Comparative benefits of orange heirloom tomatoes over red tomatoes for bone health

A thesis presented in partial

fulfilment of the

requirements for the degree of

Doctor of Philosophy

in

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at Massey University, Manawatū

New Zealand

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MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

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Thank you for taking the time to examine this thesis, which has been undertaken during the Covid-19 pandemic. The New Zealand Government's response to Covid-19 includes a system of Alert Levels which have impacted upon researchers. Our University's pandemic plan applied the Government's expectations to our research environment to ensure the health and safety of our researchers, however, research was impacted by restrictions and disruptions, as outlined below.

For a six-week period from March 26 to April 27 2020, New Zealand was placed under very strict lockdown conditions (Level 4 – Lockdown), with students and staff unable to physically access University facilities, unless they were involved in essential research related to Covid-19. All field work ceased and data collection with humans was restricted to online methods, if appropriate. The restrictions were partially lifted on April 27, but students and staff were not generally allowed back into University facilities until May 13.

Ongoing disruptions have also been encountered for some students due to uncertainties over the potential for future Covid-19-related restrictions on activities, and a Covid-19 cluster outbreak based in Auckland in New Zealand on 12 August 2020 led to the imposition of rolling Level 2 (Reduce) and Level 3 (Restrict) conditions until 23 September 2020. Auckland campus based students remained on Level 2 until 7 October 2020. This Alert Level system continues to be utilised throughout 2021.

These changing Alert Levels have meant that some research students had experimental, clinical, laboratory, field work, and/or data collection or analysis interrupted, and consequently may have had to adjust their research plans. For some students, the impacts of Covid-19 stretched far beyond the lockdown period in April/May 2020, as they may have had to significantly revise their research plans.

Overseas travel is not permitted by the University and restrictions have been placed on the New Zealand borders which are closed to non-New Zealand citizens and permanent residents. This meant that international students who were based offshore at the time of lockdown, were unable to return to New Zealand. A small number of offshore students were provided permission to return to New Zealand in early 2021. Many students have also suffered from anxiety and stress-related issues, and have had financial impacts, meaning their research progress has been significantly delayed.

This form, as completed by the supervisor and student, outlines the extent that the research has been affected by Covid-19 conditions.

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Student Name:	Umani Walallawita	ID Number:	16348775
Supervisor Name:	Prof Julian Heyes	Date:	06-Dec-21

Thesis title:

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The candidate was analyzing samples from her rat trials when 2020 lockdown happened. Due to the sudden closure of the university, candidate had to pause the lab experiments until students were allowed to enter the university. Candidate wanted to get some samples freeze dried from the Nutrition laboratory-Massey University, however, as the laboratory did not accept samples from outside due to Covid restrictions, candidate had to wait until the country moved to alert level 2 to drop the samples in contactless way to the laboratory. However, candidate could manage to do some data analysis and writing with the available data during the lockdown. But as this unexpected university closure affected lab experiments, candidate started the lab experiments again when the students were allowed to enter the university.

Candidate was engaging in cell culture experiments at the time when New Zealand had early 2021 lockdown. Although the duration of lockdown was not as long as in 2020, unfortunately candidate had to stop culturing cells for the experiment as students were not allowed to enter the university. Candidate started cell culture experiments again when university opened after lockdown.

Both lockdowns affected the uHPLC analysis carried out in Plant and Food Research. Due to the alert level, outsiders were not allowed to enter Plant and Food laboratories. Therefore, candidate had to postpone the carotenoid analyses carried out in Plant and Food Research and start again when New Zealand moved to alert level 2.

To compensate for these time loses candidate took two months of suspension.

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Abstract

Bone loss, common in women after menopause, is characterized by reduced bone mineral density with disruption of bone microarchitecture, leading to higher fracture risk and osteoporosis. There are few treatments, and most have adverse side effects. Intervention studies have shown protective effects of lycopene against bone loss in animal models. Lycopene is a lipid-soluble carotenoid found mainly in tomatoes. The molecule exists in all-*trans*- and a variety of *cis*- configurations; and *cis*- lycopene isomers have been reported to be more bioavailable than all-*trans*-. 'Moonglow', an orange heirloom tomato, contains >90% of its lycopene in *cis*- isomeric form and thus may be a better source of naturally bioavailable lycopene than red tomato, which contains all-*trans*-lycopene. We hypothesised that consumption of moderate daily doses of whole 'Moonglow' tomato would reduce bone loss more effectively than red tomato in rat and cell models of osteoporosis. *In vivo*, 'Moonglow' tomato supplementation delivered physiologically relevant plasma lycopene concentrations within four days of feeding. The post-ovariectomy rat model was successful in accelerating bone loss. 'Moonglow' tomatoes delivered a higher plasma lycopene concentration than red tomatoes and were beneficial in reducing a bone turnover marker compared to red tomatoes, even when fed after initiation of bone loss, but did not improve bone mineral density. Ovariectomy reduced gut bacteria abundance; compared to red, 'Moonglow' tomato feeding restored the numbers of *Lactobacillus*, *Enterococcus*, *Bacteroides* and *E. coli*. *In vitro* cell culture studies showed that both red and 'Moonglow' tomato hexane extracts induced a significant decrease in the number of bone-resorbing TRAP-positive osteoclasts at 10 µmol/L lycopene concentration. In conclusion, there were slight differences in bone measurements between red or 'Moonglow' tomato feeding, except for the significant reduction of one bone turnover marker. This suggests that a higher dose and longer intervention period may be needed to provide clinically important improvement against bone loss. However, significantly higher plasma lycopene followed 'Moonglow' feeding compared to red tomatoes, indicating its better bioavailability and demonstrating value for future research on health benefits from 'Moonglow' tomatoes. In addition, findings from this study provide support for the importance of exploring the potential prebiotic-like effect of tomatoes and the mechanisms associated with changes in gut bacteria.

Acknowledgements

A PhD is a great journey in one's life, and for me it was a full of life changing experience. But I would not be able to reach my destination without the enormous support I was given by the number of people I met during my journey. Therefore, I would take this opportunity to express my gratitude to all of them who supported me during this journey. I would like to start by expressing my sincere gratitude to my supervisory panel. It was a great privilege to work with you all. I'm blessed to have you all, with expertise knowledge in different areas related to my project. At the outset, I wish to express sincere gratitude to my chief supervisor, Professor Julian Heyes for his motivation, extensive advice and constructive comments. It was a great opportunity to work with Julian, the understanding and encouragement I received from the very beginning has been remarkable. I wish to express my sincere gratitude to my co supervisor Dr Fran Wolber, who always being really helpful to me, not only with her excellent guidance in the animal trials but also her constructive comments and encouragement throughout my research journey, especially during my thesis writing. I am also extremely grateful to Professor Marlena Kruger, who always provide motivation in me and insightful comments, and also for her excellent support, advice and all the worthwhile discussions throughout this journey. I would also like to express my sincere gratitude to Dr Ayelet Ziv-Gal for her thoughtful comments, friendly advice and encouragement. Thank you, Julian, Fran, Marlena and Ayelet, for being a biggest strength in my PhD journey.

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I'm extremely grateful for the expertise support that I received from Tony McGhie, Sarah Cordiner and Martin Hunt (Plant and Food Research, Palmerston North, New Zealand) with uHPLC analysis. I would like to extend my sincere gratitude to the staff in Massey University for their expertise technical support. Specially the Massey University Small Animal Production Unit (SAPU) for providing rats and housing/husbandry facilities. I also cannot thankful enough to Aimee Hamlin and Anne Broomfield for their specialist assistance given during rat trials. Anne Broomfield, I feel so sad that you can't see my acknowledgment today for your enormous help during my study. You are always loved and never forgotten. May your soul rest in peace. I also render my sincere gratitude to Gabrielle Plimmer, Shampa De, Louise Shaw and Corrin Hulls for their special assistance during rat surgeries, dissections, DXA scans, bone biomechanical test and ELISA, I don't know where I would be without your support.

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Last but not the least, I would like to thank my family: my parents and to my sister and brother-in-law for supporting me spiritually throughout this PhD and my life in

general. Finally, I would like to express my greatest thank to my beloved husband, Harshana Jayarathna, for his never-ending support throughout my research journey. Without them, I would not have had that courage to complete my PhD successfully.

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List of abbreviations

‘Post-MG’	Post-surgery ‘Moonglow’ tomato supplementation
‘Post-R’	Post-surgery red tomato supplementation
‘Pre-MG’	Pre-surgery ‘Moonglow’ tomato supplementation
‘Pre-R’	Pre-surgery red tomato supplementation
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BCO2	B-carotene-9’,10’-oxygenase
BHT	Butylated Hydroxy Toluene
BMC	Bone mineral content
BMD	Bone mineral density
BMU	Basic multicellular unit
BSA	Bovine serum albumin
BV/TV	Bone volume density; Bone volume per total volume
BW	Body weight
CKD	Chronic kidney disease
CM	Chylomicron
COL1A	Collagen type 1 alpha
COL1A1	Collagen type 1 alpha 1
CRTISO	Carotenoid isomerase
CRTL-b	β-ring hydroxylase
CRTL-e	ε-ring hydroxylase
CTX-1	Carboxy terminal crosslinked telopeptides of type 1 collagen
CV	Coefficient of variation
CYP19A1	Aromatase genes
d	Day
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DXA	Dual-energy X-ray absorptiometry
E2	Estrogen; 17ß-Estradiol; Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay
ERs	Estrogen receptors
ERα	Estrogen receptor alpha genes
FCS	Foetal calf serum
FDA	Food and Drug Administration
g	Relative centrifugal force (RCF)
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
h	Hours
H_2O_2	Hydrogen peroxide
HDL	High Density Lipoprotein
HED	Human Equivalent Dose
$HO_2^{\cdot}$	Hydroperoxyl
HPLC	High Performance Liquid Chromatography
HRT	Hormone Replacement Therapy
IDL	Intermediate density lipoprotein

IFN	Interferon
IL-1	Interleukin-1
IL-6	Interleukin-6
LCY-b	β-cyclase,
LCY-e	Lycopene-ε-cyclase,
LDL	Low Density Lipoprotein
LS	Lumbar spine
mBar	Millibar
M-CSF	Macrophage colony-stimulating factor
MEMα	Minimum Essential Medium Alpha Modification
MHC	Major histocompatibility complex
min	Minute
MQ	Milli-Q
MRI	Magnetic Resonance Imaging
mS	Milliseconds
MSCs	Mesenchymal Stem Cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor kappa beta
NTx	N-telopeptide of type 1 bone biomarker
NXS	Neoxanthin synthase
OC	Osteocalcin
OCLs	Osteoclasts
OPG	Osteoprotegerin
OVX	Ovariectomized
OVX-C	Ovariectomized Control
P	Probability
PBS	Phosphate Buffered Saline
PDS	Phytoene desaturases
PGE2	Prostaglandin E2
PINP	Procollagen type 1 N-terminal propeptide
PTH	Parathyroid hormone
QC	Quality control
qPCR	Quantitative real-time polymerase chain reaction
R	Correlation coefficient
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
ROS	Reactive Oxygen Species
RXR	Retinoid X receptor
SAPU	Small Animal Production Unit
SCFAs	Short-chain fatty acids
SD	Standard deviation
sec	Seconds
SEM	Standard error of mean
Sham-C	Sham-Control
SOD	Superoxide dismutase
SPSS	Statistical Analysis Software
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
THF	Tetrahydrofuran

TNF- α	Tumour necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase
TRAP+ OCLs	TRAP-positive osteoclasts
uHPLC	Ultra High-Performance Liquid Chromatography
VDE	Violaxanthin de-epoxidase
VLDL	Very Low-Density Lipoprotein
VLDL	Very low-density lipoprotein
VOI	Volume of Interest
WAT	White Adipose Tissue
ZDS	ζ-carotene desaturase,
μCT	Micro-computed tomography
ρ-NPP	Para-nitrophenyl phosphate

CHAPTER 1

Introduction

1.1 Research background

Phytochemicals from fruits and vegetables are reported to aid in the maintenance of bone health. Particularly, carotenoids such as α-carotene, β-carotene, canthaxanthin and lycopene have shown beneficial effects on skeletal health and there is a clear positive association between lycopene intake and reduced bone loss (Hunter et al., 2008). Lycopene is an acyclic carotenoid, containing 11 conjugated double bonds in its all-*trans*-isomeric form and its various *cis*-configurations (Perveen et al., 2015). Tomatoes are rich sources of lycopene and they represent more than 80% of typical dietary sources containing lycopene. Depending on the variety, maturity, and other environmental conditions, the average lycopene content of tomatoes ranges from 0.7-20 mg/100 g of fresh fruit (Shi and Maguer, 2000). Dietary sources of lycopene are primarily found in the all-*trans*- isomeric form. Red tomatoes contain almost 90% of total lycopene in its all-*trans*- form (Cooperstone et al., 2016, Unlu et al., 2007a). In contrast to red tomatoes, orange heirloom tomatoes naturally contain *cis*-isomers of lycopene, with up to 90% being in *cis*- isomeric form (Cooperstone et al., 2016, Burri et al., 2009, Unlu et al., 2007a). This is due to a mutation known as *tangerine* in the carotenoid *cis-trans*-isomerase gene which gives a deep orange colour to the tomatoes (Cooperstone et al., 2016, Englert et al., 1979). As the *tangerine* mutant lacks the ability to convert tetra-*cis*-lycopene into all-*trans*- lycopene, it accumulates predominantly the tetra-*cis*- isomer, along with phytoene, phytofluene, ζ-carotene and neurosporene which are precursors of all-*trans*- lycopene (Cooperstone et al., 2016, Kopec et al., 2015). *Cis*- isomers of lycopene are estimated to be 8.5 times more bioavailable than all-*trans*- lycopene (Cooperstone et al., 2015).

Lycopene is not known to have any adverse effects in humans, even when eaten at very high doses. Olmedilla *et al* reported that lycopene supplementation at 13.3 mg/day for 20 weeks, which was the longest period reported for a human lycopene intake study, did not cause any observable adverse or side effects (Olmedilla et al., 2002). The median lethal dose (LD50, representing the dose that is lethal for 50% of the animals in a

treatment group) in rats for synthetic lycopene was 500 mg/kg body weight (BW) (Bend et al., 2007). Therefore the World Health Organization (WHO) expert committee on food additives has established 50 mg/kg BW/day as the highest acceptable daily intake (ADI) for synthetic lycopene (Bend et al., 2007). Published survey data show that dietary intake of lycopene ranges from 1-10 mg/person/day, equating to 0.016 – 0.16 mg/kg BW/day for the average adult (Bend et al., 2007). However, many dietary intervention studies on bones have used daily doses up to 25-30 mg of lycopene (0.4 mg/kg BW/day) as a physiologically relevant dose for bone health. A study performed to investigate the effects of lower levels of lycopene showed that 5-10 mg of daily lycopene intake (0.08 – 0.16 mg/kg BW/day) is adequate for the maintenance of average plasma lycopene levels in adults (Rao and Shen, 2002). However, considering the poor bioavailability of all-*trans*-lycopene, this would equate to an adult eating up to 1.5 kg of fresh red tomatoes daily.

The prevalence of age-related diseases is increasing with the aging world population (Wright et al., 2014). Osteoporosis is a metabolic disease characterized by reduced bone mineral density along with disruption of bone microarchitecture and alteration of non-collagenous protein in the bone, which leads to higher fracture risk (Kini and Nandeesh, 2012) as osteoporotic bone is more fragile (Rao and Rao, 2013). An initial fracture increases the risk of subsequent fractures where approximately one in eight osteoporotic patients have a second fracture within a year after the first fracture (Ferdous et al., 2016). Thus, this condition results in a reduction of overall quality of life with severe morbidity, disability and mortality. An imbalance between bone resorption and formation initially leads to osteopenia and results in low mineralization. This can progress further to osteoporosis. Low bone mineral density, bone mineral content and bone mineral area are the distinguishing features of people with osteoporosis compared to healthy people (Kruger and Morel, 2016). The World Health Organization (WHO) classifies bone mineral density (BMD) using a T-score system, with a BMD $\geq$ 2.5 standard deviations below the young adult mean BMD (equivalent to a T-score of <2.5) as osteoporotic. The National Bone Health Alliance added a classification, in which a T score between -2.5 and -1.0 equates to osteopenia, identifying patients who have low bone mass and are prone to becoming osteoporotic (Adler, 2018). The T score for normal BMD ranges between 1 and -1.0 (Rao and Rao, 2013).

1.2 Importance of the research

Osteoporosis is more common in women after menopause and it has become a public health issue in the ageing population. To date, medications used to prevent and treat bone loss may increase bone mass up to 10% over 3-5 years (Nieves and Cosman, 2015). However, there are many side effects along with these medications such as increased risk of cancers (Porch et al., 2002) and stroke (Hendrix et al., 2006, Wassertheil-Smoller et al., 2003). With the current trend of seeking natural ways to minimize bone loss, people may undertake several lifestyle changes including diet, exercise and supplements to reduce the risk of osteoporosis. Lycopene has an inverse relationship between intake and bone loss which has been demonstrated *in vitro*, in animal models of osteoporosis, and in human clinical studies (Pandey et al., 2018, Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014, Dai et al., 2014). Due to the relatively low bioavailability of lycopene *trans*- isomers in normal tomatoes, several techniques are being used to increase bioavailability such as canning, mixing with oil or making into sauces and pastes. This processing can increase bioavailability of lycopene 2-2.5 fold (Kamiloglu et al., 2013, Colle et al., 2010). An adult would have to consume unreasonably high amounts of raw red tomatoes to get the reported beneficial amount of lycopene. However, lycopene from orange heirloom tomatoes has a higher bioavailability in the fresh fruit, due to the *tangerine* mutation resulting in the lycopene being present as *cis*- rather than *trans*- isomers.

'Moonglow', an orange heirloom variant tomato, is rich in lycopene and contains >90% in *cis*- isomeric form and may be a better source of naturally bioavailable lycopene than conventional red tomato and may be of benefit to reduce postmenopausal bone loss while requiring a more reasonable intake. In addition to its beneficial effects on bone, tomato lycopene has also been reported to have a positive effect on intestinal microbiota through acting as a prebiotic (Wiese et al., 2019, García-Alonso et al., 2017). Prebiotics stimulate the growth of gut microbiota (Markowiak and Śliżewska, 2017) while gut microbes could be involved in the modulation of bone metabolism (Rettedal et al., 2021) via the production of metabolic signalling molecules (Cox-York et al., 2015). Therefore, the present study was carried out to determine whether the *cis*- isomers of lycopene in 'Moonglow' are more bioavailable than *trans*- isomers in red tomatoes. Following this, the study compared red versus 'Moonglow' tomatoes for bone health-promoting and

prebiotic effects in a post-menopausal rat model. This study further investigated the cellular mechanisms exerted by red and 'Moonglow' tomato extracts.

1.3 Research hypothesis and objectives

We hypothesized that the consumption of small and manageable daily doses of raw 'Moonglow' tomatoes would reduce bone loss and turnover in ovariectomized rats. To reach our main goal, the following research questions were addressed.

Research Question 1

What is the dose-dependence, and time-course, appearance of lycopene in plasma, liver and excreta in rats, after consumption of 'Moonglow' tomatoes?

Hypothesis: Absorption of lycopene by rats from 'Moonglow' tomatoes is significantly higher than that reported for conventional red tomatoes, but there is a similar time-course of absorption, tissue distribution, and excretion of lycopene to humans.

Research Question 2

Are 'Moonglow' tomatoes more effective to reduce bone loss in ovariectomized rats compared to red tomatoes over a long-term feeding period?

Hypothesis: Consumption of 'Moonglow' tomatoes significantly reduce bone resorption of ovariectomized rats compared to consumption of red tomatoes.

Research Question 3

Is there an ability of 'Moonglow' tomato extract to inhibit osteoclastogenesis, compared to red tomato extract?

Hypothesis: 'Moonglow' tomato extract exposure can significantly inhibit osteoclast formation and activity compared to red tomato extract in differentiated RAW 264.7 cells.

Research question 4

Will the levels of lycopene change in plasma and liver of ovariectomized rats following supplementation of 'Moonglow' and red tomato?

Hypothesis: 'Moonglow' tomato feeding both before and after ovariectomy can increase lycopene concentrations in plasma and liver, with the isomeric profile differing to those in rats fed red tomato.

Research question 5

Will ‘Moonglow’ or red tomato produce a prebiotic-like effect on gut microbiota?

Hypothesis: ‘Moonglow’ tomato feeding can increase the abundance of beneficial microbes in the gut.

1.4 Thesis overview

This thesis includes seven chapters as follows.

Chapter 1 describes the general introduction of the thesis including background and the importance of the research. This chapter also outline the organisation of the thesis with research hypotheses and the objectives.

Chapter 2 describes the review of literature related to this research. This chapter includes three sections. The first part explains the literature on bone biology and pathophysiology of postmenopausal osteoporosis. The second part explains the various aspects of lycopene including chemistry, metabolism, absorption, and prebiotic-like effects. The last part reviews the potential role of lycopene in the prevention of postmenopausal bone loss using evidence from molecular, animal and clinical studies.

Chapter 3 reports the results on dose-dependence and time-course of absorption, tissue distribution, and excretion of 'Moonglow' tomato lycopene (*in vivo*) when fed to young adult rats for up to one week; these findings helped in determining the appropriate dose selected for the study described in chapters 4 and 5.

Chapter 4 presents the influence of red and 'Moonglow' tomato consumption on bone properties in the rat model of post-ovariectomy osteoporosis using different parameters such as bone markers, bone density measures, biomechanical properties, and bone microarchitecture.

Chapter 5 describes the differences in lycopene content in the plasma and liver following red versus 'Moonglow' tomato feeding in adult female ovariectomized or ovary-intact rats. This chapter also evaluates the changes of body composition and gut bacteria following tomato feeding which assess the prebiotic like effects of red and 'Moonglow' tomatoes.

Chapter 6 describes the anti-osteoclastogenic effects of red and 'Moonglow' tomato extracts on RANKL-induced osteoclast differentiation in RAW 264.7 cells assessed by tartrate resistant acid phosphatase staining and assay (TRAP).

Chapter 7 summarises the main findings of this study through discussing overall results and identifying limitations. This chapter also poses directions for future research work.

1.5 Thesis output

Publications

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2020). Potential role of lycopene in the prevention of postmenopausal bone loss: Evidence from molecular to clinical studies. International Journal of Molecular Sciences, 21(19), 7119.

Conference presentations

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2019). Evaluation of Lycopene from orange heirloom ('Moonglow') tomatoes. Plant Science Central, Massey University, Palmerston North - New Zealand (Poster presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2019). Small daily doses of orange heirloom tomatoes ('Moonglow') dose-dependently increase plasma and liver lycopene concentrations in rats. International Conference of Food Structures, Digestion and Health-Rotorua, New Zealand (Poster presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2020). Importance of *tangerine* tomato lycopene over red tomato lycopene. Centre for Metabolic Health Research (CMHR) annual symposium (Oral presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2020). Lycopene from orange heirloom tomatoes ('Moonglow') supress bone turnover in OVX rats against red tomatoes. Nutrition Society of Australia Virtual Conference (Poster presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2021). Daily lycopene accumulation in plasma and liver after supplementation of 'Moonglow' tomato in female rats. NZIFST Annual conference, Palmerston North-New Zealand (Poster presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2021). Lycopene from orange heirloom tomatoes ('Moonglow') increase bone biomechanical properties and supress bone turnover in OVX rats against red tomatoes. International Carotenoid Society Virtual Conference (Oral poster presentation).

Walallawita, U. S., Wolber, F. M., Ziv-Gal, A., Kruger, M. C., & Heyes, J. A. (2021). Changes in plasma and liver lycopene concentrations, body composition and gut bacteria following red versus 'Moonglow' tomato feeding in ovariectomized rats. Nutrition Society New Zealand Virtual Conference (Pre-recorded poster presentation).

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STATEMENT OF CONTRIBUTION
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CHAPTER 2

Literature review

2.1 Bone biology, modelling, and remodelling

Bone is a specialized connective tissue that is responsible for the framework of the body. Primarily, the skeleton provides support for the body and assists its movements. Bones also act as a major mineral reservoir, carrying 99% of calcium, 85% of phosphorus, and 65% of magnesium body stores, and they are the main repository of growth factors and cytokines (Kini and Nandeesh, 2012). Moreover, bone plays an important role in acid-base balance and hormonal functions related to phosphate metabolism, blood glucose, and fat deposition in the body (Kini and Nandeesh, 2012). Bone also helps in the detoxification of heavy metals and other waste materials by removing them from blood circulation (Kini and Nandeesh, 2012, Bartl and Frisch, 2009, Morgan, 2008). Bone generally comprises three components: organic matrix, inorganic salts, and water. Approximately 90% of the organic matrix consists of collagenous protein, non-collagenous protein, and growth factors. The inorganic matrix contains mainly calcium and phosphorus in the form of hydroxyapatite crystals (Florencio-Silva et al., 2015). Depending on the degree of porosity, bones are categorized as cortical bone (compact) or trabecular bone (cancellous). Cortical bone is denser, while trabecular bone is more porous. The porosity of cortical bone is approximately 3–5% and may increase with age (Burr and Akkus, 2014, Bartl and Frisch, 2009). Cortical bone represents 80% of the adult skeleton. Trabecular bone is more metabolically active than cortical bone. This difference occurs due to the higher surface area of trabecular bone compared to cortical bone (Bartl and Frisch, 2009).

Bone tissue contains four different cell types: osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts originate from mesenchymal stem cells and are responsible for bone formation (Alves, 2012). Osteoclasts are derived from mononuclear hematopoietic stem cells and are responsible for bone resorption (McCormick, 2007). The majority of mature osteoblasts may undergo apoptosis, while a minority re-differentiate into osteocytes or lining cells (Jilka et al., 1998). Approximately 90–95% of bone cells are osteocytes, which have a long life span of nearly 25 years (Florencio-Silva et al., 2015,

Horcajada and Offord, 2012). Bone marrow, found in the bone cavity, consists of two types of cells: hematopoietic and stromal. Hematopoietic stem cells produce osteoclasts, immune cells, platelets, and red blood cells, while mesenchymal stem cells produce osteoblasts, cartilage, and adipocytes (Horcajada and Offord, 2012, McCormick, 2007).

Bone tissue undergoes two major physiological processes: modeling and remodeling. Bone modeling is characterized by a change in the shape of the bone as a result of physiological influences or mechanical forces (Clarke, 2008). For example, bone modeling widens the bones with aging (Kini and Nandeesh, 2012). Moreover, bone modeling is upregulated in hypoparathyroidism, chronic kidney disease (CKD), and medical treatments containing anabolic agents (Iñiguez-Ariza and Clarke, 2015). During bone modeling, the changes in the shape of bones are regulated by independent actions of osteoblasts and osteoclasts (Langdahl et al., 2016).

In contrast, bone remodeling occurs throughout life and is responsible for the removal of older bone and its replacement with new bone structure (Kini and Nandeesh, 2012, Baron and Hesse, 2012, Rao et al., 2003). Bone remodeling involves a sequence of cellular activities that occur within a specialized multicellular unit (Dempster and Raisz, 2015). The bone multicellular unit is predominantly comprised of osteoclasts, osteoblasts, and osteocytes (Florencio-Silva et al., 2015). There are 3–4 million basic multicellular units (BMU) produced each year, and approximately one million among them actively participate in the bone remodeling process (Manolagas, 2000). Bone remodeling has four major stages: activation, resorption, reversal, and formation (Dempster and Raisz, 2015). In the first step, osteoblastic stromal cells or lining cells are activated via lining cell retraction and endosteal membrane digestion by collagenase. Following this, osteoclasts initiate bone resorption by dissolving the mineral matrix (Dempster and Raisz, 2015, Kini and Nandeesh, 2012). At the end of the resorption phase, mononuclear cells such as monocytes, osteocytes, and preosteoblasts are found in resorption cavities (Kini and Nandeesh, 2012). During the reversal phase, a cement lining rich in mucopolysaccharides is deposited between old and new bone, as well as signaling molecules that can activate osteoblast precursors. Therefore, the reversal phase is considered a transitional phase between resorption and formation of new bone (Dempster and Raisz, 2015). Lastly, a new organic matrix is produced by osteoblasts, which eventually mineralizes into new bone (Langdahl et al., 2016). The resorption and reversal phases last for 2 weeks and 4–5 weeks, respectively. The formation phase is the longest and lasts approximately 4–6

months until the new bone is completely formed (Shetty et al., 2016, Clarke, 2008, Hadjidakis and Androulakis, 2006).

Approximately 90% of cortical bone is calcified; thus, it contains a low surface area to volume ratio. This leads to a slower rate of remodeling in cortical bone compared to trabecular bone (Bartl and Frisch, 2009). Approximately 25% of the body's trabecular bone is remodeled each year compared to only 2.5% of cortical bone (Bartl and Frisch, 2009). Bone remodeling is regulated by various systemic and local factors. Genetics, mechanical factors, vascular factors, nutrition, and hormones are considered systemic regulators, while growth factors, matrix proteins, and cytokines act as local regulators (Siddiqui and Partridge, 2016, Florencio-Silva et al., 2015, Dempster and Raisz, 2015, Kini and Nandeesh, 2012, Hadjidakis and Androulakis, 2006). At menopause, bone remodeling increases and continues at a higher rate for 5–10 years due to the decrease in levels of estrogen (Iñiguez-Ariza and Clarke, 2015).

2.1.1 Postmenopausal osteoporosis: a silent disease

Postmenopausal osteoporosis is a common metabolic disease among older women ($\geq$50 years). It is characterized by reduced bone mineral density along with disruption of bone microarchitecture and alteration of non-collagenous protein in bone, which together lead to higher fracture risk (Kini and Nandeesh, 2012). This disease reduces women's overall quality of life by significantly increasing their rates of morbidity, disability, and mortality. Osteoporotic bone tends to be more fragile and easily fractured (Rao and Rao, 2013). In general, one in eight people will experience a second fracture within a year after their first osteoporotic fracture (Ferdous et al., 2016).

FRAX is a computer-based algorithm which estimates 10-year probability of fracture risk in hip, vertebrae, wrist and proximal humerus. FRAX uses the clinical risk factors to estimate probable fracture risk and bone mineral density (BMD) can be used to enhance the prediction (Kanis et al., 2009). Bone mineral density, bone mineral content (BMC), and the quantity and quality of bone are the distinguishing factors between people with osteoporotic versus healthy bones (Kruger and Morel, 2016). The risk of osteoporosis is primarily evaluated through bone mineral density, which is predominantly measured by dual-energy X-ray absorptiometry (DXA) scanning (Shetty et al., 2016). Other methods include magnetic resonance imaging (MRI), ultrasound, and microcomputed tomography (Kruger and Morel, 2016, Annapoorna et al., 2004). Bone

density is a quantitative measure, but its limitation is that it does not measure bone quality (Siris et al., 2012). Therefore, measurements of bone turnover markers as proxies for bone formation and bone resorption are used to evaluate the quality of bone in tandem with DXA scanning (Shetty et al., 2016).

For the diagnosis of osteoporosis, BMD values are converted into a T-score (Szulc and Bouxsein, 2011). The T-score is calculated by dividing the difference between a female patient's BMD and the mean BMD of young, healthy women by the standard deviation of the reference population (Szulc and Bouxsein, 2011). A T-score between −1 and +1 standard deviation (SD) is considered to be normal BMD. A T-score between −1 and −2.5 SD is categorized as osteopenia. A T-score of −2.5 SD is considered to be osteoporotic (Adler, 2018, Rao and Rao, 2013, McCormick, 2007, Annapoorna et al., 2004). An imbalance between bone resorption and formation initially leads to osteopenia, which is characterized by low mineralization and is likely to further progress to osteoporosis (Feng and McDonald, 2011). The most common pharmacological treatments currently being used for osteopenia and osteoporosis are bisphosphonates, denosumab, anabolic agents, and hormone replacement therapy (Ferdous et al., 2016). Most of these treatments have been shown to increase bone mass by up to 10% over 3–5 years (Nieves and Cosman, 2015). Postmenopausal women are advised to take these medications with calcium and vitamin D supplements to increase their effectiveness (IOF, 2020).

2.1.2 Risk factors of postmenopausal osteoporosis

Risk factors for osteoporosis can be primarily categorized as modifiable and nonmodifiable (Table 2.1). Heredity is the major nonmodifiable factor of osteoporosis, and children of parents with osteoporosis and fractures are themselves more prone to develop osteoporosis (Ferrari and Karasik, 2015). Osteoporosis is a polygenic disease that involves several genes. In rare instances, osteoporosis can be inherited due to mutations in single genes. Mutations of two genes of type 1 collagen (COL1A1 and COL1A2) are responsible for the dominant osteoporotic disease called "osteogenesis imperfecta", which is characterized by low bone mass and increased bone fragility (Ralston, 2010, Marini et al., 2007, Prockop et al., 1993, Spotila et al., 1991). Osteoporosis inheritance has also been linked with inactivating mutations in the aromatase (CYP19A1) and estrogen receptor alpha genes (ERα) (Morishima et al., 1995, Smith et al., 1994).

Table 2.1 Risk factors of postmenopausal osteoporosis.

Fixed Risk Factors	Modifiable Risk Factors
Menopause age (Sullivan et al., 2017)	Inadequate calcium and vitamin D intake (Dawson-Hughes et al., 2005)
Menopause and hysterectomy (Sullivan et al., 2017, Melton III et al., 2007)	Alcohol consumption (Kanis et al., 2005a)
Estrogen deficiency and amenorrhea (Fazeli and Klibanski, 2018)	Cigarette smoking (Kanis et al., 2005b)
Family history of osteoporosis (Kanis et al., 2004a, Seeman et al., 1989)	Low body mass index (<20 kg/m^2) (De Laet et al., 2005)
Previous fractures (Kanis et al., 2004b)	Eating disorders (Misra et al., 2016)
Height loss (>0.5 cm per year) (Moayyeri et al., 2008)	Inadequate physical exercise (Kemmler et al., 2004, Bass et al., 2002)
Ethnicity (Caucasian and Asian population are at high risk) (IOF, 2020)	Frequent falls (Tinetti, 2003)

Estrogen deficiency is the primary risk factor of postmenopausal osteoporosis; other contributors besides the genetics mentioned above include modifiable factors such as nutrition, certain medications, and lifestyle (Sipos et al., 2009). Bone cells (osteoblast, osteoclast, and osteocytes) contain estrogen receptors on their surface (Becker, 2006). Stimulation of estrogen receptors, particularly on osteoblasts, may inhibit the activation of osteoclasts and thus reduce bone resorption and protect bones from osteoporosis (Jahanian et al., 2016). Oxidative-stress-generating factors such as poor nutrition, low levels of antioxidants in the body, smoking, alcohol intake, and excessive caffeine intake can be modified through lifestyle changes (Rao and Rao, 2015, Rao and Rao, 2013). For example, a diet low in calcium may induce secretion of parathyroid hormone, which activates osteoclasts and bone resorption (Jahanian et al., 2016).

Interestingly, some cross-sectional studies have found an inverse relationship between sleep duration and BMD in elderly women (Kim et al., 2014, Kobayashi et al., 2012). It has been hypothesized that a shorter waketime reduces the daily mechanical

loading that induces bone remodeling, and thus reduces BMD (Kobayashi et al., 2012). Lower melatonin levels due to reduced light exposure may lead to fewer interactions between estrogen and its receptors and, thus, negatively impact BMD (Hill et al., 1992). However, other studies report either positive or null relationships between sleep duration and BMD in postmenopausal women (Ochs-Balcom et al., 2020, Wang et al., 2015, Niu et al., 2015, Fu et al., 2011). The conflicting evidence in this area may be due to confounding study participant factors that are not uniformly controlled for across the studies, such as age, body composition, diet, and nighttime-only sleep duration versus inclusion of daytime naps.

2.1.3 Pathophysiology of postmenopausal osteoporosis

The occurrence of postmenopausal osteoporosis is dependent mainly on body estrogen levels. Estrogen regulates bone turnover either by directly interfering with osteocytes and osteoclasts or by regulating T-lymphocyte function and the formation of osteoblasts (Khosla et al., 2012). Estrogen has both skeletal and non-skeletal functions, and, due to the former deficiencies, can cause bone-related diseases (Figure 2.1). Women are at higher risk of developing osteoporosis 3–5 years after the onset of menopause (Okman-Kilic, 2015). The mechanism by which estrogen deficiency causes postmenopausal osteoporosis is complex. Estrogen can influence bone remodeling through inhibiting cell differentiation and increasing osteoclast apoptosis (Okman-Kilic, 2015).

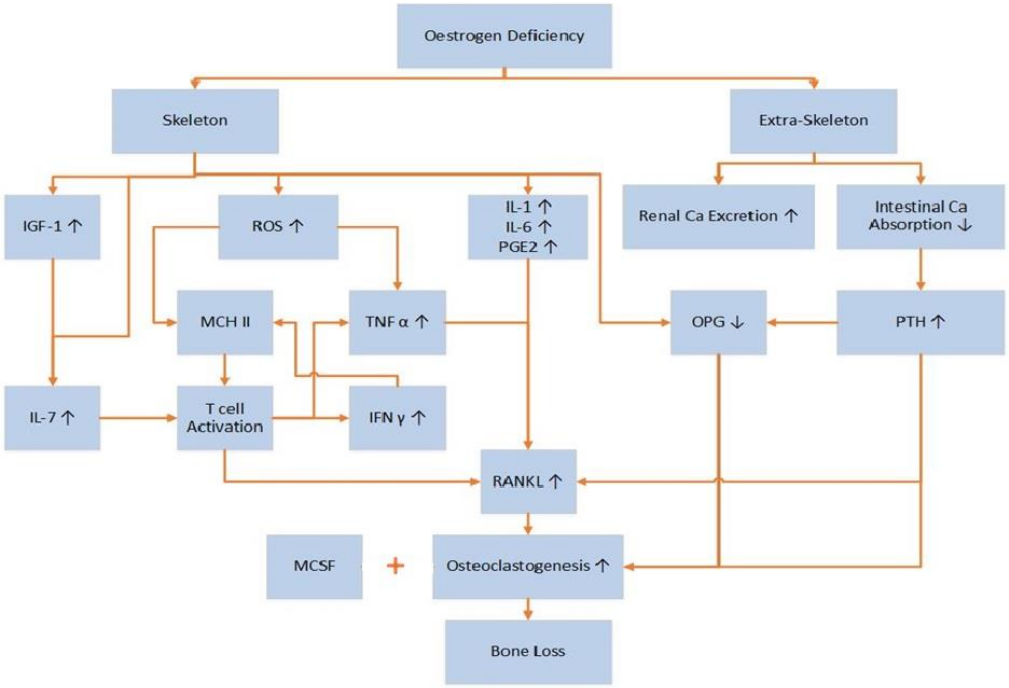

Figure 2.1 Occurrence of bone loss through estrogen deficiency (Redrawn using the reference from (Sipos et al., 2009, Weitzmann and Pacifici, 2006)).

Estrogen deficiency increases the production of IL-7 directly and via increased production of IGF-1. IL-7 activates T-cells to produce IFN-γ and TNF-α. Reactive oxygen species (ROS), along with IFN-γ, upregulate MHC II, located in antigen-presenting cells that may further activate T-cells. Activated T-cells produce RANKL and TNF- α. Other cytokines, IL-1, IL-6, and PGE2, also increase the production of RANKL. Decreased osteoprotegerin (OPG) due to insufficient estrogen directly influences osteoclastogenesis. Beyond the skeletal activities, estrogen deficiency may increase renal calcium excretion while decreasing intestinal calcium absorption. This stimulates the parathyroid glands to produce PTH, which can reduce the production of OPG and increase the production of RANKL and, therefore, increase bone resorption. All these actions together are involved in postmenopausal bone loss.

Molecular markers of bone metabolism are widely used to assess bone-related disorders. These markers include enzymes and nonenzymatic peptides produced by bones. Bone formation or resorption markers correlate with the metabolic phase in which they are produced (Shetty et al., 2016). Short-term estrogen deficiency (3 weeks) is associated with low levels of bone formation markers in early postmenopausal women (Charatcharoenwitthaya et al., 2007). Interestingly, studies have shown that long-term estrogen deficiency increased both bone resorption and bone formation markers in postmenopausal women, suggestive of enhanced bone turnover with increased net bone loss (Wu et al., 2014, Iki et al., 2004, Garnero et al., 1996, Dresner-Pollak et al., 1996). Estrogen deficiency increases renal calcium excretion while decreasing intestinal calcium absorption (Sipos et al., 2009), and the resultant fall in calcium levels can activate various bone resorption mechanisms that include PTH, osteocalcin, OPG, and the

RANK/RANKL system (Tariq et al., 2019, Kalaiselvi et al., 2013, Jagtap et al., 2011, Iki et al., 2004, Yasuda et al., 1998). These bone resorption markers are, therefore, found in the blood in higher concentrations in osteoporosis. Conversely, osteocalcin, which is secreted by osteoclasts, directly binds calcium and enables bone mineralization by increasing hydroxyapatite absorption and, thus, is a marker of bone formation (Zoch et al., 2016). However, insufficient calcium and phosphorus stores in osteoporotic women reduce hydroxyapatite crystal formation, leaving more osteocalcin free to circulate in the blood (Kalaiselvi et al., 2013, Jagtap et al., 2011). The molecular mechanisms responsible for these complex changes are not yet fully elucidated (Garnero et al., 1996).

As mentioned earlier, oxidative stress can be coupled with osteoporosis. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and their neutralization by antioxidants. Reactive oxygen species are formed as a result of cellular respiration, enzymatic activities in mitochondria, and cellular responses to cytokines induced by external stimuli (Jahanian et al., 2016). Reactive oxygen species include both highly reactive oxygen-containing molecules and free radicals such as hydroxyl (OH), superoxide (O_2^-), and hydrogen peroxides (H_2O_2) (Rao and Rao, 2013). Free radicals can oxidize lipids and proteins, thus causing cell damage and altered function (Domazetovic et al., 2017, Wauquier et al., 2009). Reactive oxygen species suppress differentiation and proliferation of osteoblasts and are significantly involved in osteoclast differentiation and bone resorption (Ardawi et al., 2016, Callaway and Jiang, 2015). Menopause increases oxidative stress; thus, the oxidized microenvironment produced by ROS plays a major role in causing postmenopausal osteoporosis (Ardawi et al., 2016, Okman-Kilic, 2015, Mendoza and Zamarripa, 2013). Antioxidants are directly involved in the scavenging process of ROS. A lack of antioxidants may increase proinflammatory cytokines, especially tumor necrosis factor alpha (TNF-α), and thereby induce bone loss (Domazetovic et al., 2017).

There are two cytokines primarily responsible for osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL), which are produced by bone marrow stromal cells and osteoblasts during bone remodeling (Iñiguez-Ariza and Clarke, 2015). The RANK (receptor activator of nuclear factor kappa B), RANKL, and OPG system has been identified as a primary regulator of the bone remodeling process. Osteoprotegerin (OPG), which is produced by osteoblasts, is considered to be a decoy receptor for RANKL. RANKL binds to its

receptor RANK on osteoclast precursors in the presence of M-CSF. Upon this, osteoclast precursors differentiate and combine to form multinucleated osteoclasts, which can start bone resorption (Okman-Kilic, 2015). Due to lower estrogen levels in postmenopausal women, OPG is downregulated and RANKL activity upregulated, thereby increasing osteoclastogenesis (Sipos et al., 2009).

2.2 Carotenoid lycopene

2.2.1 Chemistry and its isomers

To date, more than 700 carotenoids (Britton et al., 2012) have been identified, of which 40–50 are present in the human diet in fruits and vegetables (Khachik, 2006). However, only 20 have been found in human tissues or blood (Fiedor and Burda, 2014, Rao and Rao, 2007). There are two classes of carotenoids: nonoxygenated carotenoids and oxygenated carotenoids. Nonoxygenated carotenoids are unsaturated hydrocarbons such as lycopene, α-carotene, β-carotene, γ-carotene, and ζ-carotene, whereas oxygenated carotenoids are the xanthophylls (Shi and Maguer, 2000).

The major carotenoids found in the human body and human diet are β-carotene, α-carotene, α-cryptoxanthin, lutein, zeaxanthin, and lycopene (Bacanli et al., 2017, Viuda-Martos et al., 2014). Carotenoids are localized within chloroplasts and chromoplasts in plant cells. In chloroplasts, the carotenoids are found in association with proteins, whereas chromoplasts contain a crystalline form of carotenoids (Schieber and Carle, 2005). Lycopene, a member of the carotenoid pigment family, is responsible for the specific red color in many fruits and vegetables, best typified by fresh tomatoes and tomato products (Viuda-Martos et al., 2014). It is a lipid-soluble antioxidant produced by plants and some microorganisms. Unlike β-carotene, lycopene does not contain a terminal β-ionone ring and thus does not have provitamin A activity. Lycopene is an acyclic carotenoid containing 11 conjugated double-bonds in its all-*trans*-isomeric form or in various *cis*- configurations (Figure 2.2) (Burri et al., 2009). Having 11 conjugated double-bonds, lycopene is theoretically assumed to have 2,048 possible *cis*–*trans*-conformations in nature, but only a few have been identified so far, predominately 5-*cis*, 9-*cis*-, 13-*cis*-, and 15-*cis*-. The most stable isomeric form is 5-*cis*-, followed by all-*trans*-, 9-*cis*-, 13-*cis*-, 15-*cis*-, 7-*cis*-, and, finally, 11-*cis*- as the least stable (Rao and Rao, 2007).

Phytochemicals from fruits and vegetables are reported to aid in the maintenance of bone metabolism. In particular, carotenoids such as α-carotene, β-carotene,

canthaxanthin, and lycopene have demonstrated beneficial effects on skeletal health; there is a clear positive association between lycopene intake and reduced bone loss in humans (Hunter et al., 2008). Recently, an inverse relationship between hip fracture risk and consumption of carotenoids from fruits and vegetables was reported in men aged 45–74 (Dai et al., 2014). Lycopene may suppress the formation of preosteoclasts from osteoprogenitor cells, thereby disrupting the osteoclast formation pathway (Pandey et al., 2018).

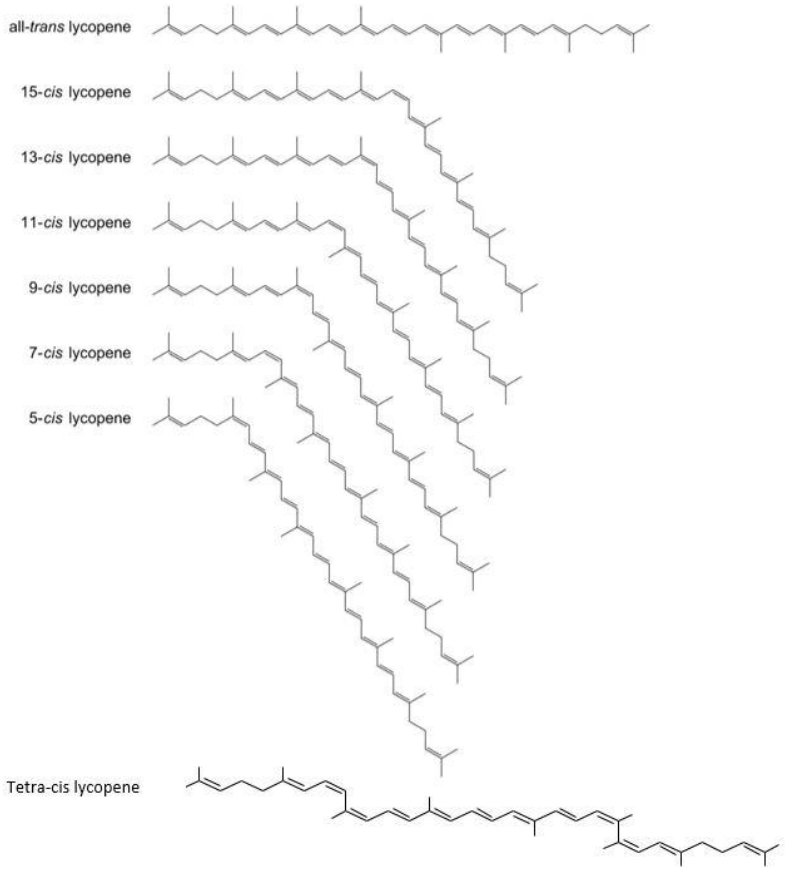

Figure 2.2 All-*trans*-lycopene and geometrical isomers .

2.2.2 Lycopene bioavailability, absorption, and metabolism

The bioavailability of ingested lycopene is dependent on the dose of lycopene consumed, linkages between molecules in the food matrix, incorporation of fats, level of dietary fiber, interactions of lycopene with other carotenoids, and genetic factors (Shi and Maguer, 2000, Castenmiller and West, 1998). Moreover, the bioavailability of lycopene differs depending on the isomeric form. In fact, *cis*-isomers of lycopene have been estimated to be 8.5 times more bioavailable than all-*trans*- lycopene (Cooperstone et al., 2015). The higher bioavailability of *cis*-isomers compared to all-*trans*- could be due to the former's increased solubility in mixed micelles (Unlu et al., 2007b). Compared to all-*trans*-, *cis*-isomers are less likely to crystallize, highly soluble in oil, preferentially micellarized, readily taken up by intestinal cells, and easily transported within cells as well as across plasma and tissue matrices (Cooperstone et al., 2015, Ishida et al., 2007). It is suggested that due to the polar nature and kinked forms of *cis*-isomers, they are less prone to crystallize (Meléndez-Martínez et al., 2014, Shi and Maguer, 2000). Moreover, their preferential solubility is likely due to the smaller chain length of *cis*-isomers based on the bending of their structures, which may not be found in *trans*-lycopene isomers (Boileau et al., 1999). Lastly, thermal processing and mixing with oil can further increase the bioavailability of *cis*-lycopene present in tomatoes (Shi and Maguer, 2000).

Being lipid-soluble, lycopene is absorbed into the body following the same pathway as fats. The foremost step of lycopene absorption is the breakdown of the food matrix and the release of carotenoids into the gastrointestinal lumen. Mechanical alteration of the food structure by cooking and other methods of food processing may improve carotenoid release from the food matrix (Wang, 2012). Lycopene then enters intestinal mucosal cells through the formation of bile acid micelles (Furr and Clark, 1997). However, bile production depends on the amount of fat present in the diet; therefore, it is necessary to incorporate fat with the lycopene-containing food in order to increase its solubility (Boileau et al., 2002). In general, lycopene absorption from dietary sources ranges from 10–30% (Rao and Rao, 2007) and, according to previous studies, a minimum of 5–10 g of fat in a meal is required to ensure better absorption of carotenoids (Anese et al., 2013). Carotenoid absorption by the enterocytes was assumed to be via passive diffusion based on previous research studies, however, recent studies suggested that carotenoid absorption could also occur via an active process which involves scavenger receptor class B type protein (SR-B1)(Lobo et al., 2010). SR-B1 can be found in the small

intestine and different organs including liver, adrenal glands, kidney and ovaries, thus partially responsible for the transportation of carotenoids between lipoproteins and tissues (Von Lintig, 2010). Once lycopene enters the enterocytes, it is either cleaved by β-carotene−9',10'-oxygenase (BCO2) to produce lycopenoids or incorporated into chylomicrons and secreted into lymphatic and blood circulation (Anese et al., 2013, Wang, 2012). Absorbed lycopene can either accumulate in the liver or be packed into VLDL and HDL and thereby re-enter the blood. Via blood circulation, lycopene is deposited in extrahepatic organs, mainly the adrenal glands, adipose tissue, prostate, and testes (Bramley, 2000). Non-absorbed lycopene and the excess metabolic products are excreted from the body in urine and feces (Figure 2.3).

Lycopene concentrations in body tissues are higher than those of other carotenoids (Boileau et al., 2002, Shi and Maguer, 2000, Rao et al., 1998). Half of the carotenoids in human serum are lycopene, and, among them, *cis*-isomers account for 58–73% of total lycopene in serum (Cooperstone et al., 2015, Marković et al., 2006, Schierle et al., 1997). In the human body, lycopene is present at 1 nmol/g in adipose tissues and is found in higher concentrations of up to 20 nmol/g in testes, adrenal, and prostate glands (Stahl and Sies, 1996). In addition, lycopene appears to have a long half-life; a recent study indicated that lycopene and its metabolites could be detected in the skin of humans up to 40 days after consumption (Ross et al., 2011).

The distribution of lycopene isomers is similar between plasma and tissues. Regardless of the isomeric forms of lycopene consumed, plasma and other tissues contain more *cis*-isomers, mainly the 5-*cis*-form (Boileau et al., 2002, Schierle et al., 1997). Lycopene metabolites, or lycopenoids, can be the products of lycopene metabolism and oxidation. Kopec et al**.** were the first to identify the series of lycopenoids present in human blood, but they are found in plasma in only negligible amounts (Kopec et al., 2010). Similarly, lycopene metabolites can be found in some foods, but at 1000-fold lower concentrations than that of lycopene itself (Kopec et al., 2010). Some studies suggest that metabolites of lycopene may play a role in the biological activities of lycopene. However, only a limited number of studies have investigated the role of lycopene metabolites *in vivo* (Cichon et al., 2018, Ip et al., 2013, Chung et al., 2012, Gajic et al., 2006, Sicilia et al., 2005).

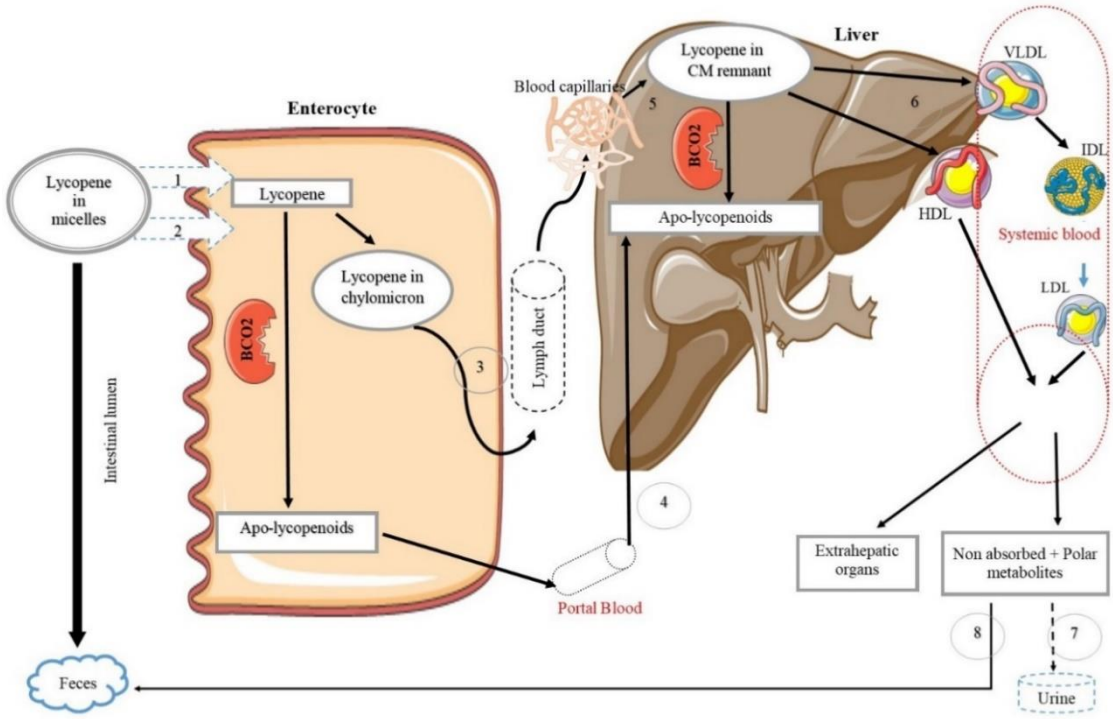

Figure 2.3 Simplified diagram of lycopene metabolism in the body (created using the reference from (Papachristodoulou et al., 2014, Anese et al., 2013, Wang, 2012, Boileau et al., 2002, Parker, 1996, Olson, 1994, Wang et al., 1992)).

Lycopene enters the enterocytes by an active process via scavenger receptor class B type protein (SR-B1) (1) and passive diffusion (2). There, it is packed in chylomicrons or converted to apo-lycopenoids by BCO2. Then, the chylomicrons or apo-lycopenoids are transferred to the liver via the lymphatic (3) and the portal venous (4) systems. Chylomicron remnants (CM) pass to the blood capillaries and are then absorbed by the liver via receptor-mediated endocytosis (5). Lycopene is packaged in very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL) by the liver and released to the systemic circulation (6). Lycopene travels to the extrahepatic organs through the systemic blood and is available there for its biological action. Polar metabolites are excreted in the urine by the kidneys (7), and non-absorbed lycopene is excreted through biliary excretion in feces (8).

2.2.3 Lycopene isomerization

2.2.3.1 *In vivo* isomerization of lycopene

There are many stages in the digestion, absorption and secretion processes where *in vivo* isomerization of lycopene could occur. The biology behind *in vivo* isomerization of lycopene is not yet fully understood. However, Chasse and co-workers identified structure and stability of the most common isomeric forms to be a major requirement for lycopene isomerization. They concluded that 5-*cis*- isomers of lycopene have the highest stability, followed by all-*trans*-,9-*cis*-,13-*cis*-,15-*cis*-,7-*cis*- and 11-*cis*- (Chasse et al.,

2001). Some *in vitro* studies indicate that *cis*- isomers are more easily taken up by mixed micelles in the intestine and hence are more bioavailable (Ross et al., 2011, Burri et al., 2009). The relatively greater levels of *cis*- lycopene found in body tissues could be due to both better absorption of *cis*- isomers into the body and the conversion of *trans*- to *cis*- isomers inside the body. One study suggested that the acidic environment in the stomach may be responsible for the production of *cis*- isomers (Re et al., 2001). However, Moraru and Lee simulated a gastric environment *in vitro* to investigate the stability of isolated lycopene from tomato-based sources when subjected to gastric pH. Their results showed that all-*trans*- lycopene has the highest stability at gastric pH. This indicates that pH dependent isomerization in the stomach is only partially responsible for the *cis*- lycopene content present in the body (Moraru and Lee, 2005). Another study was carried out giving rats lycopene micelles or lycopene chylomicrons prepared using a micro-emulsion technique. This revealed that lycopene micelles are more important than chylomicrons for the bioavailability of lycopene, as evinced by a greater maximum plasma concentration of lycopene in the rats given micelles (Chen et al., 2014).

Interestingly, another study revealed that the majority of *in vivo* lycopene isomerization occurs inside intestinal cells rather than in the stomach, duodenum or mixed micelles (Richelle et al., 2010). Another study reiterated that no *cis*-to-*trans*- lycopene isomerization occurs in the human stomach. However, the stomach is responsible for initiating the movement of food matrix to the lipid phase of the meal, and it has been argued that lycopene *cis*- isomers are of post-enterocyte origin since only a small amount of *cis*- isomers are secreted in the chylomicron (Tyssandier et al., 2003). Accelerated mass spectrometry was used to further study lycopene bioavailability and its metabolism in humans; the results indicated that isomerization of lycopene occurs after food ingestion and that isomers are rapidly metabolized. This suggests that at least a portion of absorbed lycopene is metabolized by β-oxidation into CO_2 as metabolites were found in urine (Ross et al., 2011).

Higher *cis*-, *trans*- and total lycopene contents of TRL fractions were found following the consumption of *cis*- isomer-rich sauce compared to *trans*- isomer-rich sauce (Unlu et al., 2007b). A broad distribution of *trans*- and *cis*- lycopene isomers may not occur with modest thermal treatment but incorporating oil and applying high temperatures may improve distribution. Therefore, it is likely that the primary lycopene isomer in most processed tomato products is all-*trans*-, but other isomers can be formed through

additional processing. For instance, one study found an increased amount of *cis*-isomers from 6% to 29% after heating tomato juice for 1 hr with 10% maize oil. Similarly, a 55% increase in *cis*-isomers was obtained after heating tomato juice paste at 100 ^{0}C for 30 min in the presence of 10% maize oil; a lesser effect was observed when 5% oil was used (Unlu et al., 2007b). In human subjects who ate tomato purée for two weeks, the isomeric composition of lycopene in plasma was predominantly *cis*-isomers (5- *cis*-, 13- *cis*- and 9- *cis*-) (Holloway et al., 2000). Tomato sauce enriched with *cis*-isomers had higher lycopene absorption as cis-isomers of lycopene are preferentially absorbed in to human body, but 5- *cis*- isomers were found in plasma TRL even though this isomer was not present in tomato sauce (Unlu et al., 2007a). Together, these findings further demonstrate *in vivo* isomerization of lycopene from *trans*- to *cis*-forms.

2.2.3.2 Isomerization during food processing

Isomerization and oxidation are the changes that affect lycopene during processing and storage (Shi and Maguer, 2000). Deliberate isomerization is a common practice in the food industry, used to produce nutraceuticals, pharmaceuticals, cosmetics and different food supplements. Different thermal methods (heating, microwaving) and non-thermal methods (irradiation, light and acid treatments) can cause *trans*- to *cis*-conversion of lycopene (Honest et al., 2011). However, no further *trans*- to *cis*-isomerization is reported to occur during storage; instead, retro-isomerization (*cis*-to-*trans*- isomerization) occurs (Honest et al., 2011, Shi and Maguer, 2000). Evidence suggests that standard home-based methods of cooking, heating, freezing and canning does not induce lycopene isomerization, although *trans*- to *cis*- isomerization of all-*trans*-β-carotene will occur under these conditions (Nguyen et al., 2001, Nguyen and Schwartz, 1998).

Lycopene isomerization is highly dependent on the time and intensity of heat processing: approximately 200^{0}C temperature for at least 45 minutes is needed to obtain successful *trans*- to *cis*- isomerization (Shi and Maguer, 2000, Nguyen and Schwartz, 1998), as lycopene loses stability at temperatures above 150^{0}C (Mayeaux et al., 2006). Another study showed that an initial temperature of 60 - 70^{0}C is needed to induce cell wall disruption in tomato products and to release lycopene from the matrix. Upon cell destruction, oxidation and enzymatic reactions can initiate lycopene release (Page et al., 2014). Both isomerization and degradation of lycopene occur simultaneously, but

temperature and light intensity may determine the rate of isomerization and degradation, as lycopene degradation is more rapid than isomerization at 100^0C and 150^0C (Lee and Chen, 2002). Thermal processing thus can increase the amount of *cis*-lycopene present in a tomato product, thereby increasing lycopene bioavailability, but it does decrease the amount of total lycopene present in the food (Shi and Maguer, 2000).

Tetra-*cis*- (5-*cis*-) lycopene, which is naturally present in fresh *tangerine* tomatoes (Cooperstone, 2012, Ishida et al., 2007), is thermodynamically more unstable and may be converted to other isomeric forms. Therefore, its presence increases the proportion of *cis*-lycopene isomers compared to all-*trans*- but may ultimately decrease total lycopene content. Hackett et al analysed the isomerization of lycopene in semi-solid, oil-based oleoresins made from various tomato varieties at different storage temperatures, and found that as the temperature increased, the amount of all-*trans*- isomers decreased and *cis*- isomers correspondingly increased. Oleoresin made from *tangerine* tomatoes showed the highest loss of tetra-*cis*- lycopene among the tomato varieties tested (Hackett et al., 2004). Lambelet *et al* developed a more stable tomato extract containing a high amount of *cis*-isomers which could be stored up to one year at room temperature without losing total lycopene content. This isomerized tomato oleoresin contained minimal 13-*cis*- lycopene, which is the most unstable form (Lambelet et al., 2009).

2.2.4 Lycopene intake and plasma levels

Lycopene is not known to have any adverse effects in humans, even when eaten at very high doses. Olmedilla *et al* reported that lycopene supplementation at 13.3 mg/day for 20 weeks, which was the longest period reported for a human lycopene intake study, did not cause any observable adverse or side effects (Olmedilla et al., 2002).The median lethal dose in rats for synthetic lycopene was 500 mg/kg BW. Therefore the World Health Organization (WHO) expert committee on food additives has established 50 mg/kg BW per day as the highest acceptable daily intake (ADI) for synthetic lycopene (Bend et al., 2007). Published survey data show that dietary intake of lycopene ranges from 1-10 mg/person/day (Bend et al., 2007). However, many dietary intervention studies have used daily doses up to 25-30 mg of lycopene. A study performed to investigate the effects of lower levels of lycopene showed that 5-10 mg of daily lycopene intake is adequate for the maintenance of average plasma lycopene levels and reduction of lipid peroxidation in adults (Rao and Shen, 2002). There is no significant difference in serum lycopene levels

in men versus women, although in women it can be affected by estrus cycle (Rao and Agarwal, 1999). Likewise, serum lycopene levels are not subject to seasonal variation in either men and women (Olmedilla et al., 1994). In contrast, serum levels of pro-vitamin A carotenoids such as β-carotene, α-carotene and β-cryptoxanthin do vary significantly by sex and season (Olmedilla et al., 1994). As shown in Table 2.2, lycopene intake differs widely by geographical region, with northern European countries having quite low intakes compared to their southern counterparts such as Italy. This is likely due to the fact that tomatoes play a major role in the Mediterranean diet, which is abundant with plant sources and seafood (Viuda-Martos et al., 2014).

Table 2.2 Daily lycopene intakes of selected countries.

Country	Lycopene Intake (mg/day)	Reference
USA	3.7-16.2 mg	(Rao and Rao, 2007)
	3.1 mg (postmenopausal women)	(Stahl and Sies, 1996)
New Zealand	1.2 mg	(Pohar et al., 2003)
UK	0.7 mg	(Rao and Rao, 2007)
	1.1 mg	(Rao et al., 2018)
Italy	7.4 mg	(Rao et al., 2018)
Australia	3.8 mg	(Rao et al., 2018)
Finland	0.7 mg/day for men	(Stahl and Sies, 1996)
	0.9 mg/day for women	
Croatia	3 mg	(Marković et al., 2006)

2.2.5 Tomatoes and lycopene

Tomatoes are a popular food in much of the world and have a high economic value (Raiola et al., 2014). In terms of consumption, tomato is the second most important vegetable crop after potato (Bergougnoux, 2014). Tomatoes were introduced to Europe by Spanish conquistadores in 1521 (Gentilcore, 2010). Tomato and tomato-based products are rich sources of lycopene, representing>80% of dietary sources containing lycopene (Shen et al., 2012, Mackinnon et al., 2011b). Watermelon, apricot, papaya and pink grapefruit are lesser sources of lycopene (Honest et al., 2011). Lycopene content in

tomatoes is influenced by growing conditions, predominately temperature and light intensity, with the most favourable temperature range for the formation of lycopene being 12-20^{0}C; lycopene production is inhibited when the fruit temperature exceeds 30^{0}C, and lycopene can be degraded by direct solar radiation (Brandt et al., 2006).

It is believed that the first tomatoes were orange in colour, as an Italian herbalist in 1544 described the tomato as a 'golden apple' which was green at first and become golden in colour with ripening (Smith, 2001). After the *tangerine* gene responsible for the golden colour and the predominantly *cis*- form of lycopene was discovered, the orange tomatoes were categorised as a mutant tomato (Giuliano et al., 2002) compared to the common red tomato. However, it is believed that the wild type tomatoes were originally orange *tangerine* tomatoes but with cross pollination the recessive *tangerine* gene disappeared (Trust, 2019). This is also supported by the evidence that the first tomato arrived to Italy was yellow in colour (Blancard, 2019). *Tangerine* tomatoes naturally contain *cis*-lycopene isomers, mainly in the tetra-*cis*- form, which is also known as prolycopene (Cooperstone et al., 2016, Unlu et al., 2007a). This isomeric form was first identified by Zechmeister and co-workers in 1941 using nuclear magnetic resonance (NMR) spectroscopy (Zechmeister et al., 1941).

Tetra-*cis*-isomers of lycopene show an unusual visible absorption spectrum where the maximum absorption is at 35 nm wavelength below the all-*trans*- isomer's maximum. *Tangerine* tomatoes predominately contain tetra-*cis*- isomeric form of lycopene due to presence of *tangerine* mutant in the carotenoid *cis*-*trans*- isomerase gene (Kachanovsky et al., 2012, Burri et al., 2009). Two alleles of *tangerine* have been identified so far, as *tangerine*^{3183} and *tangerine*^{mic}. *Tangerine*^{3183} accumulates similar amounts of prolycopene and pro-ζ-carotene, while *tangerine*^{mic} predominately produces pro-ζ-carotene (Giuliano et al., 2002). 'Moonglow', is an orange heirloom tomato, and a variety similar to *tangerine* tomatoes. It contains a relatively high amount of *cis*-lycopene isomers (McGhie, 2013) and thus could have several health benefits compared to conventional red tomatoes.

2.2.6 Animal models for carotenoid absorption studies

The selection of an optimal animal model for carotenoid research depends on several factors such as absorbance of different carotenoids at physiological levels similar to humans, the disease state of interest, and carotenoid distribution patterns in tissue and

serum. For example, gerbils and ferrets absorb β-carotene at rates similar to human but only gerbils convert β-carotene to vitamin A with similar efficacy as humans and therefore can be considered as a good model for β-carotene studies (Kim and Kim, 2011). Rats absorb carotenoids including β-carotene, canthaxanthin and lycopene and accumulate β-carotene in tissues in a dose dependant manner at supraphysiological level (≥0.02% of diet). In contrast, humans absorb small, physiological doses of different carotenoids (Lee et al., 1999). As rats efficiently convert β-carotene to vitamin A at the level of the enterocyte, they do not readily absorb carotenoids intact (Lee et al., 1999) thus cannot be considered as the best animal model for β-carotene studies. However, as lycopene is a non-provitamin A carotenoid, rats can be considered as a better animal model for lycopene absorption studies (Clark et al., 1998). Also, several rat studies have demonstrated that plasma lycopene concentrations in rats are similar to those observed in human trials (Boileau et al., 2002, Boileau et al., 2000, Ferreira et al., 2000). Finally, availability, manageability and affordability support the use of rats as the best model for carotenoid studies (Lee et al., 1999).

With respect to lycopene in particular, humans store more lycopene in adrenal and testes, whereas hepatic storage dominates in rats, and gerbils store 60 times and several thousand time more lycopene in the liver than in adrenals and testes respectively (Mills et al., 2007). The range of plasma lycopene is 290 nmol/L to 350 nmol/L in humans, and 226 nmol/L in ferrets supplemented with 15 mg/day lycopene, indicating similar absorption and accumulation to humans (Kim and Kim, 2011). Breed or strain can also affect lycopene metabolism: one study found significantly higher levels of serum lycopene in F344 rats fed a diet containing 0.5 g/kg lycopene for 10 weeks compared to Sprague-Dawley rats fed the same diet of lycopene for 18 weeks (Kim and Kim, 2011, Cohen, 2002). Clark et al found lymph cannulated male Holtzman albino rats a useful animal model for the absorption study of non-pro-vitamin A carotenoids. Both lycopene and canthaxanthin showed similar dose-dependent absorption rates, although canthaxanthin was more efficiently absorbed than lycopene. These carotenoids did not affect each other when they were ingested together (Clark et al., 1998).

2.2.7 Animal models for bone research

Pearce et al reviewed studies that investigated the suitability of animal models to test bone implant interactions. Researchers have used dogs, sheep, goats, pigs and rabbits

to examine microstructure, macrostructure, bone composition and bone remodelling. Dogs were found to have the most similar bone structure mimicking the human skeleton, while rabbits have the least similar (Pearce et al., 2007). The very short femoral neck, small amount of cancellous bone at the femoral neck, vertebral body and mandible make the rabbit an undesirable animal model for research on bone physiology. However, dogs are far more expensive to maintain, and there is less public support for the use of dogs in research. Rodents, particularly rats, are a better alternative for bone research (Bagi et al., 2011). Rats are considered an appropriate research animal to model human osteoporosis, although the rate and magnitude of bone loss depends on the method used to induce osteoporosis. Further, it depends on the skeletal site and whether bone loss is measured in cancellous versus cortical bone (Lelovas et al., 2008). Ovariectomized (OVX) rats fed a low calcium diet demonstrate the most rapid bone loss. This model is considered a gold standard for the evaluation of drugs for the treatment of osteoporosis. OVX model is recommended for research in the pathophysiology and therapeutic efficacy of treatments (Gao et al., 2014).

A weakness of this model is the fact that the indices of cancellous bone turnover do eventually return to the value of sham controls. Sham-operated rats, which undergo a matching anaesthesia and surgical procedure but are left with their ovaries intact, are normally used in OVX studies as a control group. However, Kruger and Morel have found no difference between BMD and BMC in sham-operated versus non-manipulated rats over a 2-6 month period. These findings suggest that it is scientifically valid to use unmanipulated rats as controls, a choice which reduces the ethical impact on the animals, unless a true sham-operated group is required by a regulatory body such as the USA Food and Drug Administration for pre-clinical trials (Kruger and Morel, 2016). Moreover, the effect of increased skeletal size on cancellous and cortical bones is less predictable in aged post-OVX rats. Therefore, studies with OVX rat models for the evaluation of therapeutic agents should be restricted to a duration of less than 12 months and probably not more than 6 months. As the responsiveness of growing rats to OVX is higher than that of aged rats, the OVX model to assess bone turnover in cancellous bones should not be initiated in rats >18 months old. Overall, the OVX rat model is ideal for examining postmenopausal cancellous bone loss and evaluating potential therapeutic agents for the prevention of osteoporosis over a short period of time (Thompson et al., 1995).

2.2.8 Evidence of the effect of lycopene on bone health

Recently, the effect of lycopene on bone health has received additional attention from researchers (Eggersdorfer and Wyss, 2018). The beneficial effects of lycopene on bone health have been studied using animal models, cell cultures, and epidemiological/clinical studies, as described below and in Tables 2.3–2.5, in the context of postmenopausal osteoporosis.

2.2.8.1 Epidemiological and clinical studies

Human epidemiological studies have investigated the specific effects of tomato/lycopene on bone health, and the majority have shown a positive correlation between tomato/lycopene consumption and the prevention of bone loss, with minimal beneficial doses varying from 3.5 to 30 mg/d depending on the observed markers (Table 2.3). The Framingham Osteoporosis study evaluated associations between total and isolated carotenoids with BMD in older adults (~75 years old) (Sahni et al., 2009b). An inverse relationship between lycopene levels and four-year bone loss in the lumbar spine in older women (~75 years old) was observed (Sahni et al., 2009b), and a follow-up study reported a protective effect of lycopene against hip fractures (Sahni et al., 2009a). Mackinnon et al. reported a notable increase in a clinically relevant bone resorption marker, the crosslinked N-telopeptide of type 1 bone biomarker (NTX), as well as oxidative stress markers in postmenopausal women after one-month restriction of lycopene in the diet (Mackinnon et al., 2011b). This also led to a drastic reduction in serum lycopene along with other carotenoids such as α-carotene, β-carotene, lutein, and zeaxanthin (Mackinnon et al., 2011b). Similarly, 30 mg/d lycopene supplementation in postmenopausal women in either juice or capsule form for four months decreased serum NTX level (Mackinnon et al., 2011a). An epidemiological study in premenopausal women, which evaluated the effect of dietary carotenoids on bone mineral status, showed a positive correlation between lycopene intake and total body BMC and BMD (Wattanapenpaiboon et al., 2003). Another study revealed lower levels of serum lycopene in postmenopausal women with osteoporosis compared to non-osteoporotic women (Yang et al., 2008).

Table 2.3 The effect of lycopene on postmenopausal bone loss based on human trials.

Author and Year	Cohort	Lycopene Formulation and Study Duration	Outcome
(Russo et al., 2020)	Postmenopausal women (n = 39) Age: 63 ± 7 years	3.9 mg/day as tomato sauce 3 months	Patients who consumed tomato sauce did not showed a significant loss of BMD compared to control group
(Mackinnon et al., 2011a)	Postmenopausal women (n = 60) Age: 50–60 years	30 mg/day (regular tomato juice), 70 mg/day (lycopene-rich tomato juice), 30 mg/day (Lyc-O-Mato capsules) 4 months	Lycopene intervention in capsule or juice form supplying at least 30 mg/day led to decreased oxidative stress and bone resorption markers
(Mackinnon, 2010)	Postmenopausal women (n = 45) Age: 55 years	43.33 mg/day supplementation 4 months	Lycopene supplemented group showed significantly lower levels of bone resorption marker (NTX)
(Mackinnon et al., 2011b)	Postmenopausal women (n = 23) Age: 50–60 years	Lycopene intake at baseline and after one month of lycopene restriction was 3.5 mg/d and 0.13 mg/d, respectively (using 7-day dietary records)	Bone resorption marker (NTX) was increased after a month of lycopene restriction Endogenous antioxidant enzymes (SOD and catalase) were decreased after a month of lycopene restriction
(Rao et al., 2007)	Postmenopausal women (n = 33) Age: 50–60 years	Lycopene intake categorized into four groups as ranged from 1.76 to 7.35 mg/day (using 7-day dietary records)	Serum NTX values dose-dependently decreased Postmenopausal women who consumed 7.35 mg lycopene/day had lower serum NTX compared to the other three groups No difference in bone formation markers

2.2.8.2 Animal trials

Ovariectomy (OVX) is the most widely used surgical technique for the induction of osteoporosis in rodents and other animal models to mimic the hormonal and skeletal status of postmenopausal women (Calciolari et al., 2017). Along with ovariectomy, most trials incorporate “sham”-operated animals as controls, which undergo surgery without the ovaries being removed (Kruger and Morel, 2016). Female rats are considered an excellent animal model for postmenopausal osteoporosis, but the age of the animals and site selection for harvesting the bones must be defined with care. Other experimental protocols related to bone loss have used interventions such as hormonal, dietary, and immobilization in rats, which had more variable effects on the rate of bone loss (Lelovas et al., 2008). Ovariectomized rats demonstrated rapid bone loss, supporting the use of this model as a gold standard for the evaluation of drugs for the treatment of osteoporosis (Ozsahin et al., 2017, Gao et al., 2014). Higher serum levels of osteocalcin, a biomarker of bone turnover, were measured in ovariectomized rats compared to sham-operated rats (Shetty et al., 2016, Gao et al., 2014). Ovariectomized rats are considered most suitable for the evaluation of preventative agents for postmenopausal osteoporosis, although not for evaluation of treatment of osteoporosis over a long period of time (Kruger and Morel, 2016, Thompson et al., 1995). This is because the rate of bone turnover in OVX rats become similar to their sham counterparts in studies >12 months, and also because the exact parameters for expected increases in the skeletal size of trabecular and cortical bone in the rat with long term OVX are not yet established (Thompson et al., 1995).

Lycopene has been shown in multiple studies to dose-dependently increase BMD in ovariectomized rats (Liang et al., 2012). Similarly, lycopene reduced bone fragility in ovariectomized rats and improved femoral bone energy, as assessed using a mechanical breaking test (Iimura et al., 2015). More recently, lycopene supplementation in ovariectomized rats was found to significantly alter levels of biomarkers of bone turnover in blood and urine, reducing bone resorption and increasing osteoblast activity. Simultaneously, lycopene treatment increased the enzyme action of glutathione peroxidase, catalase, and superoxide dismutase, and downregulated oxidative stress (Ardawi et al., 2016). Taken together, these animal studies suggest that lycopene has bone-protective benefits when supplied at at least 10 mg/kg BW (Table 2.4-Dose conversion from ppm to mg/kg BW was done according to Table 7.12 in Toxicological risk assessment of chemicals: A practical guide (Nielsen et al., 2008)).

Table 2.4 The effects of lycopene on postmenopausal bone loss based on rodent trials.

Author and Year	Animal Strain	Lycopene dose and Study Duration	Outcome
(Oliveira et al., 2019)	Female Wistar rats	10 mg/kg BW/day 4 weeks pre-OVX and 8 weeks post-OVX	Decreased bone loss in femur epiphysis in the OVX + lycopene group compared to the OVX control group
(Li et al., 2018)	Female Sprague-Dawley rats	50 mg/kg BW/day 12 weeks	Higher bone volume and trabecular thickness with low trabecular spaces in the OVX + lycopene group compared to the OVX control group Increased bone contact and bone area around the implant were in the lycopene-treated group compared to controls
(Ardawi et al., 2016)	Female Wistar rats	15, 30, 45 mg/kg BW/ day 12 weeks	Lycopene treatment dose-dependently enhanced BMD and BMC at the lumbar spine and humerus compared to OVX control group Lycopene (30 and 45 mg/kg BW) increased bone formation markers (serum-OC and serum PINP) while bone resorption markers (serum-CTx-1 and urine-DPD) were decreased
(Iimura et al., 2015)	Female Sprague-Dawley 6-week-old	0, 5, 10, 20 mg/kg BW/day 9 weeks	Lycopene (10 mg/kg BW) increased lumbar spine BMD and femoral-breaking force compared to OVX control group Bone resorption markers were low in all lycopene-treated groups
(Iimura et al., 2014)	Female Sprague–Dawley 6-week-old	0, 5, 10, 20 mg/kg BW/day 9 weeks	Lycopene (10 mg/kg BW) increased BMD of the lumbar spine and the tibial proximal metaphysis
(Liang et al., 2012)	Female Wistar rats 8 week old	20, 30, 40 mg/kg BW/day 8 weeks	Lycopene (30 and 40 mg/kg BW) dose-dependently increased BMD and BMC in OVX rats compared to OVX control group

2.2.8.3 Bone cell culture studies

Multiple *in vitro* studies have demonstrated that lycopene and other carotenoids directly affect both osteoclasts and osteoblasts (Table 2.5). Ishimi and coworkers reported that osteoclast-like cell formation induced by 1α, 25(OH) 2 D3 (Calcitriol), IL-1β, and parathyroid hormone was inhibited by retinoic acid and carotenoids, including β -carotene, canthaxanthin, and lycopene, with retinoic acid being most effective (Ishimi et al., 1999). Park et al**.,** also found retinoic acid to have the highest activity in inducing differentiation of the osteoblastic cell line MC3T3-E1, with retinol, β -carotene, lycopene, and canthaxanthin showing lesser but similar effectiveness (Park et al., 1997). Lycopene inhibited osteoclast formation and bone resorption by rat bone marrow cells in a model of parathyroid hormone-induced osteoclastogenesis (Rao et al., 2003). More recently, human osteoclast and osteoblast precursor cells, treated with 500 nmol/L lycopene for 21 days, demonstrated reduced osteoclastogenesis while increasing osteoblastogenesis (Costa-Rodrigues et al., 2018). Lack of nuclear factor kappa B (NFκB) may lead to a reduction in osteoclast precursors differentiating into mature osteoclasts, thereby reducing bone loss. Interestingly, some studies suggest that derivatives of carotenoids can downregulate the activity of NFκB activity, a regulator of cytokine expression (Linnewiel-Hermoni et al., 2014, Kini and Nandeesh, 2012, Boyce et al., 2010), suggesting NFκB modulation is a candidate pathway for lycopene's protective effects against osteoporosis. Taken together, evidence from *in vitro* studies suggest that lycopene in the range of 1-10 μmol/L has beneficial effects on bone health via molecular mechanisms that are summarized in Figure 2.4.

Table 2.5 The effects of lycopene on bone cells (osteoblasts and osteoclasts).

Author and Year	Cell Line	Lycopene Concentration	Outcome
(Russo et al., 2020)	Human osteoblast-like cell line Saos-2	5 and 10 µmol/L	Lycopene suppressed RANKL expression indicating the reduction of bone resorption Lycopene reduced the stimulatory effect of ALP within 24 h indicating possible role in mineralization
(Oliveira et al., 2019)	Osteoblastic cells from femur medullary canals of ovariectomized female rats	1 µmol/L	Lycopene upregulated the genes associated with bone metabolism of osteoblastic cells within 3–10 days
(Costa-Rodrigues et al., 2018)	Osteoblastic cells (human mesenchymal stem cells bone-marrow-derived) Osteoclastic cells (human peripheral blood mononuclear cells)	5 nmol/L −50 µmol/L	Lycopene (≥500 nmol/L) increased osteoblastic cell proliferation and differentiation Lycopene (≥500 nmol/L) significantly decreased osteoclast differentiation
(Marcotorchino et al., 2012)	RAW 264.7 cells	0.5, 1, 2 µmol/L	Lycopene dose-dependently reduced the lipopolysaccharides (LPS) mediated activation of inflammatory cytokine (TNF-α) produced by macrophages
(Feng et al., 2010)	RAW 264.7 cells	1–10 µmol/L	Lycopene dose-dependently inhibited the increase of nitric oxide production and the secretion of IL-6 when RAW cells were stimulated by LPS
(De Stefano et al., 2007)	RAW 264.7 cells	5, 10, 20 µmol/L	Lycopene (20 µmol/L) significantly inhibited the ROS accumulated due to addition of gliadin Lycopene (20 µmol/L) significantly inhibited increase in nitric oxide synthase levels
(Rao et al., 2003)	Osteoclast were generated from bone marrow cells	0.01, 0.1, 1, 10 µmol/L	Lycopene (10 µmol/L) significantly inhibited PTH stimulated resorption by osteoclasts

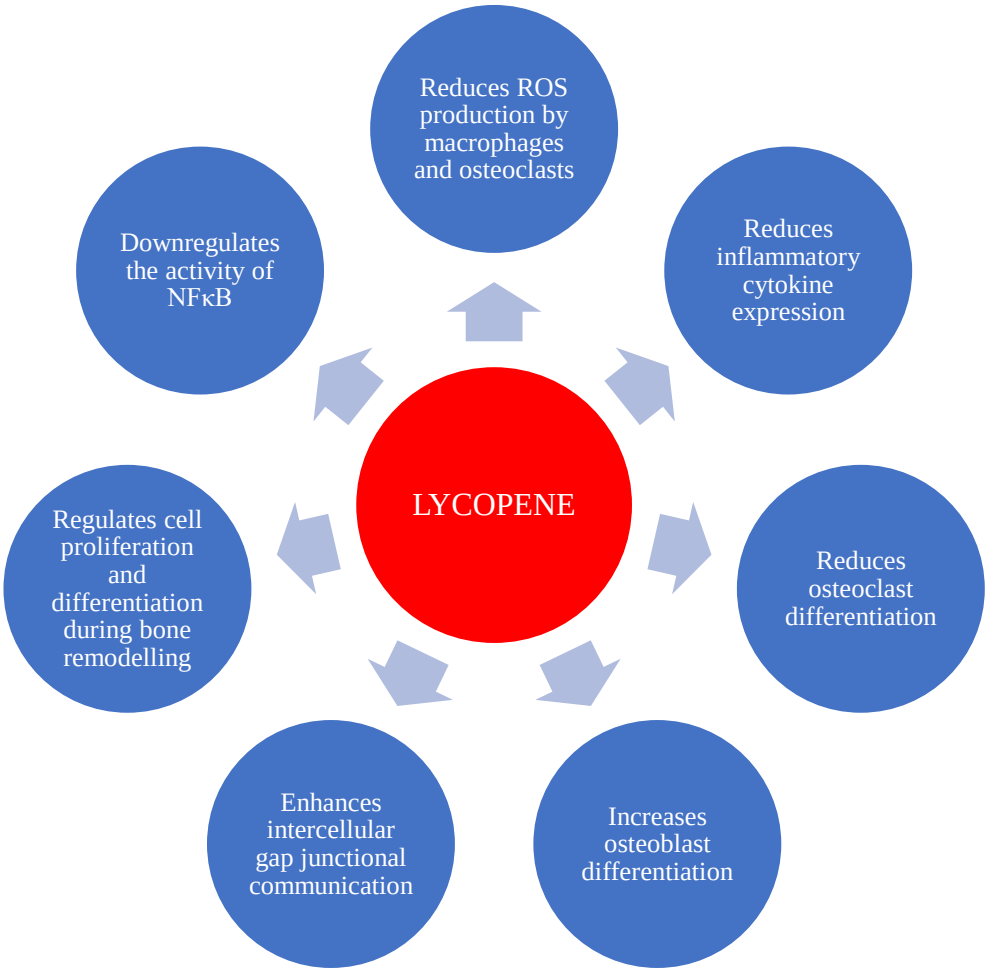

Figure 2.4 Schematic representation of the potential effects of lycopene on bone cells (created using the reference from (Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014)).

2.2.9 Gut microbiota and prebiotic-like effect of tomatoes

The human large intestine contains up to a thousand different bacterial species, which can vary due to delays in transit time, nutrient availability and a favorable pH for the growth of bacteria (Cummings and Macfarlane, 1991). Gut bacteria, acting in tandem with the gut immune system, form a barrier to prevent pathogenic bacteria from colonizing or invading the gut tissue (Slavin, 2013). Gut microbiota therefore maintain a symbiotic relationship with the host (Kazimieras Malys et al., 2015). The composition of gut microbiota varies during the periods from youth through adulthood (Radjabzadeh et al., 2020). A study in healthy rats found that the gut microbiota changes over time, passing through three distinct profiles during the periods of pre-weaning, year one, and year two, with the final period demonstrating the greatest bacterial diversity (Flemer et al., 2017).

Probiotics are live bacteria that can deliver a health benefit to the host when present in adequate numbers (Fijan, 2014). Probiotic bacteria confer several advantages in humans. They maintain the balance between pathogens and the beneficial bacteria in the body (Oelschlaeger, 2010). Importantly, probiotic bacteria can protect the host from activities of pathogenic bacteria that may enter the gut with contaminated food. Their beneficial effects are applicable to a variety of health conditions including digestive disorders, food allergies, candidiasis and dental caries (Oelschlaeger, 2010, Marković et al., 2006). Probiotics are also used to restore the gut microbiota after antibiotic treatment. Their mode of action can be divided into four main categories: production of antimicrobial substances; immune modulation of the host; inhibition of bacterial toxin production; and blocking the adhesion pathogens to the epithelium for nutrient uptake (Markowiak and Śliżewska, 2017). Mouse and rat models are widely used to evaluate the interaction between host and gut microbes (Nagpal et al., 2018). A study demonstrated that rats have more similar gut microbiota profiles to humans, compared to mice (Flemer et al., 2017).

Prebiotics are food ingredients that induce the growth and/or activity of probiotic bacteria and thus are indirectly beneficial for host health (Slavin, 2013). Ideally they should be chemically stable under different food processing conditions such as heat treatment, low pH levels and Maillard reactions (Wang, 2009). Only a limited number of food components are classified as prebiotics (Wang, 2009). The most important criterion shared across prebiotics is that they are not able to be digested in the upper gastrointestinal tract (GIT). And as a result, prebiotics enter into the colon intact, where colonic probiotic bacteria selectively ferment the prebiotics; this in turn can change the composition and activity of the microbial population in the gut (Macfarlane, 2008). Fermentation increases the production of small chain fatty acids, particularly acetate, butyrate and propionate while decreasing the colonic pH, nitrous end products and fecal enzymes.

Tomatoes are a good source of dietary fiber, which acts as a prebiotic (Koh et al., 2010) and provides a favorable environment for the growth of beneficial gut microbiota (Periago et al., 2016). However, the pre-biotic-like potential of tomatoes is primarily depend on their phenolic content rather than the fiber (García-Alonso et al., 2017). Specifically, lycopene, which is abundant in tomatoes, has demonstrated a prebiotic-like effect through increasing the abundance of *Lactobacillus* and *Bifidobacterium* species in human gut (Wiese et al., 2019). Supplementation of tomato powder to mice also increased

Lactobacillus and *Bifidobacterium* as well as other beneficial bacteria (Xia et al., 2018). In another study, supplementation with the carotenoid fucoxanthin, a carotenoid, similarly increased the abundance of *Lactobacillus* and *Bifidobacterium* (Sun et al., 2020).

In conclusion, lycopene has a protective effect against bone loss; this has been demonstrated in *in vitro* studies, in animal models of osteoporosis, and in human clinical studies. Hence, the consumption of tomatoes can be considered a valid dietary approach in the management and prevention of bone loss. In addition, lycopene from tomato or other sources has also demonstrated a prebiotic-like effect on gut microbiota. Therefore, consumption of tomatoes can also be considered a safe and economical approach for the management of gut health as well as bone health.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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- Describe the contribution that the candidate has made to the manuscript/published work:

Involved in planning and designing of animal trials, uHPLC analysis and data analysis,compiled and interpreted the results, reviewed and co-edited.

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

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CHAPTER 3

Absorption, tissue distribution, and excretion of 'Moonglow' tomato lycopene (*in vivo*)

Abstract

Lycopene in red tomatoes is primarily found in its all-*trans*-isomeric form, which tends to occur in a less-bioavailable crystalline state. It is calculated that an adult human would need to consume unreasonably high amounts of red tomatoes (ca. 1.5 kg per day) to achieve physiologically effective plasma lycopene concentrations (>0.45 μmol/L). *Cis*-isomers of lycopene have been reported to be more bioavailable than all-*trans*-lycopene. 'Moonglow', an orange heirloom tomato, contains a relatively high amount of *cis*-lycopene isomers. We hypothesized that consumption of small daily doses of raw 'Moonglow' tomatoes could produce physiologically relevant levels of plasma and liver lycopene in a rat model. Female Sprague-Dawley rats (8 weeks old; n=3 per test group) were supplemented with 'Moonglow' tomato powder. In the first study, rats housed in metabolic cages were given lycopene at 0, 0.05 (low), 0.35 (mid), or 2.6 (high) mg/kg body weight (BW) once daily for five days, after which plasma, liver, urine and feces were collected. In the second study, rats housed in conventional shoe-box cages were supplemented with the lycopene mid dose (0.35 mg/kg BW) once daily, and plasma and liver collected after 1, 2, 3, 4 or 5 days. Plasma, liver, feces and urine samples were analysed for total and *cis/trans*-isomers of lycopene by ultra-high-performance liquid chromatography. Both plasma and liver lycopene concentrations were increased with dose and over time. After five days of feeding, the average total lycopene concentrations for low, mid and high doses were 0.06, 0.60, 1.87 μmol/L in plasma and 1.59, 15.6, 121.9 μmol/kg in liver, respectively. Plasma concentrations of lycopene reached 0.42 μmol/L after four days of feeding, which is similar to the reported physiological beneficial concentration in humans. On average 48-58% of the lycopene ingested was apparently in the body regardless of dose. These results suggest that the consumption of 'Moonglow' to achieve an effective plasma concentration could be achieved with a reasonable daily intake of fresh 'Moonglow' tomatoes.

3.1 Introduction

Lycopene is a lipid soluble carotenoid produced by plants and some micro-organisms (Viuda-Martos et al., 2014, Rao and Ali, 2007). The molecule exists in all-*trans*- and a variety of *cis*-configurations in nature (Richelle et al., 2012). However, *cis*-lycopene isomers have been reported to be more bioavailable than all-*trans*-lycopene (Srivastava and Srivastava, 2015). Lycopene in raw red tomatoes predominantly exists in all-*trans*- configurations. Further, lycopene in red tomatoes exists in large crystalline aggregates showing poor solubility in micelles during absorption (Cooperstone et al., 2015). Compared to all-*trans*-, *cis*-lycopene is dissolved in lipid droplets, rather than in crystalline form, and is thus highly bioavailable (Unlu et al., 2007b, Ishida et al., 2007). Lastly, *cis*- isomers of lycopene possess smaller chain lengths and an overall chemical structure that facilitate its solubility. *Cis*-isomers of lycopene are estimated to be 8.5 times more bioavailable than all-*trans*-lycopene (Cooperstone et al., 2015, Unlu et al., 2007b).

Recent clinical and animal studies have found an inverse relationship between dietary intake of lycopene and the progression of certain non-communicable diseases including cancer (Fraser et al., 2020), hypertension, cardiovascular diseases (Zeng et al., 2019, Ferreira-Santos et al., 2018) and bone loss (Iimura et al., 2015, Iimura et al., 2014). Many dietary intervention studies in humans have used daily doses up to 25-30 mg of lycopene as a physiologically relevant dose for the reduction of certain non-communicable disorders (Mackinnon et al., 2011a, Ried and Fakler, 2011, Grainger et al., 2008, Bowen et al., 2002). However, in Western countries, daily lycopene intake by adults has been reported to range from 1.1 – 10.5 mg per day (Story et al., 2010). Based on the reported levels of lycopene in red tomatoes and the percent absorption in the body, a daily dose of 25 mg lycopene would equate to an adult eating 1.5 kg of fresh red tomatoes or 600 grams of tomato sauce (ketchup) daily (Story et al., 2010, Rao and Rao, 2007, Rao and Shen, 2002, Shi and Maguer, 2000), neither of which would be easily achievable. This requirement is largely due to the poor absorption of lycopene in the body (Anese et al., 2013).

Orange heirloom tomatoes naturally contain *cis*-lycopene isomers, mainly in tetra-*cis*- form, which is also known as prolycopene (Cooperstone et al., 2016, Unlu et al., 2007a). This isomeric form was first identified by Zechmeister and colleagues in 1941 using Nuclear Magnetic Resonance (NMR) spectroscopy (Zechmeister et al., 1941).

Tetra-*cis*-isomers of lycopene show an unusual visible absorption spectrum with a maximal absorption wavelength 35 nm below that of the all-*trans*-isomers (Cooperstone et al., 2015). Almost 90% of lycopene in red tomatoes is present in all-*trans*-isomeric form, whereas 90% of lycopene in orange heirloom tomatoes is in a *cis*- form (Burri et al., 2009). This is due to a mutation known as *tangerine* in the carotenoid *cis*-*trans*-isomerase gene (Kachanovsky et al., 2012), which gives a deep orange colour to these tomatoes (Cooperstone et al., 2016, Englert et al., 1979). As the *tangerine* mutant lacks the ability to convert poly-*cis*-lycopene into all-*trans*-, orange heirloom tomatoes predominantly accumulate tetra-*cis*-lycopene along with other precursors of lycopene such as phytoene, phytofluene, ζ-carotene and neurosporene (Cooperstone et al., 2016) (Appendix i, Figure 1).

The bioavailability of lycopene is defined as the amount absorbed by enterocytes, incorporated into the blood circulation, and made available for tissue uptake; this can also be expressed as "absorption efficiency" (Gustafson and Bradshaw-Pierce, 2011, Toutain and Bousquet-Mélou, 2004, Parker et al., 1999). It is partly dependent on the amount of lycopene that is expected to be released from the food format in which it is present, which is termed bio-accessibility (Anese et al., 2013). Bioavailability of ingested lycopene is dependent on the dose of lycopene consumed, linkages between molecules in the food matrix, incorporation of fats, level of dietary fiber, interactions of lycopene with other carotenoids, and genetic factors (Shi and Maguer, 2000, Castenmiller and West, 1998). However, the fate of lycopene inside the body is not fully understood, thus this is an area which is actively being researched, employing a number of methodologies. The mass balance method is used to compare lycopene intake and excretion and to determine absorption kinetics of lycopene. Moreover, time-course and dose-dependent studies are important to identify optimal doses and delivery methods, and to predict possible physiological effects of the compound (Bassingthwaighte et al., 2012).

However, rat studies have only been conducted using lycopene from conventional red tomatoes or synthetic lycopene in all *trans*-isomeric form. The comparative bioavailability of *cis*- isomers in rats has not been reported. Therefore, the main objectives of the present study were to identify the daily dose of lycopene from 'Moonglow' tomatoes and the time period required to achieve a physiologically relevant level of lycopene in the plasma and to determine whether the *cis*- and *trans*-isomer ratios were altered after digestion, absorption and metabolism in rats.

3.2 Materials and methods

3.2.1 Tomatoes

Fresh ripe 'Moonglow' tomatoes were provided by Mark Christensen, Heritage Food Crops Research Trust, Whanganui, New Zealand. Upon receipt, fresh tomatoes were cut into medium sized pieces and stored at -20^{0}C prior to freeze drying. Frozen tomatoes were freeze-dried (Cuddon FD18, Blenheim, New Zealand) at 1.8 mbar for 3 days. Temperature was held at -30^{0}C for 1 hour and then increased to +20^{0}C over 2 hours. Then the temperature was held at +20^{0}C approximately 3 days until dry. Tomatoes were ground using a food processor (Kenwood 800W, Havant, UK) into a fine powder with pulse mode on. Tomato powder was sieved using a stainless-steel sieve (mesh no. 200). The coarse particles retained after sieving were ground into fine powder using a Breville coffee grinder. Batches of 'Moonglow' freeze-dried tomato powder were separately packed in zip lock bags with inert nitrogen gas and again covered with aluminium resealable bags to protect from the light and kept at -20^{0}C until use.

3.2.2 Chemicals

All the chemicals used in this study were of analytical grade unless otherwise stated. Ethanol was purchased from Thermofisher Auckland, NZ and hexane, acetone, toluene, butylated hydroxytoluene, and potassium hydroxide pellets were purchased from Sigma-Aldrich® Auckland, NZ. For HPLC analysis, HPLC grade methanol, 2-propanol, methyl tetra-butyl ether, acetone, ethanol, and tetrahydrofuran were purchased from Thermofisher Auckland, NZ. All-*trans*-lycopene, β-carotene and lutein standards were purchased from Extrasynthase, Geney Cedex, France.

3.2.3 Lycopene dose

Three different doses of 'Moonglow' tomato lycopene were calculated using the conversion factor from human to rat equivalent dose based on metabolic body weight (Reagan-Shaw et al., 2008) and using reported bioavailability of *cis*-lycopene being 8.5 times greater than all-*trans*- (Cooperstone et al., 2015) (Appendix i, Table 2). 'Moonglow' tomato powder in the appropriate dose was mixed daily with peanut butter (Sanitarium; New World, Palmerston North, NZ) and raw honey (provided by Horowhenua apiary H5206) to increase palatability and to form a solid treat that could be easily given to the rats for voluntary consumption. The control group received peanut

butter and honey without tomato powder. Rats were trained to eat control treats for two days prior to the tomato treats.

Table 3.1 Ingredient ratio of different lycopene doses[1].

Ingredient (per rat), mg	Lycopene dose (mg/kg BW)			
	Control (0)	Low (0.05)	Mid (0.35)	High (2.6)
'Moonglow' tomato powder	0	25	175	1,301
Peanut butter + honey (2:1)	600	575	625	699
Total gram of a treat mixture per day	0.6	0.6	0.8	2

[1] Ingredients were mixed daily to prepare the appropriate dose in mg/kg BW of rats. The amount of peanut butter + honey increased with increasing tomato powder to make it more palatable to rats and to ensure even mixing of tomato powder.

3.2.4 Animals and diet

Twenty-seven 4 week old female Sprague-Dawley rats were obtained from Animal Resource Centre, Australia. Following importation, rats were quarantined for one month at the Small Animal Production Unit (SAPU), Massey University (Palmerston North, New Zealand) according to the requirements of the Ministry of Primary Industries (MPI, New Zealand). Rats were weighed and transferred to clean standard plastic shoe-box cages with wood bedding upon arrival. Temperature and humidity were set at 22 ^{0}C and 45%-50%, respectively with a 12-12 hours light-dark cycle. All rats had *ad libitum* access to standard chow pellets and distilled water. Rats were monitored for their health and behaviours. Ethical approval for this study was obtained from Massey University Animal Ethic Committee (Protocol No: 18/40). A power analysis was carried out using PS (Power and Sample Size; Vanderbilt) based on published data for lycopene bioavailability studies for different species (Faisal et al., 2010, Mills et al., 2007, Sicilia et al., 2005). A sample size of 3-4 was necessary for a power of 0.8 to detect a significant difference between groups when p=0.05. Therefore, we selected n=3 rats per group and we assessed three doses at a single time point, plus one dose at six time points; so we needed a total of 27 rats. At the end of the quarantine period 12 rats were individually housed in separate metabolic cages for study #1 (dose-response) and 15 rats were

individually housed in shoe-box cages for study #2 (time-course). Rats were given a two-day adaptation period in their cages with *ad libitum* chow diet and water. Following this period the 'Moonglow' tomato feeding was initiated.

3.2.5 Animal trial 1 (dose-response)

Twelve (n=12) 8 week old female Sprague-Dawley rats were randomly assigned into four experimental groups (n=3). Each rat was housed separately in a metabolic cage to facilitate a daily collection of urine and feces, which were weighed, placed into labelled tubes and stored at -20^{0}C until analysis. Four different groups were fed treats with 'Moonglow' tomato powder containing total lycopene at a concentration of 0 (no dose), 0.05 (low dose), 0.35 (mid dose) and 2.6 (high dose) mg/kg BW over five days (Table 3.1). Rats received one treat per day on day 1, 2, 3, 4 and 5 between 9 a.m. to 10 a.m. and feces and urine of the previous day were collected between 9 a.m. and 10 a.m. on the following day. On day six, all rats were deeply anaesthetized between 9 a.m. to 11 a.m. with an intraperitoneal injection of 0.12 mL/100 g BW of acepromazine (2 mg/mL): ketamine (100 mg/mL): xylazine (10%): and sterile water = 2:5:1:2. Blood was collected from each rat by cardiac puncture into heparin sulphate-containing vacutainers. Euthanasia from exsanguination was confirmed with pneumothorax and the livers dissected, weighed, snap frozen and kept at -80^{0}C until further analysis. Blood samples were centrifuged at 3,500 *g* for 10 minutes at 20^{0}C (Allegra 64R, Beckman Coulter, NSW, Australia) to obtain the plasma.

3.2.6 Animal trial 2 (time-course)

Fifteen (n=15) 8 week old female Sprague-Dawley rats were housed separately in shoe-box cages and fed with lycopene mid dose (0.35 mg/kg BW) once daily as above. At selected time points (n=3 rats per time point) animals were euthanized, and blood and livers were collected and processed as above. Rats were acclimatized for two weeks prior to the experimental feeding period.

3.2.7 Analysis of carotenoids

'Moonglow' tomato, rat plasma, liver and excreta were analysed for carotenoid composition using ultra high-performance liquid chromatography-diode array detection (uHPLC-DAD).

3.2.7.1 Extraction of carotenoids from tomatoes

Carotenoids were extracted under minimum light, using the method previously described (Brown et al., 2004). Briefly, 5 g of tomato powder was added to a 50 mL plastic test tube. This was mixed with a mixture of 500 mg Na_2CO_3, 5 g anhydrous Na_2SO_4 with 20 mL of tetrahydrofuran (THF): methanol (2:1). Samples were shaken in genogrinder (2010 Geno/Grinder) at 1,750 rpm for 4 min. After 60 min of incubation in the dark, samples were centrifuged at 1,620 *g* for 10 min (Eppendorf centrifuge 5804, with rotor A-4) and the supernatant was collected into a 50 mL glass bottle. The extraction process was repeated on the remaining solid fraction and the supernatants were pooled together and the total volume was recorded. Extracts were analysed using uHPLC-DAD as given in 3.2.7.4.

3.2.7.2 Carotenoid extraction from plasma and urine of rats

Plasma and urine carotenoids were extracted using the methods previously described (Cooperstone et al., 2017, Kopec et al., 2010). Briefly, plasma (500 µL) or urine (1 mL) were added to the same volume of ethanol with 0.1% Butylated Hydroxy Toluene (BHT) and 0.5 mL of saturated NaCl. The mixture was vortexed for 15 s followed by addition of 2 mL of 10:6:7:7 (v/v/v/v) hexane: ethanol: acetone: toluene (HEAT). The mixture was vortexed again for 15 s and centrifuged at 1,000 *g* for 5 min at -20^{0}C (Allegra 64R, Beckman Coulter, NSW, Australia). The top layer was transferred to a new Kimax tube using a soda lime pasteur pipette. Each biological sample was extracted thrice. The extracts were dried under nitrogen gas and stored at -20^{o}C until analysis, which was carried out within two days. Samples were re-dissolved in 75 µL of THF: methanol (1:3) before analysis.

3.2.7.3 Carotenoid extraction from liver and feces of rats

Liver and feces carotenoids were extracted using the methods previously described (Zaripheh et al., 2003, Boileau et al., 2000). Briefly, the frozen livers and feces were freeze-dried using freeze dryer (Cuddon FD18, Blenheim, New Zealand). Drying pressure was maintained between 0.1-0.3 mBar during the run. Vacuum temperature was -30^{0}C to -40^{0}C. Freeze-dried livers/feces were crushed to a powder using a porcelain mortar and pestle. 0.1 g of freeze-dried liver/feces were dissolved in 3 mL of KOH (60%) /ethanol (1:5) solution containing 1 g BHT/L. Samples were vortexed and kept in a water

bath at 60^0C for 30 min for complete saponification. Samples were again vortexed after adding hexane (3 mL) and distilled water (1 mL) and centrifuged at 1,000 *g* for 5 min (Allegra 64R, Beckman Coulter, NSW, Australia). Top layer was collected as mentioned previously. Samples were extracted thrice, and dried extracts were stored at -20^{o}C until analysis, which was carried out within two days. Samples were re-dissolved in 100 µL of THF: methanol (1:3) before analysis by HPLC. Weights of liver and feces were measured before and after freeze drying. Lycopene concentration was measured in 0.1 g of freeze-dried liver/feces and back calculated for fresh liver/feces weight.

3.2.7.4 Ultra-high-performance liquid chromatography

Analysis of carotenoids was performed at Plant and Food Research, Palmerston North. Extracts of 'Moonglow' tomatoes and rat biological samples were analysed for their carotenoid composition using ultra high-performance liquid chromatography-diode array detection (uHPLC-DAD). The Ultimate ^{TM} 3000 HPLC system (Dionex, USA) consisted of an Ultimate ^{TM} HPG-3400RS rapid separation binary pump (Dionex, USA), Ultimate ^{TM} WPS-3000RS auto sampler, and the Ultimate ^{TM} DAD -3000RS detector (Dionex, USA). A 150 x 2.1 mm, 2.6 µm pore size C30 Accucore carotenoid column was used for the separation of carotenoids. The system was flushed with 100% acetonitrile before each sequence. Solvent A was comprised of methanol/milliQ water (50:50, v/v), and solvent B was comprised of methanol/isopropanol/methyl tetra butyl ether/milliQ water (100:100:80:2, v/v) with a flow rate 0.4 mL/min. Column temperature was set at 40^{o}C with a CH-150 column heater (ESA, USA). Acetone was used as a needle wash solution between each injection. The linear gradient was applied as follows: 60% B to 100% B over 12 min, held at 100% B over the next 4 min, to 60% B for 0.5 min, held at 60% B for last 3.5 min.

3.2.8 Data processing

Data processing was performed using Chromeleon^{TM} 7.2 SR4 software (ThermoScientific^{TM}). All-*trans*-lycopene was identified based on the retention time of the standard and its spectral characteristics and quantified by the standard curve created. Tetra-*cis*-isomer was identified by its distinctive absorption spectrum at 435 nm (Figure 3.1b). Since there are no commercial standards available for tetra-*cis*-lycopene, the quantification was done through the calculation of response factor using extinction coefficients of both tetra-*cis*- and all-*trans*-lycopene to yield a relative slope (see formula

below). *Cis*-lycopene isomers except tetra-*cis*- were collectively quantified as other-*cis*-lycopene, equivalent to all-*trans*- as done in other studies (Fröhlich et al., 2007, Moraru and Lee, 2005).

$$Extinction\ coefficient\ ATLyc\ (436nm) = \left(\frac{Average\ ATlyc\ area\ (436\ nm)}{Average\ ATLyc\ area\ (470\ nm)}\right) x\ Extinction\ coefficient\ of\ ATlyc\ (470\ nm)$$

$$Response\ factor = \left(\frac{Extinction\ coefficient\ of\ TClyc\ (436\ nm)}{Extinction\ coefficient\ of\ ATlyc\ (470\ nm)}\right)$$

3.2.9 Statistical analysis

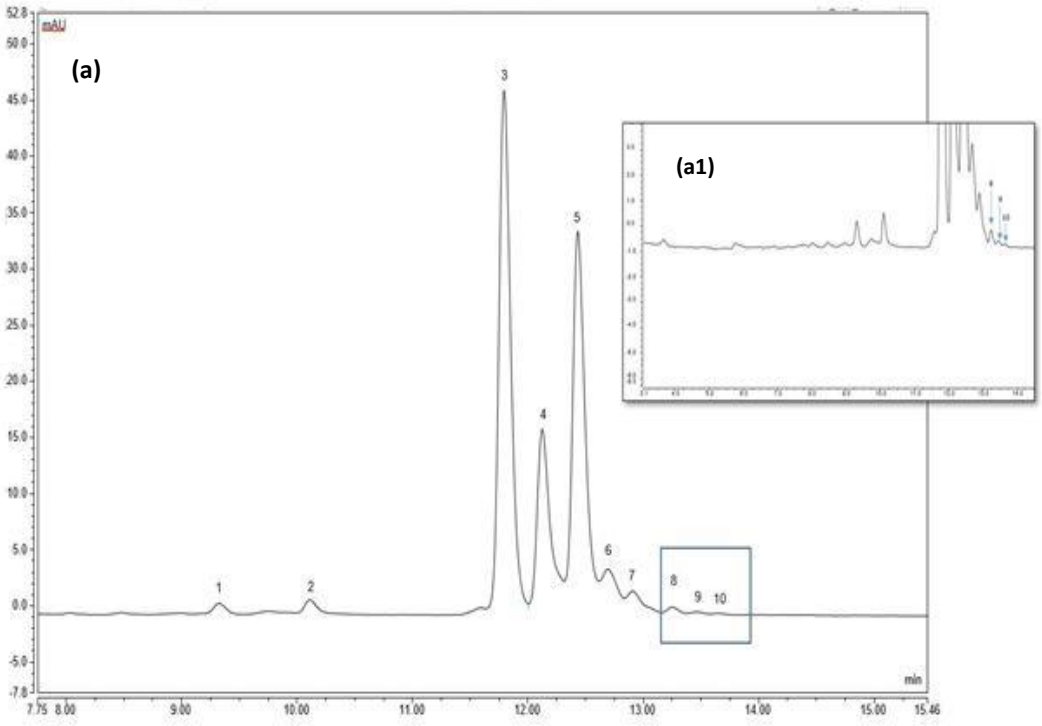
Results are presented as means with standard error of the mean (means ± SEM). All statistical analyses were conducted using SPSS statistical software version 25. All the data were tested for normality using Shapiro-Wilk test and homogeneity of group variances were assessed by Levene's test. The differences between group means were analysed using one-way ANOVA followed by post-hoc Tukey test. Two-way ANOVA was conducted to analyse the effect of dose and time on the lycopene concentration in urine and feces and the apparent absorption. A difference was considered statistically significant when $p<0.05$.

3.3 Results

3.3.1 Analysis of lycopene in 'Moonglow' tomatoes

'Moonglow' tomato lycopene isomers were analysed using uHPLC-DAD (Figure 3.1(a)). Peaks of the tomato chromatogram were matched against available standards of lutein, β- carotene and all-*trans*-lycopene. All-*trans*-lycopene peaks were identified and quantified according to the standard. 'Moonglow' tomatoes do not contain β-carotene. These 'Moonglow' tomatoes are rich in tetra-*cis*-lycopene isomers which was identified based on its special absorption spectrum where the maximum absorption occurs at 436 nm (Figure 3.1(b)) and quantified relative to all-*trans*-lycopene. There are other unidentified peaks in the chromatogram, but it was not possible to quantify other peaks due to lack of standards. However, peak absorption maxima (λ_{max}) and UV-spectra were recorded and identified as detailed in the Table 3.2. Other than tetra-*cis*-lycopene, phytoene, phytofluene, ζ-carotenes and neurosporene were identified using their spectral characteristics. Smaller amounts of other *cis*- and all-*trans*-lycopene were identified and

quantified by using the all-*trans*- calibration curve as discussed in other studies (Ishida et al., 2007, Moraru and Lee, 2005). Total lycopene content of these 'Moonglow' tomatoes was 6.76 mg/100 g of fresh weight (FW), comprising tetra-*cis*- 6.57 mg/100 g FW, all-*trans*- 0.03 mg/100 g FW, and other-*cis*- forms 0.16 mg/100 g of FW. Thus, the tested 'Moonglow' tomatoes contained >97% lycopene in its *cis*-isomeric forms.



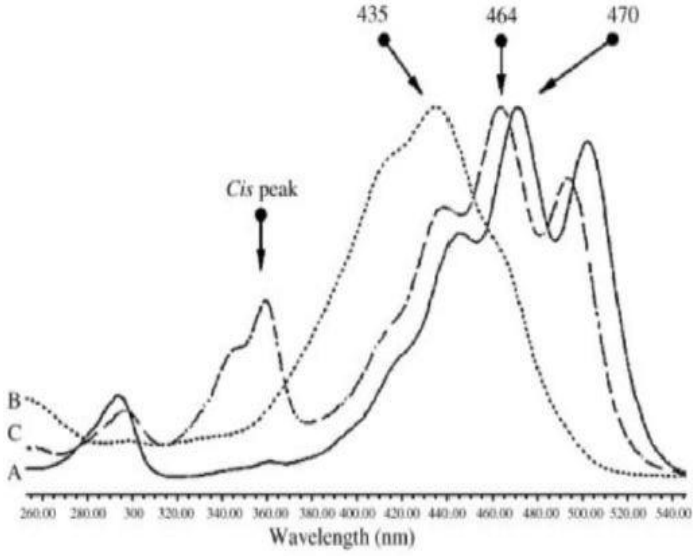


Figure 3.1 (a) Representative ultra-high-performance liquid chromatography of 'Moonglow' tomato using C30 reverse phase column, presenting the separation of carotenoids at 430 nm. a1 represents the closer view of the same chromatogram with clear separation of peak 8,9 and 10. Table 3.2 identifies each numbered peak. (b) Absorption spectra of lycopene isomers. Solid line (A): all-*trans*-lycopene; dotted line (B): tetra-*cis* lycopene; dashed line (C): 13-*cis* lycopene.

Table 3.2 HPLC-DAD parameters for carotenoid identification.

Peak No.	Tentative identification	λ max (nm)	HPLC-DAD UV/Vis spectrum λ_{max} (nm)
1	Phytoene	286	276-286-297
2	Phytofluene isomer	348	331-347-366
3	Tetra-*cis*-lycopene	436	437
4,5	ζ-Carotene isomers	400	348-366-430 382-399-424
6,7	Neurosporene isomers	440	416-440-468 413-440-467
8,9	*Cis*- isomers of lycopene	470	463-465-468 464-467-469
10	All-*trans*-lycopene	470	467-470-500

3.3.2 Study 1- dose-response lycopene accumulation in rats

3.3.2.1 Lycopene intake, weight gain and other related parameters

Body weights of individual rats were monitored throughout the study (Table 3.3). All rats gained weight during the five-day intervention period; however, there were no significant differences in body weights, weight gain, or liver weights between the groups. Additionally, there were no differences in the amount of feces or volume of urine excreted between test groups. Data were normalized to final body weight, but again no significant differences were observed between groups. The control group rats gained half the proportional amount of body weight compared to the other groups, but this was not statistically significant and likely reflects the small number of animals and short time period.

Table 3.3 Body weights, liver weights, and daily amounts of urine and feces excreted for each experimental group in a five-day feeding trial.

Parameter	Control	Low	Mid	High
Initial body weight (g)	230.5 ± 5.8	229.4 ± 3.1	228.9 ± 3.6	229.8 ± 2.8
Final body weight (g)	248.6 ± 17.4	251.7 ± 8.7	248.3 ± 15.9	246.9 ± 13.4
% weight gain	7.9 ± 6.1	9.7 ± 3.4	8.4 ± 5.2	7.4 ± 5.4
Liver weight (g)	8.5 ± 0.4	9.2 ± 0.6	9.2 ± 0.7	8.6 ± 0.4
Liver weight (g/kg BW)	35.9 ± 1.2	36.4 ± 1.6	37.0 ± 1.4	34.7 ± 2.0
Feces excreted (g/d)	4.4 ± 0.8	3.9 ± 1.3	5.7 ± 0.3	3.9 ± 1.1
Feces excreted (g/d/kg BW)	18.6 ± 3.5	15.4 ± 5.2	23.0 ± 1.9	15.7 ± 4.4
Urine excreted (mL/d)	5.9 ± 2.7	7.3 ± 2.5	7.3 ± 1.6	5.0 ± 2.7
Urine excreted (mL/d/kg BW)	23.2 ± 4.4	31.1 ± 6.7	29.6 ± 3.0	22.3 ± 1.2

Values are means ± SD, n=3. Statistical analysis was performed between groups using one-way ANOVA and post-hoc Tukey test and there were no significant differences within the data in each row.

3.3.2.2 Dose dependent plasma and liver lycopene concentration

This trial was carried out to identify the appropriate dose to produce plasma and liver lycopene concentrations that have been reported to be beneficial in preventing certain non-communicable diseases in human. The peak for tetra-*cis*-lycopene was prominent in all plasma chromatograms; however, all-*trans*- and other-*cis*-isomers were detected only in plasma of high dose rats. Plasma chromatogram of the high dose also shows a considerably higher amount of ζ-carotene (Figure 3.2).

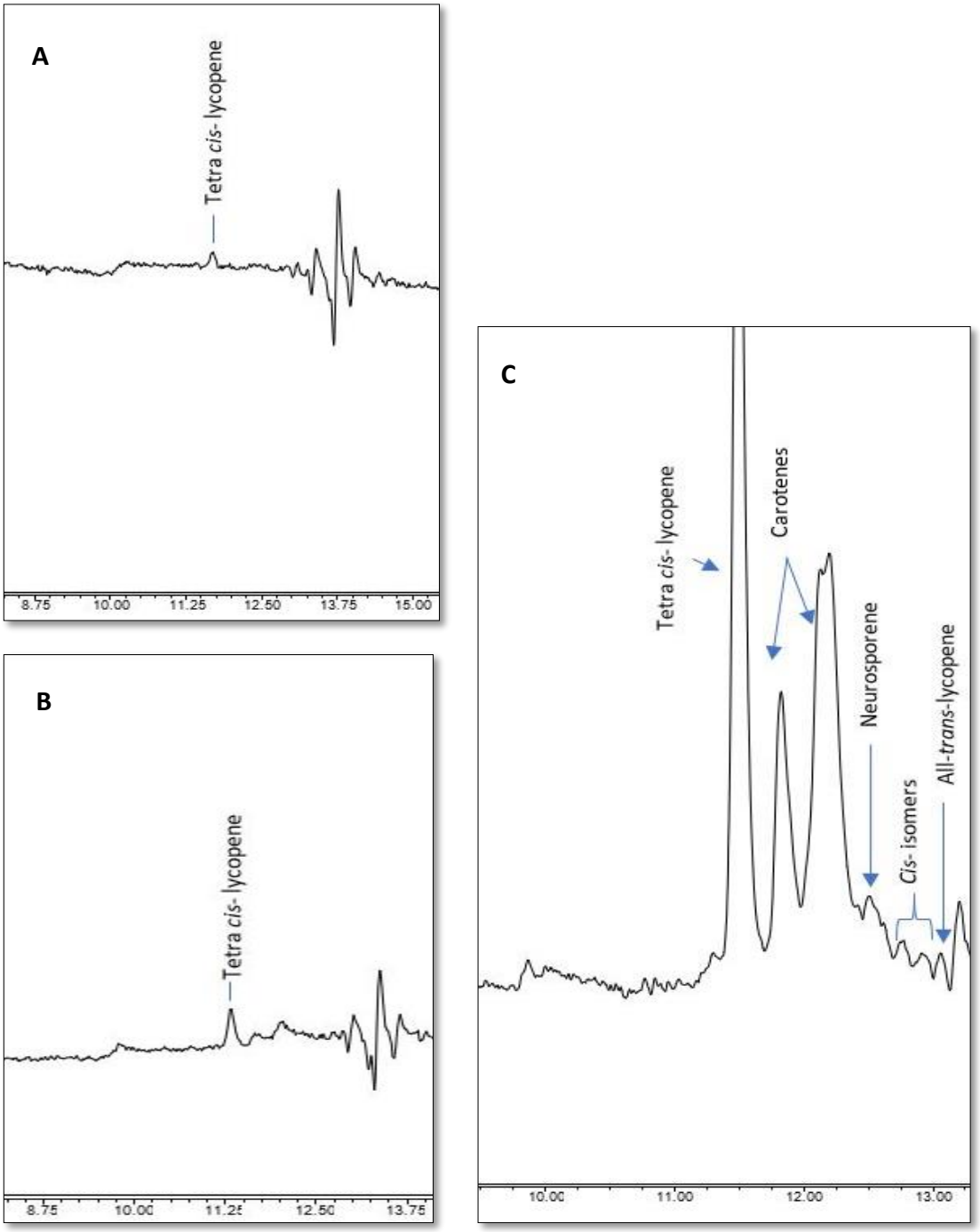


Figure 3.2 Plasma lycopene isomer profile of rats fed 'Moonglow' tomato doses containing 0.05 (A), 0.35 (B) and 2.6 (C) mg/kg BW lycopene. The only detectable peak in A & B is tetra-*cis*-lycopene. Chromatogram C shows higher tetra-*cis* and ζ-carotene along with minute amounts of all-*trans*- and other-*cis* isomers.

Lycopene was not detected in plasma and liver of control rats. Plasma lycopene concentrations in low, mid and high doses were 0.06, 0.60 and 1.87 µmol/L respectively and the values were significantly different at $p<0.05$ (Figure 3.3 (a)). Among total lycopene in high dose, 96% was in the form of tetra-*cis*- whereas 3.4% was from all-*trans*- and other-*cis*-isomers. In contrast to plasma, liver accumulated much higher quantities of lycopene in all-*trans*- and *cis*-isomeric forms (Figure 3.3 (b) & Table 3.4). The total lycopene concentrations in liver were 1.59, 15.6, and 121.9 µmol/kg for low, mid and high doses respectively (Figure 3.3 (b)). Liver lycopene also showed a dose response pattern with increasing dose. Liver contained more than 70% of lycopene in *cis*-isomeric forms (tetra-*cis*- and other-*cis*-) from all three doses, with the remainder being all-*trans*-. The proportion of *cis*- isomers in liver increased significantly ($p<0.05$) with the increase of dietary lycopene intake. Total *cis*-lycopene concentration was 75% for rats fed 0.05 mg/kg, 84% for rats fed 0.35 mg/kg and 90% for those fed 2.6 mg/kg. The all-*trans*-lycopene concentration was 25% for rats fed 0.05 mg/kg, 16% for rats fed 0.35 mg/kg and 10% for those fed 2.6 mg/kg (Table 3.4).

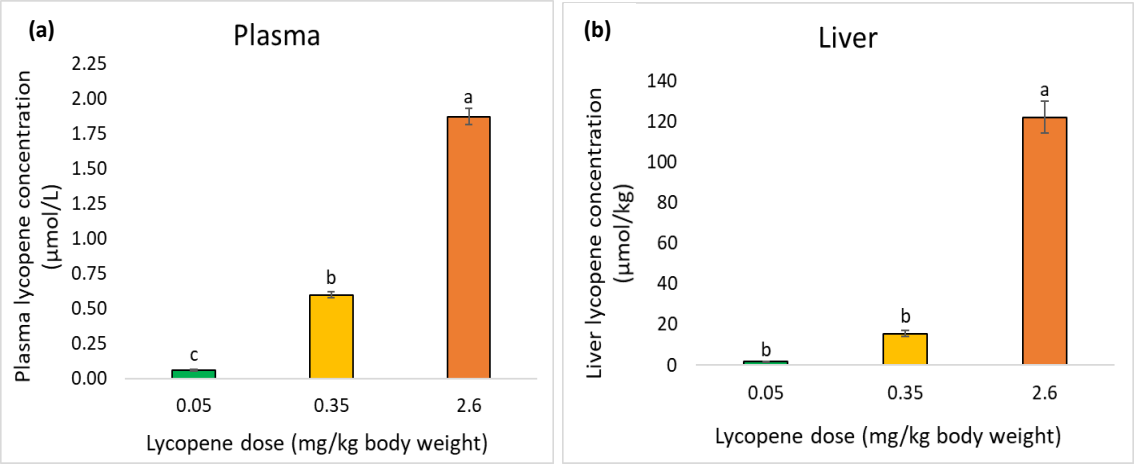


Figure 3.3 Dose-dependent effect of 'Moonglow' tomato on lycopene concentration in (a) plasma and (b) liver after five days. Data shown as mean ± SE (n=3). Columns with a different letter are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test.

Table 3.4 Lycopene isomer composition in plasma and liver of rats after 'Moonglow' tomato supplementation for 5 days[1].

Lycopene isomers	Plasma (µmol/L)			Liver (µmol/kg)		
	Low	Mid	High	Low	Mid	High
Cis-lycopene	0.06±0.01^{c}	0.60±0.02^{b}	1.84±0.06^{a}	1.18 ± 0.09^{b}	13.15±1.02^{b}	109.36±7.40^{a}
All-*trans*-lycopene	n. d.	n. d.	0.03±0.00	0.40 ± 0.07^{b}	2.42 ± 0.50^{b}	12.57±1.16^{a}

[1] Values are means ± SEM, n=3. Statistical analysis was performed between three doses within a single tissue using one-way ANOVA and post-hoc Tukey test. Different superscripts within liver groups or within plasma groups are significantly different at $p<0.05$. n.d. indicates not detected.

We also calculated the total lycopene in whole plasma and the whole liver of rats. Approximate plasma volume was calculated based on each rat's body weight (Bijsterbosch et al., 1981) and the total lycopene in the liver was calculated using whole liver weight. Figure 3.4 (a) and (b) represent the amount of lycopene in whole plasma after the last dose and the amount of lycopene accumulated over 5 days in the whole liver respectively. The three doses were significantly different in the amount of lycopene in whole plasma and the liver.

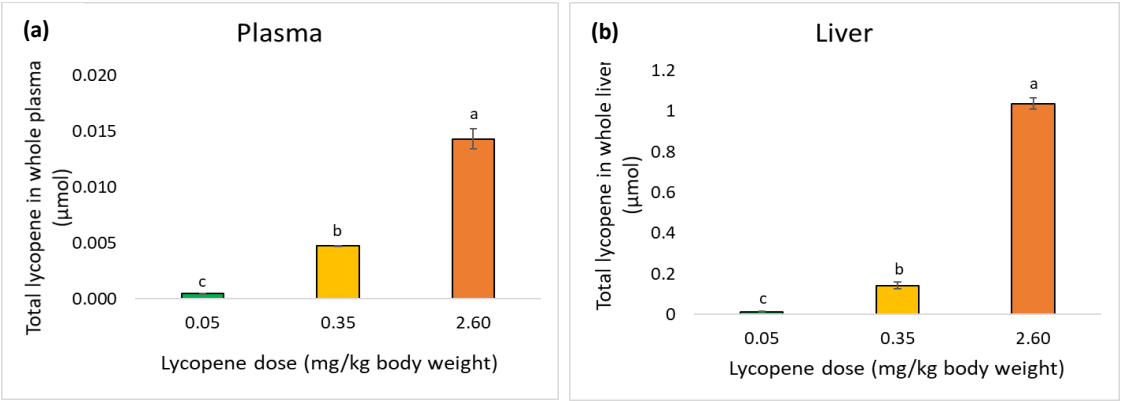


Figure 3.4 Dose-dependent effect of 'Moonglow' tomato on total lycopene in (a) whole plasma and (b) whole liver after five days. Data shown as mean ± SE (n=3). Columns with a different letter are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test.

3.3.3 Lycopene excretion, retention, and apparent absorption

Rats were fed with three doses of 'Moonglow' tomato powder, and feces and urine were collected daily to evaluate lycopene retention and apparent absorption (Tables 3.5 and 3.6). There was no significant difference in the mean volume of urine and amount of feces excreted between test groups and between days within test groups (Appendix i, Table 4 & 5). In this study, we noticed that the high dose rats failed to eat the majority of their dose on day 1. This could be due to rats' natural inclination to not fully consume a new feed. To be consistent with all three groups, the day 1 data were excluded from all three doses and only day 2 to 5 data were considered during calculation of lycopene retention and apparent absorption. Minimal lycopene was excreted in urine. The majority of lycopene was found in feces where the average over four days were 10.6, 70.7 and 635.4 nmol/day for low, mid and high doses respectively (Table 3.5). The amount of lycopene in feces and urine as a proportion of rats' daily intake was also calculated (Table 3.5). Lycopene in feces as a percentage of daily intake increased over time in all three doses. Average amount of lycopene over time was significantly different amongst the three doses. At the end of the study period an average 43% - 53% of lycopene from rats' daily intake was found in feces in all three doses (Table 3.5). Average fecal recovery of lycopene as a percentage of dose was not significantly different between three doses and this suggest that regardless of the dose, nearly half of the administered lycopene was recovered in feces.

Only minute amounts of lycopene were detected in the urine of all doses, indicating that absorbed lycopene was retained in the body. The amount of lycopene in urine as a proportion of daily intake was 1.2%, 0.19% and 0.03% for low, mid and high doses respectively (Table 3.6). In contrast to the feces, average amount of lycopene over time was not significantly different in urine among the three doses, although the values did increase with increasing dose.

Table 3.5 Daily lycopene concentration in feces in each dose[1].

	Lycopene excreted in feces (nmol/day ± SE) (Percentage of daily intake)[2]			
Day	Control	Low	Mid	High
1[#]	0.00	6.07 ± 0.76 (25 ± 3%)	69.97 ± 8.76 (43 ± 5%)	114.25 ± 18.22 (9 ± 2%)
2	0.00	6.48 ± 0.76 [b] (27 ± 3%) [b]	58.94 ± 24.20 (36 ± 15%)	576.19 ± 113.06 (48± 9%)
3	0.00	8.66 ± 1.19 [ab] (36 ± 5%) [ab]	68.68 ± 12.61 (42 ± 8%)	628.06 ± 66.00 (52 ± 5%)
4	0.00	13.43 ± 1.62 [a] (55 ± 7%) [a]	64.03 ± 22.41 (39 ± 14%)	662.57 ± 74.96 (55 ± 6%)
5	0.00	13.92 ± 2.42 [a] (57 ± 10%) [a]	91.10 ± 20.77 (52.9 ± 1.3%)	675.20 ± 159.11 (56 ± 13%)
Mean feces lycopene (4d)		10.64 ± 1.17[c] (43.9 ± 4.9%)	70.70 ± 9.48[b] (43.1 ± 5.8%)	635.43 ± 48.06[a] (52.5 ± 4.0%)

[1] Values are means ± SEM, n=3. Statistical analysis was performed between days in each dose and the average over four days in each dose using one-way ANOVA and post-hoc Tukey test. Data in columns with different superscripts are significantly different at $p<0.05$. [2] Lycopene excretion via feces as a proportion of rats' daily intake. # High dose rats did not fully consume the dose in day 1, therefore, to be consistent with all three groups, day 1 data were excluded from all groups and days 2-5 were considered during statistical analysis.

Table 3.6 Daily lycopene concentration in urine in each dose[1].

	Lycopene concentration in urine (nmol/day ± SE) (Percentage of daily intake)[2]			
Day	Control	Low	Mid	High
1[#]	0.00	0.20 ± 0.04 (0.83 ± 0.18%)	0.24 ± 0.12 (0.15 ± 0.08%)	0.18 ± 0.03 (0.01 ± 0.00%)
2	0.00	0.19 ± 0.12 (0.79 ± 0.50%)	0.27 ± 0.01 (0.16 ± 0.01%)	0.26 ± 0.06 (0.02 ± 0.00%)
3	0.00	0.22 ± 0.12 (0.89 ± 0.48%)	0.24 ± 0.06 (0.15 ± 0.04%)	0.31 ± 0.08 (0.03 ± 0.01%)
4	0.00	0.34 ± 0.08 (1.42 ± 0.34%)	0.36 ± 0.09 (0.22 ± 0.06%)	0.47 ± 0.07 (0.04 ± 0.01%)
5	0.00	0.41 ± 0.15 (1.69 ± 0.60%)	0.44 ± 0.09 (0.27 ± 0.06%)	0.50 ± 0.15 (0.04 ± 0.01%)
Mean urine lycopene (4d)		0.27 ± 0.05 (1.20 ± 0.24%) [a]	0.31 ± 0.04 (0.19 ± 0.02%) [b]	0.35 ± 0.05 (0.03 ± 0.003%) [c]

[1] Values are means ± SEM, n=3. Statistical analysis was performed between days in each dose and the average over four days in each dose using one-way ANOVA and post-hoc Tukey test. Different subscripts are significantly different at $p<0.05$. [2] Lycopene excretion via urine as a proportion of rats' daily intake. # High dose rats did not fully consume the dose in day 1, therefore, to be consistent with all three groups, day 1 data were excluded from all groups and days 2-5 were considered during statistical analysis and to calculate mean urine lycopene.

We also performed two-way ANOVA to analyse the interaction effect of dose and the day (from day 2- day 5) on the proportion of lycopene excreted. Results for both feces and urine revealed that there was no significant interaction between dose and day (Table 3.7).

Table 3.7 Two-way ANOVA for daily lycopene concentration in urine in each dose as a proportion of daily intake.

Effect	Feces	Urine
	p value	*p* value
Dose	0.333	<0.0001
Day	0.305	0.408
Dose*Day	0.432	0.682

Data were analysed using two-way ANOVA, n=3 rats/group.

Apparent absorption of lycopene was calculated in each day using the difference between daily dose of lycopene and the amount of lycopene present in feces.

$$Apparent\ absorption\ of\ lycopene = \left(\frac{Lycopene\ intake - Lycopene\ in\ feces}{Lycopene\ intake}\right) * 100$$

Apparent absorption of lycopene decreased over time with continuous feeding; however, the mean apparent absorptions at the end of the study were 56%, 58% and 48% for low, mid, and high doses respectively (Table 3.8). There was a ~7-fold difference among three doses of lycopene given daily to the rats; however, the mean percent apparent absorption was not significantly different (p<0.05) among the groups.

Table 3.8 Percent absorption of lycopene in each group in each day[1].

Day[2]	Apparent absorption percentage (%) of lycopene[2]			
	Control	Low	Mid	High
2	0.00	73.2 ± 3.1[a]	64.0 ± 14.8	52.4 ± 9.4
3	0.00	64.2 ± 4.9[ab]	58.1 ± 7.7	48.2 ± 5.5
4	0.00	44.5 ± 6.7[b]	60.9±13.7	45.3 ± 6.2
5	0.00	42.5 ± 9.9[b]	47.2 ± 1.3	44.2 ± 13.2
Mean apparent absorption over four days	0.00	56.1 ± 4.8	57.5 ± 5.0	47.5 ± 4.0

[1] Values are means ± SEM, n=3. Values in a column with a different superscript letter are significantly different at $p<0.05$. [2] Because of the exclusion of day 1 data for feces and urine, apparent absorption was calculated and presented only for days 2-5.

We also performed two-way ANOVA to analyse the interaction effect of dose and the day (from day 2- day 5) on the apparent absorption of lycopene. Results revealed that there was no significant interaction between dose and day (Table 3.9).

Table 3.9 Two-way ANOVA for apparent absorption percentage (%) of lycopene.

Effect	*p* value
Dose	0.253
Day	0.286
Dose*Day	0.368

Data were analysed using two-way ANOVA, n=3 rats/group.

3.3.4 Study 2: time-course of lycopene accumulation in rats

This study was designed to evaluate the time-course of a daily feeding of mid dose (0.35 mg/kg BW) of 'Moonglow' tomato on total lycopene concentration in plasma and liver, over five consecutive days. Plasma lycopene concentration increased significantly through the five-day supplementation period (Figure 3.5 (a)) and did not reach a plateau. Lycopene accumulated significantly in livers of rats over time up to day four and then reached a steady-state level ($p<0.05$) (Figure 3.5 (b)). Only tetra-*cis*-lycopene was detected in the plasma in each day. Liver accumulated both *cis*- and *trans*-isomers but

there was around four-six times more *cis*-lycopene each day compared to all-*trans*- (Table 3.10).

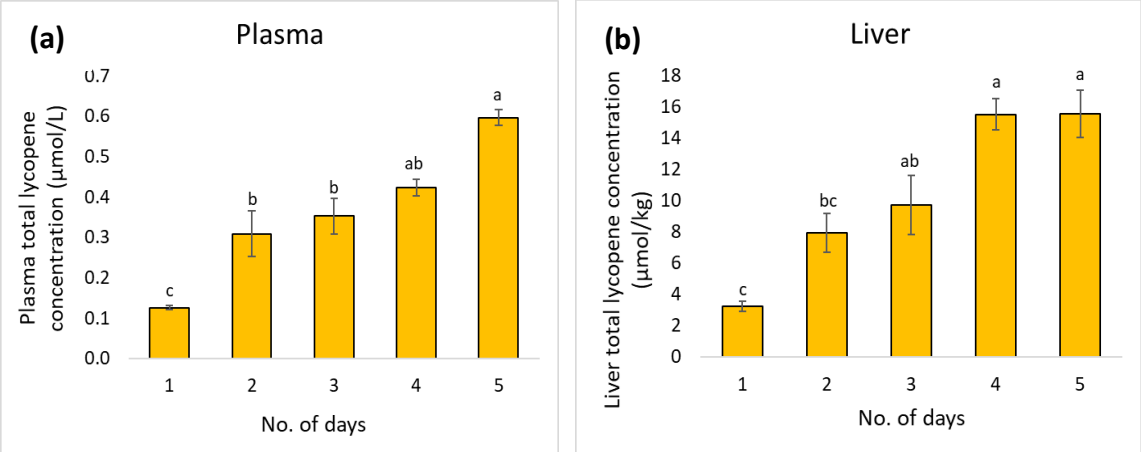


Figure 3.5 Time-course of mid dose (0.35 mg/kg BW) of 'Moonglow' tomato on total lycopene concentration in (a) plasma and (b) liver daily over a five-day. Data shown as mean ± SE (n=3). Bars within a tissue with a different letter (a, b, c) are significantly different (p<0.05) as determined by one-way ANOVA and post-hoc Tukey test.

Table 3.10 Lycopene isomer composition in plasma and liver of rats after 'Moonglow' tomato supplementation for 5 days[1].

Day	Plasma (µmol/L)		Liver (µmol/kg)	
	Cis- isomers	All-*trans*- isomers	*Cis*- isomers	All-*trans*- isomers
1	0.13 ± 0.01^{c}	n.d.	2.68 ± 0.29^{c}	0.56 ± 0.06^{b}
2	0.31 ± 0.06^{b}	n.d.	6.24 ± 0.96^{bc}	1.71 ± 0.42^{ab}
3	0.35 ± 0.04^{b}	n.d.	8.33 ± 1.62^{b}	1.39 ± 0.30^{ab}
4	0.42 ± 0.02^{ab}	n.d.	13.27 ± 0.65^{a}	2.27 ± 0.34^{a}
5	0.60 ± 0.02^{a}	n.d.	13.15 ± 1.02^{a}	2.42 ± 0.50^{a}

[1] Values are means ± SEM, n=3. Statistical analysis was performed between days using one-way ANOVA and post-hoc Tukey test. Data with different superscript letters within a tissue are significantly different at p<0.05. n.d. indicates not detected.

Since our study 2 evaluated the time-course of lycopene accumulation, we also calculated the amount of lycopene retained in the body at each time point following supplementation of mid dose and the liver accumulation of retained lycopene (Table 3.11). Retention of lycopene was calculated each day using difference between

accumulated daily dose of lycopene fed to rats and total amount excreted in feces and urine.

$$Lycopene\ retention\ in\ the\ body = (Lycopene\ intake - (Lycopene\ in\ feces + urine))$$

Amount of lycopene retained in the body was significantly increased with the time. Percent lycopene accumulation in the liver did not significantly different throughout the study period (Table 3.11).

Table 3.11 Lycopene retention and liver accumulation over time[1].

Day[2]	Amount of lycopene retained in the body (nmol)	Amount of lycopene in the liver (nmol)	Proportion of retained lycopene accumulated in the liver (%)
2	268.65 ± 24.20[d]	21.32 ± 3.01[b]	7.85 ± 0.46
3	422.87 ± 12.66[c]	26.99 ± 5.62[ab]	6.34 ± 1.19
4	591.34 ± 22.39[b]	40.98 ± 1.51[a]	6.94 ± 0.23
5	732.59 ± 2.08[a]	43.84 ± 3.95[a]	5.98 ± 0.53

[1] Values are mean ± SEM, n=3. Values in a column with a different superscript letter are significantly different at $p<0.05$. [2] Because of the exclusion of day 1 data for feces and urine, lycopene retention in the body was calculated and presented for days 2-5.

3.4 Discussion

3.4.1 'Moonglow' lycopene isomer composition

'Moonglow' tomatoes were rich in tetra-*cis*-lycopene, which made up approximately 97% of total lycopene. Other-*cis*- and all-*trans*-lycopene made up 2.3% and 0.44% of total lycopene respectively. Similar to our results, *tangerine*, another orange heirloom tomato variety, has been shown to contain 99% of tetra-*cis*- isomers in its lycopene (Ishida et al., 2007). In contrast to tomatoes carrying the *tangerine* mutation, common red tomatoes contain >90% of lycopene in all-*trans*-isomeric form (Burri et al., 2009, Nguyen and Schwartz, 1998). The analysed lycopene profile of 'Moonglow' tomatoes matched previous findings in which tetra-*cis*-lycopene content in 'Moonglow' tomatoes ranged from 3.46 to 5.36 mg per 100 g of fresh weight (McGhie and Cordiner, 2018, Cordiner et al., 2017, McGhie, 2013). Small variations in the content would be due mainly to the maturity stage of tomatoes and the harvesting season (Brandt et al., 2006). In addition to tetra-*cis*-lycopene, 'Moonglow' tomatoes contained considerably higher amounts of ζ-carotene and smaller amounts of phytoene, phytofluene and neurosporene. β-carotene was not identified in 'Moonglow' tomatoes. These tomatoes accumulate more *cis*-isomers and the precursors of lycopene (Kopec et al., 2015, Kachanovsky et al., 2012, Giuliano et al., 2002) due to the presence of a non-functional form of the carotenoid isomerase enzyme that converts *cis*-lycopene to all-*trans*-; the lack of this functional enzyme may block the downstream production of other carotenoids, explaining why β-carotene was absent in 'Moonglow' tomatoes (Appendix i, Figure 1).

3.4.2 Dose response plasma and liver accumulation of lycopene

A clear dose-response of plasma and liver lycopene was observed in our study when doses increased from 0.05 to 2.6 mg/kg body weight. Liver lycopene accumulation was significantly higher ($p>0.05$) in the high dose than the other two doses. The three doses used in this study achieved several fold higher plasma and liver lycopene concentration within five-day period compared to similar rat studies with lycopene in all-*trans*-isomeric form. For example, the mid dose of 'Moonglow' achieved a *cis*-lycopene plasma concentration after five days comparable to that measured in a similar time frame with a 70-fold higher dose of all-*trans*- (Zaripheh *et al*, 2003). Similarly, the mid dose of 'Moonglow' after five days achieved a *cis*-lycopene concentration in liver comparable to that measured in rats fed 14-fold (Boileau *et al*, 2000) and 13-fold (Ferreira *et al*, 2000)

higher amounts of all-*trans*- for 8 – 9 weeks. This was not unexpected, as *cis*-lycopene has been shown to be approximately 8.5 times more bioavailable than all-*trans*- (Cooperstone et al., 2015). In comparison with our results, reported plasma and liver lycopene concentrations are given in Table 3.12.

Table 3.12 Plasma and liver lycopene concentrations.

Author	Lycopene dose	Type of lycopene	Duration	Plasma lycopene (μmol/L)	Liver lycopene (μmol/kg)
Boileau et al, 2000	5 mg/kg BW	All-*trans*-lycopene (beadlets)	8 weeks	0.082	16
	50 mg/kg BW		8 weeks	0.295	61
Ferreira et al, 2000	4.6 mg/kg BW	All-*trans*-lycopene (as tomato oleoresin)	9 weeks	0.022	14
Zaripheh & Erdman, 2005	25 mg/kg BW	All-*trans*-lycopene (90%)		0.314 (5 h after dosing)	116
				0.600 (24 h after dosing)	107
Zaripheh et al, 2003	25 mg/kg BW	All-*trans*-lycopene (90%)	7 days	0.536	138
Ardawi et al, 2016	30 mg/kg BW	All-*trans*-lycopene	12 weeks	0.038	23.2
	45 mg/kg BW		12 weeks	0.071	33.6
(Current study)	0.35 mg/kg BW	97% *cis*-lycopene	5 days	0.600	15.5

Several rat studies have demonstrated that plasma lycopene concentrations in rats are similar to those observed in human trials (Boileau et al., 2002, Boileau et al., 2000, Ferreira et al., 2000). Interestingly, the plasma lycopene concentrations achieved in our study was similar to the reported human studies even at the low dose of feeding (Mackinnon et al., 2011a, Gerster, 1997).

The isomeric composition of lycopene differed in plasma and the liver of rats supplemented with 'Moonglow' tomato powder for 5 days. Plasma chromatograms of low and mid dose rats had prominent tetra-*cis*-lycopene peaks but no detectable peaks of all-*trans*-lycopene, whereas plasma of high dose rats contained small amounts of all-*trans*-and other-*cis*-isomers. The proportions of *cis*- lycopene in the liver after five days of 'Moonglow' feeding were 75%, 84% and 90% for low, mid and high doses respectively. These percentages matched a recent finding where feeding *cis*-isomers of lycopene at a dose of 5 or 10 mg/kg BW of mice produced 81.6 or 81.0% of total *cis*-isomers and 18.4 or 19.0% all-*trans*-lycopene in the liver after 4 weeks of supplementation (Honda et al., 2020). This study further showed that the feeding of a diet rich in *cis*- isomers could produce three times higher levels of total lycopene in the liver compared to all-*trans*-lycopene. It suggests that the consumption of *cis*- isomers will enhance lycopene accumulation in the liver.

Despite the 'Moonglow' tomato containing 97% of lycopene in *cis*-isomeric form, 10-25% of lycopene accumulated in the liver was all-*trans*- regardless of dose. In the single comparative study available assessing isomeric composition of lycopene in diet versus liver, intake of lycopene with 97% *cis*-isomers similarly resulted in liver lycopene of 81% *cis*- and 19% all-*trans*- isomers (Honda et al., 2020). Part of the reason could be if the diet contains high amounts of *cis*-lycopene it can convert into more stable isomeric forms such all-*trans*- or 5-*cis*-lycopene during absorption (Honda et al., 2020, Richelle et al., 2012). Another possible explanation is that the incorporation of fat into the diet can increased the absorption of all-*trans*- lycopene to the body. In our study, peanut butter was used as a source of fat containing high amount of unsaturated fatty acids which can be preferably incorporated into lipoproteins (Unlu et al., 2007a, Roodenburg et al., 2000). Therefore, it possibly could facilitate the absorption of all-*trans*- lycopene. Further, metabolism of lycopene isomers into apo-lycopenals in the liver also could change the isomeric composition in the liver (Gajic et al., 2006).

Liver is a major site of lycopene metabolism and accumulation. In the liver, lycopene is stored mainly in the lipid droplets of hepatic stellate cells (Teodoro et al., 2009). In the post-prandial state the liver tends to store or secrete carotenoids in very low density lipoproteins (VLDL) and low density lipoproteins (LDL) while in the fasting state liver carotenoids can be mainly found in LDL in the plasma. Specific factors may contribute to the tissue carotenoid concentrations through recycling of carotenoid back to

the liver and the excretion of carotenoids (Wang, 2012); however, these factors are not yet fully understood (Canene-Adams and Erdman, 2009). As reviewed by the Elvira-Torales et al., lycopene stores also benefit the liver itself, as studies have found that the lycopene may protect liver from non-alcoholic fatty liver disease and the liver cancer associated with this disorder (Elvira-Torales et al., 2019).

3.4.3 Lycopene excretion, retention, and apparent absorption

Lycopene excretion has been shown to occur mainly through feces while only a minute amount is eliminated in the urine (Zaripheh et al., 2003). Minute amounts of some carotenoids absorbed after digestion could also be excreted through sloughing of keratinized skin cells and expired air (Olson, 1994). The percentage of administered dose found in urine showed an inverse relationship with the doses; however, the concentration of lycopene in urine did not differ significantly between groups and the amount of lycopene excreted by this route was minimal. The calculated apparent absorption of lycopene from 'Moonglow' tomatoes was 56%, 58% and 48% for low, mid and high doses respectively. While not statistically significant, less absorption from the high dose suggests the possibility of a dose-dependent lycopene absorption saturation. A human study feeding lycopene at 0.2, 0.5, 1, 1.5, and 2 mg/kg BW reported that optimal absorption (33.9%) occurred at the lowest dose but did not significantly differ between doses (Diwadkar-Navsariwala et al., 2003). Apparent absorption of lycopene in rats has been reported to be 44.5% after daily supplementation for five weeks of 14 mg/kg BW via tomato juice containing 5% *cis*-isomers (Navarro-Gonzalez et al., 2014), 44% following lycopene supplementation of 14 mg/kg BW containing 10% *cis*-isomers (Martín-Pozuelo et al., 2015). The slightly higher absorption achieved in our study may be due to our using lower doses and to 'Moonglow' being rich in the more bioavailable *cis*-isomers (Cooperstone et al., 2015).

The three doses used in our study did not show significant differences in the proportions excreted from each dose (Table 3.5). Two-way ANOVA further revealed that there was no significant interaction between dose and day (Table 3.7). We chose our three doses based on the reported bioavailability of *cis*- lycopene, and therefore our selected doses were several times lower than reported doses in the literature that have shown dose-dependent saturation (Diwadkar-Navsariwala et al., 2003, Stahl and Sies, 1992). Moreover, independent of the dose administered, lycopene accumulation can be very

specific to the subjects tested. For instance, regardless of the dose, there are some people who are high accumulators while others are low accumulators of lycopene, and this may be true in rats as well. Therefore, inter-individual variation may affect lycopene absorption in the body. In addition, we had a small number of rats per test group; a larger group size may be needed for more conclusive evidence on the absorption saturation of lycopene at higher doses (Diwadkar-Navsariwala et al., 2003).

Incorporation of fat plays a major role in facilitating the lycopene absorption. In this study, we used matching fat percentages for all three doses and increased the amount of peanut butter proportional to the dose, ranging from 384 mg at low dose to 466 mg at high dose. Therefore, it is unlikely the low absorption observed with the high dose was due to an insufficiency of fat. Another study in rodents demonstrated that much of lycopene was unabsorbed and excreted in feces within 48 hours of lycopene administration (Moran et al., 2013). Similarly, rats fed lycopene at 0.2 and 2 mg/kg BW showed no significant differences in the amounts recovered in feces (McClain and Bausch, 2003). Lycopene in urine was minimal overall. The amount of lycopene in urine did not differ between lycopene doses, but the proportion of lycopene dose excreted in urine was significantly reduced with the high lycopene dose compared to the lower doses. This may reflect the fact that we measured total lycopene recovered in urine but not the metabolites of lycopene. Lycopene is rapidly metabolized into polar metabolites and these are excreted in urine (Ross et al., 2011). It is likely that the high dose of lycopene resulted in more lycopene metabolic products in the urine, and this may be of interest in future to explore.

3.4.4 Time-course accumulation of lycopene in plasma and liver

We observed that total lycopene accumulation increased in both plasma and liver daily over time. Liver lycopene reached a plateau after four days of feeding whereas plasma lycopene continued to rise. Porrini *et al* studied the absorption of lycopene in humans from daily intake of raw tomatoes containing 16.5 mg lycopene for nine days and showed a steady daily increase of plasma lycopene up to day seven when it peaked at ~0.58 μmol/L, after which it declined (Porrini et al., 1998); in comparison, plasma lycopene concentration in our mid dose rats was 0.6 μmol/L on day 5. These concentrations are similar enough and thus it is likely that a longer feeding period would not have achieved a significant increase in plasma lycopene in the rats. In other studies,

plasma lycopene was found to stabilise at 0.58-0.72 µmol/L in gerbils fed 20 mg/kg BW lycopene for 20 days (Huang et al., 2006), at 0.78-0.98 µmol/L in dogs fed 30 mg/kg BW all-*trans*-lycopene for 28 days (Korytko et al., 2003), and at 0.602 µmol/L in postmenopausal women supplemented with 30 mg lycopene per day for four months (Mackinnon et al., 2011a). Another human trial found plasma lycopene plateaued at 0.75 µmol/L after two weeks of supplementation of lycopene at 25 mg/day (Richelle et al., 2002). Interestingly, our mid dose (0.35 mg/kg BW) resulted in a plasma concentration of 0.42 µmol/L after only four days of feeding, which is similar to the reported physiological beneficial concentration in humans (Hanson et al., 2018, Mackinnon et al., 2011a, Fielding et al., 2005, Walfisch et al., 2003). Extrapolating this finding to humans suggests that consumption of ~24 g 'Moonglow' tomato daily for less than one week could produce a plasma lycopene concentration at the level reported to be beneficial in the reduction of certain diseases. However, further human trials are required to extrapolate these findings to humans and draw conclusions.

This is the first report on daily accumulation of lycopene in liver in any animal model. We found that lycopene stored in the liver increased daily until day 4, at which point the levels plateaued. Approximately 6-8 % of the lycopene retained in the body was present in the liver in each day for five days of *cis*-lycopene rich 'Moonglow' tomato feeding. In mice given dose-matched supplementation with *cis*-isomers of lycopene versus all-*trans*- for four weeks, the former resulted in three-fold more liver lycopene, attributed to its higher bioavailability (Honda et al., 2020). It is thus likely that consumption of 'Moonglow' tomato compared to red tomato would result in higher liver stores and greater health benefits.

3.5 Conclusions

This study shows that lycopene from 'Moonglow' tomatoes is highly bioavailable and even low doses result in measurable lycopene isomers in plasma and liver. Regardless of the dose, 48-58% of the lycopene ingested was apparently absorbed in the body. For the first time we report daily lycopene accumulation in the liver of rats following intake of *cis*-isomers from 'Moonglow' tomato, with nearly 6-8% of the lycopene retained in the body being present in liver in each day of feeding. Based on these results, we conclude that consumption of even small amounts of 'Moonglow' tomatoes by humans is likely to result in beneficial plasma levels of lycopene. In future animal model and human

intervention studies, both absorption kinetics and health benefits of *cis*- lycopene should be examined. It will also be of interest to carry out direct comparisons between red and 'Moonglow' tomatoes delivered in matching formats and doses.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Umani Walallawita
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In which chapter is the manuscript /published work:	4

Please select one of the following three options:

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- Describe the contribution that the candidate has made to the manuscript/published work:

Involved in planning and designing of animal trials, rat treat preparation, feeding and handling, carried out all experiments and data analysis,compiled and interpreted the results, reviewed and co-edited.

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

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CHAPTER 4

The effect of orange ('Moonglow') tomato on bone loss in ovariectomized rat model of postmenopausal osteoporosis

Abstract

Lycopene is a carotenoid that exists in all-*trans*- and *cis*- forms in nature. In red tomatoes >90% of lycopene is *trans*-lycopene, whereas in orange heirloom tomatoes such as the 'Moonglow' variant >90% of lycopene is the more bioavailable *cis*-lycopene. Lycopene has shown protective effects against bone loss. However, no work has been carried out so far to study the effect of consumption of raw orange tomatoes on their preventive and protective effect on bone loss. Therefore, this study aimed to compare red and 'Moonglow' tomatoes as freeze-dried powder on bone protective properties using a rat model of osteoporosis. Female Sprague-Dawley rats underwent Sham or ovariectomy (OVX) surgery at age 16 weeks to induce osteoporosis. Sham control (Sham-C) and OVX control (OVX-C) groups received no dietary supplement; 'Post-R' and 'Post-MG' received tomato for 8 weeks post-surgery; 'Pre-R' and 'Pre-MG' received tomato for 8 weeks prior to plus post- surgery (n=15/group), where 'R' and 'MG' refer to red and 'Moonglow' tomatoes respectively. Rats were fed tomato powder containing 0.172 mg lycopene (~ 0.35 mg lycopene/kg body weight/day). *In vivo* bone mineral measurements were taken using dual energy x-ray absorptiometry at baseline, week 6, 12 and 16. At the end of the study, rats were euthanised to collect blood, femurs, lumbar spine, and uterus. Effects of red and 'Moonglow' tomatoes on bones were evaluated by analysing bone markers, biomechanical properties, *in vivo* and *ex vivo* dual x-ray absorptiometry, and bone microarchitecture. OVX significantly increased CTx-1 (bone resorption) and osteocalcin (bone turnover) compared to Sham-C. 'Pre-R' (247.0 ± 17.4 ng/mL), 'Pre-MG' (260.2 ± 10.1 ng/mL), and 'Post-MG' (260.3 ± 10.4 ng/mL) had significantly reduced serum osteocalcin levels compared to OVX-C (337.6 ± 19.6 ng/mL). Serum CTx-1 was not significantly different among OVX tomato treatment groups. No significant effect was shown by any of the treatments on bone mineral density, biomechanical properties and bone microarchitecture. Our results showed that compared to red tomato, 'Moonglow' feeding reduced bone turnover that occurs due to estrogen

loss. However, these results need to be further examined using human intervention studies to confirm their applicability.

4.1 Introduction

Osteoporosis is a major health concern among women after menopause. The disorder is mainly associated with skeletal fragility and the deterioration of bone microarchitecture and ultimately reduces the quality of life (Black and Rosen, 2016, Cooper et al., 1992), with significant morbidity and mortality (Kanis, 1994). Osteoporotic bones are more brittle thus fracturing easily (Rao and Rao, 2013). The T-score is calculated by dividing the difference between a female patient's BMD and the mean BMD of young, healthy women by the standard deviation of the reference population. Osteoporosis is diagnosed clinically when bones low in bone mineral density have a T score of -2.5 or below (Szulc and Bouxsein, 2011).

Post-menopausal women have the highest incidence of osteoporosis, as the disease is caused in part by decreased estrogen levels; according to statistics from Europe and the United States, approximately 30% of post-menopausal women are affected by osteoporosis (Szulc and Bouxsein, 2011). Reduction of bone mass occurs asymptomatically throughout adult life and thus is often only diagnosed after the first fracture (Rachner et al., 2011). The risk of second fracture occurrence increases after the first fracture (Ferdous et al., 2016). The most common fractures in post-menopausal women occur at the hip, spine, wrist and ribs (Rao and Rao, 2015, Khosla, 2010, Kanis, 1994). There is predicted to be a demographic-related 32% increase by 2030 in the incidence of osteoporosis and low bone mass in older adults (aged $\geq$ 50) (Wright et al., 2014). Further, treatment cost for osteoporosis is annually increasing and many countries may face a huge economic burden due to fractures in elderly populations (Pandey et al., 2018, Cauley et al., 2014).

Pharmacological approaches are the standard treatment for osteoporosis and the pharmaceuticals are mainly targeted to increase bone formation or reduce bone resorption (Åkesson, 2003) through regulating the activity of osteoblasts and osteoclasts (Langdahl, 2020). Pharmacological treatments developed to prevent bone loss include bisphosphates and, more recently, Denosumab, which is a monoclonal antibody targeting receptor activator of nuclear factor kappa-B ligand (RANKL), and Odanacatib, which is an inhibitor of cathepsin K (Ferdous et al., 2016, Nieves and Cosman, 2015).

Pharmacological therapies could increase bone mass up to 10% over 3-5 years (Nieves and Cosman, 2015). A recent meta analyses showed that even relatively small changes in BMD over time could significantly reduce fracture risk indicating that pharmacological therapies are effective in improving/ maintaining bone mass (Bouxsein et al., 2019). Also, some individuals who has a genetic risk in developing osteoporosis need personalized pharmacological therapies (Pavone et al., 2017). Therefore, the dietary interventions combined with pharmacological therapies could give more benefits for the patients with osteoporosis (Moschonis et al., 2010). Hormone replacement therapy (HRT) was used as a standard care for post-menopausal women diagnosed with osteoporosis until 2002 (Langer et al., 2021). However, the use of HRT is not very common currently due to its side effects such as increases in the risk of breast cancer (Porch et al., 2002) and stroke (Hendrix et al., 2006, Wassertheil-Smoller et al., 2003).

There are both modifiable and non-modifiable risk factors associated with osteoporosis. Globally, consumer interest is growing in identifying and utilising natural rather than pharmaceutical methods to minimize the risk factors for osteoporosis. Lifestyle changes include diet, exercise, and supplements. In particular, dietary phytochemicals play an important role in prevention of some non-communicable diseases including osteoporosis. Among the phytochemicals, lycopene has been demonstrated to have an inverse relationship between its intake and bone loss (Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014).

Lycopene, a major carotenoid in tomatoes, exists mainly in two different isomeric forms: all *trans*- and *cis*-. *Cis*- isomers of lycopene are said to be more bioavailable than all *trans*-. However, the conventional red tomatoes contain >90% of lycopene in its all-*trans*- isomeric form, which has low bioavailability. Orange heirloom tomatoes such as 'Moonglow' have >90% of lycopene in *cis*- isomeric form. We have shown (Chapter 3) that we can achieve the beneficial plasma concentration of lycopene from lower doses of 'Moonglow' tomatoes than red tomatoes. Clinical and animal studies have used purified lycopene in extract or capsule form, or tomatoes processed into a juice, sauce or purée, to demonstrate the protective effects of lycopene on various health parameters (Russo et al., 2020, Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014, Liang et al., 2012, Mackinnon et al., 2011a, Mackinnon, 2010), and there is evidence that lycopene may be bone-protective (Walallawita et al., 2020). To date no studies have been published evaluating the effect of lycopene intake on bone measures using unfractionated and/or

uncooked tomatoes. Considering the research gap and the positive results obtained from our pilot trials, we hypothesise that the consumption of 'Moonglow' tomatoes rich in *cis*-isomers could more effectively protect against bone mineral density loss in ovariectomized rats compared to red tomatoes. Therefore, this study evaluated bone loss and bone strength via different measures such as *in vivo* and *ex vivo* dual x-ray absorptiometry (DXA), bone biomechanical tests, bone resorption and absorption markers and bone microarchitecture. This study also assessed both preventive and protective effect of red and 'Moonglow' tomatoes on bone loss induced by ovariectomy in rats.

4.2 Material and methods

4.2.1 Tomatoes

'Moonglow' tomatoes were grown at Shane's Greenhouses, Whanganui, New Zealand, and transported to Massey University as soon as possible after the ripe tomatoes were harvested. Red tomatoes ('Merlice') were purchased from T&G Global, Palmerston North, New Zealand. Upon arrival, tomatoes were diced and frozen at -80^{0}C. Tomatoes were then freeze-dried (Cuddon FD18LT, Blenheim, New Zealand), ground using a food processor (Kenwood 800W, Havant, UK) and sealed in aluminium zip lock bags with nitrogen gas. Freeze-dried pouches of tomatoes were stored at -80^{0}C until use.

4.2.2 Supplement "treat" preparation

Pure plain flour (Pams) and peanut butter (Sanitarium) were bought from New World, Palmerston North, NZ and raw honey was kindly provided by Horowhenua apiary H5206. Dried tomatoes were incorporated into other ingredients as shown in Table 4.1. According to the amount of total lycopene, tomato powders were mixed with peanut butter, honey and wheat flour to make the desirable dose of 0.35 mg/kg. The rationale for selection of lycopene dose was based on a pilot study which showed physiologically beneficial plasma lycopene concentration (>0.45 μmol/L) at the dose of 0.35 mg/kg BW from 'Moonglow' tomatoes. All ingredients were thoroughly mixed manually and made into a non-sticky paste. The mixture (100 g) was flattened on a 13 cm^2 plastic tray and chilled in a refrigerator. Then the block was dusted in wheat flour and cut into 100 pieces by pressing it through a 1 cm^2 wire mesh. Random pieces from each batch were weighed to ensure the weight of each piece was within the range of 1.0 – 1.2 g. The pieces were

lightly coated with wheat flour to reduce sticking, counted, placed into small plastic zip-lock bags (50 pieces per bag), labelled with the date and stored at -20^{0}C (Figure 4.1). Fresh batches of treats were made once every four weeks; one day prior, the diet formulation was recalculated according to the amount of lycopene present in the batch of tomato powder to be used.

Table 4.1 Ingredients for 100 g of treat mixture.

Ingredients for 100 g *	Control treat	Red tomato treat	'Moonglow' tomato treat
Tomato powder	-	9.2 g	12.8 g
Wheat flour	20 g	10.8 g	7.2 g
Peanut butter	20 g	20 g	20 g
Honey	60 g	60 g	60 g

*Amount of tomato powder added was changed over time when preparing treats according to lycopene content present.

Figure 4.1 Photographs of (a) control (b) red tomato and (c) 'Moonglow' tomato treats.

4.2.3 Animals and diet

All animal procedures were carried out under the ethical approval obtained for this study from Massey University Animal Ethics Committee (Protocol No: 19/12). Ninety, 4 week old female Sprague-Dawley rats were obtained from Animal Resource Centre, Australia. Upon arrival, rats were quarantined for one month at the Small Animal Production Unit (SAPU), Massey University (Palmerston North, New Zealand) according

to the requirements of the Ministry of Primary Industries (MPI, New Zealand). Rats were weighed and transferred to clean standard plastic shoe-box wire-lidded cages with aspen chip bedding upon arrival, housing 2 – 3 rats per cage. Temperature and humidity were set as 22 ^{0}C and 45%-50%, respectively with a 12-12 h light/dark cycle. We have determined from data collected in prior rat ovariectomy studies that to achieve significant differences, n=15 rats per group are needed for a power of 0.8 for the key parameters of bone mineral density and bone breaking energy three months after OVX when p=0.05. Rats were weighed once weekly and were monitored daily for health and behaviour. Throughout the study all rats had *ad libitum* access to standard chow pellets and distilled water, except for the final day of the study, for which the rats were fasted overnight prior to cull. The composition of the rat chow diet is shown in Table 4.2.

Table 4.2 Dietary composition of standard chow pellets

Component*	Unit	Amount
Dry matter	%	91.0
Moisture	%	9.0
Ash	%	6.3
Protein	%	17.9
Fat	%	7.6
CHO	%	59.2
Calcium	mg/kg	9300
Magnesium	mg/kg	3100
Phosphorus	mg/kg	6600
Zinc	mg/kg	142
Gross energy	kJ/g	17.0

*Nutritional composition of rat chow pellets was analysed by the IANZ-accredited Nutrition Laboratory, Massey University, using the following methods: fat analysis by acid hydrolysis using Mojonnier extraction (AOAC 922.06); protein analysis by Dumas method (AOAC 968.38); moisture analysis (AOAC 930.15/925.10/942.05); minerals by plasma emission spectrometry; total carbohydrates calculated by difference; gross energy by bomb calorimeter.

At the end of the quarantine period rats were DXA-scanned as described in 4.2.4.1, randomised into test groups by body weight, whole body bone mineral density and percent body fat, and housed in shoe-box cages as pairs. Rats were given two weeks of adaptation period in their cages with control treat supplements (peanut butter + honey + wheat flour) containing no tomato, to train them to accept and eat the treats offered by hand by the researchers. Throughout the study, rats received their treats between 9 a.m. and 10 a.m. daily for five days per week (Monday to Friday). Following the adaptation

period, tomato supplementation was initiated by switching the rats to tomato powder-containing treats as appropriate by test group. Tomato powder was fed at 0.35 mg lycopene/kg initial body weight/day throughout the trial as humans do not generally increase their tomato intake as body weight increases. Sham-C and OVX-C received control treats containing no tomato; 'Post-R' and 'Post-MG' received tomato-containing treats for 8 weeks post-OVX surgery; 'Pre-R' and 'Pre-MG' received tomato-containing treats for 8 weeks prior to, plus 8 weeks post-OVX, surgery (n=15/group). OVX surgery was performed as described below in 4.2.4.2, and rats underwent periodic DXA scans as shown in Figure 4.2 and described in 4.2.4.1.

4.2.4 Experimental techniques

	DXA						DXA		OVX				DXA				DXA	
Week (age)	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Week (Treatment)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Group A (n=15) Sham-C	Control supplement								Sham	Control supplement								Negative control
Group B (n=15) OVX-C	Control supplement								OVX	Control supplement								Positive control
Group C (n=15) Post-R	Control supplement								OVX	Red tomato supplement								Protective effect of red tomato on bone loss
Group D (n=15) Pre-R	Red tomato supplement								OVX	Red tomato supplement								Preventive effect of red tomato on bone loss
Group E (n=15) Post-MG	Control supplement								OVX	‘Moonglow’ tomato supplement								Protective effect of ‘Moonglow’ tomato on bone loss
Group F (n=15) Pre-MG	‘Moonglow’ tomato supplement								OVX	‘Moonglow’ tomato supplement								Preventive effect of ‘Moonglow’ tomato on bone loss

Figure 4.2 Experimental design for the evaluation of bone properties using ovariectomized (OVX) or non-ovariectomized (sham) rats. Group A = Sham control (Sham-C); group B = OVX control (OVX-C); group C = ‘Post-R; group D = ‘Pre-R’; group E = ‘Post-MG’; group F = ‘Pre-MG’

4.2.4.1 Evaluation of *in vivo* bone mineral density using DXA scan

DXA measurements were taken using Hologic Discovery A bone densitometer (Bedford, MA, USA). A quality control (QC) scan was performed on each DXA day to ensure the precision of the machine through checking the standard coefficient of variance (CV), which ranged from 0.98% -1.01%. The spine phantom was scanned at the beginning and the end of each DXA day to verify the calibration. An ultrahigh-resolution mode of acquisition was used for rat bones. Rats were weighed and anesthetised via intraperitoneal injection (25 G x 5/8" needle) of 0.12 mL/100 g body weight (BW) using an injectable anaesthetic mixture of acepromazine (2 mg/mL): ketamine (100 mg/mL): xylazine (10%): and sterile water at ratios of 2:5:1:2. Following anaesthesia, rats were DXA scanned as described elsewhere (Kruger and Morel, 2016) and this was repeated periodically as shown in Figure 4.2. For the third DXA scan, the anaesthetic cocktail was replaced with ketamine at 60 mg/kg BW and medetomidine at 0.2 mg/kg BW, which after scanning was partially reversed with aptipamezole at 1 mg/kg BW administered via intraperitoneal injection; this reverser shortened time to recovery and lessened the ethical impact of the procedure on the animals. Bone mineral content and bone mineral density for whole body (Appendix i, Table 10 & 11, Figure 16), lumbar spine, left and right femurs were evaluated.

Post-mortem, frozen right femurs and lumbar spine were allowed to thaw to room temperature and then individually scanned *ex vivo* using Hologic Discovery A bone densitometer (Bedford, MA, USA). Similar to *in vivo* scans, quality control (QC) and spine phantom scans were performed according to manufacturer's guidelines to ensure precision and verify system calibration.

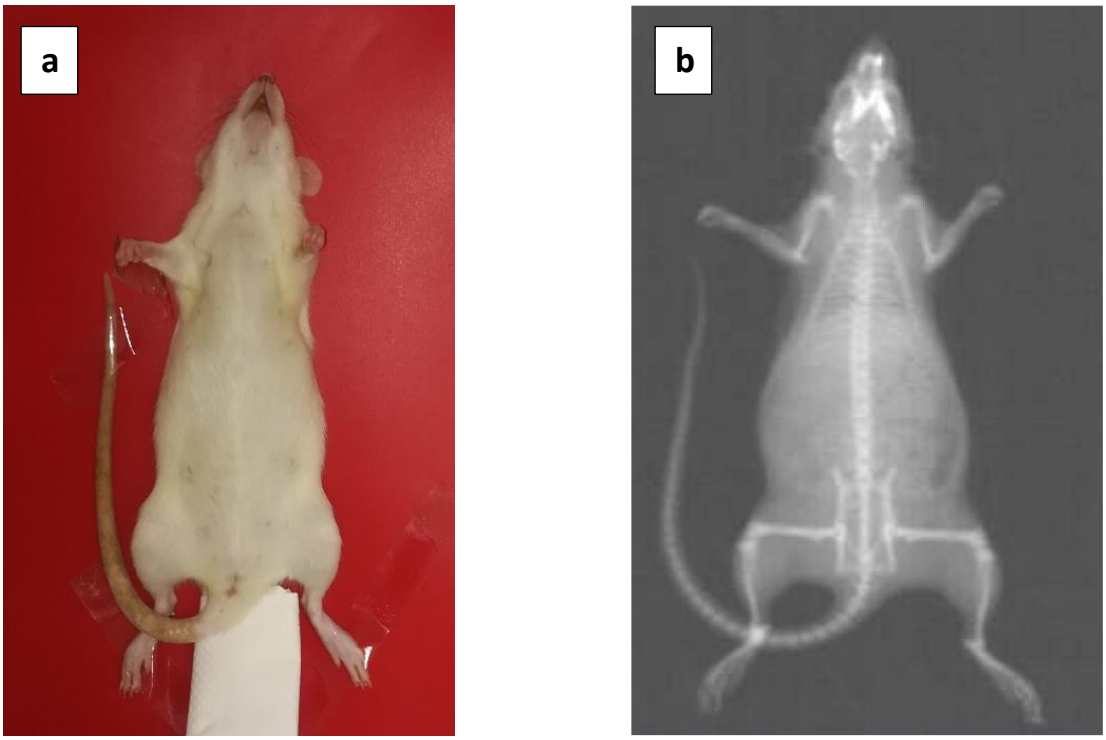

Figure 4.3 Representative images of a female Sprague-Dawley rat (a) secured on an acrylic mat after anaesthesia and (b) *in vivo* scanned.

4.2.4.2 Ovariectomy surgery

Rats in group A underwent no surgery. Ovariectomy surgery was performed as described elsewhere (Kruger and Morel, 2016) with some modifications. Briefly, rats (group B,C,D,E,F) were lightly anesthetised by intraperitoneal injection (25 G x 5/8" needle) of 0.12 mL/100 g body weight using an injectable anaesthetic mixture of acepromazine (2 mg/mL): ketamine (100 mg/mL): xylazine (10%): sterile water at 2:5:1:2. Once the rat reached the required depth of anaesthesia, the surgical site was prepared by shaving and scrubbing with surgical prep solution and alcohol. Subsequently, the rats were deeply anaesthetized by inhalation of isoflurane. A <2 cm scalpel incision was made on the ventral surface of the skin between the hump of the back and the base of the tail. The skin was loosened, and a small incision made through the peritoneal muscles. One peri-ovarian fat pad was identified, grasped with forceps, exteriorised, and the ovary removed. Haemostat pressure was applied for ~30 seconds to stop any bleeding. Using the same incision site, the procedure was carried out for the second ovary. Warm sterile saline (3 mL) was injected into the peritoneal cavity through the incision to compensate for lost fluids. The incision site was closed using 3-4 surgical clips followed

by subcutaneous injection of saline for hydration and butorphanol for analgesia. Rats were kept warm under a heat lamp and on a heat blanket for 30 minutes to 1-hour post-surgery in cages with shredded paper bedding. The ambient temperature did not exceed 25^0C. Rats were then returned to their home cages and monitored at least four times daily for the next two days. Seven to ten days after surgery, surgical clips were removed.

4.2.4.3 Euthanasia, blood collection, and dissection

Blood samples were collected from each rat, directly from the heart under completed anaesthesia after overnight fasting. Following the final DXA scan with anaesthesia as described in 4.2.4.1, rats received a second injection of acepromazine/ketamine/xylazine anaesthetic. Blood was collected by cardiac puncture as described in 3.2.5 and placed into vacutainer tubes containing either no anticoagulant (for serum) or sodium heparin (for plasma) and subsequently separated by centrifugation as described in 3.2.5. Plasma or serum aliquots placed into labelled 1 mL Eppendorf tubes and stored at -80^0C until use. Following exsanguination, euthanasia was ensured by pneumothorax and tissue samples were then collected by dissection. Femurs, lumbar spine and uterus were removed. Bone samples were placed into pottles containing phosphate buffered saline (PBS) and held in an ice bath; later in the day, they were stored at -20^0C. The uterus weight was measured and recorded to verify successful OVX.

4.2.4.4 Evaluation of bone formation and resorption markers in serum

The bone formation marker osteocalcin, and the bone resorption marker carboxyl-terminal crosslinks telopeptide of type I collagen (CTx-1), were evaluated in fasted blood serum. Osteocalcin was measured using IDS Rat Osteocalcin ELISA kit (IDAC12F1, Abacus dx Ltd, NZ) and CTx-1 using IDS RatLaps ELISA (CTx-1) kit (IDAC06F1, Abacus dx Ltd, NZ) according to manufacturer's instructions. Absorbances of duplicate test wells for each sample were measured at 450 nm using a microplate reader (Multiskan™ FC, Thermo Scientific™).

4.2.4.5 Evaluation of biomechanical properties of long bones

Femurs were thawed, and remaining soft tissues were removed using a scalpel blade. The length of the femurs (a) was measured using an electronic vernier calliper (150 mm Mitutoyo Digimatic 500-19, Kanagawa, Japan). The midpoint of the femur was marked using a permanent felt-tip marker, and the width and thickness were measured at

the midpoint (Figure 4.4). Biomechanical properties were evaluated by a three-point bending test using a texture analyser (Shimadzu AGS-H/EZTest, Columbia, Maryland, USA). Femurs were placed on the bending jig with a fixed distance of 12 mm between two supporting rods. Constant deformation rate of 50 mm/min was applied at femur midpoint until the bone fractured. Values for break force (N), break stroke (mm), break stress (N/mm^2), break strain (%), elasticity (N/mm^2) and energy (J) were obtained by the load-deformation curve generated by TrapeziumX software. These terms are described in (Appendix i, Figure 17)

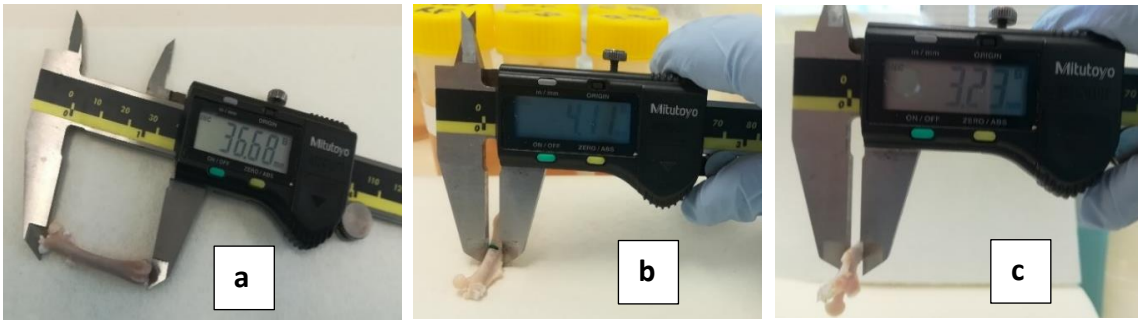

Figure 4.4 Measuring of femur length (a), width (b), and thickness (c).

4.2.4.6 Assessment of trabecular microarchitecture by microcomputed tomography

The effects of tomato supplementation on microarchitectural changes in the fourth lumbar (L4) vertebra in female Sprague-Dawley rats were evaluated using micro-computed tomography (micro-CT) (Skyscan 1172, Bruker, Kontich, Belgium) at Auckland Bioengineering Institute, Auckland University, New Zealand. Briefly, soft tissues were removed from the spine and the third to fifth lumbar units dissected as a unit and stored in 70% ethanol. Three samples were mounted on top of each other, separated by foam disks, in a sealed tube maintaining a saturated vapour for imaging. Scanning was performed with x-ray energy settings of 72 kV, 138 µA, 700 mS exposure, 2x frame averaging, 4 x random movement, 1 mm Al filter, 2000 x 1332 camera pixels at a pixel resolution of 10.02 µm. Vertebrae were scanned using a rotational step of 0.36^0 for 180° requiring 40 minutes per sample. The images were reconstructed using InstaRecon CBR Server Premium 15K (InstaRecon Inc.,1800 S. Oak St, Champaign, Illinois) and subsequently visualized in 3D using CTVox V 3.3 (Bruker, Kontich, Belgium). Data View V 1.5.4.0 (Bruker, Kontich, Belgium) was used to adjust rotational and tilt

orientation to give comparable views of all samples and transverse planes. Analysis was done using CTAnal v.2.0.0.5 (Bruker, Kontich, Belgium). For the analysis of bone parameters, 500 planes (+250 & -250) from the midpoint of the 4th vertebrae was used as a volume of interest (VOI) (Jadzic et al., 2020).

Bone volume fraction, bone surface density, trabecular number, trabecular thickness, trabecular separation and structure model index were calculated. These key parameters are expressed in the conventional way and brief definitions, are presented in Table 4.3. Stronger trabecular bone will have higher bone volume and bone surface ratios to total volume. In osteoporosis the thickness of trabeculae remains unchanged, but the number of trabeculae will decrease, resulting in a larger distance between them. Structural model index is included as this parameter is commonly presented when reporting bone microstructure, although it often gives a negative value for trabecular bone due to its concave surface as opposed to a 0 for plates, 3 for rods, and 4 for spheres.

Table 4.3 Defined microarchitectural parameters of trabecular bone.

Parameter	Abbreviation	Definition
Bone volume fraction	BV/TV	Segmented bone volume:total volume ratio
Bone surface density	BS/TV	Segmented bone surface:total volume ratio
Trabecular number	Tb. N	Average number of trabeculae per mm
Trabecular thickness	Tb. Th	Mean trabecular thickness
Trabecular separation	Tb. Sp	Mean distance between trabeculae
Structure model index	SMI	Structural indicator of trabeculae

4.2.5 Statistical analysis

IBM SPSS statistics version 25 was used for statistical analysis. Results are presented as means with standard error of mean (mean ± SEM). Analysis for the *in vivo* DXA data was done using repeated measures of ANOVA focusing on actual data with body weight at each time point considered as a covariate. Repeated measures of ANOVA were conducted separately for pre-surgery period (week 0-6) and post-surgery period (week 6-16). A secondary analysis focused on percent change in *in vivo* DXA measures between weeks 0-6, 6-12, 12-16 and 6-16 analysed using one-way ANOVA and post-hoc Tukey tests. Body weight, uterus weight, serum CTx-1, serum osteocalcin, three-point bending test and bone microarchitecture were analysed using one-way ANOVA. Each

time, the Sham-C (non-OVX) was excluded from the analysis and separately tested using independent t-test to compare with the OVX-C group.

4.3 Results

4.3.1 General observations

Rats were healthy throughout the quarantine (1 month) and study (4 months) periods, and no adverse effects were observed following tomato feeding such as behaviour change, illness, or deaths. After the first week of treat-training and thereafter throughout the study, all rats would stand erect in their cages and actively seek to take the treats as the researchers provided them. Three rats (rats #25, #77 and #79) failed to recover from anaesthesia after DXA scan. One rat (rat #88) was euthanised at week 14 due to a leg/spine injury. The deaths of these rats were not related to the tomato treatment and the post-mortem examination results are attached in Appendix ii. Transient haematuria was observed in a few of the rats during the study; the Animal Welfare Officer could not determine a cause but as there was no time or treatment pattern discernible, it was deemed unrelated to the tomato treatments.

4.3.2 Effect of ovariectomy on uterus weight of female Sprague-Dawley rats

Rats in group B, C, D, E and F (Figure 4.2) underwent ovariectomy at 16 weeks of age while rats in group A underwent no surgery (Sham-C). At the end of the study, the uterus was collected from each rat and weighed. As seen in Figure 4.5, the uteri weight of Sham-C rats was significantly higher ($p<0.0001$) than the OVX-C. This finding was expected as the rat uterus atrophies following OVX. Uterus weight did not differ among OVX groups ($p=0.806$). However, uteri weights were higher in three rats (rat #6, 1.705 g; rat #86, 0.997 g; rat #8, 0.756 g) compared to the others. This indicates the incomplete removal of one or both ovaries during OVX surgery. On this basis, these three rats were excluded from all subsequent analyses.

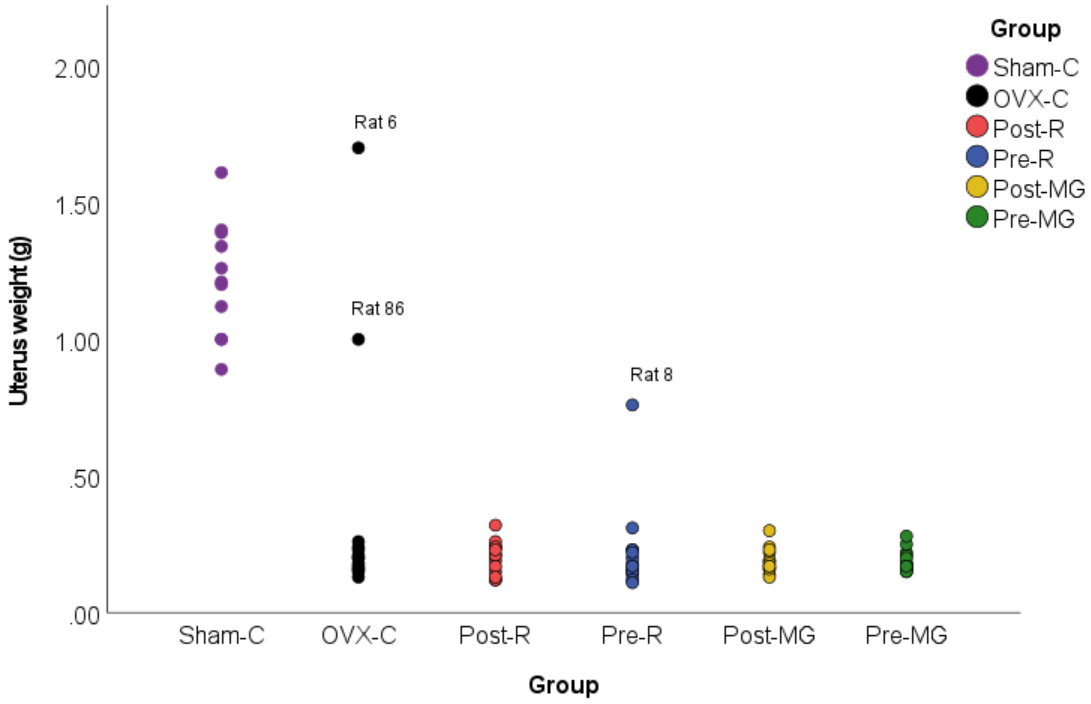

Figure 4.5 Uteri weights of 24-week-old female Sprague-Dawley rats. Coloured points represent each rat in each group.

4.3.3 Effect of red and 'Moonglow' tomato feeding on body weight

Weekly body weight measurements and proportional weight gain from week 0 to week 16 are shown in Figures 4.6 and 4.7. Initial body weight at the baseline ranged from 227.6 ± 22.7 to 241.3 ± 16.8 g and there was no significant difference (p=0.773) between groups at the baseline indicating our randomization based on body weight was similar in all groups. Also, there was no significant difference (p=0.245) of body weight of OVX groups at the end of the study. 'Post-R' group maintained their higher body weight throughout the feeding period due to their higher baseline body weight (Figure 4.6). All rats including rats with no surgery (Sham-C) increased body weight throughout the study period. The weight gain trajectories of OVX rats were significantly different (p=0.001) from Sham-C.

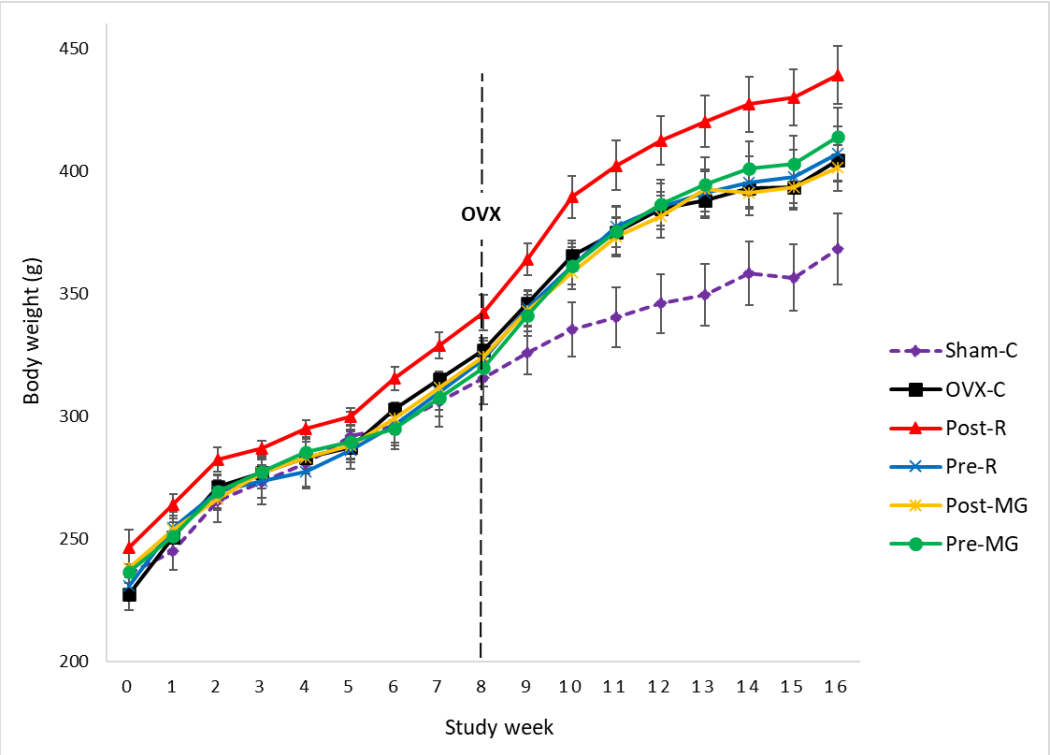

Figure 4.6 Mean weekly body weight measurements in female Sprague-Dawley rats. Data are mean ± SEM, n=12-15 rats/group.

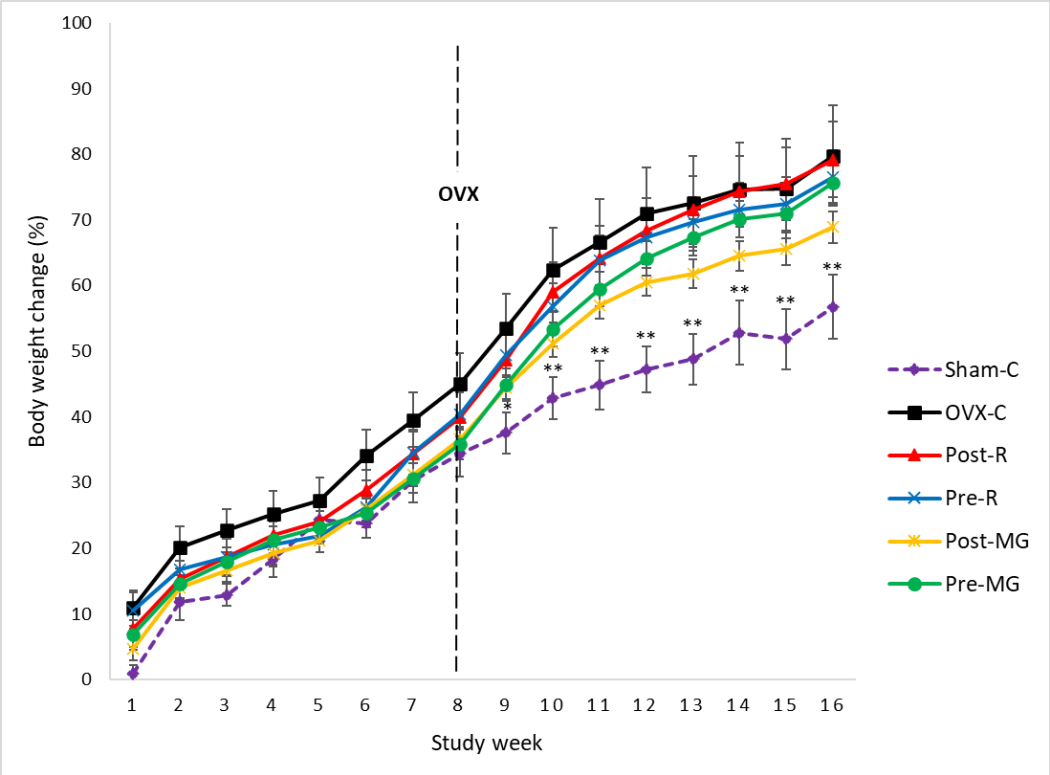

Figure 4.7 Percent body weight change from baseline in female Sprague-Dawley rats. Data are mean ± SEM, n=12-15 rats/group. *indicates significant difference from OVX-C: *p<0.05; **p<0.01.

4.3.4 Effect of tomato extracts on serum osteocalcin concentration

Serum osteocalcin, produced by osteoblasts, is correlated with the bone formation process and is expected to rise following ovariectomy. Compared to Sham-C, serum osteocalcin in OVX-C rats was significantly higher ($p=0.02$) when assessed 8 weeks after surgery. As shown in Figure 4.8, the serum osteocalcin level of rats ranged from 247.0 ± 17.4 to 350.8 ± 26.8 ng/mL. Serum osteocalcin concentrations in OVX rats in both 'Moonglow' groups, but only the 'Pre-R' group, were significantly lower than that of OVX-C. This indicates that 'Moonglow' tomato significantly reduce bone turnover regardless of pre or post-OVX whereas similar dose of red tomatoes showed significant reduction of bone turnover when it is given before the OVX.

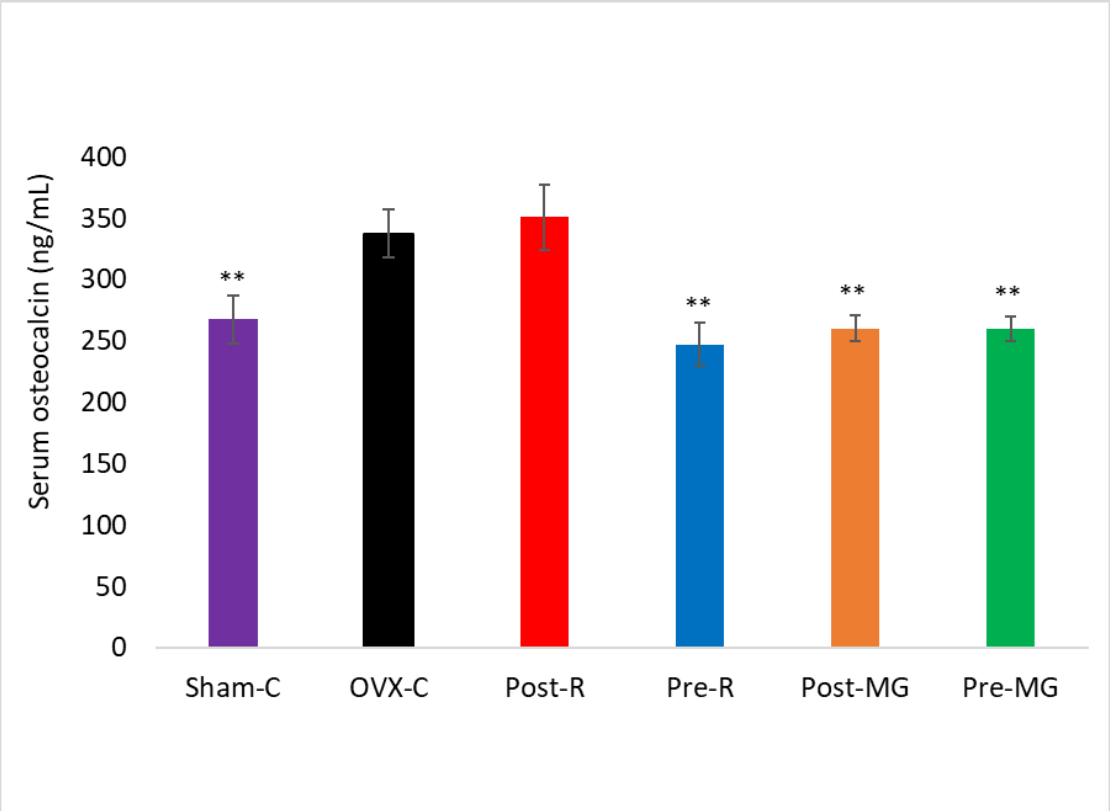

Figure 4.8 Effect of red and 'Moonglow' tomato consumption on serum osteocalcin concentrations in 24-week old female Sprague-Dawley rats. Data are mean ± SEM, n=12-15 rats/group. * indicates significant difference from OVX-C: ** $p<0.01$.

4.3.5 Effect of tomato feeding on serum CTx-1 concentration

CTx-1, the biomarker of bone resorption, was significantly higher in OVX-C rats (p=0.038) compared to Sham-C rats (Figure 4.9), as expected. OVX rats in all of the tomato groups had lower CTx-1 compared to OVX-C. These data suggest that both red and 'Moonglow' feeding can reduce the bone resorption that occurs after ovariectomy.

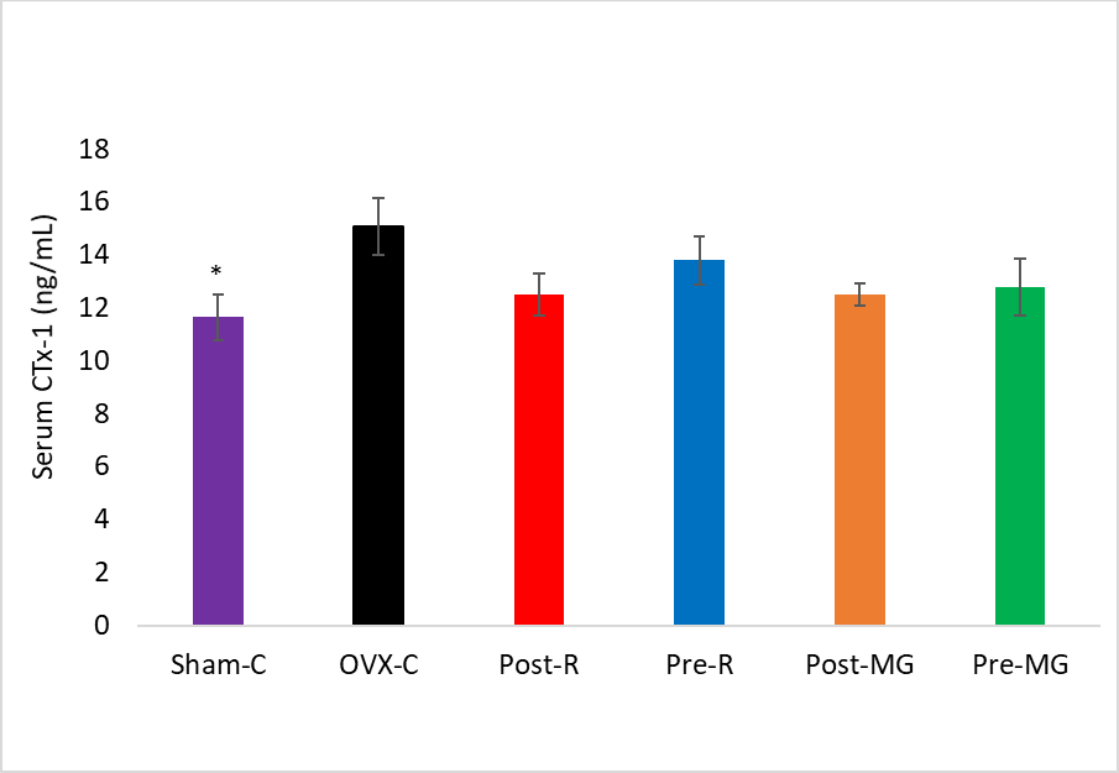

Figure 4.9 Effect of red and 'Moonglow' tomato consumption on serum CTx-1 concentrations in 24-week old female Sprague-Dawley rats. Data are mean ± SEM, n=12-15 rats/group. * indicates significant difference from OVX-C: *p<0.05.

4.3.6 Effect of experimental diets on femur BMD

There was no significant difference of diet on right femur BMD. However, the significant 'week' effect indicates that the right femur BMD increased overtime from 0-6 weeks but did not change thereafter (Figure 4.10). There was a significant group and week interaction effect (Table 4.4). This indicates the changes of BMD over different time points. Femoral BMD of all groups increased during pre- surgery period from week 0-6. 'Post-R' feeding group slightly increased BMD than the other treatments from 0-6 weeks, from week 6-16, all the groups had slight increase in BMD except for those receiving 'Post-R' or 'Post-MG' feeding (Figure 4.10). During week 12-16, the percent change in BMD was significantly different between 'Post-MG' and OVX-C. However,

there was no real difference in the percent change of BMD between OVX groups during pre (0-6 weeks) and post (6-16 weeks) surgery period (Table 4.4). OVX model was successful as percent change of right femur BMD of OVX rats was significantly lower (p=0.002) than that of Sham-C rats during post-surgery period (Table 4.5). Changes in femur BMC are given in Appendix i, Table 6 & 7, Figure 12).

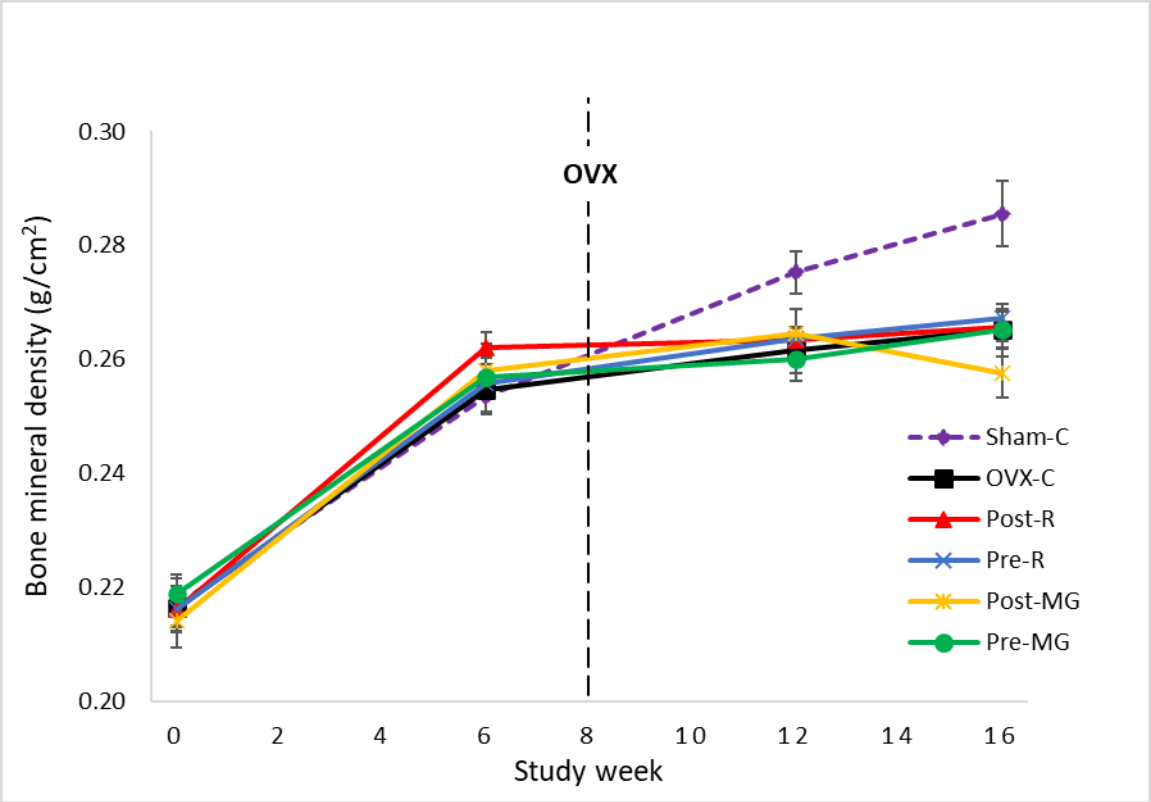

Figure 4.10 Right femur BMD in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Data are mean ± SEM, n=12-15 rats/group.

Table 4.4 Repeated measures ANOVA for right femur BMD during pre- and post-surgery period.

	Pre- surgery period (0-6 week)	**Post- surgery period (6-16 weeks)**
OVX only	**BMD**	**BMD**
Group	p=0.795	p=0.825
Week	p=0.036	p=0.824
Group x week	p=0.037	p=0.012

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.

Table 4.5 Percent change of right femur BMD in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	Week 6-12	Week 12-16	Week 6-16
Sham-C	17.20 ± 1.86	9.17 ± 0.90***	4.58 ± 1.47	14.25 ± 2.04*
OVX-C	17.69 ± 1.86	2.62 ± 0.64	1.85 ± 1.39	4.46 ± 1.89
'Post-R'	19.50 ± 1.17	0.60 ± 0.80	0.79 ± 1.05	2.53 ± 1.80
'Pre-R'	18.83 ± 1.11	1.82 ± 0.63	1.31 ± 1.09	4.23 ± 0.83
'Post-MG'	20.36 ± 0.98	1.67 ± 0.47	-2.64 ± 1.12*	-0.62 ± 1.51
'Pre-MG'	17.57 ± 1.47	1.60 ± 0.75	0.71 ± 0.90	3.47 ± 1.42

Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significant difference from OVX-C *p<0.05, ***p<0.00001.

4.3.7 Effect of experimental diets on lumbar spine BMD

There was no significant group or week effect on lumbar spine BMD from week 0-6 or 6-16. But there was a significant interaction effect between group and week indicating changes in BMD over different DXA time points (Table 4.6). During week 6-16, red tomato groups slightly increased BMD while 'Moonglow' groups slightly decreased in BMD (Figure 4.11). Only during week 6-12, 'Pre-MG' group showed significantly lower percent change in BMD compared to OVX-C. The percent change in BMD was highest for 'Post-MG' during pre- surgery and lowest during post- surgery period. However, the percent change in BMD was not significantly different amongst OVX groups during pre (0-6 weeks) and post (6-16 weeks) surgery period. OVX model was successful with percent change in BMD of lumbar spine being significantly lower (p=0.002) than that of Sham-C group (Table 4.7). Changes in lumbar spine BMC are given in Appendix i, Table 8 & 9, Figure 14).

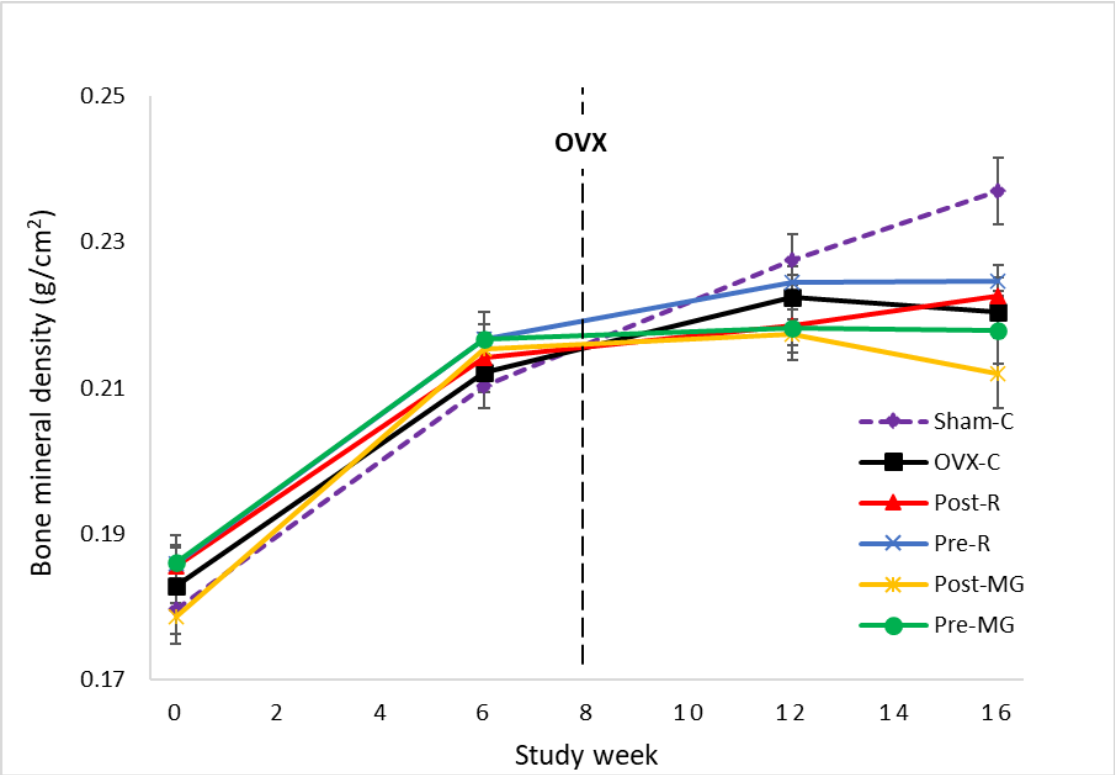

Figure 4.11 Lumbar spine BMD in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Data are mean ± SEM, n=12-15 rats/group.

Table 4.6 Repeated measures ANOVA for lumbar spine BMD during pre- and post-surgery period.

	Pre- surgery period (0-6 week)	**Post- surgery period (6-16 weeks)**
OVX only	**BMD**	**BMD**
Group	p=0.592	p=0.279
Week	p=0.313	p=0.558
Group x week	p<0.001	p=0.049

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.

Table 4.7 Percent change of lumbar spine BMD in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	Week 6-12	Week 12-16	Week 6-16
Sham-C	17.30 ± 1.18	8.00 ± 1.04	4.67 ± 1.59*	12.92 ± 2.26*
OVX-C	15.54 ± 1.04	5.46 ± 0.72	-2.38 ± 1.46	2.92 ± 2.11
'Post-R'	15.67 ± 1.23	2.07 ± 0.85	2.00 ± 1.87	3.73 ± 1.49
'Pre-R'	16.77 ± 1.09	3.69 ± 0.90	0.31 ± 0.79	4.00 ± 0.88
'Post-MG'	20.00 ± 1.25	1.20 ± 0.55	-2.93 ± 1.16	-1.79 ± 1.55
'Pre-MG'	16.57 ± 1.27	0.71 ± 0.70**	-0.33 ± 1.41	0.57 ± 1.37

Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using students' t-test. OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significant difference from OVX-C: *p<0.01, **p<0.01.

4.3.8 Effect of tomato feeding on femoral bone strength

Biomechanical properties were assessed in both right and left femurs using three-point bending test and the pooled analysis of combined paired femur data are presented in Table 4.8. Separate left and right femur results are attached in Appendix i, Table 16 & 17. Sham-C rats did not differ from the OVX-C rats in femur length. Femur midpoint width and thickness were ~3 % lower in Sham-C rats compared to OVX-C (p<0.05). In contrast, break stress, the most relevant biomechanical parameter that correlates with stronger and more resilient bone, was significantly higher in Sham-C rats compared to OVX-C (p<0.05). Three were no significant differences amongst tomato treatment groups for each variable.

Table 4.8 Mechanical properties of left and right femurs of female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* values [b]
Bone length (mm)	36.31±0.30	36.40±0.24	36.61±0.15	36.50±0.12	36.37±0.21	36.34±0.13	0.772
Midpoint width (mm)	4.01±0.04*	4.15±0.06	4.11±0.03	4.10±0.04	4.15±0.04	4.12±0.04	0.906
Midpoint thickness (mm)	3.09±0.03*	3.18±0.03	3.14±0.02	3.17±0.02	3.17±0.03	3.16±0.03	0.710
Break force (N)	165.05±2.55	170.69±5.50	166.10±2.56	169.17±3.08	176.43±4.19	165.68±3.61	0.390
Break stroke (mm)	1.50±0.05	1.53±0.05	1.43±0.04	1.44±0.03	1.53±0.04	1.40±0.03	0.100
Break stress (N/mm^2)	105.18±4.56*	91.30±2.28	92.70±1.56	93.22±2.66	94.8±2.82	91.25±2.40	0.834
Break strain (%)	12.35±0.41	13.01±0.52	11.92±0.36	12.18±0.27	13.10±0.43	11.83±0.30	0.125
Elasticity (N/mm^2)	1257.28±45.30	1159.82±38.93	1210.82±29.54	1181.79±29.09	1188.76±43.65	1232.68±37.38	0.717
Energy (J)	0.16±0.01	0.16±0.01	0.15±0.01	0.16±0.01	0.17±0.01	0.15±0.01	0.123

Data are mean ± SEM, n=12-15 rats/group. [a]Asterix mark in Sham-C column shows significant differences between Sham-C and OVX-C (Student's t-test). [b] *p* values of one-way ANOVA followed by post-hoc Tukey test between OVX groups; *$p<0.05$.

4.3.9 Assessment of trabecular microarchitecture by microcomputed tomography

The five variables were computed to describe the microarchitecture of L4 vertebrae. As shown in Figure 4.12, OVX-C rats had less trabecular bone compared to Sham-C. Analysis of micro-CT data (Table 4.9) showed the OVX-C group underwent a significant reduction of trabecular bone volume fraction (20.2%; p=0.004), bone surface density (19.6%; p=0.002) and trabecular number (21.2%; p=0.001). In addition, OVX-C showed significant increases in trabecular separation (p=0.012) and structural index (p=0.003) when compared to Sham-C. There was no significant difference in trabecular thickness between OVX-C and Sham-C groups. Compared to OVX-C, all tomato groups showed slight although not statistically significant differences. 'Post-R' and 'Pre-R' increased BV/TV by 9.2 – 9.3%, increased BS/TV by 6.7 – 7.0%, increased Tb. N by 7.5 – 8.3%, and reduced Tb. Sp by 11.3 – 11.6. 'Post-MG' and 'Pre-MG' induced similar changes although at approximately half the magnitude, and increased BV/TV by 2.2 – 7.0%, increased BS/TV by 1.2 – 3.5%, increased Tb. N by 1.9 – 4.0%, and reduced Tb. Sp by 6.2 – 8.7%.

Table 4.9 *Ex-vivo* micro-CT analysis of the L4 vertebral body trabecular bones of female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

Group	Sham-C	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* values
BV/TV (%)	41.30±1.53**	32.25±1.56	35.26±0.68	35.23±0.77	32.95±0.57	34.50±0.67	0.180
BS/TV (1/mm)	13.96±0.25**	11.23±0.53	11.98±0.33	12.02±0.22	11.37±0.63	11.62±0.17	0.596
Tb. N (1/mm)	4.74±0.09***	3.74±0.18	4.02±0.11	4.05±0.08	3.81±0.22	3.89±0.06	0.531
Tb. Th (mm)	0.09±0.00	0.09±0.00	0.10±0.00	0.09±0.00	0.09±0.00	0.10±0.00	0.784
Tb. Sp (mm)	0.17±0.02*	0.25±0.02	0.22±0.01	0.22±0.00	0.25±0.01	0.26±0.01	0.375
SMI	-0.21±0.08**	0.23±0.08	0.23±0.07	0.20±0.05	0.25±0.09	0.22±0.05	0.658

Data are shown as mean ± SEM of n=6 rats/group. * in Sham-C column indicates significant difference between Sham-C and OVX-C by Student's t-test (*p<0.05, **p<0.01, ***p<0.001). Differences in OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test and the p values are given in the final column of the table. BV/TV, bone volume fraction; BS/TV, bone surface density; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; SMI, structure model index.

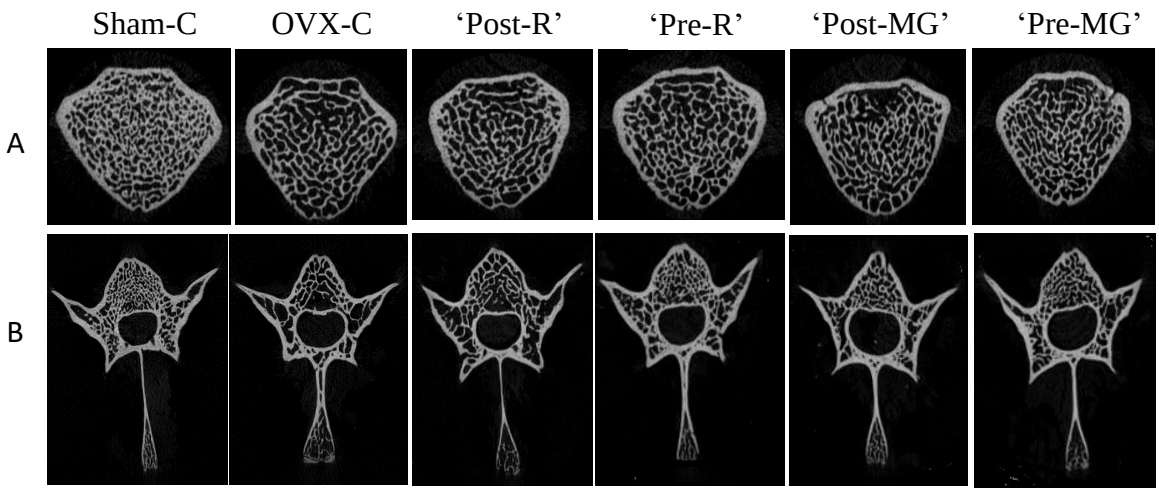

Figure 4.12 Representative two-dimensional images of (A) trabecular body and (B) reconstructed transaxial planes of L4 vertebrae of female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

4.4 Discussion

This study was carried out to compare the effects of red and 'Moonglow' tomato powders on bone loss in ovariectomized female rats. Bone mass was evaluated using i*n vivo* and *ex vivo* DXA, biomechanical analysis, bone turnover markers and bone microarchitecture to assess both preventive and protective effects. Ovariectomized rats were used as a model of postmenopausal osteoporosis in this study where it is considered as a well-established model to mimic estrogen deficient condition in humans (Kharode et al., 2008). Sham surgery rats have been using to mimic the placebo effect of ovariectomy and in particular to control for any short- or long-term effects that might result from the animals being subjected to general anaesthesia, surgical incisions, laparotomy and post-surgical analgesia and healing (Wolf and Buckwalter, 2006). However, a recent definitive study demonstrated no difference between sham-surgery and non-manipulated rats in terms of BMD and BMC (Kruger and Morel, 2016), which are the key parameters measured in osteoporosis models. Therefore, while some regulatory bodies such as the FDA still require rats in sham groups to undergo a sham surgery in which the ovarian fat pads are exteriorized and then replaced intact, studies not intended to form part of an application to a regulatory body need not include the sham surgery. The moral duty to minimize animal harm by "replacement, reduction and refinement" of animal use was recognized over half a century ago and has become a growing global issue (Lee et al., 2020). Avoiding unnecessary surgery in the sham group of rats minimizes the ethical impact on the animals, fulfilling the mandate known as the three Rs, and also ensures that staff time and resources are not wasted. In this study, the sham rats with intact ovaries were not subjected to any surgery. However, the control group was identified as "sham" due to reader familiarity with this terminology.

4.4.1 Effectiveness of the OVX rat model in inducing osteoporosis

Ovariectomized rats are considered a good preclinical animal model of post-menopausal osteoporosis for the evaluation of therapeutic agents (Jee and Yao, 2001). The age of the rat at OVX is an important factor to consider as it affects the osteoporotic response. Skeletally mature rats are considered appropriate for inducing a model of post-menopausal osteoporosis (Lelovas et al., 2008). In rats, two stages of skeletal growth have been standardized: mature at 3-6 months, and aged at >6 months (Kalu, 1991). The responsiveness of growing rats for OVX is higher than in older rats (Thompson et al.,

1995). Thus, most studies carry out ovariectomy surgery in rats aged 3-6 months to induce a stronger and more reproducible osteoporotic response (Kruger and Morel, 2016, Ardawi et al., 2016, Iimura et al., 2015, Mathavan et al., 2015, Francisco et al., 2011). The duration of assessment post-surgery is generally brief, as osteoporotic changes can be observed in as little as 4 weeks. According to FDA guidelines, OVX rat models for the evaluation of osteoporosis therapeutic agents should be restricted to a duration of less than 12 months and probably not more than 6 months (Thompson et al., 1995) as the skeletal changes become unclear and the cancellous bone turn over becomes similar to the sham control after a longer period after OVX (Thompson et al., 1995). Our study performed OVX surgery on rats aged 4 months and followed them post-surgery for additional two months.

The uterus size is highly dependent on estrogen levels in the body, and post-OVX rats have significantly reduced uteri weights compared to sham rats due to the uterus atrophying post-surgery (Lemini et al., 2015). Confirmation of a successful OVX surgery is therefore determined by final uterine weight. All Sham-C rats in this study had uterine weights of >0.5 g, whereas all OVX rats had uterine weights of <0.5 g except three rats; those three rats demonstrating incomplete OVX were discarded from the data set. The overall model was demonstrated to be successful as evinced by the OVX rats having significant decreases in femur and lumbar spine BMD, bone break stress, and significant increases in body weight, serum osteocalcin and CTx-1, and femur thickness, compared to ovary-intact control rats. These are discussed in more detail below.

4.4.2 Effect of tomato feeding on bone markers

Evaluation of bone turnover markers is a reliable, sensitive and non-invasive method to detect changes in bone remodelling in terms of bone formation and resorption (Shetty et al., 2016, Naylor and Eastell, 2012). Among the available biomarkers, serum osteocalcin (OC) and carboxy-terminal crosslinks telopeptide of type 1 collagen (CTx-1) are most commonly reported in bone-related research (Ardawi et al., 2016, Wheater et al., 2013, Eastell and Hannon, 2008). CTx-1 is a sensitive marker of bone resorption, and the circulating levels of CTx-1 increase as a result of increased bone resorption (Shetty et al., 2016, Coleman, 2002). Osteocalcin is a bone-specific protein produced by osteoblasts and an increased level of osteocalcin reflects higher rates of bone formation (Garnero, 2008). However, serum osteocalcin is elevated after menopause because the bone matrix

releases osteocalcin during bone resorption, and this contributes to the total amount of osteocalcin in the circulation (Kini and Nandeesh, 2012, Ivaska et al., 2004). Therefore, osteocalcin is regarded as a sensitive biomarker of bone resorption after ovariectomy (Ardawi et al., 2016, Gao et al., 2014) and in this context is considered to be a bone turnover biomarker rather than indicating healthy bone formation (Clarke, 2008, Ivaska et al., 2004).

In clinical studies, CTx-1 measurements have been shown to vary with circadian changes, reaching peak levels in early morning and falling to their lowest in the afternoon (Qvist et al., 2002, Hannon and Eastell, 2000). Fasting status also affects the variation in serum CTx-1, with post-prandial CTx-1 levels being lower when compared to fasting state (Clowes et al., 2002). To a lesser extent, bone formation markers such as osteocalcin also show circadian variation both in humans and animals (Heshmati et al., 1998). Morning blood collection after overnight fasting is recommended for the measurement of bone turnover markers (Clowes et al., 2002). For this reason, the rats in this study were fasted overnight and blood samples were collected as early in the day as possible. Early versus late morning blood collections were spread evenly across all treatment groups to avoid circadian changes selectively impacting the results of a test group.

In our study, both CTx-1 (p=0.038) and osteocalcin (p=0.018) were significantly reduced in Sham-C rats compared to OVX-C. These results are in line with the reported increase in bone turnover in ovariectomized rats (Srivastava et al., 2000, Wronski et al., 1988) and in human studies where both osteocalcin and CTx-1 increased after menopause (Park et al., 2018, Haryono et al., 2017, Takahashi et al., 1999, Garnero et al., 1996). Interestingly, serum osteocalcin concentration in OVX rats in both 'Moonglow' groups and the 'Pre-R' group was significantly lower than that of OVX-C. In addition, all tomato groups had reduced levels of CTx-1 to near-normal concentrations compared to OVX-C.

Our results are in agreement with other studies which assessed the effect of lycopene on bone turnover markers in ovariectomized rats. A study by Liang *et al* reported that lycopene at 20-40 mg/kg BW dose-dependently reduced the CTx-1 levels compared to OVX control (Liang et al., 2012). Similarly, Ardawi reported that lycopene doses of 30 and 45 mg/kg BW significant reduced bone turnover (Ardawi et al., 2016). Interestingly, our results indicate that 'Moonglow' tomato fed either after or before OVX significantly reduced bone turnover, whereas a matching dose of red tomatoes had a

similar effect only if it was given before OVX. This finding suggests that even for women who did not consume tomatoes in earlier life, introducing 'Moonglow' tomato supplementation at the time of menopause may at least partially protect against post-menopausal bone loss as seen by the reduction of bone turnover markers.

4.4.3 Effect of tomato feeding on bone mineral measures

In vivo serial DXA measures were not sensitive enough to detect any significant differences between groups for femoral and lumbar spine BMD. Therefore, we evaluated the proportional change over consecutive time points as a secondary analysis. Use of percent changes has also been reported in previous studies as a better indication to evaluate the differences between groups in both animal (Tousen et al., 2014, Poulsen and Kruger, 2006) and human studies (Alekel et al., 2000, Pruitt et al., 1995), since it reduces inter-subject variability by enabling each individual's readings to be normalised to their own baseline. As expected, OVX-C rats showed a marked decrease in percent change in BMC and BMD compared to Sham-C rats for lumbar spine and femur. These results are consistent with previous studies (Tousen et al., 2014, Tousen et al., 2012) and support the paradigm that the significant decrease of bone mineral measures after ovariectomy surgery, paralleled by elevated bone turnover, is due to estrogen deficiency (Kharode et al., 2008, Jee and Yao, 2001, Kalu, 1991). However, no significant differences were observed between OVX treatment groups, and no protective effects of tomato on BMD post-OVX were found. This contrasts with several studies that found a significant increase in BMD in OVX rats fed with lycopene at 10-15 mg/kg BW/day for 8-12 weeks (Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014, Liang et al., 2012). Those reported doses are 30- to 40-fold higher than our tested dose of lycopene (0.35 mg/kg BW/day). It is likely that the more physiologically relevant but markedly lower dose used in our study contributed to the lack of an observed significant tomato effect on BMD.

4.4.4 Assessment of trabecular microarchitecture by microcomputed tomography

Microcomputed tomography (micro-CT) is a sensitive, non-destructive method which can be used to assess bone microarchitecture (Bouxsein et al., 2010). We evaluated the microarchitecture of rat L4 vertebrae. A study which evaluated the microarchitecture of femur and vertebrae sections found reduced bone mass in ovariectomized rats compared to sham rats (Palumbo et al., 2009, Laib et al., 2001). Similarly, we also found

significant deterioration of bone microarchitecture in OVX rats compared to sham rats which indicated that our model successfully induced osteoporosis. We did not see any statistically significant differences between tomato treatment groups for the variables assessed.

The volume of the vertebral bodies (L1-L5) is the highest among thoracic and caudal spinal segments where more bone is sampled. Therefore, it is recommended to use lumbar spine for the evaluation of bone microarchitecture (Bouxsein et al., 2010). Several studies have used L4 for microarchitecture as a representative segment of lumbar vertebrae in rats (Morton et al., 2018, Wu et al., 2015, Yoon et al., 2012, Abe et al., 2007), as no significant differences were found between L4 and L5 in terms of microarchitecture (Wu et al., 2015). Interestingly, it has also been reported that both site specificity and the age of female rats at the time of OVX affects the changes and the severity of the deterioration of bone microarchitecture upon ovariectomy (Francisco et al., 2011). Trabeculae in the spine become thinner in OVX rats compared to sham whereas the results are reversed in tibia (Kishi et al., 1998). Older rats who underwent OVX at age 24 and 44 weeks had osteoporotic responses in all sites where only the spine of younger rats who underwent OVX at 12 weeks of age showed osteoporotic responses similar to older rats (Francisco et al., 2011).

We evaluated the trabecular regions of lumbar spine, because the decrease of trabeculae bone mass starts earlier than in cortical bones (Ferretti et al., 2010) and is also more comparable with human studies of osteoporosis (Thompson et al., 1995). Similar to postmenopausal women, bone loss in ovariectomized rats is mainly due to trabecular bone loss (Kim et al., 2011). In order to support a normal mechanical load, bone tissue compensates for the loss of bone mass caused due to ovariectomy which is why, as expected, no significant difference was found in trabecular thickness between OVX-C and Sham-C (Rhee et al., 2009). However, OVX-C rats had significantly higher SMI compared to Sham-C, which indicates the flattening of concave trabeculae and possible reformation into rod-like structures at the onset of osteoporosis induced by ovariectomy (Ahmad et al., 2017, Mathavan et al., 2015, Boyd et al., 2006).

While no statistically significant effects of tomato supplementation on bone microarchitecture were observed, red tomato and to a lesser degree ‘Moonglow’ tomato reduced the decrease in BV/TV, BS/TV, and Tb. N, and reduced Tb. Sp. It is likely that

these beneficial effects were due to the lycopene in the tomato powder. As reported by Ardawi *et al* lycopene supplementation at 15-45 mg/kg BW per day significantly increased bone volume, trabecular number and thickness while significantly reduced trabecular separation (Ardawi et al., 2016). Compared to this study the lycopene dose that we used (0.35 mg/kg BW) was approximately 100-fold lower but still showed a positive trend in many variables tested, which suggests that a higher dose may provide clinically important improvement.

4.4.5 Effect of tomato feeding on biomechanical properties of bones

Measuring biomechanical properties of bones is considered a highly accurate parameter to assess the resistance of bone for fractures caused by external forces (Havill et al., 2014, Kim et al., 2004). A bone bending test can measure properties of whole bone that can be described using different variables (Ferretti, 1995). We used a three-point bending test to evaluate whole bone strength using six different variables: break force, break stroke, break stress, break strain, elasticity and energy. A detailed discussion of each of these variables can be found in Appendix i, Figure 17.

Compared to the Sham-C, OVX-C rats had larger femurs that were significantly wider and thicker at the midpoint. However, their bones, while larger, demonstrated a significantly lower break stress: this is the most relevant biomechanical parameter, as a higher break stress directly correlates with stronger, more resilient bone. Elasticity was also lower in the OVX-C rats compared to Sham-C, although this did not reach statistical significance. Wider, thicker femurs with reduced elasticity and break stress have been reported elsewhere in OVX rats (Kruger et al., 2009, Kruger et al., 2008, Kruger et al., 2006, Kruger et al., 2005). We did not observe the expected decrease in other parameters such as energy and break stroke, but this may be due to the high degree of variation of low bone mass in rats after ovariectomy, compounded by the fact that the femurs that we used for three point bending tests are mainly composed of cortical bone (Clarke, 2008) which has slower bone turnover than trabecular bone (Jee and Yao, 2001). There is a reported increase in femoral bone strength during the three month period after ovariectomy, followed by a decline afterwards, when rats undergo ovariectomy surgery between 3-6 months of age (Shimizu et al., 2018, Yoshitake et al., 1999, Mosekilde et al., 1993, Danielsen et al., 1993). One suggested reason for this transient increase in bone strength following ovariectomy is an increased rate of periosteal bone formation and bone

matrix apposition of femoral bone (Aerssens et al., 1993, Turner et al., 1990, Turner et al., 1989, Turner et al., 1987), as it is known that adult rats, unlike humans, retain growth plate function (Roach et al., 2003). It may also be explained by increased mechanical loading of bone due to increased weight gain (Iwaniec and Turner, 2016, Shapses and Sukumar, 2012). We did not observe significant difference in bone biomechanics between OVX-C and tomato-fed rats. There are few studies demonstrating significant effects of lycopene on bone strength, and in these the lycopene was administered at doses of 20-45 mg/kg BW/day for 8-12 weeks (Ardawi et al., 2016, Liang et al., 2012). The low doses and the feeding time we used during this study were insufficient to induce significant changes in bone strength, but it would be of interest to compare lycopene from red versus 'Moonglow' tomatoes at higher doses for a longer duration to assess their relative effects on bone biomechanics.

4.5 Conclusions

Both red and 'Moonglow' tomatoes reduced bone turnover in rats with simulated post-menopausal bone loss. However, the benefits of 'Moonglow' feeding on the reduction of bone turnover was observed even after OVX-initiated osteoporosis. These results demonstrate that 'Moonglow' *cis*- lycopene is more effective than red tomato *trans*- lycopene in reducing bone turnover after estrogen loss. This is the first report demonstrating effects of tomato lycopene on bones at such a low dose (0.35 mg/kg BW/day); prior to this the lowest effective reported dose in the rat OVX model was 10 mg/kg BW/day (Walallawita et al., 2020). Our dose was insufficient to induce the measurable improvements in femur or lumbar spine BMD reported with 30-40-fold higher doses (10 – 15 mg/kg BW) and the results suggest that low, physiologically beneficial doses of 'Moonglow' tomato lycopene could reduce bone turnover even when intake is initiated after menopause. Studies with higher doses are required to confirm and possibly lead to improvements in the other bone parameters. Further investigation with clinical trials is also warranted to determine how well findings in animal models will extrapolate to humans.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Umani Walallawita
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Involved in planning and designing of animal trials, rat treat preparation, feeding and handling, carried out all experiments and data analysis,compiled and interpreted the results, reviewed and co-edited.

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CHAPTER 5

Changes in plasma and liver lycopene, body composition and gut bacteria following red versus 'Moonglow' tomato feeding in ovariectomized rats

Abstract

Cis- isomers of lycopene have been reported to be more bioavailable than all-*trans*-lycopene. 'Moonglow' is an orange heirloom tomato variety with >90% of its lycopene in the more bioavailable *cis*-isomeric form, compared to red tomatoes with all *trans*- lycopene. Estrogen deficiency after menopause changes body composition and gut microbes. This study evaluated differences in lycopene stability, bioavailability and retention, and lycopene effects on body composition and gut microbiota, by comparing red versus 'Moonglow' tomato feeding in adult female ovariectomized rats. Female Sprague-Dawley rats underwent no surgery (sham) or ovariectomy (OVX) surgery at age 16 weeks to induce osteoporosis. Sham-C and OVX-C groups received a daily dietary supplement 'treat' containing no tomato powder; 'Post-R' and 'Post-MG' received tomato for 8 weeks post-surgery; 'Pre-R' and 'Pre-MG' received tomato for 8 weeks prior to surgery plus 8 weeks post-surgery (n=12-15/group), with the tomato powder incorporated into the supplements. Rats were fed tomato powder containing 0.172 mg lycopene (~ 0.35 mg lycopene/kg body weight/day). After 8 or 16 weeks of tomato supplementation, mean plasma lycopene concentrations in 'Pre' and 'Post' 'Moonglow' groups were ~8X higher than 'Pre' and 'Post' red groups, but liver lycopene stores did not differ. Caecal pH in rats ranged from 6.79 ± 0.08 to 7.05 ± 0.11 and was not significantly different between groups. Ovariectomy reduced the abundance of gut bacteria; both 'Pre-R' and 'Pre-MG' restored the numbers of bacteria in all five genera. 'Post-MG' increased the *Lactobacillus, Enterococcus, Bacteroides* and *E. coli* whereas 'Post-R' only increased *Lactobacillus*. A significant increase in fat mass and reduction of lean mass was found in OVX rats compared to Sham-C after 16 weeks, and individual fat pad weights strongly correlated with total body fat. These results demonstrate that 'Moonglow' *cis*- lycopene is significantly more bioavailable than red *trans*- lycopene and 'Moonglow' tomato has a more consistent prebiotic- like effect.

5.1 Introduction

Lycopene has recently gained attention of researchers due to its beneficial health effects against certain non-communicable diseases (Fraser et al., 2020, Zeng et al., 2019, Ferreira-Santos et al., 2018, Iimura et al., 2014, Viuda-Martos et al., 2014). Consumption of lycopene-rich foods can increase total lycopene in the plasma and body tissues (Liu et al., 2009, Unlu et al., 2007b). Tomatoes and tomato-based products represent more than 80% of dietary sources of lycopene (Storniolo et al., 2019, Shen et al., 2012). Lycopene exists in two major isomeric forms: all *trans*- and various *cis*- (Burri et al., 2009). Conventional red tomatoes contain >90% lycopene in the all *trans*- isomer form (Singh and Goyal, 2008). However, *cis*-lycopene has been found to be 8.5 times more bioavailable than all *trans*- (Cooperstone et al., 2015) and orange heirloom ('Moonglow') tomatoes naturally contain >90% lycopene in *cis*- isomeric forms (see chapter 3.3.1).

Regardless of the isomeric forms present in food, mammalian plasma and solid organ tissues contain more *cis*-lycopene isomers (Boileau et al., 2002, Schierle et al., 1997). Initially it was unclear whether endogenous all *trans*- to *cis*- isomerization, or bioavailability differences in different isomers from food, were responsible for the higher amount of *cis*- isomers observed in human plasma and tissues. However, a kinetic study revealed that endogenous all *trans*- to *cis*- isomerization is the major cause for the high amount of *cis*-isomers present in body tissues (Moran et al., 2015). Similarly, our previous study (chapter 3) showed that the changes in isomeric composition in the liver compared to the diet. The physiological mechanisms responsible for *in vivo* isomerization of lycopene are not fully understood yet. Recent *in vitro* studies have begun to identify locations where isomerization could happen in the body (Chen et al., 2014, Richelle et al., 2012, Moraru and Lee, 2005, Tyssandier et al., 2003, Re et al., 2001).

Menopause is a turning point at which women experience an increased frequency of certain non-communicable diseases (Navarro-Pardo et al., 2017). Changes in body composition via accelerated gain in fat mass are reported after menopause (Greendale et al., 2019, Kozakowski et al., 2017). Estrogen regulates the expression of enzymes involved in glucose/energy metabolism (Lizcano and Guzmán, 2014, Hart-Unger and Korach, 2011) and its deficiency after ovariectomy transiently increases food intake by regulating the activity of hormones involved in appetite (Lizcano and Guzmán, 2014). Studies have also found a relationship between changes in gut bacteria and menopause

(Rettedal et al., 2021, Kaliannan et al., 2018, Cox-York et al., 2015). Gut microbes play a vital role in estrogen metabolism and a strong corelation between gut microbes and the progression of metabolic diseases has been found both in humans and animals (Zhang et al., 2013, Howitt and Garrett, 2012, Larsen et al., 2010, Joyner et al., 2000). Further, gut microbes are also involved in the modulation of bone metabolism (Rettedal et al., 2021). Prebiotics can stimulate the growth of gut microbiota (Markowiak and Śliżewska, 2017) and, interestingly, studies have found a prebiotic-like effect of tomatoes, or lycopene, which modulated gut bacteria (Wiese et al., 2019, García-Alonso et al., 2017).

To date there are no published studies that have been carried out to compare the lycopene levels in the body after intake of red versus orange 'Moonglow' tomatoes for long term. Also, no studies have evaluated the gut microbiota and body composition of ovariectomized (OVX) rats after supplementation with different isomeric composition of lycopene. This study aimed to evaluate the differences in lycopene content in the plasma and liver following red versus 'Moonglow' tomato feeding in adult female OVX or ovary-intact (Sham) rats. We also evaluated the changes of body composition and gut bacteria following tomato feeding to compare prebiotic-like properties of red and 'Moonglow' tomatoes.

5.2 Material and methods

Tomatoes were obtained as described in chapter 3.2.1. Chemicals used for carotenoid extraction and HPLC analysis were described in 3.2.2. Tomato carotenoid extraction and analysis were described in chapter 3.2.7. Animal, diet, and treat preparation were described in 4.2.2. & 4.2.3. All the experimental techniques were described in chapter 4.2.4.

5.2.1 Carotenoid extraction from rat plasma

Plasma carotenoids were extracted using the methods previously described (Cooperstone et al., 2017, Kopec et al., 2010). Briefly, plasma (500 µL-1 mL) was added to the same volume of ethanol with 0.1% BHT and 0.5 mL of saturated NaCl (10 g NaCl in 100 mL MQ water). The mixture was vortexed for 15 s followed by addition of 2 mL of 10:6:7:7 (v/v/v/v) hexane: ethanol: acetone: toluene (HEAT). The mixture was vortexed again for 15 s and centrifuged at 1,000 *g* for 5 min at -20^{0}C (Allegra 64R, Beckman Coulter, NSW, Australia). The top layer was transferred to a new Kimax tube

using a soda lime Pasteur pipette. Each biological sample was extracted thrice, and extracts were pooled together. The extracts were dried under nitrogen gas and stored at -20^{o}C until analysis, which was carried out within two days. Samples were re-dissolved in 75 µL of tetrahydofuran: methanol (1:3) before analysis. Frozen livers were freeze-dried (Cuddon FD18, Blenheim, New Zealand). Drying pressure was maintained between 0.1-0.3 mBar during the run. Vacuum temperature was -30^{0}C to -40^{0}C.

5.2.2 Carotenoid extraction from supplement treats and rat liver

Liver and supplement treat carotenoids were extracted using the methods described previously (Conlon et al., 2012, Zaripheh et al., 2003, Boileau et al., 2000). Briefly, frozen livers/supplement treats were freeze-dried (Cuddon FD18, Blenheim, New Zealand). Drying pressure was maintained between 0.1-0.3 mBar during the run. Vacuum temperature was -30^{0}C to -40^{0}C. Freeze-dried livers/ supplement treats were crushed to a powder using a porcelain mortar and pestle. Representative samples of 0.1 g of freeze-dried liver were dissolved in 3 mL of KOH (60%) /ethanol (1:5) solution containing 1 g BHT/L. Samples were vortexed and kept in a water bath at 60^{0}C for 30 min for complete saponification. Samples were again vortexed after adding hexane (3 mL) and distilled water (1 mL) and centrifuged at 1,000 *g* for 5 min (Allegra 64R, Beckman Coulter, NSW, Australia). Top layer was collected as mentioned previously. Samples were extracted thrice, and dried extracts were stored at -20^{o}C until analysis, which was carried out within two days. Samples were re-dissolved in 100 µL of THF: methanol (1:3) before analysis by HPLC. Weights of liver/supplement treats were measured before and after freeze drying. Lycopene concentration was measured in 0.1 g of freeze-dried liver/ supplement treats and back calculated to fresh weights.

5.2.3 Evaluation of caecal pH

Caecal samples were thawed to room temperature and the pH of each caecal sample was tested using a pH meter (HANNA-HI99121, Woonsocket, USA). Triplicate measurements were taken from each sample by inserting the electrode of pH meter into the caeca.

5.2.4 DNA extraction from rat caeca

Frozen caecal samples were kept at 5^{o}C overnight to thaw. The following day, DNA was extracted from caeca using the Bioline Isolate II Fecal DNA Kit according to

the manufacturer's instructions. Briefly, caecal content (150 mg) was mixed with 750 µL of lysis buffer in a bashing beads lysis tube. Tubes were fixed in a vortex mixer (Total Lab Supply, Auckland, New Zealand) and processed at maximum speed for 5 min. Lysis tubes were centrifuged in a microcentrifuge (Eppendorf Hamburg, Germany) at 10,000 *g* for 1 min. The supernatants (400 µL) were transferred to a spin filter, placed in a collection tube and centrifuged at 7000 *g* for 1 min. DNA binding buffer (1200 µL) was added to the filtrate in the collection tube. The mixtures (800 µL) were transferred to a spin column in a collection tube and centrifuged at 10,000 *g* for 1 min. Flow through from the collection tube was discarded and the previous step was repeated for the remaining mixture. DNA pre-wash buffer (200 µL) was added to a spin column and centrifuged at 10,000 *g* for 1 min. DNA wash buffer (500 µL) was added to a spin column and centrifuged at 10,000 *g* for 1 min. Spin column was transferred to a clean 1.5 mL microcentrifuge tube and DNA elution buffer (100 µL) was added directly to the column matrix. Tubes were then centrifuged at 10,000 *g* for 30 seconds to elute DNA. Eluted DNA was then transferred to a spin filter in a clean 1.5 mL microcentrifuge tube and centrifuged at 8000 *g* for 1 min. DNA concentration was measured using nanodrop at 260 nm (IMPLEN Nanophotometer P300, München, Germany) according to the method described in the user guide and the samples were kept at -80^{0}C until use.

5.2.5 Real-time quantitative PCR

All PCR experiments were performed in duplicate in 96 well plates using a Light cycler 480 (Roche Diagnostics International AG, Rotkreuz, Switzerland). The targeted bacteria, annealing temperatures and primer sequences are given in Table 5.1. A total volume of 20 µL/tube was composed of 10 µL Light cycler 480 SYBR green master (Roche Diagnostics, Mannheim, Germany), 1 µL of each primer and 2 µL of template DNA. The total reaction consisted of four stages as pre-incubation, amplification, melting curve and cooling. Pre-incubation consisted of 95^{0}C for 10 min, 40 cycles of 95^{0}C for 15 sec, annealing temperature for 1 min, then extension at 72^{0}C for 30 sec. Melting curve analysis was carried out to evaluate the specificity of the PCR reaction following amplification by increasing temperature to 95^{0}C for 5 sec and then cooling to 65^{0}C for 1 min. Standard curves were prepared from pure DNA of each bacterial group using 10-fold serial dilutions. The amount of DNA was quantified using a standard curve generated for each DNA.

Table 5.1 Target bacterium, primer sequences and the annealing temperature.

Target bacterium	Primers	Annealing temperature
Lactobacillus	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	56^0C
Bifidobacterium	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	60^0C
Enterococcus	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	50^0C
Bacteroides/ Prevotella	F: GAAGGTCCCCCACATTG R: CAATCGGAGTTCTTCGTG	60^0C
E. coli	F: GTTAATACCTTTGCTCATTGA R: ACCAGGGTATCTAATCC TGTT	51^0C

5.2.6 Evaluation of body composition

Body composition data including fat mass, fat percentage and lean mass parameters were measured as part of the DXA scanning procedures described in chapter 4.2.4.1.

5.2.7 Statistical analysis

Results are presented as means with standard error of mean (mean ± SEM). All statistical analyses were conducted using SPSS statistical software version 25. All data were tested for normality using the Shapiro-Wilk test. Data were log transformed when the normality assumption was violated. Homogeneity of variance was tested using Levene's test. The differences between groups were compared using one-way ANOVA followed by post-hoc Tukey test. Welch test was used in lieu of one-way ANOVA when the assumption of homogeneity of variances was not met and when the sample size was not equal, and this test was followed by Games-Howell post-hoc test. For body composition analysis, repeated measures of ANOVA were conducted separately for pre-surgery period (week 0-6) and post-surgery period (week 6-16). A secondary analysis focused on percent change in body composition measures between weeks 0-6, 6-12, 12-16 and 6-16 and were analysed using one-way ANOVA and post-hoc Tukey tests. Pearson correlation coefficient (R) test was performed to determine the relationship between whole body fat and individual fat pads. A difference was considered as statistically significant when $p<0.05$.

5.3 Results

5.3.1 Lycopene content in red and 'Moonglow' tomatoes

Lycopene content in fresh and freeze-dried red versus 'Moonglow' tomatoes is given in Table 5.2. Dry matter content of red (5.9%) was significantly lower ($p<0.001$) than 'Moonglow' (7.0%) tomatoes. Red and 'Moonglow' tomatoes contained 16.1 and 11.8 mg lycopene/100 g fresh weight equivalent respectively with red being significantly ($p<0.001$) higher than 'Moonglow'. Lycopene content reduced to 10.9 (red) and 9.3 ('Moonglow') mg/100 g after freeze drying and the decrease was significantly different ($p<0.05$). Red tomatoes had significantly higher ($p<0.0001$) levels of all *trans*- lycopene (15.04 ± 0.15) compared to 'Moonglow' (0.10 ± 0.03) whereas 'Moonglow' contained significantly higher ($p<0.0001$) amounts of *cis*-lycopene (11.70 ± 0.46) than red tomatoes (1.01 ± 0.01). After freeze drying the amount of all *trans*- in red and amount of *cis*-lycopene in 'Moonglow' was significantly reduced ($p<0.0001$).

Table 5.2 Isomeric composition of frozen and freeze dried red and 'Moonglow' tomatoes.

Lycopene isomer	**Red tomato**		**'Moonglow' tomato**	
	Frozen (mg/100g fresh weight)	Freeze-dried (mg/100g fresh weight)	Frozen (mg/100g fresh weight)	Freeze-dried (mg/100g fresh weight)
All *trans*-	15.04 ± 0.15	10.30 ± 0.34	0.10 ± 0.03	0.08 ± 0.00
Cis-	1.01 ± 0.01	0.61 ± 0.13	11.70 ± 0.46	9.21 ± 0.08

Data are presented as mean ± SEM of three replicates. Statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. Tomato chromatograms are included in Appendix i, Figure 7-8.

5.3.2 Lycopene in the diet

Rats were fed with standard commercial chow diet *ad libitum* throughout the study period and the proximal analysis of the rat chow diet is given in Table 4.2 of this thesis. Dietary supplement treats were formulated based on the lycopene content in freeze-dried tomato powder. Control, red and 'Moonglow' tomato supplements were extracted and analyzed for lycopene. Control supplements did not contain any lycopene. Appropriate weights of freeze-dried tomatoes were mixed with wheat flour, peanut butter and honey as described in Section 4.2.2. As this trial ran for four months, we analyzed the lycopene

once a month and prepared a new batch of supplement treats once a month. Rats were provided with their respective treats (control/ red /'Moonglow') daily between 9 a.m. and 10 a.m. for five days per week (Monday to Friday). According to the initial analysis, red tomato powder had 94% all *trans*- and 6% *cis*- in freeze-dried tomato powder while 'Moonglow' had 1% all *trans*- and 99% *cis*-. However, after the powder was incorporated into the diet, the red tomato isomer proportions remained unchanged while 'Moonglow' had slightly altered to 6% all *trans*- and 94% *cis*- (Table 5.3).

Table 5.3 Isomeric composition of tomato diets.

Lycopene	Red tomato treats (mg/kg Diet) [a]	'Moonglow' tomato treats (mg/kg Diet) [b]
All *trans*- lycopene	157.10 ± 13.78	9.33 ± 1.45
Cis- lycopene	9.67 ± 0.83	158.97 ± 8.82
Total lycopene	166.77 ± 14.36	168.30 ± 8.81

[a] Values are presented as means ± SEM in 9.207 g red tomato powder.
[b] Values are presented as means ± SEM in 12.827 g 'Moonglow' tomato powder.

5.3.3 Plasma lycopene concentration

As expected, the plasma of ovary-intact rats which received control treats with no tomato powder (Sham-C) and ovariectomized rats who received control treats with no tomato powder (OVX-C) contained no measurable lycopene. Plasma lycopene concentrations in 'Post-R', 'Pre-R', 'Post-MG' and 'Pre-MG' were $5.45x10^{-3}$ ± 0.001, $5.15x10^{-3}$ ± 0.001, $43.01x10^{-3}$ ± 0.014, $39.75x10^{-3}$ ± 0.006 µmol/L respectively. With the selected dose (0.35 mg/kg BW), we could detect very minute amount of lycopene in plasma (Figure 5.1 (a) & Table 5.4). Despite all supplemented rats receiving equivalent amounts of total lycopene, plasma lycopene concentrations of both 'Pre' and 'Post' 'Moonglow' fed groups were significantly (p<0.0001) higher than red tomato groups. After 8 or 16 weeks of tomato supplementation, mean plasma lycopene concentrations in 'Pre' and 'Post' 'Moonglow' groups were ~8X higher than 'Pre' and 'Post' red groups. We also calculated the total lycopene in whole plasma of rats (Figure 5.1(b)). Approximate plasma volume was calculated based on each rat's body weight (Bijsterbosch et al., 1981).

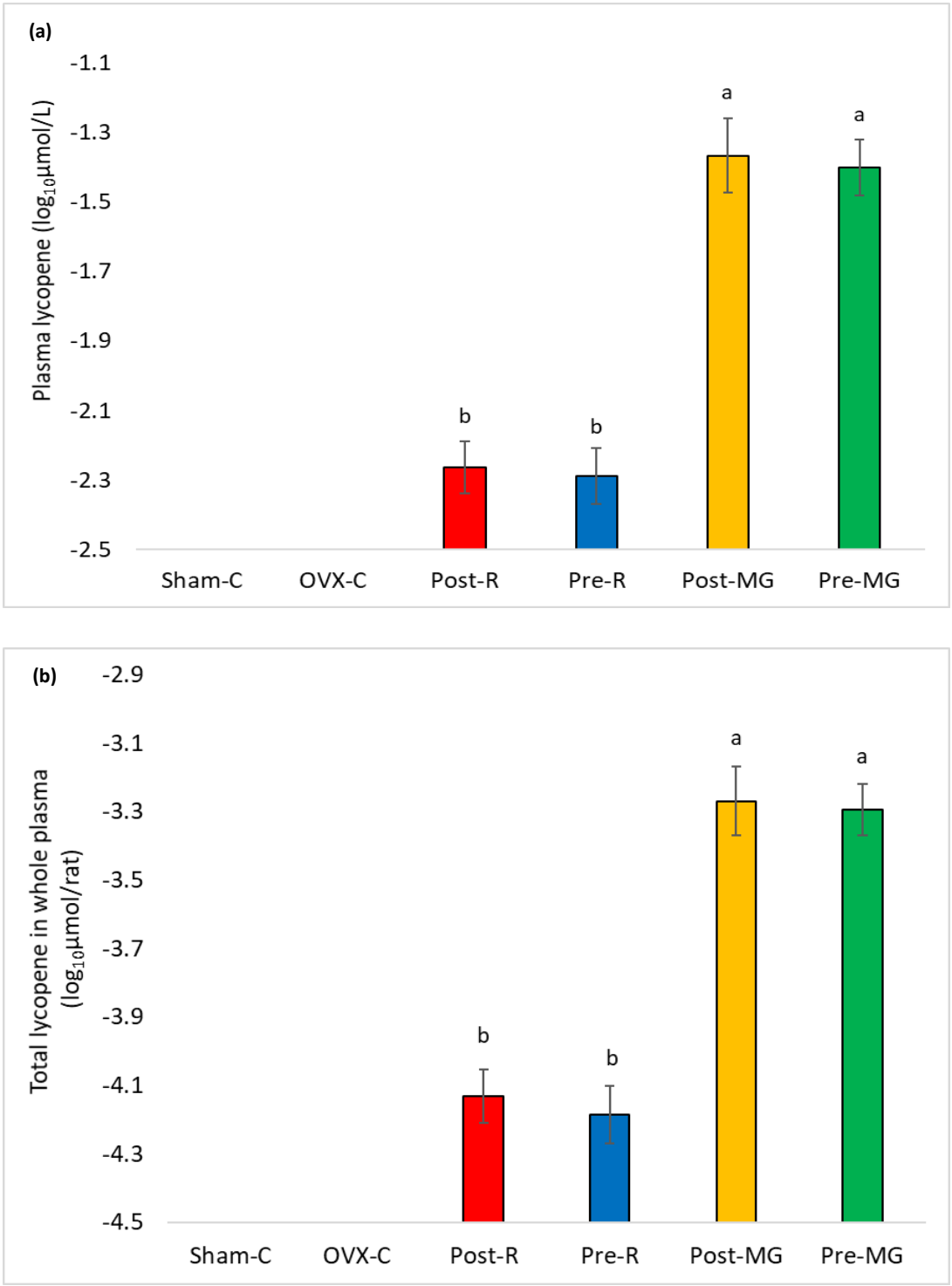


Figure 5.1 (a) Plasma lycopene concentration and (b) calculated total lycopene in whole plasma extrapolated by body weight following red and 'Moonglow' tomato feeding for 16 weeks. Data are shown as mean ± SEM (n=12-15). Different letters indicate significant difference (p<0.05) as determined by one-way ANOVA and post-hoc Tukey test.

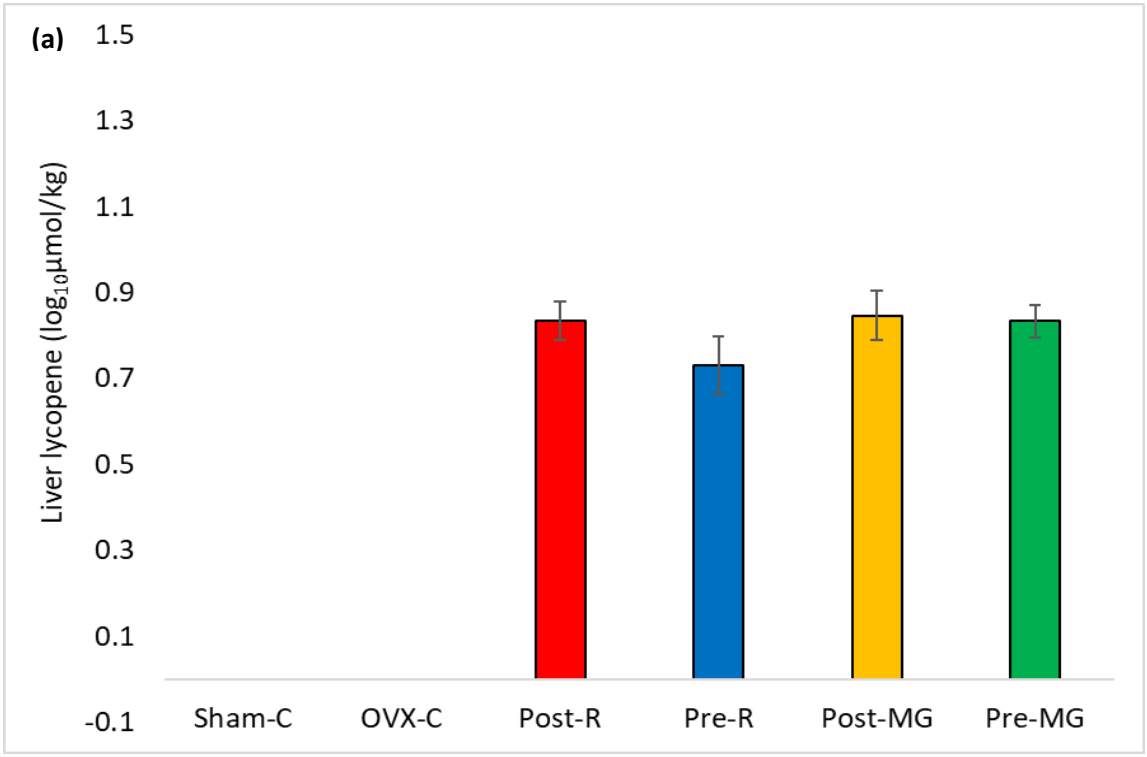
Table 5.4 Isomeric composition of plasma lycopene.

Lycopene isomers	Plasma (µmol/L)					
	Sham-C	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'
Cis-lycopene	n. d.	n. d.	n. d.	n. d.	$43.01 \times 10^{-3} \pm 0.014$	$39.75 \times 10^{-3} \pm 0.006$
All *trans*-lycopene	n. d.	n. d.	$5.45 \times 10^{-3} \pm 0.001$	$5.15 \times 10^{-3} \pm 0.001$	n. d.	n. d.

Data represent mean ± SEM, n=12-15 rats/group. Statistical analysis was carried out amongst the four tomato treated groups using one-way ANOVA ; n.d. = not detectable.

5.3.4 Liver lycopene

Mean liver weights for 'Post-R', 'Pre-R', 'Post-MG' and 'Pre-MG' were 9.68 g, 9.13 g, 8.81 g and 9.04 g respectively and did not differ between the groups. Liver lycopene accumulation was not significantly different between red and 'Moonglow' tomato groups (Figure 5.2 (a)). Yet, all *trans*- lycopene isomer concentrations were significantly ($p<0.00001$) higher in red than in 'Moonglow' tomato groups. Also, *cis*-isomer concentrations were significantly higher ($p<0.00001$) in 'Moonglow' compared to red tomato groups. 'Post-R' and 'Pre-R' groups showed 72 and 76% of lycopene in all *trans*- isomeric form and 28 and 24% in *cis*- isomeric forms respectively. 'Post-MG' and 'Pre-MG' groups showed 38 and 32% of lycopene in all *trans*- isomeric form and 62 and 68 % in *cis*- isomeric forms (Table 5.5). Total lycopene in the liver was calculated using whole liver weight (Figure 5.2 (b)).



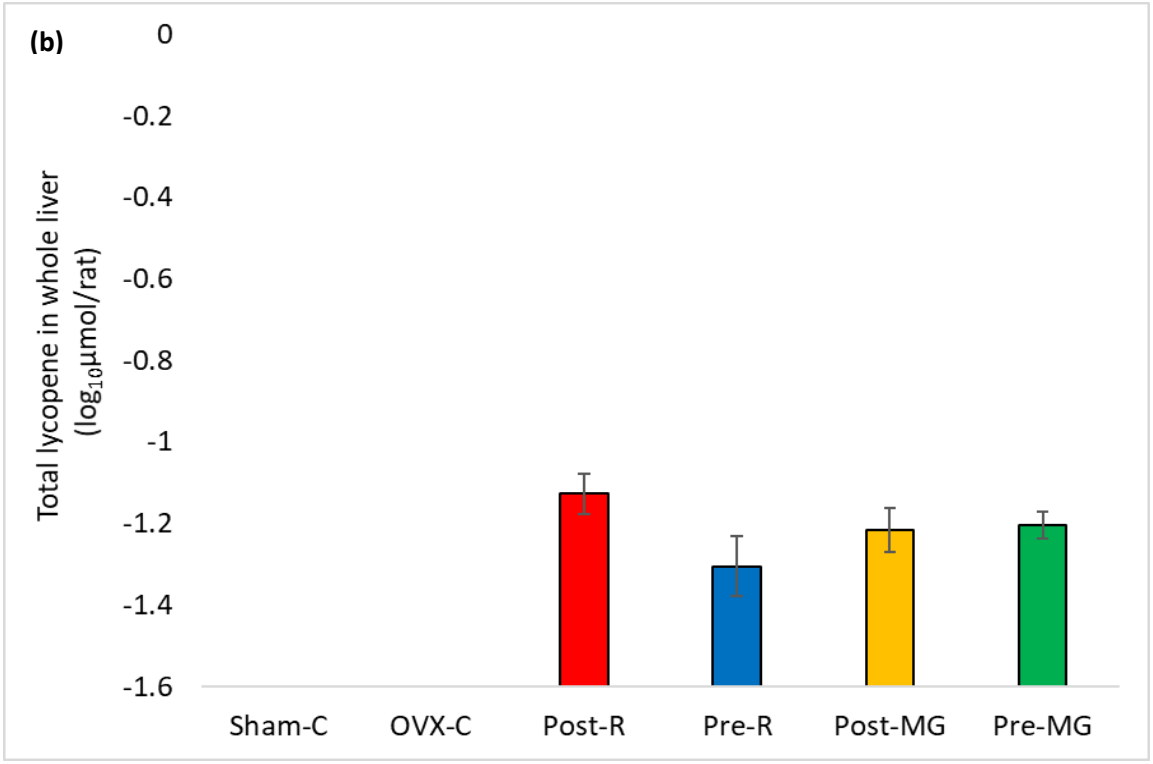


Figure 5.2 (a) Liver lycopene concentration and (b) total lycopene in whole liver following red and 'Moonglow' tomato feeding for 16 weeks. Data shown as mean ± SEM (n=12-15).

Table 5.5 Isomeric composition of liver lycopene.

Lycopene isomers	Liver (µmol/kg)					
	Sham-C	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'
Cis-lycopene	n. d	n. d	1.92 ± 0.33[b]	1.33 ± 0.31[b]	4.23 ± 0.91[a]	4.71 ± 0.47[a]
All-*trans*-lycopene	n. d	n. d	4.82 ± 0.67[a]	4.20 ± 0.65[a]	2.59 ± 0.31[b]	2.22 ± 0.26[b]

Data represent mean ± SEM, n=12-15 rats/group. Statistical analysis was carried out between four tomato test groups using one-way ANOVA. Different letters in the same row indicates significant difference from others.

5.3.5 Abundance of gut microbiota

As presented in Figure 5.3, tomato consumption resulted in a significant increase in the five bacterial genera evaluated in this study. Abundance of *Lactobacillus* was not significantly different between Sham-C and OVX-C groups; however, all four tomato treatment groups significantly (p=0.004) increased the *Lactobacillus* population compared to OVX-C. *Bifidobacterium* population was significantly (p=0.01) reduced in OVX-C rats compared to Sham-C rats. Among the tomato treatment groups, both 'Pre-R' and 'Pre-MG' groups had significantly (p=0.006) increased *Bifidobacterium* populations compared to OVX-C. Although there was no real difference in the abundance of *Enterococcus* in Sham-C and OVX-C, both 'Moonglow' and 'Pre-R' group showed significant (p=0.001) increases in the abundance of *Enterococcus* in the caecum compared to OVX-C. Similarly, *Bacteroides* population was significantly (p<0.00001) increased in both 'Moonglow' and 'Pre-R' groups compared to OVX-C. OVX-C group significantly (p=0.05) reduced the abundance of *E. coli* compared to Sham-C, but both 'Moonglow' and 'Pre-R' group increased (p=0.001) the level of *E. coli* compared to OVX-C. We also calculated the relative abundance of five bacterial genera in each experimental group. We found that *Bacteroides* predominates in each group, making up >70% among the five bacterial genera. Relative abundance of *Bifidobacterium*, *Enterococcus* and *E. coli* was <0.2% compared to *Bacteroides* and *Lactobacillus*.

Compared to red groups ‘Moonglow’ feeding relatively increased *Bacteroides* by 21-28% and decreased *Lactobacillus* by 20-28% (Appendix i, Figure 19).

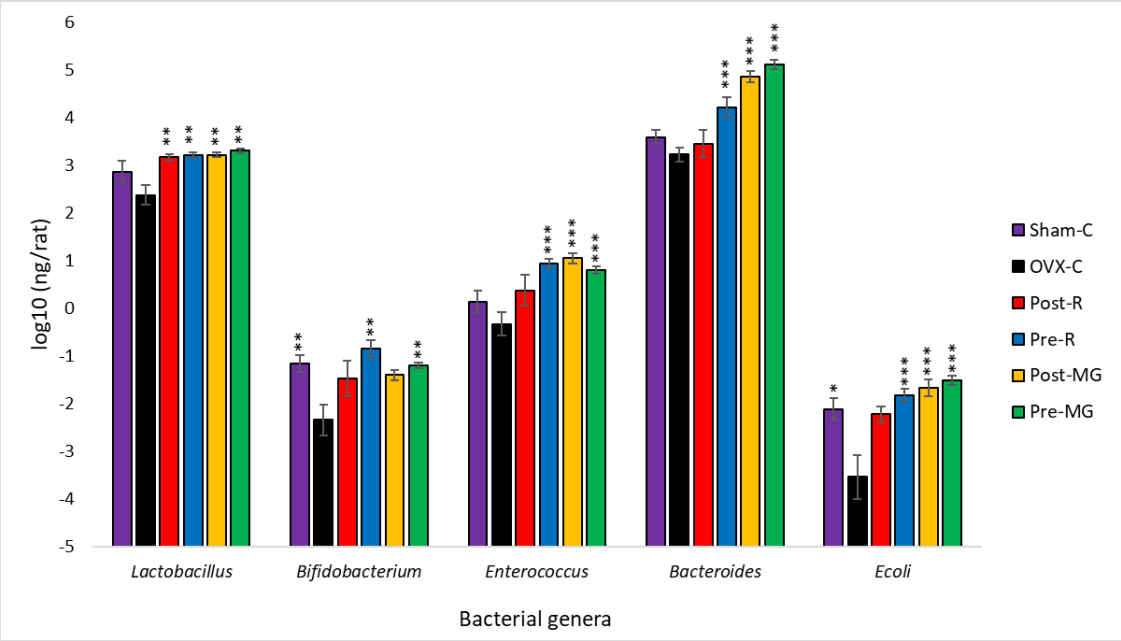


Figure 5.3 Abundance of (a) *Lactobacillus* (b) *Bifidobacterium* (c) *Enterococcus* (d) *Bacteroides* and (e) *E. coli* in rat caeca with supplementation of red and ‘Moonglow’ feeding before and/or after ovariectomy. Data are shown as mean ± SEM of n = 12-15. Sham-C vs OVX-C groups were analysed using Students’ t-test. All OVX groups were separately analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *p<0.05, **p<0.01, ***p<0.001.

5.3.6 Caecum pH and correlation with gut microbes

Caecal pH values in Sham-C and OVX-C rats ranged from 6.79 ± 0.08 to 7.05 ± 0.11 and the pH was not significantly different amongst groups (p=0.343; Figure 5.4). However, the caecal pH was highest in ‘Post-R’ and lowest in ‘OVX-C’. In ‘Post-R’ group, pH was significantly negatively correlated in each bacterial genus: *Lactobacillus* (p=0.05); *Bifidobacterium* (p=0.05); *Enterococcus* (p=0.01); *Bacteroides* (p=0.01); *E. coli* (p=0.01). *Bifidobacterium* in ‘Post-MG’ significantly positively correlated with pH whereas *Bacteroides* in ‘Pre-MG’ significantly negatively correlated with pH (Table 5.6).

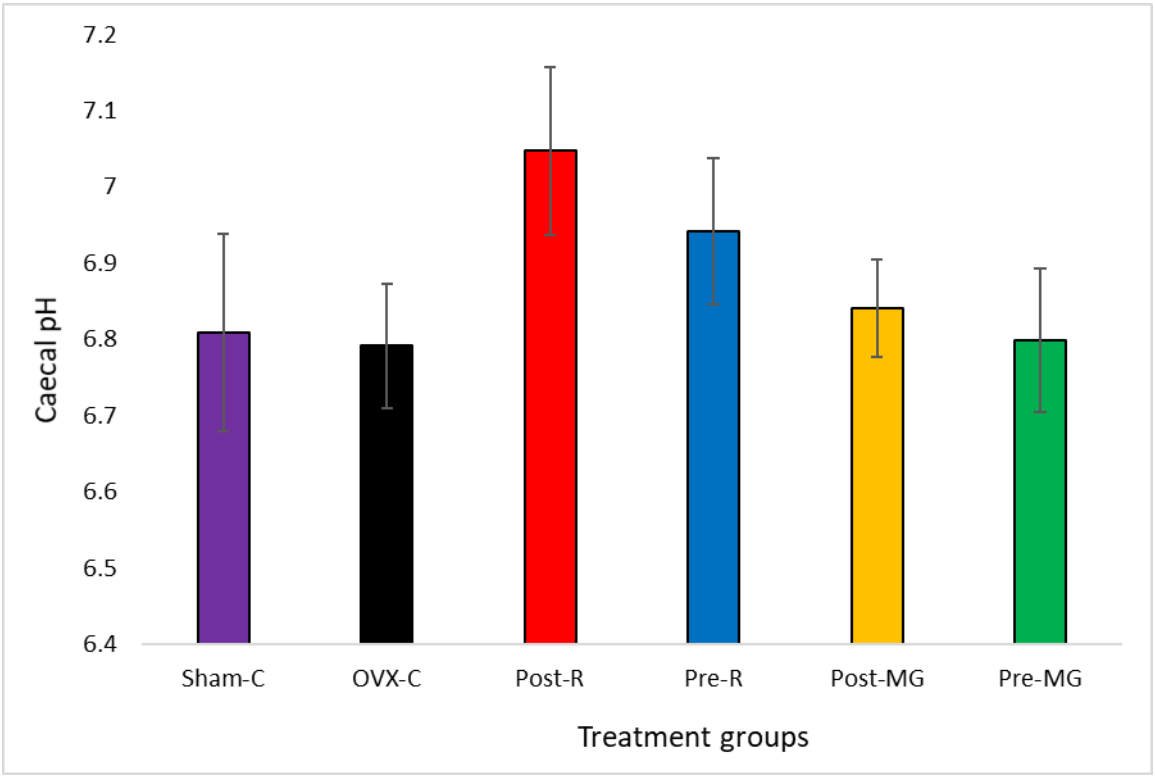


Figure 5.4 Rat caecal pH of six experimental groups. Data are shown as mean ± SEM of n =12-15. Sham-C vs OVX-C groups were analysed using Students' t-test. All OVX groups were separately analysed using one-way ANOVA followed by post-hoc Tukey test.

Table 5.6 Correlation between pH and different bacterial genera in each experimental group.

	pH vs *Lactobacillus*	pH vs *Bifidobacterium*	pH vs *Enterococcus*	pH vs *Bacteroides*	pH vs *E.coli*
Sham-C	0.036	0.052	0.221	0.238	0.406
OVX-C	0.223	0.506	0.400	-0.008	0.296
'Post-R'	-0.589*	-0.656*	-0.702**	-0.675**	-0.795**
'Pre-R'	0.460	-0.176	0.255	-0.005	0.296
'Post-MG'	0.174	0.703**	0.173	-0.085	-0.240
'Pre-MG'	-0.434	-0.160	-0.402	-0.719*	-0.056

Each cell in the table shows Pearson correlation coefficient R-values, minus indicates negative corelation. * indicates significance differences in each pair: *p<0.05, **p<0.01.

5.3.7 Whole body fat mass

There was a near-significant (p = 0.056) group effect on fat mass during the pre-surgery feeding period in OVX groups. However, there was no real difference in fat mass of OVX groups during post-surgery feeding period. There was no significant group and week interaction effect (Table 5.7). Four weeks after ovariectomy, rate of gain in fat mass was significantly higher in 'Post-R' group compared to OVX-C (Table 5.8). Fat mass of the Sham-C group increased until week 12 and thereafter remained relatively unchanged up to the end of the study (Figure 5.5).

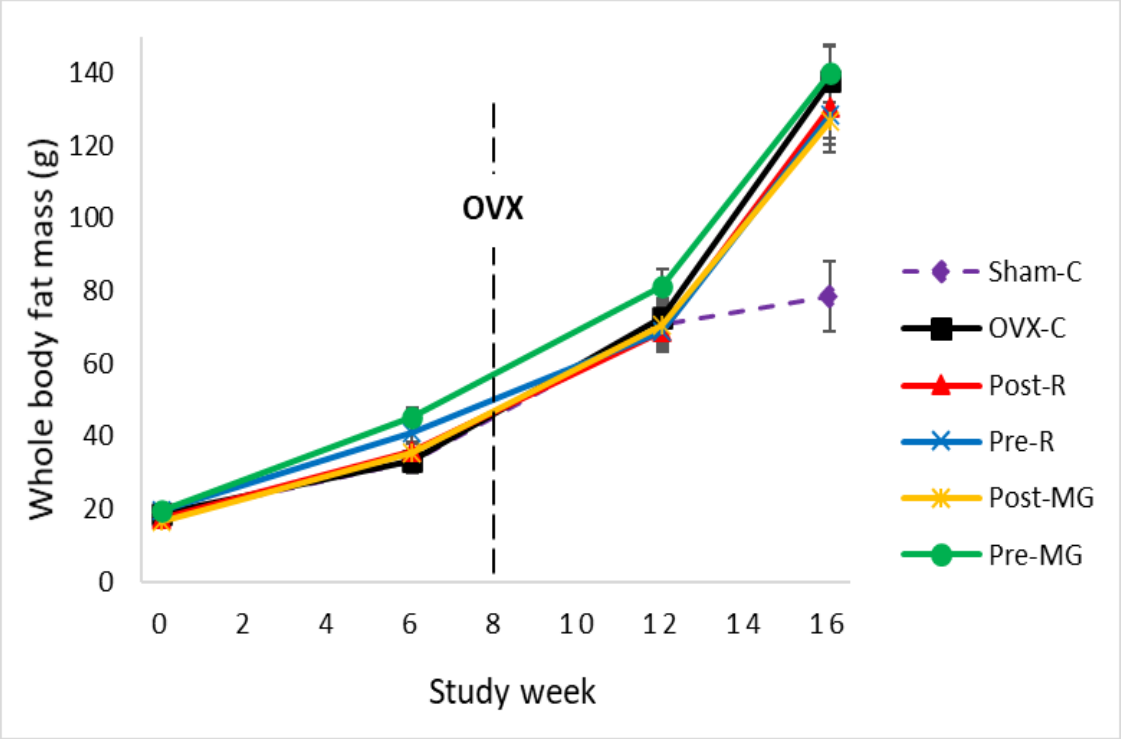


Figure 5.5 Whole body fat mass in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Data are mean ± SEM, n=12-15 rats/group.

Table 5.7 Repeated measures ANOVA for whole body fat mass during pre- and post-surgery periods.

	Pre- surgery period (0-6 weeks)	**Post- surgery period (6-16 weeks)**
OVX only	**Fat mass**	**Fat mass**
Group	*p*=0.056	*p* =0.175
Week	*p* =0.236	*p* =0.847
Group x week	*p* =0.068	*p* =0.706

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.

Table 5.8 Percent change of whole body fat mass in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	Week 6-12	Week 12-16	Week 6-16
Sham-C	64.45 ± 14.80	101.64 ± 11.26	33.08 ± 8.38**	183.50 ± 27.55*
OVX-C	98.00 ± 19.54	122.08 ± 17.54	71.17 ± 4.58	283.23 ± 27.40
'Post-R'	129.93 ± 6.36	104.73 ± 11.22	96.33 ± 6.65*	298.47 ± 21.66
'Pre-R'	109.85 ± 14.13	87.08 ± 10.80	81.00 ± 4.44	240.15 ± 22.98
'Post-MG'	132.71 ± 31.39	115.64 ± 14.72	75.71 ± 8.61	280.79 ± 33.62
'Pre-MG'	137.13 ± 18.76	92.33 ± 13.57	76.27 ± 4.01	236.60 ± 21.68

Percent change in fat mass was calculated between each consecutive time point and also for whole post-surgery period. Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *$p<0.05$, **$p<0.001$.

5.3.8 Whole body fat percentage

Group effect on whole body fat percentage was significant during the pre-surgery feed period where 'Pre-MG' rats had relatively higher values at week 6 compared to OVX-C (Table 5.9). Yet, there was no difference during post-surgery feeding period. Whole body fat percentage continued to increase in all OVX groups throughout the feeding period. After week 12, the whole body fat percentage of Sham-C group remained relatively unchanged (Figure 5.6). The percent change of whole body fat percentage in OVX groups exceeded Sham-C group after eight weeks post-ovariectomy. However, there was no difference in the percent change during pre- and post-surgery feeding periods (Table 5.10).

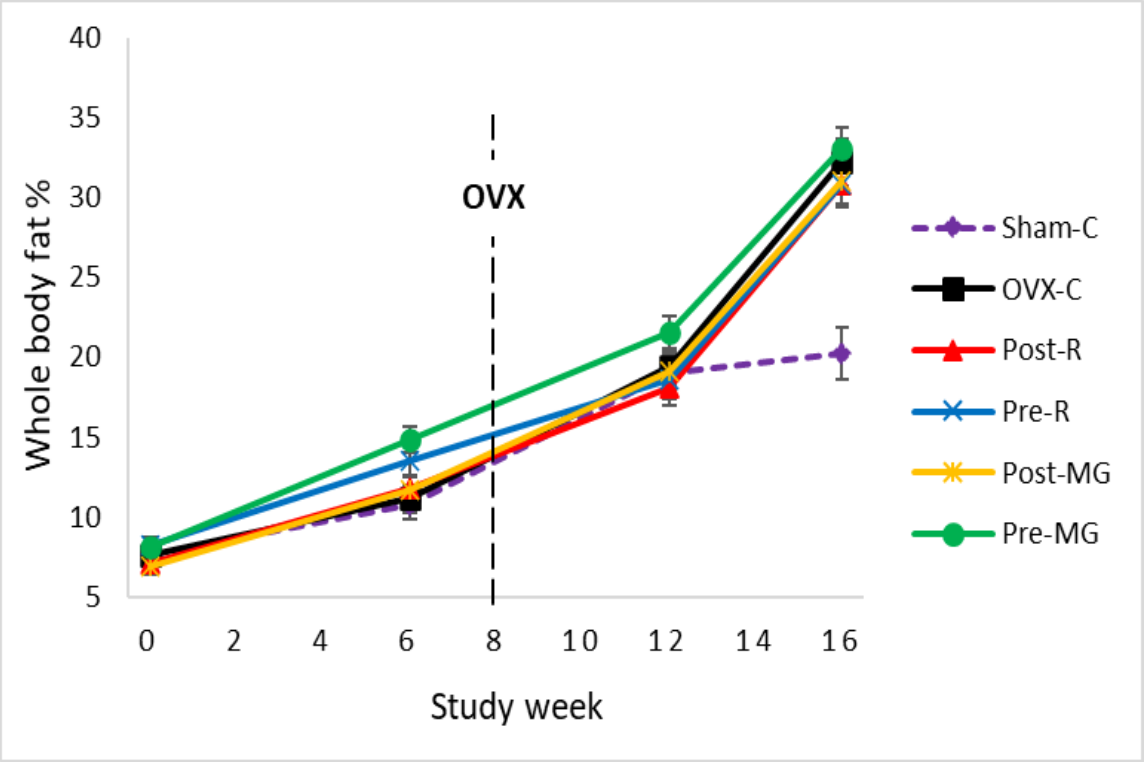


Figure 5.6 Whole body fat percentage in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Data are mean ± SEM, n=12-15 rats/group.

Table 5.9 Repeated measures ANOVA for fat percentage during pre- and post-surgery period

	Pre-surgery period (0-6 week)	**Post-surgery period (6-16 weeks)**
OVX only	**fat %**	**fat %**
Group	p=0.040	p=0.140
Week	p=0.999	p=0.188
Group x week	p=0.091	p=0.512

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.

Table 5.10 Percent change of whole body fat percentage in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	Week 6-12	Week 12-16	Week 6-16
Sham-C	31.55 ± 10.25	74.64 ± 8.99	21.08 ± 6.62**	122.67± 19.84
OVX-C	55.46 ± 13.46	78.31 ± 13.47	59.23 ± 5.65	180.08 ± 19.80
'Post-R'	81.13 ± 18.50	60.47 ± 8.13	74.87 ± 5.50	178.93 ± 14.31
'Pre-R'	65.77 ± 9.22	47.85 ± 7.77	62.50 ± 3.25	143.46 ± 14.30
'Post-MG'	53.50 ± 8.89	72.79 ± 10.31	53.42 ± 4.55	179.86 ± 21.84
'Pre-MG'	87.87 ± 13.77	53.20 ± 9.903	57.00 ± 3.27	138.73 ± 13.42

Percent change in fat percentage was calculated between each consecutive time point and also for whole post-surgery period. Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: ** $p<0.001$.

5.3.9 Whole body lean mass

Whole body lean mass increased in all groups between weeks 0 and 6 (rats ages 8 and 14 weeks) and between weeks 6 and 12 (2 weeks pre-surgery and 4 weeks post-surgery) as shown in Figure 5.7. In older rats during the period between weeks 12 and 16, all treatment groups of OVX rats had shown reduction in lean mass. In contrast, the Sham-C rats during this period continued to gain lean mass. There was no tomato group effect on lean mass during pre- or post- surgery feeding periods (Table 5.11). Between weeks 6 and 12, the rate of increase of lean mass was significantly higher in 'pre-R' group compared to OVX-C (Table 5.12). During the period between 2 weeks pre-surgery and 8 weeks post-surgery (6-16 weeks), the Sham-C group showed a significantly higher rate of increase in lean mass compared to OVX-C.

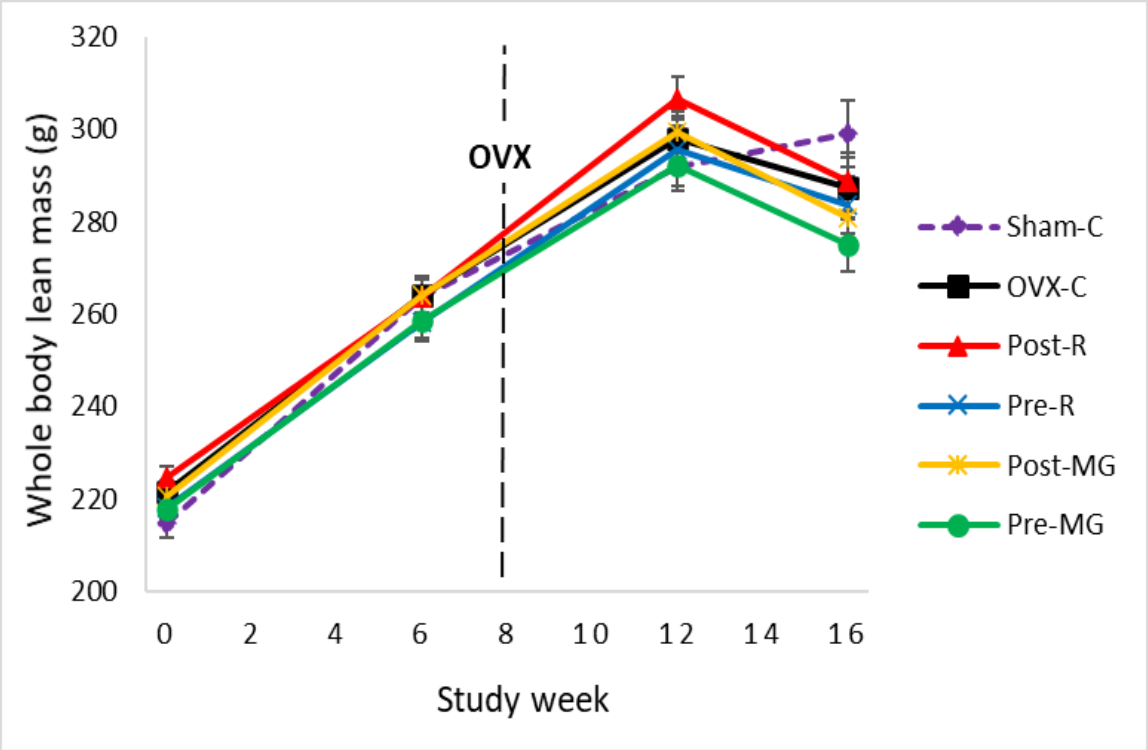


Figure 5.7 Whole body lean mass in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Data are mean ± SEM, n=12-15 rats/group.

Table 5.11 Repeated measures ANOVA for lean mass during pre- and post-surgery period.

	Pre-surgery period (0-6 week)	**Post-surgery period (6-16 weeks)**
OVX only	**Lean mass**	**Lean mass**
Group	p=0.502	p=0.740
Week	p=0.002	p=0.163
Group x week	p=0.663	p=0.278

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate

Table 5.12 Percent change of whole body lean mass in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks.

	Week 0-6	**Week 6-12**	**Week 12-16**	**Week 6-16**
Sham-C	20.91 ± 2.14	6.33 ± 1.29*	5.17 ± 1.01***	11.42 ± 1.12*
OVX-C	21.00 ± 2.13	12.23 ± 1.65	-5.31 ± 0.92	6.38 ± 1.78
'Post-R'	17.93 ± 1.49	17.07 ± 1.13	-7.27 ± 0.71	8.60 ± 1.50
'Pre-R'	18.77 ± 1.57	18.08 ± 1.40*	-6.08 ± 0.54	10.62 ± 1.31
'Post-MG'	18.50 ± 1.46	13.64 ± 1.41	-6.36 ± 1.09	6.36 ± 1.65
'Pre-MG'	16.73 ± 0.95	14.60 ± 1.69	-5.00 ± 0.58	8.67 ± 1.50

Percent change in lean mass was calculated between each consecutive time point and also for whole post-surgery period. Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Student's t-test. OVX groups were analysed using one-way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *$p<0.05$, *** $p<0.00001$.

5.3.10 Correlation between whole body fat and gonadal adipose tissue

We also collected and measured the weights of gonadal white adipose tissues (WAT) of female Sprague-Dawley rats. Gonadal WAT weight was strongly correlated with both total body fat and percent body fat at R=0.665, $p<0.014$ and R=0.618, $p<0.01$ respectively (Figure 5.8 (a) & (b)).

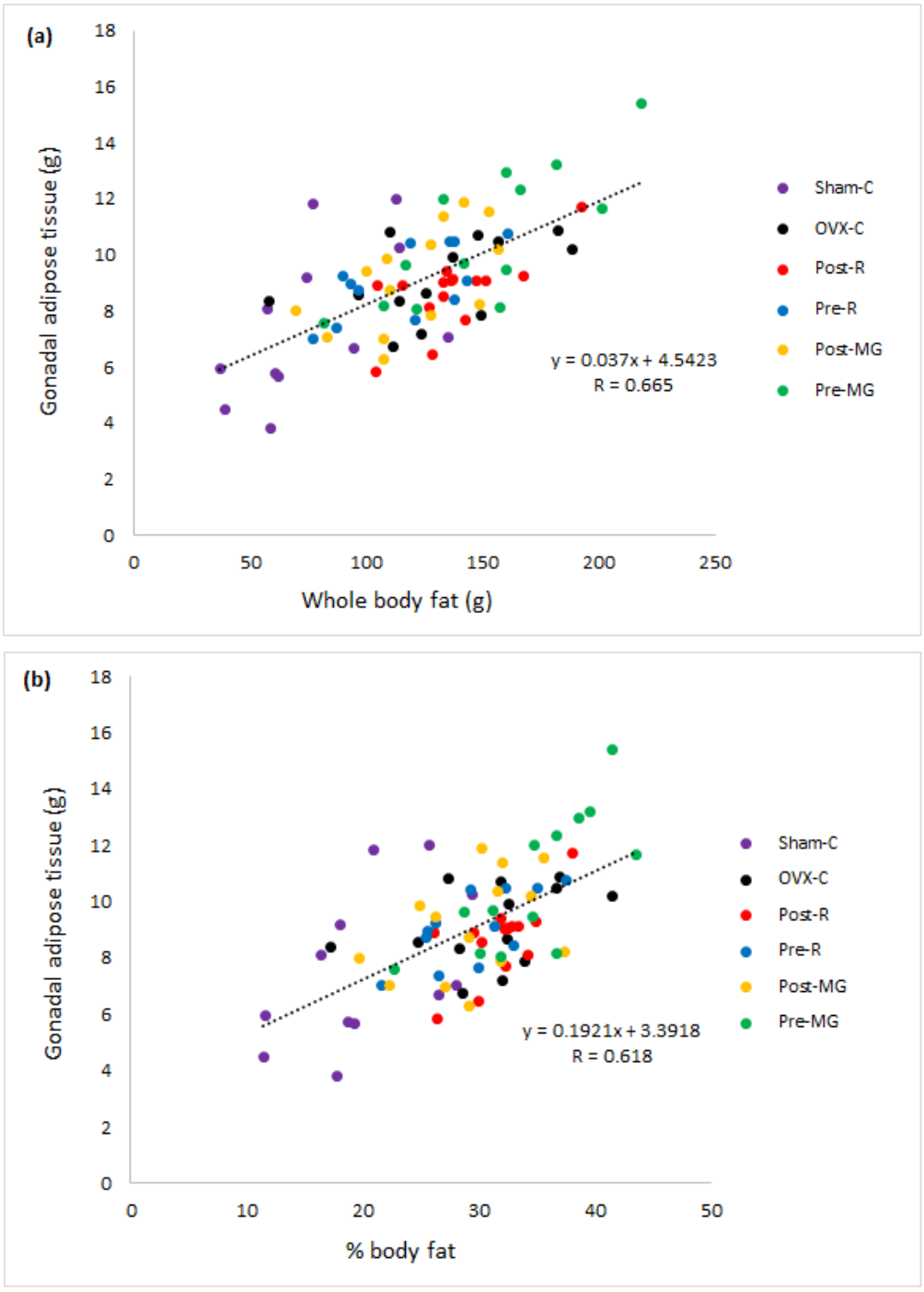


Figure 5.8 Correlation between (a) whole body fat and gonadal adipose tissue and (b) whole body fat percentage and gonadal adipose tissue in female Sprague-Dawley rats. 'R' represents Pearson correlation coefficient.

5.4 Discussion

5.4.1 Effect of tomato feeding on plasma and liver lycopene concentration

In this study, rats fed 'Moonglow' tomato had higher plasma lycopene levels than rats fed red tomato at the dose of 0.35 mg/kg BW. 'Pre' and 'Post' 'Moonglow' groups showed 8-fold higher lycopene concentrations than 'Pre' and 'Post' red groups after 8 or 16 weeks of tomato feeding. All four tomato groups accumulated considerably higher amounts of lycopene and the groups did not differ between tomato types. It is of interest to note that in a previous study, rats fed all *trans*- lycopene daily at 30 mg/kg BW for 12 weeks had a mean plasma lycopene concentration of 0.038 µmol/L, which was nearly identical to our mean plasma lycopene after 16 weeks of 'Moonglow' *cis*- lycopene at a 100-fold lower dose. In addition, the liver lycopene concentrations achieved in the current study from all four tomato groups (5.526-6.935 µmol/kg) were greater than the range of those reported for humans where the liver lycopene range was 0.1- 4.5 µmol/kg (Stahl et al., 1992, Kaplan et al., 1990).

Our previous trial (chapter 3) conducted by feeding 0.35 mg/kg BW lycopene from 'Moonglow' tomato to rats for 5 days resulted in lycopene concentrations in plasma of 0.596 µmol/L and liver of 15.570 µmol/kg, while the current study showed lycopene in plasma at 0.043-0.040 µmol /L and liver at 6.824-6.935 µmol/kg for 'Post-MG' and 'Pre-MG' respectively. While it appears surprising that a longer feeding period would result in two- to ten-fold lower plasma and liver lycopene concentrations, similar findings have been published elsewhere. For instance, 25 mg/kg BW lycopene fed to rats for 7 days had plasma lycopene at 0.536 µmol/L and liver at 138 µmol/kg, while almost similar dose (30 mg/kg BW) fed to rats for 12 weeks had plasma at 0.038 µmol/L and liver at 23 µmol/kg (Ardawi et al., 2016, Zaripheh et al., 2003). In addition, the data in Chapter 3 indicate liver accumulation stopped at day 4 or 5, suggesting an upregulation of metabolic capacity in the liver.

Zaripheh *et al* evaluated the time-course distribution of a single dose of lycopene following pre-feeding of lycopene for 30 days and the results showed that pre-feeding reduced lycopene absorption and tissue distribution and increased lycopene excretion (Zaripheh and Erdman Jr, 2005, Zaripheh et al., 2003). It has also been suggested that continuous feeding upregulates enzymes involved in lycopene metabolism and clearance (Zaripheh et al., 2003). Another study reported that the enzymes involved in

detoxification can be induced by prolonged lycopene feeding (Breinholt et al., 2000) and also noted elevation of phase II enzyme in liver (Zaripheh et al., 2005). Also, increased production of lycopene metabolites with lycopene feeding was observed after pre-feeding of tomato products (Khachik et al., 2002). These lycopene metabolites are biologically active and have a number of beneficial effects (Ford and Erdman, 2012, Aust et al., 2003). Therefore, with the long term feeding we used, lycopene metabolites may have been more prevalent in the plasma than total lycopene; it is a limitation of the current study that due to costs we did not measure lycopene metabolites using HPLC analysis.

5.4.2 Effect of tomato powder treatment, storage and feeding on isomeric composition

Lycopene concentration was reduced by freeze drying, with red tomato total lycopene decreasing by 32% and 'Moonglow' by 21%. The relative proportions of lycopene *trans*- and *cis*- isomers were only slightly affected by the freeze drying process: red tomato went from 6.3 to 5.6% *cis*-, and 'Moonglow' went from 99.2 to 99.1% *cis*-. However, once incorporated into the peanut butter and honey treats, 'Moonglow' but not red tomato lycopene underwent slight isomeric changes. Red tomato lycopene remained at 5.8% *cis*-; 'Moonglow' *cis*- was reduced to 94.5%. This difference could be due to *cis*- to *trans*- re-isomerization, which has been shown to occur during certain storage conditions (Anguelova and Warthesen, 2000). *Cis*- lycopene is less stable than *trans*- and tends to be lost more quickly (Ishida et al., 2007). Therefore, the decrease of total lycopene with higher proportional losses of *cis*- isomers in 'Moonglow' could change the isomeric profile.

Interestingly, in the livers of the red tomato groups accumulated lycopene as 72-76% all *trans*- and 24-28% *cis*-, while 'Moonglow' groups contained 32-38% all *trans*- and 62-68% *cis*- lycopene. As this implies the occurrence of *trans*- to *cis*- isomerization in rats fed with red tomato as well as *cis*- to *trans*- isomerization in rats fed with 'Moonglow', we cannot calculate the relative impacts of endogenous all *trans*- to *cis*- isomerization versus increased bioavailability of *cis*- lycopene that led to the higher amount of *cis*- isomers present in body tissues. However, Morgan and co-workers, using compartmental and non-compartmental modelling for absorption, isomerization and distribution kinetics, determined that endogenous all *trans*- to *cis*- isomerization is the major cause for the high amount of *cis*- isomers present in the body tissues, rather than the different bio-availabilities of isomers (Moran et al., 2015).

It is also not yet fully understood in which locations in the body isomerization occurs. According to Re et al, all *trans*- to *cis*- isomerization happens during digestion and the suggested possible cause for this conversion is the acidic pH in the stomach (Re et al., 2001). Another kinetic study carried out by simulating the gastric environment *in vitro* showed gastric pH is partially responsible for the *trans*- to *cis*- isomerization in the body (Moraru and Lee, 2005). Contrastingly, another human intervention study showed isomerization is not significant in the stomach and suggested that, as the stomach is only responsible for the initiation of isomerization, lycopene *cis*- isomers are of post-enterocyte origin (Tyssandier et al., 2003). In validation of this, another *in vitro* study showed that lycopene isomerization happens at the level of the enterocytes (Richelle et al., 2012).

Given the nearly pure *cis*- isomeric composition of 'Moonglow' tomatoes, it was also interesting to see the *cis*- to *trans*- isomeric changes in the liver. Studies have suggested that the action of β-carotene 9',10' oxygenase (BCO2) is more effective on *cis*-lycopene isomers than on all *trans*- lycopene as BCO2 showed preferential cleavage of *cis*- lycopene isomers (Wang, 2012); this may contribute to our observed differences in isomeric composition between 'Moonglow' in the dietary supplement versus liver. Another study suggested that feeding more *cis*- isomers can increase efficiency of absorption of *trans*- lycopene (Burri et al., 2009), and therefore the all *trans*- lycopene in 'Moonglow' may have been preferentially absorbed at the level of the gut due to the high level of *cis*- lycopene present.

5.4.3 Gut microbiota

Gut microbiota play a vital role in digestion and the health of the host (Sommer and Bäckhed, 2013). Furthermore, recent studies have identified beneficial effects of gut microbes on the prevention of bone loss that can lead to osteoporosis (Scholz-Ahrens et al., 2016, McCabe et al., 2015, Britton et al., 2014). The caecum in the rat is located distal to the small intestine and proximal to the large intestine. Studies have shown differences in the microbiota pattern between caecal contents and feces, because of specific ecologies in the microenvironment of the caecum (Tanca et al., 2017, Cross et al., 2017). Also, the microbiota in the caecum can be modulated via molecules released from small intestine. Considering these facts, it is advantageous to evaluate caecal microbiota during a dietary intervention study in animal species possessing a caecum (Guo et al., 2019).

All five bacterial groups evaluated in this study – *Lactobacillus, Bifidobacterium, Enterococcus, Bacteroides,* and *E. coli* – have probiotic capabilities (Azad et al., 2018). A reduced abundance of gut microbiota in the OVX-C group was observed in most of the bacterial genera. This can be attributed to the loss of ovaries and estrogen, rather than diet, as both OVX-C and Sham-C were fed the same diet and received the same tomato powder-free treats. Ovariectomy surgery has been reported to cause dysbiosis in gut microbiota (Org et al., 2016, Cox-York et al., 2015). Studies have shown that the interaction between estrogen and gut microbiota can affect the microenvironment and the metabolism of the host (Sommer and Bäckhed, 2013, Tremaroli and Bäckhed, 2012). It has been further reported that as gut microbiota can be regulated by estrogen in the body, estrogen status may directly affect the gut microbial composition (Baker et al., 2017). Moreover, gut microbiota produce the short chain fatty acids (SCFA) which act as metabolic signalling molecules and these SCFA are significantly reduced in OVX rats (Cox-York et al., 2015). In addition to these findings in animal models, human trials have also revealed that the total urinary estrogen was highly correlated with gut microbe diversity and the species richness (Flores et al., 2012).

Prebiotics facilitate the growth of gut microbiota which in turn can provide health benefits to the host (Markowiak and Śliżewska, 2017). A limited number of studies have revealed a prebiotic-like effect of tomatoes on some gut microbiota (Wiese et al., 2019, García-Alonso et al., 2017). The current study advanced the knowledge in this field with the novel finding that the abundance of all five bacterial genera evaluated from digestive tract contents was increased in tomato treatment groups compared to OVX-C; as all rats received peanut butter and honey treat supplements, this change can only be attributed to the tomato powder. The prebiotic effects of red and 'Moonglow' on increasing *Lactobacillus* were similar regardless of the feeding regimen. Both 'Pre-MG' and 'Pre-R' groups showed significantly higher abundance of all gut microbes tested compared to OVX-C; in contrast, 'Post-MG' but not 'Post-R' significantly increased *Enterococcus, Bacteroides,* and *E. coli*. There is a still scarcity of studies related to the effect of carotenoids on gut microbiota (Bohn et al., 2015). However, a recent study has found a significant corelation between bacterial groups and the production of SCFA, which indicates the changes of SCFA profile is determined by the microbiota in the gut (García-Alonso et al., 2017). Gut microbiota contain both gram positive and gram negative bacteria (Xia et al., 2018). Although some studies showed that changes of gut microbiota

depend on the dietary fiber (Fåk et al., 2015, Weitkunat et al., 2015), a recent study showed a strong positive corelation between intake of phenolic compounds and *Lactobacillus* (gram positive bacteria) while a negative correlation with *Enterobacteriaceae* (gram negative bacteria) (García-Alonso et al., 2017).

In our study, we found a clear difference in relative abundance of *Lactobacillus* and *Bacteroides* following red and 'Moonglow' tomato feeding (Appendix i, Figure 19). As *Lactobacillus* are gram positive and *Bacteroides* are gram negative (Tan et al., 2019, Fijan, 2014), the changes in relative abundance of *Lactobacillus* and *Bacteroides* following red and 'Moonglow' feeding could possibly be due to the amount of phenolic content along with fiber present in these tomatoes. Supporting this evidence, a similar relationship was also observed between bacterial groups and phenolic catabolites in rat feces (García-Alonso et al., 2017). Tomatoes are a good source of insoluble fiber (Koh et al., 2010), thus poorly fermented by the gut microbes (Lattimer and Haub, 2010). Interestingly, *Bacteroides* have an ability to produced SCFA via fermenting complex carbohydrate (Cullen et al., 2015). As we saw the increased relative abundance of *Lactobacillus* in red tomato supplemented groups, another study found that the dose dependent increase of *Lactobacillus* along with changes in SCFA in the caecum following different diets containing red cherry tomatoes (Hwang et al., 2014). However, we did not evaluate phenolic compounds and SCFAs in our study. Therefore analysis of SCFA and the microbial phenolic catabolites following red and 'Moonglow' tomato feeding would help to better understand their relationship with gut bacteria. While differences in fiber content between red and 'Moonglow' tomato powders were not evaluated in the current study, tomato powder intake was ~0.1 g per rat per day, representing <0.5% of total food intake from standard chow containing 20% fiber (Pellizzon, 2016). Therefore, it is likely that lycopene along with other phenolics and fiber were the cause of improving the abundance of gut microbiota in OVX rats in tomato groups.

5.4.4 Correlation between pH and gut microbiota

Caecal pH is very important in terms of bacterial growth (Ilhan et al., 2017). We did not see statistically significant differences in caecal content pH between any of the experimental groups; however, the red tomato groups showed a mean pH ~0.2 points higher than the pooled control and 'Moonglow' groups. We also found a significant negative correlation between pH and the abundance of all five bacteria in the 'Post-R'

group. Gut bacteria produce SCFA by fermenting resistant dietary fiber (Scott et al., 2008), and high levels of SCFA correlate with more acidic pH (Lupton and Kurtz, 1993). Therefore, the negative correlation observed between gut bacteria and pH could be due to less production of SCFA when there is a lower number of gut bacteria. Studies suggest that fiber and phenolic compounds are effective promoters of gut bacteria populations and this in turn will facilitate production of SCFA (García-Alonso et al., 2017). It has also been found that phenolic compounds are not absorbed in the stomach but rather reach the large intestine, where they can promote the abundance of gut bacteria (Dueñas et al., 2015b). Phenolics, their catabolites produced *in vivo*, and their overall accumulation can all affect gut microbial profile (Pérez-Conesa et al., 2009, Crozier et al., 2009). As we utilised whole tomato powder in the current study, it is likely that phenolic compounds and fiber, along with lycopene may have contributed to the beneficial effects observed.

5.4.5 Effect of tomato feeding on body composition

The OVX model successfully induced increased weight gain to a state similar to obesity in humans, via accelerated fat mass gain and lean mass loss. Rats gained significantly more fat mass post-OVX compared to Sham-C, as reported previously (Kurrat et al., 2015, Zoth et al., 2010, Isken et al., 2008, Wu et al., 2004) and this is widely believed to be due to the low level of estrogen after menopause/ovariectomy (McCarthy et al., 2013). The mechanism behind this phenomenon remains unclear and is only partially explained by increased energy intake. Some studies suggest that the presence of estrogen receptors in adipocytes may have a role in the increase of fat mass (Brown and Clegg, 2010, Pallottini et al., 2008). A study carried out with adipose tissue-specific androgen receptor knock-out mice showed increased subcutaneous obesity and hyperleptinemia due to an increased level of intra-adipose estradiol (Yu et al., 2008). In addition, changes in energy homeostasis due to low estrogen could result in accumulation of intra-abdominal body fat (Lizcano and Guzmán, 2014). It has also been found that estrogen affects the nuclei of the hypothalamus which control energy homeostasis and it also regulates the activity of hypothalamic neurones via gene regulations and neuronal excitability (Roepke, 2009). Estrogen regulates the expression of enzymes involved in glucose/energy metabolism (Lizcano and Guzmán, 2014, Hart-Unger and Korach, 2011) and estrogen deficiency after ovariectomy induces additional food intake by regulating the activity of molecules involved in appetite (Lizcano and Guzmán, 2014).

Although the full mechanisms of the relationship between decreased estrogen level and muscle loss are not fully understood, it has been suggested that the low estrogen may increase proinflammatory cytokines which can lead to lower muscle mass (Messier et al., 2011). In addition, muscle tissues contain estrogen receptors (β) on cell membranes, cytoplasm and nuclear membranes (Brown, 2008). White adipose tissues are reported to be very important in regulating the metabolic functions of adipocytes (Wang et al., 2005), increasing fat oxidation (Orci et al., 2004) and decreasing lipogenesis (Zhang et al., 2008) due to their production of the hormone leptin, thus regulating body weight at the systemic level. Also, some human studies have found a strong relationship between whole body fat and adipose tissues (Swainson et al., 2017, Saelens et al., 2007). Similarly, in this study the mass of adipose tissues was strongly correlated with whole body fat mass and fat percentage.

Several studies have examined the relationship between lycopene intake and body composition. One study showed that lycopene intake at 5.855 mg/day is associated with low levels of visceral and subcutaneous fat in elderly men (Sluijs et al., 2009). Similarly, another study demonstrated an inverse relationship between lycopene intake and fat mass in healthy adults (Grolier et al., 2000). A third study observed a reduction in body fat in young females after supplementation of tomato juice containing 32.5 mg of lycopene/day for two months (Li et al., 2015). In contrast, a study carried out with healthy premenopausal women did not show any association between dietary intake of lycopene and fat mass (Nuss et al., 2017). A recent article highlighted the need for a better understanding of the relationship between the gut microbiome and the changes in body composition that result from menopause (Becker and Manson, 2021). In addition, animal models are of value to explore the more complex cellular and molecular mechanisms underlying the observations in human trials. For example, a recent study found that while lycopene supplementation in male mice did not alter lean mass, it significantly increased slow-twitch muscle fibres and decreased fast-twitch fibres, altering both muscle potential and the activity of specific enzymes associated with muscle function (Liu et al., 2021).

Surprisingly, the ‘Pre-R’ group significantly increased lean mass gain one month after OVX and ‘Post-R’ group significantly increased fat mass gain two months after OVX compared to the OVX-C. This contrasting finding could be due to differences in feed intake and the level of physical activity. We did not measure the feed intake of rats which was a limitation of this study and we also did not measure physical activity as this

was beyond our objectives and technical capabilities. However, physical activity strongly correlates with the reduction of fat mass and the increase of muscle mass in rats (Shinoda et al., 2002); thus changes in energy expenditure could be a reason for a significant increase in lean mass in the 'Pre-R' group. It would be of interest to further explore this phenomenon and its potential mechanisms in future pre-clinical studies.

5.5 Conclusions

These results confirm that 'Moonglow' is rich in *cis*- lycopene whereas red lycopene is mainly in all *trans*- form, but both types are partially degraded by the freeze drying process. Feeding 'Moonglow' resulted in significantly higher plasma lycopene concentrations compared to feeding matching doses of red tomato. The liver is an organ where lycopene accumulates and feeding both tomato types resulted in rats storing similarly high amounts of lycopene in the liver. The lycopene isomer liver profiles were skewed compared to the starting material fed, indicating compositional changes in *cis*- and *trans*- isomers occur *in vivo*. Both tomato types demonstrated a significant prebiotic effect, although 'Moonglow' was more effective when fed for a shorter time period. The OVX model was successful in accelerating fat mass gain and lean mass loss, but tomato treatments overall did not induce permanent, significant differences in body composition. Overall, 'Moonglow' *cis*- lycopene is significantly more bioavailable than red *trans*- lycopene, and 'Moonglow' tomatoes showed a greater potential as a prebiotic.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Umani Walallawita
Name/title of Primary Supervisor:	Prof Julian Heyes
In which chapter is the manuscript /published work:	6

Please select one of the following three options:

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- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 90.00

- Describe the contribution that the candidate has made to the manuscript/published work:

Involved in planning and designing of cell culture experiments,carried out all experiments and data analysis,compiled and interpreted the results, reviewed and co-edited.

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Umani Walallawita Digitally signed by Umani Walallawita Date: 2021.12.06 06:38:56 +13'00'
Date:	06-Dec-2021
Primary Supervisor's Signature:	Julian Heyes Digitally signed by Julian Heyes Date: 2021.12.07 18:04:16 +13'00'
Date:	7-Dec-2021

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CHAPTER 6

The effect of red and ‘Moonglow’ tomato extracts on RANKL-induced osteoclastogenesis in murine macrophage RAW 264.7 cells

Abstract

Osteoclasts are multinucleated cells responsible for bone resorption which, when excessive, causes significant bone loss leading to osteoporosis. Therapeutic strategies for osteoporosis target the reduction of osteoclast generation and osteoclast activity. Lycopene, a major carotenoid in tomatoes, has been studied for potential bone-protective effects. However, *cis*- versus *trans*- lycopene isomers have never been compared in osteoclastogenesis. In this study, red and orange heirloom ‘Moonglow’ tomato extracts were evaluated for their effect on osteoclastogenesis using the murine macrophage RAW 264.7 cells activated by receptor activator of NFκB ligand (RANKL). Osteoclast differentiation was assessed by quantifying cell viability, tartrate-resistant acid phosphatase (TRAP) activity in conditioned medium, and visually enumerated TRAP-positive osteoclasts. Both red and ‘Moonglow’ tomato extracts with lycopene at 0.01 - 1 µmol/L increased the number of viable cells after 1 or 5 days, with red showing more significant enhancement. At 10 µmol/L lycopene there was no effect, but at 100 µmol/L the extracts were significantly cytotoxic after 5 days. TRAP enzyme activity was not significantly affected by the extracts with lycopene at 0.01 - 10 µmol/L. In contrast, the number of TRAP-containing osteoclasts was slightly reduced with lower concentrations of lycopene and significantly reduced by both extracts with 10 µmol/L lycopene. Our results showed for the first time equivalent anti-osteoclastogenic effects of tomato extracts containing vastly different lycopene isomeric profiles. The degree of anti-osteoclastogenesis observed was much greater than reported elsewhere for this concentration of lycopene, suggesting that the effects could be due to lycopene derivatives, regardless of the isomeric form of lycopene.

6.1 Introduction

Bone is a highly dynamic structure that undergoes constant remodelling (Datta et al., 2008). Bone remodelling allows bone to repair damage and maintain the integrity of the skeletal system (Raggatt and Partridge, 2010). Bone remodelling occurs throughout life (Kini and Nandeesh, 2012, Baron and Hesse, 2012, Rao et al., 2003), and governs the function and formation of the skeleton as well as the maintenance of calcium and phosphate homeostasis (Rao and Rao, 2015). Equilibrium in the opposing processes of bone formation by osteoblast cells and bone resorption by osteoclast cells plays a major role in terms of bone health (Feng and McDonald, 2011), whereas excessive osteoclast activity leads to skeletal disorders such as osteoporosis (An et al., 2016, Das et al., 2010). Clinical treatments for osteoporosis, such as bisphosphates, induce inhibition of osteoclast activity (Drake and Cremers, 2010). Osteoclasts are large multinucleated cells originating from haematopoietic lineage; their main function is degrading the mineralized bone matrix (Marino et al., 2014). Two major cytokines, macrophage-colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL), are produced by osteoblasts and are involved in the regulation of osteoclast differentiation, function and survival (Pallottini et al., 2008, Matsuo and Irie, 2008). Therefore RANKL and its receptor RANK play a vital role in differentiation and activation of osteoclasts (Ha et al., 2004).

Tartrate resistant acid phosphatase (TRAP) is an enzyme expressed by osteoclasts, inflammatory macrophages and dendritic cells (Rissanen et al., 2008). Its presence and concentration correlate with the number of osteoclasts present rather than changes in resorptive activity of individual osteoclasts. The measurement of TRAP is a useful marker of bone resorption (Rissanen et al., 2008) and is used to monitor the effectiveness of antiresorptive treatments in clinical settings (Civitelli et al., 2009, Halleen et al., 2006). It is also used in cell studies to assess osteoclast generation and activity (Rantlha et al., 2017). The use of *in vitro* models is a practical way of predicting *in vivo* bone resorption. The RAW 264.7 macrophage cell line has the capacity to differentiate into mature osteoclasts in the presence of RANKL (Wattel et al., 2004, Hsu et al., 1999). Therefore RAW 264.37 cells are commonly used as a model to study osteoclast formation and function (Collin-Osdoby and Osdoby, 2012), including the production of TRAP and its enzymatic activity.

Lycopene, the major carotenoid in tomatoes, has been proven to be an effective antioxidant. Compared to other carotenoids, lycopene has very high antioxidant and free radical scavenging activities (Srivastava and Srivastava, 2015, Heber and Lu, 2002). It has also been shown to have inhibitory effects on osteoclastogenesis and bone resorption (Rao et al., 2003, Ishimi et al., 1999) and therefore lycopene supplementation may be a nutritional approach for the management of bone loss. Studies have found that carotenoid derivatives downregulate RANKL activity, and that reduction of RANKL can inhibit the differentiation of osteoclast precursors into mature osteoclasts; together, these suggest that modulation of RANKL is a candidate pathway for the protective effect of lycopene against bone loss (Walallawita et al., 2020). Lycopene is present in red tomatoes largely as *trans*- isomers, whereas in orange tomatoes such as the heirloom 'Moonglow' variant the lycopene is predominantly found in the *cis*- isomeric form (chapter 3). It has been shown that *cis*- lycopene is more highly bioavailable than *trans*- (Walallawita et al., 2020, Cooperstone et al., 2015), but little is known about putative differences in function between the isomeric forms. To date no study has been conducted to compare the effect of *cis*- versus *trans*- lycopene as part of tomato extracts on osteoclastogenesis. Therefore, the purpose of the current study was to evaluate the effect of red and 'Moonglow' tomato hexane extracts on osteoclast formation and TRAP enzyme activity using RANKL-stimulated RAW 264.7 cells.

6.2 Material and methods

6.2.1 Chemical reagents

Cell culture medium (Dulbecco's Modified Eagle Medium-DMEM) was purchased from GE Life Sciences (Pittsburgh, PA, USA). Heat inactivated fetal bovine serum (FBS), bovine serum albumin (BSA) and antibiotic/antimycotic 15240-062 were purchased from Gibco, Life Technologies, Auckland, New Zealand). RANKL (RDS462-TEC010) was purchased from In Vitro Technologies (Summit Road, Victoria, Australia). 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and 1α,25-dihydroxycholecalciferol (calcitriol) were obtained from Sigma-Aldrich (Merck, Auckland, NZ). Napthol AS-BI-phosphate, 4-nitrophenyl phosphate, pararosaniline and haematoxylin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

6.2.2 Preparation of tomato hexane extracts

Freeze-dried tomato powder (1 g) was mixed with 40 mL hexane and the mixture was agitated for 30 min using a magnetic stirrer. The solution was filtered using 3HW filter paper in minimum light. The hexane extract was separated using 2 mL of milliQ water. The non-polar layer was separated and centrifuged at 3400 *g* for 10 min. Hexane was evaporated under nitrogen gas. The dried extract was wrapped in foil and stored at -20^{0}C until use.

6.2.3 Quantification of lycopene and reconstitution

Dried tomato extracts were reconstituted in a 1:3 mixture of tetrahydrofuran:methanol. The lycopene concentration was measured using uHPLC (see section 3.2.7.4 of this thesis). Based on HPLC analysis, the appropriate volume of medium was added to dried extracts and vortexed to make a stock solution. Dried extracts were firstly dissolved in 1% DMSO (500 µL) and vortexed before topping up with media to get final concentration of 0.1% DMSO. Stock solution was filter sterilised using sterilised 0.20 µm syringe filters. Highest concentration of lycopene (100 µmol/L) was prepared from stock solution and diluted further to make 10, 1, 0.1 and 0.01 µmol/L.

6.2.4 Cell culture maintenance

The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC; In Vitro Technologies, Auckland, NZ). RAW 264.7 cells were grown in the Dulbecco's Modified Eagles Medium (DMEM, Gibco, Life technologies, New Zealand) supplemented with 10% foetal calf serum (heat-inactivated FCS, Invitrogen, New Zealand) with 1% antibiotic-antimycotic (15240-062; Gibco, Life Technologies, New Zealand), designated as 'culture medium'. Cells were seeded at 1×10^5 cells/mL in 75 cm^2 flasks, and culture medium was replaced every 3-4 days. Cells were passaged when 90% confluence was reached. All cultures and assays were maintained in an incubator at 37^{0}C with 95% humidified air and 5% CO_2. All cell culture techniques were carried out in a laminar flow cabinet under aseptic conditions. Non-sterile solutions, pipette tips, and troughs were autoclaved before use; non-autoclavable solutions were sterilised through 0.20 µm syringe filters prior to use.

6.2.5 Preparation of cell culture for the assay

Cell confluency (90%) was visually confirmed under the microscope. Spent culture medium was discarded and 10 mL of warmed fresh culture medium was added into the flask. Cells were then scraped gently using sterile rubber scraper (BIO70-1180, Lab Supply, Dunedin, New Zealand) and mixed with a pipette to break up cell clumps. Viable cells were counted using a haemocytometer with trypan blue exclusion. A new 75 cm^2 maintenance flask was seeded at a density of 1×10^5 cell/mL. Cell concentrations were adjusted to 1.5×10^3 or 2×10^4 cells/mL and seeded in either 96 microwell plates or 24 microwell plates for MTT and TRAP assay respectively.

6.2.6 Cell viability assay

The MTT assay was used to estimate the metabolic activity of RAW 264.7 cells as an indicator for cell viability over either 24 hours or 5 days. One day after seeding, spent culture medium was replaced with DMEM containing 0.1% bovine serum albumin (BSA) and incubated for 24 hours to synchronise the cell cycle. Tomato extracts were dissolved in DMEM containing 0.1% DMSO at the required concentration and filter sterilised. Each tomato extract was added into n = 5 replicate wells (Appendix i, Layout 1) and incubated for 24 hours. DMSO (10%) was used as a positive cytotoxic control; the DMEM with 0.1% DMSO was used as a vehicle control. Next, 10 µL of MTT (5 mg/mL in PBS) was added and incubated at 37^0C for 3-4 hours. Wells were checked from 3 hours onward for the formation of purple-blue crystals. The reaction was stopped between 3-4 hours. Medium was removed carefully without disturbing the cells or crystals from each well, and the crystals were dissolved by adding 100 µL of DMSO. Plates were allowed to sit for 5-10 min without shaking and the absorbance was measured at 550 nm using an ELX 808 Ultra Microplate Reader (KC4 version 3.01 software Bio-Tek. Instruments Inc, USA). For the 5-day viability assay, the media were replaced at day 3 with the matching tomato or control substances, and the absorbances were read at day 5 as described above. Cell viability was expressed as percentage of vehicle control.

6.2.7 Tartrate-resistant acid phosphatase (TRAP) experimental techniques

The effect of red and ‘Moonglow’ tomato extracts on osteoclastogenesis was assessed in a TRAP assay. RAW 264.7 cells were cultured with tomato extracts at a range of concentrations in the presence of RANKL. Briefly, cells were seeded at 2×10^4 cells/mL

(1 mL/well) into triplicate wells of a 24 microwell plate in culture medium containing 15 ng/mL of RANKL ± tomato extracts at four different lycopene concentrations (0.01, 0.1, 1.0 and 10.0 µmol/L) and incubated for 5 days. Lycopene concentrations were chosen based on previously reported studies (Feng et al., 2010, De Stefano et al., 2007). Calcitriol (10^{-8} M) was used as a positive control and DMEM (10% FBS) with 0.1% DMSO was used as a vehicle control (Appendix i, Layout 2). Cell culture media were replaced after 72 hours with fresh media DMEM containing treatments matching the originals. After 5 days of exposure to the tomato extracts, medium in each well was removed in to 2 mL Eppendorf tubes and stored at -80^{0}C until analysis. Each experiment was repeated thrice to provide experimental replicates.

6.2.8 Tartrate-resistant acid phosphatase (TRAP) activity assay

Eppendorf tubes containing conditioned medium were completely thawed and 30 µL of the test material was added to triplicate wells in 96 well plates (Appendix i; Layout 3) with 170 µL of acetate (100 mM) - tartrate (26.67 mM) buffer containing *p*-nitrophenyl phosphate (pNPP) at a final concentration of 5 mM. After incubation at 37^{0}C for 1 hour, the reaction was stopped by adding 50 µL 1M NaOH. After colour formation, absorbance was recorded at 405 nm.

6.2.9 Tartrate-resistant acid phosphatase (TRAP) staining assay

After removing the media on day 5, the TRAP staining assay was carried out for counting TRAP-positive multinucleated osteoclast cells. TRAP staining method was performed according to method described by Boeyens *et al* with slight modifications (Boeyens et al., 2014). Briefly, cells were washed with warm PBS and fixed with 3% Formaldehyde/66% Acetone/Citrate (fixative buffer) for 30-60 sec and washed with warm MQ water three times. Naphthol AS-BI-phosphate (12.5 mg/mL) in acetate (100 mM)-tartrate (26.67 mM) buffer (500 µL) was added in each well and incubated at 37^{0}C. After 30 min, this was removed and warm pararosaniline dye in acetate (100 mM)-tartrate (26.67 mM) buffer (500 µL) was added and incubated at 37^{0}C. After 20 min, this was removed, and the plate was rinsed with warm MQ water three time. Haematoxylin was added to each well to cover the cells and left for 30-60 sec for counter staining. Images were taken with Olympus Camedia C-5060 camera using 20X objective lens of an inverted microscope (Olympus CK40). Stained cells with more than four nuclei were identified as TRAP-positive multinucleated cells.

6.2.10 Statistical analysis

Results are presented as means with standard error of the mean (means ± SEM). All statistical analyses were conducted using SPSS statistical software version 25. Data were tested for normality using Shapiro-Wilk test and homogeneity of group variances was assessed by Levene's test. The differences between group means were analysed using one-way ANOVA followed by post-hoc Tukey's test. Differences were considered statistically significant at $p<0.05$.

6.3 Results

6.3.1 Effect of tomato extracts on cell viability

Effects of tomato extracts on RAW 264.7 cell viability were assessed, and the means calculated from triplicate experiments, each conducted with n=5 wells per treatment. The presence of metabolically active cells, referred to here as cell viability, was normalised relative to the vehicle control with no added tomato extracts, which was set to be 100% viability. Cells treated with 10% DMSO (dimethyl sulfoxide) were the positive control and DMSO demonstrated the expected cytotoxicity, as <10% of cells survived (Figures 6.1 and 6.2). After 24 hours, high doses of the tomato extracts (10 µmol/L and 100 µmol/L of red; 1,10 and 100 µmol/L of 'Moonglow') resulted in cell viability similar to vehicle control (Figure 6.1). However, at low doses (0.01, 0.1, and1 µmol/L of red; 0.01 and 0.1 µmol/L of 'Moonglow') the tomato extracts showed significant increases in cell viability compared to vehicle control.

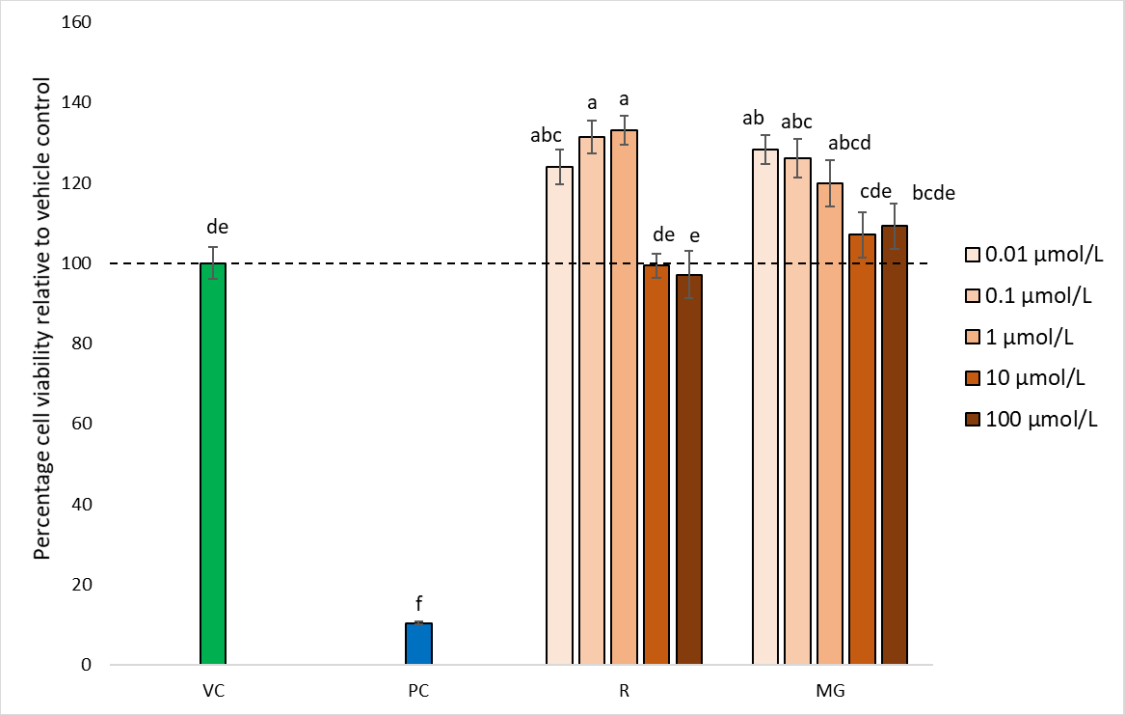

Figure 6.1 Dose-dependent effect of red (R) and 'Moonglow'(MG) tomato extracts (0.01, 0.1, 1.0, 10, and 100 µmol/L) on RAW 264.7 cell viability after 24 hours as measured by MTT assay, compared to negative vehicle control (VC; 0.1% DMSO) and positive control (PC; 10% DMSO). Data are shown as mean ± SEM of triplicate experiments, each conducted with five replicate wells per treatment. Different letters are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test

After 5 days incubation with tomato extracts, significant decreases in cell viability were observed at the highest concentration (100 µmol/L) of both tomato extracts (Figure 6.2). This dose reduced the proportion of viable cells to 24.4% and 5.5% in red and 'Moonglow' groups respectively; for 100 µmol/L of 'Moonglow' extract, the cell viability was reduced to a level similar to that observed with 10% DMSO (positive control). The remaining 'Moonglow' concentrations had no significant effect on cell viability. In contrast, while 10 µmol/L red tomato did not affect cell viability, at 1 – 0.01 µmol/L it resulted in a significant increase in viable cells.

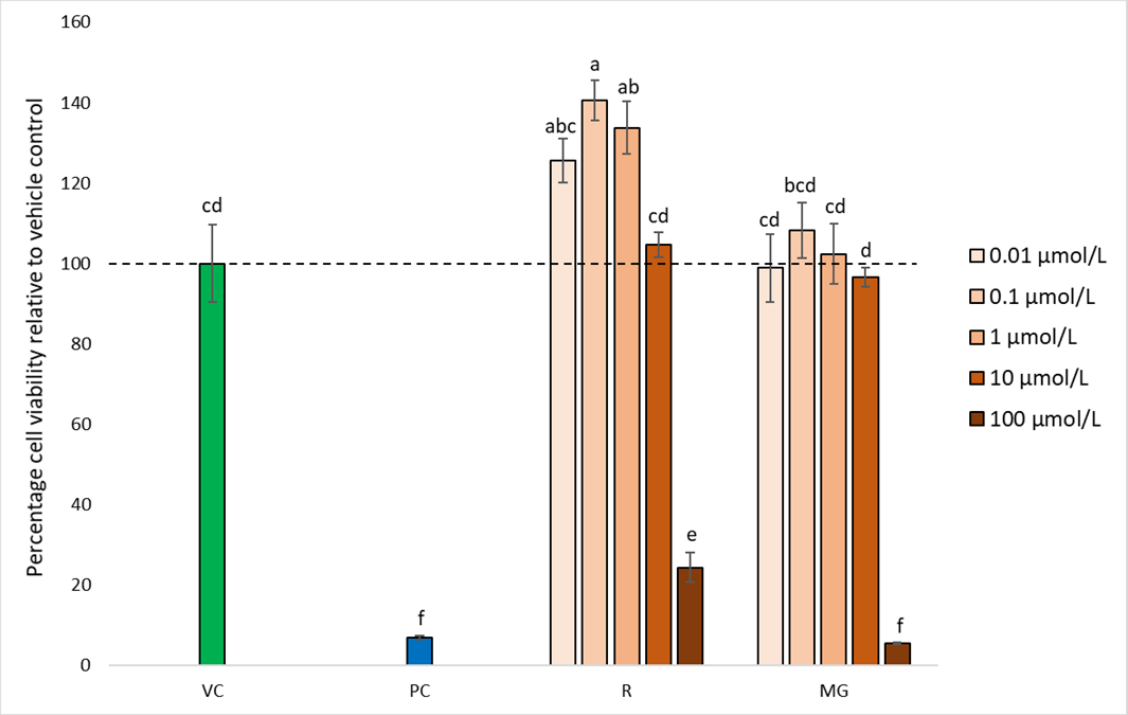

Figure 6.2 Dose-dependent effect of red (R) and 'Moonglow'(MG) tomato extracts (0.01, 0.1, 1.0,10.0, and 100 µmol/L) on RAW 264.7 cell viability after 5 days of incubation as measured by MTT assay, compared to negative vehicle control (VC; 0.1% DMSO) and positive control (PC; 10% DMSO). Data are shown as mean ± SEM of triplicate experiments, each conducted with five replicate wells per treatment. Different letters are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test.

6.3.2 TRAP activity assay

The cell viability assay demonstrated that the highest dose of 100 µmol/L was significantly cytotoxic after five days. Therefore, the TRAP activity assays, which also required a five-day culture period, were performed using the lower four lycopene concentrations (0.01-10 µmol/L). Figure 6.3 shows the biphasic effect of four concentrations of red and 'Moonglow' tomato extracts on osteoclast differentiation. No TRAP activity was detected in the conditioned medium of control wells from cells cultured with no RANKL present. As expected, TRAP activity of RAW 264.7 cells induced by RANKL (vehicle control) was significantly ($p<0.0001$) higher than that of positive control which inhibited the osteoclast differentiation. The positive control, RANKL combined with calcitriol, showed a 72% reduction of TRAP activity compared to vehicle control. Both red and 'Moonglow' tomato extracts from 0.01-10 µmol/L lycopene concentration did not show any significant difference in TRAP activity compared to vehicle control. However, 10 µmol/L concentration of both tomato extracts led to significantly lower activity than with the matching extract at 1 µmol/L.

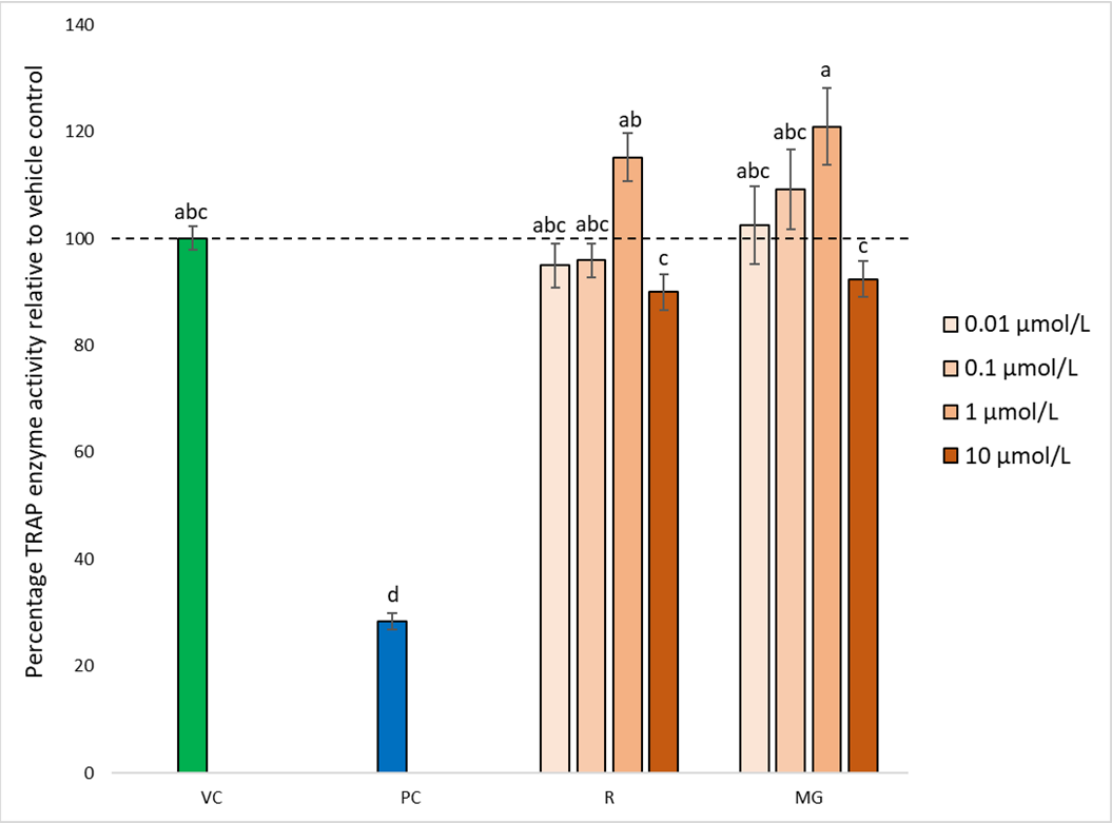

Figure 6.3 TRAP enzyme activity of RANKL-induced osteoclasts following incubation with red (R) and 'Moonglow' (MG) tomato extracts at 0.01-10 µmol/L, compared to the vehicle control (VC; RANKL alone) and positive control (PC; RANKL + calcitriol). Data are shown as mean ± SEM of triplicate experiments, each conducted with nine replicate wells per treatment. Different letters are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test.

6.3.3 TRAP-positive cell count

RAW 264.7 cells did not differentiate into mature osteoclasts without RANKL being present (Figure 6.4). The cells remained as small, dark-staining amorphous shapes. In contrast, with RANKL present, mature osteoclasts were easily identified by their large size, definitive cell membrane, pink-stained cytoplasm, and multiple nuclei. Every well, except for the negative control with no RANKL present, contained at least one identifiable osteoclast. The inclusion of calcitriol, as expected, appeared to reduce the number of visible osteoclasts, with fewer large, pink osteoclasts visible and more small, dark, undifferentiated cells present. In addition, the osteoclasts appeared to be smaller and contained fewer nuclei. A similar effect, although to a lesser degree, was seen in wells containing tomato extracts at the highest dose.

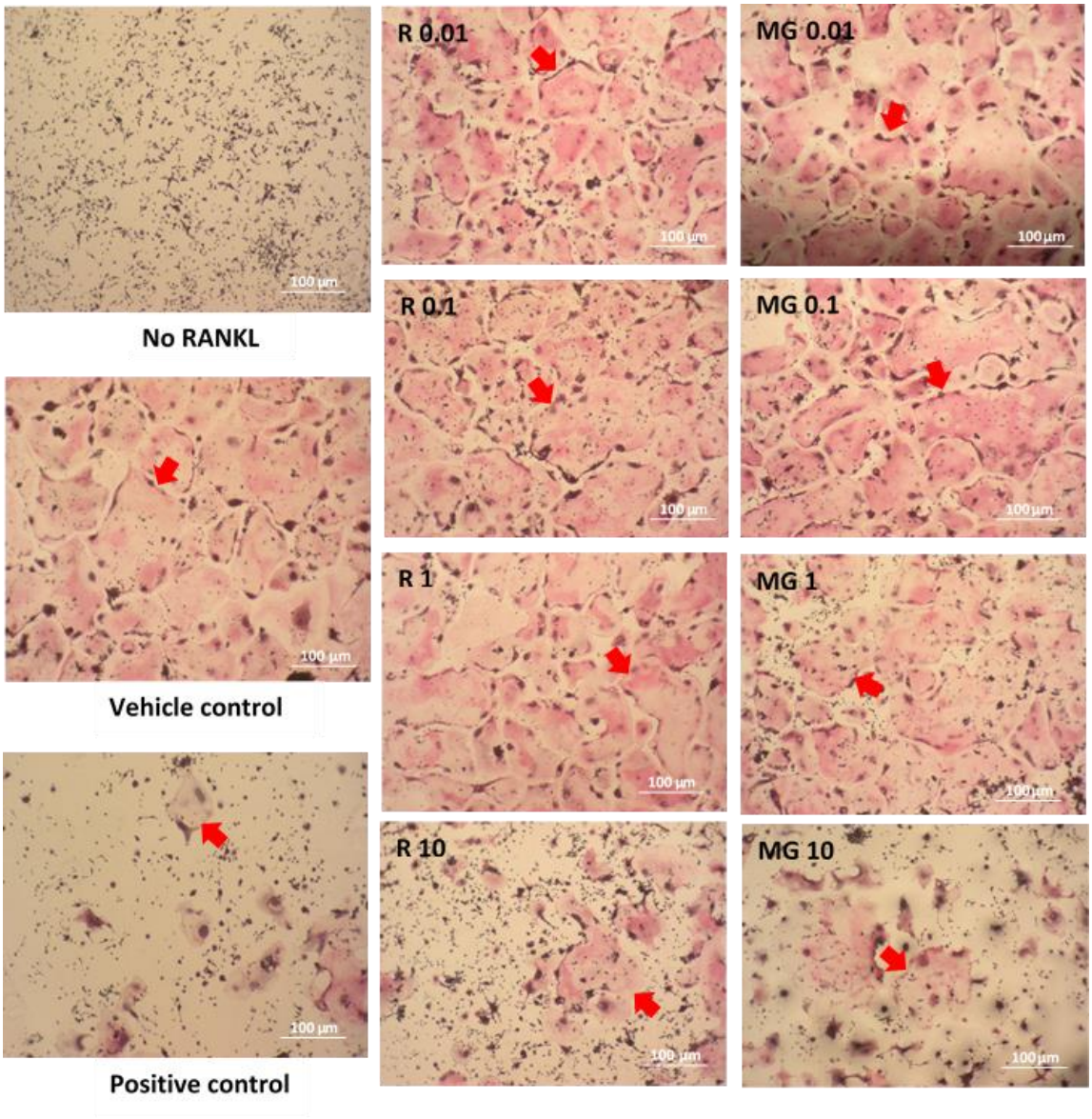

Figure 6.4 Representative images of RANKL-induced osteoclast differentiation from RAW 264.7 cells, following incubation with red (R) and 'Moonglow' (MG) tomato extracts at 0.01-10 µmol/L, compared to the vehicle control (RANKL alone) and positive control (RANKL + calcitriol). Red arrows identify an osteoclast in each image.

The numbers of osteoclasts per well were then quantified. The morphological changes observed in the photomicrographs were supported by the cell count findings. RANKL induced osteoclast formation, but the addition of calcitriol reduced osteoclast numbers by 89% (Figure 6.5). Tomato extracts at 0.01, 0.1, and 1.0 µmol/L did not significantly alter osteoclast numbers. However, both red and 'Moonglow' tomato extracts at 10 µmol/L significantly reduced osteoclast differentiation by 60% and 63% respectively.

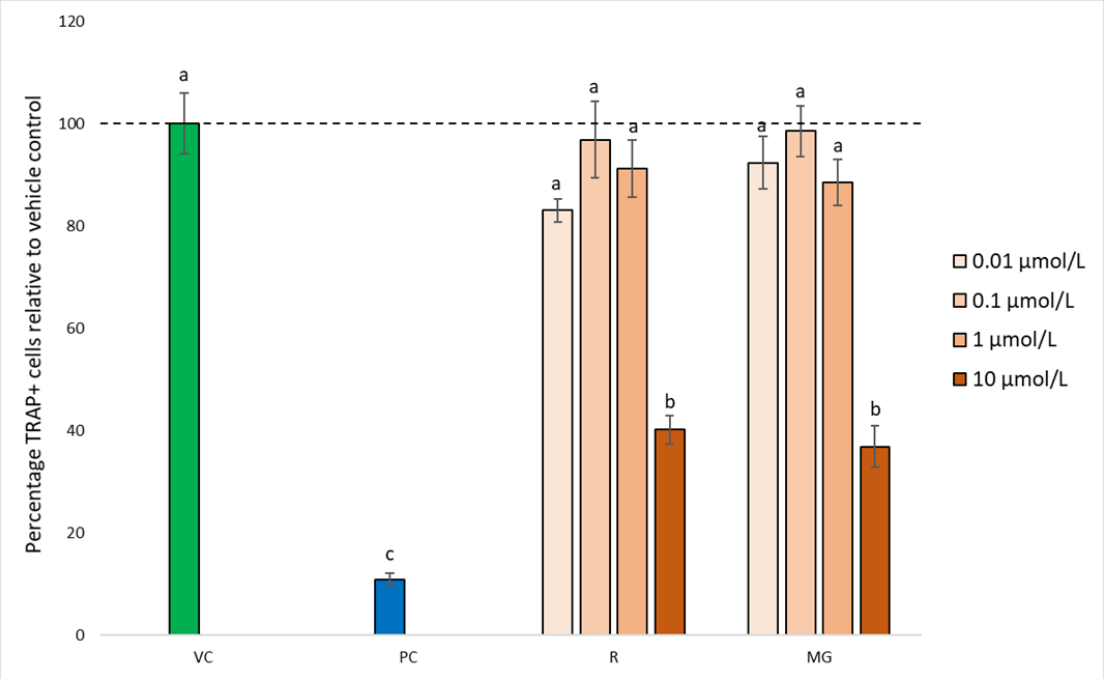

Figure 6.5 Quantification of RANKL-induced osteoclast differentiation from RAW 264.7 cells, following incubation with red (R) and 'Moonglow' (MG) tomato extracts at 0.01-10 µmol/L, compared to the vehicle control (VC; RANKL alone) and positive control (PC; RANKL + calcitriol). Data are shown as mean ± SEM of triplicate experiments, each conducted with three replicate wells per treatment. Different letters are significantly different ($p<0.05$) as determined by one-way ANOVA and post-hoc Tukey test

6.4 Discussion

Potential strategies for the treatment for osteoporosis are to increase bone formation by osteoblasts or to inhibit bone resorption by osteoclasts. However, the principle of most current clinical therapies for osteoporosis is based on the inhibition of osteoclast activity (An et al., 2016). This could be achieved by decreasing osteoclastogenesis, inducing osteoclastic apoptosis, or blocking osteoclast function. *In vitro* cell culture studies have shown that lycopene can inhibit osteoclast differentiation

(Rao et al., 2003, Ishimi et al., 1999) and play a vital role in bone remodelling via gap junctional communication between osteocytes, osteoblasts and osteoclasts (Batra et al., 2012). In the current study, we demonstrated that tomato powder from either red or 'Moonglow' tomatoes was capable of reducing osteoclastogenesis *in vitro* by ~60%, while having no effect on cell viability, when present with a lycopene concentration of 10 µmol/L. The development of therapeutic drugs has been mainly based on the understanding of RANK signalling pathways (Jules et al., 2010, Tanaka et al., 2005). In this study we evaluated the effect of red and 'Moonglow' tomato extracts on RANKL-induced osteoclastogenesis in RAW 264.7 cells. The concentrations tested were based on the lycopene content present. We also assessed the effect of tomato extracts on cell viability.

In one-day cultures, the tomato extracts had no effect on viability when present at lycopene concentrations of 10 – 100 µmol/L, but significantly increased cell viability at 0.01 – 1.0 µmol/L. This may have been due to the fact that the assay was conducted under nutrient-deficient conditions of culture medium with 0.1% albumin but no serum. In five-day cultures, red tomato extract at 0.01 – 1.0 µmol/L again increased cell viability, whereas the effect was no longer present in 'Moonglow' tomato extract at these concentrations suggesting that the possible differences in nutritional composition of two tomato types could contribute to long-term cell proliferation and viability. Increased cell viability compared to the vehicle control observed in this study could possibly be due to increased metabolic activity of cells rather than increased cell numbers (Riss et al., 2016). Both nutrient deficiency and excess nutrients in the media can cause cellular stress which ultimately leads to increased cell metabolism (Wellen and Thompson, 2010). At 10 µmol/L, neither extract had a significant effect, as observed in the one-day cultures. However, at 100 µmol/L, both extracts were significantly cytotoxic.

A similar study assessing prostate cancer cell survival showed that inhibition of cell growth depends on the duration of exposure to lycopene as well as the dose (Hwang and Bowen, 2005). Lycopene can be oxidized rapidly due to light, oxygen or incubation temperature (Hwang and Bowen, 2005). Different oxidation products of lycopene have been identified (Khachik et al., 1998); however, its effect on cell proliferation and increase of gap junctional communication is poorly understood. A study showed that cancer cells supplied with an oxidative mixture of lycopene resulted in >96% inhibition of cell growth, whereas purified lycopene showed no significant effect at the same dose

(Nara et al., 2001). It is also unclear whether the effect of lycopene on cellular function and signalling pathways is caused by intact lycopene or its derivatives (Wang, 2012). Studies have demonstrated biological effects of lycopene derivatives (Landrum, 2009, Mein et al., 2008). Among identified lycopene metabolites, acyclo-retinoic acid, an analogue of lycopene, has been demonstrated to inhibit cell proliferation and induce apoptosis in several cell lines (Mein et al., 2008). Several studies have found that the formation of lycopene metabolites and oxidation products does occur *in vitro* (dos Anjos Ferreira et al., 2003, Caris-Veyrat et al., 2003, Zhang et al., 2003, Kim et al., 2001). This suggests that the lycopene, rather than other components, was responsible for the observed cytotoxic effect of tomato powder in the current study. As we used lipid extracts of tomatoes where the predominant bioactive compound is lycopene (Hwang and Bowen, 2005), it is less likely that this cytotoxicity was due to the ionic or macronutrient components of tomato powders.

Osteoclast activity was assessed by tartrate resistant acid phosphatase (TRAP) enzymatic activity in conditioned medium. At low concentrations, neither tomato extract induced a significant difference in TRAP activity. However, a significant difference was observed between the treatments of 1 versus 10 µmol/L lycopene in tomato powder, with the higher dose resulting in lower TRAP activity. This is in accordance with Rao *et al* who found a significant functional inhibition of PTH-stimulated resorption by osteoclasts at a lycopene concentration of 10 µmol/L (Rao et al., 2003). Similarly, Stefano *et al* demonstrated significant inhibition of production of reactive oxygen species (ROS) with lycopene concentration at 20 µmol/L (De Stefano et al., 2007). Feng *et al* found that lycopene from 1-10 µmol/L dose-dependently inhibited the production of nitric oxide, a ROS, by RAW 264.7 cells (Feng et al., 2010). It is suggested in the literature that oxidative stress caused by ROS is involved in the bone resorption process caused by osteoclasts (Silverton, 1994, Zaidi et al., 1993).

Osteoclastogenesis was assessed by quantifying the number of TRAP-positive osteoclasts present after RANKL-induced differentiation. These results did not mirror those observed for TRAP enzymatic activity. At 1 µmol/L, both tomato extracts slightly increased TRAP activity but slightly decreased osteoclast numbers compared to vehicle control. At 10 µmol/L, both tomato extracts slightly reduced TRAP activity but significantly reduced osteoclast numbers by >50% compared to vehicle control. It is suggested that TRAP activity increases with increasing TRAP-positive cells (Rissanen et

al., 2008, Alatalo et al., 2000). However, TRAP activity does not always correlate with the osteoclast number, because the round and spindle shaped mononucleated cells derived from osteoclast precursors also have some TRAP activity (Bernhardt et al., 2016, Glenske et al., 2014). TRAP activity therefore may not correlate with functional resorption by osteoclasts, as it is considered a marker of osteoclast number rather than osteoclastic activity (Rissanen et al., 2008, Alatalo et al., 2000). Nevertheless, increased bone resorption is associated with increased number of osteoclasts (Henriksen et al., 2007). The significant decrease in osteoclast numbers observed with our tomato powders did not differ between red and 'Moonglow'; there are no published studies comparing the effects of all *trans*- and *cis*- lycopene isomers on osteoclastogenesis. However, Varghese *et al* found that both all *trans*- and 9-*cis*- isomers of retinoic acid equally increased the secretion of procollagenase in primary osteoblasts (Varghese et al., 1994).

In a previous study, Ishimi and co-workers observed a minimal decrease of TRAP positive cells after treatment with lycopene at 0.1-10 μmol/L concentration (Ishimi et al., 1999); this may reflect the different cell type and differentiation factor used by that group, or it may reflect differences in purified lycopene versus a complex tomato powder containing not only lycopene but also other carotenoids. Carotenoids with pro-vitamin A activity such as β-carotene produce retinoic acid during metabolism (Wang, 2012). Ishimi and co-workers reported that osteoclast cell formation was inhibited by retinoic acid and other carotenoids including β-carotene and canthaxanthin (Ishimi et al., 1999). Retinoic acid moderates the inhibitory effect of osteoclastogenesis via retinoid acid receptors (retinoid acid receptor; RAC and/or retinoid X receptor; RXR). However, lycopene does not have pro-vitamin A activity, and it has been speculated that carotenoids in this class may express their inhibitory activity through unknown orphan receptors (Ishimi et al., 1999).

Some studies have noted that derivatives of carotenoids have an ability to downregulate NFkB activity. Lack of NFkB may lead to a reduction of osteoclast precursor differentiation into mature osteoblasts, thereby reducing bone loss (Linnewiel-Hermoni et al., 2014, Kini and Nandeesh, 2012, Boyce et al., 2010). In addition to carotenoid derivatives, phytonutrients such as flavonoids and polyphenols also exert an inhibitory activity on osteoclast differentiation. It has been suggested that these carotenoid derivatives and other phytochemicals have electrophilic groups that can

interact with reactive proteins in the NFkB system or signalling pathways involved in osteoclast differentiation (Odes-Barth et al., 2020).

As we used tomato hexane extracts, other carotenoids in addition to the lycopene likely affected osteoclastogenesis. A study carried out by Navarrete *et al* using aqueous tomato extracts which were free from carotenoids showed that their tomato extract inhibited the expression of inflammatory cytokines via inhibiting the activity of NFkB. (Navarrete et al., 2015). Several studies have reported that ferulic acid, a flavonoid present in tomatoes, as well as its derivatives can inhibit the expression of inflammatory cytokines (Kim et al., 2012, Ronchetti et al., 2006, Hirata et al., 2005, Sakai et al., 1999). Fucoxanthin, an oxygenated carotenoid, significantly reduced TRAP-positive osteoclast numbers at 2.5 and 5 µmol/L (Ha et al., 2021) The hydrolysed form of fucoxanthin induced osteoclast cell apoptosis, suggesting that the hydrolysed form of fucoxanthin is more effective than fucoxanthin itself in terms of cell apoptosis inducing ability (Das et al., 2010). As well as all-*trans*- lycopene, red tomato extracts contained β-carotene and small amount of lutein. 'Moonglow' extracts predominantly contained tetra-*cis*- lycopene and considerable amount of ζ-carotene, which is a precursor of lycopene. β-carotene or lutein were not detected in 'Moonglow' extract. Therefore, amount of β-carotene and lutein was calculated to check any synergetic/cooperate effect on osteoclastogenesis. Amount of β-carotene and lutein were 0.067 and 0.0086 µmol/L at the highest dose (10 µmol/L) that we used to assess osteoclastogenesis. According to the published studies, the concentration of β-carotene and lutein at 0.1 (Wang et al., 2017, Tadaishi et al., 2014) and 10 µmol/L (Tominari et al., 2017) respectively was effective in inhibition of RANKL-induced osteoclastogenesis. Therefore, it is highly unlikely that our results were from additive or synergetic effects of the other carotenoids.

6.5 Conclusions

We summarize that regardless of the isomeric form of lycopene, both red and 'Moonglow' tomato hexane extracts induced a significant decrease in the number of TRAP-positive osteoclasts at 10 µmol/L lycopene concentration compared to vehicle control. As we used tomato hexane extracts instead of lycopene itself, there are numerous other lipophilic phytochemicals that also could affect the biological activity of RAW 264.7 cells. We were unable to measure the oxidation products of lycopene, which could be involved in osteoclastogenesis in addition to or instead of lycopene itself. We expect

that future studies would identify specific genes that regulate osteoclastogenesis when RAW 264.7 cells are treated with red and ‘Moonglow’ tomato extracts, and aid in identifying the putative bioactive components within these powders.

CHAPTER 7

General discussion and future research directions

7.1 Overall discussion

There is a growing interest among people in preventing and managing bone diseases thorough the dietary approaches along with combination of pharmacological therapies (Sahni et al., 2015). Calcium and vitamin D along with proteins, other vitamins (K & A), minerals (P, Mg, Zn, Cu, Fe, F), and various bioactive factors in foods contribute to maintenance of bone health (Sugiura et al., 2012, Morgan, 2008). Among modifiable and non-modifiable risk factors of osteoporosis (Snelling et al., 2001), the most common disorder associated with loss of bone structure, changes in diet can be considered as a major modifiable risk factor in preventing osteoporosis. Several intervention studies have been conducted to evaluate the effect of dietary and nutritional supplements in minimizing the adverse effects of osteoporosis. Lycopene is a major carotenoid representing 80-90% of total carotenoids present in tomatoes (Shi and Maguer, 2000). Tomato and tomato-based products represent more than 80% of dietary sources containing lycopene (Shen et al., 2012, Mackinnon et al., 2011b). Recent literature provides substantial evidence on the beneficial effects of lycopene against bone loss (Walallawita et al., 2020).

To date, published clinical and animal studies have used lycopene as purified lycopene extracts or in processed forms of tomatoes such as juices, sauces, or purées (Russo et al., 2020, Ardawi et al., 2016, Iimura et al., 2015, Iimura et al., 2014, Liang et al., 2012, Mackinnon et al., 2011a, Mackinnon, 2010). Lycopene has therefore been provided mainly in the all-*trans*- form of lycopene. Bioavailability of lycopene differs between *cis*- and *trans*- isomeric forms, with *cis*- lycopene reported to be 8.5 times more bioavailable than *trans*- lycopene (Cooperstone et al., 2015). ‘Moonglow’, an orange heirloom tomato variety, contains >90% lycopene in *cis*- isomeric form whereas conventional red tomatoes contain >90% lycopene in all-*trans*- isomeric from. However, to date no studies have been conducted to compare the effects of *cis*- versus *trans*- isomeric forms of lycopene when consumed in whole tomato form *in vivo* or to assess cultured bone cells supplemented with whole tomato extracts *in vitro*. Hence the aim of

this study was to identify any bone health-promoting properties of red and 'Moonglow' tomatoes. This study also evaluated red and 'Moonglow' tomato for potential prebiotic-like effects. This is the first study that compared the beneficial effects of red and orange ('Moonglow') tomatoes on bone health through *in vivo* and *in vitro* assays to compare the bioactivity of *cis*- and *trans*- isomeric forms of lycopene present in tomatoes. Several studies have reported that the *cis*- lycopene found in orange (*tangerine*) tomatoes is more bioavailable than the all-*trans*-lycopene in red tomatoes (Cooperstone et al., 2015, Burri et al., 2009, Unlu et al., 2007b, Unlu et al., 2007a). Previous reports had suggested that the higher bioavailability of *cis*- lycopene isomers is due to its lipid-dissolved structures, whereas all-*trans*- isomers are located in crystalline structures (Cooperstone et al., 2015). Our work has also confirmed the presence of round shaped non-crystalline carotenoid containing structures in 'Moonglow' while in red tomatoes the lycopene was visible in needle-like crystalline structures (Appendix i, Figure 2-4).

7.2 Changes in lycopene content with tomato maturity stages and lycopene stability during storage

We confirmed a major decline in lycopene concentration over time, that led to a requirement to recalculate the dose of powder supplied each month. Lycopene is liable to degrade in the presence of high temperature, light, and oxygen, which affects bioavailability (Shi and Maguer, 2000). As we used freeze-dried tomato powder for the preparation of tomato supplement treats for rats over four months, we tested the stability of freeze-dried tomato powder stored at -20 ^{0}C for 90 days with or without nitrogen (N_2) while protected from light. Interestingly, there was no significant difference in the stability of red vs 'Moonglow' derived lycopene in long-term storage ($p>0.05$). N_2 flushing was effective in reducing lycopene loss at 60 days in both tomato varieties ($p<0.05$) but the benefit from N_2 did not continue to 90 days (Appendix i, Figure 5, Table 1). These results are in line with reported data where powdered tomato had poor lycopene stability during storage and N_2 flushing also had a limited effect on the stability (Shi and Maguer, 2000). Another study has found that lycopene degradation is higher in freeze-dried tomato samples than oven dried samples (Sharma and Le Maguer, 1996). We conclude that long-term storage reduces the content of *cis*- and *trans*- lycopene in freeze-dried tomato powder, and storage under N_2 can partially mitigate this loss. This information will inform storage of lycopene samples for use in animal or human clinical trials and emphasises the need to re-assess composition just before use. Therefore, we

analysed the lycopene content of our freeze-dried tomato samples once every four weeks prior to preparation of tomato supplements for rats.

Tomato colour is an important indicator of the lycopene content. Tomato colour changes with maturation; the changes in tomato colour from green to red are the result of both lycopene synthesis and chlorophyll degradation (Bui et al., 2010). Changes in lycopene content in red tomatoes with different ripening stages have been extensively studied (Gastélum-Barrios et al., 2018, Namitha et al., 2011) and for red tomatoes lycopene content was found to increase up to firm red stage with no additional lycopene synthesis thereafter (Arias et al., 2000). Therefore, in red tomatoes a more intense red colour appears to be primarily due to degradation of chlorophyll rather than accumulation of lycopene. We analysed the lycopene content of orange 'Moonglow' tomatoes at seven maturity stages and found that the lycopene content increased significantly with the colour and found the highest amount of lycopene when the tomato skin was >90% in orange in colour, indicating that the increase of tomato colour in this orange variant was highly dependent on the lycopene content (Appendix i, Figure 6). The sample size was a limitation of this analysis as three experimental replicates were used from one tomato to represent each maturity stage. However, these pilot data indicate the importance of additional research to analyse lycopene content against the colour index parameters, in order to gain a better understanding of the relationship between tomato colour, maturity stage, and lycopene synthesis and accumulation.

7.3 Carotenoid compositions: from tomatoes to body tissues

A comparison of tomato carotenoids demonstrated that the red tomato ('Merlice') we used for our study predominantly contained all-*trans*-lycopene and β-carotene and small amount of lutein, whereas the orange 'Moonglow' predominantly contained tetra-*cis*- lycopene and a mixture of ζ-carotenes (Appendix i, Figure 7-8). In addition to the all-*trans* lycopene, β-carotene and lutein were quantified in red tomatoes. Based on the amount of tomato powder fed per rat per day, the received doses are calculated to be 2.6 µg of lutein and 6.5 µg of β-carotene /day/rat. No β-carotene or lutein were detected in 'Moonglow' tomato powder other than a considerable amount of ζ-carotene, which is a precursor of lycopene.

A standard rat chow diet prepared to ensure maximum growth and well-being of rats was used in the main feed, and therefore it had to be considered that the nutrients in

rat chow diet could mask beneficial effects of tomatoes. We analysed both the standard chow diet and the control treat-supplement (wheat flour + peanut butter + honey) used to deliver the tomato powders, to confirm that they were free from lycopene. We did not identify any lycopene present in either preparation, but a few other carotenoids were found. We could identify a very small amount of lutein in the control supplement and also tentatively identified zeaxanthin (Gupta et al., 2015) according to absorption spectra (Appendix i, Figure 10). The presence of zeaxanthin and lutein could be due to incorporation of wheat flour, as zeaxanthin and lutein are major carotenoids in wheat (Abdel-Aal et al., 2007, Adom et al., 2003). β-cryptoxanthin, zeaxanthin and α-carotene were tentatively identified based on absorption spectra in rat chow (Appendix i, Figure 9). This carotenoid composition in standard chow could be from the corn starch and wheat.

One limitation of this study was that we did not measure the daily feed intake, as this is extremely labour-intensive. Instead, we estimated the quantities of β-carotene and lutein consumed by rats based on the reported average feed intake of female Sprague-Dawley rats (Siriarchavatana, 2021). We found the measured β-carotene and lutein content in our standard rat chow were 0.015 and 0.34 mg/day/rat respectively. According to published studies, a significant bone health promoting properties were observed with β-carotene intake at 3.75 mg/day/rat (Matsumoto et al., 2018) and lutein intake at 66 mg/day per rats (Takeda et al., 2017). Therefore, it is unlikely that the chow's contribution of these carotenoids, calculated to be ~200-fold lower than the published effective doses, contributed to the results of our study.

Our *in vivo* study 1 (chapter 3) found that 'Moonglow' tomato at 0.35 mg lycopene /kg BW/day could achieve a lycopene plasma concentration comparable to that measured in a similar time frame with a 70-fold higher dose of all-*trans*- (Zaripheh *et al*, 2003). Also, after only four days of feeding our dose achieved a plasma concentration of 0.42 μmol/L, which is similar to the reported physiologically beneficial concentration in humans (Hanson et al., 2018, Mackinnon et al., 2011a, Fielding et al., 2005, Walfisch et al., 2003) (Chapter 3). Supporting the literature reporting 8.5-fold higher bioavailability of *cis*-lycopene (Cooperstone et al., 2015), we also found an 8-fold higher plasma lycopene concentration in rats fed 'Moonglow' tomatoes compared to red tomatoes. However, the plasma lycopene concentration we achieved from this dose in our *in vivo* study 2 was several times lower compared to our *in vivo* study 1. This appears to reflect

gradual stabilisation of circulating lycopene levels over time, resulting in the fact that we did not observe the expected physiologically beneficial plasma concentration after 8 or 16 weeks of tomato feeding. The plasma concentration we achieved in our *in vivo* study 2 (chapter 5) following 'Moonglow' feeding after 8 or 16 weeks (0.040-0.043 µmol/L) was >10-fold lower than the plasma concentration achieved in our *in vivo* study 1 (0.6 µmol/L; chapter 3). Similarly, the liver lycopene concentrations achieved in study 2 (chapter 5) following 'Moonglow' feeding after 8 or 16 weeks were 6.824-6.935 µmol/kg respectively, but we found 15.570 µmol/kg lycopene in the liver in study 1. As discussed in chapter 5, continuous feeding of lycopene for longer period may reduce absorption and tissue distribution and increase excretion of lycopene mainly through increased metabolism via upregulation of enzymes involved in lycopene metabolism (Zaripheh and Erdman Jr, 2005, Zaripheh et al., 2003, Khachik et al., 2002) .

Although plasma lycopene concentrations were significantly different between red and 'Moonglow', both tomato types resulted in rats storing similarly amounts of lycopene in the liver (Chapter 5). Unlike plasma, liver is a major site of lycopene metabolism and accumulation. In the liver, lycopene is stored mainly in the lipid droplets of hepatic stellate cells (Teodoro et al., 2009). According to the physiological pharmacokinetic model describing the disposition of lycopene, liver is considered as a fast turnover pool, while other tissues are considered as a slow turnover pool (Appendix i, Figure 11). Being a fast turnover pool, liver can supply lycopene to the blood circulation upon acute demand; thus, a low lycopene diet can drastically reduce the liver lycopene stores (Diwadkar-Navsariwala et al., 2003). Specific factors are thought to contribute to tissue carotenoid concentrations through recycling of carotenoid back to the liver and excretion of carotenoids (Wang, 2012); however, these factors are not yet fully understood (Canene-Adams and Erdman, 2009). For instance, a recent finding from a study in which lycopene isomers were fed to mice at 5 or 10 mg/kg BW produced, after 4 weeks of supplementation, total liver lycopene ~3 times higher total liver lycopene in mice fed with *cis*- compared with *trans*- at both doses. Our dose of at 0.35 mg lycopene /kg BW/day, being >10-fold lower, was insufficient to produce isomer-dependent differences in liver lycopene stores. However, our data support the reversibility of lycopene from liver to plasma. As discussed in chapter 3, we evaluated the proportion of retained lycopene accumulated in the liver and found that although the retained lycopene did increase incrementally over time the kinetic changes in liver accumulation were not

statistically significant (Chapter 3). It is not possible to compare the liver lycopene findings in the rat model to the human, as there are no data available for this. It must be acknowledged that rat and human physiology and metabolic processes are not identical, and findings in the rat may not extrapolate to the human.

7.4 Effects of lycopene on bone properties

As reported in Chapter 3, we evaluated the bone health-promoting properties of red and 'Moonglow' tomatoes in ovariectomized rats. We chose freeze-dried whole tomato powder as the source of lycopene to more closely mimic the consumption of whole tomatoes by humans. However, in terms of bone health-promoting properties, 'Moonglow' *cis*- lycopene was effective only in reducing bone turnover compared to all-*trans*-lycopene in red tomatoes. To date, the lowest lycopene dose reported to have beneficial effects on bone health in rats was 10 mg/kg BW (Walallawita et al., 2020). Our study for the first time reported that 0.35 mg/kg BW lycopene, a dose 28-fold lower, could inhibit bone turnover. In particular, 'Moonglow' tomatoes resulted in improved outcomes even when intake was initiated after ovariectomy and the initiation of osteoporosis.

Other variables tested, such as bone mineral density measures, mechanical properties and bone microarchitecture, showed no significant differences between red and 'Moonglow' tomatoes (Chapter 4). Our lycopene dose may have been insufficient to make measurable improvements in these bone properties, despite achieving significant lycopene plasma concentrations. We chose to restrict the post-OVX period to 8 weeks as previous rat studies showed a better osteoporotic response 3 - 8 weeks after OVX (Oliveira et al., 2019, Iimura et al., 2015, Liang et al., 2012). Studies which attained more significant osteoporotic responses performed OVX surgery on rats aged 3 - 6 months (Kruger and Morel, 2016, Ardawi et al., 2016, Iimura et al., 2015, Mathavan et al., 2015, Francisco et al., 2011); therefore, we carried out the OVX in rats at age of 4 months and our OVX model was successful in inducing osteoporosis as measured by most of the bone parameters tested. We based our experimental design on earlier trials but selected a lycopene dose that would equate to an achievable intake of fresh tomato by consumers. Although our study design was properly planned, we did not see any significant changes in bone parameters following tomato feeding except for bone markers. The lower dose used for our study likely explains the lack of observed osteoporosis prevention by tomato supplementation.

In our study, the rats may have neared but not reached the plasma concentration of lycopene required for beneficial bone health effects. As an example, a study carried out to evaluate bone properties following lycopene feeding at 45 mg/kg BW, >100-fold higher than ours, showed significant improvements of bone measurements, and the plasma lycopene concentration following this dose was 0.071 µmol/L. Compared to this study, the plasma concentration we found from 'Moonglow' tomato feeding for 8 and 16 weeks was 0.040 and 0.043 µmol/L respectively. This suggests that plasma lycopene may not have a dose-dependent effect below a certain threshold, which our animals failed to achieve. Considering the plasma lycopene concentrations achieved in our two rat studies held for 5 days and 16 weeks respectively, and the previously reported beneficial concentrations for lycopene in rats for bone health promoting properties, we suggest that feeding our high dose (2.6 mg/kg/BW) for 12 weeks after OVX could possibly reveal more significant changes in bone measures and we would recommend this for a future study.

In the *in vitro* study, the effect on osteoclastogenesis was not significantly different between red and 'Moonglow' tomato extracts. However, both tomato varieties significantly reduced the number of mature osteoclasts at the highest lycopene dose of 10 µmol/L (Chapter 6). Studies have found that derivatives of carotenoids, but not intact molecules, regulate the inhibition of NFκB via directly interacting with the proteins involved in the NFκB pathway (Linnewiel-Hermoni et al., 2014). Similarly, the inhibition of RANKL-induced osteoclast differentiation is regulated by the oxidation products of lycopene but not by intact lycopene (Odes-Barth et al., 2020). For this reason, it is more likely that the similar inhibition effects produced by both red and 'Moonglow' extracts on osteoclasts differentiation could be due to lycopene derivatives, regardless of the isomeric form of lycopene supplied with the media. The fact that red and 'Moonglow' tomato extracts differed in their magnitude of efficacy *in vivo* but not *in vitro* is likely due to the fact that only in the *in vivo* situation is lycopene bioavailability in the gut a relevant factor.

For the *in vitro* cell studies we used a tomato hexane extract which predominantly contained carotenoids (Hwang and Bowen, 2005). Hexane was identified as the preferred solvent for maximum extraction of total carotenoids from tomatoes (Tanambell, 2019). Preparation of tomato extracts using hexane as a solvent minimised the interference from water-soluble compounds in tomatoes. Due to this phase separation, the water-soluble

compounds such as vitamin C and phenolics were concentrated in the polar phase and subsequently removed. This phase separation eliminated possible additive or synergetic effects that might be exerted by water soluble compounds present in tomatoes. We analysed and confirmed the red and 'Moonglow' tomato hexane extracts did not contain any polyphenols (Appendix i, Figure 20). Therefore, it is unlikely that any effect observed in the cell studies can be attributed to tomato polyphenols.

Various other carotenoids have been shown to have bone health-promoting properties. A cross-sectional study revealed a positive association between increased bone mineral density and the serum levels of lutein and zeaxanthin in humans (Bovier and Hammond, 2017). A cohort study in Japan demonstrated beneficial effects of β-carotene and β-cryptoxanthin on bone loss in post-menopausal women (Sugiura et al., 2012). In another study β-carotene showed a slight inhibition of bone loss at the proximal tibia and the findings suggested that inhibition of osteoclast formation caused the observed reduction in bone loss (Yu Matsumoto, 2018). A study using rat femoral tissue to measure the effects of the carotenoids β-carotene, lutein, lycopene, and β-cryptoxanthin on alkaline phosphatase activity and calcium content showed a significant effect of β-cryptoxanthin on bone calcification whereas lycopene had no effect (Yamaguchi and Uchiyama, 2003). Wattanapenpaiboon *et al* studied the relationships between bone mineral status and dietary carotenoid intake and found a positive relationship between β-carotene intake and increased bone mineral density (BMD) in post-menopausal women while lycopene, lutein and zeaxanthin intake was positively correlated with BMD of perimenopausal women (Wattanapenpaiboon et al., 2003). As shown in Fig 3.1, 'Moonglow' tomatoes contain a considerably higher amount of ζ-carotene, but there are no studies that specifically investigated the effect of ζ-carotene on bone health. Therefore, there is a possibility that ζ-carotene itself may have had an effect in the observed results in our study, however, further work is needed to evaluate the role of ζ-carotene in bone health promoting properties. It is also reported that consumption of a mixture of carotenoids is more effective than isolated compounds and a marked synergistic effect was found when lycopene and lutein were present in the mixture (Heber and Lu, 2002). However, as discussed above, except for ζ-carotene, it is highly unlikely that the results of this study were from additive or synergetic effects of the other carotenoids as they made a minimal contribution to the daily intake.

7.5 Correlation between BMD, body composition and gut microbiota

Measurement of bone mineral density (BMD) is considered the most clinically relevant indicator of fracture risk (Nguyen et al., 2005, Marshall et al., 1996) and thus is an effective way to diagnose the onset of post-menopausal osteoporosis. The combination of fat mass and lean mass (fat free mass) are collectively termed body weight (Ho-Pham et al., 2010). BMD is directly correlated with body weight (Nguyen et al., 1994) indicating that people with higher body weight have stronger bones (Nguyen et al., 1994). There are a number of large epidemiological studies reporting a positive relationship between body weight and BMD (Nguyen et al., 1997, Edelstein and Barrett-Connor, 1993, Felson et al., 1993), although it is not fully established whether the benefit is due to lean mass, fat mass, estrogen produced by adipose cells, or a combination of these factors (Kremer and Gilsanz, 2016). The mechanisms by which adipose tissues influence BMD are less clear. However, adipokines and molecules secreted by osteoclasts and osteoblasts may play a role in the relationship between bone and fat (Salamat et al., 2013). It is also suggested that obesity may improve bone mass due to conversion of androgen into estrogen by adipocytes (El Hage et al., 2009). The relationship between increased BMD and lean mass can be due to mechanical loading of the skeleton as higher lean mass is related to physical activity (Chubak et al., 2006). Muscle contractions exert a higher force on bones than other weight-associated gravitational forces (Burr, 1997). Several cross-sectional studies of pre- and post-menopausal women have reported a positive relationship between fat mass and BMD (Reid et al., 1994, Reid et al., 1992), while other studies reported both fat and lean mass positively correlate with BMD (Seeman et al., 1996, Lindsay et al., 1992). However, these relationships may depend on the method by which BMD is measured (Khosla et al., 1996). The relationship between fat and lean mass and BMD can also be influenced by environmental factors (Nguyen et al., 1998).

We observed that BMD in the right femur and lumbar spine of OVX rats was positively correlated with body weight (BW), fat mass and lean mass (Appendix i, Table 18-23). However, the relationship between BW and BMD becomes more complex during the postmenopausal period or post-ovariectomy, as estrogen deficiency may induce both fat deposition and bone loss (Pitroda et al., 2009, Bolland et al., 2006). In addition, estrogen deficiency increases the loss of calcium from bones (O'Loughlin and Morris, 1998). Moreover, a correlation between elevated levels of parathyroid hormone and body weight was found in obese menopausal women (Bolland et al., 2006), and another study

identified low levels of circulating vitamin D in obese people (Drincic et al., 2012). A study in obese, ovariectomised rats showed that although there were significant correlations between fat and lean mass, BW and fat mass, and BW and lean mass, the data overall showed that lean mass was more strongly correlated with BMD than BW and fat mass itself (Siriarchavatana, 2021). This finding validated the multiple linear regression analysis in postmenopausal women that revealed lean mass is the strongest predictor of BMD in all sites (Ho-Pham et al., 2010). However, increased BW after menopause/ovariectomy is usually due to a proportionally greater increase in fat mass than lean mass. Therefore, the relationships between BMD, fat mass, lean mass, and body weight in ovariectomized rats have not yet been fully defined.

Gut microbiota may affect bone health in several ways, as they can break down macromolecules into micro-components which may improve bone health via increasing bone mineral density (Quigley, 2013). For instance, *Lactobacillus* and *Bifidobacterium* can improve BMD via increasing the absorption of minerals such as calcium, magnesium and phosphorus (Rodrigues et al., 2012). Gut microbe composition also regulates gut pH which is important in calcium absorption (Palmer and Rolls, 1981). Further, gut microbes regulate BMD via both alteration of immune system and regulation of hormones and neurotransmitters (He et al., 2020, Ding et al., 2020). Our current study showed an increased abundance of five bacterial species tested (*Lactobacillus, Bifidobacterium, Enterococcus, Bacteroides* and *E. coli*), and the tomato feeding prior to ovariectomy led to significantly higher abundance of all gut microbes (Chapter 5). However, we did not see a significant relationship between BMD and the abundance of gut microbiota (Appendix i, Table 18-23). There remains a scarcity of studies related to the effect of carotenoids on gut microbiota. (Bohn et al., 2015). Gut microbes produce short chain fatty acids (SCFAs) as fermented end products, which regulate intestinal pH and growth of harmful bacteria and also maintain the homeostasis of the intestinal environment (Sun et al., 2020). Therefore, changes in microbiota are associated with the SCFA profile (García-Alonso et al., 2017). As our study found no significant correlation between lycopene content and bacterial groups or SCFA, we hypothesise that the observed changes in microbiota and SCFA were induced by phenolic compounds and/or fiber in tomatoes, rather than lycopene (García-Alonso et al., 2017). Tomatoes are a good source of dietary fiber (Koh et al., 2010), although the dietary fiber present in tomatoes is mainly insoluble and thus poorly fermented by gut microbes (Lattimer and Haub, 2010).

Human studies have found that phenolic compounds are not absorbed by the stomach but instead proceed to the large intestine where they can facilitate the activity of gut microbes (Dueñas et al., 2015a, González-Barrio et al., 2011). Phenolics more effectively induce changes in microbiota and SCFA. Therefore, phenolic compounds are considered the more likely contributor for the observed prebiotic-like effect exerted by tomatoes (García-Alonso et al., 2017). In our study, the significantly higher abundance of gut microbes in 'Moonglow' compared to red tomato groups could be due to variability in phenolic content and subsequent changes in production of SCFA. We did not evaluate the phenolic compounds or SCFAs in our study, but our findings provide justification and future direction for the evaluation of these compounds following tomato feeding, to improve our understanding and ability to interpret our gut microbe results.

7.6 Implications of the research

Our study outcomes provide novel information about the beneficial effects of regular consumption of red and orange ('Moonglow') tomatoes. The study findings confirmed increased bioavailability and absorption of *cis*- isomers of lycopene compared to all-*trans*- and demonstrated that both tomato powders had some beneficial effects on bone health *in vitro* and *in vivo*. As our study results are based on an *in vitro* cell line and a rat model, they are not conclusive and may not extrapolate to post-menopausal women. Further research in this area, particularly human intervention trials, would provide support for this dietary approach on the management and prevention of post-menopausal osteoporosis. In addition, this study's findings provide tentative support for the value of exploring the potential prebiotic-like effect of tomatoes and the mechanisms associated with changes in gut bacteria.

7.7 Future research directions

The limitations of the current study could be overcome via an extended research design in the rat OVX model using higher doses of lycopene, slightly longer feeding periods, and direct comparisons between whole tomato powder and purified lycopene. Also, further examination of the effects of tomato extracts on the cellular and molecular aspects of bone cell biology could provide important new information to better understand the osteoprotective effects of tomatoes. Some of the future directions are mentioned below.

1. Several studies have suggested that the progression of osteoporosis is associated with oxidative stress produced by reactive oxygen species (Almeida, 2012, Wauquier et al., 2009). Oxidative stress can disrupt intracellular redox balance, which is crucial in cellular homeostasis. Therefore, removal of excessive ROS may be a preferred approach to maintain bone integrity (Banfi et al., 2008). As lycopene is a known antioxidant, evaluation of antioxidant enzyme activity such as plasma glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) would further our understanding of lycopene's antioxidant potential.

2. Our *in vitro* work was limited to osteoclastogenesis. Future research using tomato extracts to evaluate osteoblast and osteocyte activities would be beneficial in terms of interpretation in bone remodelling. Lycopene treatment may decrease osteoclast activity via down-regulating Nfatc1 gene expression and increase osteoblast activity by upregulating Alp, Col1a1 and Ocn gene expression (Ardawi et al., 2016). Therefore, the evaluation of key genes involved in the bone cell activity would help identify the molecular mechanisms associated with red and 'Moonglow' tomato feeding.

3. More research is needed to identify the optimum maturity stage of 'Moonglow' tomatoes against a simple colour index. Correlating changes in carotenoid composition with tomato colour would help consumers be able to choose tomatoes containing a greater amount and variety of beneficial phytochemicals.

4. Previous studies provide significant evidence on the relationship between lycopene intake and reduced prostate cancer risk (Rowles et al., 2017, Assar et al., 2016). It would be of value to expand the current study's scope and assess the effect of red and 'Moonglow' tomato extracts on the proliferation and survival of prostate cancer cell lines compared to non-cancerous cells. Subsequently, prostate cancer markers and the development and progression of prostate cancer could be evaluated in male rats supplemented with red versus 'Moonglow' tomato extracts.

7.8 Concluding remarks

In summary, we observed that the consumption of an achievable daily intake of fresh 'Moonglow' tomatoes could result in a physiologically-beneficial plasma lycopene concentration in humans after four days as explained in chapter 3. As chapter 4 & 5 described, 'Moonglow' tomato consumption produced ~8 fold higher mean plasma

lycopene concentration compared to red tomatoes. ‘Moonglow’ tomato feeding also caused a reduction of bone turnover even after OVX-initiated osteoporosis, although there were no significant changes found in bone mineral density, biomechanical properties and bone microarchitecture. We also observed that both red and ‘Moonglow’ tomato supplementation restored the numbers of bacteria in all five genera after ovariectomy, but ‘Moonglow’ tomato showed more consistent prebiotic-like effects. As described in chapter 6, our results showed similar anti-osteoclastogenic effects of tomato extracts containing significantly different lycopene isomeric profiles. We found a more significant reduction in anti-osteoclastogenesis at the concentration we used compared to what is reported elsewhere, suggesting that the effects that we observed could possibly be due to lycopene derivatives, regardless of the isomeric form of lycopene.

In conclusion, our study findings confirmed increased bioavailability and absorption of *cis*- isomers of lycopene compared to all-*trans*-. Both tomato powders had some beneficial effects on bone health *in vitro* and *in vivo*. However, further research with higher doses and longer intervention periods could possibly result in more significant changes in bone measures. Further human intervention trials would provide support for this dietary approach for the management and prevention of post-menopausal osteoporosis. In addition, this study’s findings demonstrated the value of exploring the potential prebiotic-like effect of tomatoes and the mechanisms associated with changes in gut bacteria.

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Appendices

Appendix i

Supplementary material

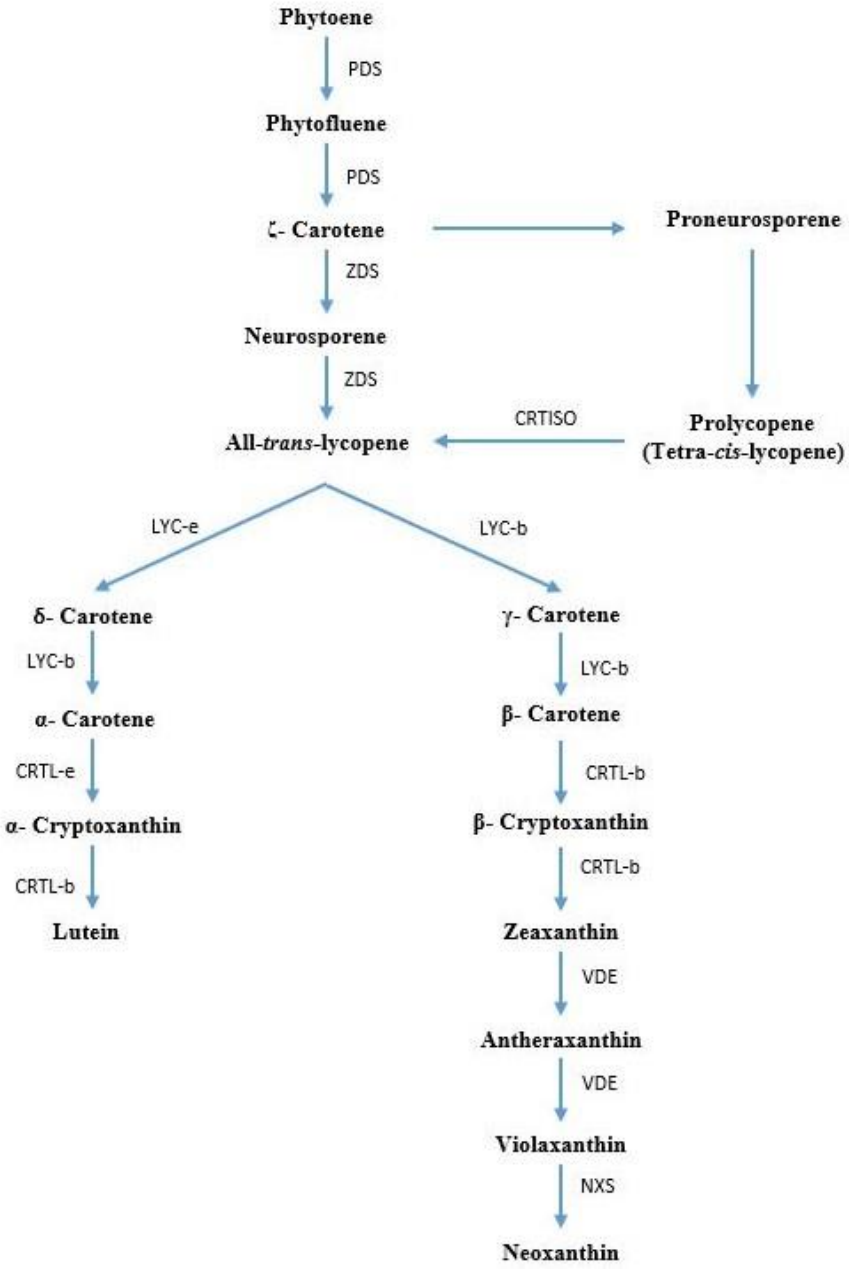


Figure 1 Carotenoid biosynthesis pathway in plants (adapted from (Fraser and Bramley, 2004)).

Abbreviations: PDS: Phytoene desaturases, ZDS: ζ-carotene desaturase, CRTISO: carotenoid isomerase, LCY-e: lycopene-ε-cyclase, LCY-b: β-cyclase, CRTL-e: ε-ring hydroxylase, CRTL-b: β-ring hydroxylase, VDE: violaxanthin de-epoxidase NXS: neoxanthin synthase.

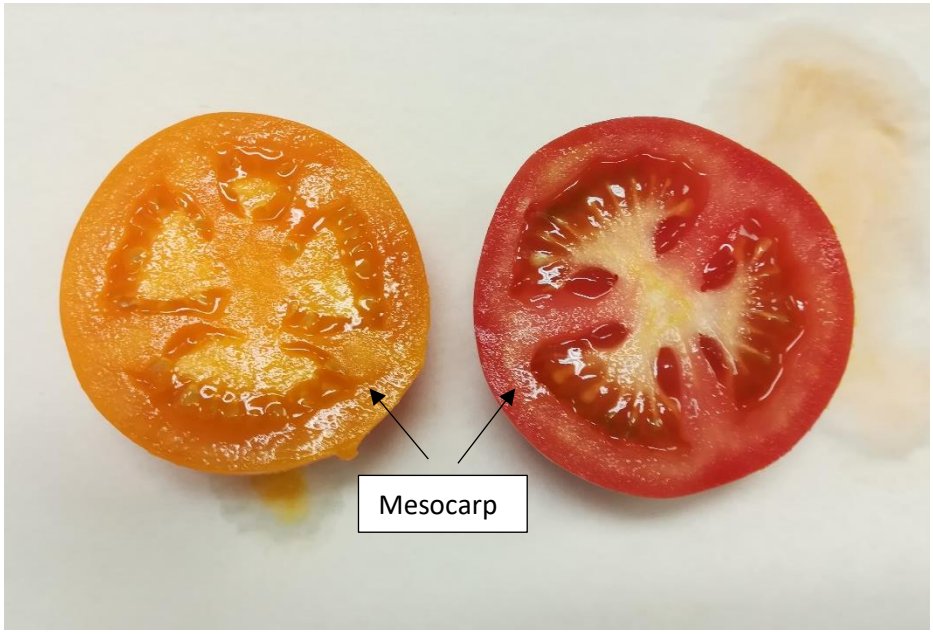


Figure 2 Cross sections of orange ‘Moonglow’ and red ‘Merlice’ tomatoes**.**

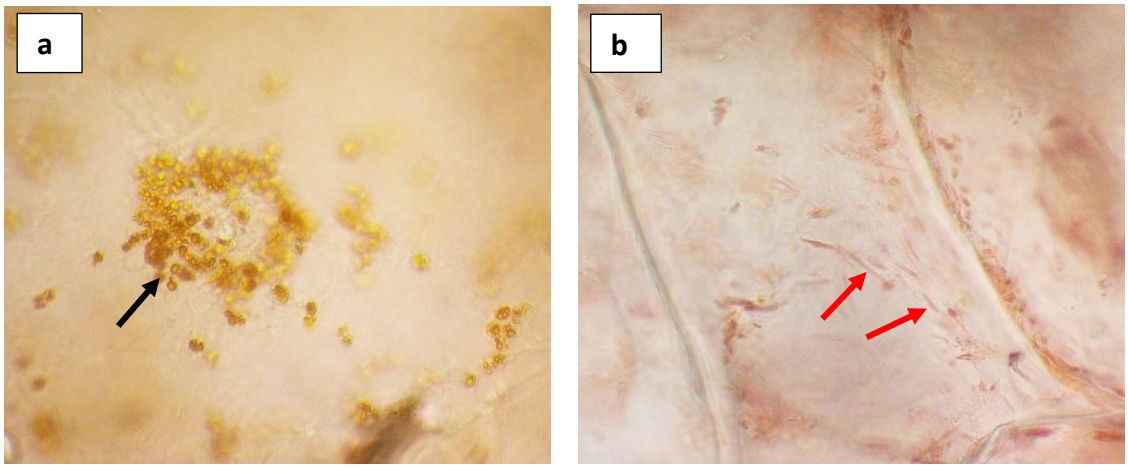


Figure 3 Light microscopic images of mesocarp of orange ‘Moonglow’ (a) and red ‘Merlice’ (b) at 400x magnification. Black and red arrows show non-crystalline and crystalline structures containing lycopene respectively.

Figure 4 Photographs of red (A1) and *tangerine* (*Solanum lycopersicon* L. hybrid FG10-314) tomatoes (B1) with corresponding light micrographs at 400× magnification of fresh red tomato (A2) and *tangerine* tomato (B2) mesocarp. Arrows and arrowheads denote crystalline and non-crystalline carotenoid containing structures, respectively (Cooperstone et al., 2015)(Copyright permission is granted for using this image).

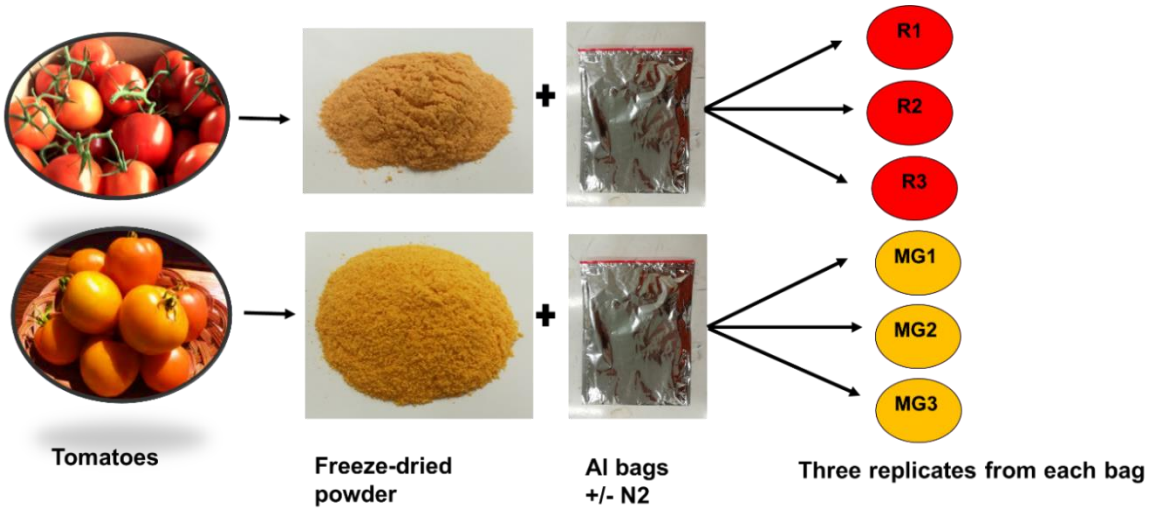


Figure 5 'Merlice' and 'Moonglow' tomatoes were freeze-dried, and the powder was packed in separate Al foil bags with and without N2 and stored at -20^{0}C. Lycopene content was measured at 0, 30, 60 or 90 days using uHPLC.

Table 1 Percent retention of lycopene in 'Merlice' (M) and 'Moonglow' (MG) tomatoes stored at -20^{0}C with and without N_2 for 30, 60 and 90 days.

% retention of freeze-dried	M (%)	M+N_2 (%)	MG (%)	MG+N_2 (%)
30 days	57	59	77	78
60 days	31	38	28	41
90 days	29	35	18	21

M represents red 'Merlice', MG represent orange 'Moonglow', +N_2 represent with nitrogen.

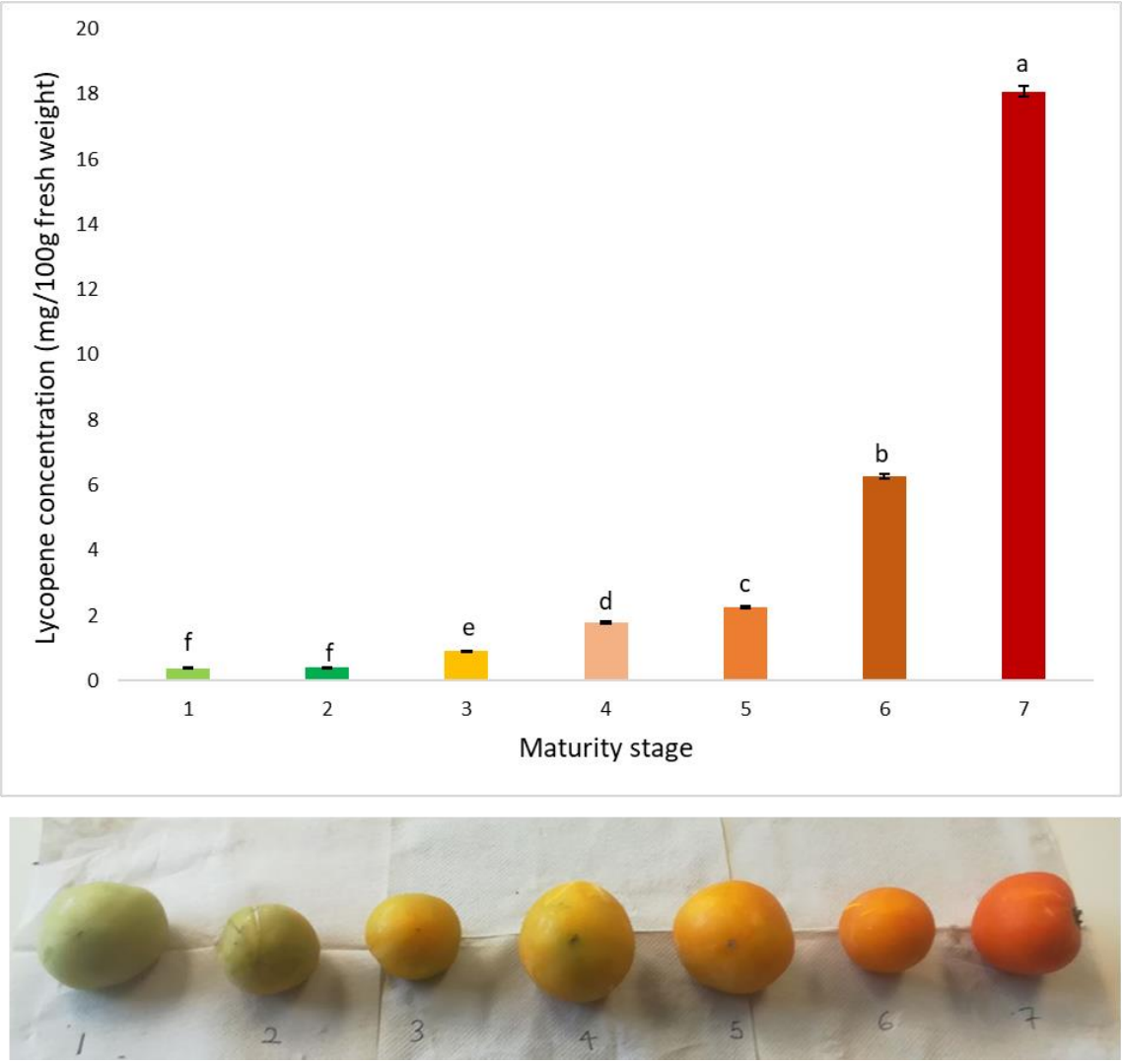


Figure 6 **‘**Moonglow’ tomato lycopene concentration (mg/100g fresh weight) in different maturity stages. Three experimental replicates were analysed from each tomato at each maturity stage. Bars with different letters (a, b) are significantly different ($P < 0.05$) as determined by one way ANOVA and post- hoc Tukey test.

Maturity stages (1-7), defined by the approximate percentage of colour.

1. Surface of tomato is completely green
2. ~10% of the surface is pale yellow colour
3. 10-30% of the surface is yellow-orange colour
4. 30-40% of the surface is yellow-orange colour
5. ~50% of the surface is orange colour
6. >70% of the surface is orange in colour
7. >90 % of the surface is orange in colour (over ripe)

Table 2 Dose calculation for the pilot studies.

Human recommended lycopene intake (mg/d) according to the intervention studies	25 mg/day
Human weight (assume)	60 kg
Bioavailability of *cis*- lycopene (assume)	8.5x than all-trans
Human recommended lycopene intake (mg/kg BW)	25/60=0.417
Rat simple body weight conversion factor from human	1
Rat metabolic conversion factor from animal dose to human equivalent dose (based on body surface area)	6.2
Rat simple body weight matched recommended lycopene intake (mg/kg BW)	0.417*1=0.42 (1)
Rat metabolic matched recommended lycopene intake (mg/kg BW)	0.417*6.2=2.60 (2)
assume 'Moonglow' lycopene is 8.5X more bioavailable, by simple body weight	0.417/8.5=0.05 (3)
assume 'Moonglow' lycopene is 8.5X more bioavailable, with metabolic conversion factor	2.583/8.5=0.30 (4)

There are two methods that can be used to convert human equivalent doses to animal doses: the first is based on body weight (BW), and the second is based on body surface area (BSA). According to Food and Drug Administration (FDA), the most accurate conversion of animal dose to human dose should be based on the normalization to body surface area (Nair and Jacob, 2016, Reagan-Shaw et al., 2008, Food and Administration, 2005). Further, BSA correlates with different biological parameters such as blood volume, circulating plasma proteins and renal functions, which makes BSA based conversion more reliable (Reagan-Shaw et al., 2008). However, there are some occasions where dose conversion based on body weight is appropriate such as the calculation for no observed adverse effect level (NOAEL) (Food and Administration, 2005). Therefore, we calculated and converted human doses to equivalent animal doses considering both simple body weight and body surface area.

Considering the bioavailability of *cis*- lycopene, metabolic conversion factor from rat to human and simple body weight conversion from rat to human, we calculated four doses as given in the above table. According to four values calculated based on conversion factors and the bioavailability of lycopene, (2) was selected as our high dose, (3) was selected as our low dose and mid dose (0.35 mg/kg BW) was selected by averaging (1) and (4).

Assumptions for the conversion of lycopene doses to grams of tomatoes.

- Bioavailability of *cis*- lycopene was assumed 8.5x than all-*trans*- (Cooperstone et al., 2015).
- Average lycopene in red tomato varieties range from 1.58-10.8 (mg/100 g fresh weight) (Park et al., 2020, Shi and Maguer, 2000). Therefore, average lycopene content for the calculation was assumed to be 5.5 mg/100 g fresh weight.
- Lycopene absorption in humans from dietary sources were assumed 30% (Rao and Ali, 2007, Rao and Rao, 2007).

Table 3 Conversion of lycopene dose to equivalent grams of tomatoes.

Rat doses	Lycopene concentration mg/kg BW rat	Human equivalent dose (HED) mg/kg BW	Equivalent to human's daily intake mg/d	Equivalent to grams of red tomatoes	Equivalent to grams of 'Moonglow' tomatoes
High (H)	2.60	0.421	25.26	1530 g	180 g
Mid (M)	0.35	0.056	3.36	204 g	24 g
Low (L)	0.05	0.008	0.48	29 g	3 g

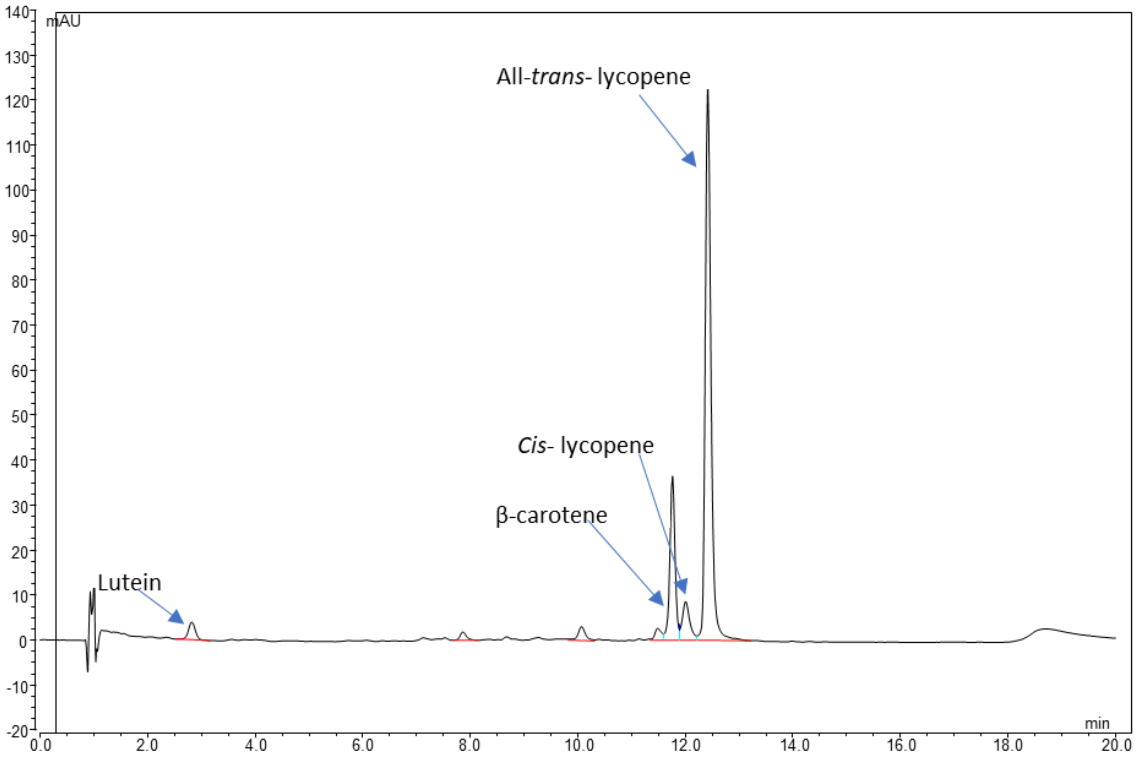


Figure 7: Carotenoid chromatogram of red 'Merlice 'tomato.

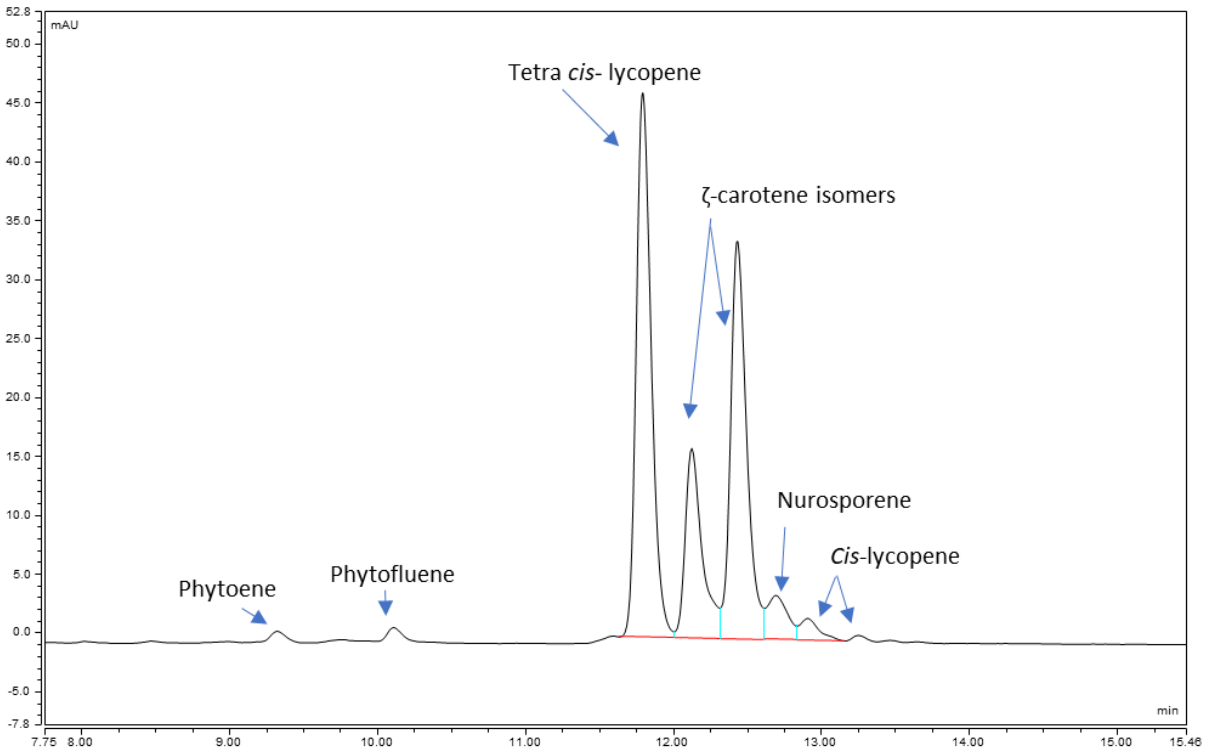


Figure 8 Carotenoid chromatogram of orange 'Moonglow 'tomato.

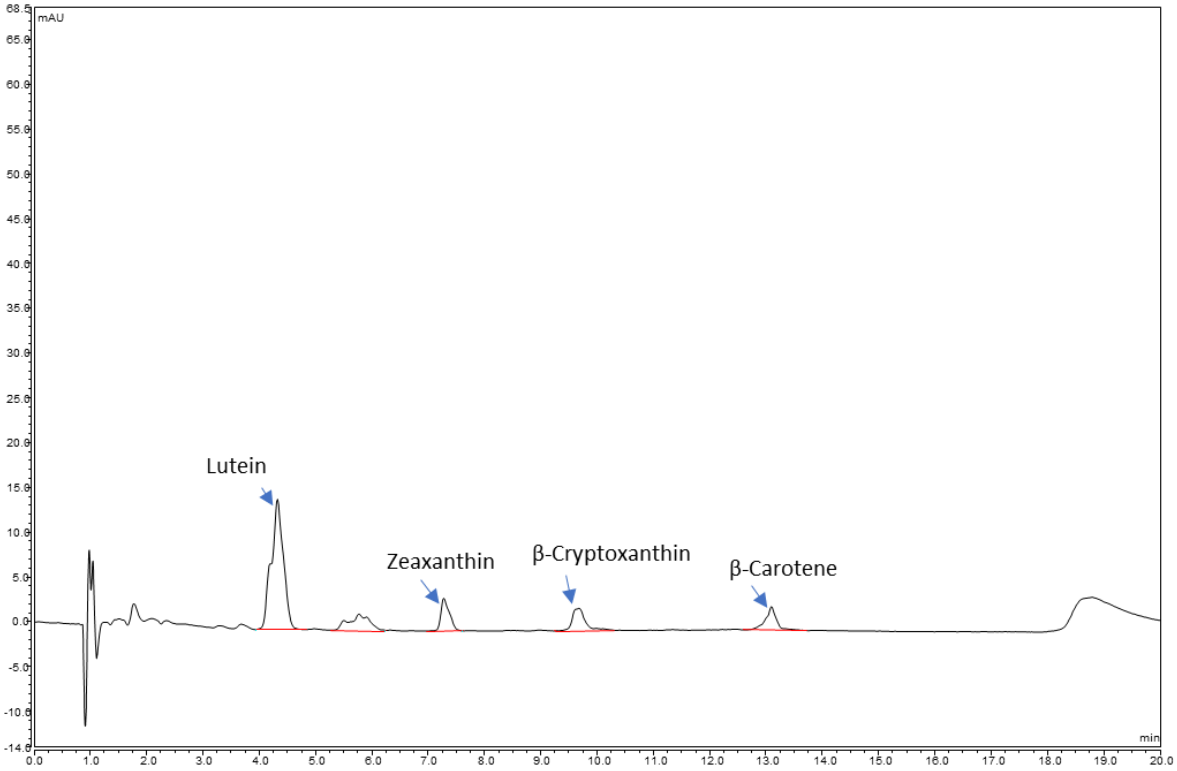


Figure 9 Carotenoid chromatogram of standard rat chow.

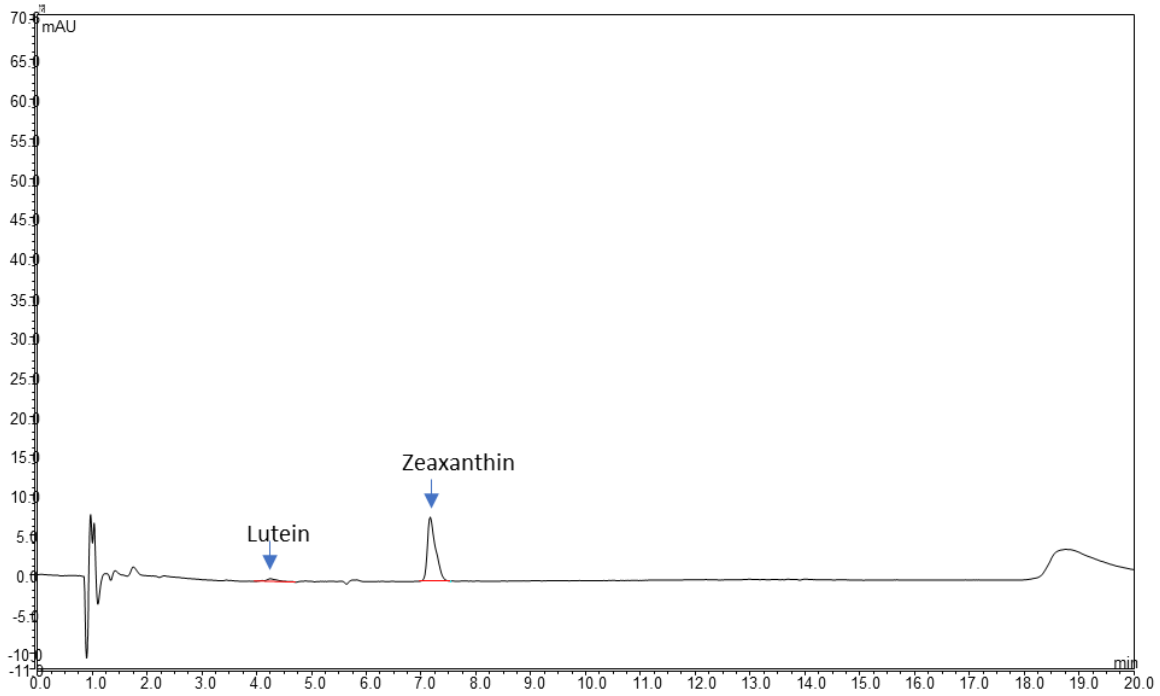


Figure 10 Carotenoid chromatogram of control supplements (wheat flour + peanut butter + honey).

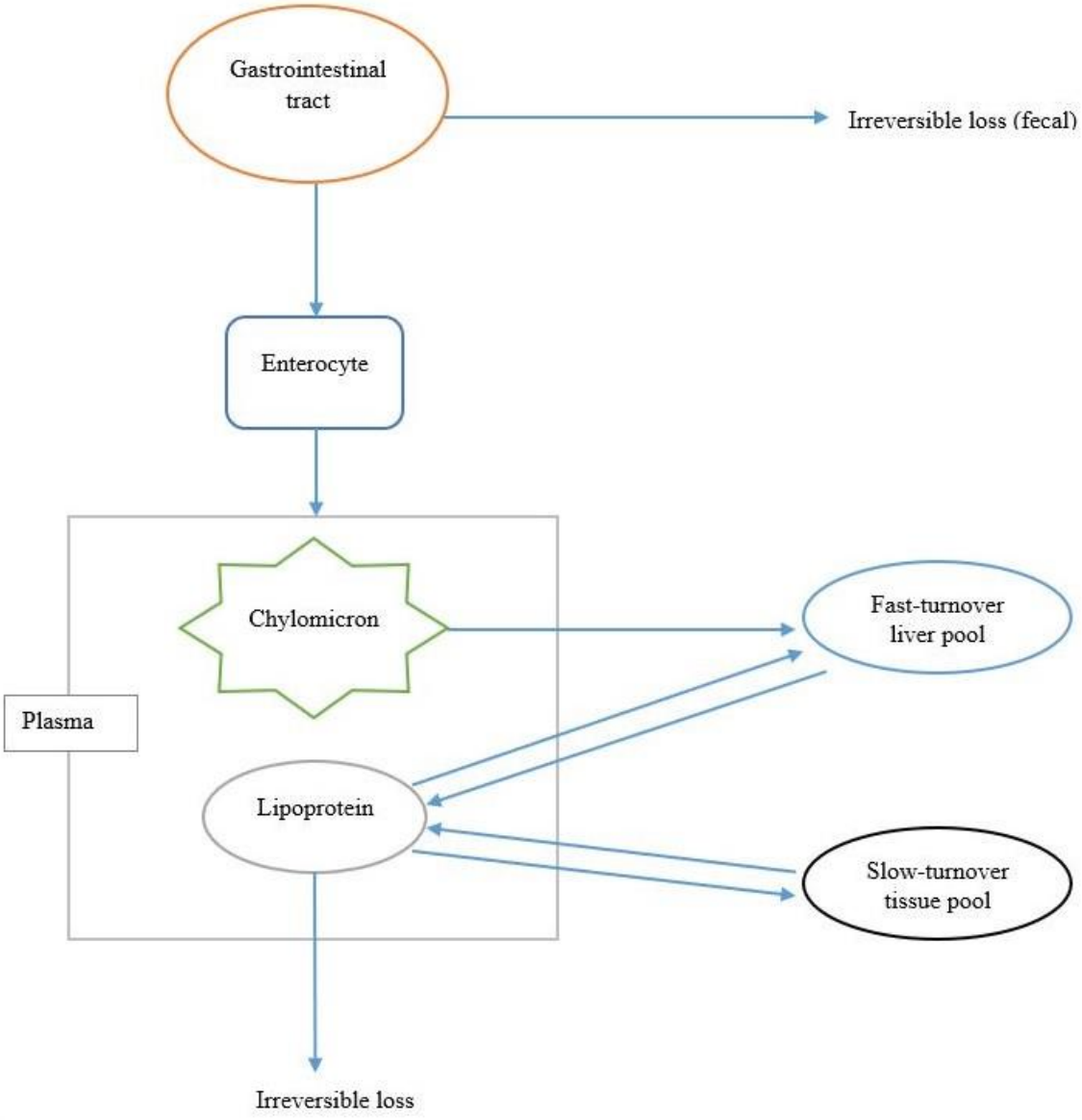


Figure 11 Pharmacokinetic model of carotenoid disposition (adapted from (Diwadkar-Navsariwala et al., 2003).

Table 4 Amount of feces excreted from day 1-5.

	Feces (mg/day)			
Day	Control	Low	Mid	High
1	3.25 ± 0.06	4.68 ± 0.13	3.52 ± 0.97	4.26 ± 0.60
2	4.68 ± 0.89	4.19 ± 0.68	3.30 ± 0.98	3.83 ± 0.93
3	4.16 ± 0.85	6.16 ± 0.63	5.86 ± 0.22	3.34 ± 0.58
4	3.84 ± 0.38	4.45 ± 1.03	6.36 ± 1.16	3.38 ± 0.54
5	4.41 ± 0.87	3.88 ± 1.34	5.69 ± 0.30	3.86 ± 1.07

Values are means ± SEM, n=3. Statistical analysis was performed between days in each dose using one-way ANOVA and post-hoc Tukey test.

Table 5 Amount of urine excreted from day 1-5.

	Urine (ml/day)			
Day	Control	Low	Mid	High
1	4.33 ± 0.73	7.83 ± 0.93	7.33 ± 1.30	5.00 ± 0.58
2	5.00 ± 1.76	5.17 ± 1.09	6.50 ± 1.53	4.50 ± 0.58
3	5.50 ± 1.04	6.33 ± 1.76	8.00 ± 0.29	4.50 ± 0.76
4	5.50 ± 1.61	6.50 ± 1.04	7.20 ± 0.91	4.67 ± 0.88
5	6.00 ± 1.32	6.00 ± 1.26	7.33 ± 0.73	5.50 ± 0.29

Values are means ± SEM, n=3. Statistical analysis was performed between days in each dose using one-way ANOVA and post-hoc Tukey test.

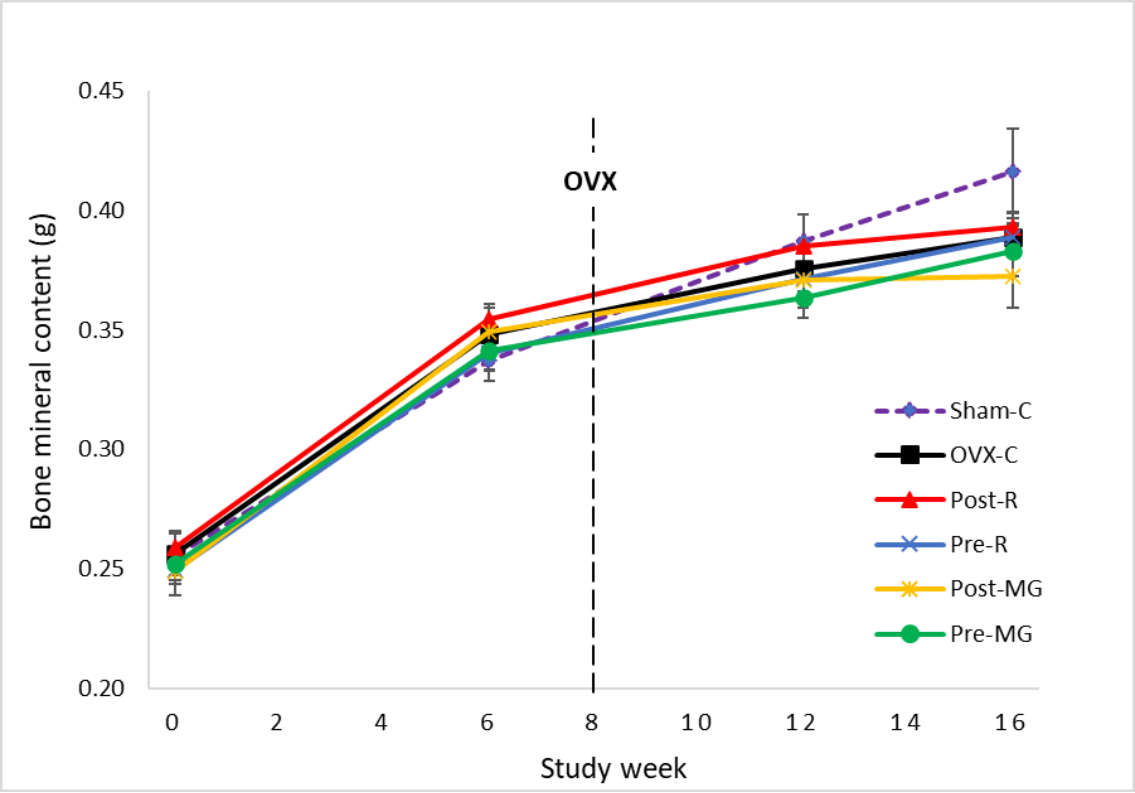


Figure 12 Right femur BMC in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks.

Table 6 Repeated measures ANOVA for right femur BMC during pre- and post-OVX feed period.

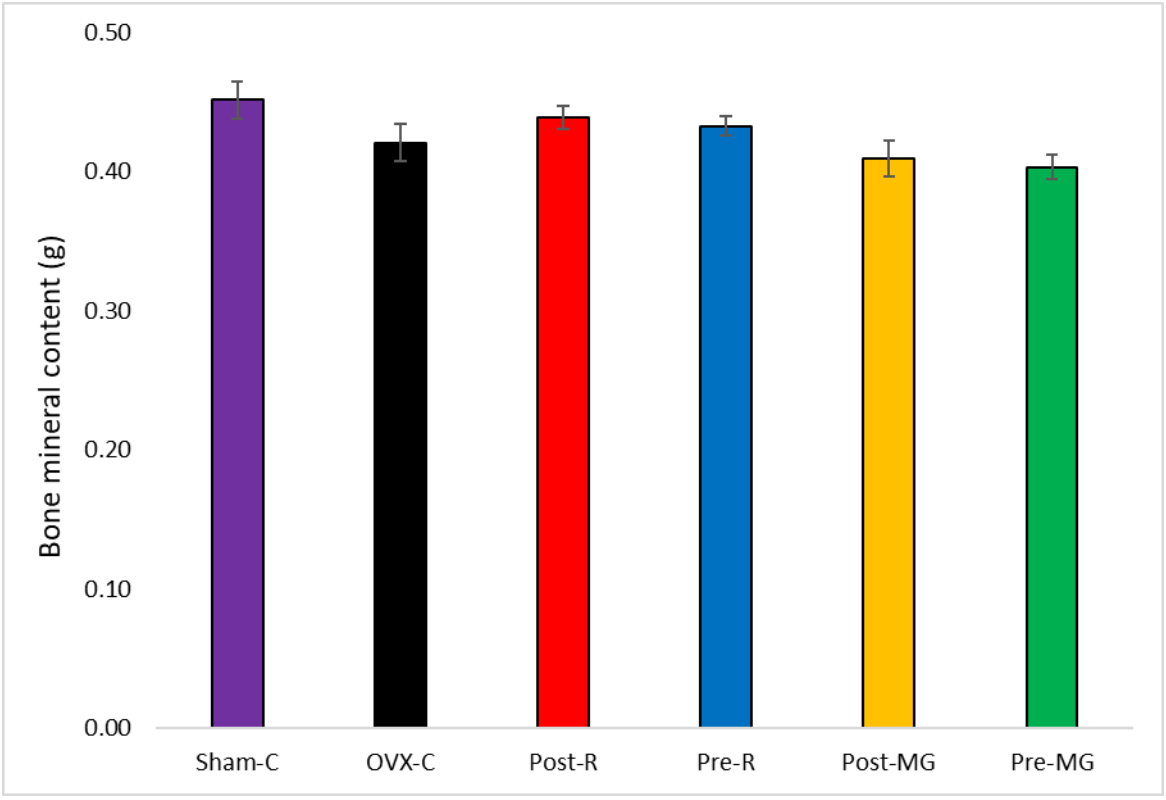
	Pre-surgery period (0-6 week)	**Post-surgery period (6-16 weeks)**
OVX only	**BMC**	**BMC**
Group	p=0.950	p=0.972
Week	p=0.078	p=0.400
Group x week	p=0.052	p=0.061

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.

Table 7 Percent change of right femur BMC in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	**Week 6-12**	**Week 12-16**	**Week 6-16**
Sham-C	32.80 ± 3.15	15.42 ± 1.36**	9.17 ± 2.99	26.25 ± 4.03*
OVX-C	35.54 ± 1.66	6.18 ± 0.59	7.00 ± 1.67	13.15 ± 3.45
'Post-R'	36.07 ± 1.83	7.21 ± 0.92	4.87 ± 1.93	13.47 ± 2.30
'Pre-R'	37.67 ± 2.06	9.23 ± 1.44	4.69 ± 1.48	14.00 ± 1.36
'Post-MG'	39.57 ± 1.66	6.29 ± 0.72	0.07 ± 1.17	6.23 ± 1.71
'Pre-MG'	36.07 ± 2.67	6.60 ± 0.85	5.60 ± 2.29	12.47 ± 2.48

Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one -way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *p<0.05, **p<0.001.



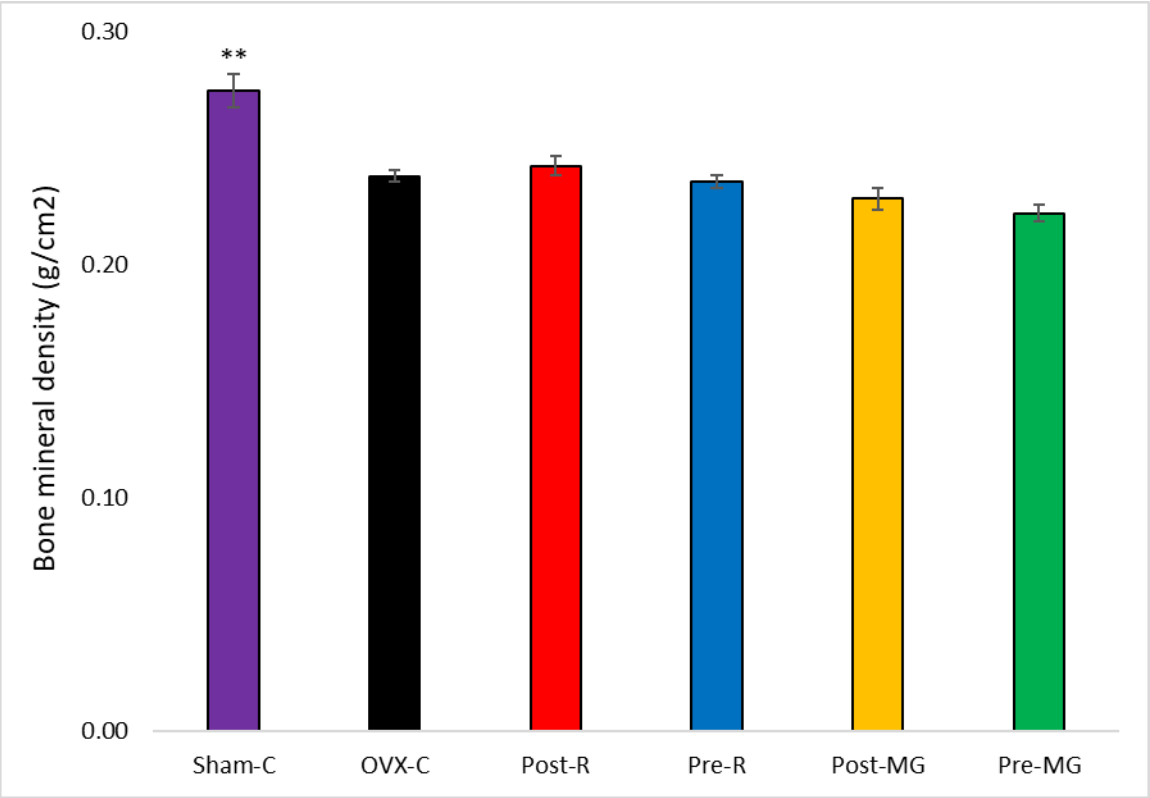


Figure 13 *Ex vivo* right femur BMC and BMD in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks. Statistical significance between OVX-C and experimental diet groups is indicated by asterisks ** $p<0.01$.

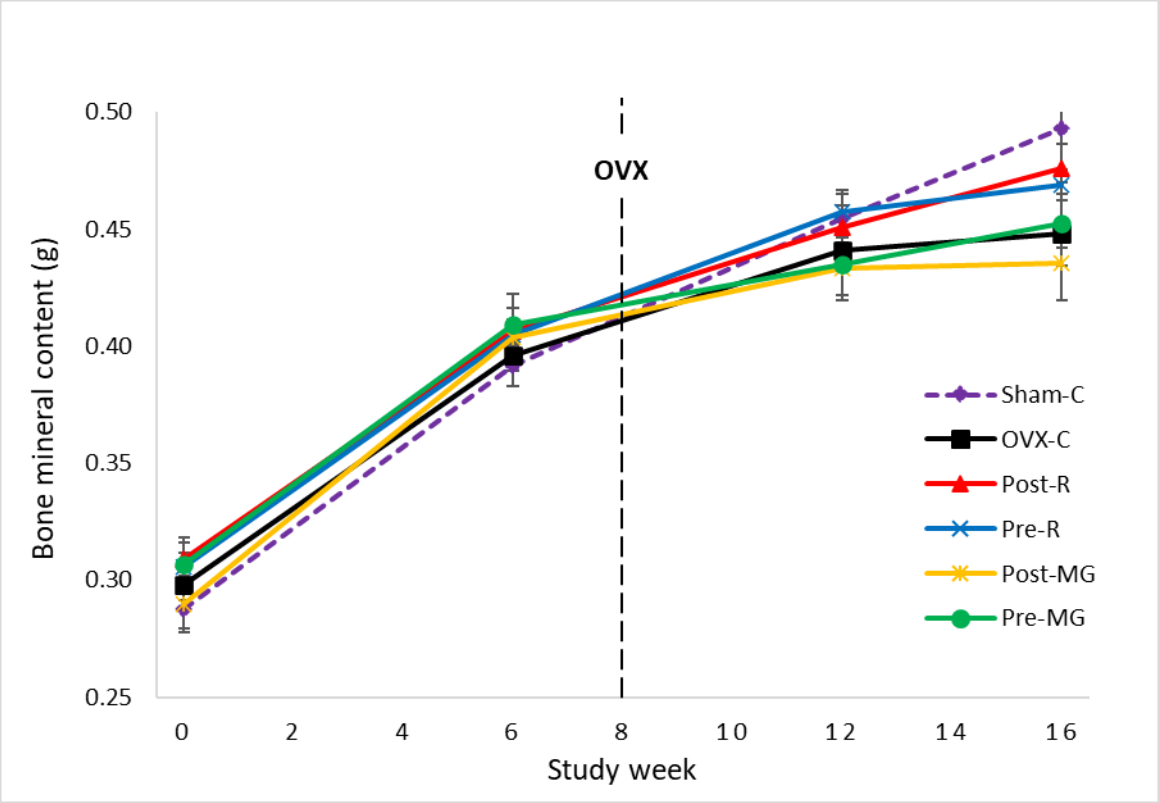


Figure 14 Lumbar spine BMC in female Sprague-Dawley rats (n=12-15) fed control or experimental supplements for 16 weeks.

Table 8 Repeated measures ANOVA for lumbar spine BMC during pre- and post-surgery period.

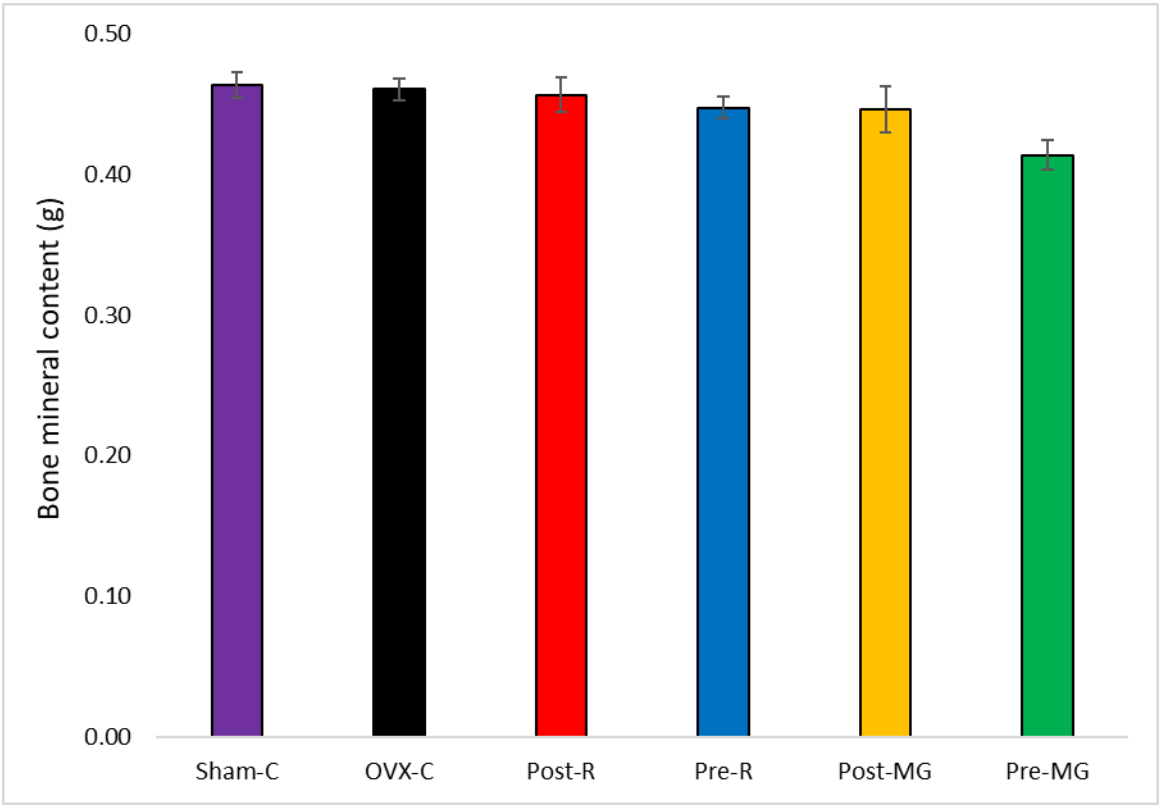
OVX only	**Pre-surgery period (0-6 week)** **BMC**	**Post-surgery period (6-16 weeks)** **BMC**
Group	p=0.701	p=0.636
Week	p=0.813	p=0.353
Group x week	p=0.002	p=0.103

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was used as covariate.

Table 9 Percent change of lumbar spine BMC in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	**Week 6-12**	**Week 12-16**	**Week 6-16**
Sham-C	36.50 ± 2.51	16.25 ± 2.08	8.42 ± 2.64*	26.33 ± 4.58*
OVX-C	32.00 ± 2.18	12.38 ± 1.44	-0.54 ± 2.08	12.00 ± 3.18
'Post-R'	31.87 ± 2.19	10.47 ± 1.55	5.07 ± 2.66	15.93 ± 2.94
'Pre-R'	34.31 ± 2.95	12.85 ± 1.85	3.54 ± 1.91	16.54 ± 1.26
'Post-MG'	38.33 ± 1.99	7.40 ± 0.99	-0.14 ± 2.14	7.43 ± 2.41
'Pre-MG'	34.29 ± 2.71	6.43 ± 1.46**	4.40 ± 2.54	10.79 ± 2.99

Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one -way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *p<0.05, **p<0.01.



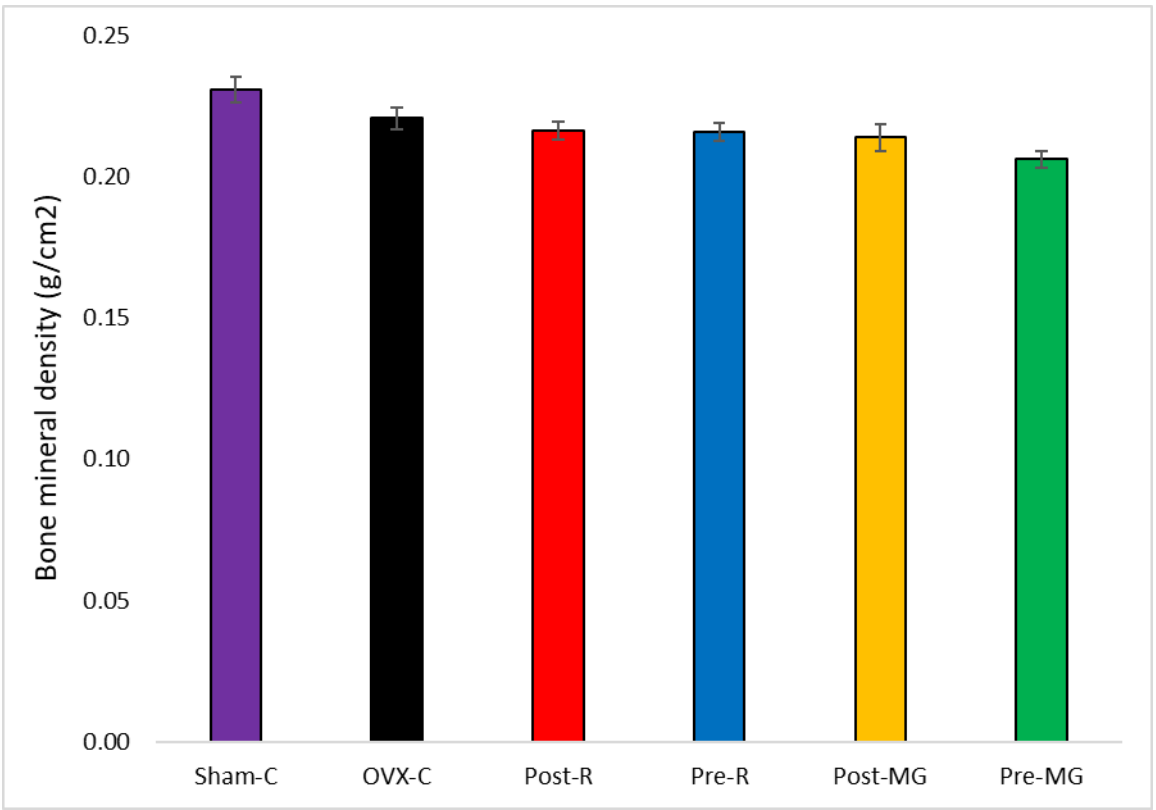
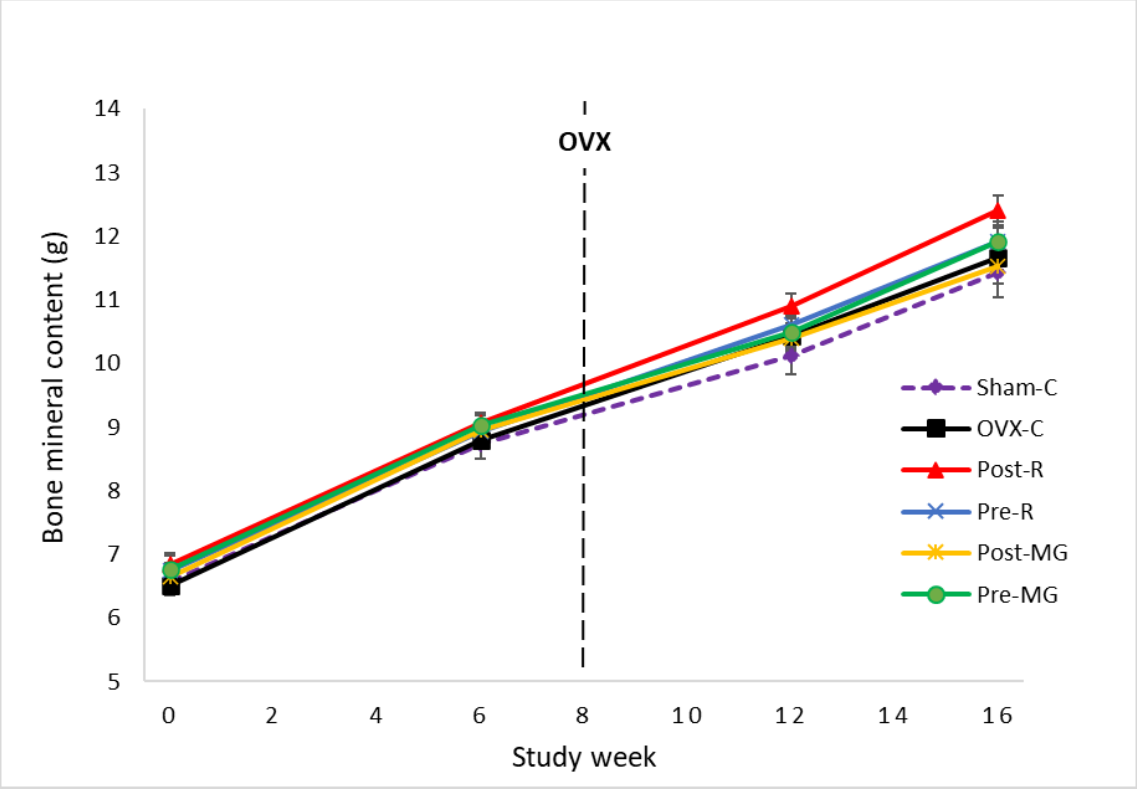


Figure 15 *Ex vivo* lumbar spine BMC and BMD in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

Table 10 Repeated measures ANOVA for whole body BMC and BMD during pre- and post-surgery period.

OVX only	Pre-surgery period (0-6 week)		Post-surgery period (6-16 weeks)	
	BMC	**BMD**	**BMC**	**BMD**
Group	p=0.186	p=0.808	p=0.384	p=0.829
Week	p=0.972	p>0.001	p=0.854	p=0.309
Group x week	p=0.013	p=0.009	p=0.011	p=0.001

Data were analysed using repeated measures of ANOVA, n=12-15 rats/group. Body weight was taken as covariate.



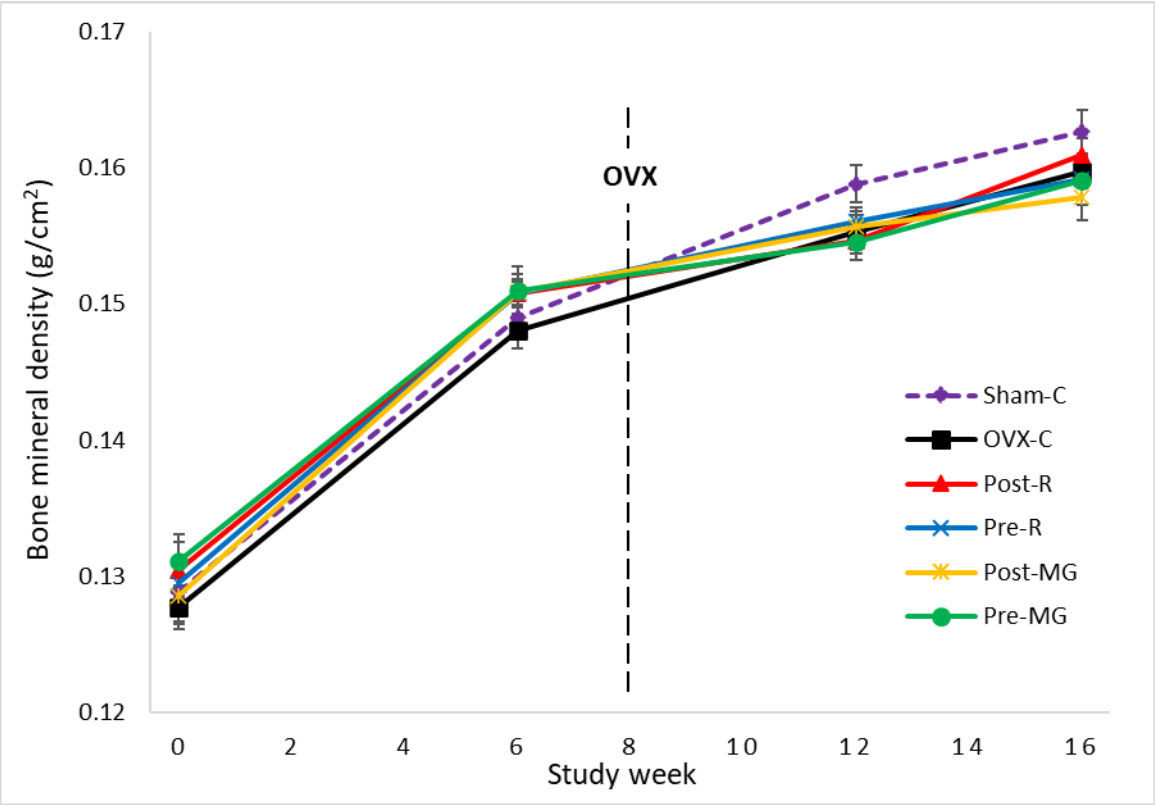


Figure 16 Whole body BMC and BMD in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

Table 11 Percent change of whole body BMC and BMD in female Sprague-Dawley rats fed control or experimental supplements for 16 weeks.

	Week 0-6	**Week 6-12**	**Week 12-16**	**Week 6-16**
Whole body BMC				
Sham-C	33.36 ± 2.22	15.50 ± 0.56	12.42 ± 1.23	29.83 ± 1.83
OVX-C	34.08 ± 2.43	18.31 ± 1.40	11.85 ± 0.53	32.31 ± 1.56
'Post-R'	33.33 ± 2.55	20.27 ± 1.10	13.73 ± 0.80	36.73 ± 1.41
'Pre-R'	33.00 ± 2.33	19.23 ± 0.99	12.00 ± 0.71	33.62 ± 1.37
'Post-MG'	34.33 ± 1.59	17.08 ± 0.92	10.14 ± 1.16	29.00 ± 1.31
'Pre-MG'	33.87 ± 2.25	17.38 ± 0.90	13.73 ± 0.96	32.13 ± 1.85
Whole body BMD				
Sham-C	15.91 ± 1.52	6.58 ± 0.72*	2.58 ± 0.43	9.08 ± 0.83
OVX-C	15.31 ± 1.22	4.77 ± 0.40	2.92 ± 0.50	7.46 ± 0.58
'Post-R'	15.93 ± 1.66	2.92 ± 0.27	4.07 ± 0.52	6.73 ± 0.58
'Pre-R'	16.31 ± 1.23	4.00 ± 0.51	2.15 ± 0.36	5.85 ± 0.69
'Post-MG'	17.00 ± 1.00	3.54 ± 0.69	1.07 ± 0.45	4.36 ± 0.68*
'Pre-MG'	15.47 ± 1.30	2.57 ± 0.37*	3.00 ± 0.54	5.27 ± 0.56

Data represent mean ± SEM, n=12-15 rats/group. Sham-C vs OVX-C groups were analysed using Students' t-test. OVX groups were analysed using one -way ANOVA followed by post-hoc Tukey test. *indicates significance difference from OVX-C: *$p<0.05$.

Table 12 *In vivo* BMC and BMD in right femur of female Sprague-Dawley rats.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
BMC (g)							
Week 0	0.256 ± 0.010	0.256 ± 0.009	0.259 ± 0.006	0.250 ± 0.006	0.249 ± 0.010	0.252 ± 0.007	0.886
Week 6	0.337 ± 0.008	0.348 ± 0.011	0.355 ± 0.004	0.340 ± 0.008	0.349 ± 0.012	0.341 ± 0.008	0.874
Week 12	0.387 ± 0.011	0.376 ± 0.011	0.385 ± 0.003	0.372 ± 0.005	0.371 ± 0.012	0.364 ± 0.009	0.686
Week 16	0.416 ± 0.018	0.389 ± 0.008	0.393 ± 0.006	0.389 ± 0.005	0.372 ± 0.013	0.383 ± 0.010	0.326
BMD (g/cm^2)							
Week 0	0.217 ± 0.005	0.216 ± 0.004	0.218 ± 0.003	0.216 ± 0.003	0.214 ± 0.005	0.219 ± 0.003	0.981
Week 6	0.253 ± 0.003	0.255 ± 0.004	0.263 ± 0.003	0.256 ± 0.002	0.258 ± 0.005	0.257 ± 0.003	0.690
Week 12	0.275 ± 0.004*	0.262 ± 0.004	0.263 ± 0.002	0.264 ± 0.002	0.264 ± 0.004	0.260 ± 0.004	0.915
Week 16	0.286 ± 0.006**	0.265 ± 0.003	0.263 ± 0.003	0.267 ± 0.001	0.258 ± 0.004	0.265 ± 0.005	0.316

Data represent mean ± (SEM), n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test. *indicates significance difference from OVX-C: *$p<0.05$, **$p<0.01$.

Table 13 *In vivo* BMC and BMD in lumbar spine of female Sprague-Dawley rats.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
BMC (g)							
Week 0	0.288 ± 0.010	0.298 ± 0.007	0.309 ± 0.007	0.305 ± 0.006	0.290 ± 0.010	0.307 ± 0.012	0.553
Week 6	0.392 ± 0.009	0.396 ± 0.006	0.406 ± 0.005	0.405 ± 0.005	0.404 ± 0.012	0.409 ± 0.013	0.922
Week 12	0.454 ± 0.010	0.441 ± 0.007	0.451 ± 0.009	0.457 ± 0.009	0.433 ± 0.013	0.435 ± 0.013	0.466
Week 16	0.493 ± 0.018*	0.448 ± 0.006	0.476 ± 0.011	0.469 ± 0.006	0.435 ± 0.016	0.452 ± 0.018	0.180
BMD (g/cm^2)							
Week 0	0.180 ± 0.003	0.183 ± 0.002	0.186 ± 0.003	0.186 ± 0.002	0.179 ± 0.004	0.186 ± 0.004	0.369
Week 6	0.210 ± 0.003	0.212 ± 0.003	0.214 ± 0.002	0.217 ± 0.001	0.215 ± 0.003	0.217 ± 0.004	0.945
Week 12	0.228 ± 0.003	0.222 ± 0.003	0.219 ± 0.003	0.224 ± 0.002	0.217 ± 0.003	0.218 ± 0.003	0.417
Week 16	0.237 ± 0.005**	0.220 ± 0.003	0.223 ± 0.003	0.225 ± 0.002	0.212 ± 0.005	0.218 ± 0.005	0.133

Data represent mean ± SEM, n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test. * indicates significance difference from OVX-C: *$p<0.05$, **$p<0.01$.

Table 14 *In vivo* BMC and BMD in whole body of female Sprague-Dawley rats.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
BMC (g)							
Week 0	6.569 ± 0.219	6.513 ± 0.123	6.841 ± 0.175	6.743 ± 0.111	6.643 ± 0.163	6.769 ± 0.202	0.704
Week 6	8.737 ± 0.244	8.803 ± 0.106	9.075 ± 0.155	8.939 ± 0.151	8.949 ± 0.225	9.020 ± 0.211	0.881
Week 12	10.116 ± 0.297	10.438 ± 0.186	10.900 ± 0.191	10.615 ± 0.186	10.389 ± 0.210	10.485 ± 0.258	0.422
Week 16	11.431 ± 0.386	11.663 ± 0.204	12.404 ± 0.238	11.912 ± 0.222	11.534 ± 0.284	11.914 ± 0.308	0.162
BMD (g/cm^2)							
Week 0	0.129 ± 0.002	0.128 ± 0.002	0.130 ± 0.002	0.130 ± 0.002	0.129 ± 0.002	0.131 ± 0.002	0.756
Week 6	0.149 ± 0.001	0.148 ± 0.001	0.151 ± 0.001	0.151 ± 0.001	0.151 ± 0.001	0.151 ± 0.001	0.536
Week 12	0.159 ± 0.001	0.155 ± 0.001	0.155 ± 0.001	0.156 ± 0.001	0.156 ± 0.001	0.155 ± 0.001	0.883
Week 16	0.163 ± 0.002	0.160 ± 0.001	0.161 ± 0.001	0.159 ± 0.001	0.158 ± 0.002	0.159 ± 0.002	0.635

Data represent mean ± SEM, n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test.

Table 15 *Ex vivo* BMC and BMD in right femur and lumbar spine of female Sprague-Dawley rats.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
Right femur							
BMC	0.452 ± 0.013	0.421 ± 0.013	0.439 ± 0.008	0.433 ± 0.007	0.410 ± 0.013	0.404 ± 0.008	0.071
BMD	0.275 ± 0.007***	0.238 ± 0.002	0.243 ± 0.004	0.236 ± 0.003	0.228 ± 0.005	0.222 ± 0.003	0.002
Lumbar spine							
BMC	0.464 ± 0.009	0.461 ± 0.008	0.456 ± 0.012	0.448 ± 0.008	0.446 ± 0.016	0.414 ± 0.010	0.053
BMD	0.231 ± 0.005	0.221 ± 0.004	0.216 ± 0.003	0.216 ± 0.005	0.214 ± 0.005	0.206 ± 0.003	0.097

Data represent mean ± SEM, n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test. *indicates significance difference from OVX-C: ***$p<0.001$.

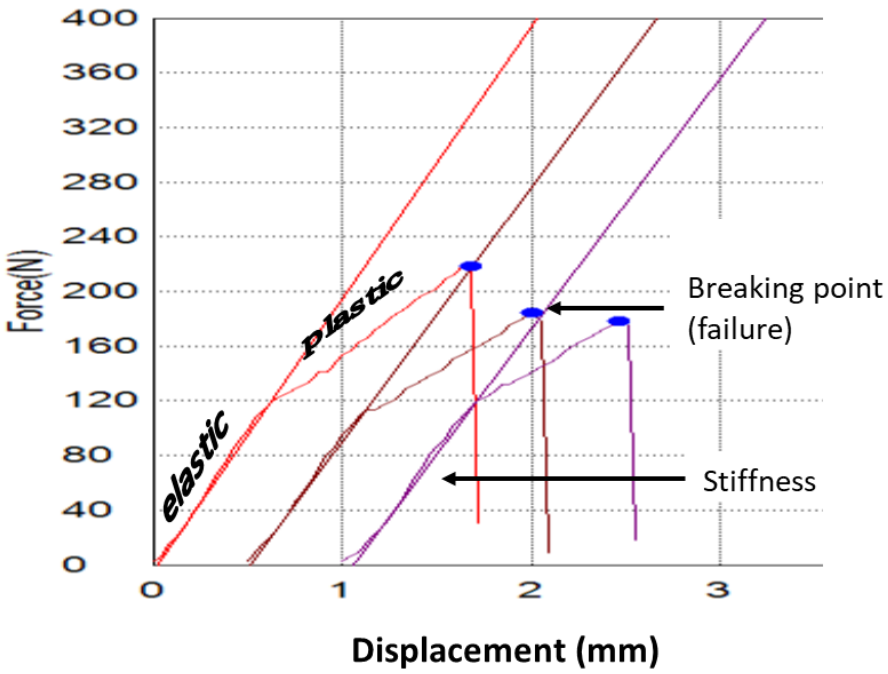


Figure 17 Force-displacement curve.

Elastic phase- slope of linear phase of curve where bone could spring back to original shape if load released.

Plastic phase- deformation increases more rapidly than force thus bone sustains permanent damage.

Breaking force (N) and break stroke (mm) are defined as the bending force and the displacement at breaking point (failure), respectively. Breaking force reflects the strength of the bone while break stroke reflects the brittleness of bone. The higher the stores, the weaker the bone is. Break stress (N/mm^2) is defined as the force per unit area where the greater the break stress the stronger the bone is. Break strain (%) is the deformation percentage of the femur just before the bone breaking point (failure) and it is measured relative to its length. Elasticity is the force needed to bend the bone and this deformation is reversible (elastic phase). Elasticity reflects the resistance of bone to deformation which is called stiffness. With the continuous force, the permeant deformation may happen, and this phase is called plastic. Breaking energy (J) is a final measure of the bone strength. It is the amount of energy required to break the bone. Energy value is calculated using the area under the force/displacement curve. bone store energy during the mechanical test and released at the point of fracture. Higher energy value reflects that bone can store more energy prior to breaking, indicating how stronger the bone is.

Table 16 Mechanical properties of right femurs of female Sprague-Dawley rats.

Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
Bone length (mm)	36.08 ± 0.48	35.85 ± 0.31	36.30 ± 0.20	36.40 ± 0.19	36.35 ± 0.29	36.33 ± 0.19	0.824
Midpoint width (mm)	3.80 ± 0.18	3.98 ± 0.09	4.04 ± 0.03	3.95 ± 0.04	4.06 ± 0.05	4.00 ± 0.04	0.371
Midpoint thickness (mm)	3.07 ± 0.06	3.13 ± 0.05	3.13 ± 0.03	3.15 ± 0.03	3.15 ± 0.04	3.12 ± 0.04	0.937
Break force (N)	164.75 ± 5.11	161.89 ± 5.70	165.78 ± 3.36	170.16 ± 3.55	177.66 ± 5.64	169.67 ± 5.41	0.298
Break stroke (mm)	1.42 ± 0.05	1.45 ± 0.05	1.37 ± 0.04	1.49 ± 0.04	1.50 ± 0.05	1.38 ± 0.04	0.107
Break stress (N/mm2)	104.46 ± 5.11	93.79 ± 3.76	94.77 ± 2.26	98.25 ± 3.32	99.49 ± 3.15	98.39 ± 2.84	0.387
Break strain (%)	11.68 ± 0.65	12.08 ± 0.57	11.42 ± 0.41	12.47 ± 0.32	12.66 ± 0.51	11.44 ± 0.42	0.123
Elasticity (N/mm2)	1308.62 ± 87.20	1262.22 ± 68.62	1276.59 ± 38.55	1258.07 ± 35.19	1248.14 ± 59.17	1346.46 ± 48.79	0.590
Energy (J)	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.177

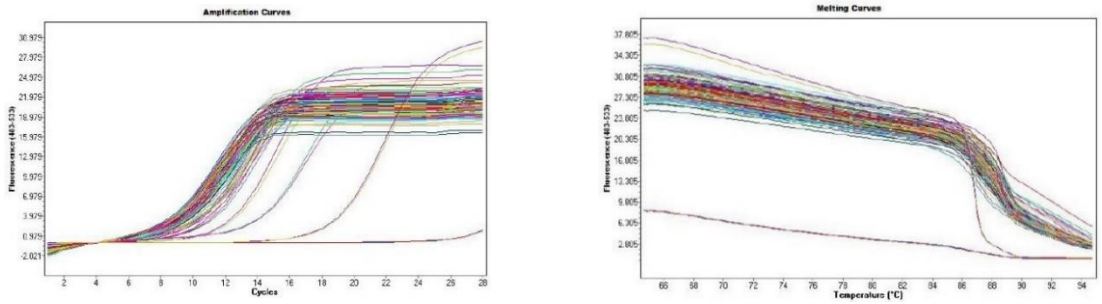
Data are mean ±SEM, n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test.

Table 17 Mechanical properties of left femurs of female Sprague-Dawley rats.

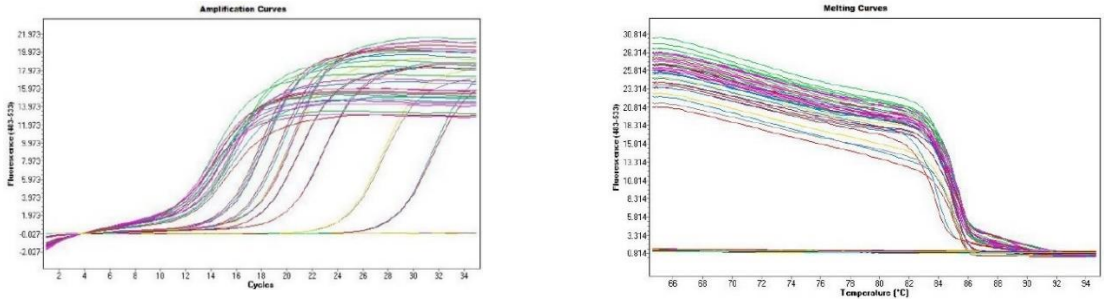
Group	Sham-C [a]	OVX-C	'Post-R'	'Pre-R'	'Post-MG'	'Pre-MG'	*p* value [b]
Bone length (mm)	36.47 ± 0.40	36.70 ± 0.30	36.99 ± 0.19	36.61 ± 0.15	36.38 ± 0.30	36.34 ± 0.19	0.452
Midpoint width (mm)	4.03 ± 0.04*	4.24 ± 0.06	4.20 ± 0.06	4.24 ± 0.03	4.23 ± 0.05	4.24 ± 0.05	0.971
Midpoint thickness (mm)	3.10 ± 0.03*	3.22 ± 0.04	3.16 ± 0.01	3.19 ± 0.03	3.18 ± 0.04	3.20 ± 0.03	0.829
Break force (N)	181.80 ± 10.15	170.29 ± 5.99	166.53 ± 4.12	165.48 ± 4.34	175.43 ± 6.29	161.89 ± 4.63	0.368
Break stroke (mm)	1.543 ± 0.08	1.530 ± 0.07	1.492 ± 0.06	1.377 ± 0.06	1.560 ± 0.05	1.440 ± 0.07	0.186
Break stress (N/mm2)	105.67 ± 7.27	89.88 ± 2.91	90.12 ± 1.95	87.79 ± 3.77	90.02 ± 4.46	84.11 ± 2.93	0.666
Break strain (%)	12.83 ± 0.47	13.53 ± 0.71	12.54 ± 0.60	12.54 ± 0.37	13.54 ± 0.68	12.21 ± 0.42	0.228
Elasticity (N/mm2)	1220.60 ± 47.87	1103.96 ± 39.75	1128.61 ± 34.22	1099.64 ± 35.75	1173.94 ± 46.90	1118.89 ± 39.48	0.688
Energy (J)	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.415

Data are mean ± SEM, n=12-15 rats/group. [a] Sham-C vs OVX-C analysed using Students' t-test. [b] One -way ANOVA followed by post-hoc Tukey test. *indicates significance difference from OVX-C: *$p<0.05$.

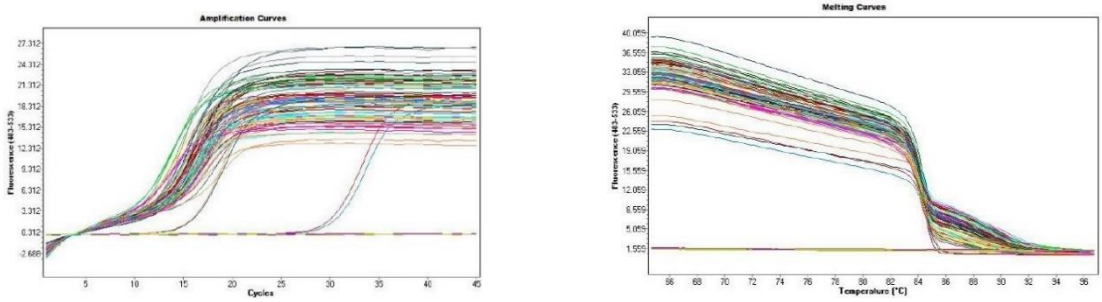
Lactobacillus



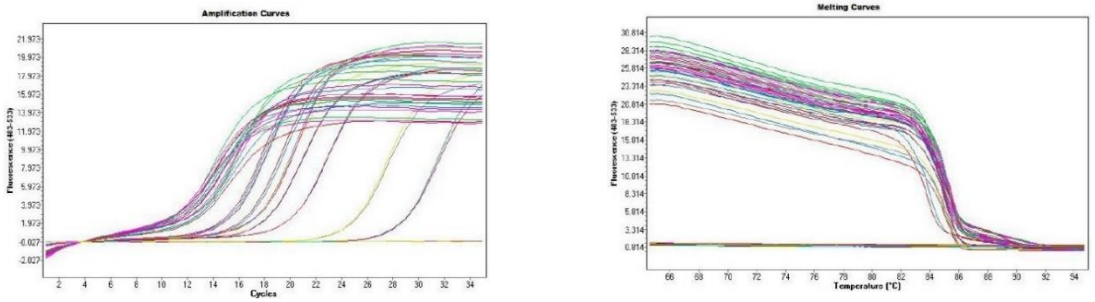
Bifidobacterium



Enterococcus



Bacteroides



E. coli

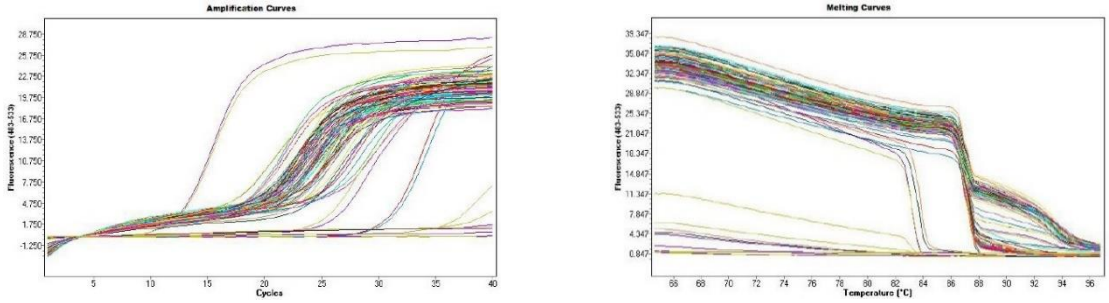


Figure 18 Amplification curves and melting curve of bacterial genera from rat caeca were assessed at the end of the RT-PCR process.

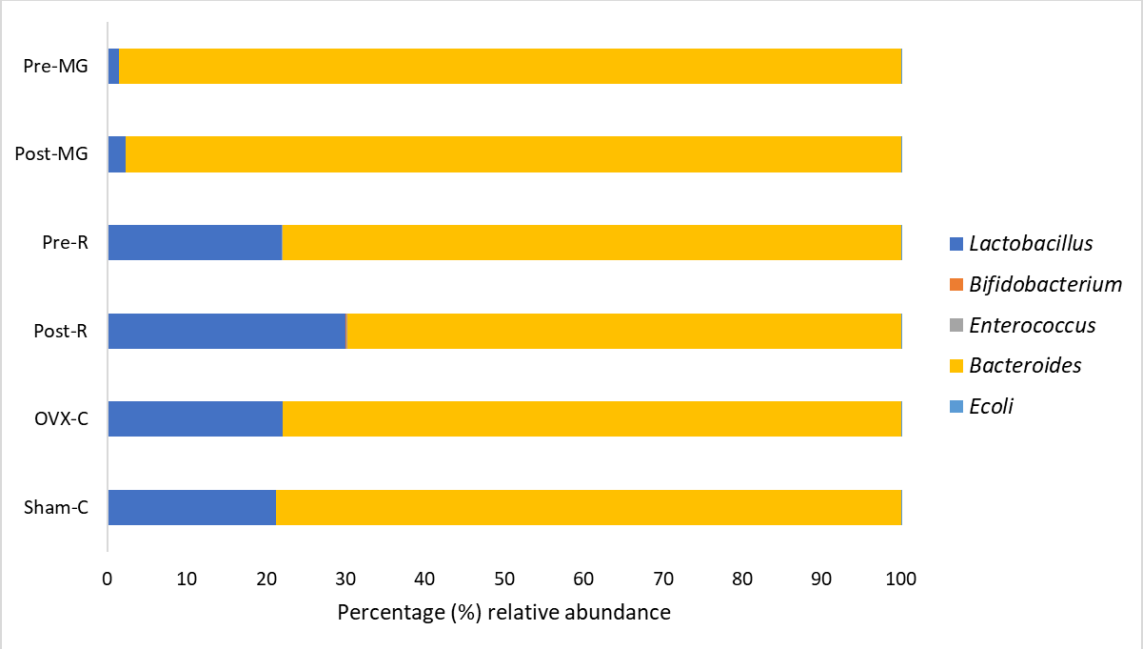


Figure 19 Relative abundance of (a) *Lactobacillus* (b) *Bifidobacterium* (c) *Enterococcus* (d) *Bacteroides* and (e) *E. coli* in rat caeca with supplementation of red and 'Moonglow' feeding before and/or after ovariectomy.

Table 18 Correlation analysis between BMD, body composition measurements and gut microbes in 'Sham-C' group. Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	0.862	0.577	0.635	0.469	0.778	0.214	0.382	0.601	0.464	0.017
	p value		p<0.01	0.104	0.027	0.124	0.003	0.504	0.221	0.039	0.128	0.961
BMD_RF	Pearson Correlation		1	0.606	0.781	0.605	0.847	0.263	0.289	0.507	0.511	-0.214
	p value			0.084	0.003	0.037	0.001	0.410	0.362	0.092	0.090	0.527
BMD_LS	Pearson Correlation			1	0.696	0.635	0.304	0.260	-0.124	0.076	0.447	0.047
	p value				0.037	0.066	0.426	0.500	0.751	0.847	0.228	0.905
Fat mass	Pearson Correlation				1	0.969	0.340	0.272	-0.045	0.027	0.261	-0.160
	p value					p<0.01	0.280	0.392	0.888	0.934	0.413	0.639
Fat %	Pearson Correlation					1	0.099	0.240	-0.173	-0.159	0.139	-0.146
	p value						0.759	0.452	0.591	0.623	0.667	0.668
Lean mass	Pearson Correlation						1	0.145	0.490	0.758	0.526	-0.130
	p value							0.652	0.106	0.004	0.079	0.703
Lactobacillus	Pearson Correlation							1	0.607	0.227	0.396	0.420
	p value								0.036	0.478	0.202	0.199
Bifidobacterium	Pearson Correlation								1	0.606	0.572	0.304
	p value									0.037	0.052	0.364
Enterococcus	Pearson Correlation									1	0.549	-0.047
	p value										0.065	0.891
Bacteroides	Pearson Correlation										1	-0.155
	p value											0.650
E. coli	Pearson Correlation											1
	p value											

Table 19 Correlation analysis between BMD, body composition measurements and gut microbes in 'OVX-C' group. Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	0.918	0.749	0.415	0.457	-0.482	-0.007	0.636	0.648	0.357	-0.303
	p value		$p<0.01$	0.020	0.233	0.184	0.158	0.986	0.048	0.059	0.311	0.466
BMD_RF	Pearson Correlation		1	0.554	0.896	0.837	0.028	-0.061	0.438	0.436	0.339	-0.221
	p value			0.122	$p<0.01$	0.003	0.938	0.876	0.205	0.241	0.337	0.599
BMD_LS	Pearson Correlation			1	-0.109	-0.095	-0.086	-0.460	0.507	0.442	0.175	0.017
	p value				0.764	0.793	0.814	0.213	0.135	0.234	0.629	0.969
Fat mass	Pearson Correlation				1	0.981	0.075	0.158	0.055	0.044	0.167	-0.244
	p value					$p<0.01$	0.809	0.624	0.859	0.897	0.586	0.470
Fat %	Pearson Correlation					1	-0.109	0.211	0.045	0.086	0.176	-0.246
	p value						0.723	0.511	0.884	0.801	0.565	0.466
Lean mass	Pearson Correlation						1	-0.335	0.034	-0.300	0.002	0.094
	p value							0.287	0.911	0.370	0.994	0.784
Lactobacillus	Pearson Correlation							1	-0.387	-0.022	0.279	0.113
	p value								0.214	0.949	0.381	0.740
Bifidobacterium	Pearson Correlation								1	0.773	0.504	0.653
	p value									0.005	0.079	0.029
Enterococcus	Pearson Correlation									1	0.654	0.709
	p value										0.029	0.022
Bacteroides	Pearson Correlation										1	0.575
	p value											0.064
E. coli	Pearson Correlation											1
	p value											

Table 20 Correlation analysis between BMD, plasma lycopene, body composition measurements and gut microbes in 'Post-R' group. Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	Plasma lycopene	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	0.507	0.032	0.532	0.148	0.207	-0.320	0.167	-0.290	0.023	-0.388	-0.197
	p value		0.092	0.922	0.075	0.645	0.518	0.310	0.604	0.361	0.943	0.213	0.562
Plasma lycopene	Pearson Correlation		1	0.084	0.375	0.107	0.099	0.038	-0.486	-0.269	-0.213	-0.478	-0.668
	p value			0.784	0.187	0.705	0.726	0.894	0.078	0.332	0.445	0.071	0.013
BMD_RF	Pearson Correlation			1	0.098	0.951	0.882	0.466	0.185	-0.036	0.070	-0.322	0.171
	p value				0.750	$p<0.01$	$p<0.01$	0.109	0.546	0.908	0.820	0.283	0.594
BMD_LS	Pearson Correlation				1	0.132	0.146	-0.036	-0.149	-0.366	-0.258	-0.549	-0.030
	p value					0.653	0.618	0.904	0.612	0.198	0.373	0.042	0.924
Fat mass	Pearson Correlation					1	0.962	0.238	0.265	0.152	0.250	-0.250	0.063
	p value						$p<0.01$	0.392	0.360	0.589	0.369	0.369	0.839
Fat %	Pearson Correlation						1	-0.033	0.291	0.063	0.161	-0.414	0.020
	p value							0.908	0.312	0.824	0.567	0.125	0.948
Lean mass	Pearson Correlation							1	-0.026	0.333	0.351	0.513	0.346
	p value								0.929	0.225	0.200	0.050	0.247
Lactobacillus	Pearson Correlation								1	0.561	0.816	0.383	0.734
	p value									0.037	$p<0.01$	0.176	0.004
Bifidobacterium	Pearson Correlation									1	0.923	0.600	0.590
	p value										$p<0.01$	0.018	0.034
Enterococcus	Pearson Correlation										1	0.603	0.598
	p value											0.017	0.031
Bacteroides	Pearson Correlation											1	0.478
	p value												0.098
E. coli	Pearson Correlation												1
	p value												

Table 21 Correlation analysis between BMD, plasma lycopene, body composition measurements and gut microbes in 'Pre-R' group.Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	Plasma lycopene	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	0.480	1.000	1.000	0.943	0.856	0.659	-0.009	0.404	-0.479	-0.105	-0.245
	p value		0.097	*p*<0.01	*p*<0.01	*p*<0.01	*p*<0.01	0.014	0.978	0.193	0.115	0.745	0.444
Plasma lycopene	Pearson Correlation		1	0.526	0.526	0.365	0.286	0.474	0.006	-0.369	0.098	-0.297	-0.409
	p value			0.079	0.079	0.221	0.343	0.102	0.984	0.238	0.761	0.349	0.187
BMD_RF	Pearson Correlation			1	1	0.952	0.854	0.691	0.009	0.364	-0.439	-0.216	-0.199
	p value				*p*<0.01	*p*<0.01	*p*<0.01	0.013	0.979	0.271	0.177	0.523	0.556
BMD_LS	Pearson Correlation				1	0.952	0.854	0.691	0.009	0.364	-0.439	-0.216	-0.199
	p value					*p*<0.01	*p*<0.01	0.013	0.979	0.271	0.177	0.523	0.556
Fat mass	Pearson Correlation					1	0.978	0.404	0.028	0.428	-0.469	-0.074	-0.185
	p value						*p*<0.01	0.171	0.931	0.165	0.124	0.818	0.565
Fat %	Pearson Correlation						1	0.209	0.042	0.419	-0.415	-0.074	-0.150
	p value							0.494	0.897	0.175	0.180	0.820	0.642
Lean mass	Pearson Correlation							1	-0.13	0.095	-0.333	0.049	-0.266
	p value								0.687	0.769	0.29	0.879	0.404
Lactobacillus	Pearson Correlation								1	0.282	-0.123	0.073	0.498
	p value									0.374	0.703	0.831	0.099
Bifidobacterium	Pearson Correlation									1	-0.689	0.873	0.203
	p value										0.013	*p*<0.01	0.528
Enterococcus	Pearson Correlation										1	-0.603	0.144
	p value											0.050	0.655
Bacteroides	Pearson Correlation											1	-0.132
	p value												0.698
E. coli	Pearson Correlation												1
	p value												

Table 22 Correlation analysis between BMD, plasma lycopene, body composition measurements and gut microbes in 'Post-MG' group. Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	Plasma lycopene	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	-0.183	0.892	1.000	0.695	0.439	0.644	0.418	0.173	-0.027	0.177	0.067
	p value		0.515	$p<0.01$	$p<0.01$	0.006	0.117	0.013	0.121	0.537	0.923	0.527	0.819
Plasma lycopene	Pearson Correlation		1	-0.308	-0.308	-0.272	-0.319	0.164	-0.046	-0.222	0.142	0.214	0.107
	p value			0.283	0.283	0.346	0.266	0.575	0.871	0.425	0.615	0.444	0.716
BMD_RF	Pearson Correlation			1	1.000	0.751	0.466	0.709	0.364	0.383	-0.266	0.051	-0.091
	p value				$p<0.01$	0.003	0.109	0.007	0.200	0.176	0.359	0.863	0.767
BMD_LS	Pearson Correlation				1	0.697	0.441	0.644	0.425	0.326	-0.117	0.121	-0.051
	p value					0.006	0.114	0.013	0.130	0.256	0.689	0.680	0.868
Fat mass	Pearson Correlation					1	0.931	0.097	-0.064	0.308	-0.478	-0.317	-0.186
	p value						$p<0.01$	0.742	0.829	0.284	0.084	0.269	0.543
Fat %	Pearson Correlation						1	-0.270	-0.253	0.273	-0.540	-0.492	-0.260
	p value							0.350	0.384	0.344	0.046	0.074	0.390
Lean mass	Pearson Correlation							1	0.534	0.077	0.242	0.498	0.226
	p value								0.049	0.793	0.404	0.070	0.459
Lactobacillus	Pearson Correlation								1	0.001	0.480	0.467	-0.153
	p value									0.997	0.070	0.080	0.602
Bifidobacterium	Pearson Correlation									1	-0.071	-0.225	-0.373
	p value										0.800	0.421	0.189
Enterococcus	Pearson Correlation										1	0.493	0.066
	p value											0.062	0.822
Bacteroides	Pearson Correlation											1	0.627
	p value												0.016
E. coli	Pearson Correlation												1
	p value												

Table 23 Correlation analysis between BMD, plasma lycopene, body composition measurements and gut microbes in 'Pre-MG' group. Each cell represents the Pearson correlation coefficient (R) and *p* values of respective pair of variables. A difference was considered as statistically significant when $p<0.05$.

		Final BW	Plasma lycopene	BMD-RF	BMD-LS	Fat mass	Fat %	Lean mass	*Lactobacillus*	*Bifidobacterium*	*Enterococcus*	*Bacteroides*	*E. coli*
Final BW	Pearson Correlation	1	-0.423	1.000	0.044	0.885	0.711	0.699	0.413	0.079	0.485	-0.224	-0.049
	p value		0.131	*p*<0.01	0.886	*p*<0.01	0.004	0.005	0.142	0.788	0.093	0.461	0.867
Plasma lycopene	Pearson Correlation		1	-0.421	-0.087	-0.337	-0.259	-0.340	-0.416	0.087	-0.321	0.250	-0.241
	p value			0.134	0.778	0.239	0.372	0.234	0.139	0.768	0.284	0.410	0.407
BMD_RF	Pearson Correlation			1	0.037	0.883	0.709	0.701	0.408	0.081	0.477	-0.221	-0.049
	p value				0.905	*p*<0.01	0.005	0.005	0.148	0.783	0.099	0.469	0.869
BMD_LS	Pearson Correlation				1	-0.040	-0.059	0.048	0.248	0.154	0.276	-0.610	-0.133
	p value					0.896	0.847	0.875	0.415	0.615	0.361	0.035	0.665
Fat mass	Pearson Correlation					1	0.956	0.301	0.328	-0.091	0.340	-0.241	0.001
	p value						*p*<0.01	0.296	0.252	0.757	0.255	0.428	0.999
Fat %	Pearson Correlation						1	0.011	0.243	-0.187	0.220	-0.237	0.028
	p value							0.969	0.403	0.522	0.470	0.435	0.925
Lean mass	Pearson Correlation							1	0.237	0.209	0.379	-0.081	-0.146
	p value								0.415	0.474	0.201	0.791	0.619
Lactobacillus	Pearson Correlation								1	0.443	0.509	-0.155	-0.006
	p value									0.113	0.076	0.614	0.984
Bifidobacterium	Pearson Correlation									1	-0.092	-0.252	0.062
	p value										0.765	0.406	0.834
Enterococcus	Pearson Correlation										1	-0.032	0.219
	p value											0.921	0.472
Bacteroides	Pearson Correlation											1	0.300
	p value												0.319
E. coli	Pearson Correlation												1
	p value												

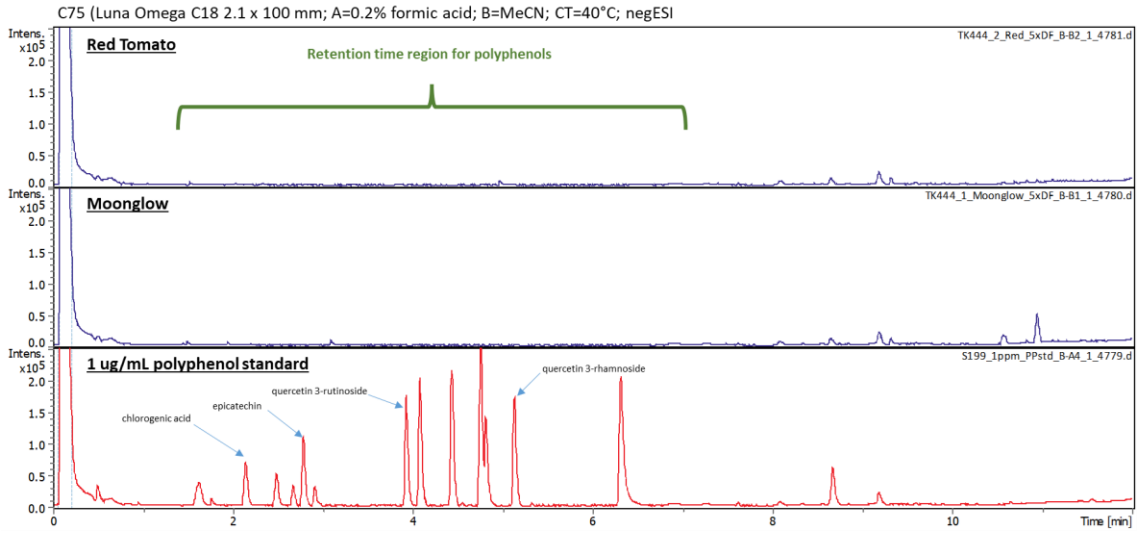
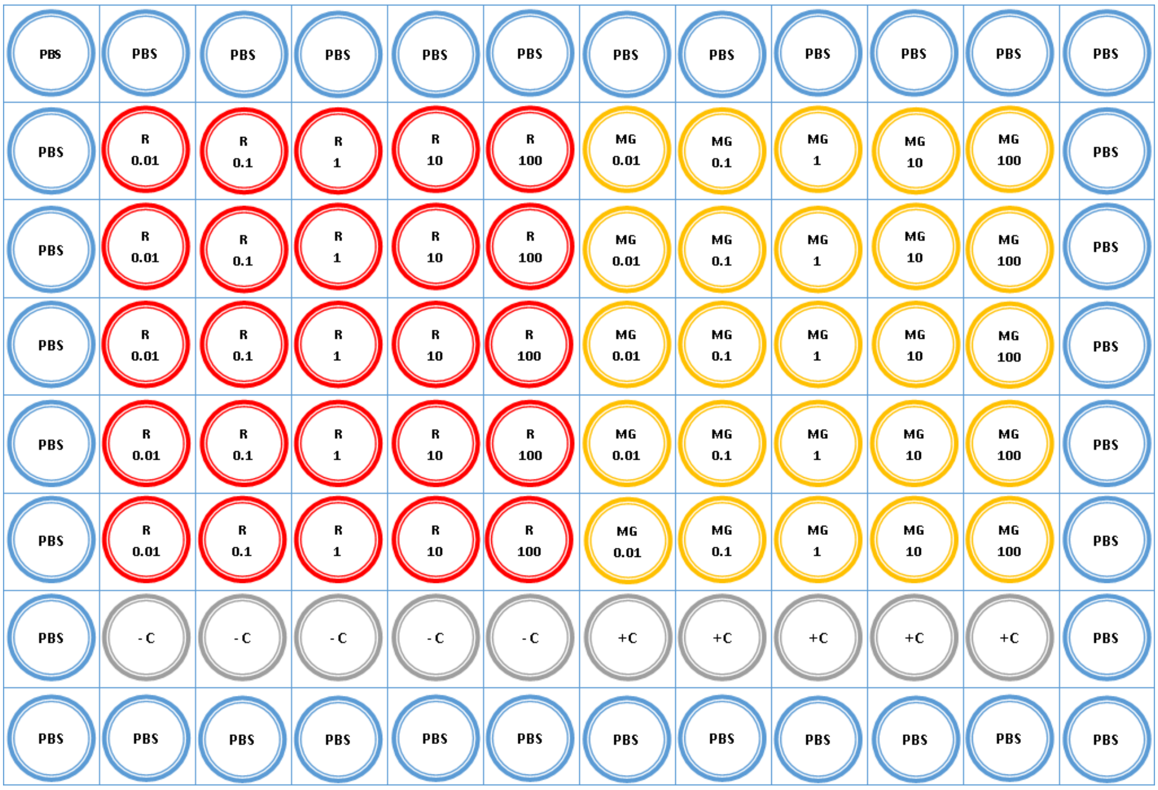
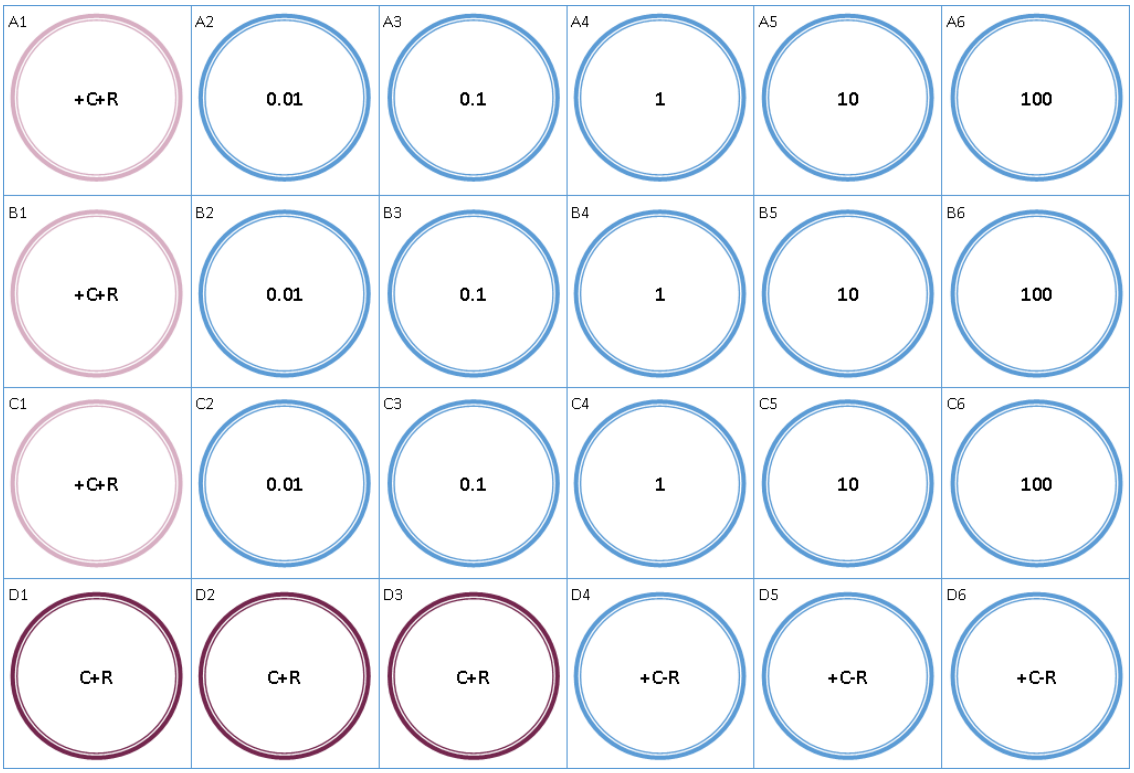


Figure 20 Polyphenol content of tomato hexane extracts.

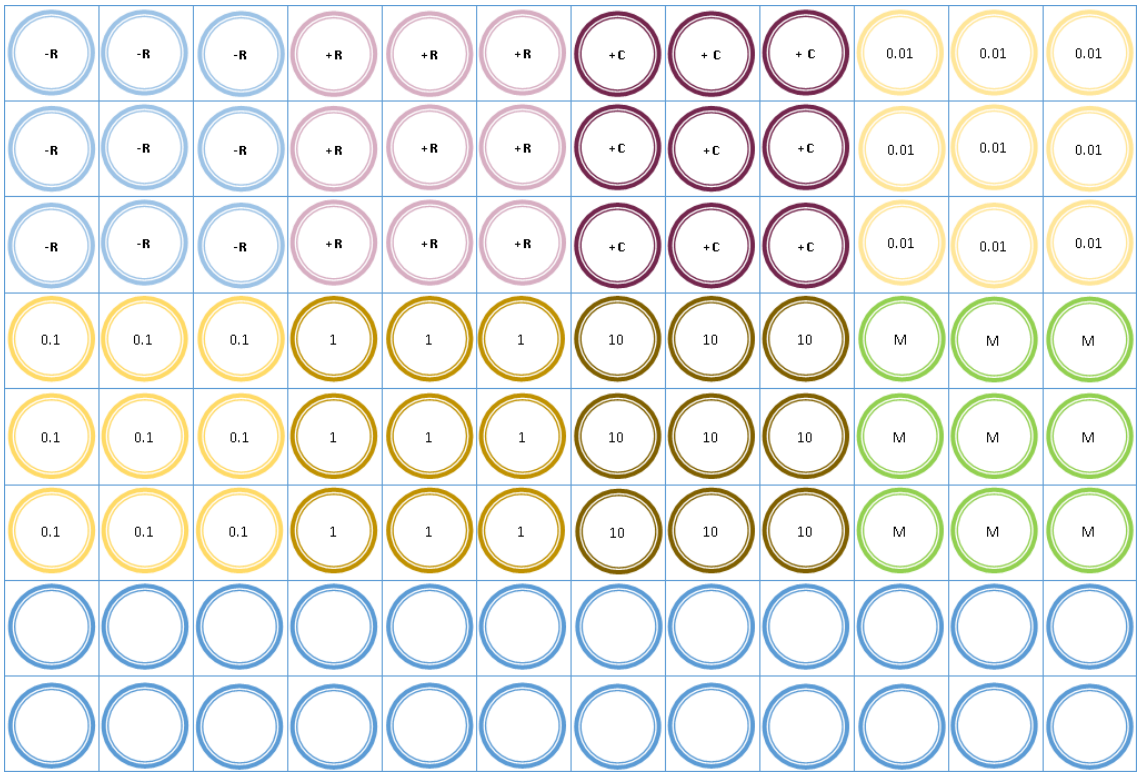
The data obtained from the analysis of red and 'Moonglow' tomato hexane extracts showed that the known tomato polyphenols were not detected. No other potential polyphenols were detected. The lack of polyphenols in these hexane extracts is to be expected. Polyphenols, especially the glycoside present in tomato, are water-soluble and insoluble in non-polar solvents such as hexane, and therefore unlikely to be extracted from tomato tissue with hexane.



Layout 1 Representative 96-well plate for cell viability assay; PBS (Phosphate buffer solution), "R" red tomato extracts, "MG" 'Moonglow' tomato extract, -C negative control (0.1% DMSO with culture media, +C positive control (10% DMSO with culture media).



Layout 2 Representative 24-well plate for TRAP assay. Positive control (+C+R) contains 0.1% DMSO+RANKL+1α,25-Dihydroxycholecalciferol (Calcitriol);Negative control (+C-R) contains 0.1% DMSO without RANKL, Control (C+R) contains 0.1% DMSO + RANKL. 1-100 represent lycopene concentration series of red or 'Moonglow' tomato extracts in triplicate.



Layout 3 Representative 96-well plate for TRAP activity assay. This assay was done by using the media collected from TRAP plate (layout 2). Control without RANKL (-R), Control with RANKL (+R), Positive control (+C), 0.01-10 represent lycopene concentration series of red or 'Moonglow' tomato extracts in replicates.

Appendix ii

Pathology and monitoring reports of rats

School of Veterinary Science

Pathology Report

Submitter Ref.:	Date Sent: 08/08/2019	Accession No.: 57700

To: Dr Fran Wolber
School of Food & Advanced Technology,
Massey University
Palmerston North

Email: f.m.wolber@massey.ac.nz

Report Sent: 27/08/2019
Copy To:

Species: Rodent		Breed: Sprague Dawley	
Age: Subadult - 8 weeks		Sex: Female	
Owner: Trial Rat			Type: Post Mortem
ID: 79 (on base of tail)			Prev. Accn.:
Submitted:	At Risk:	Affected:	Dead:

History

8-week-old rat in a food trial. Report required for ethics. Died under anaesthesia while undergoing DEXA scan.

Gross Findings

The 8-week-old female rat is presented dead. The carcass has minimal post-mortem changes. The rat is in good body condition with a total body weight of 221 g. On opening of the thoracic cavity, several multifocal, 2 mm to 3 mm, dark red to black, round blotchy lesions are seen over the surface of all lung lobes. No gross lesions are present elsewhere.

Histopathology

Sections of kidney, lung, pancreas, liver, spleen, heart, aorta, brain, tongue, stomach, and small and large intestine were examined. Lesions were present in the kidney, pancreas, lungs and liver. Proximal convoluted tubule epithelial cells in the kidney showed severe, diffuse necrosis. A few small foci of peripancreatic fat necrosis were present. The lungs were congested and oedematous and there were multiple small foci of perivascular and peribronchiolar round cell aggregates, as well as focal aggregates, predominantly macrophages, elsewhere in the pulmonary parenchyma. The liver was congested.

Diagnosis

Severe renal cortical necrosis
Mild focal peripancreatic fat necrosis
Pulmonary congestion and oedema with mild perivascular and peribronchiolar cuffing Hepatic congestion
Anaesthetic death

Comments

The renal cortical necrosis could be due to hypoxia (prolonged anaesthesia??) or a renal toxin. The exocrine pancreas was likely leaking lipase to cause the peripancreatic fat necrosis. The pulmonary oedema and congestion and hepatic congestion likely to be due to anaesthesia.

Date: 10/08/2019	Pathologists: S Brown M G Collett
Students: Emily Hooper	

School of Veterinary Science

Pathology Report

Submitter Ref.:	Date Sent:	Accession No.: 57812

To: Fran Wolber
Senior lecturer
Palmerston North
Email: F.M.Wolber@massey.ac.nz

Report Sent:
Copy To:

Species: Rodent		Breed: Rat	
Age: Juvenile - 12 weeks		Sex: Female	
Owner: fran Wolber			Type: Post Mortem
ID: # 25 and # 77			Prev. Accn.:
Submitted: 2	At Risk:	Affected:	Dead:

History

These two rats were reported to have died shortly after anaesthesia. The rats were reported to have been ambulatory, but possibly could have overheated due to being in a cage on a faulty heating pad.

Gross Findings

These rats were presented dead. They were judged to be in a good state of nutrition, adequately hydrated, and in a fair state of preservation. Small amounts of porphyrin were visible around the eyes of both rats. However, significant gross lesions were not observed within either rat.

Histopathology

Multiple sections were examined from these rats. Critical evaluation of sections of intestine is not possible due to autolysis. Examination of sections of lung reveals marked congestion. In addition, alveoli are distended by increased quantities of faintly eosinophilic material that is consistent with edema. Expansion of the connective tissues surrounding blood vessels is also visible, again consistent with pulmonary edema. In one rat there is a single small focus in which alveoli contain small numbers of degenerate neutrophils; however, there is generally little evidence of inflammation visible within the sections. Examination of sections of liver reveals marked congestion with dilation of the hepatic sinusoids with increased quantities of blood. However, there is no evidence of inflammation or necrosis visible within sections of liver. No other significant findings are present on histological examination of tissues from these rats.

Diagnosis

Pulmonary congestion and edema.
Hepatic congestion.

Comments

There was little evidence of any inflammatory or necrotizing process within the sections. Therefore, it may be most likely that the heating pad malfunction resulted in heat stroke within these rats. While the pulmonary and hepatic congestion are consistent with heat stroke, these lesions are non-specific and so no definitive cause of death can be identified in these rats.

Date: 04/09/2019	Pathologists: J S Munday
Students: Finn, Chantelle	

MONITORING REPORT

To: Miralie Thomas Vincent

Cc: Fran Wolber, Anne Broomfield

Date of visit: 03 Sep 2019

Date of report: 10 Sep 2019

Protocols: 19/12

Location: DEXA scanning room and 3rd floor laboratory SF&AT building

Two rats died on 2nd Sep after being DEXA scanned, whilst recovering from injectable anaesthesia in the recovery room (3rd floor). Other recovering rats reportedly were hunched and had piloerection. This visit was in response to those events.

Anne was administering the intra-peritoneal anaesthetic and DEXA scanning the rats. The room was reasonably warm however no additional thermal support, or supplementary oxygen, were provided.

After scanning, the rats were returned to a cage. Odine Johnstone collected the covered cage and carried it up the lift and into the laboratory (recovery) room. Yesterday, more than one rat was returned at a time, giving a longer stay in the scanning room for some rats. Transferring the rats as soon as possible to the lab where warmth and oxygen were available was a good idea.

On arrival, each rat was given 3mL warmed isotonic saline s/c by Louise Shaw. The anaesthetised rats were placed on one heat pad covered with an incontinence sheet and additional paper towels to absorb urine. The rats (up to six were lined up at one time) were covered with a towel. Oxygen was available from a tube connected to the cylinder, although this didn't effectively deliver oxygen to all the anaesthetised rats. A thermometer probe was placed between a rat and heating pad, the temperature ranged from 36.0-37.4°C. I took the rectal temperature of the newest anaesthetised arrival, and the rat that had been on the heat pad for the longest time. They were 33.3 and 35.0 °C respectively (normal rectal temperature is 36-37.5 °C). This indicated that the rats were becoming hypothermic prior to their arrival in the recovery room and that the provision of this thermal support was appropriate.

As the rats began to wake up they were transferred to group cages. The cages were also on heating pads; a thermometer indicated the cage floor temperature was 30 °C. A towel covered half the cage; yesterday it had been placed over the entire cage. Apparently, the room had also been much warmer, the caged rats (feet) were sweating and the cage environment was humid. Monitoring thermometers were not present. It is possible that hyperthermia and hypoxia contributed to the deaths.

The recovered rats will be returned to a recovery animal room at SAPU. The thermostat in this room will be raised from approximately 23 °C to approximately 26 °C. The rats will be returned to their usual animal room the following day.

Recommendations that were discussed during the visit amongst personnel present (including Aimee Hamlin and Fran Wolber) are as follows:

1. Place the cages in the DEXA room on heat pads. Have heated small blankets or woollen socks available to wrap/cover the rats immediately on completion of the scan.

2. Provide oxygen to the anaesthetised rats in the DEXA room (infrastructure to secure a cylinder is needed).
3. Modifying the oxygen delivery system to direct oxygen to each anaesthetised rat simultaneously. For example, by attaching a length of tubing to the oxygen hose, with holes made on one side at intervals consistent with the distance between rat noses when rats are lined up on the heat pad. The end should be sealed.
4. Routinely monitor the rectal temperature of at least a portion of the rats recovering from anaesthesia. Obtain lubricant for use on the end of the thermometer.
5. Monitor the temperature of the heat pads/cage floors. A temperature of 37 °C for the recovery pad is appropriate for hypothermic rats. Cage-floor temperature of 30-35°C until recovery will also provide thermal support. At that point, ensure the heat pad is under half of the cage (if still used) to provide the rats with choice regarding the floor temperature.
6. Don't cover more than half the cage top with a towel.
7. An ambient temperature of approximately 23 °C is suitable for recovery. Raising the temperature in the animal room at SAPU by several degrees, for recovering rats, should be continued.
8. Lactated ringers provides a more balanced fluid supplement than normal saline.
9. Offering recovered rats an oral sugar/water solution via syringe on recovery.
10. Updating the SOPs is required.

4th - 6th Sep DEXA scanning

Heating pads were placed under the cages in the DEXA room. The temperature of the cage floor was monitored. The anaesthetised rats were also placed in warmed socks on completion of DEXA scanning.

Raising the ambient temperature in the DEXA room was attempted however this interfered with the functioning of the DEXA machine (it's optimal temperature is 18 °C; ambient temperatures of 19-20°C are generally targeted). It was determined that bubble wrap did not interfere with the DEXA scan image. Bubble wrap, superseded by a plastic sheet (to improve centring of the scanner over the rat) were subsequently placed over the rat during scanning to provide insulation and protection from the air-conditioner draft. The airflow (cold draft) from the air conditioning unit was also re-directed away from the surface of the DEXA table. In addition, strategies for heating the DEXA table between rats will be investigated.

Rectal temperatures on arrival at the recovery room were between 33.2-36.7 °C (mean 35.0 °C, n=50 rats). Temperature monitoring probes were also used in the recovery room, on the heat pad (as they were the previous day).

Sugar water was offered once the rats had recovered, prior to their return to SAPU. Two-thirds of them readily accepted the offer.

Tubing was used to direct oxygen to each of the recovering anaesthetised rats in the recovery room.

These improvements will provide useful additional support to the rats anaesthetised for DEXA scanning.

Appendix iii

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