Effects of Cocoa Supplementation on Aging and Amyloid-β-induced Deficits in Alzheimer's Disease; Studies with *C. elegans*

Submitted by

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Abstract

Aging is the predominant risk factor for most well-recognized pathologies that limit healthspan. Diet is a key modifiable risk factor in most aging-related diseases, in particular diets rich in polyphenols. Cocoa, one of the richest dietary sources of polyphenols has been known to contribute significantly to the total antioxidant capacity of American and European diets. Even though the health-promoting effects of cocoa have been explored in various models, most of these studies are either short-term interventions or acute effects. In this study, a series of experiments was conducted to determine how long-term consumption of cocoa affects aging and Aβ-induced deficits in Alzheimer's disease using *C. elegans* as the model organism. Cocoa improved age-associated deficits in neuromuscular function as shown by improved locomotory parameters. In addition, cocoa ameliorated the age-associated decline in cognition, particularly learning and short-term memory loss. Supplementation of cocoa extended the lifespan of worms while increasing the thermotolerance at all stages of life (young, middle, and old). Cocoa supplementation starting from the first larval stage (L1) was critical and essential for the longevity-extending effects of cocoa but did not need to be continued in a long-term manner. The longevity improving effects of cocoa were mediated through the insulin/insulin-like growth factor-1 signaling pathway and mitochondrial respiration. The results showed that pan-neuronal expression of Aβ induced a reduced growth, a reduced maximum speed at old age, short-term memory deficits at middle age, and a reduced lifespan. Long-term cocoa-supplementation reversed the deficits in growth, maximum speed, short-term memory loss, and lifespan to reach similar levels to control counterparts while reducing the Aβ fibril levels. Lastly, the metabolomic changes associated with aging and with the expression of pan-neuronal Aβ in *C. elegans* and the effects of long-term cocoa supplementation were explored using gas chromatography-mass spectrometry. Pan-neuronal expression of Aβ resulted in significantly increased hypoxanthine and ornithine levels showing alterations in purine metabolism and urea cycle respectively. Cocoa seemed to interact with these metabolites to reverse the Aβ-induced alterations. Thus, bioactive compounds in cocoa appear as promising biomolecules in developing effective treatment regimens for age-related diseases.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Genetically modified *C. elegans* were maintained and all the relevant experiments were carried out with the approval of the La Trobe University Institute of Biosafety Committee (LTIBC), reference number GMSC15-017.

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I hereby certify that Munagamage Dona Mihiri Munasinghe is the first author for this publication, and she contributed greater than 75% of the publication by planning, writing, and revising the review article.

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Publications, Presentations, and Awards

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- **Munasinghe, M**., Almotayri, A., Thomas, J., Heydarian, D., & Jois, M. (2021). Early Exposure is Necessary for the Lifespan Extension Effects of Cocoa in *C. elegans*. *Nutrition and Metabolic Insights*, 14, 11786388211029443. doi:10.1177/11786388211029443 (published).
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- Almotayri, A., Thomas, J., **Munasinghe, M.**, Weerasinghe, M., Heydarian, D., & Jois, M. (2021). Metabolic and Behavioural Effects of Olanzapine and Fluoxetine on the Model Organism *C. elegans*. Saudi Pharmaceutical Journal. doi:https://doi.org/10.1016/j.jsps.2021.07.006 (published).

Conference presentations

- Poster presentation at 22nd International *C. elegans* Conference, University of California, Los Angeles in 2019 on "Cocoa reduces amyloid-β (Aβ) toxicity and increases lifespan in a transgenic *C. elegans*".
- Poster presentation at 34th Virtual International Conference of Alzheimer's Disease International in 2020 on "Long-term consumption of cocoa prevents dementia induced by amyloid-β_{1-42}($A\beta_{1-42}$) in *C. elegans*".

Symposiums

- Poster presentation and "3 min talk" at PAM Research Symposium 2019 held by PAM HDR Student Society, Department of Physiology, Anatomy, and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Victoria, Australia.

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Awards

- 22nd International *C. elegans* Conference travel award 2019, Genetics Society of America (USD 500).

List of Abbreviations

n

(4’-OH)-PVL - 5-(4’-hydroxyphenyl)-γ valerolactone

A

AAs - amino acids

Aβ - amyloid beta

ABTS - [2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)]

ACh- acetylcholine

AChE - acetylcholinesterase

AD - Alzheimer’s disease

ADP - adenosine diphosphate

APOE - apolipoprotein E

APP - amyloid precursor protein

Akt - protein kinase B

AKT-2 - *C. elegans* ortholog of human AKT2 (AKT serine/threonine kinase 2)

AMP - adenosine monophosphate

APRT - adenine phosphoribosyltransferase

ASS - argininosuccinate synthetase

ASL - argininosuccinate lyase

ATP - adenosine triphosphate

B

BACE1 - β-secretase 1

BBB – the blood-brain barrier

BCA - bicinchoninic acid

BDNF - brain-derived neurotrophic factor

BP - blood pressure

BSTFA - N,O-bis-(trimethylsilyl)trifluoroacetamide

C

CA1 - Cornu Ammonis 1

CE - catechin/ catechin equivalents

CFs - cocoa flavanols

CGC - *Caenorhabditis* genetics center

CI - chemotaxis index

C. elegans - *Caenorhabditis elegans*

CR - calorie restriction

CREB - cAMP response element-binding protein

D

DAF-2 - insulin-like growth factor 1 (IGF-1) receptor gene (*C. elegans*)

DAF-16 - sole ortholog of the FOXO (Forkhead box protein O) family of transcription factors in *C. elegans*

DBP - diastolic blood pressure

DG - dentate gyrus

DNA - deoxyribonucleic acid

DST - disposable soma theory

E

EC - epicatechin

E. coli - *Escherichia coli*

EMPs - endothelial microparticles

eNOS - endothelial nitric oxide synthase

EPM - elevated plus maze

EtOH - ethanol

ETC - electron transportation chain

F

fMRI - functional magnetic resonance imaging

FAICAR - 5-formamidoimidazole-4-carboxamide ribotide

FMD – flow-mediated vasodilation

FoxO - class O of forkhead box transcription factors

FOXO1A - the forkhead box O1A
FOXO5 - an ortholog of mammalian FOXO3 (Forkhead box transcription factors of the class O3) found only in fish
FRTA - free radical theory of aging
FUdR - Floxuridine

G
G-3P - glycerol-3-phosphate
GAE - gallic acid equivalents
GC-MS - gas chromatography/Mass spectrometry
GFP - Green fluorescent protein
GMP - guanosine monophosphate
GSH - glutathione reductase
GSK-3 - glycogen synthase kinase 3
GSSG - glutathione oxidized form

H
HF - high flavanol
HGM - high growth media
HIF - hypoxia-inducible factor
HNE - 4-Hydroxynonenal
H_2O_2 - hydrogen peroxide
HPRT - hypoxanthine-guanine phosphoribosyltransferase
Hst3 - yeast SIRT6 (sirtuin 6) homolog

I
IF - intermediate flavanol
IGF-1 – insulin-like growth factor-1
IIS - insulin/insulin-like growth factor-1 signaling
IL-1β - interleukin 6
IMP - inosine monophosphate

J
JNK - c-Jun N-terminal kinase

L
LB - Luria-Bertani
LBF - leg blood flow
LDH - lactate dehydrogenase
LF - low flavanol
LI - learning index
LOAD - late-onset Alzheimer's disease
LTP - long-term potentiation

M
MBV - microvascular blood volume
MD - Mediterranean diet
MEF2A - myocyte enhancer factor 2A
MFRTA - mitochondrial free radical theory of aging
mkk4 - mitogen-activated protein kinase kinase 4
MMSE - mini-mental state examination
MPP^+ - 1-methyl-4-phenyl-1,2,3,6- pyridine
mRNA - messenger RNA
mt DNA - mitochondrial deoxyribonucleic acid
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MURF1 - muscle ring finger 1
Myf5 - myogenic factor 5
MyoD - Myoblast determination protein 1

N
NAD - nicotinamide adenine dinucleotide
NaN_3 - sodium azide
NCDs - non-communicable diseases
NDDs - neurodegenerative diseases
NFTs - neurofibrillary tangles
NF-κB - nuclear factor-κB
NGM - nematode growth medium

NHR-8 - nuclear hormone receptor-8 (a homolog of vertebrate liver-X and vitamin-D receptors in *C. elegans*)
NO - nitric oxide
NOS - nitric oxide synthase
Nrf1/Nrf2 - NF-E2 related factor 1/2

O
•OH - hydroxyl radical
$^1O_2$ - singlet oxygen
$O_2^{\bullet-}$ - superoxide anion
O. latipes - *Oryzias latipes*
OAT - ornithine aminotransferase
OCR - oxygen consumption rate
ODC - ornithine decarboxylase
OF - open field
OS - oxidative stress
OTC - ornithine transcarbamylase

P
p38 MAPK - p38 mitogen-activated protein kinase
p70S6 kinase - ribosomal protein S6 kinase
PGC-1$_\alpha$ - PPARγ coativator-1a
PKC - protein kinase C
PP - pulse pressure
PPAR - peroxisome proliferator-activated receptor
PPs - polyphenols
PR - procyanidins
PR B2 - procyanidin B2
PRPP - phosphoribosyl pyrophosphate
PSEN1 - presenilin 1
PSEN2 - presenilin 2
PUFA - polyunsaturated fatty acids
PVLs - phenyl-γ-valerolactones
PWA - pulse wave amplitude
PWV - pulse wave velocity

R
R5P - ribose 5-phosphate
RCT - randomized controlled trial
ROS - reactive oxygen species

S
SA-β-Gal - senescence-associated β-galactosidase
SBP - systolic blood pressure
S. cerevisiae - *Saccharomyces cerevisiae*
SEM - standard error of the mean
SIR2 - silent information regulator 2
SIR 2.1 - *C. elegans* ortholog of mammalian sirtuin 1
SIRT1 - sirtuin 1
SkM - skeletal muscle
SKN-1 - skinhead-1
SOD - superoxide dismutase
SOL - soleus
SSVEP - steady-state visually evoked potentials

T
tBHP - tert-butylhydroperoxide
TF - total flavonoids
Th T - thioflavin-T
TMS - trimethyl silyl
TMT - trail making test
TNF-α - tumour necrosis factor-α
TOR – the target of rapamycin
TP - total phenolics

V
VFT - verbal fluency test

X
XOR - xanthine oxidoreductase

CHAPTER 1: Literature Review

Most of the content of this chapter has been written as a review article titled "Effects of cocoa flavanols on aging and amyloid-β-induced deficits in Alzheimer's disease: A comprehensive review" and is currently under the review of "Human Nutrition and Metabolism" journal (Elsevier).

1.1. Introduction

Aging is the time-dependent loss of physiological integrity and the predominant risk factor for most diseases and conditions that limit healthspan (Franceschi et al., 2018). In mammals, age-related degeneration is known to cause well-recognized pathologies such as sarcopenia, atherosclerosis and heart failure, neurodegeneration, and many others (Campisi, 2013). Prolonged longevity due to advances in medicine and socioeconomic development together with declining fertility has shifted the world population demographics remarkably towards a progressive increase in the older population, increasing the prevalence of age-related debilitating diseases ("Ageing well: a global priority," 2012).

Aging is thought to be a multifactorial process that involves many cellular and molecular mechanisms (MacNee et al., 2014). The free radical theory of aging which has been one of the most widely tested concepts of aging suggests that the accumulation of reactive oxygen species (ROS) induced oxidative damage is the cause of aging (Harman, 1956). When the natural anti-oxidative defense of the body cannot balance ROS, oxidative damage occurs, leading primarily to mitochondrial DNA damage and dysfunction (Schöttker et al., 2015). Alzheimer's disease (AD), the most common and devastating form of dementia in the elderly is one such disease, where oxidative stress (OS) plays a key role (Tönnies & Trushina, 2017). To date, there is no pharmaceutical intervention that has been shown to cure AD or halt the disease progression.

Diet has been reported to play a key role in modifying aging (McCay et al., 1935; Weindruch & Sohal, 1997) and the development of age-related diseases (Gaziano, 2004; Hertog et al., 1993; Miquel et al., 2002; Sacks et al., 2006; Taubert et al., 2003). The Mediterranean diet (MD), one of the most widely studied dietary patterns has been reported to be associated with higher longevity and delays the onset of the deterioration in health (Haveman-Nies et al., 2003; Kouris-Blazos et al., 1999). MD has beneficial effects on chronic diseases such as cardiovascular disease (Ciccarone et al., 2003), cancer (Bosetti et al., 2003), and AD (Scarmeas et al., 2006). The MD is comprised of abundant plant foods (fruits, vegetables, bread, other forms of cereals, pulses, nuts, and seeds). Polyphenols (PPs) in plant-based foods which act through different mechanisms play a key role in protecting against aging and related diseases (Hügel & Jackson, 2015).

Cocoa, one of the most popular food products rich in PPs is a key contributor to the total antioxidant capacity of European and American diets (Vinson et al., 2006). Therefore, this study aimed to investigate how long-term cocoa supplementation specifically affects age-associated health, lifespan, and amyloid-β (Aβ)-induced behavioral deficits, particularly in learning and memory

impairments. Moreover, we determined the effects of cocoa consumption on aging and Aβ-induced deficits at the metabolite level. Further, we investigated how critical the effect of the timing of cocoa exposure for its lifespan extension effects and the mechanisms and pathways behind lifespan extension. For all these studies, we employed *Caenorhabditis elegans* (*C. elegans*) as the model organism due to its attractive features including short lifespan, availability of mutants, and many readily observable and quantifiable aging-associated changes.

1.2. The process of aging; theories and mechanisms

Over the past couple of centuries, many theories have been proposed to explain the process of aging but, none of them appears to be fully satisfactory (Davidovic et al., 2010). Programmed theories of aging suggest that aging follows a biological timetable, possibly a continuation of the one that regulates childhood growth and development (Jin, 2010). Certain pacemakers at the organ system level or cellular level govern the aging process according to these programmed theories (Kamel et al., 2000). Finite cell division theory explains the mechanisms behind cellular aging where Leonard Hayflick (Hayflick & Moorhead, 1961) demonstrated that human diploid fibroblasts grown in culture have a finite lifespan, a phenomenon known as "Hayflick limit". Further, this theory states that a normal human cell can only replicate and divide forty to sixty times before it cannot divide anymore, and will break down by programmed cell death or apoptosis (Bartlett, 2014).

Wear and tear theory states the idea that as machines become damaged and break down after being used for a certain period, the human body also ages due to damage from accidents, diseases, radiation, toxic substances, food, and many other harmful substances when it is utilized for a long time (Park & Yeo, 2013). The rate of living theory suggests that the lifespan of an organism is inversely proportional to its metabolic rate (Kamel et al., 2000). Observations like larger animals with lower metabolic rates live longer than smaller animals with higher metabolic rates, and lifespan extension of these lower species when they are raised in cooler temperatures supports this theory (Clarke & Smith, 1961). However, these observations are not universal and there is evidence to show that metabolic rates are poorly correlated with longevity (Lints, 1989).

According to the cross-linkage/glycosylation theory, aging is caused by the progressive linking together of large vital molecules (Bjorksten, 1968). Over time, these cross-links alter the biological and chemical properties of cells and translate into significant dysfunction of body systems which result in aging (Bjorksten & Tenhu, 1990). Protein synthesis error catastrophe theory of aging (Orgel, 1963) postulates that transcriptional and/or translational errors in the synthesis of proteins that were themselves used for the synthesis of proteins could result in an exponential cascade of errors involving essentially all proteins. Accumulation of these errors eventually reaches a catastrophic level, leading to cell and organismal death (Oshima et al., 2019).

Telomere theory of aging provides another explanation for the aging process (Blackburn & Gall, 1978). Telomeres are specific DNA-protein structures found at both ends of chromosomes which play a vital role in preserving the information in our genome by protecting the genome from

nucleolytic degradation, unnecessary recombination, repair, and interchromosomal fusion. With each cell division, a small portion of telomeric DNA is lost and when the length of the telomere reaches a critical limit, the cell undergoes aging (Shammas, 2011).

DNA is susceptible to damage by exogenous as well as endogenous threats and these damages are often not perfectly repaired (López-Otín et al., 2013). The DNA damage/repair theory of aging states that this unrepaired DNA damage contributes to genomic instability and the aging process (Maynard et al., 2015).

Free radical theory of aging (FRTA)/OS theory was the most popular concept in the area of aging for many years (Harman, 1956). This theory proposes that organisms age due to the accumulation of oxidative damage to cellular macromolecules (carbohydrates, lipids, proteins, and DNA) induced by free radicals. The FRTA was extended in 1972 (Harman, 1972) with the suggestion that most of the free radical reactions (FRRs) are initiated by mitochondria at an increasing rate with age and the lifespan is determined by the rate of free radical damage to the mitochondria (Harman, 2003). The mitochondrial free radical theory of aging (MFRTA) which was posited later (Miquel et al., 1980) described mitochondria as the source of free radicals and mitochondrial deoxyribonucleic acid (mt DNA) as a critical target to explain the age-associated damage in cells (Viña et al., 2013). The idea of a single cause of aging such as a single gene or a key body system has been later replaced by the idea that aging is an extremely complex, multifactorial process that involves interactions among many different mechanisms (Kowald & Kirkwood, 1996). Therefore, these different theories of aging should not be considered mutually exclusive, but might be complementary of others in explaining some or all the aspects of the aging process (Weinert & Timiras, 2003). There are recently described nine basic molecular changes or hallmarks of aging which include four primary hallmarks, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis, three antagonistic hall-marks, deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence, and two integrative hallmarks stem cell exhaustion, and altered intercellular communication (López-Otín et al., 2013).

1.3. The role of oxidative stress in aging and related diseases

OS has been defined as the imbalance between the production and accumulation of ROS in cells and tissues and the ability of a biological system to detoxify them (Pizzino et al., 2017). Free radicals (any chemical species that contains unpaired electrons), the metabolic by-products such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) are commonly defined as ROS. Even though there are several intracellular sources of free radicals, mitochondria are believed to be the most contributory (Albers & Beal, 2000). It has been shown that there is an age-associated increase in the generation of oxidants by mitochondria (Sastre et al., 1996; Sohal & Sohal, 1991). This enhanced generation of oxidants by aged-mitochondria may itself cause damage to the mitochondrial membranes and proteins, starting a vicious cycle of mitochondrial oxidant damage and oxidant generation (Pollack & Leeuwenburgh, 2000). Moreover,

the increased mitochondrial ROS production may cause mt DNA mutations, eventually leading to a positive feedback loop of more ROS and more mt DNA mutations (Linnane et al., 1989).

OS is involved in many aging-associated acute and chronic pathological processes (Fig. 1.1) according to a compelling body of evidence. OS-induced endothelial dysfunction plays a central role in the pathogenesis of a broad spectrum of human diseases including peripheral vascular disease (Gokce et al., 2002), stroke (Cosentino et al., 2001), hypertension and, atherosclerosis (Davignon & Ganz, 2004; Panza et al., 1990), diabetes (Avogaro et al., 2011) and cancer (Franses et al., 2013). In humans, besides merely providing a lining for vessel walls, the endothelium is involved in many important functions including control of thrombosis and thrombolysis, platelet and leukocyte interaction with the vessel wall, and the regulation of vascular tone and growth of blood vessels (Verhamme & Hoylaerts, 2006). Endothelial dysfunction shifts the actions of the endothelium towards reduced vasodilation, a proinflammatory state, and prothrombotic properties (Rajendran et al., 2013). In endothelial dysfunction, the bioavailability and efficacy of a key molecular mediator of normal endothelial function which is nitric oxide (NO) is decreased, largely owing to an increase in ROS (Loscalzo, 2002). In diabetes, increased ROS production deteriorates the islets β-cells and pancreas reducing the release of insulin (Evans-Molina et al., 2013). Moreover, ROS can activate various signaling pathways such as NF-κB (nuclear factor-κB) and PKC (protein kinase C) which interfere with insulin signaling pathways, leading to developing insulin resistance (Kaneto et al., 2002; Scivittaro et al., 2000). The onset of cancer in humans is also known to induce by oxidative DNA damage (Dreher & Junod, 1996; Marnett, 2000). Even though the specific mechanism for how oxidative damage contributes to carcinogenesis is largely unknown, at least two mechanisms are thought to play a role. The first is modulation of gene expression and the second is radical-induced genetic alterations such as mutations and chromosomal rearrangements (Valko et al., 2004). Mounting evidence suggests that OS may play a critical role in neurodegenerative diseases (NDDs) like AD (Bonda et al., 2010; W.-J. Huang et al., 2016), Huntington's disease (Covarrubias-Pinto et al., 2015; Reagan et al., 2000), Parkinson's disease (Dias et al., 2013; Perfeito et al., 2012) and amyotrophic lateral sclerosis (D'Amico et al., 2013). The higher energy demand, lower rate of cellular renewal, membrane PUFA levels, as well as less active oxidative defense mechanisms in neurological systems make them extremely sensitive and vulnerable to ROS-induced damage, contributing to the onset of these NDDs (Rego & Oliveira, 2003).

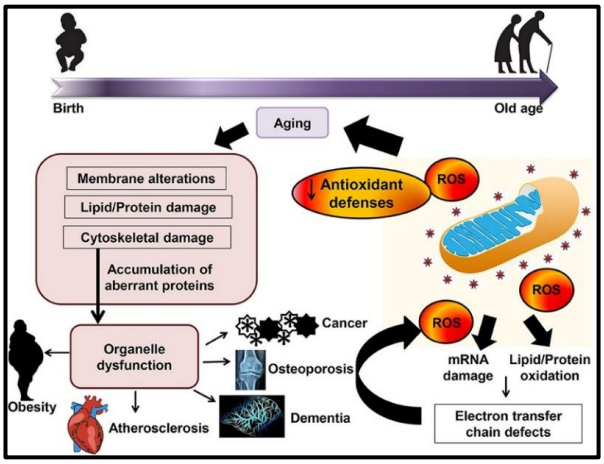

Figure 1.1. ROS-induced damage in aging and related diseases, adapted from (Tan et al., 2018). Age-related increased production of ROS in mitochondria leads to mRNA damage and lipid/protein oxidation which ultimately results in decreased mitochondrial function and more OS contributing to age-related diseases.

1.4. Alzheimer's disease and amyloid-β cascade hypothesis

AD is a progressive neurodegenerative disorder that is accountable for around two-thirds of dementia cases worldwide (Drew, 2018). The prevalence of AD increases with age and affects 10% of people over the age of 65 and about 50% of people over the age of 85 (Zvěřová, 2019). AD leads to a progressive loss of mental, behavioral, functional decline and ability to learn (Kumar et al., 2015). Late-onset AD (LOAD), the most common form of the disease is thought to be driven by a complex interplay between genetic and environmental factors. Around 70% of the risk of LOAD is now believed to come from genetic factors, where apolipoprotein E (APOE) is considered to be the single biggest risk for LOAD (Lane et al., 2018). Dominantly inherited familial AD (FAD) which accounts for only 1% of all AD cases is caused by mutations in amyloid precursor protein (APP), presenilin 1 (PSEN1), or PSEN2 genes (DeTure & Dickson, 2019).

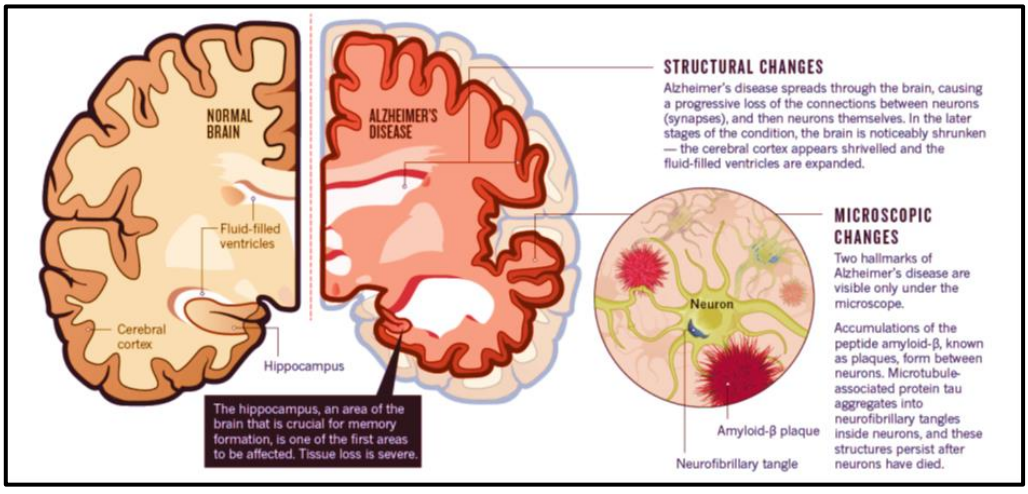

Figure 1.2. Structural and microscopic changes of the brain in AD, adapted from (Drew, 2018). Structural changes include the progressive loss of synapses and then neurons themselves. When the disease progresses, the shrinking of the brain occurs along with shriveling of the cerebral cortex

and expansion of the fluid-filled ventricles. Microscopic changes include extracellular accumulation of Aβ plaques and intracellular aggregation of neurofibrillary tangles (NFTs).

From its first introduction in 1992, the amyloid cascade hypothesis played a key role in explaining the etiology and pathogenesis of AD (Reitz, 2012). Senile plaques and NFTs were considered as the two pathological hallmarks of AD since the first description of presenile dementia by Alois Alzheimer (Newell et al., 1999). It was later identified that Aβ protein forms the plaque core in AD (Masters et al., 1985). Based on these findings, the amyloid cascade hypothesis of AD suggests that the overproduction and accumulation of Aβ peptide in the brain as plaques is the key pathological event of AD that triggers the subsequent neuropathological changes (Fig. 1.2) such as the formation of NFTs, neuronal cell death and dementia (Hardy & Higgins, 1992). Proteolytic cleavage of the APP results in Aβ which is a 4kDa, 39-43 amino acid peptide (Adeniji et al., 2017). APP is a member of a family of conserved type I membrane proteins (H. Zheng & Koo, 2011). Processing of APP occurs through two pathways namely (1) nonamyloidogenic or (2) amyloidogenic pathways (Fig. 1.3). In non-amyloidogenic processing, APP undergoes sequential proteolytic cleavage by α-secretase and γ-secretase to generate sAPPα. Alternatively, activation of β-secretase in the amyloidogenic pathway generates sAPPβ and Aβ (Y. W. Zhang et al., 2011). The length of the Aβ peptide varies according to the cleavage pattern and the $A\beta_{1-40}$ isoform is most prevalent followed by $A\beta_{1-42}$. $A\beta_{1-42}$ is hydrophobic and aggregates faster than $A\beta_{1-40}$ (Perl, 2010; Walsh & Selkoe, 2007). Aβ peptide in β-sheet conformation assembles and polymerizes into structurally different forms such as fibrillar, protofibers, and oligomers within plaques (Glenner et al., 1984; Selkoe, 1994). While the end-stage senile plaques are relatively inert, oligomers and fibrils appear to be the most neurotoxic species. Even though the production and clearance of Aβ are balanced in physiological conditions, pathological conditions such as increased production of total Aβ or increased $A\beta_{1-42}/A\beta_{1-40}$ ratio or decreased Aβ degradation/clearance led to elevated levels of $A\beta_{1-42}$. The mutations in three different genes that are responsible for familial AD (APP, PSEN1, and PSEN2) can elevate the production of more aggregatable AB_{1-42}. The decreased expression of enzymes responsible for Aβ clearance (insulin-degrading enzyme) appears as a cause for the decreased clearance of Aβ levels as found in sporadic AD. According to the Aβ cascade hypothesis, both these conditions lead to the Aβ accumulation, oligomerization, and plaque formation which further initiate a cascade of pathological events as aforementioned (Šalković-Petrišić, 2008).

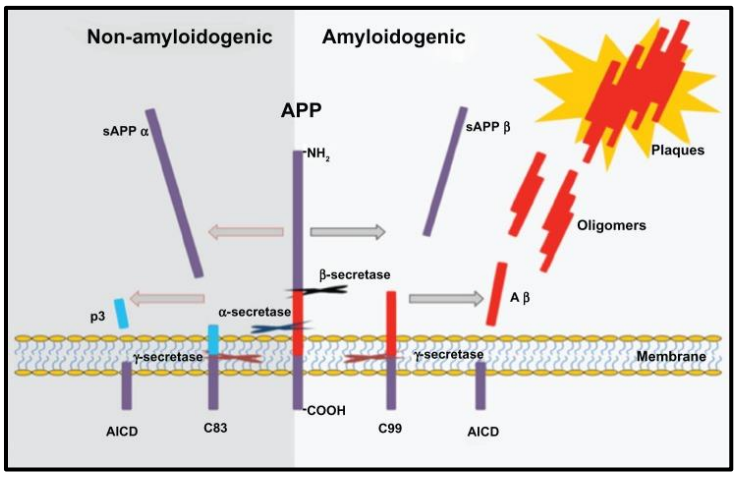

Figure 1.3. APP processing through the amyloidogenic pathway and non-amyloidogenic pathway, adapted from (S. Chen et al., 2013). Processing of APP by α-secretases forms sAPPα and p3 fragments which are non-toxic. APP processing by β-secretase 1 (BACE1) leads to the formation of neurotoxic sAPPβ and Aβ.

1.5. *C. elegans* as a model in aging research

C. elegans is a free-living nematode that feeds on microorganisms. It has a life cycle (from the egg to the egg-laying adult) of 3.5 days and a lifespan of about 3 weeks at 20 ℃ (J. Li & Le, 2013). Worms are 1 mm long at adulthood, sexually dimorphic and self-fertilizing hermaphrodites exist as the predominant form. The life cycle of *C. elegans* is comprised of an embryonic stage, four larval stages (L1-L4), and the adult stage (Corsi et al., 2015). The adult worm is composed of an invariant number of somatic cells. The worm possesses many of the tissues and organs (gastrointestinal tract, gonads, epidermis, nervous system, and muscles) present in more complex animals, but in a relatively simplified form (Jorgensen & Mango, 2002). *C. elegans* display many readily observable and quantifiable age-associated changes including tissue degeneration, decreased movement, accumulation of lipofuscin dark pigments, presence of vacuole-like structures, and cessation of reproduction (Olsen et al., 2006).

C. elegans has many convenient features that make it an attractive model in aging research. Most notable is its short and largely invariant lifespan which allows the identification of mutants that shorten or lengthen the average lifespan by as little as 10-15% but still be statistically significant. The lifespan assay of *C. elegans* is straightforward, allowing the assay of many worms in a single experiment. In addition, it is easy and cheap to maintain in the laboratory in large quantities. (Tissenbaum, 2015). Transparency of the body at all stages of life allows for anatomical observation by light and fluorescent microscopy. *C. elegans* has multiple distinct tissues that are involved in both worm and mammalian aging. Muscle function of worms declines with age, resembling human sarcopenia (Herndon et al., 2002). Aging-related neurodegeneration causes cognitive and behavioral impairments in worms (Link, 1995; Oeda et al., 2001). *C. elegans* show a high genetic homology (~83%) with humans and its genome possesses homologs of about two-thirds of all human disease genes (S. Zhang et al., 2020). The entire genome of *C. elegans* is sequenced and

annotated and an RNAi library comprising approximately 80% of the genes in the genome is available. Moreover, generating the transgenic strains is easy with *C. elegans*. These techniques have led to comprehensively surveying the worm genome. With these techniques, more than 200 genes and regimens that modulate lifespan have been identified and evolutionarily conserved pathways that modulate lifespan have been revealed in *C. elegans* (Tissenbaum, 2015). Therefore, *C. elegans* has become an invaluable model in aging research due to the combination of its short, invariant lifespan, easy assaying methods, plenty of genetic, molecular, and genomic tools, and evolutionary conservation.

1.6. Role of polyphenols in aging and related diseases

PPs are secondary metabolites of plants and are found largely in fruits, vegetables, cereals, and beverages (Pandey & Rizvi, 2009). These molecules are generally involved in defense against ultraviolet radiation or aggression by pathogens. More than 8000 polyphenolic compounds have been identified and these compounds are classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another (Manach et al., 2004). The main classes of PPs are phenolic acids, flavonoids, stilbenes, and lignans (Pandey & Rizvi, 2009). Figure 1.4 illustrates the chemical structure of the four main classes of PPs.

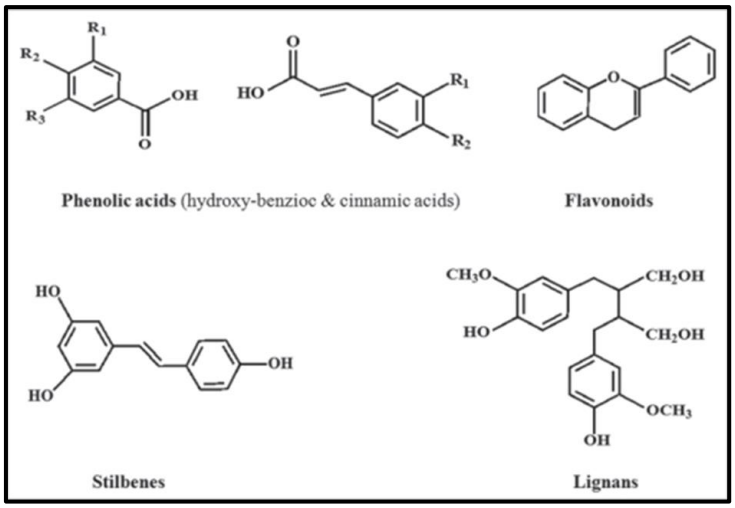

Figure 1.4. Chemical structure of different classes of PPs, adapted from (Pandey & Rizvi, 2009)
These compounds are classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. The four main classes of PPs include phenolic acids, flavonoids, stilbenes, and lignans.

Phenolic acids are further divided into two classes as the derivatives of benzoic acid and the derivatives of cinnamic acid (Tsao, 2010). Gallic, *p*-hydroxybenzoic, vanillic, and syringic acids come under the hydroxybenzoic acid derivatives. Hydroxycinnamic acids consist mainly of *p*-coumaric, caffeic, ferulic, and sinapic acids. Hydroxycinnamic acids are more common in edible plants than hydroxybenzoic acids (Pandey & Rizvi, 2009). Hydroxycinnamic acids are present at high concentrations in red-colored fruits, vegetables, tea, cocoa, wine, tea leaves, coffee, and whole

grains (Durazzo et al., 2019). Red fruits, black radish, and onions are the sources of hydroxybenzoic acids (Pandey & Rizvi, 2009).

Flavonoids are the most studied group of PPs. The basic flavonoid structure consists of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (Fig. 1.5). Flavonoids are classified into several subclasses as flavonols, flavanones, flavanols, flavones, anthocyanins, and isoflavones based on the variation in the type of heterocycle involved (Durazzo et al., 2019). Flavonols have a ketone group and, are the building blocks of proanthocyanins. They occur in a variety of fruits and vegetables including onions, kale, lettuce, tomatoes, apples, grapes, and berries. Kaempferol, quercetin, myricetin and fisetin are the most studied flavonols. Flavanones are another flavonoid group, generally present in all citrus fruits such as oranges, lemons, and grapes. Hesperitin, naringenin, and eriodictyol are examples of flavanones. In its structure, flavanones have the C ring saturated. Flavanols are the 3-hydroxy derivatives of flavanones and referred to flavan-3-ols as the hydroxyl group is always bound to position 3 of the C ring. They occur mainly in bananas, apples, blueberries, peaches, and pears. Flavones, another important subgroup of flavonoids occur in leaves, flowers, and fruits as glucosides. Luteolin, apigenin, and tangeritin are examples of flavones. Major sources of this subgroup of flavonoids are celery, parsley, red peppers, chamomile, mint, and ginkgo biloba. The structure of flavones is composed of a double bond between positions 2 and 3 and a ketone in position 4 of the C ring. The pigments responsible for colors in plants, flowers, and fruits are anthocyanins. They can be found in the outer cell layers of fruits such as cranberries, red grapes, raspberries, strawberries, blueberries, and blackberries. The most commonly studied anthocyanins include cyanidin, delphinidin, malvidin, pelargonidin, and peonidin. Isoflavones are mainly found in the leguminous family of plants including soybean. (Panche et al., 2016). Genistein and daidzein are the two main isoflavones found in soy (Tsao, 2010).

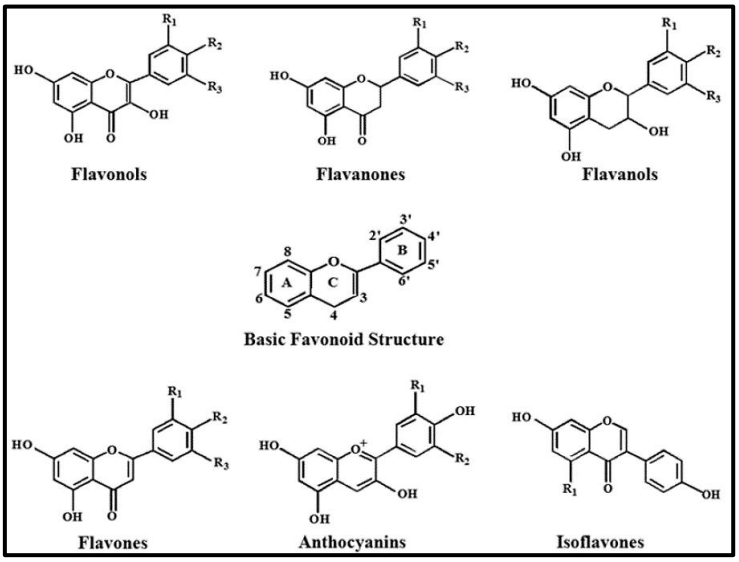

Figure 1.5. Subclasses of flavonoids and their chemical structure, adapted from (Pandey & Rizvi, 2009)

Stilbenes occur in many plants species including grapes, blueberries, bilberries, almonds, beans, peanuts, cranberries, and plums. The chemical structure of stilbenes is composed of two phenyl moieties connected by a two-carbon methylene bridge (Fig. 1.4). The occurrence of stilbenes in the human diet is quite low. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is one of the most studied stilbene compounds, found mainly in the skin of grapes (Reinisalo et al., 2015).

Lignans are found in relatively low quantities in various seeds, grains, fruits, and vegetables (Rodríguez-García et al., 2019). Linseed is the richest dietary source of lignans which contains secoisolariciresinol (up to 3.7g/kg dry weight) and low quantities of matairesinol (El Gharras, 2009). Lignans are phenolic dimers and have the structure of 2,3-dibenzylbutane (Fig. 1.4) (Zitterman, 2003).

Epidemiological studies have shown an inverse association between the consumption of a diet rich in PPs and chronic human diseases (Beckman, 2000; Graf et al., 2005; Scalbert et al., 2005; Spencer et al., 2008). In aging-associated neurological dysfunction, resveratrol (a PP present in red wine, blueberries, cranberries, etc.) and tea-derived catechin gallate esters have been demonstrated neuroprotective effects (Bastianetto et al., 2007). The beneficial effects of resveratrol in aging are not limited to its radical scavenging activity as it can activate the protein sirtuin 1(SIRT1) (Alcaín & Villalba, 2009; Okawara et al., 2007). SIRT1 plays a crucial role in many cell signaling pathways and involves the regulation of lifespan (Rahman & Islam, 2011). It has been reported that catechin-rich green tea consumption improved the postprandial glucose status in humans (Takahashi et al., 2014; Takahashi et al., 2019). Chronic consumption of flavanone-rich orange juice is known to associate with improvements in cognition in older subjects (Kean et al., 2015). Supplementation of blueberry extracts and powder, strawberry extracts and raspberry juice has been reported to attenuate atherosclerosis in animal models (Alarcón et al., 2015; Ströher et al., 2015; Suh et al., 2011; X. Wu et al., 2010). In several *in vitro* studies, procyanidins (PR) have shown protective effects against different cancer cells including lung cancer cells, colorectal cancer cells, and Caco-2 colon cancer cells (Choy et al., 2016; Gorlach et al., 2011; Kin et al., 2013).

1.7. Cocoa in human health and disease

Cocoa consumption has a long history which goes back to at least 460 AD (Seligson et al., 1994). Historically, cocoa has been consumed as a medicine to induce weight gain in emaciated patients, stimulate the nervous system, and improve digestion and elimination (Katz et al., 2011). Cocoa is rich in PPs, mainly flavanols, to which most of the health-promoting effects of cocoa have been attributed (Martin & Ramos, 2021). Various studies have been demonstrated that cocoa intake can alleviate the symptoms of chronic disease conditions including cardiovascular disease, insulin resistance, cancer, and obesity (Katz et al., 2011). Moreover, evidence suggests that cocoa consumption can improve skin tone and elasticity, cognition, and tooth health (Andújar et al., 2012). The mechanisms underlying these biological actions associated with disease-modifying effects include antioxidant, anti-carcinogenic, anti-diabetic, anti-inflammatory, anti-obesity, and anti-

allergic activities which are connected with the regulation of numerous signaling pathways (Martin & Ramos, 2021).

1.7.1. Components of cocoa and classification of cocoa phytochemicals

The term “cocoa” is defined as the dried and fully fermented fatty seed of the cacao tree (Badrie et al., 2015) and is also used to refer to cocoa powder, which is the dry, powdered, reduced-fat component prepared by removing some of the cocoa butter from cocoa liquor (Ellam & Williamson, 2013; Katz et al., 2011).

The chemistry of cocoa beans is very complex and changes throughout its life, mainly depending on the processing of the bean. Proteins constitute 10-15% of the dry weight of fermented cocoa beans and represent 60% of the total nitrogen. Protein is the second most abundant constituent after cocoa fat. Fractionation of cocoa results in different types of proteins including albumin (water-soluble), globulins (salt soluble), glutelins (soluble in dilute acids and alkali), and prolamins (alcohol soluble) (Zak & Keeney, 1976). Of the total proteins in the cotyledons of ripe cocoa beans, albumin constitutes 52% and the storage protein vicilin (7S)-class globulin constitutes 43% of the (Kumari et al., 2016). The concentrations of glutelins and prolamins are lower, representing 5 % and 1 % respectively. However, the fermentation process alters protein concentrations resulting in an increase in albumin and glutelin from 52% to 79% and a decrease in globulins from 43% to 8.3%. The non-protein nitrogen in cocoa beans is available in the form of amino acids (about 0.3% presents in amide form and 0.02% as ammonia). Even though the fresh cacao bean contains a lot of enzymes including β-glucosidase, β-fructosidase, α-amylase, β-galactosidase, proteinase, alkaline and acid phosphatases, lipase, polyphenol oxidase, pectinesterase, catalase, and peroxidase, most of them are inactivated during the production process.

The fat found in cocoa beans which is responsible for the melting properties of chocolate is called cocoa butter. Cocoa butter accounts for 50% to 57% of the dry weight of cocoa beans (Steinberg et al., 2003). The predominant fatty acids in cocoa butter are monounsaturated (oleic; 18:1, 35%) and saturated (stearic; 18:0, 35% and palmitic; 16:0, 25%) fatty acids. The rest is primarily polyunsaturated linoleic (3%) (Bracco, 1994).

Cocoa beans contain mono-, oligo- and polysaccharides (Bertazzo et al., 2013). Raw cocoa beans contain around 2% to 4% (dry weight) free sugars including fructose, glucose, sucrose, galactose, sorbose, xylose, arabinose, mannitol, and inositol. Polysaccharides that represent 12% (dry weight) include starch, pectins, cellulose, pentosans, and mucilage. Starch is the major digestible polysaccharide, ranging from 3% to 7%. Cellulose, one of the predominant polysaccharides in the cell wall is about 12% in fermented/dried cocoa beans. Hemicelluloses are also present in lesser amounts in cocoa shells (Bertazzo et al., 2013). Sucrose represents about 90% of the total sugars in unfermented beans. During the fermentation, invertase converts sucrose into reducing sugars (fructose and glucose) (Aprotosoaie, Luca, et al., 2016). The cocoa bean is known to be an extremely rich source of many essential minerals, including magnesium, copper, potassium, and iron (Katz et al., 2011).

Cocoa and its derived products are composed of a wide range of phytochemicals mostly flavonoid and non-flavonoid phenols, and methylxanthines (Table 1.1) (J. Kim et al., 2014). Cocoa powder contains up to 50 mg of PPs per gram (Katz et al., 2011) and cocoa has more PPs and a higher antioxidant capacity than teas and red wine (K. W. Lee et al., 2003). Therefore, cocoa significantly contributes to the total antioxidant capacity of European and American diets (Vinson et al., 2006). The three major groups of flavonoid PPs in cocoa beans are called catechins (CE, 37%), PR (58%), and anthocyanins (4%) (Andújar et al., 2012). CE are the monomeric units that are classified under flavan-3-ols (Scapagnini et al., 2014). Monomers account for 5% to 10% of the total cocoa PPs and polymers for ≥ 90% of the total cocoa PPs. CE are composed of mainly monomeric (–)-epicatechin (EC, up to 35% of the total PPs) and (+)-CE. In addition, (+)-gallocatechin and (–)-epigallocatechin are found in smaller amounts. PR in cocoa seeds are represented by dimers, trimers, or oligomers of flavan-3,4-diols linked by 4→8 or 4→6 bonds (Aprotosoaie, Luca, et al., 2016). The main PR in cocoa are B1, B2, B3, B4, B5, C1, and D. Leucoanthocyanins L1, L2, L3, and L4, cyanidin-3-α-L-arabinoside, and cyanidin-3-β-D-galactoside are the main anthocyanins in cocoa (Andújar et al., 2012). Other bioactive compounds in cocoa beans include methylxanthines (4%) with theobromine as the main compound (Aprotosoaie, Luca, et al., 2016). Flavanols (CE and EC) are the predominant polyphenolic compounds in cocoa powder (Maleyki & Ismail, 2010; Natsume et al., 2001). In chocolates, EC is the major compound with a ratio of 1:0.1 compared to CE (Cooper et al., 2007).

Table 1.1. Flavonoid and non-flavonoid phenols in cocoa, adapted from (Katz et al., 2011)

Class	Compounds
Cocoa non flavonoid phenols	
Flavanols	(–)-Epicatechin
	(+)-Catechin
	(–)-Epicetechin-3-O-gallate
	(–)-Epigallocatechin
	Procyanidin B1 (epicatechin-(4b (8)-catechin
	Procyanidin B2 (epicatechin-(4b (8)-epicatechin
	Procyanidin B2-O-gallate (epicatechin-3-O-gallate-(4b (8)-epicatechin
	Procyanidin B2-3,3-di-O-gallate (epicatechin-3-O-gallate-(4b (8)-epicatechin-3-O-gallate
	Procyanidin B3 (catechin-(4b (8)-catechin)
	Procyanidin B4 (catechin-(4b (8)-epicatechin)
	Procyanidin B4-3-O-gallate (catechin-(4b (8)-epicatechin-3-O-gallate
	Procyanidin C1 (epicatechin-(4b (8)-epicatechin-(4b (8)-epicatechin)
Flavonols	Quercetin
	Isoquercitin (quercetin-3-O-glucoside)
	Quercitin-3-O-arbinoside
	Quercitin-3-O-galactoside
Anthocyanins	3-alpha-L-Arabinosidyl cyanidin

	3-beta-D-Galactosidyl cyanidin
Flavones	Luteolin
	Luteolin-7-O-hyperoside
	Iso-orientin
	Vitexin
Flavanones	Naringenin
	Naringenin-7-O-glucoside
Cocoa non-flavonoid phenols	
Phenolic acids	Chlorogenic acid
	Vanillic acid
	Coumaric acid
	Phloretic acid
	Caffeic acid
	Ferulic acid
	Phenylacetic acid
	Syringic acid
Others	Clovamide
	Deoxyclovamide
Methylxanthines	Theobromine
	Caffeine

1.7.2. Bioavailability of cocoa phytochemicals

The biological activity of cocoa PPs and methylxanthines is largely dependent on their bioavailability (Ramiro-Puig & Castell, 2009). Cocoa PPs are known to have a relatively low bioavailability with a low plasma C_{max}, a short half-life, and rapid excretion (Manach et al., 2005). Monomers and dimeric or trimeric PR are quite stable in the gastric environment and can travel to the small intestine where they are absorbed (Fraga et al., 2011). These compounds are rapidly absorbed when cocoa products are consumed in doses close to a habitual diet (Nasiruddin Khan et al., 2014). It has been shown that PR and monomers present in plasma as early as 0.5 h after ingestion and reach the maximal plasma concentration at about 2 h (Actis-Goretta et al., 2012; Holt et al., 2002; Pearson et al., 2002; Rein et al., 2000; Richelle et al., 1999; Schramm et al., 2001; Steinberg et al., 2002). Detected cocoa flavanols (CFs) normally did not exceed a plasmatic T_{max} of 3 h and in most cases, their elimination from plasma was achieved 6 h after the consumption (Holt et al., 2002; Mullen et al., 2009; Richelle et al., 1999). The peak concentration achieved by CFs in plasma is typically in the nanomolar or low micromolar range, depending on the dose consumed (Goya et al., 2016; Rimbach et al., 2009).

The absorption of flavanols depends on several factors including flavanol chemistry, structural isomerism, and stereoisomerism (Ottaviani et al., 2011). Therefore, the absorption characteristics of EC and CE vary depending on these factors (Holt et al., 2002; Steinberg et al., 2002). Moreover, (+) and (−) forms of CE in plasma also report different concentrations (Donovan et al., 2006; Ritter et al., 2010). Bioavailability of (–)-EC is higher compared to the other flavanols such as CE and galloylated derivatives (Steffen et al., 2008). In addition, as the absorption depends on the range of polymerization, flavanols only up to trimers pass through the small intestine (Deprez et al., 2001; Spencer et al., 2001). Absorption of dimeric and trimeric PR is about 10-100 times less compared to EC and CE (D. Field & Newton, 2013). PR B2 was detected only in trace amounts in human plasma after cocoa products were consumed (Holt et al., 2002; Steinberg et al., 2002). Oligomeric PR and polymeric flavanols which are not absorbed in the small intestine are metabolized by the local microbiota in the colon within 48 h into various low-molecular weight compounds (Keen et al., 2002; N. Khan et al., 2012). The metabolism of simple CFs in mammals occur in the liver and small intestine (D. Field & Newton, 2013). In addition, flavonoid metabolism can occur in the vascular endothelial cells (Aprotosoaie, Miron, et al., 2016). Theobromine is absorbed extensively in the small intestine (Ellam & Williamson, 2013) and the metabolites formed are 7-methylxanthine (around 30% of the dose), 3-methylxanthine (around 20% of the dose), and 7-methyluric acid (around 4% of the dose) (Cornish & Christman, 1957).

1.7.3. Lifespan extension by cocoa

Cocoa has been shown to extend the lifespan in non-mammalian as well as mammalian models. In a study, supplementation of cocoa powder (5%) significantly increased the average lifespan of *Drosophila melanogaster* under normoxia, but at 10% had no significant effects (Bahadorani & Hilliker, 2008). This suggests that moderate consumption of cocoa under normoxia may be beneficial to health. Moreover, the longevity-extending effects of cocoa appeared to be selective. Under hyperoxia or in a Cu/Zn-superoxide dismutase (SOD)-deficient background, cocoa (at both 5% and 10%) significantly increased the average life span of flies, exhibiting a strong antioxidant activity. However, cocoa in an Mn-superoxide dismutase-deficient background increased the mortality rate which was accompanied by a loss in climbing activity in flies. These results indicated a dual oxidative property of cocoa with an antioxidant effect in the cytoplasm and a pro-oxidant effect in mitochondria. Surco-Laos et al. (2012) demonstrated that long-term supplementation of methylated EC derivatives (3′-O-methylepicatechin and 4′-O-methylepicatechin) increased the mean lifespan of *Caenorhabditis elegans* (*C. elegans*) by 6-12%. However, no enhancement in mean lifespan was found in both CE and EC treated worms. Moreover, maximum lifespan was extended with EC and its methyl ethers, but not with CE. Further, worms treated with all the CE types showed increased resistance to juglone-induced OS and thermal stress with greater protective effects in older worms (at the 6^{th} day of adulthood compared to the 1^{st} day of adulthood). The lifespan extension and increased resistance to stress were believed to be mediated, at least in part, through the antioxidant and radical-scavenging activity of CEs. Moreover, CE modulated NHR-8,

AKT-2, SIR 2.1 and DAF-2 mediated stress resistance and repair mechanisms to elicit beneficial effects. In another study, exposure of *Oryzias latipes* (Medaka fish) embryos to a cocoa extract resulted in a significant 2-day delay in hatching (Sánchez-Sánchez et al., 2018). Moreover, the incubation of fish embryos with the cocoa extract significantly reduced the lethality induced by menadione. Similarly, cocoa extract protected the embryos against hydrogen peroxide (H_2O_2) induced OS. These protective effects were mediated through the regulation of genes involved in response to OS including catalase and SOD (SOD1, SOD2, and SOD3) and longevity (FOXO5). In the same study, the long-term feeding of *O. latipes* with a diet supplemented with cocoa extract increased the mean fish lifespan by 18%. In both embryos and adult fish, cocoa extract induced the activity of the SOD enzyme, indicating that cocoa extract may act similarly through the regulation of SOD function during embryonic development and adulthood. It has been reported that a polyphenol-rich cocoa extract increased the chronological lifespan of *Saccharomyces cerevisiae* (yeast) during the stationary phase in a dose-dependent manner (Baiges & Arola, 2016). The lifespan extension mechanism of the cocoa extract was not SOD2-dependant. The same cocoa extract further extended the yeast lifespan under severe caloric restriction conditions. However, none of the major components in the cocoa extract (theobromine, caffeine, maltodextrin, (**-)**-EC, (+)-CE, and PR B2) extended the lifespan of yeast. A separate study reported the supplementation of two different types of cocoa powders on the lifespan of *S. cerevisiae* and *C. elegans* (P. Martorell et al., 2011). The study utilized a conventional cocoa powder (4% PP content) and a flavanol-enriched cocoa powder (12% PP content). Flavanol-enriched cocoa powder supplemented *C. elegans* showed a resistance against OS induced by H_2O_2 and an extension of lifespan by 17%. The beneficial effects were mediated through SIR2.1 and DAF-16 dependent mechanisms in *C. elegans*. The same cocoa powder increased the percent survival of *S. cerevisiae* (from 2.63% to 15.39%) exposed to H_2O_2 depending on sirtuins Hst3. Saul et al. (2009) demonstrated that CE increased both the mean and median lifespan of *C. elegans* while eliciting a significant reduction in body length. In addition, CE showed enhanced OS and thermal stress resistance. The lifespan-extending effects of CE are explained in terms of disposable soma theory (DST) together with NHR-8, AKT-2, and DAF-2 dependent stress resistance and repair mechanisms which include broad-spectrum detoxification rather than a simple antioxidative action. DST suggests that the amount of energy available for an organism is distributed into 3 parts as maintenance, growth, and reproduction (Kirkwood 1977, 1988). In this study, the additional energy required to increase the stress response and defense resulted in an energy imbalance that reduced the body size of *C. elegans* by utilizing energy involved in growth mechanisms. Supplementation of a cocoa polyphenolic extract increased the lifespan in aged rats (Bisson et al., 2008). However, the underlying mechanisms for the prolongation of the lifespan were not described in the study. Another study demonstrated that EC supplementation increased the survival rate of old mice from 39 to 69% (Si et al., 2019). Nicotinamide adenine dinucleotide (NAD) metabolic pathway could primarily mediate the lifespan

extension effects of EC as evident by restored 2 NAD metabolites (nicotinate ribonucleoside and nicotinamide).

Table 1.2. Cocoa improves age-associated health, a summary of findings

Study	Design	Treatment	Duration	Population	Outcome
Cocoa on skeletal muscle					
Mafi et al. (2019)	RCT	EC (1 mg/kg/day)	8 weeks	Sarcopenic adults (68.63 ± 2.86 years)	Follistatin, follistatin/myostatin ratio ↑ Muscle strength and physical capacity ↑
Gutierrez-Salmean et al. (2014)	Animal study and a pilot study with humans	EC (1 mg/kg/day)	2 weeks	Young (6 months) and old mice (26 months); human subjects (41±5 years)	Myostatin levels in both young and old mice ↑ Follistatin levels in old mice ↑ MEF2A, Myf5, and myogenin in both young and old mice ↑ MyoD in old mice ↑ SA-β-Gal levels in old mice ↓ Hand strength in humans ↑ Plasma follistatin/myostatin levels in humans ↑
Munguia et al. (2020)	Animal study	Cocoa beverage enriched with flavanols (2 mg EC + 12. 8 mg PR/kg/day) or EC (2 mg/kg/day)	5 weeks	Sarcopenic obesity model of middle-aged mice (64 weeks)	Physical performance ↑ Follistatin/myostatin ratio and the expression of MEF2A ↑ Expression of FOXO1A and MURF1 markers of the SkM ubiquitin-proteasome degradation pathway ↓
Si et al. (2019)	Animal study	EC (0.25% w/v in drinking water)	37 weeks	Old mice (20 months)	Physical activity ↑ SkM degeneration ↓ Age-altered mRNA and protein expressions of extracellular matrix and PPAR pathways in SkM and the age-induced declines of the nicotinate and nicotinamide pathway in both serum and SkM ↓
Nogueira et al. (2011)	Animal study	EC (1 mg/kg) twice a day	15 days	Old mice (1 year)	Exercise capacity ↑

					Number of capillaries in the hindlimb muscle ↑ Amount of muscle mitochondria and signaling for mitochondrial biogenesis ↑
(Munguia et al., 2019)	RCT	Natural cocoa powder rich in flavonoids (179 mg) once a day	12 weeks	Middle-aged and older subjects (62.4 ± 3.2 years)	Mobility and physical performance ↑
B. E. Phillips et al. (2016)	Acute study	CFs (350 mg)	Single-dose	Healthy older men (72.3 ± 1.4 years)	Leg blood flow and muscle microvascular blood volume ↑
Ito et al. (2017)	Animal study	CFs (50 mg/kg/day)	2 weeks	Mice	Progression of atrophy of gastrocnemius, tibialis anterior, and SOL induced by hindlimb suspension ↓ Muscle mass of the extensor digitorum longus with or without hindlimb suspension ↑ The increase in ubiquitin ligase and MURF1 in the SOL by hindlimb suspension ↓ The decreased protein expression of the p70S6 kinase in the SOL by hindlimb suspension ↑
Cocoa on brain					
Stringer et al. (2015)	Animal study	EC (4 mg daily)	2 weeks	Adult mice (14 weeks)	Anxiety ↓ Hippocampal and cortical tyrosine hydroxylase ↑ Cortical monoamine oxidase-A levels ↓ Hippocampal BDNF and pro-BDNF ↑
Brickman et al. (2014)	RCT	CFs (900 mg CFs and 138 mg of EC per day)	3 months	Healthy, older adults (50-69 years)	DG function ↑ Modified Benton Task performance ↑
Mastroiacovo et al. (2015)	RCT	A cocoa drink containing either HF (993	8 weeks	Healthy, elderly subjects	TMT A and B ↑ by HF and IF groups VFT ↑ by all 3 treatment groups

		mg), IF (520 mg), or LF (48 mg) once daily			
Nurk et al. (2009)	Cross-sectional study	Flavonoid intake from chocolate, wine, and tea	-	Cognitively intact older adults (70-74 years)	Mean test scores for Kendrick object learning test, TMT A, modified versions of the digit symbol test, block design, mini-mental state examination, and controlled oral word association test ↑ Poor cognitive performance ↓
Letenneur et al. (2007)	Prospective study	Flavonoid intake from multiple foods including chocolates	10 years	Older adults (65 years or older) free from dementia	Cognitive performance at baseline as well as over time for three psychometric tests (MMSE, Benton's visual retention test, ''Isaacs'' set test) ↑
Camfield et al. (2012)	RCT	A chocolate drink containing 250 mg or 500 mg CFs once daily	30-day period	Healthy middle-aged subjects (40-65 years)	Neural efficiency in spatial working memory function ↑
Neshatdoust et al. (2016)	RCT	Cocoa drinks containing 494 mg (HF) and 23 mg (LF) once daily	28-day period	Older subjects (62-75 years)	BDNF serum levels and global cognitive scores ↑ by HF group
Suominen et al. (2020)	RCT	Treatment group: 50 g dark chocolate/day containing 410 mg total of flavanols, of which 85 mg was EC Control group: 50 g dark chocolate/day containing 86 mg total of flavanols, of	8 weeks	Cognitively healthy adults (65-75 years)	No significant effect in cognitive parameters (VF and TMT A and B)

		which 26 mg was EC			
Moreira et al. (2016)	Prospective study	Chocolate intake	A median of 48 months follow-up	Cognitively healthy, the elderly population (65 years or over)	Cognitive decline as evident by MMSE ↓
Bisson et al. (2008)	Animal study	Cocoa polyphenolic extract (24 mg/kg/d of Acticoa powder)	1 year	Aged rats	Cognitive performances in light extinction and water maze paradigms ↑
Cocoa on oxidative stress					
Q. Huang et al. (2005)	*In vitro* study	CE	-	N9 cells	Oxidative agent tBHP induced cell death ↓ DNA damage ↓ Levels of intracellular •OH radical ↓
Dani et al. (2008)	Animal study	CE (10 μg/ml)	1 hour	*S. cerevisiae*	Tolerance against the OS induced by H_2O_2, CCl_4, and Cd^{2+} ↑
Xiao et al. (2018)	Animal study	0.2% PR B2	7 weeks	D-galactose induced mimetic aging model of mice	Spacial memory of mice in Morris water maze test ↑ PR B2 remodeled the gut microbiota
Cho et al. (2008)	*In vitro* study	Cocoa PR fraction (1 and 5 µg/ml) and PR B2 (1 and 5 µg/ml)	-	PC12 rat pheochromocytoma cells	Cell death induced by H_2O_2 ↓
Cho et al. (2009)	*In vitro* study	Cocoa PR fraction (5 and 10 µg/ml) and PR B2 (10 and 20 µM)	-	PC12 rat pheochromocytoma cells	HNE induced apoptosis ↓

Rozan et al. (2007)	Animal study	Cocoa polyphenolic extract (22.9 mg/kg/d of Acticoa powder)	2 weeks	Rats	Free radical production ↓ Spatial learning in rats as measured by Morris water maze test ↑
Cocoa on vascular health					
Ramirez-Sanchez et al. (2018)	*In vitro* study and animal study	EC (1 μM) for the *in vitro* study and EC (1 mg/kg/day) for the animal study	48 hours for the *in vitro* study and 15 days for the animal study	Primary bovine coronary artery endothelial cells (*in vitro* study) and rats (animal study)	*In vitro* NO production ↑ Acetylcholine induced aged aorta vasodilation and NO levels in mice ↑ BP ↓
Sorond et al. (2008)	RCT	Flavanol rich cocoa (900 mg flavanols daily)	2 weeks	Healthy older adults (72 ± 6 years)	Mean blood flow velocity in the middle cerebral artery ↑
Fisher et al. (2003)	-	Flavanol rich cocoa (821 mg total flavanols daily)	5 days	Healthy older subjects	Peripheral vasodilation, endothelial function ↑
Heiss et al. (2015)	RCT	CFs containing drink (450 mg CF daily)	2 weeks	Healthy older men (50-80 years)	Endothelial function ↑ PWV, aortic augmentation index, SBP, and total peripheral resistance ↓ Arteriolar and microvascular vasodilator capacity, red cell deformability, and DBP ↑
Fisher and Hollenberg (2006)	-	Flavanol-rich cocoa (230 ml dose four times a day which provides 821 mg total flavanols/day)	4-6 days	Young (<50 years) and older subjects (>50 years)	FMD and PWA ↑
Gröne et al. (2020)	RCT	CFs (450 mg of total flavanols twice daily)	2 weeks	Healthy young (<35 years) and elderly (50-80	Concentrations of $CD31^+/41^-$, $CD144^+$, and $CD62e^+$ EMPs ↓ in both young and elderly subjects

				years) subjects	
Engler et al. (2004)	RCT	High-flavonoid (213 mg PR, 46 mg EC) or low-flavonoid dark chocolate bars (46 g, 1.6 oz)	2 weeks	Healthy adults	Endothelium-dependent FMD of the brachial artery ↑
Okamoto et al. (2016)	RCT	Cocoa powder (17 g of cocoa either once daily except Sundays or twice daily every other day)	12 weeks	Postmenopausal women (64±12 years)	Central and peripheral arterial stiffness ↓
Cocoa on Aβ					
Cox et al. (2015)	Animal study	EC in water (3 mg/ml) which was estimated to be close to 15 mg EC per day	3 weeks	TASTPM transgenic mice (7 months old)	Aβ pathology and Aβ levels ↓
Diaz et al. (2019)	Animal study	EC (200 mg/kg/day)	4 days	Rats	Deterioration of spatial memory and OS induced by the $A\beta_{25-35}$ ↓ Enzymatic activity of SOD and catalase ↑ IL-1β and TNF-α ↓ HSP-60, HSP-70, and HSP-90 ↓
S.-M. Choi et al. (2014)	*In vitro* study	CE and EC at a concentration of 30 µM	24 hours of exposure	Cultured mouse cortical neurons	Neuronal death induced by $A\beta_{25-35}$ ↓ by both treatments
S. B. Lee et al. (2020)	*In vitro* study	EC and CE	-	Human microvascular endothelial cells	$A\beta_{1-42}$ fibril formation ↓ and destabilized the preformed $A\beta_{1-42}$ fibrils by both treatments
Z. Zhang et al. (2016)	Animal study	EC (50 mg/kg daily)	4 months	APP/PSEN1 mice (a moderate pathology phase; 8 months old)	OS ↓ BDNF protein levels in the hippocampus ↑ Phosphorylation of Akt/GSK-3/CREB ↑

Zeng et al. (2014)	Animal study	EC supplemented diet (40 mg/kg/day)	9 months	APP/PSEN1 transgenic mice (3 months old)	Total Aβ in brain and serum ↓ by 39% and 40% respectively and microgliosis and astrocytosis in the brain of Alzheimer's mice by 38% and 35% respectively TNF-α in the plasma ↓
J. Wang et al. (2014)	*In vitro* study	Three different cocoa extracts (Natural, Dutched, and Lavado)	-	-	Aβ oligomer formation of both $A\beta_{42}$ and $A\beta_{40}$ ↓ LTP response ↑
Cimini et al. (2013)	*In vitro* study	Cocoa polyphenolic extract	-	SH-SY5Y cells	Cell viability and cell morphology from Aβ injury ↑
Heo and Lee (2005)	*In vitro* study	A cocoa extract, EC, and CE	-	Rat pheochromocytoma PC12 cells	Viability of neuronal cells ↑ Aβ induced cell membrane damage ↓
Ruotolo et al. (2020)	*In vitro* study and an animal study	A comprehensive set of phenyl-γ-valerolactones (PVLs), the main circulating metabolites of flavan-3-ols and related dietary compounds in humans	-	An acute mouse model of Aβ oligomers induced memory impairment	Yeast cell growth inhibition caused by cytotoxic artificial polypeptide (β23) ↓ Monohydroxylated metabolite 5-(4'-hydroxyphenyl)-γ valerolactone [(4'-OH)-PVL] elicited a dose-dependent neuroprotective and anti-neuroinflammatory effect in mice

↑=increased, ↓=decreased; EC, Epicatechin; RCT, randomized controlled trial; MEF2A, myocyte enhancer factor 2A; Myf5, myogenic factor 5; MyoD Myoblast determination protein 1; SA-β-Gal, senescence-associated β-galactosidase; FOXO1A, forkhead box O1A; MURF1, muscle ring finger 1; p70S6 kinase, ribosomal protein S6 kinase; SkM, skeletal muscle; PPAR, peroxisome proliferator-activated receptor; CF, cocoa flavanol; SOL, soleus; BDNF, brain-derived neurotrophic factor; DG, dentate gyrus; HF, high flavanol; IF, intermediate flavanol; LF, low flavanol; TMT, trail making test; VFT, verbal fluency test; MMSE, mini-mental state examination; tBHP, tert-butylhydroperoxide; CE, catechin; PR, procyanidin; HNE, 4-Hydroxynonenal; NO, nitric oxide; BP, blood pressure; PWV, Pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow mediated vasodilation; PWA, basal pulse wave amplitude; EMPs, endothelial microparticles; Aβ, amyloid-β; SOD, superoxide dismutase; IL-1β, interleukin 6; TNF-α, tumour

necrosis factor-α; APP/PSEN1, amyloid precursor protein/presenilin 1; OS, oxidative stress; Akt, protein kinase B; GSK-3, glycogen synthase kinase 3; CREB, cAMP response element-binding protein; LTP, long-term potentiation

1.7.4. Cocoa reverses age-associated deficits in skeletal muscle

Age-related loss of skeletal muscle (SkM) mass and strength is generally known as sarcopenia. In humans, starting from approximately the 4th decade of life till 80 years of age, sarcopenia decreases around 30-50% of skeletal muscle mass and function (Lexell et al., 1988). Sarcopenia in humans is thought to develop via multiple mechanisms (Demontis et al., 2013). These processes include a decrease in protein synthesis (Welle et al., 1995), dysregulation of proteasomal degradation pathways (Chondrogianni et al., 2000), a decline of both the number and the regenerative ability of satellite cells (Shefer et al., 2010), defects in the neuromuscular junction and denervation (Delbono, 2003; Jang & Van Remmen, 2011), altered hormonal status (Szulc et al., 2004), increased production of catabolic cytokines (Visser et al., 2002), mitochondrial dysfunction (Short et al., 2005) and increased ROS production (Broome et al., 2006). Given the complex etiology of the disease, finding an effective solution for the management of sarcopenia is yet to be resolved (Calvani et al., 2013). Some of the existing beneficial remedies for sarcopenia include physical activity and exercise (Belavy et al., 2014; Caiozzo et al., 2009), resistance training (Maltais et al., 2016; Tsuzuku et al., 2018), aerobic training (Konopka et al., 2014; Schwartz et al., 1991), increase in protein intake (Genaro Pde et al., 2015; Norton et al., 2016) and calorie restriction (Joseph et al., 2013; C. M. Lee et al., 1998). Among these therapies, PP supplementation is emerging as an effective therapy for sarcopenia (J.-A. Kim et al., 2019; Meador et al., 2015).

EC, the major flavanol compound found in cocoa has been shown to improve the age-associated loss of muscle strength and physical capacity through the reversal of age-related deleterious effects on modulators of muscle growth/differentiation (Gutierrez-Salmean et al., 2014; Mafi et al., 2019). Aging significantly increased the myostatin and senescence-associated β-galactosidase (SA-β-Gal) while significantly decreasing follistatin, myoblast determination protein 1 (MyoD), and myogenin levels in humans (Gutierrez-Salmean et al., 2014). EC supplementation increased the follistatin levels and follistatin/myostatin ratio in humans (Gutierrez-Salmean et al., 2014; Mafi et al., 2019). In a parallel animal study, EC supplementation increased follistatin levels in old mice as well (Gutierrez-Salmean et al., 2014). In addition, the aging of SkM of mice was associated with an increase in myostatin and SA-β-Gal levels and a decrease in myogenic factor 5 (Myf5) levels. EC decreased myostatin levels in both young and old mice while decreasing SA-β-Gal levels in old mice. Moreover, the levels of myocyte enhancer factor 2A (MEF2A), Myf5, and myogenin were also increased in both young and old animals with EC. In old animals, EC increased the MyoD. Another study supported these findings of the role of EC as well as PR on muscle growth and differentiation (Munguia et al., 2020). The consumption of EC and a cocoa beverage enriched with EC and PR increased the follistatin/myostatin ratio and the expression of MEF2A in a sarcopenic obesity model of middle-aged mice. Moreover, both treatments decreased the expression of

forkhead box O1A (FOXO1A) and muscle ring finger 1 (MURF1) markers of the SkM ubiquitin-proteasome degradation pathway. Additionally, both treatments improved the physical performance of mice evaluated with the hang-wire, inverted-screen, and weight-lifting tests and dynamometry. In another study, EC improved the physical activity, delayed SkM degeneration, reversed age-altered mRNA and protein expressions of extracellular matrix and peroxisome proliferator-activated receptor (PPAR) pathways in SkM, and the age-induced declines of the nicotinate and nicotinamide pathway in both serum and SkM in old mice (Si et al., 2019). Another study demonstrated that EC increased the exercise capacity which was indicated by increased treadmill performance and enhanced *in situ* muscle fatigue resistance in old mice (Nogueira et al., 2011). These improvements were accompanied by an increased number of capillaries in the hindlimb muscle, an increased amount of muscle mitochondria, and signaling for mitochondrial biogenesis.

In addition to the individual components in cocoa, some studies demonstrated the beneficial effects of the CF fraction or the whole cocoa powder rich in flavonoids in sarcopenia. Consumption of natural cocoa powder rich in flavonoids improved the mobility and physical performance of middle-aged and older subjects as shown by improvements in 6 minutes-walk test, the step test, the sit-up test, up and go test, skeletal muscle index, and the quality-of-life indices in the visual analog scale (Munguia et al., 2019). B. E. Phillips et al. (2016) reported that the improved muscle macro-and microvascular responses to nutrition, independently of modifying muscle protein anabolism in healthy older men by acute CF supplementation. In young and middle-aged people, a well-defined response to nutrient intake is the increased limb arterial blood flow (Raitakari et al., 2000). However, this response is reduced or not present in older subjects (B. Phillips et al., 2012). Age-related reductions in leg blood flow (LBF) are further associated with impairments in muscle microvascular blood volume (MBV). In this study, CF increased both LBF and muscle MBV in older subjects. A period of SkM disuse is common in aging as a result of a sedentary lifestyle or bed rest and can lead to muscle atrophy (Magne et al., 2013). The hindlimb suspension disuse model is a common model used to induce disuse muscle atrophy (Brooks & Myburgh, 2014). In a study, supplementation of CFs delayed the progression of atrophy of gastrocnemius, tibialis anterior, and soleus (SOL) induced by hindlimb suspension in mice (Ito et al., 2017). Additionally, CFs increased the muscle mass of the extensor digitorum longus with or without hindlimb suspension. The protein level of the ubiquitin ligase and muscle ring finger 1 in the SOL was significantly increased by hindlimb suspension. The increase of these protein levels which are involved in muscle protein degradation was inhibited by CFs. Further, the protein expression of the 70-kDa ribosomal protein S6 kinase (p70S6 kinase) in the SOL was significantly decreased by hindlimb suspension. Flavanol treatment inhibited this change in the protein level which increased protein synthesis. Studies demonstrating the effects of cocoa on SkM are summarized in Table 1.2.

1.7.5. Cocoa prevents brain aging

The impact of age itself on cognition not including dementia, mild cognitive impairment, or other specific cognitive decline syndromes is a huge and neglected problem for current science (Deary et

al., 2009). While some cognitive abilities such as vocabulary are resilient to aging and may even improve with age, other abilities such as conceptual reasoning, memory, and processing speed decline gradually over time (Harada et al., 2013). Even though these declines are not yet well understood, researchers have identified some of the underlying mechanisms of cognitive decline. These mechanisms include declines in grey and white matter volume (Meier-Ruge et al., 1992), changes in white matter (Meier-Ruge et al., 1992), and declines in neurotransmitter levels (Peters, 2006). Emerging evidence suggests that healthy lifestyle choices can control the impact of aging on cognition up to a certain extent. Some of these healthy lifestyle choices include eating a healthy diet, avoiding excessive alcohol consumption, regular exercising, participating in cognitively stimulating activities, managing emotional stress, and managing medical problems such as hypertension, diabetes, depression, and obstructive sleep apnoea (Murman, 2015). In dietary intervention studies, PPs have been shown to ameliorate the age-related cognitive deficits in both animals (Andres-Lacueva et al., 2005; Haque et al., 2006; Williams et al., 2008) and humans (R. Krikorian et al., 2012; R Krikorian et al., 2010; Letenneur et al., 2007).

We found both human and animal studies that demonstrated the effects of CFs on age-associated cognitive decline. EC intake has been reported to reduce anxiety in adult mice as measured by elevated plus maze (EPM) and open field (OF) tests (Stringer et al., 2015). These beneficial effects were accompanied by elevated hippocampal and cortical tyrosine hydroxylase, downregulated cortical monoamine oxidase-A levels, as well as increased hippocampal brain-derived neurotrophic factor (BDNF) and pro-BDNF. Mastroiacovo et al. (2015) reported that the consumption of CFs improved the verbal fluency and trail making test score (which determines visual attention and task switching ability) in elderly subjects, but another with CFs reported contrary results where no improvement was achieved with both tests (Suominen et al., 2020). However, in another study, consumption of CFs improved cognition in older subjects as evidenced by improvements in global cognitive scores (Neshatdoust et al., 2016). The BDNF serum levels which are important particularly for long-term memory was also increased following CF consumption. Camfield et al. (2012) showed that the middle-aged participants who received CFs had an improved working memory function as indicated by changes in steady-state visually evoked potentials (SSVEP) average amplitude and phase across several posterior parietal and centro-frontal sites. It has been shown that the dysfunction of the dentate gyrus (DG) which is a part of the hippocampus drives the age-associated cognitive decline in humans and the consumption of flavanols (CFs and EC) enhanced the DG function in older adults as determined by functional magnetic resonance imaging (fMRI) and improved Modified Benton Task performance (DG-dependent memory task) (Brickman et al. (2014). In a prospective study, Moreira et al. (2016) determined the association between chocolate consumption and cognitive decline in healthy adults. The global cognitive function at baseline and follow-up (a median of 48 months) was assessed by using mini-mental state examination (MMSE) which includes questions on orientation, registration, attention and calculation, recall, language, and visual construction, and chocolate consumption was associated

with a lower risk of cognitive decline. In the PAQUID (Personnes Agées Quid) study, which is the other prospective study, Letenneur et al. (2007) examined the flavonoid intake from multiple foods including chocolates about cognitive function and decline in adults. Cognitive function was assessed through three psychometric tests (Mini-mental state examination, Benton's visual retention test, ''Isaacs'' set test) at each visit for a total of 4 visits over 10 years. People with higher flavonoid intake showed better cognitive performance at baseline as well as a better evolution over time. In a cross-sectional study, Nurk et al. (2009) studied the association between flavonoid intake from chocolate, wine, and tea and cognitive performance in older adults. The cognitive tests included were Kendrick object learning test, trail making test, part A (TMT-A), modified versions of the digit symbol test, block design, mini-mental state examination, and controlled oral word association test. Flavonoid consumers reported significantly better mean test scores and a lower prevalence of poor cognitive performance in a dose-dependent manner. In aged rats, a cocoa polyphenolic extract improved the cognitive performances in light extinction and water maze paradigms and preserved high urinary free dopamine levels (Bisson et al, 2008). Studies demonstrating the effects of cocoa on brain aging are summarized in Table 1.2.

1.7.6. Cocoa flavanols mitigate mitochondrial and DNA damage as suppressors of oxidative stress

Free radical theory of aging which is later termed as OS theory of aging suggests that age-associated functional losses are the result of the accumulation of oxidative damage to macromolecules (Liguori et al., 2018). Dietary PPs are powerful antioxidants *in vitro* which are capable of neutralizing free radicals (H. Zhang & Tsao, 2016). *In vitro*, CE has been reported to preserve N9 cells from an oxidative agent, tert-butylhydroperoxide (tBHP) induced cell death (Q. Huang et al., 2005). The DNA damage was diminished and the levels of intracellular $^{\bullet}OH$ radical was reduced after the treatment. CE inhibited the tBHP-induced translocation of nuclear factor-kappa B (NF-*κ*B) to survive cells. In another study, CE improved the tolerance against the OS induced by H_2O_2, CCl_4, and Cd^{2+} in *S. cerevisiae* (Dani et al., 2008). The increased tolerance for H_2O_2 was associated with catalase. In addition, OS tolerance was correlated with a reduction in lipid peroxidation, indicating that the antioxidant property of CE involves protection against membrane oxidation. Xiao et al. (2018) used the D-galactose induced mimetic aging model of mice to study the effects of PR B2 supplementation. PR B2 improved the spatial memory of mice assayed by the Morris water maze test. PR B2 regulated the citrate cycle, fatty acid transport, biosynthesis of unsaturated fatty acids, saturated fatty acid metabolism, bile acid metabolism, and other metabolic pathways to improve the protection against the OS induced by D-galactose. In addition, PR B2 remodeled the gut microbiota, possibly showing beneficial effects against aging. *In vitro*, cocoa PR fraction and PR B2 reduced the PC12 rat pheochromocytoma cell death which was induced by H_2O_2 as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue exclusion assays (Cho et al., 2008). These protective effects were achieved through the inhibition of the phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38

MAPK). According to the findings, cocoa PR may protect neurons against OS-induced neurodegeneration. Another study reported the effects of cocoa PR fraction and PR B2 on 4-Hydroxynonenal (HNE) induced apoptosis of rat pheochromocytoma (PC12) cells (Cho et al., 2009). Results indicated that both cocoa PR fraction and PR B2 protected PC12 cells from HNE induced apoptosis in a dose-dependent manner by blocking mitogen-activated protein kinase kinase 4 (mkk4) activity and ROS accumulation. Rozan et al. (2007) studied the preventive effect of a cocoa polyphenolic extract supplementation on free radical production by leucocytes and cognitive performances after heat exposure in rats. Supplementation of the cocoa polyphenolic extract significantly reduced the free radical production and improved the spatial learning as measured by the Morris water maze test. Studies demonstrating the effects of cocoa on mitochondrial and DNA damage are summarized in Table 1.2.

1.7.7. Cocoa flavanols improve vascular health

With aging, complex and diversified changes in the cardiovascular structure and function are occurred (Ferrari et al., 2003). Structural changes occur throughout the vascular system and significant changes occur within the wall of large elastic arteries. The intimal medial thickness of these arteries is known to increase with age (Nagai et al., 1998), thereby leading to increased systolic blood pressure (SBP), decreased diastolic blood pressure (DBP), and widened pulse pressure (PP)(Kovacic et al., 2011). Age-associated endothelial dysfunction is another change resulting from the changes in the microcirculation linked to aging (Ferrari et al., 2003). Endothelial dysfunction further contributes to the vascular stiffness in both large and peripheral arteries which hinders the normal contractile capability of vascular smooth muscle (Lakatta & Levy, 2003). Aging also affects the venous vessels, leading to an overall increase in stiffness and a decrease in venous compliance (Hernandez & Franke, 2004). Aging-associated physiological changes further influence these structural changes in blood vessels. Aging increases SBP, PP and mean arterial pressure, with an early rise (till 50 years of age) and a late fall (after 60 years of age) in DBP (Franklin et al., 1997). PPs from various sources are known to exert beneficial effects on vascular health via multiple mechanisms (Borriello et al., 2010; Spanier et al., 2009; Steffen et al., 2008; Xia et al., 2010). *In vitro*, treating with EC restored the aged endothelium and nitric oxide (NO) production to levels comparable to those of young cells (Ramirez-Sanchez et al., 2018). These beneficial effects were associated with increased levels of endothelial nitric oxide synthase (eNOS) phosphorylation, reduced acetylation, and improved mitochondrial function as evidenced by increased levels of citrate synthase activity, mitofilin, and oxidative phosphorylation complexes. Moreover, treatment of rats with EC enhanced acetylcholine-induced aged aorta vasodilation and stimulated NO levels while reducing blood pressure. It has been reported that CFs induced peripheral vasodilation, improving endothelial function in a NO-dependent manner in healthy older subjects (Fisher et al., 2006). The vasodilator response was reversed by NG-nitro-L-arginine methyl ester (an arginine analog that blocks nitric oxide synthesis). Administration of CFs to older subjects yielded more robust improvements in flow-mediated vasodilation (FMD) and basal pulse wave amplitude (PWA)

compared to young subjects (Fisher & Hollenberg, 2006). CFs improved the mean blood flow velocity in the middle cerebral artery in healthy older adults by 8% at one week and by 10% at two weeks in another study (Sorond et al., 2008). Gröne et al. (2020) determined the age-associated changes in endothelial functional integrity using endothelial microparticles (EMPs) and the effects of CF supplementation. Elderly subjects showed significantly increased concentrations of $CD31^{+}/41^{-}$, $CD144^{+}$, and $CD62e^{+}$ EMPs compared to young subjects. CFs decreased these EMPs in both young and elderly subjects. There were significant inverse correlations between changes in EMPs and FMD. In addition, changes in central SBP and PWV were correlated with the changes in EMPs. Healthy older men who consumed a CF-containing drink reported a significant improvement in endothelial function as measured by FMD (Heiss et al., 2015). Further, CF intake decreased the pulse wave velocity (PWV), aortic augmentation index, SBP, and lowered total peripheral resistance, increased arteriolar and microvascular vasodilator capacity, red cell deformability, and DBP. Engler et al. (2004) investigated the effects of dark chocolate consumption on endothelial function in adults using two cocoa products as high-flavonoid or low-flavonoid (trace flavanols) dark chocolate. The high-flavonoid group reported a significantly increased FMD of the brachial artery compared to the low-flavonoid group. Okamoto et al. (2016) demonstrated that the consumption of cocoa powder reduced central and peripheral arterial stiffness in postmenopausal women.). Studies demonstrating the effects of cocoa on vascular health are summarized in Table 1.2.

1.7.8. Amyloid-β modifying effects of cocoa flavanols in Alzheimer's disease

EC, CE, and CF extracts have been shown to reduce the Aβ induced toxicity *in vitro* and in animals. *In vitro*, screening of dietary flavonoids in primary neurons has shown EC as a potent (nM) inhibitor of amyloidogenic APP processing (Cox et al., 2015). An animal study conducted alongside further supported these findings where the oral administration of EC to TASTPM transgenic mice reduced Aβ pathology and Aβ levels. According to *in vitro* mechanistic studies, these reductions have been achieved through the indirect inhibition of the β-secretase 1 (BACE1) enzyme. By using the water maze paradigm, it has been shown that EC treatment reduced the deterioration of spatial memory induced by the $A\beta_{25-35}$ in rats (Diaz et al., 2019). In addition, EC treatment reduced the OS induced by $A\beta_{25-35}$ as indicated by decreased lipoperoxidation and ROS levels in the hippocampus. Moreover, EC treatment increased the enzymatic activity of SOD and catalase while decreasing the levels of proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Animals treated with EC showed a reduced immunoreactivity of the three chaperone proteins HSP-60, HSP-70, and HSP-90 which coincided with a decrease of dead neurons in the Cornu Ammonis 1 (CA1) region of the hippocampus. S.-M. Choi et al. (2014) demonstrated that CE and EC reduced neuronal death induced by $A\beta_{25-35}$ in cultured mouse cortical neurons. In another study, EC and CE disturbed the $A\beta_{1-42}$ fibril formation and destabilized the preformed $A\beta_{1-42}$ fibrils in human microvascular endothelial cells in a dose-dependent manner as detected by thioflavin T assay (S. B. Lee et al., 2020). Oral administration of EC to amyloid precursor protein/presenilin 1 (APP/PSEN1)

mice (a moderate pathology phase) attenuated the OS as demonstrated by reduced lipoperoxidation levels and increased glutathione reductase (GSH), SOD, catalase, and GSH/glutathione oxidized form (GSSG) ratio (Z. Zhang et al., 2016). EC treatment showed elevated BDNF protein levels in the mouse hippocampus, a pathway that is known to support neuronal survival, regulate neuroplasticity, and mediates memory fixation. EC treatment reversed the decreased phosphorylation of protein kinase B (Akt)/glycogen synthase kinase 3 (GSK-3)/cAMP response element-binding protein (CREB) pathway which shows disturbances in AD brains. In another study, feeding APP/PSEN1 transgenic mice with an EC supplemented diet reduced the total Aβ in the brain and serum by 39% and 40% respectively (Zeng et al., 2014). Furthermore, EC reduced the microgliosis and astrocytosis in the brain of Alzheimer's mice by 38 and 35% respectively as determined by immunohistochemistry. EC treated mice showed a reduced level of proinflammatory cytokine TNF-α in the plasma. J. Wang et al. (2014) studied the effects of 3 different cocoa extracts (Natural, Dutched, and Lavado) on $A\beta_{42}$ and $A\beta_{40}$ oligomerization *in vitro,* using photo-induced cross-linking of unmodified proteins technique. Lavado extract showed the highest total PP content which was 100 GAE (gallic acid equivalents) followed by natural cocoa (62 GAE) and Dutched cocoa (21 GAE). All 3 extracts reduced the Aβ oligomer formation of both $A\beta_{42}$ and $A\beta_{40}$, with Lavado extract being the most effective. Exposure to oligomeric Aβ induced a reduced long-term potentiation (LTP) response in hippocampal slices of young mice. However, LTP response was significantly increased with Lavado extract but, not with Dutched extract, suggesting that PPs are the active components that rescue synaptic transmission. In a study, acute treatment with both Aβ plaques ($A\beta_{25–35}$) and Aβ oligomers ($A\beta_{1-42}$) resulted in a loss of neurite branching and a decrease of cell viability of SH-SY5Y cells (Cimini et al., 2013). Treatment with a cocoa polyphenolic extract protected the cell viability and cell morphology from Aβ injury by modulating the BDNF signaling pathway. Heo and Lee (2005) demonstrated that $A\beta_{25–35}$ decreased the viability of rat pheochromocytoma PC12 cells. Treatment with a cocoa extract, EC, and CE increased the viability of these neuronal cells by reducing the Aβ induced cytotoxicity. The protection is partially due to the mitochondrial protection mechanisms as indicated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Additionally, cocoa extract, EC, and CE protected the cells from Aβ induced cell membrane damage as measured by lactate dehydrogenase (LDH) release and trypan blue exclusion assays. Ruotolo et al. (2020) investigated the ability of a comprehensive set of phenyl-γ-valerolactones (PVLs), the main circulating metabolites of flavan-3-ols and related dietary compounds in humans to prevent Aβ oligomers induced toxicity. These metabolites are known to originate from EC and PR B2. In this study, multiple PVLs prevented the yeast cell growth inhibition caused by a highly cytotoxic artificial polypeptide (β23) preferentially forming Aβ oligomeric species. In particular, the monohydroxylated metabolite 5-(4'-hydroxyphenyl)-γ valerolactone [(4'-OH)-PVL] showed the most effective detoxifying effects in yeast and human cells over-expressing β23 as well as in other assays involving human $A\beta_{42}$-derived Aβ oligomers, but not fibrils. In an acute mouse model of Aβ oligomers induced memory

impairment, [(4'-OH)-PVL further elicited a dose-dependent neuroprotective and anti-neuroinflammatory effect. Studies demonstrating the effects of cocoa on Aβ in AD are summarized in Table 1.2.

1.8. Therapeutic potential of cocoa in aging and related diseases

The findings of the above-discussed studies indicate that cocoa has multiple disease-modifying effects in aging-associated disease conditions. The consumption of major flavanol compounds in cocoa, cocoa flavanol extracts, and cocoa powder has been shown beneficial effects in improving longevity in several animal models. We found that CFs improved the age-associated deficits in the SkM in both animals and humans through different mechanisms including the reversal of age-related deleterious effects on modulators of muscle growth/differentiation, decreasing the protein degradation, improving the muscle microvascular blood volume, and increasing the number of muscle mitochondria and signaling for mitochondrial biogenesis. CFs attenuated the age-associated cognitive decline by improving the dentate gyrus function and neural efficiency. Additionally, EC reduced anxiety by modulating pro-BDNF and BDNF. CFs alleviated the OS induced by different oxidative agents while improving vascular health by reducing age-related endothelial dysfunction. CFs seemed to reduce the Aβ-induced toxicity via multiple mechanisms including the reduction of Aβ oligomer and fibril formation. CFs appear as potent dietary components to improve age-associated health and to reduce Aβ toxicity in AD, thus promising biomolecules in developing effective treatment regimens for age-related diseases.

1.9. Aims and rationale

The overall aim of this study was to gain insight into how cocoa, one of the richest dietary sources of PPs modifies aging and related diseases. Cocoa powder, cocoa extracts rich in PPs and the major flavanol components in cocoa have been shown to improve aging-related diseases and extend the lifespan as discussed under the literature review. However, almost all of these studies are either short-term interventions or acute effects of cocoa supplementation. Effects of long-term consumption of cocoa starting from early life on age-associated health and lifespan are not well-defined. Moreover, the impact of the timing of cocoa exposure on lifespan has not been explored. Even though cocoa has been shown to reduce Aβ-induced toxicity in AD in several animal models and *in vitro* studies, how long-term cocoa supplementation specifically affects the early and most significant signs of human AD such as memory loss and learning ability have not been properly investigated. Further, the effects of cocoa consumption on aging and Aβ-induced toxicity in AD are not described at the metabolite level. Therefore, the specific focus of each chapter describes in this thesis is as follows.

The experiments described in chapter three show how long-term consumption of cocoa affects age-associated health and lifespan in *C. elegans*. *C. elegans* is considered an exceptionally valuable model in aging-related research as of many advantages including its genetic tractability, short lifespan, and readily observable and quantifiable age-dependent physiological changes. In addition,

human gene homologs exist for ~83% of the *C. elegans* proteome and the *C. elegans* genome possesses about two-thirds of all human disease genes. Therefore, wild-type *C. elegans* was selected to investigate how long-term consumption of cocoa affects its health at different stages of life including young age (day 4), middle age (day 8), and old age (day 12). It was assumed that being filter-feeders, worms eat cocoa particles. Therefore, cocoa powder suspended in buffer was added to the bacterial diet of worms. However, the previous studies have added cocoa directly to the culture medium of worms when preparing the medium. This study describes age-associated health in terms of worm locomotion, thermotolerance, learning, short-term associative memory, and mitochondrial respiration. In addition, experiments were conducted to study the effects of cocoa on lifespan. As commercial processing is known to reduce the phenolics in cocoa powder, the total phenolics content, total flavonoids content, and the antioxidant activity of cocoa powder were determined.

It has been previously shown that early nutrition is an important factor that determines the risk of developing non-communicable diseases in late life. Moreover, nutritional supplementations at different stages of life have been reported to exhibit different effects on lifespan. As stated in the third chapter, cocoa extended the lifespan of *C. elegans* when supplemented starting from the first larval stage. Based on these findings, how the timing of cocoa exposure is important for its longevity improving effects was investigated. Therefore, the fourth chapter of the thesis reports the lifespan experiments conducted with *C. elegans* when they were supplemented with cocoa at different stages of life. Moreover, the research reported in this chapter aimed to discuss the mechanisms and pathways behind the lifespan extension effects of cocoa when supplemented starting from the first larval stage.

Memory loss and learning impairments are the first and the most crucial behavioral deficits observed in human AD. Even though some studies have shown that cocoa reduces toxic Aβ forms, evidence to show how cocoa affects behavioral deficits in AD is scanty. To study how cocoa supplementation affects these behavioral deficits, a transgenic *C. elegans* strain that expresses pan-neuronal $A\beta_{1-42}$ and middle-age onset behavioral dysfunction was employed. The fifth chapter of the thesis reports the characterization of behavioral deficits in this *C. elegans* strain and how cocoa supplementation affects these deficits.

The experiments reported in the sixth chapter discuss the key metabolomic changes associated with aging and pan-neuronal Aβ expression in *C. elegans* and the effects of cocoa supplementation using the untargeted gas chromatography-mass spectrometry (GC-MS) approach.

CHAPTER 2: General Materials and Methods

This chapter is a general summary of materials and methods used throughout the experiments reported in this thesis. Detailed methods for each experiment can be found in respective chapters.

2.1. Materials, devices, and equipment used in experiments

Commonly used equipment, devices, and consumables used throughout *C. elegans* experiments included: Thermoline Scientific refrigerated incubators (15 ºC and 20 ºC); Thermo Scientific 4 ºC fridge; Fisher and Paykel (-20 ºC) and Thermo Fisher (-80 ºC) freezers; Precellys®24 Homogenizer (Bertin technologies) and Precellys® lysing kit: 03961-1-004 (0.5mm glass beads); Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific); CLARIOstar multi-mode plate reader (BMG LABTECH); Spectro Corning Cell culture plates (35 mm, 60 mm and 90 mm); Global Science centrifuge tubes (1.5 ml, 15 ml, and 50 ml); Thermo Scientific pipettes; Interpath Aerosol Barrier Pipette Tips; Pioneer weighing scale; Thermo Scientific centrifuge; Thermo Scientific vortex; Alcohol burners; Lab made worm picks; J. Melvin Freed Brand microscope slides. Nikon upright and inverted microscopes fitted with a Nikon DS-Fi2 camera with bright light and fluorescence capabilities were utilized for monitoring the development and data collection of *C. elegans* experiments.

2.2. Maintenance of *C. elegans* strains

Wild type (N2, Bristol) *C. elegans*, mutant strains of *C. elegans* [GRU101 gnals1 (myo-2p::yfp), GRU102 gnals2 (myo-2p::YFP + unc-119p::Abeta$_{1-42}$), DA1116 *eat-2* (*ad1116*) *II*., VC199 *sir-2.1* (*ok434*) *IV*., CB4876 *clk-1* (*e2519*) *III*., EU1 *skn-1* (*zu67*) *IV*., CB1370 *daf-2* (*e1370*) *III*., ZG31 *hif-1* (*ia4*) *V*., TJ356 *daf-16(zls356) IV*. and GR1307 *daf-16* (*mgDf50*) *I*.] and *Escherichia coli* (*E. coli*) OP50 were obtained from *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). The required approval to maintain genetically modified organisms was granted by La Trobe University Institute of Biosafety Committee (LTIBC), reference number GMSC15-017. *C. elegans* were maintained at the appropriate temperatures (15 °C or 20 °C) as per instructions on nematode growth medium (NGM) plates seeded with *E. coli* OP50. NGM was prepared by dissolving 3 g NaC1, 2.5 g Bactopeptone (Difco) and 25 g Bacto-agar in 1000 ml distilled water. The mixture was autoclaved and cooled to 55 ºC, to which 1 ml of cholesterol in absolute ethanol (5 mg/ml), 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4$, and 25 ml 1M KPO_4 buffer (pH 6.0) were added in order. The bacterial food source was prepared in Luria-Bertani (LB) broth (10 g Bacto-tryptone, 5 g yeast extract, and 10 g NaCl were dissolved in 1000 ml distilled water and the pH was adjusted to 7.0 using 1M NaOH. The mixture was autoclaved and cooled to 37 °C) by adding loopful of *E. coli* OP50 and incubating at 37 °C overnight. Bacteria were pelletized by centrifugation (3500 g for 20 min) and a concentrated *E.coli* pellet was obtained. The pellet was resuspended in M9 buffer as appropriate. The two most used buffers in the experiments were M9 and S-buffer. M9 buffer was made of 6 g Na_2HPO_4, 3 g

KH_2PO_4, 5 g NaCl, and 0.25 g $MgSO_4.7H_2O$ per liter of distilled water. S-buffer was composed of 0.1M NaCl and 0.05M KPO_4 and pH was adjusted to 6.0.

2.3. Obtaining synchronous cultures of *C. elegans*

Age-synchronized worms were obtained by bleaching gravid adults. Briefly, gravid hermaphrodites were washed off from an NGM plate with M9 buffer and made the final volume to 3.5 ml. Bleaching solution (bleach: 5N NaOH = 2:1) was added to the worm suspension at a volume of 1.5 ml. The suspension was mixed by vortexing the tube for five seconds and keeping further 30 seconds on rest until all the worm bodies got dissolved. The suspension was centrifuged (1300 g for 2 min) to pellet the released eggs and the supernatant was poured off. M9 buffer was added to a final volume of 5 ml, centrifuged again and the supernatant was removed. This step was repeated at least three times to completely get rid of the bleaching solution. The egg pellet was resuspended in 3 ml of M9 and kept for 48 hours on a shaker for hatching.

2.4. Body length, area, and locomotion

Age synchronized worms were washed off from plates and collected in a 15 ml falcon tube. Once worms settled to the bottom, the supernatant was removed and 5 ml of M9 was added to wash off the food and repeated twice. Thereafter, 100 µl of worm suspension was placed on plain agar plates in the absence of food. Plates were left in the laminar flow cabinet for 5 minutes to dry out the liquid. Then, they were placed under a stereomicroscope (SMZ745T; Nikon, Japan equipped with G-AL 1.0x objective) and 30 seconds videos were captured using NIS Elements imaging software (version 5.01, Japan) and a digital camera (DS-Fi2; Nikon, Japan) with settings at 50 frames per second, 30-second duration per video and resolution of 640 x 480 pixels. The scale bar (µm) in the imaging software was used to convert pixels into µm. Worms were tracked and analyzed via WormLab 3.1 software (MBF Bioscience, Williston, VT USA). Worms tracked over a minimum of 10 seconds were included in the final analysis.

2.5. Determination of motility classes at old age

Motility classes of worms were determined as previously described (Herndon et al., 2002). Age synchronized worms were sorted by class (A, B, or C) at day 12 and re-scored for class every day until death. Animals move constantly away from the touch stimulus and leave sinusoidal tracks in the *E. coli* lawn were categorized into the class "A". Worms do not move unless prodded and leave non-sinusoidal tracks were categorized as class "B". Worms do not move even prodded but, exhibit head and/or tail movements or twitch in response to touch were categorized as class "C".

2.6. Lifespan assay

Lifespan assay was performed as described in reference (Sutphin & Kaeberlein, 2009). Age-synchronized L1 worms were added to *E.coli* seeded NGM plates with or without cocoa (the day worms were plated was considered as day 0). Worms were maintained at 20 °C. Floxuridine (FUdR) was not used to avoid progeny production and instead, worms were transferred every day onto fresh plates until they stop laying eggs (until day 9). Thereafter, worms were transferred every other day.

Both dead and live worms on each plate were counted and recorded daily until all the worms die. The plate was gently tapped and made the worms move before counting. If they did not move, the head area of the worms was tapped with a platinum transfer pick. The worm was scored as dead if it did not respond to the tapping by moving its head. All the dead worms were removed from plates daily. Around 100 worms were used per treatment and the experiments were performed in triplicates. Missing worms (due to crawling off the plates) were excluded from the analysis. Survival curves were plotted. Mean, median, and maximum lifespan was reported.

2.7. Thermotolerance assay

Age synchronized worms were grown on NGM plates until they reached the required age. Approximately 75 worms per treatment were distributed among two NGM plates seeded with *E. coli* OP50 and maintained at 35 °C. Survival was measured every hour by touch provocation. Survival curves were plotted. Mean and median survival times were reported.

2.8. *C. elegans* positive butanone learning and short-term memory assay

The assay was performed as described previously (Kauffman et al., 2011). Age-synchronized L1 worms (around 500-600 worms per plate) were cultivated on high growth media (HGM; 3 g/L NaCl, 20 g/L bactopeptone, 30 g/L agar, 5 mg/ml cholesterol in ethanol, 1 M $MgSO_4$, 1 M $CaCl_2$, 1 M K_2HPO_4 and 1 M KH_2PO_4) plates (60 mm) seeded with 1 ml of concentrated *E. coli* OP50. Once they reached adulthood (at day 4; day 1 was the day that L1 were plated), worms were washed off from the plates with M9 buffer into a 15 ml conical tube and allowed to settle by gravity. Worms were washed two more times and some worms (around 200) were immediately used for naive chemotaxis assay (described below). The rest of the worms were starved in M9 for 1 hour. At the end of the starvation period, 2 µl of 10% Butanone (in 95% ethanol) was streaked on the inside of the lid of an NGM plate (60 mm) seeded with 500 µl of *E. coli* OP50. The supernatant was removed from the 15 ml conical tube and worms were placed on the butanone-streaked plate. The plate was incubated at 20 °C for 1 hour. Thereafter, worms were washed off from the plate and collected into the 15 ml conical tube. After washing two more times, some worms (around 200) were used for the chemotaxis assay to test 1x (massed) associative learning of the food-butanone association (pairing food and butanone for a single conditioning period is meant by massed training which produces short-term associative memory that lasts ~2 hours) immediately after conditioning (0-hour time point). The remaining worms were transferred equally to two NGM plates (60 mm) seeded with 500 µl of *E. coli* OP50 and incubated at 20 °C. After 1 hour, one plate was taken, and performed the chemotaxis assay (1-hour time point) was. After two hours, the other plate was taken and performed the chemotaxis assay (2-hour time point).

2.8.1. Chemotaxis assay

The bottom of unseeded 100 mm NGM plates were marked with spots on the bottom and each side of the plate (Fig. 2.1A). At the odorant and control spot, 1 µl of 2 M NaN_3 was spotted (Fig. 2.1B). While the worms are settling in the conical tube after several M9 buffer washes as aforementioned,

1 µl each of 95% EtOH and 10% butanone were spotted at the appropriate spots on the marked assay plate (Fig. 2.1C) on top of the previously spotted NaN_3. By using a pipette with a pre-cut tip, around 200 worms were delivered to the origin of the marked assay plate (Fig. 2.1D). Excess M9 buffer was blotted out with a KimWipe to release the worms onto the assay plate (Fig. 2.1E). The chemotaxis assay plate was incubated for 1 hour at room temperature. Upon reaching the butanone and EtOH spots, worms were paralyzed (Fig. 2.1F). The number of worms at EtOH and butanone spots as well as the total number of worms on the assay plate was counted. Chemotaxis index (CI) was calculated as, CI = [(Number of worms at butanone) – (Number of worms at ethanol)]/ (Total number of worms). The learning index (LI) at different time points was calculated as, LI = Chemotaxis $index_t$ – Chemotaxis $index_{Naive}$. Dementia index (short-term associative memory loss) was calculated after 1 hour and 2 hours from the food-butanone association (Dementia $index_t$ = Learning $index_0$ - Learning $index_t$). Experiments were performed in triplicates.

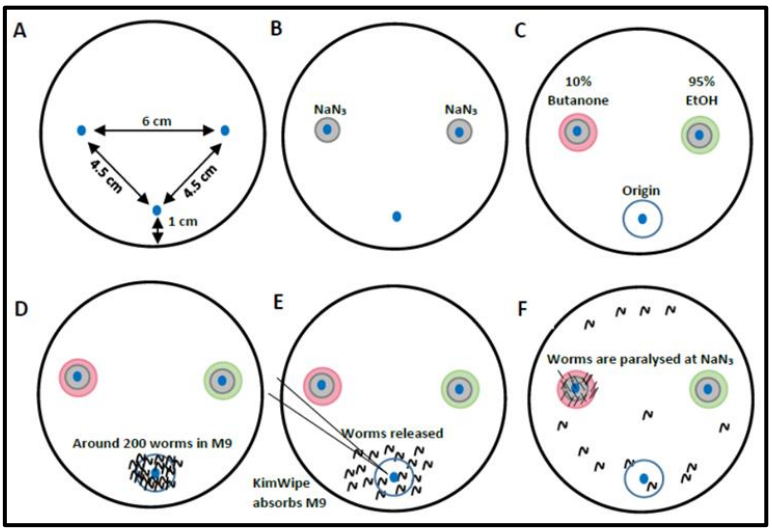

Figure 2.1. Chemotaxis assay setup, adapted from the reference (Kauffman et al., 2011). (A) Chemotaxis assay plate with spots marked for the origin of worms (bottom), butanone, and EtOH (left and right respectively). (B) 1 µl of 2M NaN3 is added to the butanone and EtOH spots. (C) 1 µl of 10% Butanone in 95% EtOH and 95% EtOH are added to the butanone and EtOH spots (left and right) (D) Worms (200) in M9 buffer are added to the origin. (E) KimWipe absorbs M9, and releases worms (F) Worms move to either butanone or EtOH spots and get paralyzed.

2.9. Mitochondrial respiration

Oxygen consumption rate (OCR) was used to investigate the mitochondrial function of *C. elegans*. To measure OCR, XFe24 Extracellular Flux Analyzer (Seahorse Bioscience) was used as described in reference (Koopman et al., 2016) and (Luz et al., 2015) with some modifications. Briefly, age-synchronized worms were washed off from plates and allowed to settle in a falcon tube. The process was repeated twice with M9 buffer and kept on an orbital shaker for 20 minutes to ensure the excretion of live *E. coli* in the gut. OCR was measured at day 4, day 8, and day 12 with 50, 35, and 35 worms in each well respectively followed by the addition of M9 to make the final volume of 500

µl per well. Sodium azide (an inhibitor of oxidative phosphorylation via inhibition of cytochrome oxidase) was used at a final well concentration of 20mM. Measurement cycles were set up as 8 cycles for both basal and drug measurements. Mix, wait, and measure cycles were set as 3, 2, and 3 minutes respectively. OCR readings were normalized based on the number of worms added per well. Basal respiration (OCR in the absence of sodium azide), non-mitochondrial respiration (OCR in the presence of sodium azide), and mitochondrial respiration (basal-non-mitochondrial) were calculated.

2.10. Quantitative staining of Aβ with Thioflavin-T

Quantitative staining of Aβ fibrils with Thioflavin-T (Th T) was performed according to a previously described method (Xin et al., 2013) with slight modifications. On day 4 and day 8 worms were collected by washing the plate with 1 ml of M9 buffer and transferred into an Eppendorf tube. The worms were pelleted by centrifugation at 14k rpm for 2 min and the supernatant was poured off. Worms were washed two more times with M9 to get rid of the food. Worm pellet was resuspended in 500 µl of M9 in a microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80 °C until use. Worms were thawed and homogenized by using Precellys®24 Homogenizer (Bertin technologies) and Precellys® lysing kit: 03961-1-004 (0.5mm glass beads) at 6400 rpm, 2x10 secs twice for a total time of 40 secs in 500 µl of M9. Homogenized worms were centrifuged at 14k rpm for 2 min and the supernatant was collected into a new tube. The concentration of total soluble protein in each sample was quantified by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit). An equal amount of protein was used from each sample and triplicates were performed for each. Each replicate was mixed with 10 µl of M9 buffer and 2 µl of 1 mM Th T (Sigma) in a final volume of 100 µl. Fluorescence intensity was measured by a fluorescence plate reader (CLARIOstar multi-mode plate reader, BMG LABTECH) using excitation at 440 nm and emission at 482 nm and averaged from three independent experiments.

2.11. DAF-16::GFP localization assay

Control and cocoa-supplemented (3 mg/ml) TJ356 daf-16(zls356) IV. worms were scored at the L4 stage for cytoplasmic, intermediate, or nuclear Green fluorescent protein (GFP) localization as described by (X. Wang et al., 2015). About 100 control and cocoa-supplemented (from the L1 stage) worms were collected at the L4 stage, washed with M9 buffer twice, and placed on a glass slide carrying 2 µl of 1M NaN_3. The sub-cellular DAF-16::GFP distribution was observed under a fluorescence microscope at 20-fold magnification. The number of worms in above mentioned three categories were counted and expressed as percentages. Three independent experiments were performed.

2.12. GC-MS analysis of *C. elegans*

2.12.1. Sampling of worms

At each time point (day 4, day 8, or day 12) worms were collected by pelleting on ice (the day L1 worms were plated was considered as day 1). The removal of bacteria and debris was carried out

using a sucrose-flotation (60 % sucrose). After collection, the worms were washed with S-buffer 5 times and partitioned into aliquots of a maximum of 0.3 ml per tube (Precellys lysing kit, Bertin Technologies). The aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

2.12.2. Extraction, polar metabolite derivatisation, and GC-MS analysis

Sample extraction and GC-MS analysis were performed according to the method described in (Afshari et al., 2020). Worm pellet was weighed (~60 mg) into a lysing tube and 500 µl of ice-cold H_2O/MeOH/$CHCl_3$ (1:3:1 v/v), containing an external standard (100 µl of $^{13}C_6$-sorbitol/$^{13}C_5$ ^{15}N-valine in water, 0.2 mg ml^{-1}) was added. The mixture was homogenized using an MP homogenizer (FastPrep®) (1 min, 4.5 m/s) and vortexed and incubated (37 °C for 15 min) in a thermomixer at 850 rpm. Then, the mixture was centrifuged at 13000 rpm for 15 min. The supernatant was transferred to a new Eppendorf tube, and 500 µl of MeOH/H_2O/$CHCl_3$ was added into the first lysing tube containing the previously freeze-dried sample. The samples were again vortexed and centrifuged at 13000 rpm for 15 min. The resulting supernatant was then transferred into the tube containing the original supernatant from the previous centrifugation. Pooled samples were then vortexed for 30 s and 50 µl aliquots of supernatant were transferred into separate glass inserts and dried *in vacuo* for subsequent trimethylsilyl (TMS) polar metabolite derivatisation using GC-MS analysis, as described below.

The dried samples were redissolved in 10 µl of 30 mg ml^{-1} methoxyamine hydrochloride in pyridine and derivatized at 37 °C for 120 min with mixing at 500 rpm. The samples were then treated for 30 min with 20 µl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 2.0 µl retention time standard mixture [0.029 % (v/v) n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane, n-hexatriacontane dissolved in pyridine] with mixing at 500 rpm. Each derivatized sample was allowed to rest for 60 min before injection. Samples (1 µl) were injected into a GC-MS system comprised of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph, and a 7000 Agilent triple-quadrupole MS (Agilent). The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)- amine (CF43). The GC was performed on a 30 m VF- 5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250 °C, the MS transfer line at 280 °C, the ion source adjusted to 250 °C, and the quadrupole at 150 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. For the polar metabolite analysis, the following temperature program was used; start at injection 70 °C, hold for 1 min, followed by a 7 °C min^{-1} oven temperature ramp to 325 °C and a final 6 min heating at 325 °C. Mass spectra were recorded at 2 scans s^{-1} with a 50-600 *m/z* scanning range.

2.12.3. Processing and analysis of GC-MS data

Both chromatograms and mass spectra were processed using Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.07.01/Build 7.1.524.0. Mass spectra of eluted compounds were identified using the NIST08 database (http://www.nist.gov) and an in-house mass

spectral library at RMIT University. All matching mass spectra were additionally verified by determination of the retention time and index in comparison to authentic standard substances. The resulting relative response ratios (area of analyte divided by area of the internal $^{13}C_6$-sorbitol standard and sample dry weight) for each analyzed metabolite were calculated. For the metabolome datasets, zero values were replaced with half of the lowest positive value in the metabolome dataset for all analyses. If a specific metabolite had multiple trimethyl silyl (TMS) derivatives, the metabolite with the greater detector response and better peak shape within the dynamic range of the instrument was selected.

Dr. Daniel Dias and Dr. Roya Afshari (RMIT University, VIC) were involved in the technical aspects and my involvement was with sample preparation, statistical analysis of data, and data interpretation.

2.13. Statistical analysis

All statistical analyses were performed in IBM SPSS® statistics software (version 24 and 27). Differences between groups for body length, mean area, locomotion, chemotaxis, worm classes, oxygen consumption rate (OCR), and metabolomic profile were analyzed using a general linear model (multivariate test). Differences between Aβ levels were calculated using a general linear model, univariate test. Differences between the number of days to reach motility group B/C from A and the differences between groups for DAF-16 localization were determined using *t*-Test (two-sample assuming equal variances). The survival function was estimated using Kaplan-Meier curves. Survival curves were compared using the log-rank (Mantel-Cox) test. The maximum lifespan was the average lifespan of the top 10 longest-lived worms. Differences between groups for maximum lifespan were determined using one-way ANOVA with post-tests (Tukey's test). $P<0.05$ was considered significant.

CHAPTER 3: Cocoa Improves Age-associated Health and Extends Lifespan in *C. elegans*

The content of this chapter has been published.

Munasinghe, M., Almotayri, A., Thomas, J., Heydarian, D., Weerasinghe, M., & Jois, M. (2021). Cocoa improves age-associated health and extends lifespan in *C. elegans*. *Nutrition and Healthy Aging, 6*, 73-86. doi:10.3233/NHA-200100.

3.1. Abstract

Background: Cocoa, one of the richest dietary sources of polyphenols has been studied for its health-promoting effects, but how long-term consumption of cocoa affects age-associated health and lifespan is not well defined.

Objective: The objective of this study was to determine the effects of long-term cocoa consumption on age-associated health and lifespan in *C. elegans*.

Methods: The standard *E. coli* OP50 diet of wild-type *C. elegans* was supplemented with cocoa powder starting from the L1 stage until they die. Body length and area were measured as indicators of worm nutrition. Age-associated health was determined at different stages of life as day 4, day 8, and day 12 using worm locomotion, thermotolerance, cognition, and mitochondrial function. In addition, lifespan was evaluated.

Results: Cocoa improved age-associated decline in neuromuscular function. Both mean and median lifespan were extended by cocoa supplementation. However, the maximum lifespan was not affected. Cocoa showed beneficial effects on thermotolerance at all ages (more prominent effects at young (day 4) and middle (day 8) age. Further, consumption of cocoa improved age-related learning deficits, short-term memory loss, and mitochondrial dysfunction.

Conclusions: Long-term cocoa consumption seemed to improve age-associated health and extends the lifespan in *C. elegans.*

Keywords: cocoa, aging, lifespan, *C. elegans*, antioxidants, polyphenols

3.2. Introduction

Aging is the primary risk factor for many chronic diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases (López-Otín et al., 2013). Mitochondrial dysfunction which is characterized by the accumulation of mitochondrial DNA (mtDNA) mutations and the increased production of reactive oxygen species (ROS) is considered to be one of the key hallmarks in aging (Srivastava, 2017). The free radical theory of aging suggests that increased ROS production in mitochondria with age causes oxidative damage to cellular macromolecules, thereby further increasing ROS production and the accumulation of molecular damage (Harman, 1972). The imbalance between excessive ROS production and limited anti-oxidant defenses leads to oxidative stress and ultimately aging and related diseases (Turrens, 2003).

Diet-derived antioxidants have recently gained popularity for their protective/preventive roles against chronic diseases (Féart et al., 2010; Morris et al., 2015). Epidemiological studies suggest that polyphenols are the major source of antioxidants that exert the preventive/protective role (Cassidy et al., 2012; Grosso et al., 2018; Scholey et al., 2010). Cocoa and its products are known to be rich sources of polyphenols which significantly contributes to the total antioxidant capacity of the diet (K. W. Lee et al., 2003; Vinson et al., 2006). Consumption of cocoa or related products including chocolates has been reported to have aging-related health-promoting effects including improvements in cognitive function (D. T. Field et al., 2011; Mastroiacovo et al., 2015; Scholey et al., 2010), cardiovascular aging (Gröne et al., 2020), oxidative stress (Petyaev et al., 2018), physical performance, and quality of life (Munguia et al., 2019). However, most of these studies are either short-term interventions or acute effects of cocoa supplementation. Effects of long-term consumption of cocoa starting from early life on healthspan and lifespan are not well-defined.

Caenorhabditis elegans (*C. elegans*) has been extensively used in aging research due to its attractive features including short lifespan, availability of mutants, and many readily observable and quantifiable aging-associated changes including tissue degeneration, decreased movement, accumulation of fluorescent material, and reduced pharyngeal pumping (C. Huang et al., 2004). Particularly, its short lifespan of approximately 17 days at 20 °C makes *C. elegans* an attractive model to study the long-term effects of dietary interventions. Therefore, this study aimed to determine the effects of long-term cocoa consumption on age-associated health and lifespan using *C. elegans* as the model organism.

3.3. Materials and methods

3.3.1. Strains and cultural conditions

Wild type (N2, Bristol) *C. elegans* and *Escherichia coli* (*E. coli*) OP50 were obtained from *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). Worms were maintained on nematode growth medium plates carrying a lawn of *E. coli* OP50. Except for the thermotolerance assay, all the experiments were carried out at 20 °C.

3.3.2. Cocoa treatment

In this study, we used commercially available cocoa powder which was an unsweetened dry powder processed with alkali. A cocoa suspension was made to the desired final concentration in M9 buffer to make it spreadable. For all the experiments except lifespan, a single dose of cocoa which is 5 mg of cocoa powder suspended in 1 ml of M9 was used. For the lifespan experiment, five different doses of cocoa were prepared by suspending 1 mg, 2 mg, 3 mg, 4 mg, and 5 mg cocoa in 1 ml of M9 buffer. Concentrated *E. coli* OP50 was prepared by suspending 1 g of *E. coli* pellet in 16 ml of M9 buffer. Concentrated *E. coli* was first added to nematode growth medium (NGM) plates and was grown overnight at 20 °C. The cocoa suspension was added on the top of the *E. coli* lawn at a ratio of 1:2 volume, *E. coli*: cocoa suspension (for small plates: 100 µl of *E.coli* and 200 µl of cocoa,

for medium plates: 200 µl of *E .coli* and 400 µl of cocoa). Worms received cocoa starting from the L1 stage.

3.3.3. Obtaining synchronous cultures

Age-synchronized worms were obtained by bleaching gravid adults. Briefly, gravid hermaphrodites were washed off from an NGM plate with M9 buffer and made the final volume to 3.5 ml. Bleaching solution (bleach: 5N NaOH = 2:1) was added to the worm suspension at a volume of 1.5 ml. The suspension was mixed by vortexing the tube for five seconds and keeping further 30 seconds on rest until all the worm bodies got dissolved. The suspension was centrifuged (2 min at 1300 g) to pellet the released eggs and the supernatant was poured off. M9 buffer was added to a final volume of 5ml, centrifuged again and the supernatant was removed. This step was repeated at least three times to completely get rid of the bleaching solution. The egg pellet was resuspended in 3 ml of M9 and kept for 48 hours on a shaker for hatching.

3.3.4. Body length, area, and locomotion

Age synchronized worms were washed off from both treatment and control plates (at day 4, day 8, and day 12) and collected in a 15 ml falcon tube. Once worms settled to the bottom, the supernatant was removed and 5 ml of M9 was added to wash off the food and repeated twice. Thereafter, 100 µl of worm suspension was placed on plain agar plates in the absence of food. Plates were left in the laminar flow cabinet for 5 minutes to dry out the liquid. Then, they were placed under a stereo microscope (SMZ745T; Nikon, Japan equipped with G-AL 1.0x objective) and 30 seconds videos were captured using NIS Elements imaging software (version 5.01, Japan) and a digital camera (DS-Fi2; Nikon, Japan) with settings at 50 frames per second, 30-second duration per video and resolution of 640 x 480 pixels. The scale bar (µm) in the imaging software was used to convert pixels into µm. Worms were tracked and analyzed via WormLab 3.1 software (MBF Bioscience, Williston, VT USA). Worms tracked over a minimum of 10 seconds were included in the final analysis. Mean body length (µm), mean area (μm^2), mean speed, maximum speed, peristaltic speed (µm), and maximum amplitude (µm) were reported. According to the WormLab software, speed is defined as the distance per second traveled by the worm along its central axis. Peristaltic speed is defined as the peristaltic track length (length of forward motion minus length of reverse motion) divided by time. Maximum amplitude is the maximum centroid displacement over an entire track.

3.3.5. Lifespan assay

Lifespan assay was performed as described in reference (Sutphin & Kaeberlein, 2009). Floxuridine (FUdR) was not used to avoid progeny production and instead, worms were transferred every day onto fresh plates until they stop laying eggs (until day 9). Thereafter, worms were transferred every other day. Worms that crawled off from the plates were excluded from the analysis. Survival curves were plotted. Mean, median, and maximum lifespan was reported.

3.3.6. Thermotolerance

Worms were grown on NGM plates until they reached the required age (day 4 or day 8 or day 12). Approximately 75 worms per treatment were distributed among two NGM plates seeded with *E. coli* and maintained at 35 °C. Survival was measured every hour by touch provocation. Survival curves were plotted. Mean and median survival times were reported.

3.3.7. Learning and short-term associative memory

Naive chemotaxis, learning, and short-term associative memory were determined based on *C. elegans* positive butanone learning assay (Kauffman et al., 2011).

Age-synchronized worms (approximately 1000) were grown on high-growth media plates with food until they reached the desired age. Chemotaxis assay (Fig. 3.1) was performed with approximately 200 worms. Rest were starved for 1 hour and at the end of the starvation period, worms were associated with food and chemo-attractant butanone for 1 hour. Chemotaxis assay was repeated to test 1x (massed) associative learning immediately after conditioning (t=0 time point). The rest of the worms were transferred equally to two NGM plates seeded with *E. coli* and incubated at 20 °C. After 1 hour, and 2 hours from the food-butanone association, chemotaxis assay was performed with the worms on two plates (t=1 hr and t=2 hrs time points). Chemotaxis index (CI) was calculated as, CI = [(Number of worms at butanone) – (Number of worms at ethanol)]/ (Total number of worms). The learning index (LI) at different time points was calculated as, LI = Chemotaxis $index_t$ – Chemotaxis $index_{Naive}$. Dementia index (short-term associative memory loss) was calculated after 1 hour and 2 hours from the food-butanone association (Dementia $index_t$ = Learning $index_0$ - Learning $index_t$).

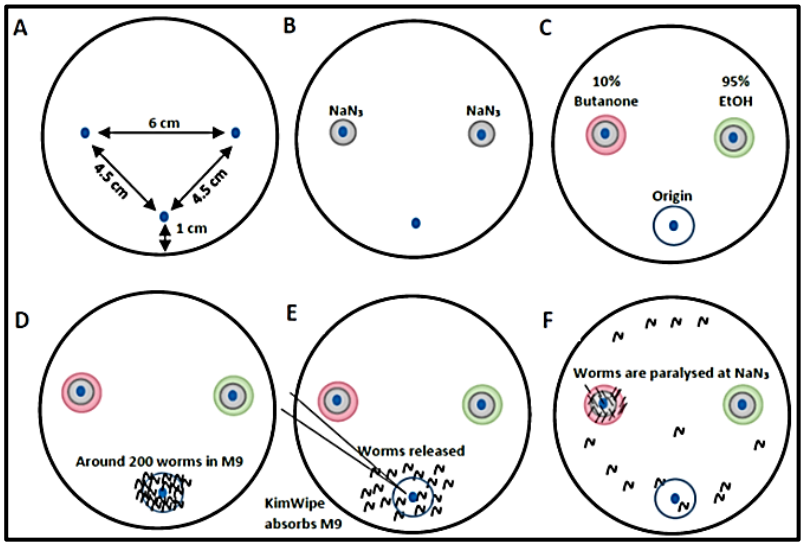

Figure 3.1. Chemotaxis assay setup, adapted from the reference (Kauffman et al., 2011). (A) Chemotaxis assay plate with spots marked for the origin of worms (bottom), butanone, and EtOH (left and right respectively). (B) 1 µl of 2M NaN_3 is added to the butanone and EtOH spots. (C) 1 µl of 10% Butanone in 95% EtOH and 95% EtOH are added to the butanone and EtOH spots (left

and right) (D) Worms (200) in M9 buffer are added to the origin. (E) KimWipe absorbs M9, and releases worms (F) Worms move to either butanone or EtOH spots and get paralyzed.

3.3.8. Mitochondrial respiration

Oxygen consumption rate (OCR) was used to investigate the mitochondrial function of *C. elegans*. To measure OCR, XFe24 Extracellular Flux Analyzer (Seahorse Bioscience) was used as described in references (Koopman et al., 2016) and (Luz et al., 2015) with some modifications. Briefly, age-synchronized worms were washed off from plates and allowed to settle in a falcon tube. The process was repeated twice with M9 buffer and kept on an orbital shaker for 20 minutes to ensure the excretion of live *E. coli* in the gut. OCR was measured at day 4, day 8, and day 12 with 50, 35, and 35 worms in each well respectively followed by the addition of M9 to make the final volume of 500 µl per well. Sodium azide (an inhibitor of oxidative phosphorylation via inhibition of cytochrome oxidase) was used at a final well concentration of 20mM. Measurement cycles were set up as 8 cycles for both basal and drug measurements. Mix, wait, and measure cycles were set as 3, 2, and 3 minutes respectively. OCR readings were normalized based on the number of worms added per well. Basal respiration (OCR in the absence of sodium azide), non-mitochondrial respiration (OCR in the presence of sodium azide), and mitochondrial respiration (basal-non-mitochondrial) were calculated.

3.3.9. Analysis of cocoa powder

Cocoa powder was analyzed for total phenolic (TP) and total flavonoid (TF) contents and antioxidant activity. Before analysis, cocoa powder was defatted by extracting it four times with hexane (AOAC, 1995). Total phenolics were determined using the spectrometric analysis with Folin-Ciocalteu's phenol reagent (D. O. Kim et al., 2003). Following the same procedure, a standard curve for TP was prepared using gallic acid standard solution (0-100 mg/l). TP in the cocoa extract was expressed as milligrams of gallic acid equivalents (GAE) per gram of cocoa powder. The sample was analyzed in six replicates. TF content was determined according to a colorimetric assay (Zhishen et al., 1999). Quantification of TF was done using catechin as the standard compound. TF was expressed as milligrams of catechin equivalents (CE) per gram of cocoa powder. The sample was analyzed in six replicates. Antioxidant activity of the cocoa extract was determined according to the ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)] assay (Re et al., 1999).

3.3.10. Statistical analysis

All statistical analyses were performed in IBM SPSS® statistics software (version 24). Data were expressed as mean ± standard error of the mean (SEM). Differences between groups for body length, mean area, locomotion, chemotaxis, and OCR were analyzed using general linear model (multivariate test). The survival function was estimated using Kaplan-Meier curves. Survival curves were compared using the log-rank (Mantel-Cox) test. The maximum lifespan was the average lifespan of the top 10 longest-lived worms. Differences between groups for maximum lifespan were

determined using one-way ANOVA with post-tests (Tukey's test). $P<0.05$ was considered significant.

3.4. Results

3.4.1. Effect of cocoa supplementation on body length and area

We measured the body length and area at two-time points as day 4 (1st day of adulthood) to represent young age and day 8 (5th day of adulthood), the middle age at which they have fully grown. Analysis of body length showed that cocoa-supplemented worms were significantly longer (Fig. 3.2A) than control worms at both day 4 and day 8 ($P<0.05$). In addition, cocoa-supplemented worms were significantly thicker (Fig. 3.2B) than control worms at day 8 as indicated by the mean area ($P<0.05$).

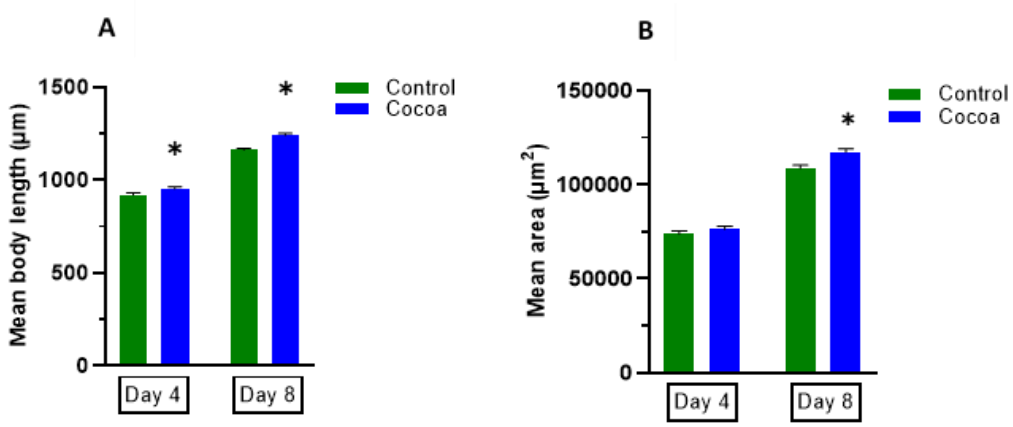

Figure 3.2. Body length (µm) and area (µm^2) of control and cocoa-supplemented wild type (N_2) *C. elegans* at day 4 and day 8. Body length (µm) and area (µm^2) were measured at two different time points as day 4 (1st day of adulthood) and day 8 (5th day of adulthood). Experiments were performed in triplicate. Values are expressed in mean ± SEM (n=52 for control at both days 4 and 8 and n=53 for cocoa at both days 4 and 8). *$P<0.05$ for the indicated comparison (calculated using the multivariate test, general linear model). (A) Cocoa supplemented worms were significantly longer than control worms at both day 4 and day 8 ($P<0.05$). (B) Cocoa-supplemented worms were significantly thicker than control worms at day 8 as indicated by the mean area ($P<0.05$).

3.4.2. Effect of cocoa supplementation on locomotion

C. elegans showed a reduction in mean speed over time (9.0% at day 8 and 29.0% at day 12 compared to day 4, Fig. 3.3A). Even though cocoa-supplementation significantly reduced the mean speed at day 8 ($P<0.05$, 9.8%,), it did not affect the mean speed at day 4 and day 12. At day 12, worms showed a reduction in maximum speed compared to both day 4 and day 8 (31.4%, 37.0% respectively, Fig. 3.3B). Cocoa supplementation increased the maximum speed of worms at a later stage of life (day 12) by 12% however, it was not statistically significant. Peristaltic speed of worms was decreased over time (47.3% at day 8 and 79.4% at day 12 compared to day 4, Fig. 3.3C). There was an improvement in peristaltic speed with cocoa supplementation (20%) in later life (at day 12).

Worms showed an increase in maximum amplitude (Fig. 3.3D) at day 8 compared to day 4 (36.6%), but it was decreased at day 12 compared to day 8 (20.6%). Interestingly, cocoa supplementation significantly increased the maximum amplitude of worms at day 12 (P<0.05, 8.8%).

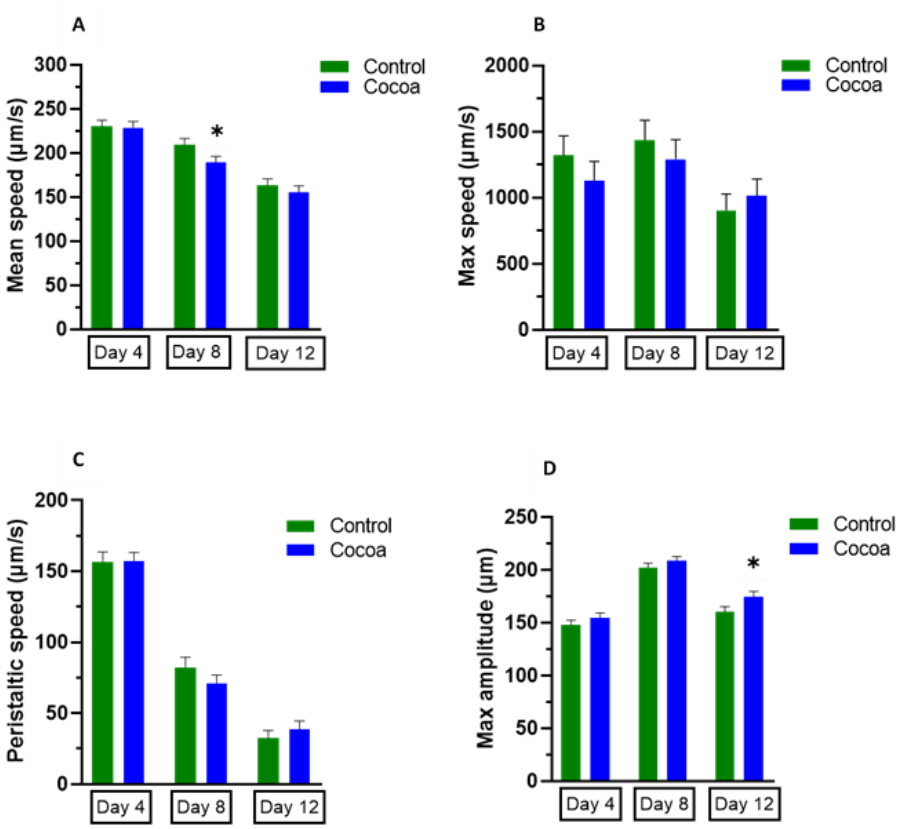

Figure 3.3. Locomotion parameters of control and cocoa-supplemented wild type (N_2) *C. elegans* at days 4, 8 and 12. Mean speed (µm/s), maximum speed (µm/s), peristaltic speed (µm/s), and maximum amplitude (µm) were measured at three different time points as day 4 (1st day of adulthood), day 8 (5th day of adulthood), and day 12 (9th day of adulthood). Experiments were performed in triplicate. Values are expressed in mean ± SEM (n=52 for control and n=53 for cocoa at all 3-time points). *P<0.05 for the indicated comparison (calculated using the multivariate test, general linear model). (A) Worms showed a reduction in mean speed over time. Cocoa-supplemented worms showed a significantly reduced mean speed at day 8 (P<0.05), but there was no effect significant at day 4 and day 12. (B) Worms showed a reduction in maximum speed at day 12 compared to both day 4 and day 8. Cocoa supplementation increased the maximum speed of worms at the later stage of life (day 12). (C) Peristaltic speed of worms was decreased over time. There was an improvement in peristaltic speed with cocoa supplementation in later life (at day 12). (D) Worms showed an increase in maximum amplitude at day 8 compared to day 4, but it was decreased at day 12 compared to day 8. Cocoa supplementation significantly increased the maximum amplitude of worms at day 12 (P<0.05).

3.4.3. Effect of cocoa supplementation on lifespan

We performed a dose-response experiment for the lifespan where the worm diet was supplemented with cocoa at 1 mg, 2 mg, 3 mg, 4 mg, and 5 mg concentrations. The wild-type (N_2) worms grown under our laboratory conditions at 20 °C had a mean lifespan of 13.5 days, a median lifespan of 13 days, and a maximum lifespan of 16.7 days (Fig 3.4, Table 3.1). The mean lifespan of worms was significantly increased by cocoa supplementation at 3 mg/ml and 5 mg/ml doses by 8.3% and 5.9% respectively (P<0.05). The median lifespan was increased by cocoa at 3 mg/ml, 4 mg/ml, and 5 mg/ml doses (P<0.05, all 3 doses by 7.8%). The maximum lifespan was not affected by any of the cocoa doses.

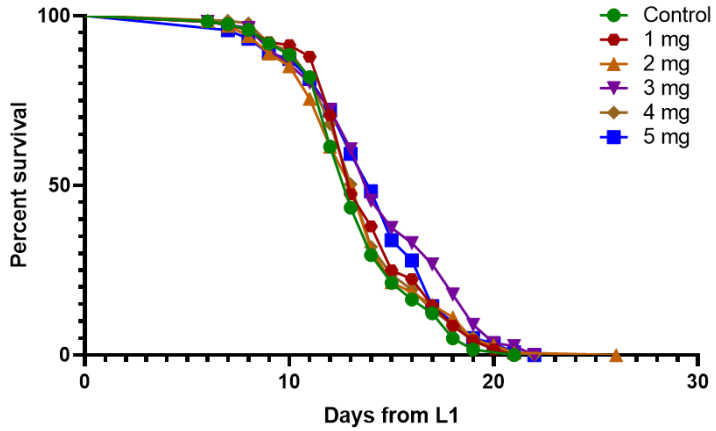

Figure 3.4. Survival curves for control and cocoa-supplemented wild type (N_2) *C. elegans*. Experiments were performed in triplicate (n=122 for control, n=116 for 1 mg/ml, n=135 for 2 mg/ml, n=112 for 3 mg/ml, n=135 for 4 mg/ml and n=118 for 5 mg/ml). Differences between groups for mean and median lifespans were calculated using the log-rank (Mantel-Cox) test and maximum lifespan using one-way ANOVA (Tukey's test). Cocoa supplementation at a dose of 3mg/ ml and 5 mg/ml significantly increased both mean (P<0.05, 8.3% and 5.9%) and median (P<0.05, 7.8%) lifespan. Cocoa at 4mg/ml dose also significantly increased the median lifespan (P<0.05, 7.8%). However, maximum lifespan was not affected by any cocoa dose.

Table 3.1. The mean, median, and maximum lifespan of different doses of cocoa-supplemented worms

Cocoa dose (mg/ml)	Mean lifespan	% extension compared to control	Median lifespan	% extension compared to control	Maximum lifespan
0	13.5±0.26		13±0.25		16.7±0.38
1	14.0±0.28		13±0.28		16.9±0.46
2	13.6±0.29		13±0.28		17.3±0.53
3	14.6±0.35*	8.3	14±0.41*	7.8	18.2±0.46
4	13.8±0.26		14±0.22*	7.8	17.4±0.43
5	14.3±0.32*	5.9	14±0.36*	7.8	18.0±0.40

*Statistically significant (P<0.05) compared to the control group

3.4.4. Effect of cocoa supplementation on thermotolerance

We measured the survival of control and cocoa-treated worms at 35 °C. At both day 4 and day 8, control worms showed a mean survival time of 8.2 hours and a median survival time of 8 hours (Fig. 3.5, Table 3.2). At day 12, the mean and median survival time for control worms was 7.1 hours and 7 hours respectively. Both mean and median survival times of control worms were significantly reduced at day 12 compared to both day 4 and day 8 (P<0.05). Cocoa supplementation significantly increased the mean survival time by 15.0% at day 4, 11.1% at day 8, and 3.3% at day 12 (P<0.05). Moreover, cocoa supplementation significantly increased the median survival time by 12.5% at both day 4 and day 8 (P<0.05). Results revealed that the cocoa effect is more prominent at the early stages of life than in later stages.

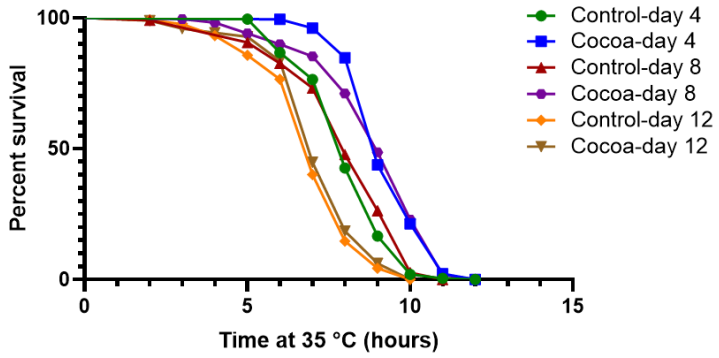

Figure 3.5. Survival curves at 35 °C for control and cocoa-supplemented wild type (N_2) *C. elegans* at day 4, day 8, and day 12. Experiments were performed in triplicate [n=204 (day 4); n=224 (day 8); n=252 (day 12) for control and n=178 (day 4); n=218 (day 8); n=194 (day 12) for cocoa]. Differences between survival curves were calculated using the log-rank (Mantel-Cox) test. Both mean and median survival times of control worms were significantly reduced at day 12 compared to both day 4 and day 8 (P<0.05). Cocoa supplementation significantly increased the mean survival time at day 4, day 8, and day 12 (P<0.05, 15.0%, 11.1%, and 3.3% respectively). Cocoa supplementation significantly increased the median survival time at both day 4 and day 8 (P<0.05, 12.5%).

Table 3.2. Mean and median survival times for cocoa-supplemented worms

Day	Treatment	Mean survival time	% extension compared to control	Median survival time	% extension compared to control
4	Control	8.2±0.09		8±0.10	
	Cocoa	9.5±0.08*	15.0	9±0.09*	12.5
8	Control	8.2±0.11		8±0.14	
	Cocoa	9.1±0.12*	11.1	9±0.14*	12.5
12	Control	7.1±0.10		7±0.09	
	Cocoa	7.4±0.10*	3.3	7±0.09	

*Statistically significant (P<0.05) compared to the control group

3.4.5. Effect of cocoa supplementation on learning and short-term associative memory

At day 8, worms showed a significant reduction in chemotaxis towards butanone even after conditioning (87.2% reduction in naive chemotaxis and 86.0% reduction in learning index). Therefore, we determined chemotaxis behavior only at day 4 and day 8 as it was not detectable at day 12. There was no significant effect of cocoa supplementation on naive chemotaxis at both day 4 and day 8 (Fig. 3.6A). However, cocoa supplementation significantly increased the learning index of worms at day 8 (P<0.05, Fig. 3.6B). As the learning index of control worms at day 8 was lower, we did not measure the short-term memory loss (dementia index) at day 8. However, cocoa-supplemented worms showed a reduction (27%) in dementia index 2 hours after conditioning at day 4 which was not statistically significant (Fig. 3.6C).

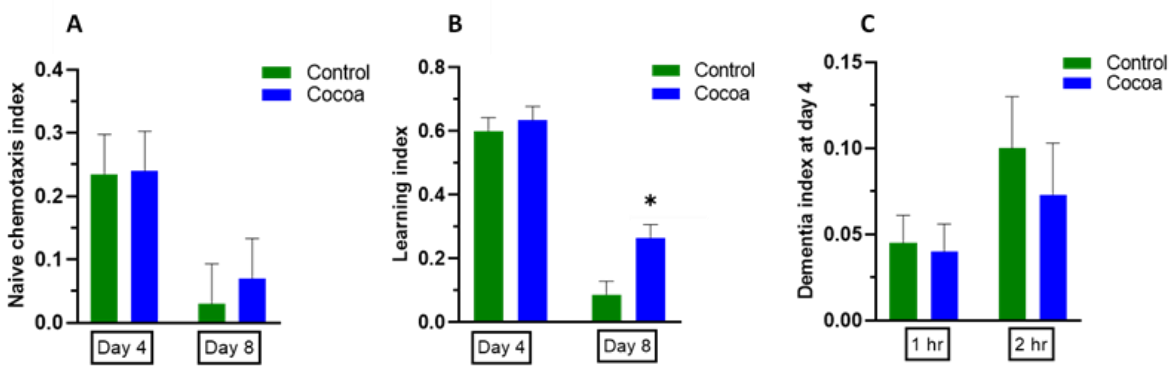

Figure 3.6. Naive chemotaxis index, learning index, and dementia index (short-term memory loss) of control and cocoa-supplemented wild type (N_2) *C. elegans* at days 4 and 8. Chemotaxis assay was performed for well-fed, synchronized worms, and naive chemotaxis index was calculated; chemotaxis index (CI) = [(number of worms at butanone) – (number of worms at ethanol)]/ [total number of worms on the plate]. Well-fed, synchronized worms were starved for 1 hour, then fed in the presence of 10% butanone for 1 hour. Worms were tested immediately after food-butanone association for 1 hour for their learning; learning Index (LI) = chemotaxis $index_t$ – chemotaxis $index_{Naive}$. Dementia index (short-term associative memory loss) was measured after 1 hour and 2 hours from the food-butanone association without exposure to butanone again (Dementia $index_t$ = Learning $index_0$ - Learning $index_t$). Four independent experiments were performed (n≥50 worms per group). Values are expressed as mean ± SEM. $^*P$<0.05 for the indicated comparison (calculated using the multivariate test, general linear model). (A) There was no significant effect of cocoa supplementation on naive chemotaxis index at both days 4 and 8. (B) Cocoa supplementation significantly increased the learning index at day 8 (P<0.05). (C) Cocoa-supplemented worms showed no significant difference in dementia index at day 4.

3.4.6. Effect of cocoa supplementation on mitochondrial respiration

Both basal (Fig. 3.7A) and mitochondrial respiration (Fig. 3.7B) were significantly decreased in control worms at day 12 compared to day 4 (P<0.05, 15.0%, and 26.5% respectively). Cocoa-

supplementation significantly increased both basal and mitochondrial respiration at both day 4 (P<0.05, 38.0% and 35.4% respectively) and day 12 (P<0.05, 19.4% and 41.4% respectively). However, cocoa supplementation significantly decreased mitochondrial respiration at day 8 (P<0.05). Non-mitochondrial respiration (Fig. 3.7C) in control worms was significantly higher at day 12 compared to day 4 (P<0.05, 48.4%). Cocoa supplementation significantly reduced the non-mitochondrial respiration at day 12 to reach similar levels to day 4 (P<0.05, 40.2%).

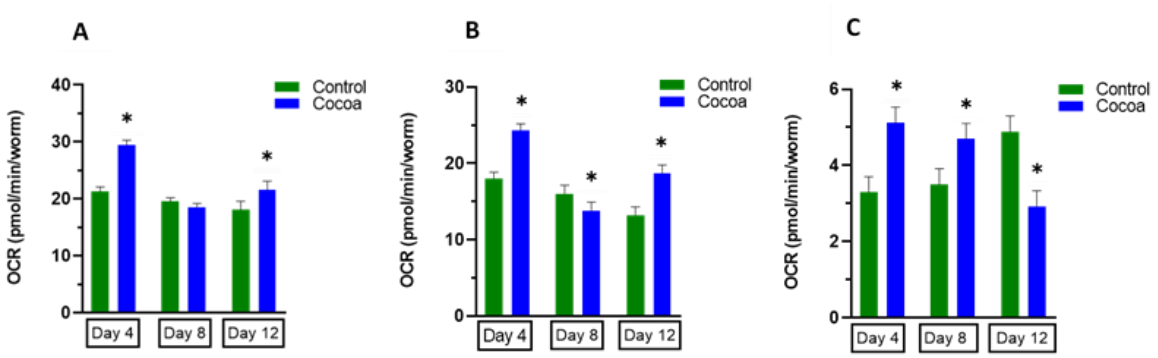

Figure 3.7. Basal respiration, mitochondrial respiration (basal-sodium azide), and non-mitochondrial respiration of control and cocoa-supplemented wild type (N_2) *C. elegans* at days 4, 8 and 12. Values are expressed in mean ± SEM (n=50 per well at days 4 and 8, n=35 per well at day 12; 4 wells/treatment at all 3-time points). $^{*}P$<0.05 for the indicated comparison compared to control at each time point (calculated using the multivariate test, general linear model). Both basal (A) and mitochondrial respiration (B) were significantly decreased in control worms at day 12 compared to day 4 (P<0.05). Cocoa supplementation significantly increased both basal and mitochondrial respiration at both day 4 and day 12 (P<0.05). However, cocoa supplementation significantly decreased mitochondrial respiration at day 8 (P<0.05). Non-mitochondrial respiration (C) in control worms was significantly higher at day 12 compared to day 4 (P<0.05). Cocoa supplementation significantly reduced the non-mitochondrial respiration at day 12 to reach similar levels to day 4 (P<0.05).

3.4.7. Total phenolics content, total flavonoids content, and the antioxidant activity of cocoa powder

The TP content of defatted cocoa powder was 27.01 mg GAE/g. The TF content was 10.13 mg CE/g, and the antioxidant activity was 7.84 mg GAE/g.

3.5. Discussion

The effect of long-term cocoa supplementation on age-associated health and lifespan was evaluated in the model organism *C. elegans*. The worm diet was supplemented with cocoa powder starting from the L1 stage until they die. Body length and area were measured as indicators of growth and development. Moreover, a reduced body size could also indicate dietary restriction in *C. elegans* (Lenaerts et al., 2008). Results indicated that cocoa provided better nutrition to worms as cocoa-supplemented worms were longer and thicker than control worms. It has been widely reported that

aging in *C. elegans* is accompanied by sarcopenia which is the progressive deterioration in muscle mass and strength (Glenn et al., 2004). In clinical settings, sarcopenia in humans is diagnosed with reduced gait speed, grip strength, and muscle mass (Zembroń-Łacny et al., 2014). In *C. elegans*, the speed of movement is analogous to the gait speed of humans. Moreover, maximum speed is believed to be one of the powerful metrics of *C. elegans* health as it is highly correlated with longevity (Hahm et al., 2015). Apart from movement speed, the maximum amplitude has been identified as a marker of *C. elegans* muscle strength. The mutants showing deficiencies in components of sarcomeres that transmit muscle contraction into locomotion have differences in bending amplitude, but not in locomotion (Nahabedian et al., 2012). Therefore, we measured mean speed, maximum speed, and maximum amplitude as well-known aging-dependent markers of *C. elegans* (Rollins et al., 2017). In addition, we observed an age-dependent reduction in peristaltic speed which is the speed of forward movement. Hence, we used it as another marker of aging. All four parameters showed an age-dependent decline. Even though it was not statistically significant, cocoa supplemented worms showed an increase in maximum speed (12%) and peristaltic speed (20%) at day 12 (old age). Cocoa supplementation improved the maximum amplitude which is an indicator of muscle strength at old age (day 12). The improvements in locomotion are consistent with previous results as epicatechin which is the most abundant flavonoid in cocoa has been reported to improve muscle performance in mice and humans by stimulating mitochondrial biogenesis (Moreno-Ulloa et al., 2018), enhancing the biosynthesis of capillaries (Nogueira et al., 2011), enhancing myogenic differentiation (Gutierrez-Salmean et al., 2014) and reversing the age-related degeneration in the skeletal muscle (Si et al., 2019). In addition, catechin which is another flavanol found in cocoa has also been reported to have similar effects on skeletal muscle (P. Li et al., 2020). Flavanol procyanidin in cocoa has also been shown to improve skeletal muscle function (M. Xu et al., 2020). Interestingly, cocoa supplementation seemed to be beneficial on locomotion only at later stages of life. We assume that this beneficial effect of cocoa supplementation at old age was achieved by reversing/reducing the age-related degeneration mechanisms in the skeletal muscle. The degenerative processes reversed/reduced by cocoa may include mitochondrial dysfunction (Short et al., 2005) and increased ROS production (Broome et al., 2006) which are known to cause sarcopenia.

Cocoa supplementation improved both the mean and median lifespan of *C. elegans*, but the improvement was not dose-dependent. Interestingly, antioxidants including N-acetylcysteine, vitamin C, and resveratrol induce an inverted U-shaped dose-response relationship between ROS levels and lifespan (Desjardins et al., 2017). No significant effect was found on the maximum lifespan. A previous lifespan study has shown cocoa-supplementation to increase the average lifespan of *Drosophila melanogaster* under normoxia (Bahadorani & Hilliker, 2008). In the same study, cocoa increased the average lifespan of *D. melanogaster* under hyperoxia or in a Cu/Zn-superoxide dismutase-deficient background, suggesting that it has strong antioxidant properties.

Moreover, it has been previously reported that polyphenol-rich extracts of cocoa powder increased the lifespan of rats (Bisson et al., 2008), *Saccharomyces cerevisiae* (Baiges & Arola, 2016), and *Oryzias latipes* (Sánchez-Sánchez et al., 2018). Conversely, catechin and epicatechin (two major flavanols in cocoa) showed no improvement in the mean lifespan of *C. elegans* in another study (Surco-Laos et al., 2012). However, epicatechin increased the maximum lifespan of *C. elegans* in the same study.

Survival under heat stress (thermotolerance) is dependent on the mechanisms contributing to proteostasis (Balch et al., 2008). Aging alters proteostasis (López-Otín et al., 2013) thereby, declining the ability to cope with heat stress. Our study demonstrated that worms at old age (day 12) have a significantly reduced survival rate compared to young age (day 4) and middle age (day 8). Exposure to cocoa powder significantly increased the survival rate at all three ages, but the effect was less prominent with old worms (day 12). Therefore, results indicate that cocoa may improve the ability of *C. elegans* to deal with poor living environments at young and middle age better than old age. Even though there are no previous studies to determine the effect of cocoa on the thermotolerance of *C. elegans*, there are few studies that show the effect of some polyphenol compounds in cocoa. In one such study, the effects of epicatechin and catechin (two major flavanols in cocoa) on the thermal stress of *C. elegans* was evaluated (Surco-Laos et al., 2012). Results indicate both epicatechin and catechin to improve survival in early adult stages (worms at 1st and 6th day of adulthood), with relatively greater protective effects in older (6th day of adulthood) than in young worms. However, our study showed better protection at the early stage (1st day of adulthood) which was gradually declined with age. In another study, the effect of epicatechin in aged worms (10th and 17th day of adulthood) was evaluated (Ayuda-Durán et al., 2019). Epicatechin significantly increased the survival of worms, but the protective effect of epicatechin against heat stress was not increased in more aged worms (at the 17th day of adulthood).

Loss of cognitive function is one of the most devastating age-related declines in humans. *C. elegans* chemotaxis behavior, a neuronally-controlled process has been widely used to study the functional changes in neurons (Kauffman et al., 2010; G. M. Stein & Murphy, 2014). Here, we used *C. elegans* positive butanone learning and short-term memory assay (Kauffman et al., 2011) to determine age-related changes in neuronal function and the effects of cocoa supplementation. Naive chemotaxis index which is the natural response of *C. elegans* towards butanone and the learning index which is an indicator of the extent that which worms learn food-butanone association was measured. Moreover, we measured the short-term memory loss/dementia index (memory loss within 2 hours after conditioning). There was no significant effect of cocoa supplementation at young (day 4) and middle age (day 8) on the naive chemotaxis index. However, Cocoa supplementation significantly improved the learning ability of *C. elegans* at middle age (day 8). As cocoa supplementation did not affect motility parameters at day 8, the improvement in learning can be independent of locomotory improvements. Even though the improvement of dementia index at young age (day 4)

with cocoa was not statistically significant, there was a 28% reduction after 2 hours from conditioning which suggests that cocoa can improve age-induced memory loss. No previous studies were found on the effect of cocoa on the age-related decline of cognition in *C. elegans*. However, several animal models and human trials have examined the effects of cocoa on the onset of age-related cognitive deficits. Administration of a cocoa polyphenolic extract improved the cognitive impairments in rats caused by heat exposure (Rozan et al., 2007). In a similar study, high-flavanol cocoa has improved cognitive performance in aged rats (Bisson et al., 2008). Consumption of cocoa-related products like chocolate has reduced the cognitive decline in adults who were not cognitively impaired (Moreira et al., 2016). Further, it has been reported that the consumption of a drink containing cocoa flavanols improves cognition in older people with mild cognitive impairment (Desideri et al., 2012).

It has been reported that the decline in mitochondrial function is related to many aging-related diseases (C. Chen et al., 2019; Weidling & Swerdlow, 2019). Accumulation of mitochondrial DNA (mtDNA) mutations and increased reactive oxygen species (ROS) production in aging is known to cause oxidative damage to cellular macromolecules which progressively results in mitochondrial dysfunction (Srivastava, 2017). In this study, we used basal respiration, non-mitochondrial respiration (measured directly in worms), and mitochondrial respiration (derived indirectly by deducting non-mitochondrial respiration from basal respiration) to assess the age-related decline in mitochondrial function and the effects of cocoa-supplementation. We have shown that both basal and mitochondrial respiration of *C. elegans* declines with age. Conversely, non-mitochondrial respiration which is an index of oxygen-consuming processes that are not associated with mitochondria increased with age. A previous study showed non-mitochondrial respiration to increase in the presence of stressors, including ROS (Dranka et al., 2010). Our results revealed that cocoa supplementation increased both basal and mitochondrial respiration and decreased non-mitochondrial respiration at old age suggesting that cocoa improves the age-associated decline in mitochondrial function. In a previous study, a cocoa bean extract was shown to improve mitochondrial biogenesis via peroxisome proliferator-activated receptor gamma ($PPAR_{\gamma}$)/PPARγ coactivator-1a ($PGC\text{-}1_{\alpha}$) dependent signaling pathway in 1-methyl-4-phenyl-1,2,3,6- pyridine (MPP^{+}) intoxicated human neuroblastoma cells (SHSY5Y) and decreased the ROS formation (Chidambaram et al., 2018). In another study, monomeric cocoa catechins significantly increased the mitochondrial respiratory function in INS-1 832/13 rat β-cells through the increased expression of components of the electron transport chain (Rowley et al., 2017). Another study showed that consumption of epicatechin-rich chocolate and a beverage improved the structural alterations in skeletal muscle mitochondria in patients with stage II and III heart failure and type 2 diabetes mellitus (Taub et al., 2012).

Even though the processing with alkali in commercial cocoa powder manufacturing is known to reduce the phenolics substantially compared to unprocessed natural cocoa powder (Andres-Lacueva

et al., 2008; Gu et al., 2006; Kealey Kirk et al., 1996; Robinson et al., 1961), there is some evidence that shows about 40% of the natural level of flavanols is retained on average for lightly Dutched powders, and an average of 22% is retained in even moderately alkali processed cocoa powders (Miller et al., 2008). Therefore, we thought to determine the TP, TF, and antioxidant activity of our cocoa powder as we attributed these beneficial effects to cocoa polyphenols. We found our cocoa powder to have 27.01 mg GAE/g TP which is about 56% compared to natural cocoa powder which contains TP around 48.84 mg GAE/g (Ünver et al., 2015). In natural cocoa powder, the TF content is around 25.55 mg CE/g (Todorovic et al., 2017). Our cocoa powder contained 10.13 mg CE/g of TF which was around 40% of the TF in natural cocoa powder. Moreover, our cocoa powder showed antioxidant activity of 7.84 (mg GAE/g). Therefore, these results confirm that the cocoa powder we used in this experiment was not devoid of beneficial flavonoids and other polyphenols.

Flavanols, the major group of polyphenols in cocoa are composed of monomeric forms, (+)-catechin and (-)-epicatechin, and their oligomeric and polymeric forms, procyanidins. In humans, both monomeric flavanols and procyanidins are stable in the gastric environment and reach the small intestine with no changes. Around 20% of monomeric flavanols are rapidly absorbed in the small intestine, undergo rapid and extensive metabolization, and circulate in the bloodstream. The majority of unabsorbed flavanols reach the colon where they get bio-transformed to secondary metabolites by gut microbiota (Sorrenti et al., 2020). Catechin and epicatechin have been shown to cross the blood-brain barrier and accumulate in the brain suggesting their direct positive action on the brain which may include cognitive improvements and neuroprotective effects (Abd El Mohsen et al., 2002; Faria et al., 2011).

As we added cocoa powder with *E. coli*, there is a possibility that *E. coli* to modify cocoa powder and produce different metabolites that are different from those present in cocoa powder. In humans, cocoa polyphenols are known to be poorly absorbed in the intestine and most of them cannot reach the systemic circulation in their natural forms (Sorrenti et al., 2020). Microbial degradation of polyphenols in the colon is considered the most effective way to produce secondary metabolites from these polyphenols which are bioavailable to enter circulation, reach the target organs and exhibit their activities (Rechner et al., 2004). Therefore, the microbial modification doesn't seem to affect the beneficial effects of polyphenols.

3.6. Conclusion

In summary, cocoa supplementation of the standard *E. coli* diet of *C. elegans* protected age-associated decline in neuromuscular function, as indicated by improved locomotor function. Moreover, cocoa improved the thermotolerance of *C. elegans* at all stages of life, showing a more prominent effect at young and middle age. Cocoa showed beneficial effects on age-related learning deficits and short-term memory loss. Further, cocoa enhanced mitochondrial function, especially in late life by significantly reducing non-mitochondrial respiration. These beneficial effects can be

attributed to the antioxidants in cocoa as evident by previous studies. However, further studies are required to identify the active compounds as well as mechanisms of action.

CHAPTER 4: Early Exposure is Necessary for the Lifespan Extension Effects of Cocoa in *C. elegans*

The content of this chapter has been published.

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4.1. Abstract

Background: We previously showed that cocoa, a rich source of polyphenols improved age-associated health and extended the lifespan in *C. elegans* when supplemented starting from the L1 stage.

Aim: In this study, we aimed to find out the effects of timing of cocoa exposure on longevity improving effects and the mechanisms and pathways involved in lifespan extension in *C. elegans*.

Methods: The standard *E. coli* OP50 diet of wild type *C. elegans* was supplemented with cocoa powder starting from different larval stages (L1, L2, L3, and L4) till the death, from L1 to adult day 1 and from adult day 1 till the death. For mechanistic studies, different mutant strains of *C. elegans* were supplemented with cocoa starting from the L1 stage till the death. Survival curves were plotted, and mean lifespan was reported.

Results: Cocoa exposure starting from the L1 stage till the death and till adult day 1 significantly extended the lifespan of worms. However, cocoa supplementation at other larval stages as well as at adulthood could not extend the lifespan, instead, the lifespan was significantly reduced. Cocoa could not extend the lifespan of *daf-16*, *daf-2*, *sir-2.1*, and *clk-1* mutants.

Conclusion: Early-start supplementation is essential for cocoa-mediated lifespan extension which is dependent on the insulin/IGF-1 signaling pathway and mitochondrial respiration.

Keywords: cocoa, polyphenols, lifespan, antioxidants, mechanisms

4.2. Introduction

As a result of the combined effects of demographic and epidemiological transitions and "modernization" over the past few decades, well-recognized non-communicable diseases (NCDs) such as cardiovascular diseases, cancers, diabetes, and neurodegenerative diseases have become the leading public health challenges globally (Gyasi & Phillips, 2020). Aging has been identified as the main risk factor for these prevalent NCDs (S.-Q. Zheng et al., 2016). A growing list of genetic, behavioral, and pharmacological interventions that have been shown to improve longevity also have proven effective in delaying the onset of age-related diseases and preserving healthspan (Barzilai et al., 2012). The well-defined pathways that regulate longevity including insulin/insulin-like growth factor-1 signaling (IIS), mitochondrial respiration, calorie restriction (CR) are also found to be involved in the onset of aging-related diseases (Chistiakov et al., 2014; Dillin et al., 2002; Hsu

et al., 2003; C. J. Kenyon, 2010; van Exel et al., 2014). Therefore, the therapies that modulate these pathways may be beneficial in delaying/preventing the onset of age-related diseases.

In recent years, dietary interventions have been proposed as alternative approaches to drugs for the prevention of aging-related diseases, particularly the consumption of foods rich in polyphenols (Rossi et al., 2008). Polyphenols have been shown to alleviate age-associated phenomena such as oxidative stress, chronic inflammation, and toxin accumulation (Queen & Tollefsbol, 2010). Resveratrol, curcumin, quercetin, catechin are some of the polyphenols well-studied for their antioxidant activity as potent compounds to mitigate the age-associated oxidative stress and damage induced by metabolic production of reactive oxygen species (ROS) (Grzesik et al., 2018; Gülçin, 2010; Sökmen & Akram Khan, 2016; M. Zhang et al., 2011). Cocoa, one of the widely consumed foods is known to be rich in polyphenols comparatively in much higher levels of total phenolics and flavonoids than tea and wine (K. W. Lee et al., 2003). Moreover, the cocoa-based product chocolate has been identified as a significant contributor to the total antioxidant capacity of European and American diets (Vinson et al., 2006).

Previously, we reported the effects of long-term cocoa supplementation (starting from the first larval stage till death) at varying doses (1, 2, 3, 4, 5 mg/ml) on the lifespan of *Caenorhabditis elegans (C. elegans)*, with 3 mg/ml dose achieving the maximum mean lifespan extension (Munasinghe, Almotayri, Thomas, et al., 2021). In addition, the same dose extended the median lifespan as well, but not the maximum lifespan. Based on these findings, in this study, we wanted to investigate the influence of timing of cocoa exposure on the longevity improving effects with the dose which previously reported the maximum lifespan extension. Additionally, we determined the pathways and mechanisms involved in the lifespan extension effect of cocoa.

4.3. Materials and methods

4.3.1. Strains and cultural conditions

All *C. elegans* strains and *Escherichia coli* (*E. coli*) OP50 were acquired from *Caenorhabditis* Genetics Center (CGC) and *C. elegans* were maintained at an appropriate temperature (15 °C or 20 °C) as per instructions. *C. elegans* strains used in this study were as follows: N2 (Bristol, wild-type), DA1116 *eat-2* (*ad1116*) *II.*, VC199 *sir-2.1* (*ok434*) *IV.*, CB4876 *clk-1* (*e2519*) *III.*, EU1 *skn-1* (*zu67*) *IV.*, CB1370 *daf-2* (*e1370*) *III.*, ZG31 *hif-1* (*ia4*) *V.*, TJ356 *daf-16(zls356) IV.* and GR1307 *daf-16* (*mgDf50*) *I.* All strains were maintained on nematode growth medium (NGM) plates seeded with *E. coli* OP50.

4.3.2. Cocoa treatment

Specifications and the composition of cocoa powder used in this study as well as the preparation of *E. coli* OP50 food source, and the addition of cocoa to NGM plates were described previously (Munasinghe, Almotayri, Thomas, et al., 2021). The major bioactive phytochemicals in cocoa are catechins including mainly monomeric (-) epicatechin and (+) catechin as well as oligomeric and

polymeric procyanidins (Ellam & Williamson, 2013). The total phenolics in the cocoa powder used for the study were 27.01 mg GAE/g which was about 56% compared to natural cocoa powder. Total flavonoids were 10.13 mg CE/g which was about 40% compared to the levels in natural cocoa powder. For all the experiments, 3 mg/ml cocoa dose was used. The influence of timing on lifespan-extending effects was determined by exposing N2 worms to cocoa at different larval stages (L1, L2, L3, and L4) till they die, L1 to adult day 1, and from adult day 1 till worms die (Figure 4.1A and B). *C. elegans* comprises 4 larval stages (L1, L2, L3, and L4) which are followed by adulthood. The larva that emerges from the eggshell has the nervous system and musculature but lacks the reproduction ability. Over four larval stages which end with a molt, gonad, and reproductive system are formed finally making the sexually mature adult worm. The larva is known to grow roughly continuously in size throughout these 4 stages with little change in overall morphology. The larval stages are mainly discriminated by their size. The L1 larvae are about 250 µm in length while the L2, L3, L4, and adult are 360 to 380 µm, 490 to 510 µm, 620 to 650 µm, and 1110 to 1150 µm respectively (Meneely et al., 2019). We supplemented the worm diet with cocoa at different larval stages as well as adulthood to represent the different stages of growth and development. To determine the pathways and mechanisms involved, mutant strains were supplemented with cocoa at a dose of 3 mg/ml starting from the L1 stage till the death.

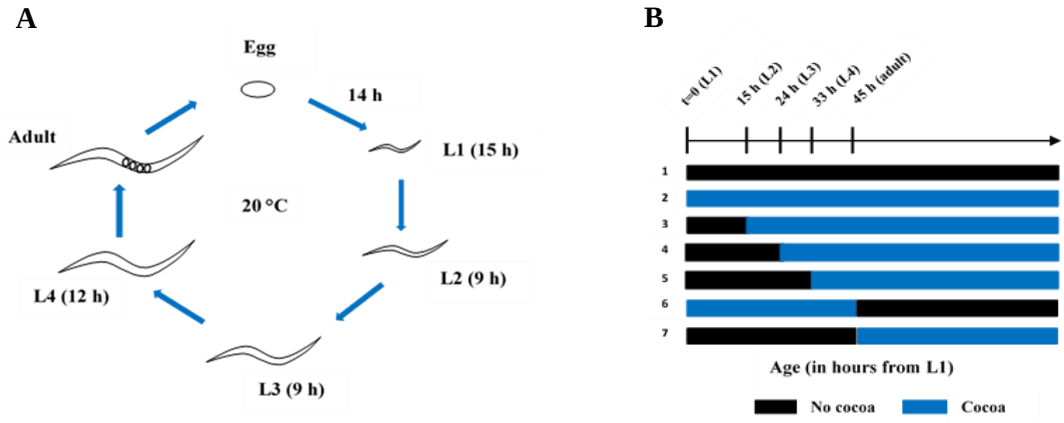

Figure 4.1. Cocoa supplementation at different growth stages of *C. elegans*. (A) Lifespan of wild-type *C. elegans* at 20 °C. (B) Schematic representation of cocoa-supplementation at different stages of life (treatment 1 = control; treatment 2 = cocoa from L1 stage; treatment 3 = cocoa from L2 stage; treatment 4 = cocoa from L3 stage; treatment 5 = cocoa from L4 stage; treatment 6 = cocoa from L1-adult day 1; treatment 7 = cocoa from adult day 1 till die).

4.3.3. Obtaining synchronous cultures

Age-synchronized worms were obtained by bleaching gravid adults. Briefly, gravid hermaphrodites were washed off from an NGM plate with M9 buffer and made the final volume to 3.5 ml. Bleaching solution (bleach: 5N NaOH = 2:1) was added to the worm suspension at a volume of 1.5 ml. The suspension was mixed by vortexing the tube for 5 seconds and keeping further 30 seconds on rest

until all the worm bodies got dissolved. The suspension was centrifuged (2 min at 1300*g*) to pellet the released eggs and the supernatant was poured off. M9 buffer was added to a final volume of 5 ml, centrifuged again and the supernatant was removed. This step was repeated at least 3 times to completely get rid of the bleaching solution. The egg pellet was resuspended in 3 ml of M9 and kept for 48 hours on a shaker for hatching.

4.3.1. Lifespan assay

Lifespan assay was performed as described in (Sutphin & Kaeberlein, 2009). Floxuridine (FUdR) was not used to avoid progeny production and instead, worms were transferred every day onto fresh plates until they stop laying eggs (until day 9). Thereafter, worms were transferred every other day. Worms that were crawled off from the plates were excluded from the analysis. Survival curves were plotted. Mean and median lifespan was reported.

4.3.2. DAF-16::GFP localization assay

Control and cocoa-supplemented (3 mg/ml) TJ356 *daf-16(zls356) IV*. worms were scored at the L4 stage for cytoplasmic, intermediate, or nuclear Green fluorescent protein (GFP) localization as described by X. Wang et al. (2015). About 100 control and cocoa-supplemented (from the L1 stage) worms were collected at the L4 stage, washed with M9 buffer twice, and placed on a glass slide carrying 2 µl of 1M NaN_3. The sub-cellular DAF-16::GFP distribution was observed under a fluorescence microscope at 20-fold magnification. The number of worms in above mentioned three categories were counted and expressed as percentages. Three independent experiments were performed.

4.3.3. Statistical analysis

All statistical analyses were performed in IBM SPSS® statistics software (version 24). Data were expressed as mean ± standard error of the mean (SEM). The survival function was estimated using Kaplan-Meier curves. Survival curves were compared using the log-rank (Mantel-Cox) test. The differences between groups for DAF-16 localization were determined using t-Test (two-sample assuming equal variances).

4.4. Results

4.4.1. Cocoa extends the lifespan of wild-type *C. elegans* when supplemented starting from the L1 stage

We supplemented the worm diet with cocoa at different larval stages (L1, L2, L3, and L4) till death, from L1 till adult day 1, and from adult day 1 till death. Worms showed a significantly extended mean lifespan when supplemented with cocoa starting from the L1 stage till they die ($P<0.05$, 8.9% increase, Fig 4.2A, Table 4.1). Similarly, cocoa extended the lifespan of worms when supplemented at L1 till only day 1 of adulthood ($P<0.05$, 6.7% increase, Fig. 4.2A, Table 4.1). However, supplementation of cocoa at L2, L3, or L4 stages could not increase the mean lifespan of worms (Fig. 4.2A, Table 4.1). Cocoa supplementation at adult day 1 till the death (treatment 7) also could

not increase (P<0.05) the mean lifespan of worms (Fig. 4.2B, -3.7%, control = 16.2 ± 0.20 and cocoa = 15.6 ± 0.19 days).

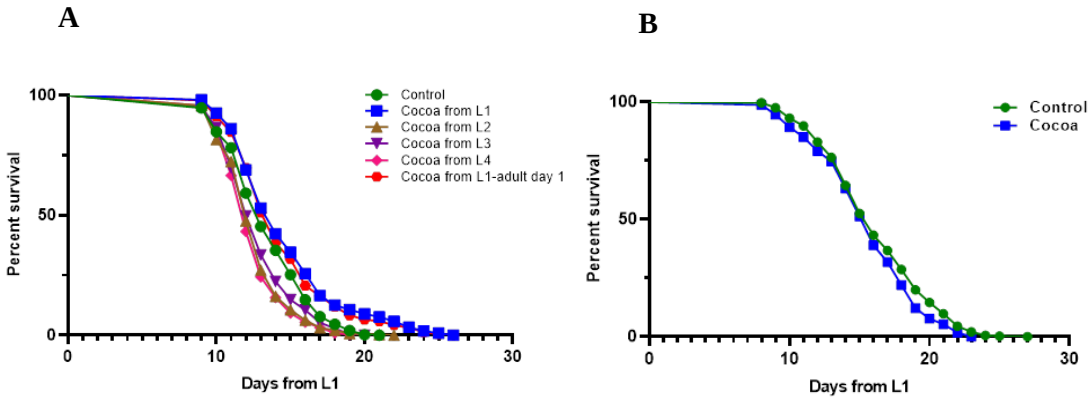

Figure 4.2. Survival curves for control and cocoa-supplemented wild type (N2) *C. elegans*. Experiments were performed in triplicate. Differences between groups for mean lifespan were calculated using the log-rank (Mantel-Cox) test. (A) Survival curves for worms supplemented with cocoa at different larval stages (L1, L2, L3, and L4) till die and from L1 to adult day 1. Cocoa significantly increased (P<0.05) the mean lifespan when worms were supplemented starting from the L1 stage. Cocoa could not extend the lifespan of worms when supplemented at other larval stages (L2, L3, and L4). (B) Survival curves for cocoa-supplemented worms from adult day 1 till die [n = 335 for control and n = 336 for cocoa-supplemented worms (treatment 7)]. Cocoa could not extend the mean lifespan of worms when supplemented from adulthood.

Table 4.1. The Mean and median lifespan of *C. elegans* when supplemented with cocoa at different stages of life

Treatment	Sample size (*n*)	Mean lifespan	% extension compared to control	Median lifespan
1 (control)	344	13.5±0.15		13±0.23
2 (cocoa from L1)	326	14.7±0.20*	8.9	14±0.21
3 (cocoa from L2)	283	12.7±0.13*	-5.9	12±0.13
4 (cocoa from L3)	209	12.9±0.17*	-4.4	12±0.19
5 (cocoa from L4)	312	12.5±0.12*	-7.4	12±0.12
6 (cocoa from L1-adult day 1)	444	14.4±0.16*	6.7	14±0.15

*Statistically significant (P<0.05) compared to the control group.

4.4.2. Cocoa extends the lifespan of *C. elegans* via DAF-16

DAF-16, the *C. elegans* homolog of the forkhead box transcription factors class O (FoxO) is a central regulator of aging, development, stress, metabolism, and immunity (Zečić & Braeckman, 2020). Therefore, we tested if DAF-16 can play a role in lifespan extension by cocoa in *C. elegans*. We found that cocoa could not extend the lifespan (P>0.05) of *daf-16* mutants (Fig. 4.4A, Table

4.2). As nuclear localization of DAF-16 is an essential prerequisite for its transcriptional activation, we used the transgenic strain TJ356 (DAF-16::GFP) to explore whether cocoa can activate the nuclear localization of DAF-16. Cocoa-treated worms showed a significantly higher (P<0.05) nuclear DAF-16 localization compared to control worms as indicated by * (Fig. 4.3A and B).

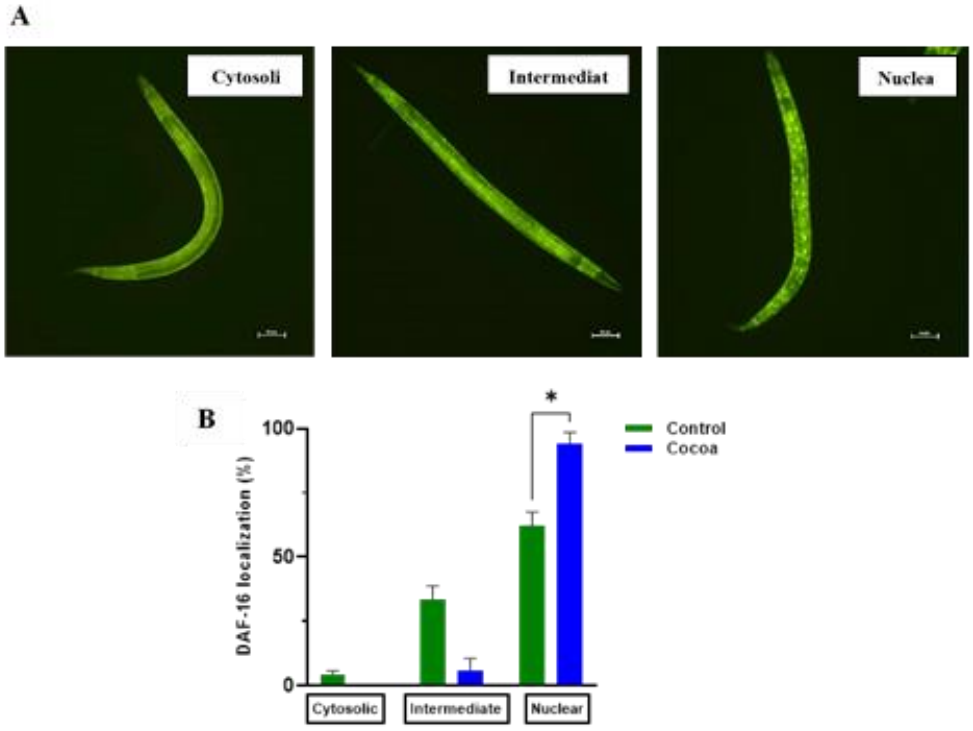

Figure 4.3. Cocoa-induced nuclear DAF-16 localization in *C. elegans*. (A) Representative images of TJ356 *C. elegans* for cytosolic, intermediate, and nuclear DAF-16::GFP localization (20-fold magnification, scale bar = 50 μm). (B) Percentage of control and cocoa-treated worms grouped for cytosolic, intermediate, and nuclear DAF-16::GFP localization. About 100 worms (at the L4 stage) per treatment were used for each replicate. The bar chart represents the mean ± SE of three independent experiments. Statistical significance was determined using t-Test (two-sample assuming equal variances). Cocoa-treated worms showed a higher nuclear DAF-16 localization compared to control worms (P<0.05).

4.4.3. Cocoa may regulate insulin/insulin-like growth factor-1 signaling (IIS) pathway

In *C. elegans*, the IIS pathway is known to regulate longevity via DAF-16 (Sun et al., 2017). Therefore, we explored whether cocoa could interact with molecules in the IIS pathway to regulate longevity. Single mutations of DAF-2 inhibit the IIS pathway and increase longevity in *C. elegans* (Zhao et al., 2017). Therefore, we used *daf-2* (*e1370*) *III.* mutants to study the involvement of the IIS pathway in cocoa-mediated lifespan extension. Cocoa supplementation could not extend the mean lifespan of long-lived insulin-like receptor mutant *daf-2* (Fig. 4.4B, Table 4.2).

4.4.4. Cocoa could not extend the lifespan of *sir-2.1* and *eat-2* mutants

The silent information regulator 2 (SIR2) can bind to DAF-16 and extend the lifespan of *C. elegans* (Berdichevsky et al., 2006). We investigated if cocoa could act on SIR-2.1 to extend the lifespan of *C. elegans*. Cocoa could not extend the mean lifespan of the *sir-2.1* mutant, indicating that SIR-2.1 is required for cocoa-mediated lifespan extension (Fig. 4.4C, Table 4.2). In addition, as SIR-2.1 is considered as a key mediator of lifespan extension by CR (Anderson et al., 2003), we used the pharyngeal pumping defective mutant *eat-2* (*ad1116*) *II*. to see if cocoa-induced lifespan extension is dependent on a CR mechanism. According to our results, cocoa could not extend the mean lifespan of *eat-2* mutants (Fig. 4.4D, Table 4.2).

4.4.5. Lifespan extension by cocoa was not dependent on stress response factors

In *C. elegans skn-1* promotes resistance to oxidative stress and extends lifespan (Tullet et al., 2017). In this study, cocoa supplementation significantly increased the mean lifespan of *skn-1* mutants ($P<0.05$, Fig. 4.4E, Table 4.2). Additionally, we determined the effects of cocoa supplementation on the lifespan of *hif-1* mutants of *C. elegans*. Hypoxia-inducible factor (HIF) enables animals to adapt to the stress caused by hypoxic (low oxygen) conditions that they experience during normal development or during disease. Both HIF-1 over-expression and *hif-1* loss-of-function mutations promote longevity by different pathways in *C. elegans* (Y. Zhang et al., 2009). Our results showed that cocoa supplementation significantly increased the mean lifespan of *hif-1* mutants ($P<0.05$, Fig. 4.4F, Table 4.2).

4.4.6. Cocoa restores the mitochondrial function to extend the lifespan of *C. elegans*

The role of mitochondrial function in aging is well-described (Harman, 1972; Linnane et al., 1989; Miquel et al., 1980). Therefore, we used electron transportation chain (ETC) mutant *clk-1* which shows an extended lifespan compared to wild-type *C. elegans* (Felkai et al., 1999) to see the involvement of mitochondria in cocoa-induced lifespan extension. Cocoa supplementation could not extend the mean lifespan of *clk-1* mutants (Fig. 4.4G, Table 4.2).

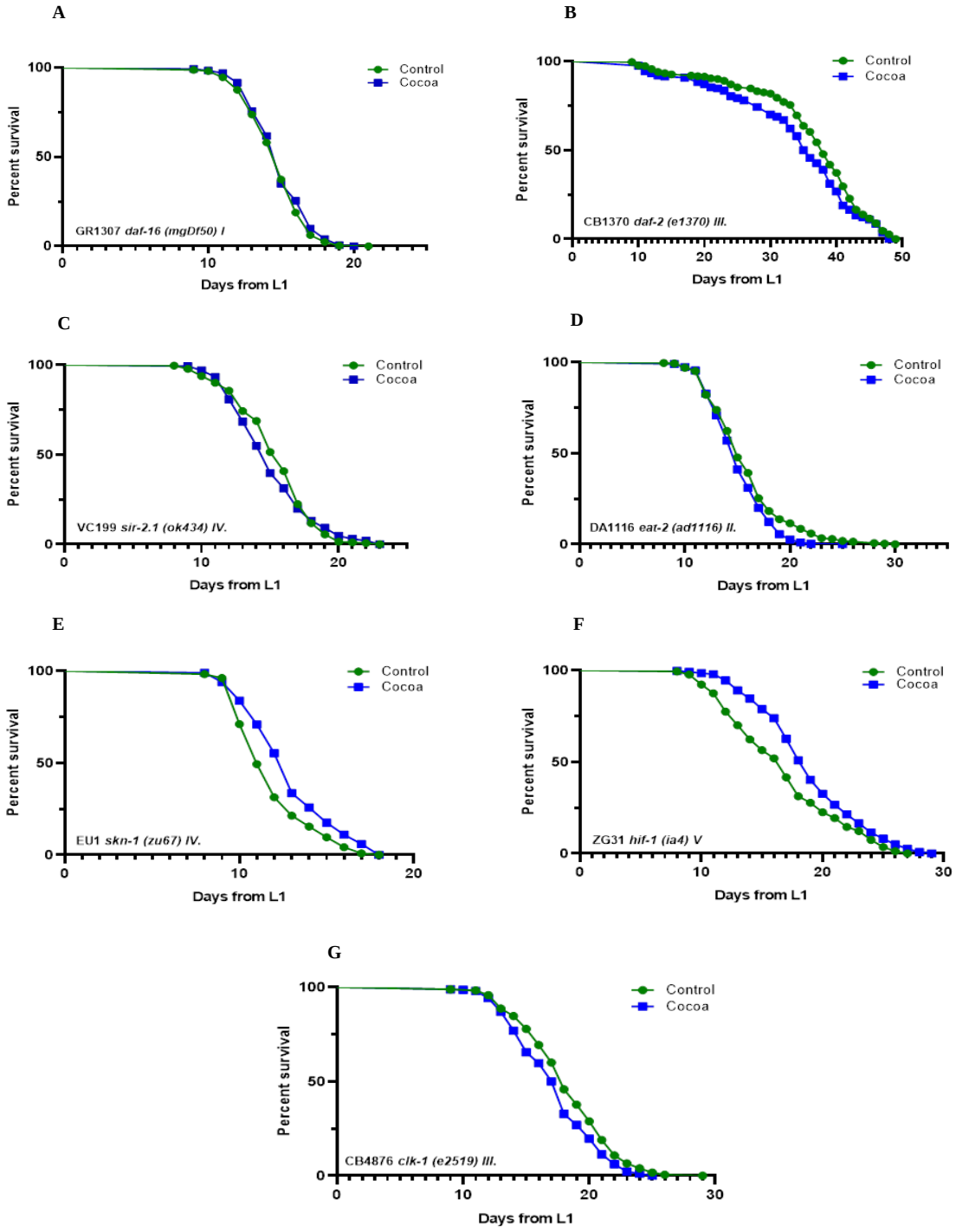

Figure 4.4. Different pathways and mechanisms are involved in cocoa-mediated lifespan extension. Experiments were performed in triplicate. Differences between groups for mean and median lifespans were calculated using the log-rank (Mantel-Cox) test. (A) Survival curves for control and cocoa-supplemented GR1307 *daf-16 (mgDf50) I.* worms. Cocoa could not extend the lifespan of *daf-16* mutants (P>0.05). (B) Survival curves for control and cocoa-supplemented CB1370 *daf-2* (*e1370*) *III*. Worms. Cocoa could not extend the lifespan of *daf-2* mutants. (C) Survival curves for control and cocoa-supplemented VC199 *sir-2.1* (*ok434*) *IV*. worms. Cocoa could not extend the lifespan of *sir-2.1* mutants. (D) Survival curves for control and cocoa-

supplemented DA1116 *eat-2* (*ad1116*) *II*. worms. Cocoa could not extend the lifespan of *eat-2* mutants. (E) Survival curves for control and cocoa-supplemented EU1 *skn-1* (*zu67*) *IV*. worms. Cocoa significantly extended the lifespan of *skn-1* mutants ($P<0.05$). (F) Survival curves for control and cocoa-supplemented ZG31 *hif-1* (*ia4*) *V*. worms. Cocoa significantly extended the lifespan of *hif-1* mutants ($P<0.05$). (G) Survival curves for control and cocoa-supplemented CB4876 *clk-1* (*e2519*) *III*. worms. Cocoa could not extend the lifespan of *clk-1* mutants.

Table 4.2. The mean and median lifespan of control and cocoa-supplemented *C. elegans*

Strain and treatment	**Sample size (*n*)**	**Mean lifespan**	***P-value***	**% extension of mean lifespan compared to the control**	**Median lifespan**
GR1307 *daf-16* (*mgDf50*) *I*.-control	339	14.8±0.11	0.12		15±0.13
GR1307 *daf-16* (*mgDf50*) *I*.-cocoa	346	15.0±0.11		1.4	15±0.10
CB1370 *daf-2* (*e1370*) *III*.-control	303	36.1±0.54	0.02		38±0.47
CB1370 *daf-2* (*e1370*) *III*.-cocoa	164	33.7±0.78		-6.6*	35±0.77
VC199 *sir-2.1* (*ok434*) *IV*.-control	260	15.7±0.16	0.03		16±0.21
VC199 *sir-2.1* (*ok434*) *IV*.-cocoa	313	15.0±0.17		-4.5*	15±0.20
DA1116 *eat-2* (*ad1116*) *II*.-control	268	15.9±0.22	0.00		15±0.26
DA1116 *eat-2* (*ad1116*) *II*.-cocoa	309	15.2±0.15		-4.4*	15±0.18
EU1 *skn-1* (*zu67*) *IV*.-control	239	12.0±0.14	0.00		11±0.16
EU1 *skn-1* (*zu67*) *IV*.-cocoa	217	13.0±0.17		8.3*	13±0.15
ZG31 *hif-1* (*ia4*) *V*.-control	310	16.8±0.27	0.00		17±0.38
ZG31 *hif-1* (*ia4*) *V*.-cocoa	383	19.0±0.22		13.0*	19±0.22
CB4876 *clk-1* (*e2519*) *III*.-control	353	18.3±0.20	0.00		18±0.24
CB4876 *clk-1* (*e2519*) *III*.-control	360	17.3±0.17		-5.5*	17±0.20

*Statistically significant extension compared to the control group ($P<0.05$)

4.5. Discussion

In humans, early life nutrition has been reported to significantly influence the risk of developing NCDs in late life, (Kelishadi & Farajian, 2014) emphasizing the importance of early nutrition on healthspan. Earlier initiation of nutritional interventions in humans has been shown to confer greater benefits in long-term cardiometabolic outputs (He & Stein, 2020). Moreover, studies suggest that early life nutrition can affect intestinal maturation and gut health in later life (Ley et al., 2019). Enhancing nutrition in early life is known to regulate the gut microbiota composition and improve infant immunity development, shaping life-long health (X. Zhou et al., 2019). Maternal intake of resveratrol, a polyphenol in Red wine has been shown to fight against the adverse effects of the high-fat diet or low-protein diet on offspring, such as glucose intolerance, obesity, cholesterol metabolic disorders, non-alcoholic fatty liver disease, or even hypothalamic leptin signaling

dysregulation in mice (L.-Y. Zhou et al., 2020). We previously reported that long-term cocoa supplementation extended the lifespan of *C. elegans* when supplemented starting from the L1 stage (Munasinghe, Almotayri, Thomas, et al., 2021). Based on the aforementioned findings on early-life nutrition on the health of individuals in later life, we wanted to investigate whether early exposure to cocoa is critical for this longevity-improving of cocoa. Our results supported the idea that cocoa supplementation starting from the first larval stage (L1) is critical and essential for the longevity-extending effects of cocoa. In addition, cocoa intervention starting from the L1 stage which continued till day 1 of adulthood increased the lifespan of worms to a similar extent as life-long exposure (L1 to the death). Therefore, these results suggest that the longevity-extending effects of cocoa require early-start exposure, but do not need to be continued in a long-term manner. Previous studies support our findings where supplementation of a cranberry extract rich in polyphenols has been prominently promoted the longevity in *C. elegans* when the supplementation was started at the early developmental stage compared to the late start. Moreover, Zuckerman and Geist (1983) reported that the antioxidant α-tocopherol supplementation early in the pre-reproductive stage significantly extended both mean and maximum lifespan in *C. elegans*. These effects that arose from early supplementation were prominent compared to the supplementation at day 4, indicating α-tocopherol was most effective if present during larval stages. However, cocoa supplementation at late larval stages (L2, L3, L4) and adulthood did not seem to play a role in lifespan extension. Additionally, supplementation of cocoa at these stages significantly reduced the lifespan. Some of the previous studies have been reported the adverse effects of polyphenols and antioxidant supplementation in animal models. Resveratrol, one of the most researched polyphenols has been reported to act as a pro-oxidant at high concentrations, promoting DNA damage while increasing oxidative stress in vitro and in animal models (Shaito et al., 2020). Supplementation of the growth medium with alpha-tocopherol increased oxidative stress and decreased cell lifespan in *Saccharomyces cerevisiae*, highlighting the pro-oxidant action of antioxidants (Lam et al., 2010). Another study reported that dietary supplementation of both vitamin E and C dramatically shortened the lifespan in voles with no exact explanation for the effect on lifespan but may be due to pro-oxidant effects (Selman et al., 2013). In humans, supplementation of antioxidants (vitamin C and E) has been reported to suppress the health-promoting effects (insulin sensitivity, reactive oxygen species defense) of physical exercise (Ristow et al., 2009). In addition, the consumption of the green tea-derived polyphenol (-)-epigallocatechin gallate or its metabolites has been reported to associate with hepatotoxicity in humans (Mazzanti et al., 2009). However, in this study, we were not able to establish the exact mechanism for the lifespan reduction effects of cocoa when supplemented at late larval stages and adulthood. Molecular mechanisms underlying aging have recently gained much attention as aging is the most significant risk factor for many chronic disease conditions (Uno & Nishida, 2016). Most of these mechanisms influencing aging are conserved across organisms (Curran & Ruvkun, 2007). Among different genetic factors that regulate aging, IIS, mitochondrial

metabolism and CR pathways have been extensively studied (G. Stein & Murphy, 2012). To find out which pathways are involved in cocoa-associated lifespan extension in *C. elegans*, we used several mutant strains related to these well-known pathways. IIS pathway, the first pathway implicated in the aging of animals has a well-established role in aging where the reduced IIS leads to lifespan extension in *C. elegans* (C. Kenyon et al., 1993). IIS pathway is a signal transduction cascade that consists of insulin-like peptides (ILPs), an insulin/IGF-1 receptor (DAF-2), a phosphoinositide 3-kinase (AGE-1/AAP-1/PI3K), serine/threonine kinases (PDK-1, AKT-1, and AKT-2), and the pivotal downstream FoxO (DAF-16) in *C. elegans* (Sun et al., 2017). In this study, we investigated whether DAF-16 was involved in the lifespan extension by cocoa. Our results showed that cocoa could not increase the lifespan of *daf-16* mutants, indicating that cocoa may regulate lifespan through DAF-16. Moreover, cocoa-treated worms showed a higher nuclear DAF-16 localization compared to their counterparts, further confirming the involvement of DAF-16. As the IIS pathway is a central regulator of DAF-16 activity (Zečić & Braeckman, 2020), we investigated whether cocoa could interact with the components in the IIS pathway to regulate the longevity of worms. According to the results, cocoa could not improve the lifespan of long-lived insulin-like receptor *daf-2 (e1370) III*. Therefore, the cocoa-induced longevity via DAF-16 may be dependent on the IIS pathway. In *C. elegans*, SIR-2.1 is a member of the SIR2 family of NAD+-dependent protein deacetylases and has been shown to regulate nematode aging via the insulin/IGF pathway transcription factor DAF-16 (Viswanathan et al., 2005). Besides, SIR-2.1 is required for lifespan extension by CR, independent of the IIS pathway (Y. Wang & Tissenbaum, 2006). In our study, cocoa treatment could not extend the lifespan in *sir-2.1* mutants, suggesting that the lifespan extension effect of cocoa is dependent on *sir-2.1*. Further, cocoa supplementation could not extend the lifespan of pharyngeal pumping defective mutant *eat-2* which mimics the functional effect of CR through the reduced food intake. This suggests that a CR mechanism is involved with the lifespan extension effects of cocoa. However, as we did not determine how cocoa affects the pharyngeal pumping rate of worms, we are unable to establish the exact mechanism for this. Moreover, we previously found that cocoa-treated worms were significantly longer and thicker than the control worms (Munasinghe, Almotayri, Thomas, et al., 2021), a result that is inconsistent with the CR effect as caloric restricted worms are thinner than the normally-fed worms (Lenaerts et al., 2008). SKN-1 (Skinhead-1) transcription factor in *C. elegans* which is the homolog of Nrf1/Nrf2 (NF-E2 related factor 1/2) in vertebrates regulates stress resistance and extends lifespan (Tullet et al., 2017). HIF-1 is another stress response molecule that regulates the lifespan of *C. elegans* (Y. Zhang et al., 2009). Cocoa extended the lifespan of both *skn-1* and *hif-1* mutants, indicating that lifespan extension by cocoa is not dependent on these stress response molecules. Mitochondrial respiration is another significant contributor to the aging process (H. C. Lee & Wei, 2001). Impairments in ETC are known to extend the lifespan of *C. elegans* (Butler et al., 2010). We tested the effects of cocoa supplementation on long-lived *clk-1* mutants. This gene encodes a hydroxylase

in the ETC that is required for ubiquinone biosynthesis (Wong et al., 1995). We found that *clk-1* mutants displayed a shorter lifespan following the cocoa supplementation, indicating that cocoa's lifespan extension is dependent on mitochondria and cocoa restores mitochondrial function.

4.6. Conclusion

In summary, we demonstrated that early-start supplementation is essential for cocoa-mediated lifespan extension in *C. elegans*. In addition, the longevity-improving effects of cocoa were mediated through the IIS pathway and mitochondrial respiration.

CHAPTER 5: Cocoa Supplementation Reduces $A\beta_{1-42}$-induced Deficits in a Transgenic *C. elegans*

The content of this chapter has been published.

Munasinghe, M., Almotayri, A., Kolivas, D., Thomas, J., Heydarian, D., & Jois, M. (2021). Cocoa supplementation reduces amyloid-beta$_{1–42}$ ($A\beta_{1–42}$) induced deficits in a transgenic *C. elegans*. *Nutrition and Healthy Aging*, 6, 117-130. doi:10.3233/NHA-200114.

5.1. Abstract

Background: Cocoa, a significant contributor of polyphenols to the western diet is effective against Aβ-induced toxicity *in vitro*. However, the effects of long-term cocoa supplementation on Aβ-induced behavioral deficits, particularly on the short-term memory loss observed in human AD are not well defined.

Objective: This study characterized the phenotype of a pan-neuronal Aβ expressing *C. elegans* strain and investigated the effects of long-term cocoa supplementation on Aβ-induced behavioral deficits including short-term memory loss and lifespan.

Methods: Cocoa powder was supplemented to the *E. coli* OP50 diet of *C. elegans* starting from the L1 stage until they die. Neuronally controlled processes including locomotion, learning, and memory were studied at different stages of the lifespan. In addition, lifespan was evaluated with different cocoa doses. Aβ fibril levels were determined with Thioflavin T.

Results: Aβ expressing worms showed reduced growth, a reduced maximum speed at old age, short-term memory deficits at middle age, and a reduced lifespan. Cocoa-supplementation reversed the deficits in growth, maximum speed, short-term memory loss, and lifespan to reach similar levels to control counterparts while reducing the Aβ fibril levels.

Conclusions: Long-term cocoa supplementation seemed to improve Aβ induced deficits in *C. elegans*.

Keywords: cocoa, Alzheimer's disease, amyloid-beta, *C. elegans*, antioxidants, polyphenols

5.2. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by a gradual loss of memory and cognitive skills (Bekris et al., 2010). Pathological hallmarks of AD include extracellular deposition of amyloid-β (Aβ) plaques and intracellular aggregation of neurofibrillary tangles in the brain. Being the most common cause of dementia worldwide, AD accounts for 50-80% of all dementia cases (Di Resta & Ferrari, 2019). Prevalence of AD increases with age and it is becoming increasingly common as the global older population is escalating. To date, there is no pharmaceutical intervention that has been shown to cure this multifactorial disease or halt the disease progression.

It has been shown that a healthy dietary pattern is one of the key protective factors for AD (W. Xu et al., 2015). Higher adherence to a diet rich in natural plant-based foods has been shown to reduce the incidence of neurodegenerative diseases (Féart et al., 2010; Morris et al., 2015). One possible explanation for this reduction is the action of polyphenols in such foods through different mechanisms (Sathya & Pandima Devi, 2018). Cocoa and its products are known to be rich sources of polyphenols which significantly contribute to the total antioxidant capacity of the diet (K. W. Lee et al., 2003; Vinson et al., 2006). Several studies report beneficial effects of cocoa peptides, cocoa extracts, and cocoa polyphenols in Aβ induced toxicity in AD (Cimini et al., 2013; Heo & Lee, 2005; Patricia Martorell et al., 2013; J. Wang et al., 2014). However, how cocoa consumption affects Aβ induced behavioral deficits, in particular, learning and memory and lifespan are not well-defined.

C. elegans is an inexpensive *in vivo* model which has been widely used to study Aβ toxicity (Fong et al., 2016; Link, 1995; Link et al., 2003; McColl et al., 2009; Y. Wu et al., 2006). Even though *C. elegans* lack Aβ and β-secretase and do not exhibit human pathological and behavioral symptoms of AD, several transgenic strains that express human Aβ have been developed for pharmacological evaluation and mechanistic studies (Alexander et al., 2014). In this study, we selected a *C. elegans* strain expressing constitutive pan-neuronal $A\beta_{1-42}$ which displays an age-related onset of behavioral dysfunction, such as that observed in human AD (Fong et al., 2016). Therefore, this study aimed to characterize the phenotype of this neuronal $A\beta_{1-42}$ expressing strain and to determine the effects of long-term cocoa supplementation on Aβ induced behavioral deficits and the lifespan.

5.3. Materials and methods

5.3.1. *C. elegans* strains and maintenance

Transgenic [GRU101 gnals1 (myo-2p::yfp), GRU102 gnals2 (myo-2p::YFP + unc-119p::Abeta$_{1-42}$)] *C. elegans* and *Escherichia coli* (*E. coli*) OP50 were obtained from *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). Worms were maintained on nematode growth medium (NGM) plates carrying a lawn of *E. coli* OP50. Worm maintenance as well as all the experiments were carried out at 20 °C.

5.3.2. Obtaining synchronous cultures

Age-synchronized worms were obtained by bleaching gravid adults. Briefly, gravid hermaphrodites were washed off from an NGM plate with M9 buffer and made the final volume to 3.5 ml. Bleaching solution (bleach: 5N NaOH = 2:1) was added to the worm suspension at a volume of 1.5 ml. The suspension was mixed by vortexing the tube for five seconds and keeping further 30 seconds on rest until all the worm bodies got dissolved. The suspension was centrifuged (2 min at 1300 g) to pellet the released eggs and the supernatant was poured off. M9 buffer was added to a final volume of 5 ml, centrifuged again and the supernatant was removed. This step was repeated at least three

times to completely get rid of the bleaching solution. The egg pellet was resuspended in 3 ml of M9 and kept for 48 hours on a shaker for hatching.

5.3.3. Cocoa treatment

A commercially available cocoa powder, which was an unsweetened dry powder processed with alkali was used for the study. A cocoa suspension was made to the desired final concentration in M9 buffer to make it spreadable. For all the experiments except lifespan, a single dose of cocoa which is 5 mg of cocoa powder suspended in 1 ml of M9 was used. For the lifespan experiment, five different doses of cocoa were prepared by suspending 1 mg, 2 mg, 3 mg, 4 mg, and 5 mg cocoa in 1 ml of M9 buffer. Concentrated *E. coli* OP50 was prepared by suspending 1 g of *E. coli* pellet in 16 ml of M9 buffer. Concentrated *E. coli* was first added to NGM plates and was grown overnight at 20 °C. The cocoa suspension was added on the top of the *E. coli* lawn at a ratio of 1:2 volume, *E. coli*: cocoa suspension (for small plates: 100 µl of *E. coli* and 200 µl of cocoa, for medium plates: 200 µl of *E .coli* and 400 µl of cocoa). Worms received cocoa starting from the L1 stage.

5.3.4. Growth and locomotion

Age synchronized worms were washed off from both treatment and control plates and collected in a 15 ml falcon tube. Once worms settled to the bottom, the supernatant was removed and 5 ml of M9 was added to wash off the food and repeated twice. Thereafter, 100 µl of worm suspension was placed on plain agar plates in the absence of food. Plates were left in the laminar flow cabinet for 5 minutes to dry out the liquid. Then, they were placed under a stereo microscope (SMZ745T; Nikon, Japan equipped with G-AL 1.0x objective) and videos were captured using NIS Elements imaging software (version 5.01, Japan) and a digital camera (DS-Fi2; Nikon, Japan) with settings at 50 frames per second, 30-second duration per video and resolution of 640 x 480 pixels. The scale bar (µm) in the imaging software was used to convert pixels into µm. Worms were tracked and analyzed via WormLab 3.1 software (MBF Bioscience, Williston, VT USA). Worms tracked over a minimum of 10 seconds were included in the final analysis. Mean body length (µm), mean area (μm^2), mean speed (µm/s), maximum speed (µm/s), and maximum amplitude (µm) were reported. According to the WormLab software, speed is defined as the distance per second traveled by the worm along its central axis. Maximum amplitude is the maximum centroid displacement over an entire track.

5.3.5. Determination of motility classes at old age

Motility classes of worms were determined as previously described (Herndon et al., 2002). Age synchronized worms were sorted by class (A, B, or C) at day 12 and re-scored for class every day until death. Animals move constantly away from the touch stimulus and leave sinusoidal tracks in the *E. coli* lawn were categorized into the class “A”. Worms do not move unless prodded and leave non-sinusoidal tracks were categorized as class “B”. Worms do not move even prodded but, exhibit head and/or tail movements or twitch in response to touch were categorized as class “C”.

5.3.6. Learning and short-term associative memory

Naive chemotaxis, learning, and short-term associative memory were determined based on *C. elegans* positive butanone learning assay (Fig. 5.1A) (Kauffman et al., 2011). Age-synchronized worms (approximately 1000) were grown on high-growth media plates with food until they reached the desired age. Chemotaxis assay (Fig. 5.1B) was performed with approximately 200 worms. The remaining were starved for 1 hr and at the end of the starvation period, worms were associated with food and chemo-attractant butanone for 1 hr. Chemotaxis assay was repeated to test 1x (massed) associative learning immediately after conditioning (t=0 time point). The rest of the worms were transferred equally to two NGM plates seeded with *E. coli* and incubated at 20 °C. After 1 hr and 2 hrs from the food-butanone association, chemotaxis assay was performed with the worms on two plates (t=1 hr and t=2 hr time points). Chemotaxis index (CI) was calculated as, CI = [(Number of worms at butanone) – (Number of worms at ethanol)]/ (Total number of worms). The learning index (LI) at different time points was calculated as, LI = Chemotaxis $index_t$ – Chemotaxis $index_{Naive}$. Dementia index (short-term associative memory loss) was calculated after 1 hr and 2 hrs from the food-butanone association (Dementia $index_t$ = Learning $index_0$ - Learning $index_t$).

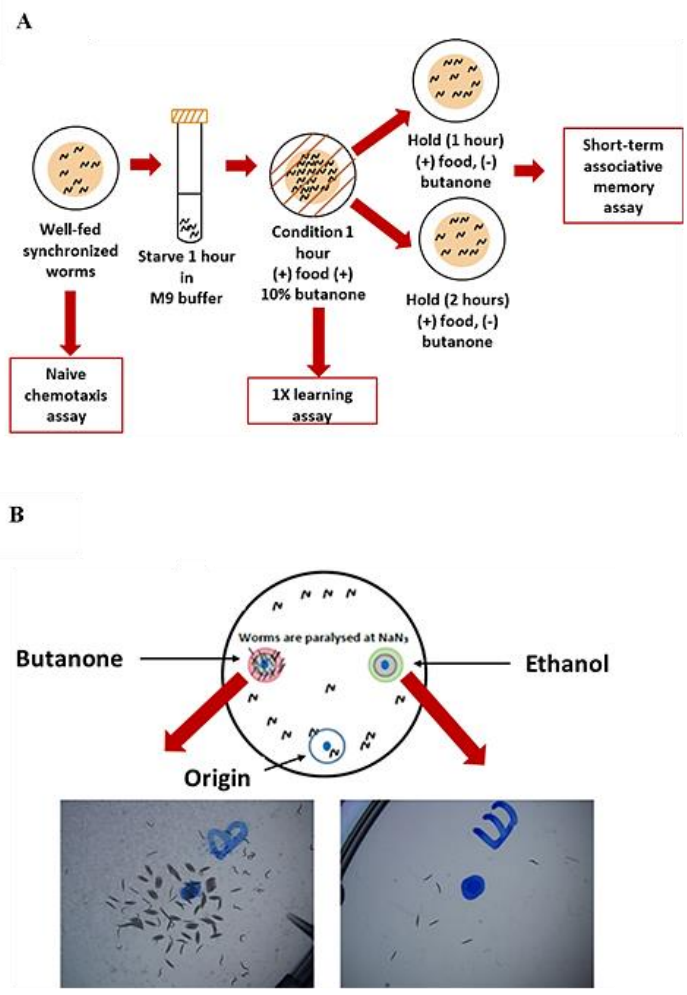

Figure 5.1. Learning and short-term associative assay workflow, adapted from (Kauffman et al., 2011). (A) Short-term associative memory assay: Starved worms were conditioned with

butanone for 1 h and some of them were immediately tested for 1X learning (t=0) and the rest were transferred onto holding plates for 1h and 2 hrs (B) Chemotaxis assay: Briefly, 1 µl of 2M NaN_3 was added to the butanone (left) and EtOH (right) spots. Then, 1 µl of 10% Butanone in 95% EtOH and 95% EtOH were added to the butanone and EtOH spots. Worms (200) in M9 buffer were added to the origin. KimWipe absorbed M9 and released worms. Worms moved to either butanone or EtOH spots and were paralyzed.

5.3.7. Lifespan assay

Lifespan assay was performed as described in reference (Sutphin & Kaeberlein, 2009). Floxuridine (FUdR) was not used to avoid progeny production and instead, worms were transferred every day onto fresh plates until they stop laying eggs (until day 9). Thereafter, worms were transferred every other day. Worms that were crawled off from the plates were excluded from the analysis. Survival curves were plotted. Mean, median, and maximum lifespan was reported.

5.3.8. Quantitative staining of Aβ fibrils with Thioflavin-T

Quantitative staining of Aβ fibrils with Thioflavin-T (ThT) was performed according to a previously described method (Xin et al., 2013) with slight modifications. At day 4 and day 8 worms were collected by washing the plate with 1 ml of M9 buffer and transferred into an Eppendorf tube. The worms were pelleted by centrifugation at 14k rpm for 2 min and the supernatant was poured off. Worms were washed two more times with M9 to get rid of the food. Worm pellet was resuspended in 500 µl of M9 in a microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80 °C until use. Worms were thawed and homogenized by using Precellys®24 Homogenizer (Bertin technologies) and Precellys® lysing kit: 03961-1-004 (0.5mm glass beads) at 6400 rpm, 2x10 secs twice for a total time of 40 secs in 500 µl of M9. Homogenized worms were centrifuged at 14k rpm for 2 min and the supernatant was collected into a new tube. The concentration of total soluble protein in each sample was quantified by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit). An equal amount of protein was used from each sample and triplicates were performed for each. Each replicate was mixed with 10 µl of M9 buffer and 2 µl of 1 mM ThT (Sigma) in a final volume of 100 µl. Fluorescence intensity was measured by a fluorescence plate reader (CLARIOstar multi-mode plate reader, BMG LABTECH) using excitation at 440 nm and emission at 482 nm and averaged from three independent experiments.

5.3.9. Statistical analysis

All statistical analyses were performed in IBM SPSS® statistics software (version 24). Data were expressed as mean ± standard error of the mean (SEM). Differences between groups for body length, mean area, locomotion, chemotaxis, and worm classes (at day 12) were analyzed using general linear model, multivariate test. Differences between Aβ levels were calculated using general linear model, univariate test. Differences between the number of days to reach motility group B/C from A was determined using t-Test. The survival function was estimated using Kaplan-Meier curves. Survival curves were compared using the log-rank (Mantel-Cox) test. The maximum

lifespan was the average lifespan of the top 10 longest-lived worms. Differences between groups for maximum lifespan were determined using one-way ANOVA with post-tests (Tukey's test). $P<0.05$ was considered significant.

5.4. Results

5.4.1. Cocoa supplementation reversed the reduced growth in pan-neuronal $A\beta_{1-42}$ expressing worms

We measured body length (µm) and area (μm^2) of worms starting from day 2 (after 24 hours from introducing L1 worms to food) till day 8 as indicators of worm growth and development. Results showed that $A\beta_{1-42}$ expressing worms (GRU102) were significantly shorter than control (GRU101) worms across all the time points starting from day 3 ($P<0.05$, Fig. 5.2A). Cocoa supplementation significantly increased the body length of GRU102 worms to reach similar levels to GRU101 worms starting from day 4 (Fig. 5.2B). GRU102 worms were significantly thinner than GRU101 worms across all the time points ($P<0.05$, Fig. 5.2C). Cocoa supplementation increased the thickness of GRU102 worms to reach similar levels to GRU101 worms at day 8 (Fig. 5.2D).

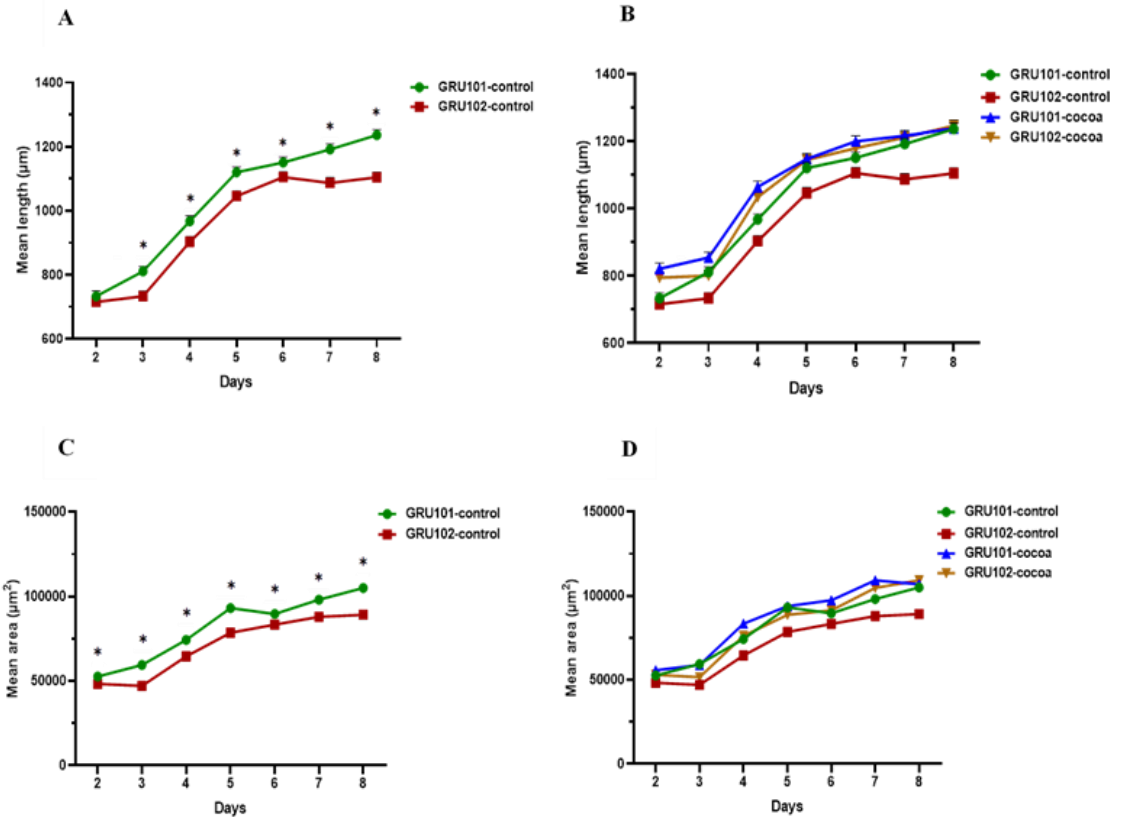

Figure 5.2. Body length (µm) and area (μm^2) of control and cocoa supplemented GRU102 ($A\beta_{1-42}$) and GRU101 (control) worms. Experiments were performed in triplicate. Values are expressed in mean ± SEM (n≥44). *$P<0.05$ for the indicated comparison (calculated using the multivariate test, general linear model). (A) GRU102 worms were shorter than GRU101 worms across all the time points, except day 2 ($P<0.05$). (B) Cocoa supplementation significantly increased the body length of GRU 102 worms to reach similar levels to GRU101 worms starting

from day 4 (C) GRU102 worms were thinner than GRU101 worms across all the time points ($P<0.05$). (D) Cocoa supplementation increased the thickness of GRU102 worms to reach similar levels to GRU101 worms at day 8.

5.4.2. Pan-neuronal $A\beta_{1-42}$ expressing worms showed a reduced maximum speed at old age which was reversed by cocoa supplementation

Mean speed (µm/s), maximum speed (µm/s), and maximum amplitude (µm) were measured as indicators of worm locomotion across the lifespan starting from day 2 to day 12. There was no significant difference in mean speed between the strains across all the time points, except day 8 (Fig. 5.3A) However, cocoa supplementation significantly increased the mean speed of both strains across most of the time points (Fig. 5.3B). The maximum speed of $A\beta_{1-42}$ expressing worms (GRU102) showed a reduction at a later stage of life starting from day 10 up to day 12 compared to control (GRU101) worms ($P<0.05$, Fig. 5.3C). Cocoa supplementation reversed this reduction in GRU 102 strain to reach similar levels to GRU101 (Fig. 5.3D). The maximum amplitude of GRU102 worms was significantly lower across most of the time points compared to GRU101 worms ($P<0.05$, Fig. 5.3E). However, Cocoa supplementation was not able to reverse this reduction in GRU102 strain at most of the time points (Fig. 5.3F).

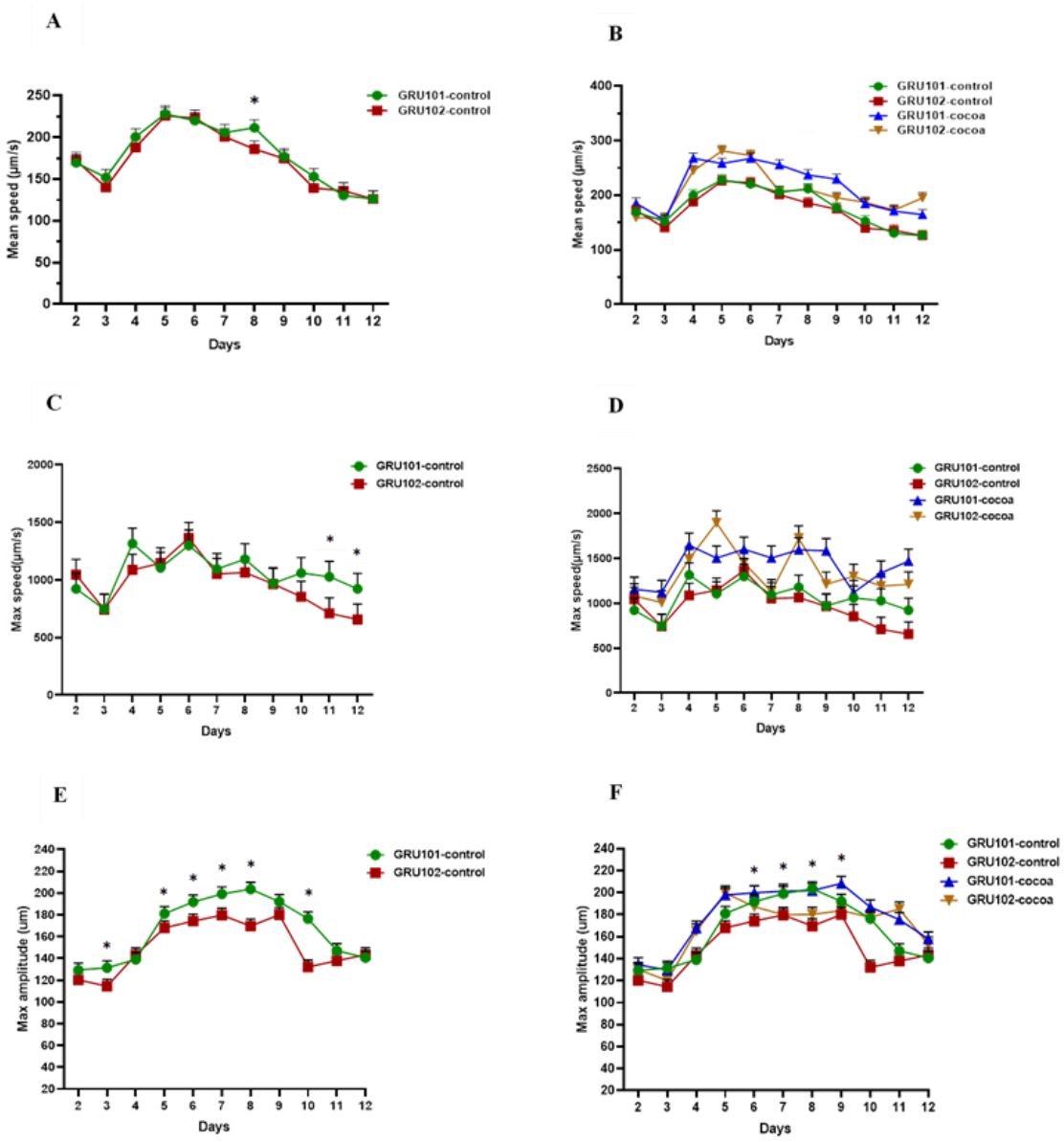

Figure 5.3. Locomotion parameters of control and cocoa-supplemented GRU102 ($A\beta_{1-42}$) and GRU101 (control) worms. Mean speed (µm/s), maximum speed (µm/s), and maximum amplitude (µm) were measured from day 2 to day 12. Experiments were performed in triplicate. Values are expressed in mean ± SEM (n≥42 per group). **P*<0.05 for the indicated comparison (calculated using the multivariate test, general linear model). (A) There was no significant difference between 2 worm strains for mean speed across all the time points, except day 8 (B) Overall, cocoa-supplementation increased the mean speed of both strains from day 4 (C) GRU102 worms showed a reduction in maximum speed at old age (day 10-12) which was significantly low at day 11 and 12 compared to GRU101 worms (D) Overall, cocoa improved the maximum speed in both strains across most of the time points and reversed the reduction (*P*>0.05) of maximum speed in GRU102 strain at old age to reach similar levels to GRU101. (E) The maximum amplitude of GRU102 worms was less across most of the time points (especially at the middle age) compared to GRU101 worms. (F) Even

though cocoa improved the maximum amplitude of GRU102 worms at some of the time points, there was no significant effect at most of the time points.

5.4.3. Cocoa supplementation maintained a similar motility reduction rate in pan-neuronal $A\beta_{1-42}$ expressing worms at old age as their control counterparts

Worms were further studied for their motility at old age by grouping them at day 12 into either class A, B, or C and re-scoring them for their class every day until they die. Class "A" represented the highly motile group. Class "B" was less motile compared to class "A" and class "C" was the least motile group. Worms showed no significant difference for their class at day 12 (Fig. 5.4A). However, cocoa-fed GRU101 (control) worms took a significantly reduced number of days to reach class B/C from A (P<0.05, Fig. 5.4B).

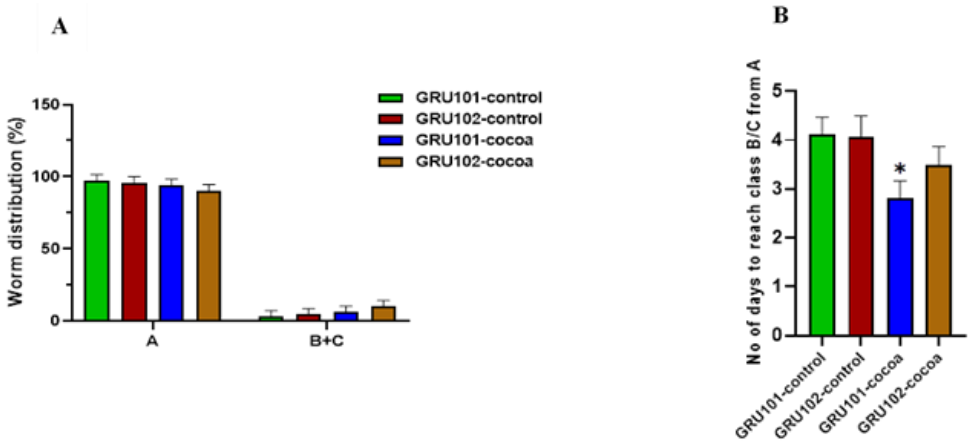

Figure 5.4. Locomotion of control and cocoa-supplemented GRU102 ($A\beta_{1-42}$) and GRU101 (control) worms at old age. Worms were categorized into either class A, B, or C at day 12 depending on their motility. They were re-scored for class every day until they die. For each group, the average number of days to reach class B/C from A was calculated. Experiments were performed in triplicate. Values are expressed in mean ± SEM (n≥24). (A) There was no significant difference between the groups for class "A" and "B+C" at day 12 (*P<0.05 for the indicated comparison calculated using general linear model, univariate analysis). (B) The number of days taken by cocoa-supplemented GRU101 worms to reach class "B/C" from "A" was significantly lower (*P<0.05 for the indicated comparison calculated using t-Test, two-sample assuming equal variances).

5.4.4. Cocoa supplementation improved the short-term memory loss in pan-neuronal AB_{1-42} expressing worms at old age

As worms showed a rapid reduction in chemotaxis and it was not detectable at day 12 (old age), we performed learning and short-term associative memory assay only at days 4 and 8 to represent young and middle age of worms respectively. There was no significant difference between GRU101 (control) and GRU102 ($A\beta_{1-42}$) worms for the naive chemotaxis at either day 4 or day 8 (Fig. 5.5A). However, cocoa supplementation improved the naive chemotaxis index in both strains at day 8 compared to their controls (P<0.05, Fig. 5.5B). Similar to the naive chemotaxis index, there was no significant difference between the strains for the learning index at both time points (Fig. 5.5C).

Cocoa supplementation improved the learning index in GRU102 worms at day 4 compared to its control (P<0.05, Fig. 5.5D). Interestingly, GRU102 worms showed a significant short-term memory loss (dementia index) compared to GRU101 worms at both 1 hr and 2 hr time points after conditioning at day 8 (P<0.05, Fig. 5.5E). GRU102 worms showed a 113.3% increase in short-term memory loss at 1 hr time point and 46.8% increase at 2 hr time point compared to GRU101 worms. Cocoa supplementation reversed this short-term memory loss in GRU102 at both 1 hr and 2 hr time points to reach similar levels to GRU101 (Fig. 5.5F). The reduction in short-term memory loss in cocoa-supplemented GRU101 worms at 2 hr time point at day 8 (46.8%) was significant compared to its control counterparts (P<0.05). At day 8, cocoa-supplemented GRU102 worms showed a 73.4% and 63.0% reduction in short-term memory loss at 1 hr and 2 hr time points respectively compared to GRU102 control worms.

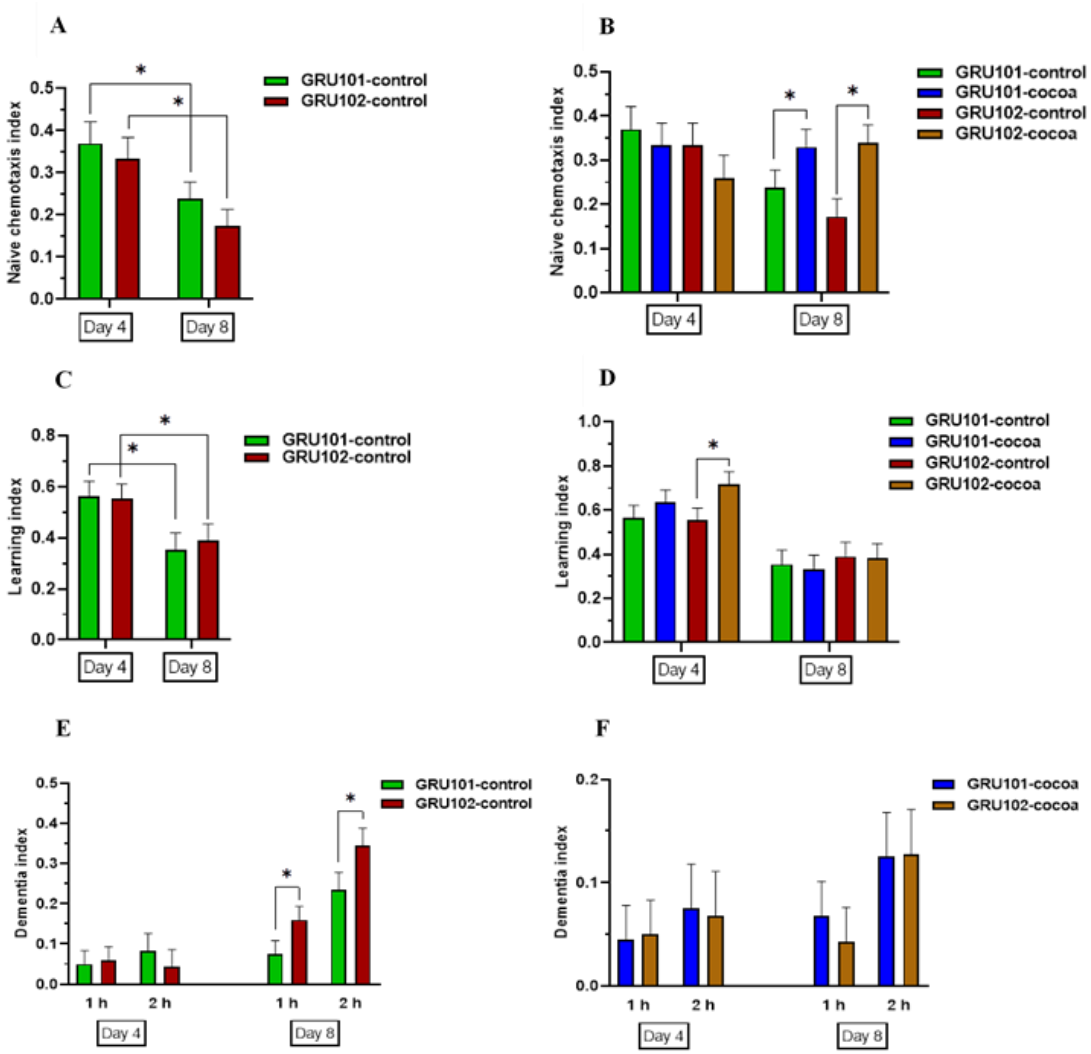

Figure 5.5. Naive chemotaxis index, learning index, and dementia index (short-term memory loss) of control and cocoa-supplemented GRU102 ($A\beta_{1-42}$) and GRU101 (control) worms at day 4 and day 8. Chemotaxis assay was performed for well-fed, synchronized worms, and naive chemotaxis index was calculated, chemotaxis index (CI) = [(number of worms at butanone) – (number of worms at ethanol)]/ [total number of worms on the plate]. Well-fed, synchronized

worms were starved for 1 hour, then fed in the presence of 10% butanone for 1 hour. Worms were tested immediately after 1 hr from the food-butanone association for their learning, learning Index (LI) = chemotaxis $index_t$ – chemotaxis $index_{Naive}$. Dementia index (short-term associative memory loss) was measured after 1 hr and 2 hrs from the food-butanone association without exposure to butanone again (Dementia $index_t$=Learning $index_0$-Learning $index_t$). Four independent experiments were performed (n≥50 worms per group). Values are expressed in mean ± SEM. $^*P<0.05$ for the indicated comparison (calculated using the multivariate test, general linear model). (A) There was no significant difference between the 2 strains at both days 4 and 8 for the naive chemotaxis index. (B) Cocoa significantly increased the naive chemotaxis in both strains at day 8 ($P<0.05$). (C) There was no significant difference between the 2 strains at both days 4 and 8 for the learning index. (D) Cocoa significantly increased the learning in GRU102 worms at day 4 ($P<0.05$). (E) GRU102 worms showed a higher dementia index (short-term memory loss) at both 1 hr and 2 hr time points from conditioning at day 8 ($P<0.05$). (F) Cocoa significantly reduced the dementia index in GRU102 worms at both time points (t=1 and t=2) at day 8 to reach similar levels to GRU101 worms.

5.4.5. Cocoa supplementation reduced Aβ fibril levels in pan-neuronal $Aβ_{1-42}$ expressing worms at both young and middle age

A significantly high fluorescence was detected in GRU102 ($Aβ_{1-42}$) worms compared to GRU101 (control) worms with ThT fluorescent dye at both day 4 and day 8 ($P<0.05$, Fig. 5.6A). With cocoa supplementation, GRU102 worms showed significantly reduced Aβ fibril levels at both day 4 and day 8 compared to untreated GRU102 worms ($P<0.05$, Fig. 5.6B).

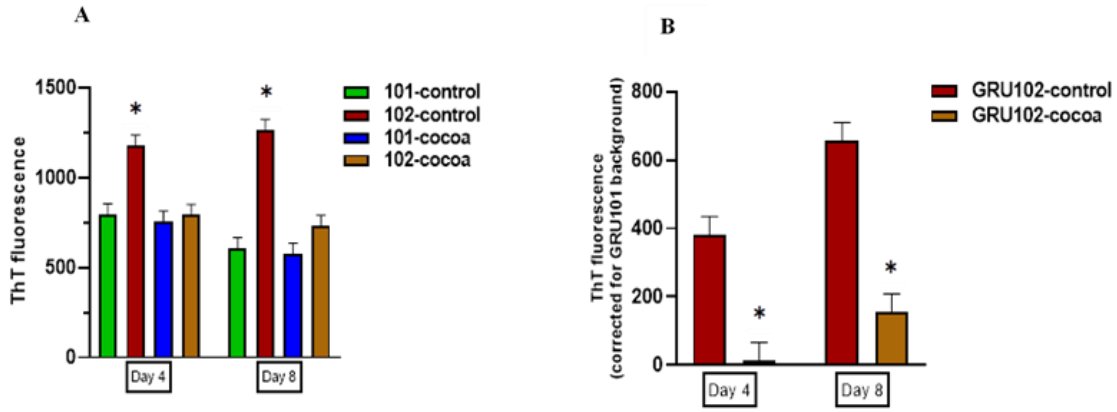

Figure 5.6. Aβ fibril levels in control and cocoa-supplemented GRU101 (control) and GRU102 ($Aβ_{1-42}$) worms at day 4 and day 8. Values are expressed in mean ± SEM of three independent experiments with at least n=100 worms per experiment. *$P<0.05$ for the indicated comparison (calculated using the univariate test, general linear model). (A) Th T fluorescence for the control and cocoa-supplemented GRU101 and GRU102 worms; untreated GRU102 (AB_{1-42}) worms showed a higher Th T fluorescence compared to GRU101 (control) worms at both day 4 and day 8 ($P<0.05$). Cocoa supplementation significantly reduced the Th T fluorescence in GRU102 (AB_{1-42})

worms at both day 4 and day 8. (B) Graph corrected for the background fluorescence of GRU101 worms; cocoa supplementation significantly reduced the Aβ fibril levels in GRU102 worms at both day 4 and day 8 (P<0.05).

5.4.6. Cocoa supplementation at a dose of 3 mg reversed the mean lifespan reduction in pan-neuronal $A\beta_{1-42}$ expressing worms

The worm diet was supplemented with cocoa at 1 mg, 2 mg, 3 mg, 4 mg, and 5 mg doses. We found GRU101 (control) worms to have a mean lifespan of 15.8 days and GRU102 ($A\beta_{1-42}$) to have a mean lifespan of 14.9 days at our laboratory conditions at 20 °C (Fig. 5.7, Table 5.1). The mean lifespan of GRU102 worms was significantly lower compared to GRU101 worms (P<0.05). The median lifespan of GRU101 and GRU102 worms were 15 and 14 days respectively and was not significantly different. Cocoa supplementation at a dose of 3 mg significantly extended the mean lifespan of GRU102 worms (8.5%) to reach similar levels to GRU101 worms. Moreover, these GRU102 worms showed a significantly increased median lifespan (P<0.05, 14.3%) compared to their control. In addition, 2 mg (7.1%) and 4 mg (7.1%) cocoa doses extended the median lifespan of GRU102 worms compared to their control. Supplementing the worm diet with cocoa at a dose of 5 mg significantly decreased the mean lifespan of both stains (P<0.05, 14.4% for GRU101 and 7.0% for GRU102) while significantly reducing the median lifespan of GRU101 worms (P<0.05, 13.3%). Besides, cocoa at 1 mg dose also decreased both the mean and median lifespan of GRU102 worms compared to GRU101 worms (P<0.05, 6.5% mean, and 7.1% median). However, none of the cocoa doses extended the maximum lifespan of GRU102 worms.

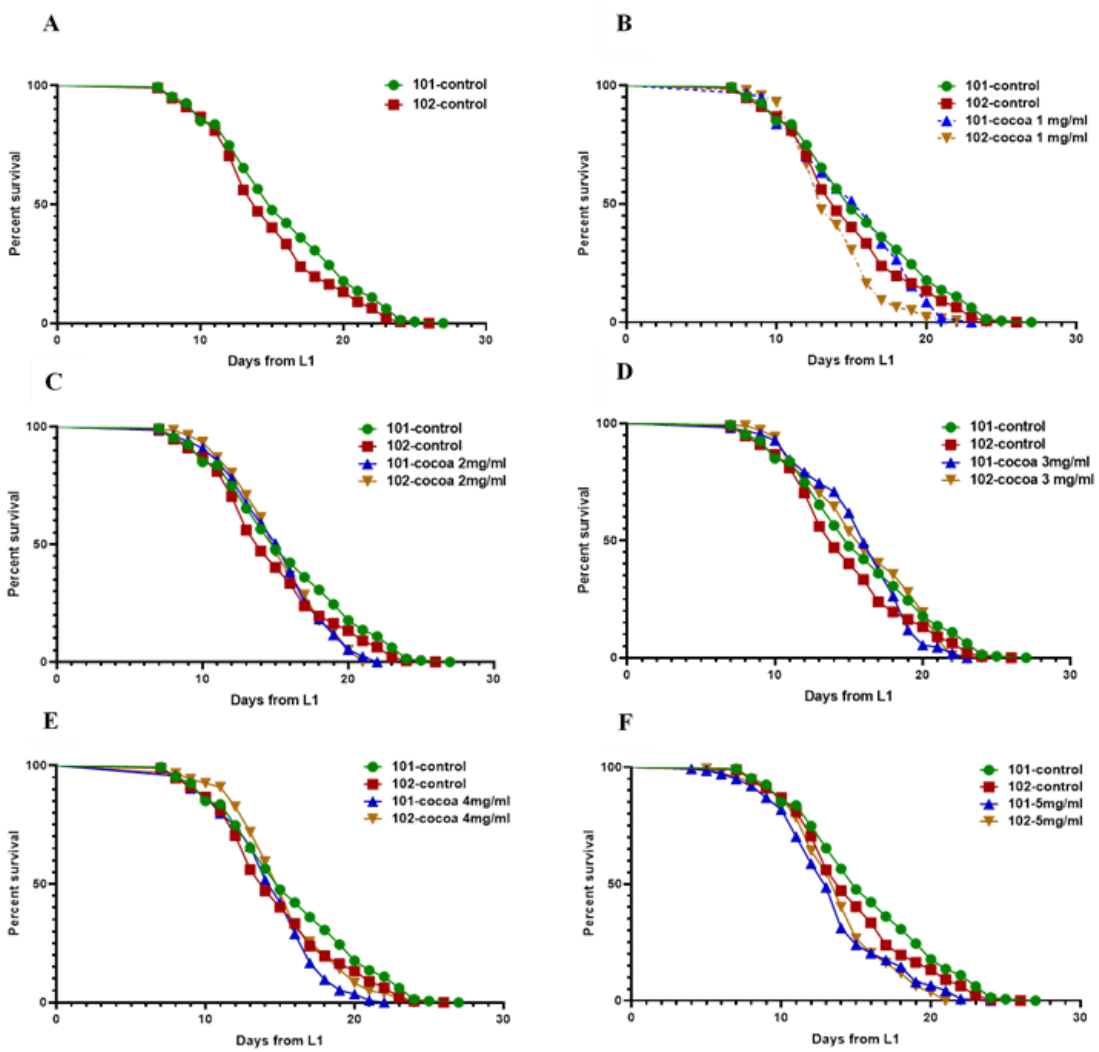

Figure 5.7. Survival curves for control and cocoa-supplemented GRU101 and GRU102 ($A\beta_{1-42}$) worms. Experiments were performed in triplicate (100<n>200 for all groups). Differences between groups for mean and median lifespans were calculated using the log-rank (Mantel-Cox) test. (A) GRU102 worms showed a significantly lower (P<0.05) mean lifespan compared to GRU101 (control strain). (B) Cocoa at a dose of 1 mg decreased the mean and median lifespan of GRU102 worms compared to GRU101 worms (P<0.05, 6.5% mean, and 7.1% median). (C) Cocoa supplementation at a dose of 2 mg increased the median lifespan of GRU102 worms compared to their control (P<0.05, 7.1%). (D) Cocoa at a dose of 3 mg significantly increased the mean (P<0.05, 8.5%) lifespan of GRU102 worms to reach similar levels to GRU101 worms. The median lifespan of GRU102 worms was significantly extended compared to their control (P<0.05, 14.3%). (E) Cocoa at a dose of 4 mg increased the median lifespan of GRU102 worms compared to their control (P<0.05, 7.1%). (F) Cocoa at 5 mg/ml dose decreased the mean lifespan of both worm strains (P<0.05, 14.4% for GRU101 and 7.0% for GRU102).

Table 5.1. The mean, median and maximum lifespan of control and cocoa-supplemented GRU101 and GRU102 worms

Treatment	Mean lifespan	% extension compared to control	Median lifespan	% extension compared to control	Maximum lifespan
GRU101-control	15.8 ± 0.38		15		20.6
GRU102-control	14.9 ± 0.30[a]		14		20.2
GRU101-cocoa, 1 mg	15.3 ± 0.36	-3.5	16	6.7	18.0[a]
GRU102-cocoa, 1 mg	13.9 ± 0.24[a,b]	-6.5	13[a]	-7.1	17.2[a,b]
GRU101-cocoa, 2 mg	15.2 ± 0.30	-4.1	16	6.7	19.0
GRU102-cocoa, 2 mg	15.4 ± 0.27	3.0	15[b]	7.1	18.9
GRU101-cocoa, 3 mg	15.9 ± 0.34	0.5	16	6.7	18.8
GRU102-cocoa, 3 mg	16.2 ± 0.39[b]	8.5	16[b]	14.3	18.8
GRU101-cocoa, 4 mg	14.5 ± 0.31[a]	-8.4	15	0	17.9[a]
GRU102-cocoa, 4 mg	15.4 ± 0.32	3.6	15[b]	7.1	19.0
GRU101-cocoa, 5 mg	13.6 ± 0.33[a]	-14.4	13[a]	-13.3	17.9[a]
GRU102-cocoa, 5 mg	13.9 ± 0.27[a,b]	-7.0	14	0	17.9[a]

[a] statistically significant (P<0.05) compared to the GRU101-control group

[b]statistically significant (P<0.05) compared to the GRU102-control group

5.5. Discussion

A transgenic *C. elegans* strain (GRU102) which expresses pan-neuronal $A\beta_{1-42}$ was used to study the effects of long-term cocoa supplementation on $A\beta_{1-42}$ induced toxicity, particularly on the short-term memory loss observed in human AD. In our study, we administered whole cocoa powder to *C. elegans* by spreading it over the *E. coli* lawn. However, E. coli can modify cocoa powder and produce different metabolites. Studies have demonstrated that polyphenols in cocoa are poorly absorbed in the intestine in their natural form and therefore, the degradation of these compounds in the colon by microbes is one of the most effective ways to produce secondary metabolites that increases their bioavailability (Rechner et al., 2004; Sorrenti et al., 2020). Therefore, we assume that even if *E. coli* modifies cocoa, it will not have any adverse impact on the beneficial effects of cocoa polyphenols.

Cocoa contains several polyphenolic compounds, in particular, it is rich in flavanols. The major flavanol compounds found in cocoa are the monomeric forms, epicatechin, catechin, and their oligomeric and polymeric forms, procyanidins. In addition to polyphenols, cocoa contains methylxanthines, predominantly theobromine about 2-3% of by weight (Katz et al., 2011). Almost all the studies attribute the health-promoting effects of cocoa to cocoa polyphenols and the role of methylxanthines on these beneficial effects of cocoa polyphenols remains unanswered (Jalil & Ismail, 2008). Processing with alkali is known to reduce the beneficial polyphenol content in natural

cocoa powder (Gu et al., 2006; Kealey Kirk et al., 1996; Robinson et al., 1961). However, the compositional analysis showed that the commercially processed cocoa powder we used in this study was not devoid of flavonoids and other polyphenols. Our cocoa powder contained 27.01 mg GAE/g total phenolics which is about 56% compared to natural cocoa powder. The total flavonoids were 10.13 mg CE/g which is about 40% of the total flavonoids in natural cocoa powder (Munasinghe, Almotayri, Thomas, et al., 2021).

Polyphenols, in general, are known to have low bioaccessibility and bioavailability, and most of them cannot be absorbed in their natural forms. Monomers and dimeric or trimeric proanthocyanidins are quite stable in the gastric environment and therefore, are traveled to the small intestine with almost no changes (Aprotosoaie, Miron, et al., 2016). Monomers can cross the gut barrier and around 20% are rapidly absorbed, undergo rapid and extensive metabolization, and circulate in the bloodstream (Sorrenti et al., 2020). Epicatechin shows higher bioavailability compared to the other monomers. Dimeric and trimeric procyanidins are known to be poorly absorbed in the gastrointestinal tract. The acidic environment in the stomach degrades some dimers to epicatechin and undergo interflavan bond conversion (Aprotosoaie, Miron, et al., 2016). Intake of cocoa with a large amount of flavanol monomers and dimers has resulted in the occurrence of B2 and B5 dimers in human plasma in modest concentrations (Keen et al., 2002). Oligomeric procyanidins (the degree of polymerization above three) and polymeric flavanols which are not absorbed in their naive form reach the colon and are being metabolized by the local microbiota to form secondary metabolites, increasing their bioavailability (Keen et al., 2002; N. Khan et al., 2012). The role of flavonoids in the modulation of neurodegeneration is often related to their antioxidant capacity, but other mechanisms are also known to be involved. These mechanisms depend on the ability of these compounds to cross the blood-brain barrier (BBB) (Faria et al., 2011). Several studies have demonstrated that catechin and epicatechin can cross the BBB therefore, indicating them as prospective compounds to exert protective effects in neurodegeneration (Abd El Mohsen et al., 2002; Faria et al., 2011).

In this study, we categorized worms as young (day 4), middle (day 8), and old (day 12) to see the effects at different stages of life. Worm body length and area were measured as indicators of worm growth and development. GRU102 ($A\beta_{1-42}$) worms showed a significantly reduced body length and area compared to GRU101 (control) worms. However, cocoa supplementation reversed this reduction in both length and area in GRU102 worms to reach similar levels to GRU101 worms. We measured well-characterized locomotion parameters of *C. elegans* including mean speed and maximum speed to see how neuronal expression of $A\beta_{1-42}$ influences worm movements. In addition, we used maximum amplitude which is known as a marker of *C. elegans* muscle strength (Nahabedian et al., 2012). Even though the mean speed of worms was not affected by Aβ toxicity, maximum speed was significantly affected at old age. Cocoa supplementation reversed this reduction at old age to reach similar levels to GRU101 worms. GRU102 worms showed a reduced

maximum amplitude across most of the time points compared to GRU101 worms. However, cocoa supplementation was not effectively reversed this reduction in maximum amplitude, particularly in mid-life. We used another method to identify the changes in locomotion at further old age starting from day 12 by classifying worms into three different classes. Results showed that there was no significant difference between the two strains in terms of the number of days that they took to reach the low motile category from the high motile category. When the worms were fed with cocoa, GRU101 worms took a significantly lower number of days to reach the low motile group. In our lifespan experiment, we observed a significant reduction in lifespan in both strains with 5 mg/ml cocoa dose and a greater reduction (about two times) in the GRU101 strain compared to GRU102. This greater reduction in lifespan in GRU101 strain may be the reason for these worms to take a lesser number of days to reach the low motile group.

C. elegans chemotaxis behavior, a neuronally-controlled process was used to study the Aβ induced deficits in neuronal function and the effects of cocoa-supplementation. Naive chemotaxis which is the natural response of *C. elegans* for butanone, learning index which is an indicator of the extent that worms learn food-butanone association, and the short-term memory loss (dementia index) were measured. Control and the Aβ expressing strain showed no difference in both naive chemotaxis index and learning index at both young age and middle age. However, cocoa supplementation significantly increased the learning index in GRU102 worms at young age. A recently published study suggests that Aβ is associated more strongly with a deficit in learning than any aspect of memory dysfunction (Lim et al., 2020). Therefore, our results suggest that cocoa might be useful in improving the ability to learn in AD. Loss of memory is among the first symptoms reported by AD patients and therefore, one of the key characteristics of AD (Jahn, 2013). Pan-neuronal expression of Aβ seemed to induce a short-term memory loss in *C. elegans* at their middle age. This short-term memory loss has not been described anywhere else before according to our knowledge and we report it here as one of the novel findings of our study. Thus, this model closely resembles human AD and therefore, is more competent in drug screening studies compared to other available *C. elegans* models of AD. Interestingly, Aβ induced memory loss was reversed by cocoa supplementation.

Fong et al. (2016) report that they detected Aβ protein in GRU102 worms in the aggregated form at day 12 by using anti-Aβ antibodies. We detected Aβ fibrils at both young and middle age of worms using ThT which is a commonly used probe to monitor *in vitro* Aβ fibril formation. Upon binding to Aβ fibrils, ThT gives a strong fluorescence signal at approximately 482 nm when excited at 440 nm (Xue et al., 2017). According to our results, cocoa supplementation effectively reduced the Aβ fibril levels in GRU102 worms at both young and middle age.

Our study was consistent with the previous study which reports that Aβ expressing worms have a significantly reduced lifespan compared to control worms (Fong et al., 2016). One cocoa dose we tested reversed the mean lifespan reduction in Aβ expressing worms. However, the lowest and the

highest doses of cocoa further reduced the lifespan in Aβ expressing worms. Lifespan reduction was observed in the control strain as well with the highest cocoa dose used. As it has been shown that caloric restriction can extend the lifespan in *C. elegans* (G. D. Lee et al., 2006), we assume that cocoa powder as a rich source of carbohydrates (more than 50% of the weight) may reduce the lifespan of *C. elegans* when worms were supplemented a high dose. The lifespan reduction with the lowest dose may be due to the availability of potential beneficial bioactive components in lesser amounts.

Aβ, an amphiphilic and partly folded molecule is prone to self-aggregate and produce intermediate soluble oligomers, protofibrils, and finally insoluble fibrils (Serpell, 2000). The neurotoxicity of Aβ is known to depend on their aggregated state, in which oligomers and protofibrils are more toxic than monomers and mature fibrils (Phan et al., 2019). A previous study found a cocoa peptide reduced the Aβ induced toxicity in a transgenic *C. elegans* (CL4176) which expresses $A\beta_{1-42}$ in body wall muscles by upshifting the temperature from 16 ℃ to 25 ℃ (Patricia Martorell et al., 2013). Aβ expression results in a concomitant progressive paralysis phenotype in this strain. They further report that this beneficial effect was achieved through the reduction of toxic Aβ levels (as determined by western blot) by reducing the Aβ oligomerization. Another study supports the beneficial effects of cocoa against Aβ toxicity by showing that cocoa polyphenols reduced the Aβ oligomerization *in vitro* and rescued the synaptic deficits induced by Aβ oligomers in a mouse model suggesting that cocoa has multiple disease-modifying properties in AD (J. Wang et al., 2014). Another study found cocoa polyphenols triggered neuroprotection by activating the brain-derived neurotrophic factor (BDNF) signaling pathway on both Aβ plaques and Aβ oligomers treated cells, resulting in the counteraction of neurite dystrophy (Cimini et al., 2013). Epicatechin, catechin, and their mixture were able to protect PC12 cells from Aβ induced neurotoxicity in another study showing more evidence for the neuroprotective effects of cocoa polyphenols (Heo & Lee, 2005). In another study, epicatechin and catechin inhibited the formation of Aβ fibrils from fresh $A\beta_{1-40}$ and $A\beta_{1-42}$ and destabilized preformed Aβ fibrils in a dose-dependent manner (Ono et al., 2003). Diaz et al. (2019) reported that epicatechin reduced the deterioration of spatial memory induced by the $A\beta_{25-35}$, in addition to reducing oxidative stress and inflammation in the hippocampus of the animals treated with epicatechin + $A\beta_{25-35}$. These previous findings support our study suggesting that cocoa can alleviate Aβ induced deficits in worm locomotion, cognition and lifespan may be through reducing the toxic Aβ levels and rescuing the neuronal damage through the action of polyphenols and peptide components.

5.6. Conclusion

Our study demonstrated that cocoa-supplementation can protect against Aβ induced toxicity in *C. elegans*. We showed that cocoa can alleviate some Aβ-induced deficits in worm locomotion, short-term associative memory, and lifespan while reducing Aβ fibril levels in worms. However, further studies are required to identify the active components and mechanisms of action.

CHAPTER 6: Effects of Cocoa Supplementation on Altered Metabolite Levels in Purine Metabolism Pathways and Urea Cycle in amyloid-β Expressing *C. elegans*

This chapter has been written as a manuscript and is ready for submission for publication.

6.1. Abstract

Aging is the primary risk factor for most of the well-recognized pathologies. Dietary interventions have been recently gained much attention as alternative therapies in preventing/delaying the onset of age-associated pathological conditions. Epidemiological studies suggest that polyphenols (PPs) play an important role in this regard. Cocoa is rich in PPs which is greater than the PPs in teas and red wine. Previously, we showed long-term cocoa supplementation can improve age-associated health and amyloid-β (Aβ)-induced deficits in Alzheimer's disease (AD) in *C. elegans*. The objective of this study was to further characterize the age-associated and Aβ-induced changes in metabolite concentrations in *C. elegans* and the effects of long-term cocoa supplementation. The untargeted Gas Chromatography-Mass Spectrometry (GC-MS) approach was used to determine the changes in endogenous metabolite concentrations. Wild-type (N2) worms were used to determine the age-associated changes at day 4 from the first larval stage (young age), day 8 (middle age), and day 12 (old age). A transgenic *C. elegans* strain expressing pan-neuronal Aβ was used at day 4 and day 8 to evaluate Aβ-induced changes. Aging altered some of the amino acid concentrations in *C. elegans*. Cocoa supplementation reduced leucine and tyrosine concentrations in wild-type worms at old age (day 12) which might be linked with the age-associated health improvements reported previously. However, most of the amino acid concentrations in wild-type worms were unaffected by cocoa supplementation. Aβ expressing worms showed higher proline and asparagine concentrations than control worms at young age (day 4) which may have a connection with cognitive deficits in AD. Cocoa reduced the elevated concentrations of these two amino acids to the levels in control worms. Purine derivative hypoxanthine concentration was higher in Aβ expressing worms than control worms at their middle age (day 8) where the elevated concentrations are known to contribute to the memory deficits in AD. Cocoa supplementation reduced the elevated hypoxanthine concentration to the normal level. Aβ expression showed alterations in the urea cycle in worms at their middle age (day 8) as indicated by increased ornithine concentration. However, the exact effects of cocoa on ornithine concentrations were uncertain due to the complicated nature of the mechanisms. Overall, cocoa seemed to interact with some of the endogenous metabolites in *C. elegans* to modify aging and Aβ-induced deficits.

6.2. Introduction

Aging is the main risk factor for most debilitating and life-threatening disease conditions including cancer, cardiovascular disease, and neurodegeneration (Niccoli & Partridge, 2012). The prevalence of aging-related diseases is expected to rise dramatically with the aging of the population worldwide (Kennedy et al., 2014). As the associated costs of diagnosis, treatment, and care for aging-related diseases are high, the overall burden on the healthcare system is significant. Therefore, preventive, lifestyle-choice approaches are needed for a sustainable, feasible, and affordable health care system (Shlisky et al., 2017).

AD is one of the most debilitating neurodegenerative diseases in aging populations (N. Li et al., 2016). AD is characterized by the extracellular accumulation of Aβ plaques and intracellular aggregation of neurofibrillary tangles in the brain. Given that there is no pharmaceutical intervention that has been shown to cure AD yet, alternative therapeutic approaches for AD have gained much attention recently.

Emerging evidence highlights the role of nutritional approaches in promoting healthy aging (Dawson-Hughes et al., 2008; Morris et al., 2015; Solfrizzi & Panza, 2014), specifically the role of dietary PPs (Arora et al., 2020; Luo et al., 2021; Sarubbo et al., 2017). It was previously shown that cocoa, one of the richest dietary sources of PPs reduced the symptoms of aging-associated disease conditions and Aβ-induced deficits in AD in the model organism *C. elegans* (Munasinghe, Almotayri, Kolivas, et al., 2021; Munasinghe, Almotayri, Thomas, et al., 2021). In addition, other studies also have been shown the beneficial effects of cocoa in aging-related diseases conditions *in vitro*, in various animal models, and in humans (Dani et al., 2008; Gröne et al., 2020; Mafi et al., 2019; Stringer et al., 2015; Zeng et al., 2014). However, the changes in the metabolic profile in *C. elegans* that occur upon long-term cocoa supplementation in aging and AD are not previously described. Therefore, this study aimed to determine the age-associated and Aβ-induced changes in the metabolome of *C. elegans* and the effects of long-term cocoa supplementation using untargeted GC coupled with MS.

C. elegans was employed as the model organism in this study due to its attractive features in aging-related studies including short lifespan (17 days at 20 °C), fully sequenced genome, and the ease of genetic intervention with RNA interference through bacterial feeding (Gao et al., 2017). The transgenic strain used to study AD expresses Aβ in neurons and shows middle-age onset behavioral dysfunction, therefore, closely resembles human AD (Fong et al., 2016). Moreover, it was previously found that this strain shows short-term memory loss at their middle age which is one of the first and most important symptoms in AD (Munasinghe, Almotayri, Kolivas, et al., 2021).

6.3. Materials and methods

6.3.1. Worm strains and culture conditions

Wild type (N2, Bristol) and GRU101 gnals1 (myo-2p::yfp), GRU102 gnals2 (myo-2p::YFP + unc-119p::Abeta$_{1-42}$) *C. elegans* and *Escherichia coli* (*E. coli*) OP50 were obtained from *Caenorhabditis*

Genetics Center (Minneapolis, MN, USA). GRU102 strain expresses pan-neuronal $A\beta_{1-42}$ under control of *C. elegans* myo-2 (myosin heavy chain structural genes) promoter. GRU101 is the control strain for GRU102. Worms were maintained on nematode growth medium (NGM) plates carrying a lawn of *E. coli* OP50 at 20 °C.

6.3.2. Cocoa treatment and sampling of worms

The treatment group received 5 mg/ml cocoa powder (unsweetened dry powder processed with alkali) suspended in M9 buffer with concentrated *E. coli* OP50. Concentrated *E. coli* was prepared by centrifuging overnight cultures of *E. coli* in Luria-Bertani (LB) broth and resuspending 1 g of *E. coli* pellet in 16 ml of M9 buffer. Concentrated *E. coli* was first added to NGM plates and was grown overnight at 20 °C. The cocoa suspension was added on the top of the *E. coli* lawn at a ratio of 1:2 volume, *E. coli*: cocoa suspension (for small plates: 100 µl of *E. coli* and 200 µl of cocoa, for medium plates: 200 µl of *E. coli* and 400 µl of cocoa). Worms received cocoa starting from the L1 stage. Floxuridine (FUdR) was not used to avoid progeny production and instead, worms were transferred every day onto fresh plates until they stop laying eggs (until day 9). Thereafter, worms were transferred every other day. At each time point (day 4, day 8, or day 12) worms were collected by pelleting on ice (the day L1 worms were plated was considered as day 1). The removal of bacteria and debris was carried out using a sucrose-flotation (60 % sucrose). After collection, the worms were washed with S-buffer 5 times and partitioned into aliquots of a maximum of 0.3 ml per tube (Precellys lysing kit, Bertin Technologies). The aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

6.3.3. Extraction

Sample extraction and GC-MS analysis were performed according to the method described in (Afshari et al., 2020). Worm pellet was weighed (~60 mg) into a lysing tube and 500 µl of ice-cold H_2O/MeOH/$CHCl_3$ (1:3:1 v/v), containing an external standard (100 µl of $^{13}C_6$-sorbitol/$^{13}C_5$ ^{15}N-valine in water, 0.2 mg ml^{-1}) was added. The mixture was homogenized using an MP homogenizer (FastPrep®) (1 min, 4.5 m/s) and vortexed and incubated (37 °C for 15 min) in a thermomixer at 850 rpm. Then, the mixture was centrifuged at 13000 rpm for 15 min. The supernatant was transferred to a new Eppendorf tube, and 500 µl of MeOH/H_2O/$CHCl_3$ was added into the first lysing tube containing the previously freeze-dried sample. The samples were again vortexed and centrifuged at 13000 rpm for 15 min. The resulting supernatant was then transferred into the tube containing the original supernatant from the previous centrifugation. Pooled samples were then vortexed for 30 s and 50 µl aliquots of supernatant were transferred into separate glass inserts and dried *in vacuo* for subsequent trimethylsilyl (TMS) polar metabolite derivatisation using GC-MS analysis, as described below.

6.3.4. Polar metabolite derivatisation

All samples were redissolved in 10 µl of 30 mg ml^{-1} methoxyamine hydrochloride in pyridine and derivatized at 37 °C for 120 min with mixing at 500 rpm. The samples were then treated for 30 min

with 20 µl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 2.0 µl retention time standard mixture [0.029 % (v/v) n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane, n-hexatriacontane dissolved in pyridine] with mixing at 500 rpm. Each derivatized sample was allowed to rest for 60 min before injection.

6.3.5. GC-MS instrument conditions for untargeted metabolite analysis

Samples (1 µl) were injected into a GC-MS system comprised of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph, and a 7000 Agilent triple-quadrupole MS (Agilent). The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)- amine (CF43). The GC was performed on a 30 m VF- 5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250 °C, the MS transfer line at 280 °C, the ion source adjusted to 250 °C, and the quadrupole at 150 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. For the polar metabolite analysis, the following temperature program was used; start at injection 70 °C, hold for 1 min, followed by a 7 °C min^{-1} oven temperature ramp to 325 °C and a final 6 min heating at 325 °C. Mass spectra were recorded at 2 scans s^{-1} with a 50–600 *m/z* scanning range.

6.3.6. Data processing and analyses of data

Both chromatograms and mass spectra were processed using Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.07.01/Build 7.1.524.0. Mass spectra of eluted compounds were identified using the NIST08 database (http://www.nist.gov) and an in-house mass spectral library at RMIT University. All matching mass spectra were additionally verified by determination of the retention time and index in comparison to authentic standard substances. The resulting relative response ratios (area of analyte divided by area of the internal $^{13}C_6$-sorbitol standard and sample dry weight) for each analyzed metabolite were calculated. For the metabolome datasets, zero values were replaced with half of the lowest positive value in the metabolome dataset for all analyses. If a specific metabolite had multiple trimethyl silyl (TMS) derivatives, the metabolite with the greater detector response and better peak shape within the dynamic range of the instrument was selected.

Dr. Daniel Dias and Dr. Roya Afshari (RMIT University, VIC) were involved in the technical aspects of GC-MS analysis and my involvement was with sample preparation, statistical analysis of data, data interpretation, and writing the manuscript.

6.3.7. Statistical analysis

IBM Statistical Package for Social Science version 27.0 (SPSS, Chicago IL) was used for the analysis of data. As the variability of three replicates was high (we assumed that the extraction efficiency was different among samples), data were transformed to percentages of the total. Metabolites were categorized as amino acids and others in the analysis. Each amino acid was expressed as a percentage of the total amino acids. Each other metabolite was expressed as the percentage of the rest of the metabolites. Shapiro-Wilk and Kolmogorov-Smirnov tests were used

to test the normality of data. Homogeneity of variance was tested with Levene's test. When data violates normality and the homogeneity of variance assumptions, variable transformations (square, square root, logarithmic) were reviewed. As the transformation did not improve the data distribution, non-parametric tests were considered for those variables [Alanine, proline, glycine, serine, threonine, methionine, glutamine, phenylalanine, glutamate, lysine, uracil, ornithine, and anthranilic acid in wild-type worms; alanine, leucine, glycine, serine, threonine, aspartate, methionine, glutamate, phenylalanine, glutamine, lysine, succinate, uracil, cytosine, ornithine, 4-Hydroxybenzoic acid, glycerol-3-phosphate (G-3P), glucose, sucrose, trehalose, and anthranilic acid in Aβ expressing worms and their control]. Metabolomic profile was analyzed using the multivariate test, general linear model. $P<0.05$ was considered significant. Kruskal-Wallis test was conducted for pairwise comparison for the skewed and the variables violating the assumption of homogeneity of variance and asymptotic significance at the 0.05 level was reported. For descriptive purposes, data were presented as Mean ± SE.

6.4. Results

6.4.1. Metabolic profile of wild type (N2) and Aβ expressing *C. elegans*

The metabolic profile of wild-type and pan-neuronal Aβ expressing *C. elegans* was determined with and without cocoa supplementation. Altogether, 28 compounds were detected in the metabolome of *C. elegans*. Of these 28, 14 were amino acids (AAs) including alanine, leucine, proline, glycine, serine, threonine, aspartate, methionine, glutamate, phenylalanine, asparagine, glutamine, lysine, and tyrosine. In addition, succinate, malate, 4-hydroxybenzoic acid, gluconic acid, adipic acid, and anthranilic acid were detected as organic acids. Glucose, sucrose, and trehalose were present as sugars. Further, two nucleobases (uracil and cytosine) and hypoxanthine which is a purine derivative, occasionally found as a nucleic acid constituent were detected. Moreover, Ornithine (a non-proteinogenic amino acid) and G-3P were identified.

6.4.2. Age-associated changes in the metabolome of wild-type (N2) *C. elegans* and the effects of cocoa supplementation

Age-associated changes in the metabolomic profile of *C. elegans* and the effects of cocoa supplementation were determined. Worms were tested on days 4, 8, and 12 to represent young, middle, and old age respectively. Serine, asparagine, and glutamine concentrations were lower at day 12 than day 4 ($P<0.05$). Conversely, glycine concentration was higher at day 12 than day 4 ($P<0.05$). Other detected AAs showed no significant difference at day 12 compared to day 4 ($P>0.05$). Cocoa-supplemented worms showed a higher asparagine concentration at day 4 than control worms ($P<0.05$). Asparagine and tyrosine concentrations were high in cocoa-treated worms at day 8 compared to their control counterparts ($P<0.05$). At day 12, the concentrations of leucine and tyrosine were lower in cocoa-supplemented worms than control worms ($P<0.05$). With organic acids, malate and adipic acid concentrations were lower at day 12 than day 4 ($P<0.05$). Gluconic and anthranilic acid concentrations were higher at day 12 than day 4 ($P<0.05$). Cocoa

supplementation resulted in a lower 4-hydroxybenzoic acid concentration in worms at day 4 than the control (P<0.05). The adipic acid concentration was higher in cocoa-treated worms at day 4 than the control (P<0.05). All three detected sugars (glucose, sucrose, and trehalose) showed no significant change at day 12 compared to day 4 (P>0.05). Trehalose concentration was higher at day 12 than day 4 in cocoa-supplemented worms (P<0.05). The nucleobase cytosine concentration was lower at day 12 than day 4 (P<0.05). However, uracil and hypoxanthine showed no significant difference at day 12 compared to day 4 (P>0.05). Cocoa supplementation did not significantly alter the concentrations of cytosine, uracil, and hypoxanthine at any of the time points (P>0.05). Non-proteinogenic AA ornithine concentration was not significantly affected by either aging or cocoa supplementation (P>0.05). G-3P concentration was lower at day 12 than day 4 (P<0.05). Cocoa supplemented worms showed higher G-3P concentrations at both day 4 and day 8 than control worms (P<0.05). Table 6.1 shows the metabolomic profile of worms at day 4, day 8, and day 12.

Table 6.1. **Untargeted metabolomic profile of control and cocoa-supplemented wild type (N2) *C. elegans* at days 4, 8, and 12**

Metabolite	Treatment	Day 4	Day 8	Day 12	Standard Error (±)
Amino acids					
Alanine	Control	2.63	2.79	2.36	0.23
	Cocoa	3.21	3.19	2.44	0.23
Leucine	Control	36.42	39.87	32.06	2.28
	Cocoa	33.07	35.97	24.40*‡‡	2.28
Proline	Control	10.70	6.81	7.86	0.87
	Cocoa	9.44	8.24	7.23	0.87
Glycine	Control	0.59	0.79	1.05*	0.10
	Cocoa	0.62	0.79	0.99*	0.10
Serine	Control	9.14	8.21	7.40*	0.40
	Cocoa	9.10	9.03	7.12*‡	0.40
Threonine	Control	4.15	4.36	3.53	0.22
	Cocoa	3.88	4.62	2.97‡	0.22
Aspartate	Control	2.96	2.78	3.53	0.39
	Cocoa	2.20	3.61*	3.17*	0.39
Methionine	Control	16.15	18.46	29.69	3.14
	Cocoa	19.00	16.47	30.16	3.14
Glutamate	Control	2.83	1.56*	2.21	0.44
	Cocoa	3.83	2.06*	2.52	0.44
Phenylalanine	Control	6.21	5.95	4.99	0.24
	Cocoa	6.33	6.29	3.89*‡	0.24
Asparagine	Control	0.17	0.09*	0.08*‡	0.01
	Cocoa	0.20†	0.12$^{*¥}$	0.07*‡	0.01

Glutamine	Control	0.16	0.10	0.08*	0.03
	Cocoa	0.22	0.17	0.06*ǂ	0.03
Lysine	Control	0.41	0.36	0.28	0.03
	Cocoa	0.44	0.38	0.19	0.03
Tyrosine	Control	7.49	7.89	7.29	0.49
	Cocoa	8.43	9.08¥	5.09*ǂ‡	0.49
Organic acids					
Succinate	Control	2.08	1.56	1.86	0.25
	Cocoa	1.92	1.46	2.40	0.25
Malate	Control	2.48	2.06	0.96*ǂ	0.40
	Cocoa	3.06	2.59	1.16*ǂ	0.40
4-Hydroxybenzoic acid	Control	2.18	1.42	1.66	0.24
	Cocoa	1.33†	1.05	1.83	0.24
Gluconic acid	Control	0.69	0.56	1.61*ǂ	0.27
	Cocoa	0.51	0.56	2.07*ǂ	0.27
Adipic acid	Control	1.69	0.90	0.30*	0.41
	Cocoa	2.74†	1.40*	0.29*ǂ	0.41
Anthranilic acid	Control	0.74	1.73	3.12*	0.27
	Cocoa	0.62	1.71	3.03*	0.27
Sugars					
Glucose	Control	65.77	63.22	53.92	6.81
	Cocoa	60.81	66.85	52.79	6.81
Sucrose	Control	11.18	17.92	23.97	6.58
	Cocoa	14.82	11.55	22.72	6.58
Trehalose	Control	2.55	4.13	4.14	0.85
	Cocoa	3.17	4.66	5.35*	0.85
Nucleobases and nucleic acid constituents					
Uracil	Control	4.40	3.06	4.06	0.48
	Cocoa	3.33	2.56	4.56	0.48
Cytosine	Control	0.09	0.05	0.03*ǂ	0.01
	Cocoa	0.07	0.07	0.02*ǂ	0.01
Hypoxanthine	Control	0.97	0.73*	0.91	0.08
	Cocoa	0.93	0.86	0.82	0.08
Non-proteinogenic amino acids					
Ornithine	Control	0.09	0.04	0.03	0.03
	Cocoa	0.12	0.08	0.08	0.03
Glycerophospholipids					

Glycerol-3-phosphate	Control	5.09	2.63*	3.43*	0.47
	Cocoa	6.58†	4.60*¥	2.87*ǂ	0.47

Each amino acid was presented as the percentage of the total amino acids. Other metabolites were presented as the percentage of the rest of the metabolites. Results are expressed as Mean ± SE. Statistical significance indicated as $P<0.05$; *significant compared to day 4 in the same group; ǂsignificant compared to day 8 in the same group; †significant between the control and cocoa treated groups at day 4; ¥significant between the control and cocoa treated groups at day 8; ‡significant between the control and cocoa treated groups at day 12.

6.4.3. Metabolome of pan-neuronal Aβ expressing *C. elegans* and the effects of cocoa supplementation

Metabolomic profile of control and cocoa-supplemented pan-neuronal Aβ expressing *C. elegans* (GRU102) and their control (GRU101) was analyzed at day 4 (young age) and day 8 (middle age). Proline and asparagine concentrations in GRU102 worms were higher than GRU101 worms at day 4 ($P<0.05$). However, there were no significant changes between the two worm strains for any of the detected AAs at day 8 ($P>0.05$). Cocoa supplementation reversed the changes in proline and asparagine concentrations in the GRU102 strain at day 4 to reach similar concentrations to GRU101. There was no significant difference between both control and cocoa-supplemented two worm strains at either day 4 or 8 for all the detected organic acids ($P>0.05$). With three sugars, there was no significant difference between both control and cocoa-treated GRU101 and GRU102 worms at either day 4 or 8 ($P>0.05$). Hypoxanthine concentration was higher in GRU102 worms than GRU101 worms at day 8 ($P<0.05$). Cocoa supplementation reduced the elevated hypoxanthine concentration in GRU102 worms to reach a similar concentration to GRU101 worms. Similarly, ornithine concentration in GRU102 worms was higher than GRU101 worms at day 8 ($P<0.05$). Cocoa supplementation significantly increased the ornithine concentration in GRU101 worms compared to their control ($P<0.05$) at day 8. Even though cocoa supplementation increased the ornithine concentration in GRU102 worms, it was not a statistically significant increase compared to both cocoa-treated GRU101 worms and GRU102 control worms ($P>0.05$). There was no significant difference between the control and cocoa-treated two strains of worms at either day 4 or day 8 for G-3P ($P>0.05$). Table 6.2 shows the metabolomic profile of Aβ expressing worms on day 4 and day 8.

Table 6.2. Untargeted metabolomic profile of control and cocoa-supplemented transgenic *C. elegans* expressing pan-neuronal Aβ (GRU102) and their control (GRU101) at days 4 and 8

Metabolite	Treatment	Day 4		Day 8		Standard Error (±)
		GRU101	GRU102	GRU101	GRU102	
Amino acids						
Alanine	Control	2.50	2.00	1.56	1.47	0.21
	Cocoa	2.30	2.21	1.69	1.31	0.21
Leucine	Control	34.07	31.27	57.50	54.94	1.18
	Cocoa	33.86	32.22	56.45	55.37	1.18
Proline	Control	10.93	13.31*	7.61	8.01	0.68
	Cocoa	12.33	11.90	7.06	8.38	0.68
Glycine	Control	0.90	0.79	0.02	0.02	0.05
	Cocoa	0.83	0.86	0.02	0.10	0.05
Serine	Control	8.42	9.56	5.54	6.26	0.51
	Cocoa	8.13	8.81	5.65	6.05	0.51
Threonine	Control	3.64	3.74	3.10	3.31	0.20
	Cocoa	3.36	3.74	3.09	3.23	0.20
Aspartate	Control	2.04	3.10	2.69	3.61	0.48
	Cocoa	2.39	2.72	3.07	3.46	0.48
Methionine	Control	18.72	15.17	7.05	7.99	1.52
	Cocoa	17.14	16.46	8.33	7.30	1.52
Glutamate	Control	3.05	3.19	0.98	1.20	0.26
	Cocoa	3.23	3.24	1.11	1.19	0.26
Phenylalanine	Control	6.61	7.25	7.89	7.58	0.57
	Cocoa	7.06	7.27	7.68	8.25	0.57
Asparagine	Control	0.22	0.32*	0.08	0.09	0.03
	Cocoa	0.24	0.29	0.09	0.09	0.03
Glutamine	Control	0.17	0.23	0.09	0.11	0.03
	Cocoa	0.19	0.22	0.11	0.09	0.03
Lysine	Control	0.59	0.73	0.00	0.00	0.04
	Cocoa	0.61	0.71	0.01	0.01	0.04
Tyrosine	Control	8.16	9.34	5.88	5.41	0.73
	Cocoa	8.34	9.36	5.64	5.11	0.73
Organic acids						
Succinate	Control	2.48	1.76	7.53	6.31	0.95
	Cocoa	2.06	1.50	6.76	5.95	0.95
Malate	Control	1.89	1.94	6.08	8.52	1.34
	Cocoa	2.01	1.43	7.91	7.78	1.34
4-Hydroxybenzoic acid	Control	1.13	0.58	4.68	4.46	0.59

	Cocoa	0.77	0.39	5.32	3.90	0.59
Gluconic acid	Control	1.16	1.25	1.14	1.63	0.36
	Cocoa	1.34	0.95	1.00	1.09	0.36
Adipic acid	Control	2.35	1.98	2.44	2.08	0.78
	Cocoa	2.53	2.34	4.33	3.71	0.78
Anthranilic acid	Control	1.09	0.61	4.63	2.41	0.66
	Cocoa	0.73	0.36	3.78	2.28	0.66
Sugars						
Glucose	Control	42.58	48.98	3.65	2.06	8.32
	Cocoa	47.67	41.50	5.68	2.02	8.32
Sucrose	Control	28.14	21.54	18.70	12.55	11.06
	Cocoa	24.00	35.69	8.42	11.52	11.06
Trehalose	Control	3.64	1.47	5.61	0.91	1.79
	Cocoa	2.88	2.07	0.63	1.63	1.79
Nucleobases and nucleic acid constituents						
Uracil	Control	5.49	5.79	25.07	28.82	3.06
	Cocoa	5.36	4.14	29.15	25.28	3.06
Cytosine	Control	0.09	0.12	0.86	0.88	0.12
	Cocoa	0.09	0.09	0.84	0.94	0.12
Hypoxanthine	Control	1.61	2.03	5.05	7.14[ǂ]	0.49
	Cocoa	2.00	1.55	6.07	6.62	0.49
Non-proteinogenic amino acids						
Ornithine	Control	0.18	0.22	0.11	0.80[ǂ]	0.14
	Cocoa	0.15	0.19	0.50[¥]	1.47	0.14
Glycerophospholipids						
Glycerol-3-phosphate	Control	8.20	11.75	14.48	21.43	2.26
	Cocoa	8.46	7.82	19.62	25.83	2.26

Each amino acid was presented as the percentage of the total amino acids. Other metabolites were presented as the percentage of the rest of the metabolites. Results are expressed as Mean ± SE. Statistical significance indicated as $P<0.05$ The control and cocoa-treated treated GRU101 and GRU102 worms were compared at either day 4 or day 8 for the differences among groups. [*]significant between GRU101 and GRU102 control groups at day 4; [ǂ]significant between GRU101 and GRU102 control groups at day 8; [¥]significant between the control and cocoa treated groups of GRU101 at day 8.

6.5. Discussion

Cocoa had long been identified as a PP-rich food. It contains more phenolic phytochemicals and a higher antioxidant capacity than teas and Red wine (K. W. Lee et al., 2003). Cocoa contains various phytochemicals, mostly flavonoid, and non-flavonoid phenols and methylxanthines (J. Kim et al., 2014). Most of the studies conducted with cocoa highlight the health-promoting effects of cocoa PPs compared to methylxanthines (Cooper et al., 2008). PPs in cocoa comprise mainly flavanols or catechins as monomers and polymers, proanthocyanidins, and anthocyanins (Aprotosoaie, Miron, et al., 2016). The cocoa powder used in this study was a commercially processed, unsweetened cocoa powder comprised of 27.01 mg GAE/g of total phenolics (TP) and 10.13 mg CE/g of total flavonoids (TF). The TP and TF were 56% and 40% respectively compared to natural cocoa powder (Munasinghe, Almotayri, Thomas, et al., 2021).

Previously, the beneficial effects of cocoa supplementation on age-associated health and Aβ-induced deficits in *C. elegans* were demonstrated with behavioral experiments (Munasinghe, Almotayri, Kolivas, et al., 2021; Munasinghe, Almotayri, Thomas, et al., 2021). In this study, age-associated and Aβ-induced changes in the metabolome of *C. elegans* and the effects of long-term cocoa supplementation were investigated. Wild-type *C. elegans* were used to determine the age-associated changes in the metabolome. The metabolomic profile was determined at day 4, day 8, and day 12 to represent young, middle, and old age worms respectively.

AAs play important roles as cell signaling molecules, regulators of gene expression, and the protein phosphorylation cascade, key precursors for syntheses of hormones and low-molecular-weight nitrogenous substances (G. Wu, 2009). There is growing evidence that apart from their main role as building blocks of proteins, some AAs regulate the key metabolic pathways that may influence the aging process (Canfield & Bradshaw, 2019). Numerous studies have been demonstrated the ability of AAs to alter the rate of aging and to ameliorate age-associated disease conditions (Powers et al., 2006; Reckelhoff et al., 1997; Z. Wu et al., 2013; Yu et al., 2013). Therefore, identifying age-associated changes in AA concentrations and finding dietary supplements to reverse or alter these changes would be beneficial in delaying the aging process and preventing age-associated pathological conditions. In this study, an age-associated decline in the concentrations of AA serine, asparagine, and glutamine was observed. Conversely, glycine showed an accumulation with aging. The accumulation of glycine in aging is known to couple to a decrease in gene expression of enzymes important for glycine catabolism (Liu et al., 2018). Previous metabolomics studies with *C. elegans* show consistent data with the findings of this study for some of the AAs, while other AAs show contrary results. One group reports that the concentrations of glutamic acid, serine, glutamine, phenylalanine, tyrosine, methionine, tryptophan, proline, asparagine, arginine, lysine, leucine, isoleucine, valine, and alanine decreased with aging *in C. elegans* (Gao et al., 2017). The same study reports an age-associated accumulation with glycine and aspartate. Another group has reported that alanine, asparagine, glutamate, and glutamine concentrations decreased with aging in

C. elegans while glycine, serine, and tyrosine showed an increase (Davies et al., 2015). Metabolomics studies of aging with humans also have reported changes in the amino acid concentrations in the plasma. One study has found significant reductions in aspartic acid, methionine, alanine, proline, phenylalanine, and tryptophan concentrations in older subjects, except significantly high cystine (Johnson et al., 2018). Another study has reported that tyrosine and glutamine concentrations increased with age while histidine, threonine, tryptophan, leucine, and serine decreased with age (Darst et al., 2019).

With cocoa supplementation, old worms showed significantly reduced leucine and tyrosine concentrations compared to their control counterparts. Even though this present study did not find any significant change in the leucine concentrations in *C. elegans* with aging, a previous metabolic study has been shown that aging causes a significant increase in leucine concentrations in the skeletal muscle of mice (J. Zhou et al., 2017). High concentrations of alpha-ketoacid of leucine (alpha-ketoisocaproic acid) are known to cause mitochondrial dysfunction in neurons of rats (Amaral et al., 2010). In *C. elegans*, TOR (target of rapamycin) deficiency is known to more than doubles its natural lifespan (Vellai et al., 2003). As leucine strongly activates the TOR signaling pathway (Edwards et al., 2015) and the inhibition of this pathway extends the lifespan in eukaryotic species, the previously observed beneficial effects of cocoa supplementation including lifespan extension (Munasinghe, Almotayri, Thomas, et al., 2021) may be due to the reduced leucine concentrations by cocoa. However, dietary supplementation of leucine has been shown to extend lifespan in various model organisms (Bennet et al., 1989; Fulks et al., 1975; Mansfeld et al., 2015).

There was no age-associated change in the tyrosine concentration in this study. However, previous studies with *C. elegans* (Davies et al., 2015), as well as humans (Darst et al., 2019), have been reported a significant age-associated increase in tyrosine concentrations. The higher concentrations of tyrosine may be the cause or can aggravate the cognitive problems in older adults as demonstrated in previous studies as it is a neurotransmitter precursor and known to compete with other large neutral amino acids for transport into the brain (Ravaglia et al., 2002). Therefore, the reduced concentrations of tyrosine at old age with cocoa supplementation might positively impact the age-associated deficits in *C. elegans* especially, the cognitive deficits. However, some studies report cognitive improvements with increased dietary tyrosine (Kühn et al., 2019) while others report no effect with moderate concentrations or decreased cognitive performance with higher concentrations in older adults (van de Rest et al., 2017). Moreover, tyrosine supplementation has been shown to moderately increase the lifespan of *C. elegans* at the lowest 1 mM dose, but only slightly at two higher doses (Edwards et al., 2015). Therefore, more experiments are required to confirm the exact effects arising from these reductions in leucine and tyrosine concentrations with cocoa supplementation.

The aging resulted in significant reductions (malate and adipic) as well as increases (gluconic acid and anthranilic acid) in the concentrations of some of the organic acids in this study. However, there

were no significant changes with the cocoa treatment in old worms with any of the organic acids. No significant age-associated changes were observed in the three sugar concentrations (glucose, sucrose, and trehalose). Similar to organic acids, cocoa did not yield any significant change in sugar concentrations in old worms. The nucleobase cytosine showed an age-associated decline, but there was no significant effect of cocoa supplementation. Uracil, hypoxanthine, and ornithine concentrations were also not significantly affected by either aging or cocoa supplementation. G-3P concentrations showed an age-related decline. Even though cocoa consumed worms showed significantly higher concentrations of G-3P at young and middle age than control worms, there was no significant effect of cocoa supplementation at old age.

In this study, a transgenic *C. elegans* strain (GRU102) which shows pan-neuronal Aβ expression and the middle-age onset behavioral dysfunction as we can see in human AD was employed to study the effects of cocoa supplementation in Aβ-induced deficits (Fong et al., 2016). This strain shows reduced growth, a reduced maximum speed at old age, short-term memory deficits at middle age, and a reduced lifespan (Munasinghe, Almotayri, Kolivas, et al., 2021). Cocoa-supplementation reversed the deficits in growth, maximum speed, short-term memory loss, and lifespan to reach similar levels to control counterparts while reducing the Aβ fibril levels. In this metabolomics study, Aβ expressing worms at young age showed significantly higher concentrations of proline and asparagine than control worms. Previous studies have been demonstrated that proline metabolism is affected in AD (Paglia et al., 2016) and therefore, the concentrations of proline in cerebrospinal fluid is a possible disease progression biomarker (Ibáñez et al., 2012). Moreover, AD patients have been shown elevated plasma proline concentrations (Chatterjee et al., 2020; G. Wang et al., 2014). Higher proline concentrations in the rat brains are known to associate with decreased acetylcholinesterase (AChE) activity (Delwing et al., 2003; Delwing et al., 2005). AChE is a cholinergic enzyme primarily found at postsynaptic neuromuscular junctions, especially in muscles and nerves (Trang & Khandhar, 2021). The primary role of AChE is the hydrolytic metabolism of the neurotransmitter acetylcholine (ACh) into choline and acetate (English & Webster, 2012). The reduced cortical AChE is reported to be associated with dementia (O'Brien et al., 2003; Petersen et al., 1999) and the reduced AChE activity has been observed in the cerebrospinal fluid (Appleyard et al., 1983) and plasma (Yamamoto et al., 1990) of AD patients. Cocoa supplementation reduced the elevated concentrations of proline in Aβ expressing worms to reach similar levels to their control worms, showing the beneficial effects of cocoa against Aβ-induced deficits. It has been further shown that treatment with antioxidants can prevent the reduction of AChE activity induced by higher proline concentrations in the rat cerebral cortex, suggesting the involvement of oxidative stress in these events (Delwing et al., 2003; Delwing et al., 2005). Therefore, the antioxidant activity of PPs in cocoa is one possible explanation for the reduction of observed higher proline concentrations in Aβ expressing worms. A positive correlation has been identified between the plasma neurofilament light chain, a marker of neurodegeneration, and plasma asparagine

concentrations in Aβ+ participants, indicating that asparagine metabolism also plays a role in AD (Chatterjee et al., 2020). However, other studies have detected lower asparagine concentrations in the cerebrospinal fluid in AD patients (Jiménez-Jiménez et al., 1998) and in plasma in patients with preclinical AD (Fiandaca et al., 2015) than controls. In this study, cocoa reduced the elevated asparagine concentrations in Aβ expressing worms to reach similar levels to control worms. Therefore, cocoa may reverse the impaired asparagine metabolism which was observed in the early stages of AD in worms. However, more studies are required to validate the results due to the inconsistency of the previously reported studies.

The elevated hypoxanthine concentrations observed in Aβ expressing worms at their middle age were restored to the concentrations in control worms by cocoa supplementation. Hypoxanthine is a purine compound and is the principal purine nucleobase involved in the salvage purine pathway in the brain (Fig. 6.1) (N. Li et al., 2016). The purine metabolism pathway is widely conserved from prokaryotes to humans (Marsac et al., 2019). It has been previously shown that purine-related metabolites and their converting enzymes are altered in AD in the human brain depending on the stage and the region (Alonso-Andrés et al., 2018). Further, previous studies have demonstrated that Aβ expression shifted the purine metabolism towards an increase in hypoxanthine/xanthine/uric acid in the 3×Tg AD mice accompanied by a decrease in adenosine monophosphate (AMP) and adenine (Esteve et al., 2017; N. Li et al., 2016). Hypoxanthine is known to contribute much to the memory deficits in AD via an AChE-related mechanism by stimulating the AChE activity which might reduce ACh levels (Y.-Y. Chen et al., 2020). Hypoxanthine is also known to promote AD development via inducing inflammation and oxidative stress (OS). Hypoxanthine contributes to OS in the brain by inducing free radical generation and reducing antioxidant defenses (Bavaresco et al., 2007). The negative impact of increased OS on learning ability and memory has been previously reported (Fukui et al., 2001). The previously reported short-term memory deficits in Aβ expressing worms (Munasinghe et al, 2021) might be due to these elevated hypoxanthine concentrations which may cause a reduction in acetylcholine levels and an increase in OS. Interestingly, cocoa-supplementation reduced the increased hypoxanthine concentrations in Aβ expressing worms to the levels in normal worms. The reduced hypoxanthine concentrations with cocoa supplementation might be one possible explanation for the improvements in short-term memory loss in Aβ expressing worms.

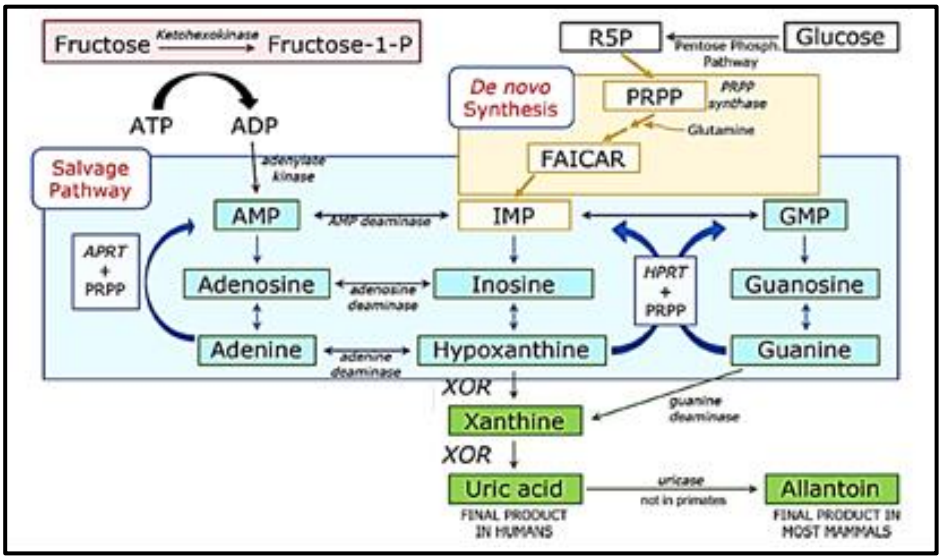

Figure 6.1. Purine metabolism pathways, adapted from (Romeo & Jain, 2020). Key reactions in the purine de novo synthesis (yellow) and salvage pathway (blue). Enzymes are indicated in italic. Abbreviations: ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; IMP: inosine monophosphate; GMP: guanosine monophosphate; R5P: ribose 5-phosphate; PRPP: phosphoribosyl pyrophosphate; FAICAR: 5-formamidoimidazole-4-carboxamide ribotide; XOR: xanthine oxidoreductase; IMP: inosine monophosphate; HPRT: hypoxanthine-guanine phosphoribosyltransferase; APRT: adenine phosphoribosyltransferase.

Aβ expressing worms showed significantly increased ornithine concentrations at their middle age. Ornithine is a non-essential amino acid that is produced as an intermediate molecule in the urea cycle from arginine (Fig. 6.2) (Muthukumaran et al., 2017). Arginine metabolism is known to be altered in AD. Aβ expression in mice brain has been shown to associate with an increased expression in arginase and a decrease in global arginine bioavailability ratio which is the ratio of arginine to its metabolites ornithine and citrulline [arginine/(ornithine + citrulline)], indicating an increase in arginine catabolism (Kan et al., 2015). In addition, an increased amount/activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine synthesis has been found in the brains in AD (Bernstein & Müller, 1995). Aβ accelerates the production of reactive oxygen species (ROS), the accumulation of which is known to induce the activity of ODC and the production of polyamines (Yatin et al., 1999). However, polyamines related to this pathway were not detected in this study. Another study has been demonstrated that there is an increase in ornithine concentrations in the mouse brain with long-term Aβ accumulation and deposition, suggesting a shift of arginine metabolism towards the arginase pathway, perhaps in response to the high load of Aβ deposition in the brain (Bergin et al., 2018). Moreover, the blockage of arginine catabolism by inhibiting key enzymes in the arginine utilization pathway (ODC and arginase) has been reported to reverse the Aβ-induced deficits in memory in mice (Kan et al., 2015). Overall, this evidence suggests that Aβ deposition shifts the arginine metabolism towards the arginase pathway as

aforementioned, which might increase ornithine concentrations. In this study, a significant increase in the ornithine concentration in control worms at middle age was observed with cocoa supplementation. In an *in vitro* study conducted with human colonic cancer cell line Caco-2, a procyanidin-enriched cocoa extract significantly decreased the activity of ODC which converts ornithine to the polyamine, putrescine (Carnésecchi et al., 2002). This inhibition of ODC might be the reason for increased ornithine concentrations with cocoa supplementation in control worms. Even though cocoa supplementation further increased the ornithine concentrations in Aβ expressing worms at their middle age, the increase was not significant compared to the cocoa-supplemented control worms (GRU101) as well as to their control without cocoa. It has been previously shown that cocoa supplementation reduced Aβ fibril levels in worms at this stage (Munasinghe, Almotayri, Kolivas, et al., 2021) which may cause to reverse the Aβ-induced effects in arginine metabolism. Therefore, this Aβ lowering effect of cocoa should also be taken into the account, in addition to the effects of cocoa on ODC when predicting the effects of cocoa supplementation in Aβ expressing worms. Due to the complicated nature of the mechanisms, it is not possible to suggest the exact effects of cocoa supplementation on ornithine in Aβ expressing worms at their middle age. Therefore, further experiments are required to confirm the effects of cocoa on ornithine.

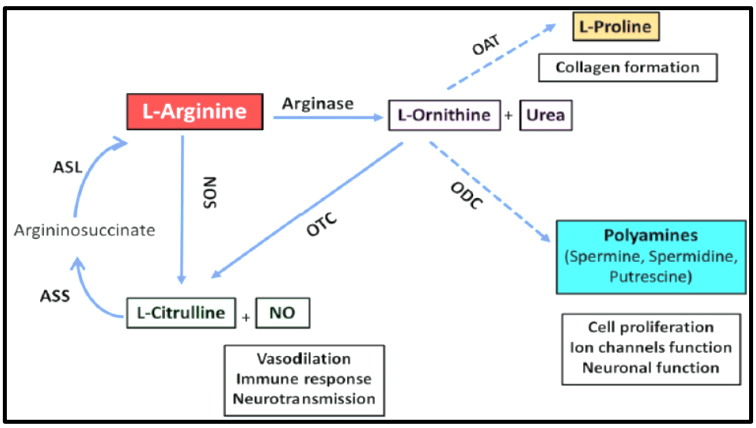

Figure 6.2. Schematic representation of L-arginine metabolism, adapted from (Shosha et al., 2020). Arginase metabolizes L-arginine to L-ornithine and urea. L-Arginine is also metabolized to L-citrulline and nitric oxide (NO) by the nitric oxide synthase (NOS). The successive actions of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) can recycle L-Citrulline back to L-Arginine. The enzymatic action of ornithine transcarbamylase (OTC) converts L-Ornithine to L-Citrulline. L-ornithine can be used for polyamines production by ornithine decarboxylase (ODC). It can also be used for L-proline production by ornithine aminotransferase (OAT).

6.6. Conclusions

In summary, amino acids, organic acids, sugars, nucleobases, nucleic acid constituents, non-proteinogenic amino acids, and glycerophospholipids were identified as the endogenous metabolites in the wild-type and a transgenic *C. elegans* strain expressing pan-neuronal Aβ. Serine,

asparagine, glutamine, and glycine concentrations were affected by aging. Leucine and tyrosine concentrations were significantly reduced by cocoa supplementation at old age which might be associated with previously observed beneficial effects of cocoa including improvements in mitochondrial function, cognition, and lifespan extension. Cocoa supplementation showed beneficial effects on impaired proline and asparagine metabolism in Aβ expressing worms at a young age by reversing the elevated concentrations to the normal levels. Pan-neuronal Aβ expression showed elevated hypoxanthine concentrations in worms at their middle age which is known to contribute to memory deficits in AD. Cocoa supplementation reduced the elevated hypoxanthine concentrations to normal levels. Alterations in the urea cycle in AD were evident as shown by increased ornithine concentrations in Aβ expressing worms at middle age. However, the exact effects of cocoa on ornithine concentrations were uncertain due to the complicated nature of the mechanisms. Overall, cocoa seemed to interact with some of the endogenous metabolites in *C. elegans* to modify aging and Aβ-induced deficits. However, more experiments with many replicates are needed to validate the results.

CHAPTER 7: General Discussion

7.1. Overview

Aging is a predominant risk factor for most well-recognized pathologies that limit healthspan in mammals, including sarcopenia, atherosclerosis and heart failure, neurodegeneration, and many others (Campisi, 2013). Aging is described as a complex, multifactorial process that involves interactions among many different mechanisms (Kowald & Kirkwood, 1996). Despite the existing pharmaceutical interventions for aging and related diseases, preventive lifestyle choices are still needed for a sustainable, feasible, and affordable health care system. Adherence to a healthy dietary pattern is beneficial in many aging and related disease conditions, including Alzheimer's disease (AD), where there is no effective pharmaceutical intervention yet (W. Xu et al., 2015). Plant-based diets such as the Mediterranean diet, MIND diet, etc. which contain a higher amount of polyphenols (PPs) are known to associate with a reduced incidence of aging-related diseases (Féart et al., 2010; Morris et al., 2015; Panagiotakos et al., 2002). In addition to the antioxidant mechanism, there are other mechanisms that PPs can act through to exert these beneficial effects (Alcaín & Villalba, 2009; Takahashi et al., 2014). In this study, cocoa which is the fourth among the 100 richest dietary sources of PPs and contains more PPs than tea and Red wine (K. W. Lee et al., 2003) was used to study how its supplementation to the diet affects age-associated health and amyloid-β (Aβ)-induced deficits in AD using *C. elegans* as the model organism. *C. elegans*, a free-living nematode shows many attractive features in aging research including a short and largely invariant lifespan (Tissenbaum, 2015) and many readily observable and quantifiable age-associated changes (C. Huang et al., 2004). The third chapter of the thesis demonstrates how long-term cocoa supplementation affects age-associated health and lifespan in wild-type *C. elegans*. Chapter four gains insight into how cocoa exposure at different stages of life affects the lifespan of *C. elegans* and whether cocoa requires life-long exposure for its lifespan-extending effects. Additionally, the pathways and mechanisms involved in the lifespan-extending effects of cocoa were studied. Chapter five characterizes the phenotypic deficits of a *C. elegans* strain that expresses pan-neuronal Aβ and closely resembles human AD, showing middle-age onset behavioral dysfunction. Further, this chapter shows how long-term cocoa supplementation affects these behavioral deficits. The sixth chapter is about how long-term cocoa supplementation affects age-associated health and Aβ-induced deficits in AD at a metabolite level.

7.2. Cocoa improves age-associated health and extends lifespan in *C. elegans*

The experiments presented in chapter three demonstrated the effects of long-term cocoa supplementation in age-associated health and lifespan in *C. elegans*. The worm diet was supplemented with cocoa powder starting from the L1 stage till their death. Cocoa-supplemented worms were significantly longer and thicker compared to their control counterparts, indicating that

cocoa provides better nutrition to worms. Sarcopenia is the age-related, involuntary loss of skeletal muscle mass and strength. It is one of the most important causes of functional decline and loss of independence in older adults (Walston, 2012). In this study, *C. elegans* speed of movement which is analogous to the gait speed of humans was used to diagnose sarcopenia. An age-associated decrease in both mean and maximum speed was observed in *C. elegans*. Cocoa supplementation improved the maximum speed of worms at their old age (day 12) by 12% compared to the control counterparts. Maximum speed is known to be one of the powerful metrics of *C. elegans* health as it is highly correlated with its longevity (Hahm et al., 2015). Similar results were observed with peristaltic speed which is the speed of forward movement of worms where there was an age-associated decline.

Cocoa improved the peristaltic speed at old age by 20% compared to control worms. Cocoa supplementation significantly improved the age-associated decline in maximum amplitude in worms at day 12 (old age), a parameter that reflects the muscle strength of worms (Nahabedian et al., 2012). The results of this study are in agreement with previous studies as epicatechin (the most abundant flavanol compound in cocoa), catechin, and procyanidins have been reported to improve muscle performance in mice as well as humans through different mechanisms (Gutierrez-Salmean et al., 2014; P. Li et al., 2020; Moreno-Ulloa et al., 2018; Nogueira et al., 2011; Si et al., 2019; M. Xu et al., 2020). Cocoa significantly improved the mean and median lifespan of worms, but not in a dose-dependent manner. It has been previously reported that antioxidants (vitamin C, N-acetylcysteine, and resveratrol) can both lengthen and shorten lifespan depending on the concentration, showing an inverted U-shaped dose-response relationship between reactive oxygen species (ROS) levels and lifespan (Desjardins et al., 2017). In addition, a previous study has been shown that low molecular weight thiols accelerate *C. elegans* aging in a dose-dependent manner (Gusarov et al., 2021). Even though ROS are potentially toxic, ROS act as important signaling molecules that regulate cellular homeostasis, differentiation, proliferation, repair, and aging (Ray et al., 2012; Schmidt et al., 2015). Therefore, scavenging naturally occurring ROS excessively can be deleterious to organisms, limiting their lifespan. Previous studies report the lifespan extension effects of cocoa, cocoa extracts, and epicatechin in *Drosophila melanogaster*, rats, *Saccharomyces* cerevisiae, Oryzias *latipes*, and *C. elegans*, supporting the findings of this present study (Bahadorani & Hilliker, 2008; Baiges & Arola, 2016; Bisson et al., 2008; Sánchez-Sánchez et al., 2018; Surco-Laos et al., 2012). However, another study reported that catechin showed no effect on the mean lifespan of *C. elegans* (Surco-Laos et al., 2012). Aging is known to alter proteostasis (López-Otín et al., 2013) and therefore, decreases *C. elegans* ability to cope with heat stress which is dependent on the mechanisms contributing to proteostasis (Balch et al., 2008). In this study, cocoa improved the ability of worms to withstand thermal stress at all stages of life, showing more prominent effects at young and middle age. There were no previous studies that used cocoa interventions to study the effects on thermotolerance. However, epicatechin and catechin have been

reported to increase the thermotolerance at mid-adulthood of *C. elegans*, but not at late adulthood (Ayuda-Durán et al., 2019; Surco-Laos et al., 2012). The impact of aging itself on cognition, not including dementia, mild cognitive impairment, or other specific cognitive decline syndromes is a huge and neglected problem for current science (Deary et al., 2009). An age-associated decline in the learning ability of *C. elegans* was observed with *C. elegans* positive butanone learning and short-term associative memory assay. Cocoa supplementation was able to significantly improve this age-associated loss in learning. Even though it was intended to determine the age-associated changes in the short-term memory loss of *C. elegans*, it was not possible to perform the assay at middle and old age as the method used was not sensitive enough at these late stages of worms. However, cocoa supplementation improved the short-term memory loss of young worms, showing neuroprotective effects. Previously, it has been shown that cocoa can exert neuroprotective effects to improve cognition in both animals and humans (Bisson et al., 2008; Desideri et al., 2012; Moreira et al., 2016; Rozan et al., 2007). Even though the role of flavanols in neuroprotection is often related to their antioxidant capacity, other mechanisms are also known to be involved which are dependent on the ability of these compounds to cross the blood-brain barrier (BBB) (Faria et al., 2011). There is evidence to show that epicatechin and catechin can cross the BBB therefore, indicating them as prospective compounds to exert protective effects in neurodegeneration (Abd El Mohsen et al., 2002; Faria et al., 2011). The decline in mitochondrial function is known to associate with many aging-related diseases (C. Chen et al., 2019; Weidling & Swerdlow, 2019). The results of this present study showed that there was an age-associated decline in both basal and mitochondrial respiration in worms. Cocoa improved both basal and mitochondrial respiration at old age while reducing the increased non-mitochondrial respiration at old age. PPs act as mild mitochondrial uncouplers of the electron transport chain (ETC) from ATP production and this uncoupling effect is described as one mechanism about their health benefits (Stevens et al., 2018). Uncoupling is known to increase respiration thereby, reducing ROS production (Mookerjee et al., 2010). Further, the "uncoupling to survive" hypothesis describes the attenuation of ROS by partial uncoupling while maintaining sufficient ATP production as a potential mechanism for delaying cellular senescence (Papa & Skulachev, 1997). Therefore, the uncoupling effect of cocoa PPs may be one possible explanation for the increased basal and mitochondrial respiration and the decreased non-mitochondrial respiration in worms.

7.3. Early exposure is necessary for the lifespan extension effects of cocoa in *C. elegans*

As mentioned in chapter three, cocoa extended the lifespan of *C. elegans* when supplemented starting from the L1 stage till the death. Chapter four describes how cocoa supplementation at different stages of life affects the lifespan of *C. elegans*. In addition, experiments were conducted to investigate whether cocoa requires life-long exposure for its lifespan-extending effects. Moreover, the mechanisms and pathways involved in the lifespan extension effects of cocoa were investigated. The results showed that cocoa supplementation starting from the first larval stage (L1)

is necessary for the longevity improving effects in *C. elegans*. In addition, cocoa supplementation till adulthood was enough to extend the lifespan and did not need to be continued in a long-term manner. Previous studies support the findings of this study where PP extracts as well as some antioxidants have been reported to improve longevity in *C. elegans* when supplemented starting from the early stages of life (Guha et al., 2014; Zuckerman & Geist, 1983). These findings are important as it has been shown that early life nutrition in humans significantly influences the risk of developing NCDs in late life (Kelishadi & Farajian, 2014). However, cocoa supplementation at other larval stages (L2, L3, L4), as well as adulthood, significantly decreased the lifespan of *C. elegans*. Even though the exact mechanism contributing to this reduction is not certain, evidence from previous studies suggests that some PPs, as well as antioxidant compounds, may have pro-oxidant effects (Lam et al., 2010; Selman et al., 2013; Shaito et al., 2020). It is also noteworthy that cocoa powder contains other nutrients including carbohydrates, proteins, and fat and the effects of these nutrients have not been explored in this study.

Insulin/insulin-like growth factor-1 signaling (IIS), mitochondrial respiration, calorie restriction (CR) pathways that regulate longevity are involved in the onset of aging-related diseases (Chistiakov et al., 2014; Dillin et al., 2002; Hsu et al., 2003; C. J. Kenyon, 2010; van Exel et al., 2014). Therefore, the interventions that can modulate these pathways are important in delaying/preventing aging and related diseases. Mutant *C. elegans* strains were used to explore the different pathways that are involved in the lifespan extension effects of cocoa. According to the results, cocoa-mediated lifespan extension was dependent on the IIS pathway and mitochondrial respiration.

7.4. Cocoa supplementation reduces Aβ-induced deficits in a transgenic *C. elegans*

The experiments described in chapter five demonstrated how long-term cocoa supplementation affects $A\beta_{1-42}$ induced toxicity in AD. The main objective of these experiments was to specifically find out how cocoa supplementation affects the early signs and symptoms of AD, including short-term memory loss and learning. Therefore, a transgenic *C. elegans* strain that expresses pan-neuronal $A\beta_{1-42}$ and middle-age onset behavioral dysfunction as we can observe in human AD was employed for the experiments. Firstly, the behavioral deficits of $A\beta_{1-42}$ expressing worms were categorized and then the effects of cocoa supplementation on these deficits were determined. $A\beta_{1-42}$ expressing worms showed a reduced growth which was reversed by the cocoa supplementation to reach similar levels to their control worms. In addition, the maximum speed of these $A\beta_{1-42}$ worms showed a significant reduction at old age. This reduction was also reversed by cocoa supplementation to reach similar levels to their control counterparts. Chemotaxis behavior of *C. elegans* which is a neuronally controlled process was used to determine short-term memory loss and learning ability. For that, *C. elegans* positive butanone learning and short-term associative memory assay was conducted. With positive butanone learning ability, pan-neuronal expression of $A\beta_{1-42}$ did not induce significant impairments in worms at either young or middle age. Loss of

memory is among the first symptoms reported by AD patients and therefore, one of the key characteristics of AD (Jahn, 2013). Interestingly, $A\beta_{1-42}$ expressing worms showed a significant short-term memory loss (dementia index) compared to control worms at both 1 hr and 2 hr time points after conditioning at their middle age. Pan-neuronal expression of $A\beta_{1-42}$ has not been previously described to induce short-term memory loss in worms according to my knowledge and therefore, I reported this finding as one of the novel findings of this study. Thus, this model closely resembles human AD and therefore, is more competent in drug screening studies compared to other available *C. elegans* models of AD. Interestingly, this memory loss was reversed by cocoa supplementation to reach similar levels to their control worms. Thioflavin (Th T) which is a commonly used probe to monitor *in vitro* Aβ fibril formation was used to detect Aβ fibrils in worms. Upon binding to Aβ fibrils, Th T gives a strong fluorescence signal at approximately 482 nm when excited at 440 nm (Xue et al., 2017). With this dye, Aβ fibrils were detected in worms at both young and middle age. Cocoa supplementation effectively reduced these Aβ fibril levels in GRU102 worms at both stages. With lifespan, the results of this present study were in agreement with previous studies where Aβ expressing worms showed a reduced mean lifespan. One of the cocoa doses used in this study reversed this reduction in mean lifespan to reach similar levels to control worms. With the lowest and the highest cocoa doses, a reduction in lifespan was observed in Aβ expressing worms. There was a reduction in the lifespan in control worms with the highest cocoa dose. It was assumed that this reduction of lifespan with the highest dose may be due to the effect of other nutrients present in cocoa such as carbohydrates as a high carb diet is known to reduce the lifespan in *C. elegans* (S. S. Choi, 2011). The lifespan reduction with the lowest dose may be due to the availability of potential beneficial bioactive components in lesser amounts. Previous studies conducted *in vitro* and with animal models support the findings of this present study where cocoa PPs, as well as some peptide components, have been reported to attenuate Aβ-induced toxicity by reducing the toxic Aβ levels and rescuing the neuronal damage (Cimini et al., 2013; Heo & Lee, 2005; Patricia Martorell et al., 2013; Ono et al., 2003; J. Wang et al., 2014).

7.5. Effects of cocoa supplementation on altered metabolite concentrations in purine metabolism pathways and urea cycle in Aβ expressing *C. elegans*

As described in chapter 6, the non-targeted GC-MS approach was used to study the aging-associated and Aβ-induced changes in the metabolome of *C. elegans* and the effects of long-term cocoa supplementation. Wild-type *C. elegans* were analyzed at young (day 4), middle (day 8), and old (day 12) age to determine aging-associated changes. A transgenic *C. elegans* strain which expresses pan-neuronal $A\beta_{1-42}$ and therefore, closely resembles human AD was analyzed at young (day 4) and middle age (day 8) to determine Aβ-induced changes. Of the 28 metabolites detected, there were amino acids (AAs), organic acids, sugars, nucleobases, nucleic acid constituents, non-proteinogenic amino acids, and glycerophospholipids. Some of the AA concentrations (serine, asparagine, and glutamine) were lower at old age than the young age. Conversely, glycine showed a significant

increase at old age. The observed results were consistent with previous *C. elegans* studies for some AAs, but for some, the results were contrary (Davies et al., 2015; Gao et al., 2017). Leucine and tyrosine concentrations were significantly lower in cocoa-supplemented old worms than the control worms. Even though aging did not yield any significant change in these two AA concentrations in this study, it has been shown that aging increases leucine concentrations in mice's skeletal muscle (J. Zhou et al., 2017) and tyrosine concentrations in *C. elegans* (Davies et al., 2015). Moreover, evidence suggests that higher concentrations of leucine can induce mitochondrial dysfunction in neurons (Amaral et al., 2010). As leucine strongly activates the TOR (target of rapamycin) signaling pathway (Edwards et al., 2015) and the deficiency of this pathway is known to increase the lifespan in *C. elegans* (Vellai et al., 2003), the previously reported beneficial effects of cocoa in age-associated health including lifespan extension (Munasinghe, Almotayri, Thomas, et al., 2021) might be influenced by this reduction in leucine. Higher concentrations of tyrosine are known to contribute to cognitive problems in older adults (Ravaglia et al., 2002). Therefore, the previously observed cognitive improvements (Munasinghe, Almotayri, Thomas, et al., 2021) might be a result of the reduced tyrosine concentrations with cocoa supplementation.

The concentrations of proline and asparagine in pan-neuronal Aβ expressing worms at the young age were significantly higher than the control. Proline metabolism is known to alter in AD (Paglia et al., 2016) and AD patients have shown elevated proline concentrations in plasma (Chatterjee et al., 2020). Higher concentrations of proline are associated with decreased acetylcholinesterase (AChE) activity (Delwing et al., 2003). Moreover, reduced concentrations and activity of AChE have been reported in dementia (O'Brien et al., 2003) and AD (Appleyard et al., 1983; Yamamoto et al., 1990). Cocoa reversed these increased proline concentrations in AD worms to reach similar concentrations to their control. Previous studies have shown that the treatment with antioxidants can reverse the proline-induced reduction in AChE activity, indicating the involvement of oxidative stress (OS) (Delwing et al., 2005). Therefore, this beneficial effect of cocoa supplementation might be due to the antioxidant activity of cocoa PPs. Asparagine concentrations are also known to be altered in AD, some studies showing elevated concentrations (Chatterjee et al., 2020) while others showing reductions (Jiménez-Jiménez et al., 1998). Cocoa supplementation reduced the elevated asparagine concentrations in AD worms to reach similar concentrations to the control worms. However, further studies are required to confirm the role of asparagine in AD due to the controversiality of previous studies and therefore, the effects of cocoa in asparagine metabolism.

Purine metabolism pathways are known to be altered in AD (Alonso-Andrés et al., 2018). The purine compound hypoxanthine which is the principal purine nucleobase involved in the salvage purine pathway in the brain (N. Li et al., 2016) was significantly higher in Aβ expressing worms at their middle age than the control. The involvement of purine metabolism in AD has been previously described which shows a shift towards an increase of hypoxanthine with the expression of Aβ (Alonso-Andrés et al., 2018; Esteve et al., 2017). Hypoxanthine is known to contribute much to the

memory deficits in AD via an AChE-related mechanism which might reduce the concentrations of the neurotransmitter, acetylcholine (Ach) (Y.-Y. Chen et al., 2020). Additionally, it contributes to AD by inducing inflammation and OS (Bavaresco et al., 2007). Therefore, the previously reported short-term memory loss in these worms (Munasinghe, Almotayri, Kolivas, et al., 2021) might be due to the increased OS and decreased Ach concentrations induced by hypoxanthine. Cocoa-supplemented worms showed a reduced concentration of hypoxanthine which was similar to their control worms. This reduction in hypoxanthine concentration with cocoa supplementation might be one possible explanation for the improved short-term memory loss in AD worms.

The non-essential amino acid ornithine which is produced as an intermediate molecule in the urea cycle from arginine was significantly higher in Aβ expressing worms at their middle age than the control. A Previous study has been demonstrated a shift in arginine metabolism towards the arginase pathway (as discussed in detail in chapter 6), perhaps in response to the Aβ accumulation which might increase ornithine concentrations (Bergin et al., 2018). Blocking arginine catabolism by inhibiting key enzymes in the arginine utilization pathway [ornithine decarboxylase (ODC) and arginase] has reversed the Aβ-induced memory deficits in mice (Kan et al., 2015). However, cocoa supplementation increased ornithine concentrations in both Aβ expressing and control worms in this study, but the increase was significant only in control worms. As it has been previously shown that a cocoa extract significantly decreased the activity of ODC which converts ornithine to polyamines (Carnésecchi et al., 2002), this appears as a possible mechanism to explain the increased ornithine concentrations in cocoa-treated control worms. The reduced Aβ fibril concentration with cocoa supplementation as previously demonstrated (Munasinghe, Almotayri, Kolivas, et al., 2021) may reverse the Aβ-induced deficits in arginine metabolism. However, the above-mentioned effect of cocoa on ODC also should be taken into the account in explaining the effects of cocoa in ornithine concentrations in Aβ expressing worms. Therefore, this study is unable to establish the exact effect of cocoa on ornithine, and further studies are needed for that.

7.6. Conclusions and future implications

Aging and related disease conditions are increasing in prevalence as a consequence of the continuously growing older population. Despite existing pharmaceutical interventions, healthy lifestyle choices are still needed to maintain an affordable health care system. Dietary interventions have been recently gained much attention in this context, in particular, polyphenol (PP) rich foods due to their proven multiple disease-modifying mechanisms. The work presented in this thesis explored the impact of the supplementation of cocoa, one of the richest dietary sources of PPs in aging-related disease conditions including Alzheimer's disease (AD) by using *C. elegans* as the model organism. Findings of this study revealed that cocoa supplementation can improve some of the aging-associated disease conditions and reduce amyloid-β (Aβ)-induced deficits in AD. The results of the lifespan experiments of this study support the idea that antioxidants can act as pro-oxidants depending on the dose. The finding that the early-start supplementation was essential for

the cocoa-mediated lifespan extension effects emphasizes the importance of further studying the role of early life nutrition on human health. Improvements in memory deficits in AD with cocoa and how cocoa affects Aβ-induced deficits at the metabolomic level are some other significant findings of the study. For future experiments, it is worth testing the hypothesis that something equivalent to cocoa "flavor", or the very act of ingesting it, against a background of a monotonous diet, is not the cause of a more vigorous nervous system, thereby "protecting" neurons, and all other tissues, of the animal against degeneration. Moreover, the specific compounds responsible for the beneficial effects of cocoa and their mechanisms of action should be further studied. Thereby, these compounds can be used in developing effective treatment regimens in aging-related diseases.

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