2	0.2773	1.2	0.9268	-1.1	0.7007
sp|Q90473|HSP7C_DANRE	Heat shock cognate 71 kDa protein	-1.5	0.8599	-1.5	0.3310	-1.1	0.8841
sp|Q6NXA4|ILF3_DANRE	Interleukin enhancer-binding factor 3 homolog	1.1	0.8127	-1.3	0.1741	1.1	0.8001
sp|Q6PC29|143G1_DANRE	14-3-3 protein gamma-1	1.3	0.0192	-2.0	0.1161	-1.2	0.1515
sp|Q9DGK4|TCTP_DANRE	Translationally-controlled tumor protein homolog	1.2	0.7959	-1.4	0.0534	-1.1	0.5207
sp|Q568F6|PURA2_DANRE	Adenylosuccinate synthetase isozyme 2	1.2	0.6880	-1.9	0.0759	1.1	0.9031
sp|A5WW21|RASEF_DANRE	Ras and EF-hand domain-containing protein	-1.0	0.6210	-1.3	0.1170	-1.1	0.6297
sp|Q6PC69|RL10A_DANRE	60S ribosomal protein L10a	1.1	0.9932	-1.4	0.2207	-1.1	0.8717
sp|Q9I8V0|PRV2_DANRE	Parvalbumin-2	2.2	0.0160	-1.5	0.5102	-1.2	0.1558
sp|Q7ZUW2|HYOU1_DANRE	Hypoxia up-regulated protein 1	1.5	0.0740	-1.6	0.0839	-2.3	0.8889
sp|Q6DG22|ADA_DANRE	Adenosine deaminase	1.3	0.2532	-3.7	0.0497	-2.1	0.2187
sp|Q7ZV82|RL27_DANRE	60S ribosomal protein L27	1.5	0.3369	1.4	0.9264	1.3	0.8761
sp|Q6PBW5|RP65A_DANRE	Retinoid isomerohydrolase	-1.3	0.8591	-5.2	0.1255	-1.7	0.2327
sp|Q5CZR5|AEP1_DANRE	Natterin-like protein	-1.3	0.0684	4.3	0.5460	-1.4	0.0076
sp|Q7T339|CHMP5_DANRE	Charged multivesicular body protein 5	-1.5	0.4612	1.4	0.4441	-4.1	0.0465
sp|Q6P5L8|HSDL2_DANRE	Hydroxysteroid dehydrogenase-like protein 2	1.3	0.6994	-1.6	0.4851	-2.4	0.5850
sp|Q6P0V6|RL8_DANRE	60S ribosomal protein L8	1.5	0.7653	-1.4	0.0156	-1.2	0.0920
sp|Q7ZTS4|K1C18_DANRE	Keratin, type I cytoskeletal 18	1.1	0.2302	-1.7	0.1735	-1.4	0.3748
sp|Q7ZUG5|RS21_DANRE	40S ribosomal protein S21	-1.3	0.0873	1.3	0.0432	-1.1	0.9210
sp|Q5XJ36|PARK7_DANRE	Protein DJ-1zDJ-1	-1.4	0.8436	-1.2	0.1383	-1.1	0.0197
sp|Q6IQM2|CYC_DANRE	Cytochrome c	-1.4	0.1095	-1.3	0.8956	1.3	0.6701
sp|Q6P7E4|PDLI7_DANRE	PDZ and LIM domain protein 7	-1.5	0.3489	-2.0	0.1419	-1.5	0.2694
sp|O42248|GBLP_DANRE	Guanine nucleotide-binding protein subunit beta-2-like 1	-1.3	0.1571	-1.2	0.4642	-1.1	0.5730
sp|Q561X9|DHRS6_DANRE	3-hydroxybutyrate dehydrogenase type 2	-1.1	0.9143	1.1	0.0339	-1.1	0.2167
sp|O57521|HS90B_DANRE	Heat shock protein HSP 90-beta	-1.2	0.7644	1.1	0.0053	1.1	0.4859
sp|Q8JH70|ALDCB_DANRE	Fructose-bisphosphate aldolase C-B	2.4	0.9718	4.2	0.4719	1.7	0.6492
sp|Q6NSN5|PURA1_DANRE	Adenylosuccinate synthetase isozyme 1	1.3	0.9900	8.9	0.7257	-1.2	0.8299
sp|Q6NUW5|AN32E_DANRE	Acidic leucine-rich nuclear phosphoprotein 32 family member E	4.7	0.5981	4.8	0.0408	1.5	0.5449
sp|P62805|H4_HUMAN	Histone H4	1.9	0.9347	1.5	0.0986	1.2	0.1625
sp|Q8UUR3|CYGB1_DANRE	Cytoglobin-1	1.9	0.5796	5.8	0.0005	-3.2	0.4853
sp|Q6PFS7|ATG3_DANRE	Ubiquitin-like-conjugating enzyme ATG3	-2.5	0.3858	1.6	0.0330	-1.2	0.5943
sp|Q4QRF4|H32_DANRE	Histone H3.2	-3.5	0.2463	17.2	0.2423	3.1	0.0167
sp|Q90486|HBB1_DANRE	Hemoglobin subunit beta-1	5.0	0.6306	1.7	0.0159	-11.3	0.3004
sp|Q71N41|GAMT_DANRE	Guanidinoacetate N-methyltransferase	-1.2	0.9141	-2.8	0.2062	-1.4	0.5393
sp|Q9DGJ3|NPC2_DANRE	Epididymal secretory protein E1	2.1	0.0837	3.2	0.0033	3.1	0.1738
sp|O42364|APOEB_DANRE	Apolipoprotein Eb	-1.2	0.8892	2.9	0.0913	1.7	0.5775
RRRRRsp|Q1RLX4|TPC11_DANRE	Trafficking protein particle complex subunit 11	-1.4	0.1554	1.3	0.0598	1.3	0.7265
sp|A0A8M2BID5|DESPA_DANRE	Desmoplakin-A	-1.2	0.4369	2.0	0.0018	1.4	0.8778
sp|Q32LQ4|BHMT1_DANRE	Betaine–homocysteine S-methyltransferase 1	-1.3	0.2984	1.7	0.0081	-1.2	0.9431
sp|Q6NZ09|ADRM1_DANRE	Proteasomal ubiquitin receptor ADRM1	-1.4	0.0787	1.4	0.0488	1.2	0.1610
sp|Q5XJ10|G3P_DANRE	Glyceraldehyde-3-phosphate dehydrogenase	3.6	0.5723	6.9	0.0019	4.6	0.0775
sp|O42363|APOA1_DANRE	Apolipoprotein A-I	-1.1	0.8540	2.8	0.5915	1.2	0.1362
sp|Q6NYG8|DPYD_DANRE	Dihydropyrimidine dehydrogenase [NADP(+)]	-4.2	0.3435	2.2	0.6862	1.2	0.0397
sp|Q7ZUY3|H2AX_DANRE	Histone H2AX	1.7	0.4868	3.0	0.7663	1.4	0.9299
sp|Q9PW80|IF2B3_DANRE	Insulin-like growth factor 2 mRNA-binding protein 3	-1.3	0.1979	1.2	0.2694	1.1	0.9576

Table 4.1: Reference table for proteomic feature names

4.3.7 Pathway Analysis

Figure 4.29 illustrates the enrichment ratios in the metabolite only pathway analysis. The enrichment ratio is calculated as the number of hits within a particular metabolic pathway divided by the expected number of hits. The figure shows that valine, leucine and isoleucine biosynthesis is the greatest pathway in terms of enrichment ratio and p-value whereas aminoacyl-tRNA biosynthesis is lesser magnitude in terms of enrichment ratio but just as significant in p-value. Other statistically significant pathways (p-value < 0.05) include purine metabolism; glycine, serine, and threonine metabolism; nicotinate and nicotinamide metabolism; valine, leucine and isoleucine degradation; tryptophan metabolism; and tyrosine metabolism.

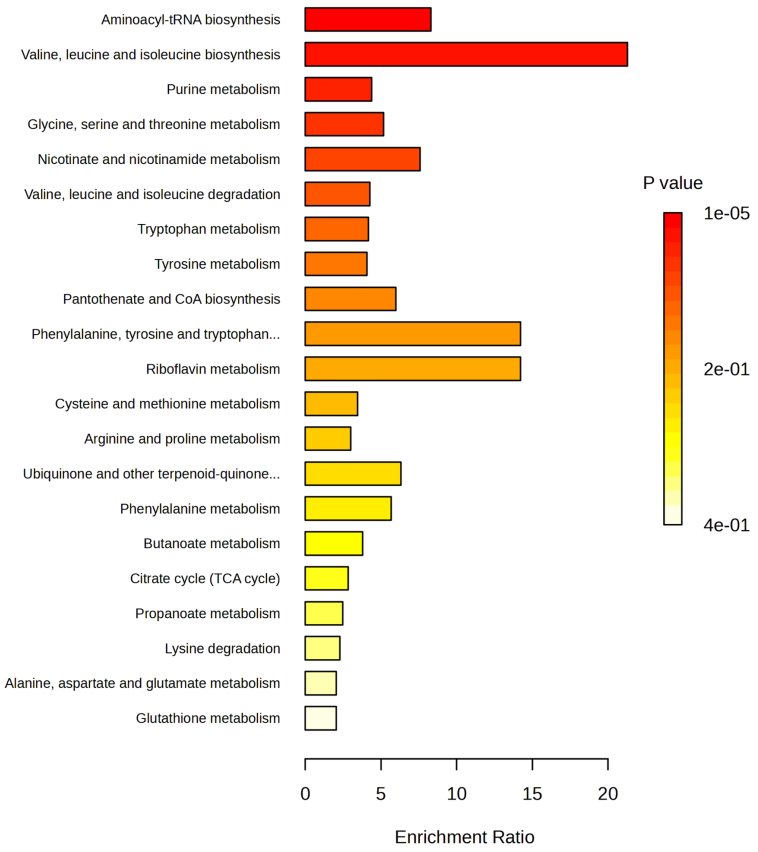

Figure 4.29: Pathway Analysis of Metabolites (untargeted and targeted)

Figure 4.30 shows the joint-pathway analysis combining the metabolites and proteins. Aminoacyl-tRNA biosynthesis pathway impact showed the most significant p-value followed by glycine, serine, and threonine metabolism; ABC transporters pathway; valine, leucine and isoleucine biosynthesis; nicotinate and nicotinamide metabolism; pantothenate and CoA biosynthesis; and purine metabolism.

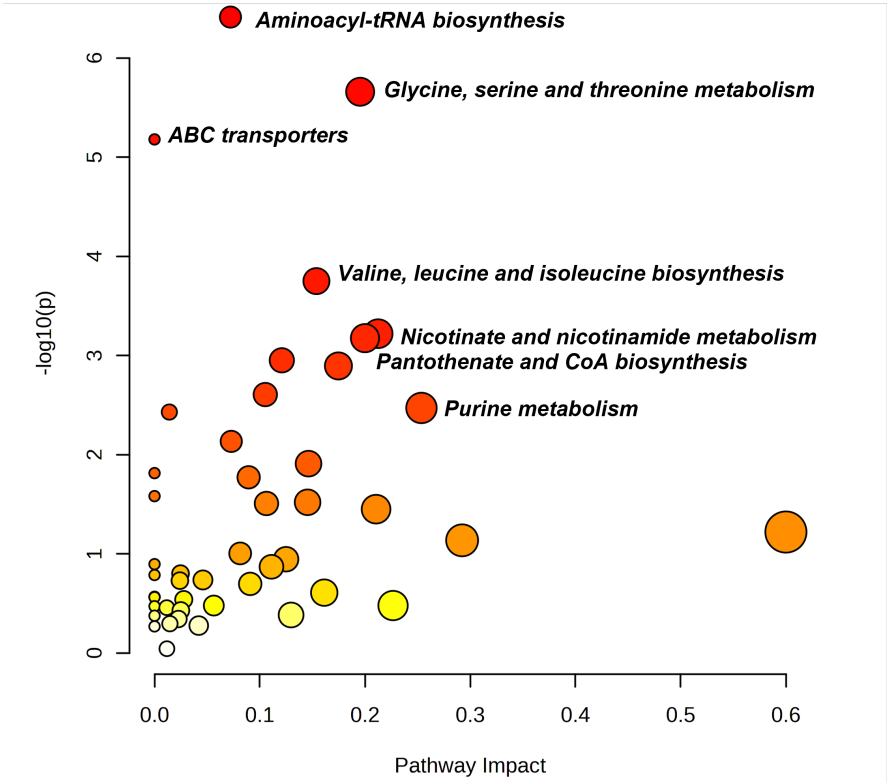

Figure 4.30: Joint-Pathway Analysis (metabolties and proteins) illustrating pathway impact and a $-\log_{10} p\text{-}value$ transformation

Combining the metabolomics and proteomics in a joint-pathway analysis provides a more systematic scope of the biological systems impacted in the VPA zebrafish model of ASD. The joint-pathway analysis revealed that VPA had the largest impact on pathways related to protein synthesis and amino acid metabolism/biosynthesis. Specifically,

aminoacyl-tRNA biosynthesis was the most significant by p-value with a pathway impact of 0.07. The following pathways were also not only significant in p-value (descending order) but also had a pathway impact greater than 0: glycine, serine and threonine metabolism; valine, leucine and isoleucine biosynthesis; tyrosine metabolism; cysteine and methionine metabolism; arginine and proline metabolism; purine metabolism; valine, leucine and isoleucine degradation; phenylalanine, tyrosine and tryptophan biosynthesis; tryptophan metabolism; beta-alanine metabolism; and alanine, aspartate and glutamate metabolism. While ABC transporter pathway was third-most significant in p-value, it had a pathway impact value of 0.

Discussion

The validation portion of this project confirmed and refined the previously described VPA-induced model of ASD in zebrafish (Messina et al., 2020; Dwivedi et al., 2019; Muhsen et al., 2021), which is still under-researched when compared to the extensive literature behind the VPA mouse model. As expected with VPA, a potent histone deacetylase (HDAC) inhibitor (Gurvich et al., 2005), concentrations greater than 5 μM were proven to be greatly teratogenic and lethal above 15 μM (Messina et al., 2020; Muhsen et al., 2021). Furthermore, in the present study cocoa polyphenol toxicology illustrated a non-toxic and beneficial 2.5 μM concentration of (-)-epicatechin for the VPA autism model, which was consistent with a study that evaluated PRE and toxicity from medicinal plants in zebrafish (Veeren et al., 2020).

Previously, *in-utero* exposure to VPA in mice was found to alter GI morphology and gut motility (J.-W. Kim et al., 2013). In a separate murine study, altered GI structure caused by VPA exposure also resulted in abnormal behaviour and social deficits (Campolongo et al., 2018). The results of this thesis have revealed that co-treatment of cocoa powder rich in (-)-epicatechin was effective in preventing the behavioural symptoms of ASD in a VPA-induced zebrafish model of autism. Additionally, cocoa powder prevented the disturbed gut morphology associated with VPA treatment. Within the context of ASD, gut dysbiosis has been identified as a causal link to behavioural deficits which, in a positive feedback loop, may worsen gut health (Taniya et al., 2022; Lu et al., 2022). However, improving gut health has been effective in alleviating behavioural deficits in human ASD

which was replicated in this study on the zebrafish ASD model. The behavioural benefits of the cocoa polyphenol co-treatment were in concert with the elimination of upregulated and downregulated metabolite and protein expression seen with the VPA treatment in addition to an improved gut structure. It is conceivable the bioactive components released from the digestion of PRE cocoa powder could have exerted effects via several mechanisms of action. Following digestion, cocoa polyphenols and their metabolites could have modulated behaviour directly as well as by promoting the proper development of the GI tract and by supporting the function of the gut-brain axis. Specific mechanisms of action include the modulation of signalling pathways to improve neuronal function by flavonoids; the protection from oxidative stress; and the ability of the gut microbiota to catabolize polyphenols into more active and better absorbed metabolites with neuroprotective effects (Martín et al., 2020). Furthermore, recent studies suggest that polyphenols benefit the brain by protecting neurons, enhancing their functions, growth, and influencing cerebrovascular systems like increased brain blood flow. Flavonoids may also promote neuroprotection by interacting with specific proteins involved in intracellular signaling crucial for neuronal survival and long-term potentiation, particularly affecting protein and lipid kinases that lead to the inhibition of neuronal death by apoptosis induced by neurotoxicants such as oxygen radicals (Lamport et al., 2012). The improved behavioral outcomes in the VPA autism model could be mediated via modulation of the gut-brain axis that connects the brain to the gut and other organs via the enteric nervous system (ENS) and the vagus nerve. Animal studies indicate that epicatechin (the main polyphenol component of CocoaVia™ cocoa powder), flavanol microbial metabolites, and theobromine which are the main metabolites of cocoa powder can cross the blood-brain barrier highlighting a bi-directional communication between the gut and brain, leading to

cognitive enhancements such as increased neuronal survival and synaptic plasticity (Martín et al., 2020). The specific aspects by which polyphenols affect this axis requires further study as this axis involves multiple systems including the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and metabolic pathways (Morais et al., 2021).

5.1 Behavioural Assessments

In our behavioural studies, VPA-treated fish exhibited greater anxiety in both calm and stress inducing environments which lead to increased movement distance and movement velocity shown in **Figure 4.12**, **Figure 4.13**, and **Figure 4.14**. Consistent elevated stress and poor management of stress could be linked, within the previous discussion on gut health and tryptophan metabolism, to dysregulated serotonin, melatonin, and niacin production and circulation resulting in hyperactive behaviour. Further, there is research on tryptophan depletion which may cause low mood and depression which can be expressed in the form of high anxiety and stress (Young, 2013; Bell et al., 2001). In terms of direct consequences of VPA exposure to anxious behaviour, there has been research to suggest that VPA-exposed astrocytes reduced the number of inhibitory synapses leading to reduced synaptic transmission by inhibitory synapses (Takeda et al., 2021). Nevertheless, ASD has been found to involve both homeostatic inhibitory and excitatory dysfunction (Nelson and Valakh, 2015). In the case of the VPA + cocoa powder treated zebrafish, however, they were able to remain calm. This observation is supported by the cocoa powder co-treated VPA zebrafish showing metabolite and protein profile as well as gut morphology similar to the control zebrafish. However, the VPA + cocoa fish were not only calm at similar levels

to control during the dark cycle of the DanioVision assessment, but they were also calmer when in the stress inducing light cycle. In fact, their swim distance and velocity remained constant through the change from dark to light whereas even the control and cocoa zebrafish exhibited some anxious response.

The seemingly anxiolytic effect of the combination of VPA and cocoa powder is counterintuitive. However, there may be behavioural and stress responses that are unlocked through this combination. Recent research in the VPA-induced ASD model in mice has shown that VPA may cause not only inhibitory, but also excitatory dysfunction (Qi et al., 2022). Specifically, they found that the expression of brain-derived neurotrophic factor (BDNF), a neurotrophin that promotes the formation and function of glutamatergic and GABAergic synapses was severely reduced. Therefore, with decreased BDNF, there would be severe imbalances between glutamatergic (excitation) and GAGBAergic (inhibition) synapses, which may lead to extreme behaviour in response to stimuli. Interestingly, research has suggested that a major cognitive benefit of cocoa polyphenols is derived from its ability to trigger neuroprotective effects through the modulation of BDNF (Socci et al., 2017; Cimini et al., 2013). It is possible in the present study that the cocoa powder and its metabolites function, via BDNF, to restore excitatory/inhibitory (E/I) homeostasis or creating a balance of E/I such that the VPA + cocoa treated fish remain calmer than control fish despite the stress inducing environment. In addition, it is possible that this unique balance in E/I can only be achieved with an initial disruption in E/I homeostasis leading to a secondary state of E/I balance where there may be more inhibitory effects. This would explain why the cocoa only treated zebrafish do not exhibit such calming effects of the cocoa treatment. In two human studies, cocoa powder treatment and specifically cocoa polyphenols such as flavonols have been positively associated with

increased activation of BDNF and its neuroprotective function in ageing adults and subjects with Alzheimer's Disease (García-Cordero et al., 2021; Cimini et al., 2013).

In addition to these possible effects to induce calming on the VPA + cocoa zebrafish, it is also possible that the fish themselves are adapting differently to the VPA exposure. Since the zebrafish is a regenerative model there may be some self-compensatory mechanisms in place (Gemberling et al., 2013). It has been noted that VPA exposure may cause decreased inhibitory synaptic transmission (Takeda et al., 2021). However, the cocoa-treated zebrafish may be compensating for this VPA-mediated loss of inhibitory synaptic transmission via neuroprotective functions such as the regeneration of neurons (Zambusi and Ninkovic, 2020; Ghosh, Hui, et al., 2016). Together, this may lead to a stronger inhibitory reaction which may subdue regular excitatory transmission. Further investigation is warranted to measure E/I circuit homeostasis under various conditions as well as quantifying in greater detail the effects of VPA exposure and subsequent cocoa polyphenol exposure on tryptophan metabolism.

5.2 Proteomics

The marked differences in metabolomic and proteomic profiles between the ASD zebrafish versus ASD zebrafish treated with cocoa powder are striking. Specifically, VPA-treated zebrafish had downregulated expression of SNAP25 (**Figure 4.25**), a protein involved in the SNARE complex responsible for regulated synaptic vesicle endo/exocytosis at the presynaptic terminal and voltage-gated calcium channels (Corradini et al., 2009). By directly engaging with various subunits of calcium channels, SNAP-25 exhibits an inhibitory effect on neuronal voltage-gated calcium channels, thereby orchestrating the regulation of intracellular calcium dynamics (Antonucci et al., 2016; Corradini et al., 2009;

Guerini et al., 2011). Significant correlations have also been established between the SNAP-25 gene and several neural disorders like Attention Deficit Hyperactivity Disorder (ADHD), schizophrenia, and bipolar disorder (Corradini et al., 2009). These associations imply the potential function of this protein as a common biological interface among a variety of "synaptopathies" (Antonucci et al., 2016). In addition, SNAP25 also oversees the trafficking of postsynaptic receptors, the formation of spine morphology, and synaptic plasticity giving it the ability to impact neuropsychiatric diseases (Antonucci et al., 2016). Given the role of SNAP25 and the behavioural benefits observed comparing the cocoa-treated VPA-treated zebrafish to the VPA-treated zebrafish, it is conceivable that the cocoa powder treatment, specifically its metabolism, influenced neurotransmitter signaling through the recovery of SNAP25 protein expression to exert a calming effect in the VPA-treated fish. However, these neurological benefits may occur at various other points in the physiology of neurotransmitters/neurotransmission disturbed in our autism model. For example, there may also be disrupted synaptic transmission of NTs through calpain-mediated SNAP25 degradation (Ando et al., 2005; Baudry et al., 2013; Ramos-Miguel et al., 2019). Indeed, this pathway has been studied previously in schizophrenia where calpain-mediated SNAP25 cleavage resulted in reduced SNARE formation causing a decrease in NT release (Ramos-Miguel et al., 2019). Previous studies have also determined that calpain-mediated SNAP25 degradation may be specific to GABAergic neurons (Baudry et al., 2013). A separate study examined calpain inhibitors and found that abolishing the Ca2+-dependent cleavage site of SNAP25 resulted in increased Ca2+-dependent glutamate (Glu) release (Ando et al., 2005). These studies imply that a reduction in SNAP25 caused by calpain cleavage would result in decreased GABA inhibition as well as increased glutamate release that could lead to uncontrolled

neuronal excitation. Such a mechanism could explain why reduced SNAP25 in the VPA-treated zebrafish could lead to increased excitation as exhibited in the behavioural assay.

In humans, VPA in small doses is used as an anti-convulsant for epilepsy. In large doses, however, VPA also becomes toxic and can cause epilepsy (Romoli et al., 2019). In the case of *SNAP25*, research has found that a substantial decrease in its expression can lead to epilepsy (Corradini et al., 2009). This is consistent with our experiments as VPA exposure in the zebrafish led to significantly decreased *SNAP25* expression. Additionally, studies in mice have also confirmed that prenatal exposure to VPA leads to a significant decrease in SNAP25 in all brain structures (Lenart et al., 2020). While previously only studied in Alzheimer's disease, ADHD, and schizophrenia, emerging research has evaluated its possible role in ASD as well. Specifically, Guerini et al. (2011) have determined that single nucleotide polymorphisms (SNPs) in *SNAP25* are associated with hyperactivity in autism spectrum disorders. This is consistent with our findings showing that VPA-treated zebrafish exhibited significant hyperactivity and anxiety behaviours which was similarly observed in a study conducted on the VPA rat model (Gąssowska-Dobrowolska et al., 2020). Furthermore, Guerini et al., 2011 found that decreased SNAP25 expression could be responsible for cognitive deficits in children affected by autism spectrum disorders. The existing literature combined with our findings that *SNAP25* is downregulated in a zebrafish model of ASD further support a causal role of SNAP25 affecting the behavioural outcomes associated with ASD.

In support of this contention, a recent study conducted in mice found that VPA treatment resulted in localized decrease of SNAP25 in all brain structures compared to control (Lenart et al., 2020). In that regard, SNAP25 is known to affect cognitive function

and the regulation of locomotor activity (Lenart et al., 2020), which may partially explain the aberrant swimming behaviour of the VPA-treated zebrafish in the present study. There have also been studies on SNAP25 deficiency leading to reduction of dopamine modulated synaptic transmission (Steffensen et al., 1999) which may also contribute to locomotor activity dysfunction and social behaviour in ASD. Interestingly, the lack of anxious-like swimming behaviours in cocoa treated zebrafish receiving VPA was also associated with no drop in SNAP25 expression. It thus appears that cocoa powder treatment induced compensatory mechanisms that overcame the VPA-induced deficit expression of SNAP25. Such mechanisms could invovle the SNARE family as previous research has shown that mice deficient in SNAP25 may have compensatory mechanisms induced by other members of the SNARE family with similar functions (Ulloa et al., 2018). Research has suggested that SNAP23 may partially substitute SNAP25 (Kádková et al., 2019). However, no such compensation was observed in the VPA zebrafish model possibly suggesting that SNAP23 alone is not enough which highlights the efficacy of the cocoa powder.

5.3 Metabolomics

Another aspect to consider in ASD is that there may be increased/decreased metabolism of amino acids, which may cause a disruption in neurotransmitter synthesis (Yao et al., 2011; Dalangin et al., 2020). Our metabolomics results highlighted an increase in both 5-MTP (marked increase in FC and significant p-value) and oxitriptan, also known as 5-hydroxytryptophan (5-HTP), (marked increase in FC) in the VPA-treated zebrafish compared to controls (**Figure 4.19**). In contrast, no such significant differences were noted between the cocoa-treated VPA-treated zebrafish and controls (**Figure 4.20**). Both

molecules are metabolites of tryptophan and 5-HTP is also a precursor to serotonin (5-hydroxytryptamine; 5-HT), an important neurotransmitter (Barik, 2020; C.-H. Chen et al., 2019). 5-HTP is produced from tryptophan by tryptophan hydroxylase (TPH) as the first rate-limiting step in serotonin synthesis (Barik, 2020). In the subsequent metabolic step, decarboxylation of 5-HTP via aromatic L-amino acid decarboxylase (AAAD) leads to the synthesis of serotonin. Further, 5-MTP is also a product of 5-HTP via hydroxyindole O-methyltransferase (HIOMT) also called N-acetylserotonin O-methyltransferase (ASMT), which converts N-acetylserotonin to N-acetyl-5-methoxytryptamine (melatonin) in the last step in the biosynthesis of melatonin from serotonin (Cho and Cowling, 2021). Interestingly, serotonin is up to 95% stored in chromaffin cells of the gut and realease in response to various gut lumen stimuli and has been identified to be a potential link in the onset of autism (Yang et al., 2014). Specifically, hyperserotonemia is a phenomenon observed uniquely in subjects with ASD and not in any other cases of intellectual disability or neuropsychiatric disorders (Mulder et al., 2004). The role of the gut in this context has been considered a key factor of the gut-brain axis (Jenkins et al., 2016). Nonetheless, currently there is some discordance in the literature regarding brain serotonin levels as human and animal studies have also shown lower serotonin levels in ASD. Specifically, human studies of ASD have reported that subjects with ASD have both decreased tryptophan metabolism and reduced brain serotonin synthesis which may contribute to the neurodevelopment and pathogenesis of ASD (Boccuto et al., 2013; Chugani et al., 1999). In a murine ASD model, gut dysbiosis was asssociated with decreased tryptophan metabolism and impaired social behaviour confirming some of the findings in human studies (Golubeva et al., 2017). The figure below highlights some metabolic and pathway impacts of VPA in zebrafish.

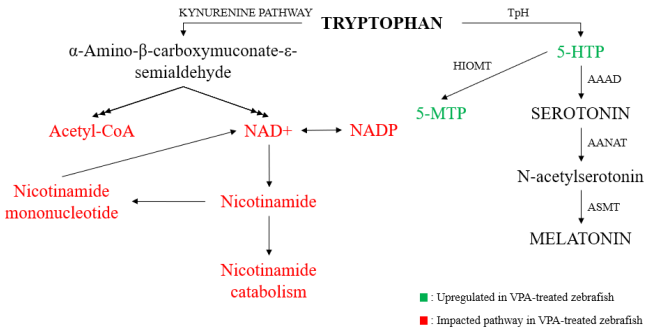

Figure 5.1: Impacts of VPA in zebrafish on tryptophan metabolism and subsequent pathways

Figure 5.1 Illustrated impacted tryptophan pathways specifically highlighting upregulated 5-HTP and 5-MTP in the biosynthesis of serotonin and melatonin as well as the effects on CoA and NAD biosynthesis in the kynurenine pathway.

In the present study, our metabolomics results are apparently conflicting with some studies as the increase in serotonin precursors points to a decreased serotonin production. Regardless, these results highlight both possible disorders in the peripheral and central metabolism of serotonin where VPA-treated zebrafish expressed more than 4-fold change increase in 5-HTP compared to control (**Figure 4.19**). Strikingly, a clinical trial conducted on children with ASD and healthy controls found that healthy subjects do not respond to 5-HTP administration whereas autistic subjects underwent significant increases in both 5-HTP and plasma serotonin (Croonenberghs et al., 2005). This illustrates the possibility that upregulated 5-HTP can occur alongside hyperserotonemia depending on the dysregulated metabolic pathway of serotonin. In this case, it is possible that tryptophan metabolism is unbalanced via TPH leading to excess 5-HTP and serotonin.

On the other hand, a large FC increase in 5-HTP may also suggest that there is reduced

serotonin production possibly as a result of a reduction in activity of AAAD, which converts oxitriptan to serotonin (Gao et al., 2020; Barik, 2020). It is also worthwhile to note that AAAD deficiency has also been associated with behaviours consistent with ASD (Blau et al., n.d.). There are also further potential downstream consequences such as a reduction in melatonin production, which is catalyzed by ASMT from serotonin (Fanciulli et al., 2020; Roth et al., 2021). Reduced production of both serotonin and melatonin may in part explain several symptoms of ASD such as mood issues, insomnia and sleep disorders. Specifically, a systematic review reported that four studies have noted a link between decreased melatonin levels and the intensity of the above behaviors associated with autism (D. A. Rossignol and Frye, 2011). Additionally, twenty clinical investigations have documented enhancements in sleep patterns with the use of exogenous melatonin supplements in ASD. These improvements consist of extended sleep time, fewer disturbances during the night, faster initiation of sleep, and improved daytime behaviours. (D. A. Rossignol and Frye, 2011).

Furthermore, in our results, 5-methoxytryptophan (5-MTP) was also significantly increased in the VPA-treated samples (**Figure 4.19** and **Figure 4.22**). 5-MTP, a metabolite of tryptophan via the enzymatic activity of HIOMT on 5-HTP (Cho and Cowling, 2021), is involved in protection against systemic inflammation (C.-H. Chen et al., 2019). Nonetheless, while serotonin cannot cross the blood brain barrier, tryptophan can traverse to act as a precursor centrally (Barik, 2020). However, both 5-MTP and 5-HTP, metabolites of tryptophan, were greatly upregulated in VPA-treated samples. This latter observation leads to the possibility that there is a dysregulation of serotonin production occurring at 5-HTP. Indeed, it is also likely that 5-HTP produced from tryptophan is getting redirected into 5-MTP production instead of serotonin thereby creating a bottleneck for serotonin production in tryptophan metabolism.

Another critical aspect in tryptophan metabolism is the metabolism of tryptophan to niacin conducted in the liver via the kynurenine pathway (Barik, 2020; Gao et al., 2020). It is well known that hepatic VPA metabolism can cause hepatotoxicity (Dreifuss et al., 1987). During gut morphological analyses, it was observed that the zebrafish liver in VPA fish was displaced and deformed. Given the role of the liver in the kynurenine pathway and the observed liver damage in the zebrafish, it is therefore possible that VPA-induced liver damage in the zebrafish may have created issues in tryptophan metabolism to niacin. Previously, VPA treatments have even been associated with nicotinic acid deficiency (Gillman and Sandyk, 1984).

In this regard, our results also highlighted high impact of VPA on nicotinate and nicotinamide as well as tryptophan patwhays in the zebrafish (**Figure 4.29**). This is also critical as niacin has been found to be involved in gene expression, cell cycle progression, DNA repair and cell death and specifically in neuronal development and survival in the central nervous system (Gasperi et al., 2019). Further, there has also been research on the role of niacin in neurodegenerative diseases such as Alzheimer's Disease as well as in other neuropathological conditions like traumatic injuries and neuropsychiatric disorders (Gasperi et al., 2019). Additionally, there has been mounting evidence on the role of the gut microbiota as a key factor in tryptophan metabolism. It is suggested that the microbes of mammalian gut can modulate tryptophan metabolism via both the serotonin and kynurenine pathways (Gao et al., 2020; Roth et al., 2021). Moreover, in a mouse model, researchers found that microbiota-related changes in tryptophan metabolism is associated with gastrointestinal dysfunction and autistic-like deficits in social interaction (Golubeva et al., 2017). With regards to the zebrafish data, similar observations can be made. Firstly, the VPA-treated zebrafish also displayed severe gastrointestinal malformations pointing to

likely gastrointestinal dysfunction. Secondly, metabolomics data displayed upregulated tryptophan metabolites 5-HTP and 5-MTP highlighting imbalances in tryptophan metabolism. Finally, the VPA-zebrafish also displayed autistic-like deficits in behaviour which was measured as anxious behaviour in response to stress. In comparison though, the cocoa-treated VPA-treated fish had normal gut structure, control-like metabolite levels, and similar locomotor activity to control fish in response to stress. Overall, the current findings in metabolomics and gut morphology surrounding tryptophan metabolism, liver health, and gut health create an interesting cross-talk linking several of our results to findings in the literature.

5.4 Pathway Analysis

5.4.1 Impact on Protein Synthesis and Amino Acids

Overall, numerous pathways in protein synthesis and amino acids are affected in the VPA model. Accordingly, the data does not point explicitly to a single biological system or pathway involved in the physiological and behavioural alterations of ASD. Nevertheless, taken together, the results indicate that dysregulated amino acids appear to be a critical feature in a systems biology framework of ASD (**Figure 4.30**). In that regard, neurotransmitter synthesis and signalling are likely affected, specifically related to SNAP-25, serotonin, melatonin, and glutamate.

5.4.2 Impact on Niacin Metabolism

Finally, based on pathway enrichment, metabolism of vitamin B3 niacin may also have been significantly impacted. Firstly, nicotinate (niacin) and nicotinamide metabolism were identified to be impacted through the identification of NAD, 6-hydroxynicotinic acid, nicotinamide, and succinic acid (**Figure 4.29** and **Figure 4.30**). The possible role of niacin in the context of tryptophan metabolism was highlighted above and its significance in pathway enrichment is further evidence of the possibility that VPA-induced hepatotoxicity is dysregulating niacin production in the liver. However, this pathway also highlights its importance in energy metabolism which may also be impacted. Indeed, nicotinate and nicotinamide are precursors for the biosynthesis of NAD and NADP (**Figure 5.1**)(Kirkland and Meyer-Ficca, 2018). These coenzymes are essential for a plethora of redox reactions in the cell. NAD is crucial for glycolysis, the citric acid cycle, and the electron transport chain, all of which are key pathways in cellular energy production (Kirkland and Meyer-Ficca, 2018). Moreover, while NAD is used in the breakdown (β-oxidation) of fatty acids for energy, NADP is involved in the synthesis of fatty acids (Kirkland and Meyer-Ficca, 2018). Research indicates that factors such as oxidative stress, weakened antioxidant defense, mitochondrial dysfunction, and disrupted energy metabolism play significant roles in the onset of ASD (M. M. Essa et al., 2013). For example, studies in mice and *C. elegans* have highlighted the interaction between genes and oxidative stress as core links in the pathogenesis of ASD (M. M. Essa et al., 2013). Furthermore, human studies have revealed significant differences in mitochondrial function and energy metabolism between healthy subjects and those with ASD where the latter exhibited elevated ratios of oxidized NADH to reduced NADH; reduced plasma ATP leading to

decreased NADPH and plasma tryptophan; and increased GSSG:GSH ratio (M. M. Essa et al., 2013). These core pathways are all connected to tryptophan metabolism which may indicate its disruption plays a central role in the neurotoxic outcomes in ASD (M. Essa et al., 2013; M. M. Essa et al., 2013).

5.4.3 Impact on Energy Metabolism

The pathway enrichment data (**Figure 4.30**) indicated possible disturbances in energy metabolism by VPA mediated through disruptions on pantothenate and CoA biosynthesis. In a recent prospective cohort study, researchers found that elevated cord pantothenate in combination with elevated cord cysteine is associated with increased risk of ASD (Raghavan et al., 2023). In the present study, we observed elevated pantothenate in the metabolomics data in the VPA treatment group. Moreover, while cysteine was not significantly elevated, cystathionine, an intermediate in the biosynthesis cysteine, was increased in the VPA treatment group. The consistent findings on the impact of pantothenate in both human and animal ASD therefore strongly suggests that energy metabolism is critical in the pathogenesis of ASD. There have also been studies which have pointed out that subjects with ASD may have altered mitochondrial function (D. Rossignol and Frye, 2012) as well as increased oxidative stress due a reduction in redox capacities specifically measured in glutathione (Bjørklund et al., 2020). Niacin metabolism is also linked to glutathione and energy metabolism through NADPH which is produced from NADP and plays a fundamental role in the regeneration of reduced glutathione (GSH) (Kirkland and Meyer-Ficca, 2018). In this study, glutathione was slightly impacted, however, the results were not statistically significant. Nonetheless, CoA derived from pantothenate is essential for both glycolysis and the citric acid cycle, converting pyruvate

into acetyl-CoA (Shi and Tu, 2015). Any disruptions in CoA biosynthesis could impact glycolysis and subsequent metabolic pathways.

In view of the present thesis findings, it appears that prebiotic cocoa polyphenols have potential as therapeutic and preventative approaches for ASD. In that regard, it is pertinent that gut dysbiosis is a predominant comorbidity that has been highlighted to not only be a cause of ASD but also contribute to the worsening of ASD symptoms (Pulikkan et al., 2019). In that context, there are various genetic bases for ASD that can interact with environmental factors such as microbiome and/or exposure to chemicals like VPA and thalidomide. Despite characterization of the above interactions with respect to the gut-microbiome-brain axis (GMBA), little success has been found in establishing reliable therapeutics. The present thesis shows that the VPA model of ASD was associated with multifaceted biological disturbances ranging from dysregulated protein synthesis and amino acid metabolism to energy metabolism and we have shown that a cocoa polyphenol-based treatment may recover many of these pathways. While there are more questions to explore on the specific implications of protein synthesis and amino acid metabolism and energy metabolism, it is evident that a nutrition-based approach may potentially present a multi-pronged solution to the symptoms of ASD. Indeed, the successful application of the high throughput zebrafish ASD model in this thesis to show protective effects of a cocoa polyphenol-based treatment has demonstrated a novel approach for assessing nutrition-based treatments for ASD.

Despite the successes of this research there remain several limitations as well. Firstly, the model employed in this study was chemically induced. While environmental factors like VPA play a significant role in the pathogenesis of ASD, genetic factors are also critical. To more accurately replicate ASD conditions, there should also be considerable research done in a

similar framework on genetic models such as *SHANK3* and *CHD8*. Secondly, while our data was able to highlight proteins, metabolites, and pathways that were impacted in the VPA model, additional attention should be accorded to studying these systems in greater detail such as the role of 5-MTP and tryptophan in serotonin pathways. Also, despite identifying that protein synthesis, amino acid metabolism, and energy metabolism were impacted in this VPA autism zebrafish model, metabolomics and proteomics with other omics data such as transcriptomics, lipidomics, and microbiomics could expand the scope of the results to provide more mechanistic details.

Conclusion and Future Research

6.1 Summary and Conclusions

In conclusion, this thesis has provided compelling evidence that cocoa powder can improve not only behavioural symptoms of ASD but also gut morphology in a VPA-induced zebrafish model of autism. Specifically, the cocoa treatment was able to impart an anxiolytic effect on autistic zebrafish while supporting the development of healthy control-like gut morphology, which was otherwise deformed in untreated VPA zebrafish. While a deformed and dysbiotic gut may cause dysregulated neurotransmitter production like serotonin and vitamins like niacin (Roth et al., 2021; Gao et al., 2020), there may also be metabolic factors such as tryptophan dysregulation that may be worsening the gut dysbiosis as reported in an autism mouse model by Golubeva et al., 2017. In fact, bacterial-related tryptophan metabolism directs immunoregulatory responses through the production of aryl hydrocarbon receptor (AhR) ligands or short-chain fatty acids which benefit gut health by improving gut barrier integrity, glucose, and lipid metabolism, regulating the immune system, the inflammatory response, and blood pressure (S.-C. Choi et al., 2020; Nogal et al., 2021). Therefore, tryptophan dysregulation might not only reduce these beneficial effects, but tryptophan deficiency has also been observed to cause increased inflammation and altered gut bacterial composition (Yusufu et al., 2021). It is noteworthy that the cocoa-treated autistic zebrafish demonstrated similar metabolic and proteomic profiles to healthy control zebrafish. These results are indicative of a

complex interaction between gut health, metabolism, protein expression, and ASD symptomatology mediated via the cocoa polyphenol treatment. Indeed the cocoa powder may help through its antioxidant and anti-inflammatory properties (Katz et al., 2011) which can help with the reduction of oxidative stress due to the metabolism of VPA (Ghodke-Puranik et al., 2013). Excess reactive oxygen species (ROS) from the metabolism of VPA can cause both hepatoxicity and neurotoxicity through, for example, mitochondrial dysfunction, issues with protein expression, and neurotransmitter dysfunction (Ghodke-Puranik et al., 2013). In addition, SNAP25 protein expression was significantly reduced in autistic zebrafish which aligns with novel up-to-date research on ASD in humans and other animal models (Guerini et al., 2011; Braida et al., 2015; Lenart et al., 2020; Antonucci et al., 2016). However, there lacks a clear understanding of the exact role of SNAP25 in the neurology of ASD as well as how a polyphenol-rich extract cocoa powder was able in our research to recover protein expression to healthy control levels. Identifying these novel factors within this framework is critical but remains only a first step. Understanding how these factors interact to create this complex pathophysiology of ASD is ongoing. Yet what is critical is that there needs to be more validated models of ASD, greater research into potential treatments, and a better more mechanistic and systemic understanding of the disorder.

6.2 Directions for Future Research

Future directions for this research should focus on identifying and quantifying gut microbial differences between autistic and healthy zebrafish using a variety of ASD models. Indeed, while VPA is a chemically-induced model that is relevant to human disease, there

are also established and less established genetic models that are worth investigating within the same framework such as *SHANK3*, *CHD8*, or even *MECP2*. Furthermore, there should be an expansion on the scope of omics included in this project. There should be consideration given to transcriptomics, lipidomics, and microbiomics as well to provide a more complete picture on the differences in omics profile between healthy controls, autistic zebrafish, and autistic zebrafish treated with cocoa powder. Additionally, more specific treatments can be administered such as tryptophan pathway enzyme modulators to observe whether there are any downstream differences. For example, production of serotonin could be induced to shunt 5-MTP away from 5-HTP by saturating with aromatic amino acid decarboxylase (AAAD). Otherwise, an attempt could be made to overcome the rate limiting step of serotonin synthesis, which involves TPH-mediated conversion of tryptophan to 5-HTP (Faria et al., 2021), by increasing L-tryptophan bioavailability through supplementation; directly supplementing 5-HTP; increasing the activity of TPH or inhibiting the activity of competing pathways such as the kynurenine pathway. Finally, to test the relationship between gut health and tryptophan metabolism, an experiment could be conducted to assess tryptophan deficiency by supplementing zebrafish with standardized diets of low, normal, and high tryptophan feed in both healthy and ASD zebrafish. These tests would help narrow down possible mechanisms involved. Also, the bioactive components in the cocoa powder apart from (-)-epicatechin need to be identified, such as procyanidins, anthocyanins, and flavonol glycosides (Rimbach et al., 2009) that may be benefitting the behaviour of the autistic zebrafish. Finally, given that the cocoa powder is a prebiotic, there is also potential in expanding the scope of autism treatment by combining it with probiotics to create a dynamic synbiotic with the framework of microbiomics to optimize gut-brain health benefits.

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